Bioreactors and the Design of the Stem Cell Niche

Yongjia Fan, Donghui Jing and Emmanuel S. Tzanakakis

1 Introduction

Stem cells—especially human pluripotent stem cells (hPSCs)—are a promising inexhaustible source of cellular material for treating ailments such as cardiovascular, diabetes and Parkinson. Greater understanding of the stem cell niche, which is the specific microenvironment where stem cells reside and function, is critical for their study and applications. Mimicking the niche in vitro is essential for the propagation of stem cells using traditional static dish cultures and scalable bioreactors [1–5].

Signals from the surrounding milieu include soluble factors (salts, steroids, amino acids, growth factors, etc.), dissolved oxygen, extracellular matrix (ECM) for cell attachment, cell–cell interactions, mechanical forces and the scaffold or microenvironment conformation (i.e. two- (2D) or three-dimensional (3D) architecture) (Fig. 1). In this chapter, these factors are discussed in connection with their effects on hPSC proliferation and differentiation. Such discussion is particularly pertinent to processes for the culture of hPSCs intended for clinical uses. Motivated by the economical production of large quantities of stem cell derivatives, various

Y. Fan · E.S. Tzanakakis (✉)
Department of Chemical and Biological Engineering, Science and Technology Center, Tufts University, 4 Colby St., Medford, MA 02155, USA
e-mail: Emmanuel.Tzanakakis@tufts.edu

Y. Fan
e-mail: yongjia.fan@tufts.edu

D. Jing
Cell & Gene Therapies, TRD, Novartis Institutes of Biomedical Research, 64 Sidney St., Cambridge, MA 02139, USA
e-mail: donghui.jing@novartis.com

E.S. Tzanakakis
Tufts Medical Center, Tufts Clinical and Translational Science Institute, Boston, MA 02111, USA

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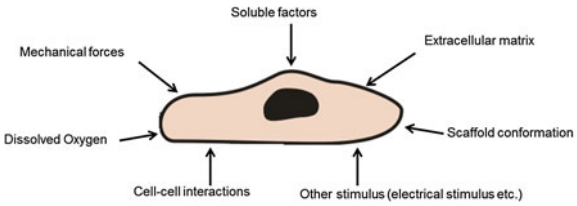

Fig. 1 Schematic representation of the microenvironment encountered by stem cells

platforms have been employed [6–8], for example, stirred suspension bioreactors [3], roller bottle systems [9] and rotating wall bioreactors [10].

2 Effects of Dissolved Oxygen

Oxygen tension is a critical factor of the physiological profile of the stem cell niche directly affecting the growth, viability and differentiation propensity of stem cells. During development, the embryo experiences a hypoxic environment in vivo [11–13] with the O_2 tension for human embryonic stem cells (hESCs) falling in the range of 1–9 % O_2, i.e. significantly lower than the ambient air O_2 fraction (21 %) [14] designated as the normoxic pO_2. Moreover, hESCs cultured under hypoxia (2–5 %) rather than normoxia exhibit reduced spontaneous differentiation and chromosomal abnormalities [15, 16].

Although sensing of O_2 tension and its influence on embryonic development and lineage specification are mediated through various processes, the family of hypoxia-inducible transcriptional factors (HIFs) is considered central to cellular responses based on the microenvironmental O_2 levels. In mouse ESCs (mESCs), HIF-2alpha (but not HIF-1alpha) binds to the promoter region of pluripotency marker gene *Pou5f1* (Oct4) inducing its expression [17]. HIF-1alpha, however, modulates Wnt/β-catenin signaling in mESCs and mouse embryonic carcinoma P19 cells by activating the expression of β-catenin and its downstream effectors LEF-1 and TCF-1 [18]. It should be noted that the effects of pO_2 on stem cell pluripotency or differentiation are species- and context-dependent. For example, hypoxia promotes the undifferentiated state of progenitor cells by blocking neuronal (in mouse neural stem cells) and myogenic (in mouse C2C12 cells) differentiation programs depending on the interaction between HIF-1alpha and the Notch-intracellular domain as shown in P19 cells [19]. Unlike the results in mouse cells, low O_2 tension (1–5 %) favors the expression of genes associated with endothelial differentiation and negative regulation of apoptosis in cultured H9 hESCs [20]. Cells grown at high O_2 tension (21 %) display more changes in genes related to division and O_2-based ATP production. Combined with media inducing differentiation, hypoxia (2 % O_2) promotes the chondrogenic differentiation of hESCs upregulating the production of collagens I & II and glucosaminoglycans [21]. Similarly, the culture of ESCs in 4 % O_2 yields an increased number of cardiac myocytes

(CMs) (3.77 ± 0.13 CMs/ESC) compared to normoxic cultures (2.56 ± 0.11 CMs/ESC) in the presence of appropriate differentiation-inducingfactors [22, 23].

Hypoxia also impacts mesenchymal stem cell (MSC) maintenance and differentiation. The self-renewal capacity of MSC populations is enhanced in hypoxic (rather than normoxic) cultures [24] concomitantly with increases in the expression of growth factors and their respective receptors. These findings suggest that the enhanced growth potential and preserved undifferentiated status can be attributed largely to the O_2-dependent gene expression in MSCs. Consequently, a lower pO_2 environment may facilitate overcoming issues including poor growth kinetics, genetic instability and poor engraftment after transplantation of hMSCs.

Thus, effective control of the pO_2 level in bioreactors becomes critical for stem cell expansion. When cells are cultured at low concentrations, the transfer of O_2 through the liquid surface (termed surface or overlay aeration) is sufficient to match the total cellular uptake rate of O_2. However, aeration through the air-liquid interface can be enhanced by O_2 enrichment of the overhead gas phase or headspace pressurization. Alternatively, the culture medium can be oxygenated via direct sparging, i.e. introducing air bubbles directly into the liquid phase. Sparging provides higher rates of O_2 mass transfer compared to the overlay aeration but may cause foaming or cell damage. This issue can be mitigated with microsparging in which a hydrophobic gas-permeable membrane is employed to provide bubble-free aeration [25]. Overall, overlay or headspace aeration is the most economical and least intrusive method generally used for low cell density/low working volume cultures or for cells exhibiting low O_2 uptake rates. Direct or open-tube sparging is generally preferable when stripping of system CO_2 is desired, whereas microsparging is most effective for cultivation at high cell densities. A schematic illustration of the aforementioned methods for supplying O_2 to cultured cells is shown in Fig. 2.

Different designs of bioreactors, which have been utilized for the culture of stem cells, offer alternatives for the large-scale culture of hPSC products [6–8]. Stirred suspension bioreactors are an appealing choice for large-scale cultures due to the

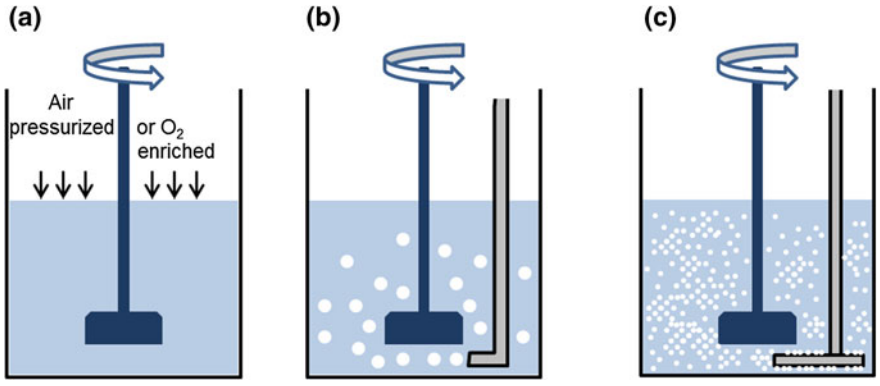

Fig. 2 Supply of O_2 to stirred-suspension bioreactors. **a** Overlay or surface aeration, **b** direct sparging or open-tube sparging, and **c** micro-sparging

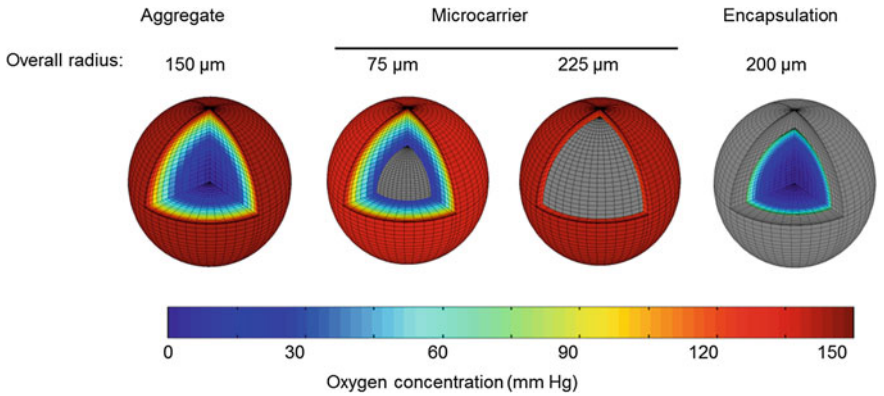

Fig. 3 Comparison of the O_2 diffusion profile within 3D cultures of hPSCs under different modes: aggregate, microcarrier and encapsulation in alginate beads. All models were run with the same volume/number of cells. Color regions represent the O_2 profile of hPSCs and *grey* regions represent different biomaterial positions (microcarriers and alginate capsule). For aggregate cultures, 150 μm hPSC clustered together to form a spherical model. For cell-loaded microcarriers, hPSC grew on the surface of microcarrier and two profiles were shown with microcarrier radii of 75 and 200 μm, respectively. For alginate encapsulation, a 200 μm bead of 1 % (w/v) alginate is shown encapsulating a 150 μm hPSC aggregate. Oxygen diffusion of 1 % alginate and hPSC were taken from Ref. [148]

homogenous environment and ease of operation and monitoring of culture. These bioreactors afford multiple culture modes including the cultivation of cells encapsulated, on microcarriers or as aggregates.

Using mathematical and computational models, the distribution of O_2 can be analyzed and predicted from experimental data. In a recent study, such data were collected from mouse and human ESC aggregates cultured in spinner flasks under different agitation rates [26]. At different time points and ultrastructural attributes (porosity and tortuosity) of aggregates, the effective diffusivity and the specific consumption rate of O_2 were calculated using a transient diffusion-reaction model coupled to a population balance equation (PBE) capturing the dynamics of cell aggregation. The model facilitated the calculation of the O_2 distribution in the medium and within the aggregates in spinner flasks. As a result, not only the fraction of cells experiencing hypoxia was predicted but also the 'residence time', i.e. the duration the cells experience O_2 concentrations within a particular range.

The availability of O_2 and nutrients to stem cells also varies depending on the culture mode (Fig. 3). Stem cells residing near the center of aggregates may experience hypoxia directly affecting their viability. The spatial gradient of O_2 can impact the proliferation and differentiation propensity of stem cells [27, 28] and their encapsulation (e.g. in alginate beads) poses an additional barrier to O_2 transport [29] reducing proliferation beyond the effect of scaffold rigidity. In contrast, cells grown on microcarriers experience O_2 and nutrient levels close to those in the medium bulk. Among different size microcarriers [30–32], those with a diameter of $\sim$200 μm expose cells to higher O_2 levels compared to those with a

size of 75 μm according to model prediction [26]. This is because stem cells on the microcarrier surface are assumed to be configured akin to cells in monolayers. In practice, cell-laden microcarriers form agglomerates posing additional restrictions to the exchange with the medium of O_2, nutrients and secreted molecules. Therefore, the culture configuration is a critical factor determining stem cell fate in addition to the chemical and biological properties of the scaffolds employed.

In conclusion, dissolved O_2 is a culture parameter affecting both stem cell growth and fate decision. Insufficient O_2 transfer can be detrimental by resulting in delayed growth rate and apoptosis. On the other hand, increased or uncontrolled O_2 supply may lead to commitment along undesirable lineages making obvious the need for fine tuning and monitoring oxygenation throughout the entire culture process.

3 Soluble Factors and the Stem Cell Niche

Soluble factors including proteins, salts, lipids, vitamins, cytokines and other small molecules play critical roles in maintaining the undifferentiated state of stem cells as well as guiding their lineage commitment. Soluble factors trigger cellular responses through multiple signaling pathways targeting gene networks which regulate the fate of stem cells [33, 34]. The transforming growth factor-beta (TGF-β) super family-activated cascades, receptor tyrosine kinase (RTK) signaling, canonical Wnt signaling [35, 36], and pathways activated by insulin or insulin-like growth factors (IGFs) [37, 38] participate in directing stem cell fate. Targeted gene networks include transcriptional factors [35, 39, 40], such as Nanog, Oct4 and Sox2 [39, 41, 42]. There is also a divergence in the cascades maintaining the pluripotency of mESCs and hESCs. Bone morphogenetic proteins (e.g. BMP4) and the JAK/STAT signaling activator, leukemia inhibitory factor (LIF), are sufficient to sustain the pluripotent state of mESCs but not of hESCs in vitro [43–46]. Instead, TGFβ signaling is important for preserving hPSC pluripotency [47–49].

Besides TGFβ signaling, basic fibroblast growth factor (bFGF) (RTK-type) signaling is another important pathway for hESC self-renewal. Basic FGF is a universal supplement in media for routine maintenance of hPSCs regardless of the use of feeder cells or serum [50, 51]. For hPSCs cultured on mouse embryonic fibroblasts (mEFs) [52] or in mEF-conditioned medium [53], the bFGF requirement (4 ng/ml) is lower than for feeder-free cultures (40–100 ng/ml) [50, 54, 55].

The roles of Wnt/β-catenin and BMP signaling have also been studied in sustaining hPSC self-renewal [56, 57]. Recombinant Wnt3a does not appear to suffice for the maintenance of undifferentiated hESCs without feeder cells [58] although caution should be exercised about the requirement for Wnt ligand supplementation given the disparate levels of endogenous canonical Wnt signaling among hPSC lines. The BMP antagonist noggin on the other hand, supports the uncommitted hESCs in non-conditioned medium containing 40 ng/ml bFGF but this effect is abolished when bFGF is supplemented at 100 ng/ml [59].

In the early days of hESC culture, those critical factors were supplemented with the addition of fetal bovine serum (FBS) or knockout serum replacer (KSR) to the medium [60]. However, the presence of undefined, non-human components (e.g. Neu5Gc [61]) in these supplements is not desirable for clinical applications and has motivated efforts toward the design of xeno-free systems for the culture of hPSCs and their products. The development of chemically defined media requires scrutiny of the stem cell niche for the identification of core elements stimulating and maintaining the propagation of stem cells in vitro [33, 62–64]. Basal media (e.g. DMEM or DMEM/F12) serve as sources of glucose, vitamins and salts at appropriate osmolarity for cell survival and proliferation. Growth factors specific for stem cell self-renewal are typically supplemented separately to the basal medium. For example, defined media consisting of DMEM/F12, 100 ng/ml bFGF and components such as TGF-β1, LiCl, insulin, gamma-aminobutyric acid (GABA) and BSA or human serum albumin (HSA) are routinely used for hPSC maintenance in vitro both in dishes and scalable stirred-suspension vessels [65, 66].

Despite the significant advances in designing and developing fully defined xeno-free media for stem cell cultivation, significant issues still remain. Almost all media for hPSC culture currently in use require daily exchanges which are costly and labor intensive. Even with frequent replacement, fluctuation of growth factor levels is unavoidable especially given the half-life of ligands in cultures. For instance, the human or zebrafish bFGF loses more than 40 % of its activity within 24 h [67]. This introduces variability to the culture impacting adversely stem cell proliferation and performance. A proposed solution to this problem is the incorporation of controlled release vehicles in the culture system facilitating the extension of growth factor or cytokine availability (and degradation) in the culture. Basic FGF-loaded PLGA microspheres can be added to the hPSC cultures reducing the frequency of medium changes from daily to every three days or biweekly [68]. Moreover, to deal with the labile nature of stem cell medium supplements and their high cost, researchers have turned to small molecules displaying similar bioactivity to native or recombinant proteins. Trimipramine and ethopropazine are two examples of small molecules with longer degradation times than bFGF and supporting hESC self-renewal in lieu of exogenously added bFGF [69, 70].

4 Extracellular Matrices for Stem Cell Cultivation

Extracellular matrix (ECM) proteins such as laminin, fibronectin, vitronectin, entactin, tenascin and collagen are critical for cell adhesion, survival, growth and differentiation [71]. Distinct domains on these molecules interact with cell surface receptors (e.g. integrins) mediating adhesion and triggering signaling cascades linked to cell fate adoption processes [72, 73].

Since first isolated, hESCs have been co-cultured with layers of mEFs which secrete various (mostly undefined) factors supporting the pluripotency of hESCs. Those cells include human fetal foreskin fibroblasts [74–77], adult epithelial cells

[78], bone marrow cells [79, 80] and placenta-derived feeder cells [81, 82]. Matrigel, which is an ECM mixture produced by Engelbreth-Holm-Swarm mouse sarcoma cells, was introduced and served as an alternative for the feeder-free maintenance of stem cells. Matrigel contains various ECMs such as laminin, collagen type IV, heparan sulfate, proteoglycans, entactin, and nidogen [45, 83], and its use in hPSC cultures is straightforward compared to feeders. However, its composition remains undefined and variable between batches paralleling issues plaguing the use of mEFs.

Whether particular ECM molecules support cultured stem cells has been the focus of multiple published studies. The arginine-glycine-aspartic acid ('RGD') motif featured in various ECM proteins (e.g. laminin, vitronectin, fibronectin [84–86]) is a binding domain for cellular integrins. A mixture of vitronectin, fibronectin laminin and recombinant human collagen IV was demonstrated to promote the growth of hESCs over multiple passages [65]. However, the capacity of individual ECM proteins to support hPSC adhesion and growth is variable and highly dependent on the culture medium utilized. For instance, laminin binds to at least 8 integrin heterodimers including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$, $\alpha7\beta1$, $\alpha9\beta1$, and $\alpha v\beta3$ [87]. Among those, $\alpha6\beta1$ is expressed in hESCs and is significant for their adhesion [83]. Indeed, natural or recombinant laminin maintains the growth and pluripotency of hESCs in mEF-conditioned medium [83, 88]. However, human placenta-derived laminin does not support hESC self-renewal beyond 3 passages in medium without serum or serum replacer [89]. Over a longer term (>10 passages), laminin failed to maintain the undifferentiated state of hESCs, which displayed reduced proliferation, widespread spontaneous differentiation and poor adhesion [90]. Similar to laminin, vitronectin and fibronectin mediate cell adhesion through the binding of integrins such as $\alpha_v\beta_5$, $\alpha_5\beta_1$ $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ [90–92]. Vitronectin from human plasma supports the growth and self-renewal of hESCs for over 20 passages without compromising their differentiation potential [93]. A chimeric glycoprotein based on vitronectin was shown to support the growth of undifferentiated hESCs in defined medium [73]. Conversely, hESCs could not be maintained on vitronectin for more than 7 days in defined medium [94]. Fibronectin isolated from human plasma promotes hESC proliferation and pluripotency in defined medium for more than 10 passages [91, 94] but others were unable to grow hESCs on fibronectin-coated surfaces in the absence of mEF-conditioned medium [90]. The discrepancies in the reported results may be due to the differences in cell lines, culture media and the length of culture (e.g. number of passages, time between cell splittings etc.). These differences also highlight the complexity of individual ECM components and their roles in supporting hPSCs in culture. They further emphasize the importance of considering multiple aspects of the culture system including the medium used.

Due to the varied performance of natural or recombinant ECM proteins as hPSC culture matrices and their high cost, efforts have been directed toward the development of synthetic ECMs. One strategy is to synthesize substrate peptides featuring known binding domain motifs such as the RGD sequence [95, 96]. Synthetic peptide sequences derived from natural ECMs like fibronectin, bone sialoprotein and vitronectin have been covalently attached onto acrylate-coated surfaces for

stem cell attachment in both feeder cell-conditioned (10 passages) and defined media (at least 5 day) [97, 98]. Synthetic peptides with binding domains require additional optimization and testing since not all resulting sequences are suitable for stem cell culture.

Besides synthetic peptides, synthetic polymers have also been investigated for hPSC maintenance due to their lower cost and higher availability compared to other alternatives [99, 100]. For example, poly(methyl vinyl ether-*alt*-maleic anhydride) [PMVE-*alt*-MA] was shown to support the long-term propagation without differentiation of three hPSC lines for five passages. Screening of 91 different polyacrylamide polymers yielded 16 candidates supporting hESC proliferation in 5-day cultures [100]. Polymers with ester ions and cyclic polymer ions were also demonstrated to promote hPSC adhesion [101].

Flat (2D) stem cell cultures afford convenience but 3D configurations mimic more closely the natural niches of stem/progenitor cells. To that end substrates which can be used with 3D hPSC culture systems are highly desirable. Hydrogels are commonly used to create 3D microenvironments in vitro. For example, scaffolds of 2.4 % (w/v) alginate and 2.4 % (w/v) chitosan prepared by lyophilization can be utilized to maintain hESCs over 21 days [102]. Indeed, scaffolds of alginate and chitin support various ESC lines for more than 10 passages [103] and alginate alone has been used for creating 3D niches for stem cell differentiation [104, 105]. Additionally, incorporation of poly(γ-glutamic acid) [γ-PGA] in alginate capsules promotes neural differentiation [106].

Hydrogels based on poly(N-isopropylacrylamide-co-acrylic acid) [p(NIPAA-co-AAc)] and Gln-Pro-Gln-Gly-Leu-Ala-Lys also support hESC growth [107]. This polymer can be degraded by collagenase facilitating downstream separation of the cells from the matrix. Hyaluronic acid, which is present during early embryo development, is used to prepare hydrogels for hESC proliferation and differentiation [108].

In addition to the composition, scaffold ultrastructure affects stem cell proliferation and differentiation. Beside gel matrices, fibrous scaffolds have also been reported to support the proliferation of stem cells. A fibrous scaffolds made from poly(desaminotyrosyl tyrosine ethyl ester carbonate) [pDTEc] and coated with poly-D-lysine was suitable for maintaining cultured hESCs for 14 days [109]. Poly (methacrylic acid)-coated carbon nanotubes, which are similar in scale to collagen and laminin moieties, also reportedly promote hESC proliferation and neuronal differentiation [110, 111].

5 Mechanotransduction and Stem Cells

Cells in the human body are constantly exposed and respond to mechanical forces and during development mechanotransduction influences differentiation and tissue morphogenesis. Similar observations are noted in vitro with external mechanical and electrical stimuli modulating the morphology, proliferation and specification of stem cells [112–114]. For example, cyclic strain not only inhibits the proliferation

of bone-marrow derived progenitor cells but induces the alignment of the F-actin cytoskeleton perpendicularly to the strain direction [113]. Under 10 % continuous cyclic strain (0.5 Hz) for 7 and 14 days, human intraoral mesenchymal stem and progenitor cells undergo osteogenic differentiation expressing markers such as type-I collagen (Col1A1), osteonectin (SPARC), bone morphogenetic protein 2 (BMP2), osteopontin (SPP1), and osteocalcin (BGLAP). Furthermore, significantly higher amounts of calcium and alkaline phosphatase (ALP) are observed in mechanically stimulated groups of cultured cells [115]. In contrast, commitment of MSCs under mechanical compressive force toward adipose cells is inhibited [116].

Apparently, the pattern of exerted mechanical forces is also important for stem cell differentiation as demonstrated by Park and co-workers [117]. The differentiation of MSCs toward vascular smooth muscle cells (SMCs) was reportedly promoted by cyclic uniaxial strain but inhibited by equiaxial strain based on the expression level of smooth muscle alpha-actin (SM α-actin) and SM 22α (a calponin-related protein). Moreover, transient expression of collagen I increased under uniaxial but not equiaxial strain. Cyclic strain also induces vascular smooth muscle cell differentiation of mESCs with the resulting cells orienting perpendicularly to the direction of strain. The authors attributed the differentiation to the activation by cyclic strain of the beta-type PDGF receptor (PDGFRB) in a ligand-independent manner. In hESCs, the tendon-specific transcription factor scleraxis (SCXA and SCXB) and mechanical stimulation synergistically promote commitment to tenocytes. This is achieved by inhibition of the osteogenic differentiation of hESC-derived MSCs through the antagonizing BMP signaling pathway [118].

The above reports illustrate that the effects of mechanical stimulation on the differentiation and proliferation of stem cells are not universal but depend on the lineage, environment, duration and magnitude of the strain and even the direction of the causative force(s). For example, in a bioreactorsystem, which was customized to study cell responses upon cyclic compressive strain in a hydrogel scaffold [119], bone marrow hMSCs expressed chondrocytic genes within 3 weeks of culture. However, under the same conditions, chondrocytic gene expression of hESC-derived cells in EBs was significantly down-regulated. After initiation of the chondrogenic differentiation, this reduction in gene expression was reversed with the addition of TGF-β1.

Moreover, Nanog in mouse ESCs is significantly downregulated while endoderm markers emerge after 2 days of exposure to cyclic stretch [120]. Unlike for mESCs however, cyclic strain appears to support the pluripotent state of hESCs. The percentage of SSEA-4^+ cells at 10 % strain of 30 cycles per minute (0.5 Hz) is reduced from 85 to 36 % with the reduction of strain to 8 % at 0.167 Hz [121]. Similarly, the fraction of $Oct4^+$ cells went from 21 % without strain to 67 % after almost 2 weeks of strain [122]. More recently, iPSCs subjected to cyclic strain showed enhanced formation of stress fibers and downregulation of Nanog, Oct4 and Sox2 [123]. These findings support the notion that hPSC self-renewal and differentiation are influenced by both mechanical forces and chemical signals. From a signal transduction viewpoint, the Rho/ROCK is a primary transducer of the effects of mechanical forces in pluripotent stem cells and acts as an upstream regulator in

pluripotency-related signaling pathways as suggested by the activation of small GTPase Rho and decreased AKT phosphorylation. The pluripotency of stem cells seems to be maintained by extended cyclic strain and this effect is reversed with the short application of higher-magnitude strain.

Application of mechanical forces to induce stem cells differentiation, for example to cardiovascular cells, has been reported. When sheep bone-marrow derived MSCs seeded onto a novel flex-stretch-flow (FSF) bioreactor, more extensive heart valve tissue formation was observed under flex-flow conditions (combined cyclic flexure and laminar flow) compared to cells subjected to cyclic flexure, laminar flow or typical static culture [124]. Others also showed that human MSCs in biaxial rotating bioreactors (BXR) have higher cellularity, confluence and more robust osteogenic differentiation than cells in spinner flasks, perfusion or rotating wall bioreactors [125].

Mechanical stimulation is caused by fluid shear stress due to agitation in stirred tank bioreactors and can potentially affect stem cell fate decisions. Although agitation is critical for ensuring a homogenous environment, a high stirring rate may result in greater shear stress to the cells. A window of acceptable agitation speeds for an operating bioreactor can be calculated based on the specific energy dissipation rate:

$$\tau\gamma = \frac{P}{V} \tag{1}$$

where γ is the average shear rate (s^{-1}) and τ is the shear stress (Pa), P is the power input (W) and V is the volume of the fluid in the vessel (m^3). The power input P is the amount of energy provided to the impeller for the rotational mixing.

By introducing the fluid's viscosity, μ ($=\frac{\tau}{\gamma}$; Pa s), the above equation yields

$$\gamma = \left(\frac{1}{\mu} \times \frac{P}{V}\right)^{0.5} \tag{2}$$

Theoretically, the average shear rate depends on the working volume in bioreactors, the viscosity of the fluid, and the power input. Several empirical equations to calculate the shear rate γ and its maximum, γ_{max}, in the impeller zone of stirred tank bioreactors are summarized by Sánchez Pérez et al. [126]. The average shear rate in Newtonian and non-Newtonian media in a stirred tank is proportional to the impeller speed N for laminar flow or $N^{3/2}$ for turbulent flow.

For bioreactor scale-up, the power input per volume (P/V) is kept fixed and calculated as:

$$\frac{P}{V} = q\rho N^3 D^5 / V \tag{3}$$

where q represents the power number. This is a property of the impeller and is generally supplied by the manufacturer. The density ρ of the fluid and the diameter

D of the impeller are also utilized in the calculations. The agitation rate after scaling up the volume of a bioreactorcan be calculated while keeping P/V constant. For example, when the working volume within one bioreactor is increased from V_1 to V_2, then agitation rate can be increased from N_1 to N_2:

$$N_2 = \left(N_1^3 V_2 / V_1\right)^{1/3} \tag{4}$$

Even if bioreactors with different impeller designs are used within a particular process, the media within both vessels can experience the same P/V. As long as the power numbers q of both bioreactors are known, the agitation rates can be determined based on Eq. 3.

In a fluid flow bioreactor, Wolfe et al. [127] demonstrated the application of steady laminar shear force in the range of 1.5–15 dynes/cm^2 to mESCs. Specification to ectodermal and mesodermal lineages depended on the magnitude of the applied force. The upregulated expression of Brachyury (T) and corresponding reduction of alpha-fetoprotein (AFP) corresponded to the increase of shear stress for 1–4 days. These changes transpired concurrently with the activation of Wnt and estrogen signaling pathways. The same group later reported that during early mESC differentiation fluid shear similarly promotes endothelial and hematopoietic differentiation for cells seeded on collagen-, fibronectin- or laminin-coated surfaces. The induction of endothelial differentiation was apparent by increasing the duration of culture under stress but hematopoiesis was less efficient at later stages. It was suggested that the membrane protein FLK1 (a VEGF receptor) is a critical regulator of fluid shear stress-induced differentiation to endothelial and hematopoietic linages [128].

6 Effects of Electrical Stimulation on Differentiating Stem Cells

Electrical stimulation has been shown to have beneficial effects for progenitor cells differentiating toward electrically active cell types including neurons and heart cells. To that end, culture (mainly 2D) of human cardiomyocyte progenitor cells has been combined with electrical stimulation leading to a significant increase in the expression of markers such as GATA4, MEF2A, structural protein genes and those related to Ca^{+2} handling [129]. Electrically stimulated adult neural stem progenitor cells give rise to neurites which are five times longer (up to 600 μm) compared to those from non-stimulated cells. Moreover, the cells display mature neuronal morphologies, expression of β-III tubulin, NeuN, organized filamentous actin (F-actin) and intracellular Ca^{2+} signaling akin to native cells [130].

Electrical stimulation in bioreactors combined with nutrient perfusion and unconstrained tissue contraction was also reported [131]. In this study, neonatal rat cardiac cells were seeded in a scaffold placed in a bioreactor with the simultaneous application of electrical stimulation and perfusion. The stimulated culture showed

improved function, expression of cardiac proteins, cell distribution and overall tissue organization over control (unstimulated) groups. Similar improvement through electrical and mechanical stimulations was also reported by Miklas et al. [132] using a microfluidic bioreactor with neonatal rat cardiomyocytes. These results indicate that elevated amplitude of contraction and improved sarcomere structure are observed in cells exposed to electrical and mechanical stimulations concurrently versus cells subjected to electrical or mechanical stimulation alone.

7 Microfluidic Technologies for Studying the Stem Cell Niche

The emergence of microfluidic technologies has opened new avenues for studying the stem cell niche. Microfluidic devices featuring patterns of tens to hundreds of micro-scale channels on customizable substrates and accessible for observation, for example, by fluorescence microscopy, allow the culture and real-time monitoring of stem cells as they proliferate and differentiate. Moreover, laminar flow profiles with well-defined and controlled dynamics can be achieved in conjunction with diffusive mixing for studying stem cell interactions in the niche [133, 134].

The miniaturized size and minute amounts of reagents required have made microfluidic platforms the tool of choice for high-throughput assays, including those for investigation of the stem cell microenvironment. Beyond screening a wide range of conditions, microfluidic devices afford greater flexibility versus traditional dish cultures as perfusion and 3D culture conditions can be incorporated [135–140]. A high-throughput microfluidic device featuring 1600 culture chambers of 4.1 nL each, was utilized for investigating the heterogeneity exhibited by hematopoietic stem cell (HSC) populations. For this purpose, proliferation was tracked at a single-cell level with dynamic medium exchange [141]. Furthermore, Steel factor (SF) was shown to regulate the survival of cytokine-activated HSCs within 16 and 24 h of being placed in vitro without an effect on the early division kinetics of the surviving cells. These results point to a regulatory role of SF when HSCs exit the G_0 phase to enter G_1. Beyond its suitability for high-throughput experimentation, the system allows medium replacement without disturbing cultured cells and makes possible the rapid and accurate generation of colony growth curves. Similarly, a microfluidic system with 96 chambers was engineered to study the effects of various combinations of cell seeding density, medium composition and feeding schedule on hMSC proliferation and osteogenic differentiation [142]. Cells treated with differentiation medium for 18 h or longer displayed significantly different motility compared to nonstimulated cells. In fact, cells stimulated for less than 96 h progressively adopted the same level of motility after stimulation ended as control cells indicating that the effects of exposure to osteogenic medium were reversible. Such studies would have been impractical to carry out in dishes or regular-scale bioreactors.

Microfluidic environments can also mimic aspects of the complex microenvironment of stem cells. Based on an Y-channel geometry, two streams of different media were combined directed toward single mouse embryoid bodies (EBs) located at the point of convergence of the channels. Cells on the EB side facing the combined streams were coaxed to neural fates but the other half of the EB remained undifferentiated [143] demonstrating that cell commitment may be controlled by simple changes in the flow of media containing differentiation cues.

In fact, microfluidic devices can be used for generating flows with a high Péclet number at low Reynolds regimes forming continuous gradients of soluble factors by diffusive mixing [144]. For example, human neural stem cell (hNSC) differentiation was studied in a microfluidic device in which concentration gradients were produced of three growth factors: epidermal growth factor (EGF), bFGF and PDGF. Cells were exposed to these gradients under continuous flow for more than 1 week. Astrocyte differentiation of the hNSCs was proportional to growth factor gradients without any threshold effects. The setup can be used to quantify the graded responses of cells to multiple concentration gradients of differentiation cues within a single chamber [145].

Given their small scale and high degree of control, microfluidic platforms are important tools for detailed studies of the stem cellniche. Outcomes from these studies can contribute to the development of strategies for the economical and effective culture of large quantities of stem cells. However, translation of findings from nano- or micro-liter scale systems to bioreactors with at least 6 orders of magnitude greater working volumes should be done with caution.

8 Conclusions

The stem cell niche comprises a complex assortment of cues driving proliferation and fate selection processes. Mimicking this microenvironment in different culture systems is a challenging task hampered not only by the large variety of growth factors and cytokines involved (with multiple remaining still elusive) but also by the combinatorial effects of their synergistic activities. Moreover, there are fewer stem cell cultivation platforms affording dynamic (as opposed to static) environments akin to those which the cells experience during embryonic development. To that end, continuous flow microbioreactor arrays may be a promising candidate system as these afford the application of statistical factorial multiplexing of multiple input factors involved in stem cell signaling hierarchies through high throughput culture chambers [146, 147]. The design and optimization of a relatively complex 3D culture system will require the systematic synthesis of the parameters and factors many of which have been summarized here. The progress noted to date in this area is deemed very promising for the development of fully automated, robust and scalable processes for the production either in the laboratory or commercially of stem cell products for regenerative medicine, tissue engineering, and drug discovery.

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