Biophysics and dynamics of natural and engineered stem cell microenvironments

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Stem cells are defined by their ability to self-renew and to differentiate into one or more mature lineages, and they reside within natural niches in many types of adult and embryonic tissues that present them with complex signals to regulate these two hallmark properties. The diverse nature of these *in vivo* microenvironments raises important questions about the microenvironmental cues regulating stem cell plasticity, and the stem cell field has built a strong foundation of knowledge on the biochemical identities and regulatory effects of the soluble, cellular, and extracellular matrix factors surrounding stem cells through the isolation and culture of stem cells *in vitro* within microenvironments that, in effect, emulate the properties of the natural niche. Recent work, however, has expanded the field's perspective to include biophysical and dynamic characteristics of the microenvironment. These include biomechanical characteristics such as elastic modulus, shear force, and cyclic strain; architectural properties such as geometry, topography, and dimensionality; and dynamic structures and ligand profiles. We will review how these microenvironmental characteristics have been shown to regulate stem cell fate and discuss future research directions that may help expand our current understanding of stem cell biology and aid its application to regenerative medicine. © 2009 John Wiley & Sons, Inc. *WIREs Syst Biol Med* 2010 2 49–64

Stem cells are defined by their ability to self-renew and to differentiate into one or more mature lineages. In the early 1960s, researchers published the first evidence of stem cells in the hematopoietic system[1–3] and the central nervous system,[4] and stem cells have since been discovered in and isolated from many adult[5–7] and embryonic[8–10] tissues. The diverse origins and unique properties of stem cells raises the relevance of a concept first proposed 30 years ago that tissues house stem cells within specific locales, or 'niches', that uniquely support stem cell homeostasis.[11] Considerable insight into extracellular regulators of stem cell behavior has since been gained from careful study of this niche or microenvironment, that is, the milieu of cells, proteins, and other factors that surrounds stem cells and provides them with the regulatory signals that control their function. In particular, two distinct but complementary approaches have proven critical to building our understanding of how niches control cell behavior: reductionist approaches to identify individual components of complex *in vivo* niches and the subsequent controlled reconstitution or engineering of these components into 'synthetic' microenvironments *in vitro*.

Although *in vivo* and *in vitro* studies differ significantly in the experimental methods used to analyze the cellular microenvironment, they have traditionally shared a common focus on the biochemical identities and properties of the cellular, soluble, and extracellular matrix (ECM) signaling

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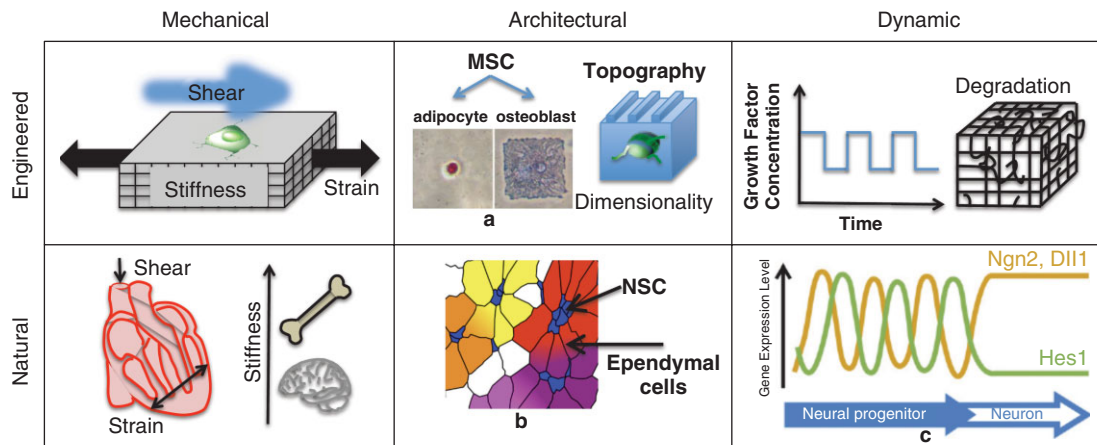
FIGURE 1 | Biophysical and dynamic characteristics of natural and engineered microenvironments regulate stem cell fate. Biomechanical characteristics such as shear, strain, and stiffness are found in diverse natural microenvironments including the heart, bone, and brain and can be recapitulated in engineered systems. In addition, unique pinwheel architectures exist in NSC niches in the ventricular zone of the brain while cellular geometry controls MSC differentiation into adipocytes and osteoblasts on small and large adhesive islands. Engineered microenvironments can also be designed with topographical and dimensional cues. Lastly, dynamic presentation of mitogens and morphogens or degradation of ECM can regulate stem cell behavior. For example, oscillations in NSC expression of neurogenic factors like Dll1 (Delta-like 1, a Notch ligand) maintain stem cell plasticity. ((a) Reprinted with permission from Ref 121. Copyright 2004 Elsevier. (b) Reprinted with permission from Ref 118. Copyright 2008 Elsevier. (c) Reprinted with permission from Ref 153. Copyright 2008 Elsevier).

factors surrounding stem cells. For example, it is well established that exposure to specific mitogens and morphogens,[12–15] as well as cell–cell[12,16,17] and cell–ECM[18,19] adhesion, regulate stem cell behavior in both natural and engineered microenvironments. These inputs have been comprehensively reviewed elsewhere.[20–32] More recently, however, the field has begun to appreciate that stem cell microenvironments also present specific *biophysical* cues that strongly influence stem cell behavior. For example, stiffness (elastic modulus) varies widely both between different tissues[33] and within individual tissues,[34] and the resulting diversity of mechanobiological inputs may be an important component of the stem cell niche. Similarly, the spatially inhomogeneous presentation of extracellular ligands and surrounding cells contributes to the induction and maintenance of cell and tissue polarity,[35] which has been shown to be relevant to cell division,[36] cell homeostasis and tumorigenesis,[37] and differential segregation of stem cell fate determinants to daughter cells.[38] Finally, the role of temporally dynamic signaling is already well recognized in developmental biology, as distinct morphogen gradients regulate tissue patterning at different stages of development. However, recent work has also shown that cells in general and stem cells in particular respond not only to static concentrations and gradients, but can also be strongly influenced by exposure to temporally evolving ligand fields.[39,40] In addition, improved imaging technologies have allowed observations of intracellular signaling fluctuations at the time scale of minutes and seconds,[41] providing further evidence that cells can track and respond to these temporally encoded signals. Here, we review the importance of these three emerging cues—biomechanics, diverse spatial architectures, and temporally dynamic structures and signals—to the regulation of stem cell behavior within both *in vivo* and engineered *in vitro* microenvironments (Figure 1).

BIOMECHANICS

Both developing and mature tissues experience a wide variety of mechanical forces, which can profoundly influence the physiology of their constituent cells. These forces are common in developmental processes requiring cellular migration and reorganization, such as gastrulation, where stem cells play a central role.[42–44] Likewise, in mature tissues, compressive impacts, muscle stretching, the movement of joints, and pulsatile blood flow are just a few examples of processes that subject cells to force, require cells to generate force, or both. For this reason, the biomechanical environment has been explored as a potential regulatory component of the stem cell niche.

TABLE 1 | The Linear Elastic Modulus or Stiffness of Mammalian Tissues Spans Over Three Orders of Magnitude, Suggesting the Biomechanical Nature of the Microenvironment May Regulate Stem Cell Fate

Tissue	Stiffness/Elastic Modulus (Pa)
Fat	17[a]
Mammary Gland	167[b]
Brain	137–786[c]
Liver	640[d]
Kidney	7500[e]
Skeletal muscle	12,000[f]
Cartilage	949,000[g]
Bone	$4-400 \times 10^6$[h]

All measurements made in compression unless otherwise indicated below.
[a]Wellman et al. 1999 Harvard BioRobotics Laboratory Technical Report.
[b]Paszek et al. 2005 Cancer Cell.
[c]Elkin et al. 2007 Journal of Neurotrauma. Gefen et al. 2003 Journal of Neurotrauma (shear).
[d]Yeh et al. 2002 Ultrasound in Medicine & Biology.
[e]Nasseri et al. 2002 Rheologica Acta.
[f]Engler et al. 2004 Journal of Cell Biology.
[g]Freed et al. 1997 PNAS.
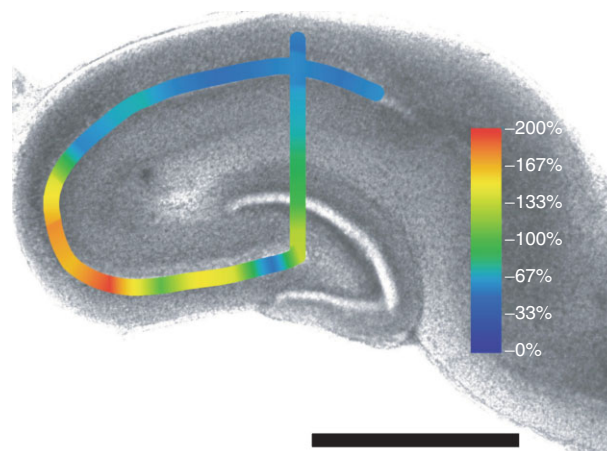[h]Goldstein et al. 1983 Journal of Biomechanics (shear).

FIGURE 2 | Elastic modulus variation within a single tissue. The hippocampus of rat brain exhibits heterogeneous elastic modulus ranging from ∼100 to ∼300 Pa (0% and 200% on the normalized color intensity scale bar, respectively). Stem cells exist in the subgranular zone of the dentate gyrus, a region of the hippocampus, and experience a specific microenvironmental stiffness. Scale bar is 1 mm. Reprinted from Elkin BS, Azeloglu EU, Costa KD, Morrison B. Mechanical heterogeneity of the rat hippocampus measured by atomic force microscope indentation. (Reprinted with permission. Copyright 2007 Mary Ann Liebert, Inc. Publishers).

Elastic Modulus

The elastic modulus of a material refers to the amount of force per unit area (stress) needed to deform the material by a given fractional amount (strain) without any permanent deformation (i.e., elastic deformation). The elastic modulus is therefore a measure of material stiffness, with a high elastic modulus corresponding to high stiffness and low deformability. The linear elastic modulus of adult tissues spans over four orders of magnitude,[33] from < 1 kPa (i.e., 1000 N/m^2) for brain,[34,45] fat,[46] and mammary tissue,[47] to 12 kPa for skeletal muscle,[48] 950 kPa for cartilage,[49] and 10 MPa for bone[50] (Table 1). Even tissues or organs that appear grossly mechanically homogeneous, such as brain (Figure 2), can contain over twofold internal stiffness variations.[34] Developmental germ layers of embryos also have different stiffnesses, in part driving their relative organization.[51] It is, therefore, likely that stem cells naturally exist in microenvironments of diverse stiffnesses both during development and into adulthood.

In addition, aging and disease processes involve profound changes in tissue stiffness, implying that stem cells in those tissues may experience varying mechanical inputs throughout adulthood. For example, although adult braincases are thicker and structurally more rigid than early postnatal ones, postnatal brains are actually stiffer than adult brains (∼800 Pa vs. 500 Pa, respectively), implying that

neural stem cells (NSCs) may experience different stiffness microenvironments at different ages.[45] Furthermore, there is evidence that vasculature stiffens with age, potentially mechanically altering stem cell microenvironments associated with vasculature throughout the body.[52,53] The mechanical microenvironment of specific tissues may also become altered in disease states,[48] such as glial scarring,[54,55] myocardial infarction,[56–59] muscular dystrophy,[60] liver fibrosis,[61–64] and cancer.[47,65] The findings that stiffness can vary widely both between different tissues and within tissues, in normal versus diseased tissues, and as a function of time during development and aging provide a strong rationale for exploring biomechanical regulation of cell and stem cell function. It is difficult to manipulate stiffness independently *in vivo*; however, engineered microenvironments can be used to rigorously study the effects of stiffness on stem cell fate *in vitro*. In fact, engineered microenvironments have been used to demonstrate that substrate stiffness regulates the growth, survival, and motility of many differentiated cell types,[33,66] and analogous approaches have been increasingly applied to stem cells.

In a landmark study, Engler et al.[67] demonstrated that, in combination with soluble cues, culturing human mesenchymal stem cells (MSCs) on

polyacrylamide gels mimicking the stiffnesses of neural, muscle, and bone tissues could induce differentiation into these respective cell types. They then showed that the ability of MSCs to sense ECM stiffness depended on nonmuscle myosin II, a motor protein that controls contractility and tension in the actin cytoskeleton. Winer et al.[68] further showed that MSCs remain quiescent on soft ECMs that mimic fat and bone marrow tissue stiffness, whereas they proliferate on stiff ECMs. Stem and progenitor cells also responded differently to matrix stiffness depending on their differentiation stage, with multipotent MSCs proliferating at similar rates on varying substrate stiffnesses but partially committed pre-osteoblastic cells proliferating at higher rates on stiffer substrates.[69] The possible mechanosensory mechanisms responsible for these substrate stiffness effects currently include nonmuscle myosin II, as mentioned above, as well as Ca^{2+} signaling. In particular, Kim et al.[70] showed that decreasing substrate stiffness decreases Ca^{2+} signaling in a RhoA/ROCK-dependent manner in hMSCs. These studies collectively show that both the defining properties of stem cells, self-renewal and differentiation into mature lineages, can be modulated by microenvironmental stiffness.

Recently, ECM elasticity has also been shown to regulate differentiation trajectories of other tissue-specific stem cells. In particular, the physiology of adult NSCs was shown to be jointly regulated *in vitro* by soluble factors and ECM stiffness. NSCs exposed to soluble factors that induce a neuronal fate achieved optimal differentiation on ECM stiffnesses mimicking that of brain tissue (~500 Pa). In addition, when the cells were given a choice to differentiate into neurons or astrocytes, ECM stiffness strongly shifted this choice from >50% astrocytes and <40% neurons on hard surfaces (10 kPa) to >90% neurons and <10% astrocytes on soft surfaces (10 Pa).[71] Stiffness has also been shown to promote differentiation of more committed progenitor cell types. Engler et al.[48] found that myoblasts formed actin/myosin striations only on stiffnesses near that of normal muscle (~12 kPa). In addition, embryonic cardiomyocytes beat optimally on two-dimensional substrates with stiffnesses similar to that of heart tissue, whereas stiffer, scar-like stiffnesses abolished both myofibrillogenesis and beating.[72] A similar trend is observed in three-dimensional cultures, where cardiomyocyte contractions are greater in amplitude and have greater synchrony with adjacent cardiomyocytes in softer gels compared with stiffer gels (25 Pa vs. 300 Pa).[73] Finally, the maintenance of appropriate mechanical inputs from the ECM can be required for preserving the differentiated state of lineage-committed cells. For example, mammary epithelial cells cultured on top of soft (~100 Pa) biomimetic substrates maintained the expression of β-casein, a milk protein. In contrast, substrates stiffer than normal mammary tissue reduced β-casein expression, a sign of dedifferentiation and possibly tumorigenesis.[74] These studies collectively highlight the potential value of matching matrix stiffness to that of target tissues to steer differentiation of stem and progenitors cells to desired lineages and to optimize their resulting function.

Shear Stress

Shear stress, the tangential force per unit area exerted by a flowing fluid against a surface, is an important biophysical regulator of a wide variety of vascular and circulating cells, including endothelial cells,[75–77] smooth muscle cells,[78–80] and leukocytes.[81–83] Thus, stem cells that are near vasculature, such as MSCs,[84,85] may be regulated by shear stress. In fact, exposure of MSCs to shear stress *in vitro* increases proliferation, endothelial differentiation, and production of angiogenic factors,[86,87] all processes necessary to form vasculature. Shear stress also promoted mesenchymal condensation of embryonic MSCs, the spatial aggregation and packing of cells that precedes musculoskeletal development and the formation of cartilage.[88] The relationship between shear and cell behavior can often be complex. For example, exposure of MSCs embedded in porous polymer or collagen scaffolds to flow induced osteogenesis at high shear[89] and promoted proliferation and maintenance of multipotency at low shear.[90]

Shear flow also induced embryonic stem cells (ESCs) to differentiate into cardiac and vascular endothelial lineages. Specifically, mouse ESCs cultured under shear exhibited increased biochemical markers of cardiovascular differentiation compared with static cultures.[91] Shear also biased mouse ESCs expressing Flk−1, a vascular endothelial marker, to differentiate toward vascular endothelial cells rather than pericytes and vascular smooth muscle cells.[92] These studies suggest that shear stress may aid in specifying stem cell differentiation into the specialized cells of niches naturally experiencing shear, such as vasculature. One subtle point to note is that ionic solutions flowing over charged surfaces of cells may generate streaming potentials on the order of 1–10 mV,[93] levels that are small but potentially significant compared with relevant biological potentials such as transmembrane potentials (resting potentials are ~80 mV). Streaming potential effects may thus be difficult to decouple from the effects of shear forces in flows, and the

development of new technologies may aid future investigations.

Cyclic Strain

Cells near vasculature also experience a ~1 Hz cyclic strain, or repetitive stretch, due to pulsatile blood flow. An organism's movements also impose strain on load-bearing tissues, and amniotic fluid pressure and flow may impart strain on embryonic cells. Furthermore, cell division and apoptosis in development as well as in adult tissue remodeling can induce strains and forces driving biological processes such as dorsal cell-sheet closure in embryos.[94] These biological processes raise the intriguing possibility that MSCs, muscle satellite stem cells, and ESCs may be sensitive to cyclic strain due to their natural locations near vasculature, muscle, and amniotic fluid, respectively. In general, stem cells may be sensitive to the strain arising from their own divisions as well as from the division or death of surrounding cells. Studies with *ex vivo* tissue sections[95] and cell culture studies of stem cells on stretchable polymeric gels or membranes provide clear support of this hypothesis.

MSCs and other stem/progenitor cells are believed to reside in tissues experiencing cyclic strains, such as muscles, tendons, ligaments, and the heart. One study showed that cyclic strain and shear stress synergistically promoted muscle tissue generation from bone marrow-derived MSCs compared with static cultures.[96] Several groups have also shown that cyclic stretch enhanced matrix remodeling and mineralization for tenogenesis and ligament tissue engineering in collagen gels,[97,98] osteogenesis on flexible silicone rubber membranes,[99] and cartilage tissue engineering on a custom compression apparatus.[100]

Strain can sometimes interact in complex ways with other ECM-encoded biophysical cues, such as microtopography. For example, MSCs oriented parallel to micropatterned microgrooves increased proliferation and smooth muscle marker expression when strain was applied parallel but not perpendicular to the microgrooves.[101] Furthermore, Terraciano et al.[102] demonstrated distinct strain responses of adult versus embryonically derived MSCs. In the absence of differentiating factors, cyclic strain induced cartilage-related marker expression in adult MSCs, yet downregulated these markers in embryonically derived MSCs. Similarly, muscle satellite cells release hepatocyte growth factor, which functions as an autocrine mitogen, upon being strained.[103,104]

Studies have also identified molecular mechanisms responsible for transducing cyclic strain into cellular behaviors. In one example, cyclic strain inhibited the spontaneous differentiation and promoted the self-renewal of human ESCs.[105] The frequency of strain had no significant effect, but strain amplitudes above 10% optimally inhibited differentiation. Cyclic strain also interacted synergistically with factors in conditioned media; however, conditioned medium derived from strained hESCs did not replicate the effect of strain, suggesting the involvement of a signaling pathway that may be directly affected by mechanical cues. A subsequent study implicated TGFβ/activin signaling in the transduction of cyclic strain into inhibition of differentiation.[106] Addition of exogenous TGFβ and activin partially rescued the effect of cyclic strain, and antibodies against transforming growth factor (TGF)β and inhibitors of activin induced hESC differentiation even in the presence of cyclic strain. Cyclic strain also induces cardiovascular differentiation and angiogenesis from embryoid bodies. Interestingly, this process was dependent on the upregulation of mitogen activated protein kinase (MAPK) signaling and the generation of reactive oxygen species, as scavengers of reactive oxygen species inhibited strain-induced differentiation.[107] These studies provide intriguing insights into key signaling pathways that may transduce mechanical signals into intracellular biochemical signals. In addition, they indicate that stem cells may respond to cyclic strain by generating cells appropriate to natural microenvironments subjected to stretch, such as muscle, cardiovasculature, and connective tissues.

In these studies of stiffness, shear stress, and cyclic strain, several intracellular signaling pathways have been implicated in biological responses to mechanical signals. However, the question remains: what molecular systems directly translate mechanical forces into biochemical signals? One potential answer is the cytoskeleton, the network of biopolymeric filaments that provides structure and shape to cells. The cytoskeleton can serve as a solid-state transducer of biomechanical cues from the extracellular microenvironment and has already been shown to communicate external mechanical signals to the cell[108] and regulate gene transcription,[109] protein localization,[110] ion channel permeability,[111] and signaling networks[112] in mature cell types. In addition, nonmuscle myosin II activation in MSCs[67] implicates the cytoskeleton in stem cell mechanosensing, as myosin acts to increase the tension of the actin cytoskeleton. Continued focus on the cytoskeleton and associated regulatory proteins like myosin and the Rho GTPases[113,114] may show additional mechanistic insights into biomechanical effects on stem cells.

ARCHITECTURE

Materials with specific mechanical properties such as elastic modulus are carefully selected when constructing a building to ensure proper function. However, the architecture or spatial arrangement of these materials—such as into beams, pillars, panels, etc.—is also necessary for the functionality of the structure. Analogously, for proper regulation of stem cell behavior, natural and engineered stem cell microenvironments present specific architectures, or spatial arrangements of ECM, cells, and ligands. Microenvironments may induce cellular and multicellular shapes and geometries through cell and ligand contacts. They may also have topographical features like discrete steps and plateaus. Finally, the microenvironment can be two-dimensional (2D) or three-dimensional (3D). These architectural details of microenvironmental geometry, topography, and dimensionality have all been shown to influence stem cell behavior.

Geometry

Natural stem cell niches have unique geometries determined by the spatial presentation of surrounding cells. For example, in *Drosophila* the germline niches of ovaries and testes have U-shaped structures determined foremost by cap and hub cells, respectively. These cells anchor the germline stem cells (GSCs) at the base of the niche through E-cadherin interactions/adherens junctions through which stem cell maintenance signals are initiated and activate β-catenin. In the testes, localization of the proteins Cnn, APC1, and APC2 to this junction controls the orientation of the mitotic spindle within stem cells and positions the daughter centrosome farthest from the hub cells during cell division.[12] Subsequent differentiation of the daughter cells in both ovaries and testes correlates with their migration outward and away from the stem cell niche. Spatial control of asymmetric stem cell division is also observed in mammals,[115] specifically for keratinocytic,[116] hematopoietic,[23] and hair follicle stem cells.[117]

Complex stem cell niche geometries have also been elucidated in the subventricular zone and ventricular zone of the mammalian brain, where NSCs are surrounded by ependymal cells in a pinwheel geometry within the VZ.[118] These NSCs were originally thought to exist exclusively in the SVZ; however, NSCs apparently maintain apical processes contacted by the pinwheel of ependymal cells at the surface of the ventricle, as well as long basal processes that extend away from the ventricular surface through the SVZ and contact blood vessels with their endfeet. At a larger spatial scale, the authors also found that the distribution of NSCs along the ventricle wall was heterogeneous, with 'hotspot' regions, and hypothesized that each hotspot may correspond to the origin of a particular neuronal subpopulation in the forebrain.

Complementing these *in vivo* studies, *in vitro* experiments have demonstrated that different microenvironmental geometries can alter stem cell behavior. Microwells ($\sim$100 µm $\times$ 100 µm) generated by microcontact printing techniques maintained hESC colony sizes without inducing spontaneous differentiation for 2–3 weeks and were capable of creating embryoid bodies of monodisperse size.[119] Microwells of similar size fabricated in different shapes also induced embryoid bodies to conform to those shapes. Furthermore, embryoid bodies of controlled size generated in microwells exhibited lower variability in differentiation compared with embryoid bodies generated in typical suspension cultures, offering potential for improving the control and reproducibility of future biological studies.[120]

In landmark work using microcontact printing techniques, Chen et al. patterned ligands into adhesive islands of either 1024 or 10,000 µm^2. They then seeded and cultured MSCs on these islands in media capable of supporting differentiation into either an osteogenic or adipogenic lineage. Cells cultured on the small islands appeared morphologically rounded and subsequently differentiated almost exclusively into adipocytes, whereas those on larger islands flattened and differentiated predominantly into osteoblasts.[121] Building on this work, multicellular MSC structures were cultured on ligand patterns of various shapes, most interestingly sinusoids. MSCs on the convex edges of the sinusoid curves differentiated predominantly into osteoblasts, whereas those on the concave side differentiated into adipocytes. Using microfabricated post array detectors (mPADs) to measure traction forces (the forces that cells exert on the substrate), the authors correlated differentiation into osteoblasts versus adipocytes with greater traction forces exerted by MSCs on the convex edge versus lower traction forces on the concave edge, respectively. This result is an interesting convergence of architecture and biomechanics, in which different multicellular shapes modulate force distributions and hence cell functions.[122] Cell shape may also modulate cell functions by altering nuclear shape and hence gene transcription, as observed with osteogenic cells where intermediate nuclear distensions promoted maximal gene expression of osteocalcin, a bone-specific differentiation marker.[123] In the micropatterning systems described above, the engineered cellular geometries influenced the

cellular mechanics of stem cells and subsequently biological differentiation processes, demonstrating how seemingly disparate factors such as geometry, traction force, and stem cell differentiation are intimately interconnected.

Topography

In addition to geometric structures, the microenvironment can also present topographies such as pores in bone marrow, undulating basement membranes as in the epidermis, cell density and packing, or grooves and ridges in engineered substrates. As with geometry, microenvironmental topography can be studied with micropatterning techniques to create raised and inset features with length scales relevant to cellular and molecular processes.[124–127]

At cellular length scales, NSCs, in cocultures with astrocytes, aligned with micron-scale grooves and formed more neuron-rich cultures than those cultured on flat substrates. Both substrates were coated with the same protein, laminin, to control for biochemical effects.[128] These topographical effects may be sensed and processed by myosin and RhoA-dependent pathways, as implicated in studies where microposts inhibit fibroblast proliferation.[129] Another study investigated the differentiation of oligodendrocytic precursors (OPCs) derived from postnatal rat brains. The authors observed that OPCs *in vivo* consistently differentiated at approximately postnatal day 8, a phenomenon typically explained by the concept of an intrinsic timer in OPCs. However, OPCs differentiated *in vitro* based on achieving a certain cell density, not time, in culture. Furthermore, the authors argued that this density-dependent differentiation was not due strictly to altered paracrine signaling or cell–cell contacts but instead resulted from the geometric constraints of physical contact with other OPCs. In support of this hypothesis, upon culturing OPCs with unfunctionalized microbeads of different sizes and densities, only high densities of beads similar in size to the precursors themselves induced oligodendrocytic differentiation.[130]

Molecular-scale topography can also control stem cell behavior. Nanopits of 100 nm diameter induced MSCs to secrete bone mineral in the absence of osteogenic media, in contrast to MSCs on unpitted surfaces. Surprisingly, disordered but not ordered arrays of nanopits induced osteogenesis,[131] implying that nanoscale asymmetry is required for this behavior. The disordered arrangement of pits may induce polarity within each cell, possibly generating intracellular gradients of signaling molecules that ordered arrays of pits may be unable to generate

due to their symmetry. At similar lengths scales, in the absence of osteogenic media, surfaces composed of vertically aligned nanotubes 70–100 nm in diameter induced hMSCs to differentiate into osteoblasts.[132] Interestingly, smaller diameter nanotubes (30 nm diameter) did not induce hMSC differentiation. The authors hypothesized that clusters of adhesion proteins on larger diameter nanotubes are farther apart than on smaller diameter tubes, requiring the stem cells to stretch to adhere to these protein clusters. This stretching may result in a similar mechanical state as MSCs cultured on stiff substrates, whereas MSCs on small diameter nanotubes would exhibit a similar mechanical state as on soft substrates. Collectively, these examples demonstrate that topographical features from the microscale to the nanoscale can modulate and regulate stem cell function.

Dimensionality

Though not generally considered fully 3D, topographical cues begin to explore the microenvironment's dimensionality by adding vertical features to flat substrates. The importance of studying stem cells in true 3D cultures arises from the fact that although there are near-2D microenvironments including sheets of endothelial and epithelial cells in vasculature and skin, most cells exist in 3D microenvironments *in vivo*. The stem cell field has accordingly begun studying stem cells in true 3D cultures, which may more accurately reflect *in vivo* microenvironments than traditional 2D cultures.[133,134] In support of this view, culturing ESC-derived embryoid bodies in 3D has been shown to improve and enhance differentiation into various lineages including chondrocytes,[135] hematopoietic cells,[136,137] and osteoblasts,[138] likely by providing the opportunity for gradients of signaling factors and/or nutrients to develop. In addition, encapsulation of human ESCs in feeder-free alginate gels allowed for long-term culture without cell passaging while maintaining an undifferentiated state.[139] 3D embryoid body aggregates have also been found to spontaneously form patterned and polarized cortical tissue, mimicking developmental corticogenesis,[140] potentially by enabling the formation of similar morphogenic gradients to those found in natural corticogenesis. These studies demonstrate the importance of studying stem cells in 3D microenvironments. 2D systems, however, enable researchers to study factors difficult to manipulate in 3D, such that a combination of 2D and 3D studies will best contribute to our understanding of stem cell control.

DYNAMICS

To orchestrate, support, and respond to the dynamic processes of organismal development, adult homeostasis, circadian cycles, and organismal aging, stem cell niches as well as stem cells themselves are likely dynamic. These biological processes span a wide range of timescales ranging from years to minutes. Starting at the largest timescales and ending with the smallest, we will review examples of dynamic stem cell microenvironments, both natural and engineered.

Aging is a gradual process that occurs, depending on the mammal, over the course of months to years.[141,142] An important set of studies by Conboy et al. examined the effects of aged muscle niches on the proliferative and regenerative capacities of both muscle satellite cells and ESCs. Muscle injuries were inflicted on both young (2–3 months) and aged (23–24 months) mice, and satellite cells in young mice were observed to proliferate more extensively in response. A similar trend was observed when cell explants from young mice generated greater numbers of myoblasts than ones from aged mice. These aging effects were first correlated with reduced expression of the Notch ligand Delta in aged mice. Specifically, pharmacological inhibition and activation of Notch signaling *in vivo* inhibited and rescued, respectively, the proliferative capacity of satellite cells from young and aged mice, suggesting that the decline in Notch stimulation by the microenvironment, rather than cell-autonomous satellite cell aging, was responsible for the aging effects in muscle.[143] A subsequent study investigated the relative roles of young and aged niches, secreted factors in young and aged sera, and the intrinsic regenerative capacity of stem cells by comparing adult muscle satellite cells and ESCs *in vitro* and *in vivo*. *In vitro*, aged sera overrode the presence of young sera and inhibited both adult satellite cell and ESC proliferative capacities. Likewise, upon transplantation into muscle tissue, the regenerative capacities of both adult satellite cells and ESCs were compromised in aged mice compared with young mice. Interestingly, ESCs exhibited a smaller decline in regenerative capacity in the aged niche and sera, suggesting ESCs may possess greater intrinsic regenerative properties than adult satellite cells.[144]

Endogenous microenvironments also change on the timescales of weeks to days. For example, the ECM protein laminin-α2, which regulates the locations of NSCs through interactions with β1−integrins, becomes increasingly restricted to the ventricular zone of the brain during corticogenesis.[145,146] Adult systems also exhibit weekly and daily dynamic changes, often through system-level changes in blood composition.[147] For example, cell division of *Drosophila* germline ovary stem cells is regulated by insulin, whose levels depend on the organism's nutritional state.[148] Moreover, there is evidence of greater neurogenic activity in the adult hippocampus during nighttime which, given the vascular niche of NSCs in this region,[149] possibly results from system-level changes in the levels of specific factors in the bloodstream.[150]

Most engineered systems for controlled release of pharmacological or genetic agents can be tuned to operate on the timescales of weeks to days. Ferreira et al. encapsulated vascular endothelial growth factor (VEGF) in poly(lactic acid co-glycolic acid) microparticles embedded in dextran gels. The resulting controlled release of VEGF over 10 days improved vascular differentiation of human ESCs compared with conventional vascular differentiation from embryoid bodies.[151] This controlled release strategy may be useful in dynamic studies of stem cell processes as well as in designing therapeutic cell replacement scaffolds.

Lastly, at the timescale of 2–3 h, recent work with NSCs has elucidated interesting oscillatory expression of the Notch ligand Delta in the stem cell niche.[152,153] Most intriguingly, neither the presence nor absence of Notch signaling was sufficient for proper maintenance of NSCs. The complete absence of Notch signaling induced neuronal differentiation through downregulation of Hes1 and upregulation of the proneural factors Neurogenin-2 and Notch ligand Delta-like 1; however, continuously high Hes1 levels inhibited NSC proliferation. The oscillatory nature of Notch-Hes1 signaling thus appears necessary for maintaining NSCs during embryonic development, possibly by providing prosurvival signals through oscillatory Neurogenin-2 and Delta-like 1 expression, but without the persistent expression of such factors that would induce differentiation. These examples demonstrate that niches can evolve on timescales ranging from hours to years, and that the effects of microenvironmental dynamics on stem cell function are beginning to be studied with engineered systems.

CONCLUSION

Biomechanical, architectural, and dynamic inputs have been studied in the context of both natural and engineered microenvironments to elucidate novel principles of stem cell regulation. Continued improvements in imaging and genetic labeling technology for tracking stem cells and their neighboring niche cells in their natural microenvironments, as illustrated recently in the elegant study of NSCs in the
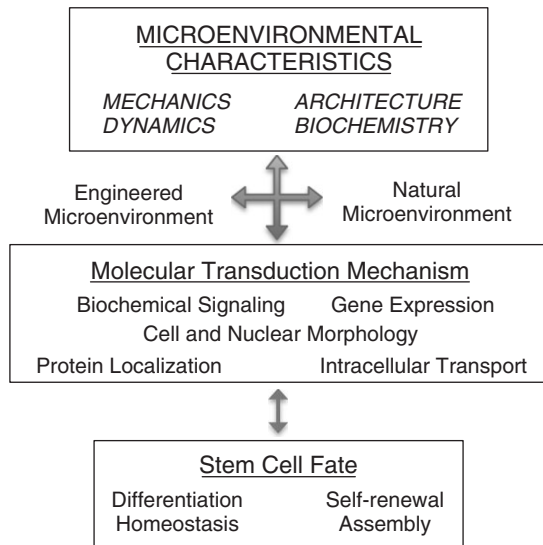
MICROENVIRONMENTAL
CHARACTERISTICS

MECHANICS　　*ARCHITECTURE*
DYNAMICS　　*BIOCHEMISTRY*

Engineered
Microenvironment

Natural
Microenvironment

Molecular Transduction Mechanism

Biochemical Signaling　　Gene Expression
Cell and Nuclear Morphology
Protein Localization　　Intracellular Transport

Stem Cell Fate

Differentiation　　Self-renewal
Homeostasis　　Assembly

FIGURE 3 | The hierarchy of microenvironmental cues and molecular transduction mechanisms regulating stem cell fate. Mechanical, architectural, dynamic, and biochemical properties define both engineered and natural microenvironments. A combination of molecular mechanisms such as biochemical signaling, gene expression, morphology, protein localization, and intracellular transport transduce these microenvironmental cues into stem cell fate choices. Microenvironmental cues may maintain stem cell homeostasis, induce differentiation, support self-renewal, or regulate assembly.

subventricular zone of the brain,[118] will greatly benefit the field's understanding of the architectural and dynamic characteristics of the microenvironment. Likewise, continued development of engineered microenvironments in 3D[154] and temporally dynamic systems,[155–157] combined with improved live cell imaging in mechanobiological studies[158–160] including in 3D systems,[161,162] will expand our understanding of biomechanical, architectural, and dynamic microenvironmental properties on stem cells. Furthermore, natural microenvironments are clearly vicoelastic in nature, and solid tissues experience predominantly shear forces in natural loading cycles, both aspects of the microenvironment that have not been explored in depth in the stem cell field due largely to technical limitations. These are important areas to begin to investigate and will be best studied by the parallel development of macro and micro-scale rheological methods in natural and engineered stem cell microenvironments.[163,164]

The continued development of these technologies will also aid a growing number of detailed studies of molecular mechanisms responsible for transducing biomechanical, architectural, and dynamic signals into biochemical and cellular phenotypes (Figure 3). For example, there are numerous molecules and signaling pathways that potentially function as stem cell mechanotransducers, including the Rho GTPase family of proteins, reactive oxygen species, activin/nodal, and calcium signaling. In addition, cadherins and β-catenin have been implicated in the shear response of osteoblasts,[165] and G-protein coupled receptors function as shear-sensors in endothelial cells.[166] These mechanosensitive pathways may also cross-talk with signaling pathways already well studied in stem cell biology, potential intersections that should be explored. Similar mechanistic strategies can also be applied to studies of microenvironmental architecture and dynamics.

These phenomenological and mechanistic studies have great biomedical potential. Therapeutic strategies such as cell and tissue replacement[167–170] will hinge upon the ability to generate large populations of stem cells *in vitro* and to precisely control their differentiation into desired cell types. Correspondingly, understanding how stem cells will react to perturbations in their *natural* microenvironments will benefit targeted gene and pharmacological therapies.[17,171,172] In addition, expanding our view of the microenvironment to include biophysical and dynamic properties may yield novel strategies that seek to prevent or correct misregulated or malignant microenvironments leading to cancer, as well as other diseases involving improper tissue development and homeostasis.[47,173–177] In fact, stiffness has already begun to be acknowledged as an important characteristic of breast tumors,[47,74,174] and other microenvironmental properties reviewed here may be implicated in additional disease states in the future. Combining an understanding of these biomechanical, architectural, and dynamic properties with the large body of knowledge of the biochemical regulation of stem cells will therefore both enhance and expand our understanding of the fundamental biology of stem cells and synergistically improve our ability to develop stem cell-based therapies.

ACKNOWLEDGEMENTS

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