Forcing Stem Cells to Behave: A Biophysical Perspective of the Cellular Microenvironment

Yubing Sun,[1,2] Christopher S. Chen,[3] and Jianping Fu[1,2,4]

[1]Integrated Biosystems and Biomechanics Laboratory, [2]Department of Mechanical Engineering, and [4]Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan 48109; email: jpfu@umich.edu

[3]Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104; email: chrischen@seas.upenn.edu

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Abstract

Physical factors in the local cellular microenvironment, including cell shape and geometry, matrix mechanics, external mechanical forces, and nanotopographical features of the extracellular matrix, can all have strong influences on regulating stem cell fate. Stem cells sense and respond to these insoluble biophysical signals through integrin-mediated adhesions and the force balance between intracellular cytoskeletal contractility and the resistant forces originated from the extracellular matrix. Importantly, these mechanotransduction processes can couple with many other potent growth-factor-mediated signaling pathways to regulate stem cell fate. Different bioengineering tools and microscale/nanoscale devices have been successfully developed to engineer the physical aspects of the cellular microenvironment for stem cells, and these tools and devices have proven extremely powerful for identifying the extrinsic physical factors and their downstream intracellular signaling pathways that control stem cell functions.

Contents

INTRODUCTION

Stem cells are critical players during development, tissue regeneration, and healthy homeostatic cell turnover, and they are an important driving force for the developing fields of functional tissue engineering and regenerative medicine owing to their self-renewal capacity and pluripotency (28). Collectively, all stem cells share the ability to self-renew and differentiate into specific lineages. Embryonic stem (ES) cells—which are derived from the inner cell mass of the developing blastocyst—are pluripotent, whereas stem cells derived from adult tissues generally maintain a more limited, tissue-specific, regenerative potential (28, 93). Owing to their ability to generate tissue de novo following disease or injury, there is a widespread hope of developing stem cell–based therapies for various degenerative diseases (71, 76). A key aspect in the enabling of these stem cell–based therapies will be the ability to manipulate interactions between stem cells and their local microenvironment (a setting in vivo known as the stem cell niche) in order to regulate and direct stem cell fate (61, 89, 113).

Microenvironment: the soluble and insoluble surroundings of a cell

Stem cell niche: specific stem cell microenvironment that regulates how stem cells participate in tissue generation, maintenance, and repair

Extracellular matrix (ECM): a meshwork of proteins, polysaccharides, and glycoproteins that provides structural and adhesive support to cells and tissues

Mechanotransduction: the processes whereby cells convert physiological mechanical stimuli to intracellular biochemical responses

hESCs: human embryonic stem cells

How the in vivo stem cell niche, which filters and presents a wide range of molecular and cellular scale physical and biological signals, acts to regulate tissue regeneration based on physiological demand and pathological state remains incompletely understood (93, 103). In vivo, stem cell niches create specialized microenvironments consisting of soluble and surface-bound signaling factors, cell-cell contacts, stem cell niche support cells, extracellular matrix (ECM), and local mechanical microenvironment (**Figure 1**). Although stem cell biologists have long appreciated the regulatory roles for soluble stem cell niche signals (e.g., growth factors and cytokines) in regulating stem cell fate, recent evidence demonstrates that regulation of stem cell fate by these soluble factors is strongly influenced by the coexisting insoluble adhesive, mechanical, and topological cues inherently contained and dynamically regulated in the stem cell niche (23, 30, 46, 129). These insoluble biophysical cues can be sensed and transduced into intracellular biochemical and functional responses by stem cells, a process known as mechanotransduction (16, 41, 96, 116, 126).

The sensory machinery of stem cells can sense and integrate multiple (soluble and insoluble) signals simultaneously from their niche and convert them to a coherent environmental signal to regulate downstream gene expression and stem cell fate. Further, different well-conserved soluble-factor-mediated signal transduction pathways and the cellular mechanosensing and mechanotransduction processes can converge to activate the elaborate intracellular signaling network in an integrated and interacting manner to regulate stem cell fate. Taking human embryonic stem cells (hESCs) as an example, basic fibroblast growth factor (bFGF)-mediated signaling, transforming

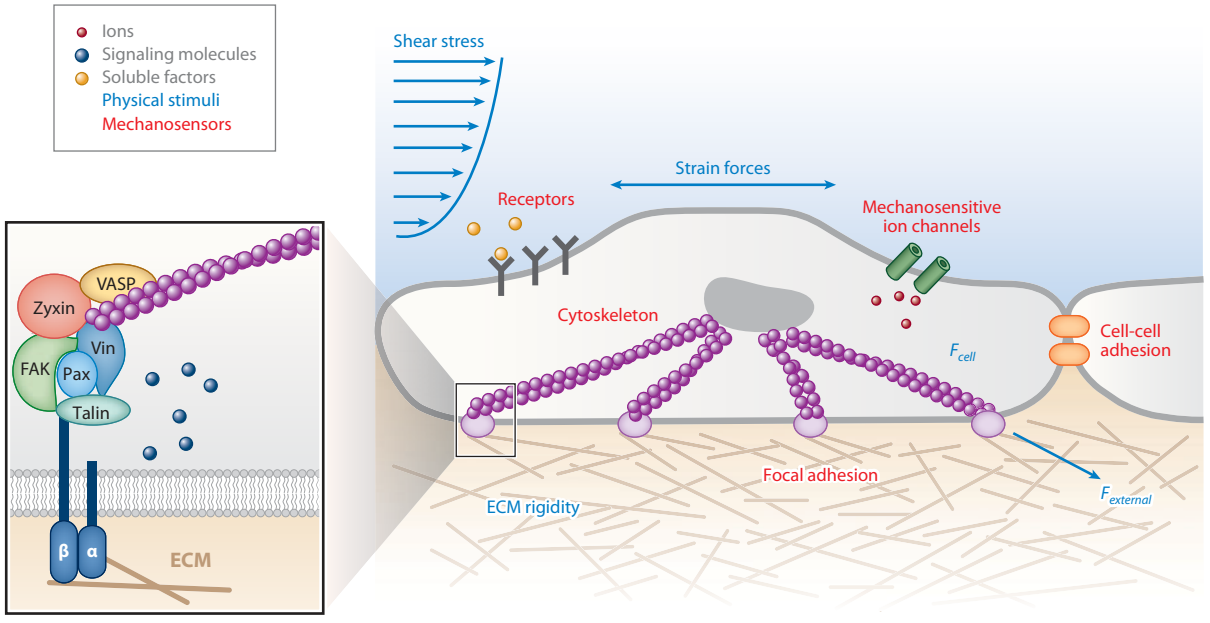


Figure 1

Schematic showing biophysical signals in the stem cell niche and the intricate reciprocal molecular interactions between stem cells and their microenvironment to regulate stem cell fate. The extracellular microenvironment of stem cells is a hydrated protein- and proteoglycan-based gel network comprising soluble and physically bound signals as well as signals arising from cell-cell interactions. Biophysical signals in the stem cell niche include matrix rigidity and topography, flow shear stress, strain forces, and other mechanical forces exerted by adjacent support cells (*blue text*). Stem cells can sense these biophysical stimuli through mechanosensors such as ion channels, focal adhesions, cell surface receptors, actin cytoskeleton, and cell-cell adhesions (*red text*). A magnified view of the focal adhesion structure is also shown, which includes transmembrane heterodimeric integrin, paxillin (Pax), talin, focal adhesion kinase (FAK), vinculin (Vin), zyxin, and vasodilator-stimulated phosphoprotein (VASP). Abbreviation: ECM, extracellular matrix.

growth factor-β (TGF-β)/activin/Nodal-mediated signaling, and canonical Wnt (wingless)/β-catenin-mediated signaling are all central for the self-renewal of hESCs, while bone morphogenetic proteins (BMPs) induce differentiation of hESCs (33, 138, 139). bFGF activates the mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (ERK) signaling cascade in hESCs, also known to be a central mechanotransduction pathway for adaptive cellular responses to mechanical stimuli from the cellular microenvironment (63, 75). Extracellular mechanical forces stimulate expressions of TGF-β, activin, and Nodal, providing an autocrine or paracrine signaling mechanism to promote maintenance of the pluripotency of hESCs (94, 110). β-catenin, which is a critical component of the canonical Wnt signaling pathway, plays an important role in cell-cell adhesions by mediating cytoskeletal attachment of E-cadherin to the actin cytoskeleton. β-catenin-mediated E-cadherin-based cell-cell adhesions are mechanosensitive and depend on nonmuscle myosin II (NMMII) activity in hESCs (74). Further, β-catenin is critical for the mechanical induction of Twist expression in *Drosophila*; Twist is a transcription factor associated with regulation of skeletal development (36).

Moreover, recent studies show that the RhoA-GTPase/Rho-associated coiled coil-containing kinase (ROCK)/myosin-II signaling axis, which is the major biochemical pathway mediating the actin cytoskeleton tension in nonmuscle cells (35, 106), plays a critical role in regulating survival and cloning efficiency of single hESCs (17, 128, 131). Blocking RhoA/ROCK-mediated cytoskeleton tension using drug inhibitors reduces dissociation-induced apoptosis of hESCs, suggesting that

TGF-β: transforming growth factor β

MAPK: mitogen-activated protein kinase

ROCK: Rho-associated coiled-coil-containing protein kinase

Nanotopography: surfaces and structures with nanoscale topological features

hyperactivation of cytoskeleton tension, triggered by hESC cell dissociation, is the upstream regulator and direct cause of hESC apoptosis. Importantly, RhoA/ROCK-mediated cytoskeleton tension plays a critical role in the mechanotransduction process (16, 58). All together, biophysical signals in the cellular microenvironment of stem cells can have extensive potential to regulate and synergize with classical signal transduction pathways induced by soluble factors to control stem cell fate.

With recent major advances in understanding how the insoluble biophysical signals in the cellular microenvironment regulate stem cell fate, tissue engineering and regenerative medicine are becoming increasingly oriented toward biologically inspired in vitro cellular microenvironments designed to guild stem cell growth, differentiation, and functional assembly (78, 127). The premise is that, to unlock the full potential of stem cells, at least some aspects of the dynamic cellular microenvironment that are associated with their renewal, differentiation, and assembly in native tissues need to be reconstructed.

A major goal of this review is therefore to offer a perspective on this new trend of designing synthetic artificial in vitro stem cell niches and their promise for stem cell research and to enable new, clinically relevant strategies for tissue regeneration. In particular, we focus on discussing the biophysical signals in the synthetic stem cell niche and their functional effects on stem cell fate. To do so, we take the approach of highlighting some illustrative examples of using bioengineering strategies for controllable synthetic cellular microenvironments developed through the interactions of stem cell biology, tissue engineering, and microtechnology/nanotechnology at multiple length scales. We first stress different biophysical factors in the cellular microenvironment that are critical for the fate decisions of stem cells, such as ECM geometry, nanotopography, and mechanics, and describe how these biophysical factors affect cell signaling and function. We discuss the mechanosensory machinery and mechanotransduction mechanisms stem cells can use to sense and respond to these biophysical factors and how these mechanotransduction pathways converge with classical signal transduction pathways to control stem cell fate. We discuss different versatile and powerful bioengineering and microtechnology/nanotechnology strategies and methods that can be used for constructing the synthetic stem cell niche. We offer some perspectives on potential research directions and opportunities for engineering stem cell functions using well-controlled cellular microenvironments.

MECHANICAL CONTROL OF STEM CELL FATE

Functional regulation of stem cells in vivo normally plays out in the context of embryonic development, tissue regeneration, and the wound healing response, in which extracellular mechanical forces abound and the mechanical environment surrounding the stem cells changes dynamically. Plenty of evidence exists to suggest that these biophysical signals from the local stem cell niche instruct the subsequent behaviors of stem cells. There are other extensive reviews on the topic of stem cell niche signals regulating stem cell fate, especially for in vivo organismal development settings (65, 93, 103); here we provide illustrative examples using novel bioengineering and microfabrication/nanofabrication approaches to control the local stem cell niche and, where evidence suggests, mechanical control of stem cell fate through synergistic regulations of stem cell shape, cytoskeleton tension, and integrin-mediated adhesion signaling.

Cell Shape and Control of Cytoskeletal Tension

Cell shape is a potent regulator of cell growth and physiology (39). Cells adapt and optimize their shape for their specific functions. For example, adipocytes are spherical in shape to maximize

lipid storage, whereas neurons have long axons to deliver signals rapidly over a long distance. In fact, many events associated with stem cell differentiation during embryonic development and tissue regeneration are designed to change cell shape, and those changes in shape can influence tissue structure and function (56, 81, 141). Thus, a natural converse question for stem cells arises: whether their fate can be regulated by their intrinsic and dynamically regulated cell shape. Compelling studies to support cell shape as a key regulator of stem cell fate came from experiments using bioengineering strategies to pattern the spreading and morphology of human mesenchymal stem cells (hMSCs). hMSCs are isolated from bone marrow and can differentiate into multiple lineages of mesenchymal tissues (15, 104). By using microcontact printing to coat flat polydimethylsiloxane surfaces with distinct patterns of adhesive ECM islands, McBeath et al. (86) reported that in response to a bipotential differentiation medium that contained inducers for both the adipogenic and osteogenic differentiations, single hMSCs confined to small ECM islands selectively underwent adipogenesis, whereas single hMSCs on large ECM islands were biased toward osteogenesis (**Figure 2*a***).

hMSCs: human mesenchymal stem cells

Actomyosin contractility: intracellular forces generated by the dynamic interaction of myosin motors and actin filaments

This osteogenic-adipogenic switch in well-spread versus poorly spread hMSCs required generation of cytoskeleton tension through RhoA-dependent actomyosin contractility. RhoA is a member of Rho family small GTPases involved in cellular signaling and cytoskeletal organization, and it stimulates cytoskeleton tension through its effector, ROCK, which directly phosphorylates both NMMII regulatory myosin light chain (MLC) and MLC phosphatase to synergistically increase MLC phosphorylation and thus myosin II contractility (6, 48). Inhibition of cytoskeleton tension using either cytochalasin D (an actin depolymerization agent) or Y-27632 (a ROCK inhibitor) promoted adipogenesis, mimicking the phenotype of poorly spread hMSCs. Moreover, manipulation of the RhoA pathway could override the effects of soluble differentiation factors, such that dominant-negative RhoA induced adipogenesis even in the context of pure osteogenic medium, whereas constitutively active RhoA triggered osteogenesis in pure adipogenic medium. These findings highlight RhoA activity as a potential convergence point for mechanical and soluble factor signaling in the control of stem cell differentiation. Importantly, McBeath et al. also demonstrated that expression of constitutively active ROCK rescued osteogenic differentiation of poorly spread MSCs, and this effect required myosin II activity, indicating that cell shape and RhoA regulate osteogenic-adipogenic switching through the development of cytoskeleton tension.

Ruiz & Chen (107) confirmed the importance of cytoskeleton tension in regulating stem cell fate in the setting of multicellular structures. Ruiz & Chen applied microscale patterning approaches to control the geometries of both two-dimensional (2D) and three-dimensional (3D) multicellular structures of hMSCs (**Figure 2*b***). The authors reported that in the presence of soluble factors permitting both osteogenic and adipogenic differentiations, hMSCs at the edge of the multicellular structures selectively differentiated into the osteogenic lineage, whereas those in the center became adipocytes. Using some microfabricated cellular traction force sensors (31, 124), Ruiz & Chen further demonstrated that a gradient of traction stress across the 2D multicellular hMSC structures could precede and mirror the patterns of multicellular differentiation, where regions of high stress were concentrated with osteogenesis of hMSCs, whereas hMSCs in regions of low stress differentiated to adipocytes. Inhibition of cytoskeleton tension using blebbistatin (a myosin II inhibitor), Y-27632, or ML-7 (an inhibitor of MLC kinase) suppressed the spatial patterns of multicellular differentiation of osteogenesis versus adipogenesis, for both 2D and 3D multicellular structures of hMSCs. Interestingly, in addition to the overall cell shape, cell geometry also plays an important role in regulating stem cell fate (66, 77, 119). Kilian et al. (66) demonstrated that in response to a bipotential differentiation medium that contained inducers for both the adipogenic and osteogenic differentiations, single hMSCs cultured in rectangles with increasing aspect ratio and in shapes with pentagonal symmetry but with different subcellular curvature—and with

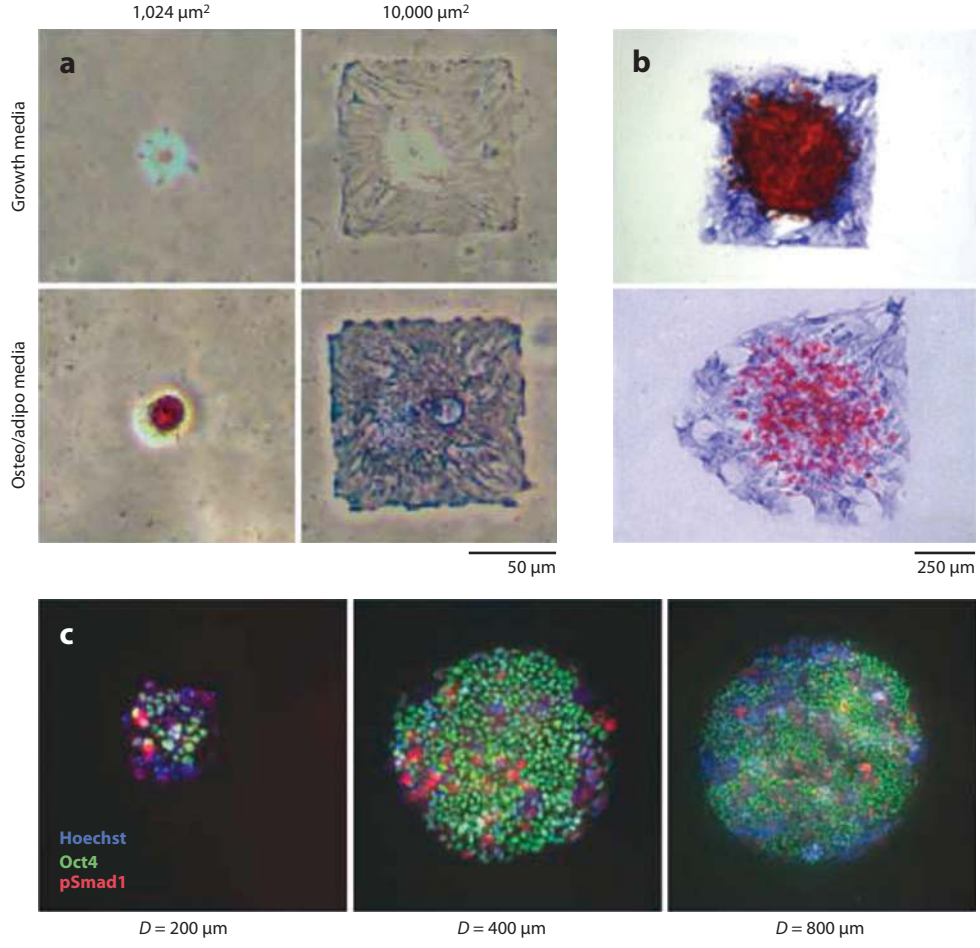


Figure 2

Microcontact printing to manipulate the cell shape and colony size of stem cells to control their fate. (*a*) Brightfield micrographs of single hMSCs plated on different sized adhesive ECM islands. hMSCs were stained for alkaline phosphatase activity (ALP, *blue*) and lipid droplet accumulation (Lip, *red*) after 7 days of culture in either the growth (*top row*) or the bipotential differentiation (*bottom row*) medium. Adapted from Reference 86, Copyright © 2004, with permission from Elsevier. (*b*) Brightfield micrographs of different shaped multicellular hMSC colonies stained for ALP (*blue*) and Lip (*red*) after 14 days of culture in the bipotential differentiation medium. Adapted from Reference 107, Copyright © 2008, with permission from John Wiley and Sons. (*c*) Immunofluorescent images showing different sized hESC colonies (H9) cultured in XV media after withdrawal of all exogenous growth factors for 48 h. hESCs were stained for Hoechst, Oct4, and pSmad1 to indicate the effect of colony size on the pluripotency maintenance of hESCs. Adapted from Reference 102, Copyright © 2007, with permission from Nature Publishing Group. Abbreviations: hMSC, human mesenchymal stem cells; ECM, extracellular matrix; hESC, human embryonic stem cell.

each occupying the same area—displayed different adipogenesis and osteogenesis profiles. Using cytoskeleton-disrupting pharmacological agents, Kilian et al. further confirmed a causal role for cytoskeleton tension in modulating the shape-based trends in lineage commitment of hMSCs. Taken together, the aforementioned studies demonstrate a causal role of cell shape and geometry in regulating stem cell fate. Importantly, there is a common theme emerging from all these studies:

Cell shape signal seems to converge with soluble inductive factors on the actin cytoskeleton and the RhoA/ROCK-mediated cytoskeleton tension to regulate stem cell fate.

Peerani et al. (102) demonstrated the effect of the cellular microenvironment on hESC fate by patterning hESC colonies onto defined adhesive islands with a controlled colony diameter (**Figure 2*c***). Peerani et al. showed that larger colonies with a high local cell density microenvironment would promote the maintenance of pluripotency in hESCs through a niche size-dependent spatial gradient of BMP-mediated Smad1 signaling generated as a result of antagonistic interactions between hESCs and hESC-derived extraembryonic endoderm. Thus, the effect of this colony size on the pluripotency maintenance of hESCs appears to be mediated by interactions between exogenously controlled parameters and autocrine and paracrine secretion of endogenously produced factors from hESCs. Even though there was no mechanotransduction mechanism revealed specifically in the study by Peerani et al. (102), combining their results together with the observations from hMSCs discussed above, it is reasonable to speculate that stem cell fate is mediated by a combination of soluble factors and insoluble biophysical signals in the local stem cell microenvironment. Thus, structural and mechanical cues associated with cytoskeletal reorganization appear to be integrated with several developmental signaling pathways critical for stem cell fate determination, and the integrated mechanochemical networks provide a mechanism for stem cells to orchestrate the many structural and mechanical changes associated with morphogenesis to direct the downstream genetic programs required to give rise to the appropriate spatiotemporal patterns of stem cell differentiation.

Focal adhesion (FA): cell adhesion sites for their attachment to ECM, where intracellular actin filaments can link to ECM proteins through transmembrane proteins such as integrins

Nanotopography: Integrins Making Sense

During embryonic development and tissue regeneration, stem cells interact not only with each other but also with the 3D porous network of the ECM, which comprises fibrillar networks of proteins such as collagen and laminin interlaced with proteoglycan. Although the characteristic pore size of the ECM might provide a direct physical constraint on the stem cell size and shape, the microscale and nanoscale topography, structure, and architecture of the fibrous ECM are also important biophysical signals that can regulate stem cell adhesion and cytoskeletal organization and thus stem cell behaviors such as proliferation, migration, and differentiation (25, 44, 120, 135). Equipped with advanced sub-100-nm microfabrication/nanofabrication techniques, materials scientists and applied physicists have successfully teamed up with stem cell biologists and tissue engineers to generate well-controlled molecular and cellular scale topography on 2D planar substrates to investigate their independent effect on stem cell fate. Existing studies on nanotopography have suggested that instead of directly affecting cytoskeleton tension as in the case for cell shape, nanotopographical cues appear to elicit their effect on stem cells by directly modulating the molecular arrangement, dynamic organization, and signaling of the cellular adhesion machinery.

Adhesion of stem cells to the nanotopographical ECM is mediated via heterodimeric transmembrane receptors, namely, α- and β-integrins (**Figure 1**). Combinations of among 18 α-chains and 8 β-chains form different heterodimers to yield a rich diversity of ECM receptors, enabling differential cell-type-specific responses to variations in the ECM. Upon binding ECM, integrins can cluster to form dynamic adhesion structures called focal adhesions (FAs). On the cytoplasmic side of FAs, integrins can interact, via their cytoplasmic tails, with different adaptor and signaling proteins. Among these molecules, talin, vinculin, paxillin, and α-actinin are adaptor proteins that provide a direct physical linkage to the actin cytoskeleton. Importantly, binding of integrins to the ECM proteins can activate tyrosine kinase and phosphatase signaling to elicit downstream biochemical signals important for regulation of gene expression and stem cell fate. Thus, it is

speculated that nanotopographical signals intrinsically contained in the ECM surrounding stem cells can regulate stem cell fate through their direct effect on integrin-mediated FA signaling.

FAK: focal adhesion kinase

As reported by Arnold et al. (4), by using block-copolymer micelle nanolithography to pattern gold nanodots coated with adhesive peptides, when the spacing between these nanodots exceeded approximately 70 nm, cell adhesion and spreading, FA, and actin stress fiber formations were significantly impaired, likely owing to the restricted clustering of integrin molecules by the distance between the adjacent gold nanodots. In another relevant study using nanoimprint lithography to pattern gold nanodots functionalized with binding ligand RGD (Arg-Gly-Asp), Schvartzman et al. (115) reported a drastic increase in the spreading efficiency of cells on arrays of different geometric arrangements of the nanodots when at least four liganded sites were spaced no more than 60 nm apart, with no dependence on global density. This interesting observation pointed to the existence of a minimum of four integrin adhesion units required for initial growth and maturation of nascent FAs on fibronectin as defined in space and stoichiometry. Together, these two studies based on well-controlled cell-ECM interactions demonstrate the molecular sensitivity and dynamic organization of FAs regulated by the local nanotopographical cue.

Given the potent influence of local nanotopography on regulating molecular organization of FAs, it is not surprising that nanotopography can significantly affect stem cell fate. Yim et al. (143) showed that hMSCs cultured on the nanoscale gratings on the polydimethylsiloxane surface tended to align and elongate their actin cytoskeleton and nuclei along the nanogratings. Gene profiling and immunostaining by Yim et al. further showed significant upregulation of neuronal markers such as microtubule-associated protein 2 for hMSCs cultured on the nanogratings, compared with unpatterned flat controls, and the combination of nanotopography and biochemical inductive factors such as retinoic acid further enhanced the expressions of neuronal markers. Importantly, Yim et al. (143) further demonstrated that nanotopography showed a stronger independent effect compared with biochemical cues (in this case, retinoic acid for neurogenic differentiation of hMSCs) alone on unpatterned control surfaces. In a follow-up study, Yim et al. (142) found that on the nanogratings, expressions of most integrins except $\alpha 3$ and $\beta 5$ were considerably downregulated and that the aligned actin cytoskeleton on the nanogratings was not as prominent or dense as on flat surface controls. Further, distributions of vinculin and focal adhesion kinase (FAK), two prominent FA proteins, on the nanogratings were different from those on flat surfaces. Combined together, studies from Yim et al. suggest that the local nanotopographical cues could affect the molecular organization and composition and signaling of FAs and that such modified FA signaling might further influence cytoskeleton structure to mediate stem cell fate.

More recently, Dalby et al. (27) applied electron beam lithography and hot embossing to pattern nanoscale pits of different symmetry and with varying degrees of disorder in the polymethylmethacrylate or polycaprolactone substrates. Dalby et al. (27) first reported that the nanoscale disorder in the nanopit array stimulated hMSCs to produce bone mineral in vitro, even in the absence of osteogenic supplements. Interestingly, Dalby et al. further showed that hMSCs plated on perfectly ordered or totally random arrays of nanopits produced much less osteoblastic differentiation. A more recent study from the same authors demonstrated another intriguing effect of nanotopography on hMSC fate regulation. McMurray et al. (87) showed that the perfectly ordered arrays of nanopits, even though not efficient to promote osteogenic differentiation of hMSCs as shown in their previous work, were conducive to hMSC growth while permitting prolonged retention of their multipotency and differentiation potential.

The aforementioned studies strongly suggest the potential of nanoscale structured surfaces as noninvasive tools to control the local stem cell microenvironment to regulate stem cell fate, even though the mechanisms by which stem cells can sense and respond to the nanotopographical signal is not yet clear. But as discussed above, molecular arrangement and dynamic organization

of integrin-mediated FAs appear to be sensitive to the local arrangement and presentation of the nanotopographical signal. Thus, it is likely that integrin-mediated FA signaling, critical for many cellular functions (42, 117) and strongly dependent on their dynamic characteristics and molecular processes (52, 101, 145), plays an important functional role in regulating the stem cell sensitivity to nanotopography.

Mechanical Forces and Matrix Mechanics: A Balanced Tensional Homeostasis

During embryonic development and tissue regeneration, cells are exposed not only to structural changes in the surrounding ECM, but also to many mechanical stresses. There are local changes in mechanical forces during development, caused by the addition or removal of cells, cell movements associated with morphogenesis, muscle contraction, and relaxation, as well as during bone compression and decompression. Therefore, stem cells are constantly subjected to and adjust to external force fluctuations from their local microenvironment. Another physical signal important for regulating stem cell fate is the intrinsic elastic modulus of the ECM surrounding stem cells. Stem cells sense and respond to changes of the elastic modulus of the ECM by modulating their endogenous cytoskeleton contractility, balanced by resistant forces generated by the deformation of the ECM, the magnitude of which is determined by the ECM elastic modulus. Thus, it appears that stem cells are mechanosensitive and mechanoresponsive to mechanical forces and matrix mechanics through a modulated delicate force balance between endogenous cytoskeleton contractility and external mechanical forces transmitted across the cell-ECM adhesions. Indeed, such tensional homeostasis in the intracellular cytoskeleton has a key role in the regulation of basic cellular functions, such as cell proliferation, apoptosis, adhesion, and migration (80, 136). Deregulation of the tensional homeostasis in cells contributes to the pathogenesis of several human diseases, such as atherosclerosis, osteoarthritis and osteoporosis, and cancer (12, 47, 57).

External forces and matrix mechanics have a key role in the regulation of stem cell fate. The detailed molecular picture of the mechanotransduction process for stem cells to sense and respond to external forces and changes in matrix mechanics has yet to be identified. The force balance transmitted across the mechanical continuum of ECM-integrin-cytoskeleton can regulate integrin-mediated adhesion signaling (such as FAK and Src signaling) to coordinate downstream integrated stem cell function. These biophysical signals are sensed at the FA sites in which integrins provide the mechanical linkage between the ECM and the actin cytoskeleton. Exposing stem cells to mechanical strain or fluid shear stress or plating stem cells on substrates with varying elastic moduli activates integrins, which promote recruitment of scaffold and signaling proteins to strengthen FAs and to transmit biochemical signals into the cell. These mechanotransduction pathways establish positive-feedback loops in which integrin engagement activates actomyosin cytoskeleton contractility, which in turn reinforces FAs (16, 41). Thus, the level of cytoskeleton contractility generated inside the cell is directly proportional to the adhesion strength and the matrix elastic modulus and dictates the cellular responses of stem cells.

Effects of external forces including mechanical strain, compression, and fluid shear stress on cellular functions have long been studied for cardiovascular tissues, skeletal muscles, and adult stem cells such as hMSCs (19, 29, 97, 129). Evidence related to regulation of pluripotent stem cell fate by mechanical forces in vitro has only recently begun to emerge. Saha et al. showed that in the presence of mouse embryonic fibroblast (MEF) conditioned medium, under cyclic equibiaxial strain, hESC differentiation was reduced and self-renewal was promoted without selecting against survival of differentiated or undifferentiated cells (109). A more recent study by the same authors further showed that the TGF-β/activin/Nodal signaling pathway played a crucial role in repressing hESC differentiation under mechanical strain (110). Saha et al. showed that mechanical

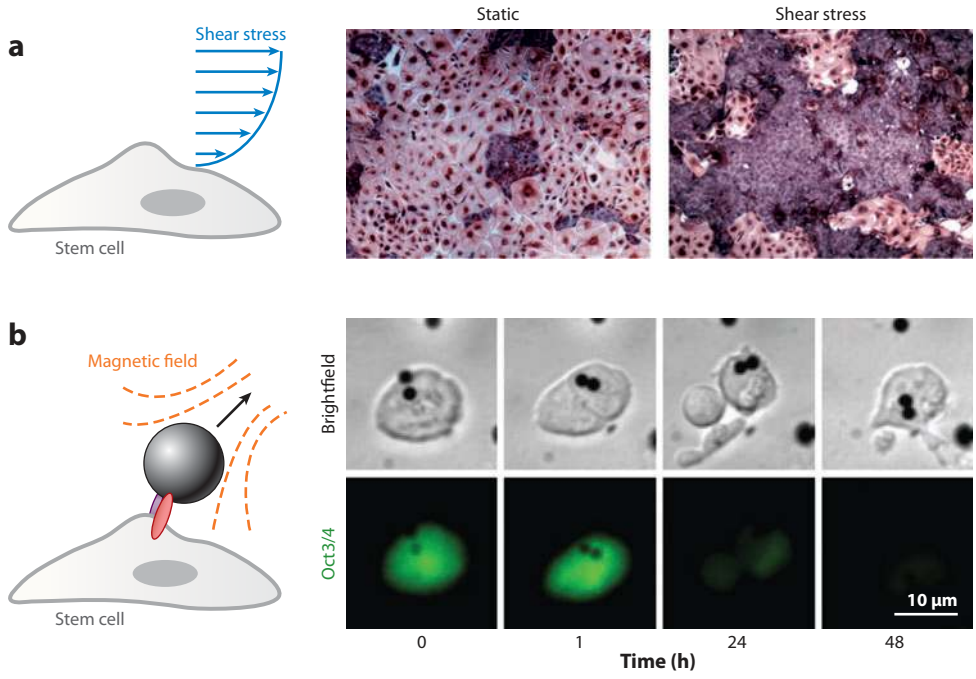


Figure 3

External mechanical forces regulate stem cell fate. (*a*) Fluid shear stress induces differentiation of Flk-1-positive mouse embryonic stem cells (ESCs) into vascular endothelial cells in vitro. Flk-1$^+$ mouse ESCs were incubated under a static culture condition (*middle*) or subjected to shear stress (5 dyn cm^{-2}) (*right*). After 24 h, the cells were stained for PECAM-1 (platelet endothelial cell adhesion molecule 1) (an endothelial cell marker, *purple*) and smooth muscle α-actin (a smooth muscle cell marker, *brown*). Shear stress induces PECAM-1-positive cell sheets. Adapted from Reference 140, Copyright © 2005, with permission from the American Physiological Society. (*b*) Brightfield images (*top row*) with corresponding fluorescence images showing Oct3/4 expression (*bottom row*) for single mouse ESCs. The cells were attached with arginine-glycine-aspartic acid (RGD)-coated magnetic beads (*black dots in the brightfield images*) and were continuously stressed for approximately 1 h. Oct3/4 expression of these stressed cells was continuously monitored over time. Adapted from Reference 21, Copyright © 2010, with permission from Nature Publishing Group.

strain induced transcription of TGF-β1, activin A, and Nodal to upregulate Smad2/3 phosphorylation in undifferentiated hESCs. Thus, the studies by Saha et al. demonstrated that TGF-β superfamily activation of Smad2/3 was required for repression of spontaneous differentiation of hESCs under mechanical strain, which further suggested that mechanical strain might induce autocrine or paracrine signaling in hESCs through TGF-β superfamily ligands (110). Inspired by the fact that in vivo blood vessels remodel and change their sizes by sensing the shear stress of blood flow (92), different researchers showed that well-controlled shear stress could be used to induce mouse embryonic stem cells (mESCs) to differentiate into the endothelial cell lineage (**Figure 3*a***) (140) as well as hematopoietic progenitor cells (1), even though the molecular mechanisms underlying these mechanoresponsive behaviors of mESCs have not been sufficiently explored. Another recent study by Chowdhury et al. (21) showed that local cyclic stress through integrin-mediated adhesions by using functionalized magnetic beads induced spreading of mESCs with a concomitant downregulation of their Oct3/4 gene expression (**Figure 3*b***).

The ability of stem cells to sense matrix mechanics has been demonstrated only recently, yet its implications for functional tissue engineering and regenerative medicine have already generated tremendous excitement. In a landmark study, Engler et al. demonstrated that in the absence of exogenous soluble cues, plating hMSCs on polyacrylamide gels of varying elasticity was sufficient to induce hMSCs to differentiate into different tissue types corresponding to the tissues' relative mechanical elasticity in vivo (**Figure 4*a*,*b***) (34). As discussed above, adherent cells sense matrix mechanics through a force balance between intracellular actomyosin contractility and the resistant force of the ECM determined by its elastic deformation. Thus, the level of cytoskeleton tension generated inside stem cells is directly proportional to the elastic modulus of the substrate stem cells adhere to. Indeed, this positive correlation between the elastic modulus of the substrate and intracellular cytoskeleton contractility was reported by Engler et al. and others for hMSCs and many other mechanosensitive adherent cells (34, 40). Importantly, to demonstrate that this cytoskeleton contractility indeed played a causal role for regulating matrix mechanics-dependent changes in hMSC differentiation, Engler et al. showed that addition of blebbistatin to block intracellular cytoskeleton tension generation in hMSCs obliterated matrix mechanics-driven differentiation. Recently, Huebsch et al. (55) extended the in vitro study of mechanobiology in MSCs to a 3D microenvironment setting by using a 3D hydrogel synthetic ECM formed by alginate polymers that presented integrin-binding RGD peptides (**Figure 4*c***) (55). Using this 3D cell culture system with well-controlled elastic modulus encapsulating mouse MSCs (mMSCs), Huebsch et al. showed that osteogenesis of mMSCs occurred predominantly at 11–30 kPa, comparable to the native tissue stiffness of precalcified bone (30). Because mMSCs were encapsulated in the 3D hydrogel, their morphology appeared to be independent of the elastic modulus of the hydrogel. Still, Huebsch et al. demonstrated that matrix stiffness regulated integrin binding as well as reorganization of adhesion ligands on the nanoscale, both of which were cytoskeleton contractility dependent and correlated with osteogenic commitment of mMSCs, again highlighting the importance of intracellular cytoskeleton contractility and its force balance with deforming surrounding ECM in regulating stem cell fate.

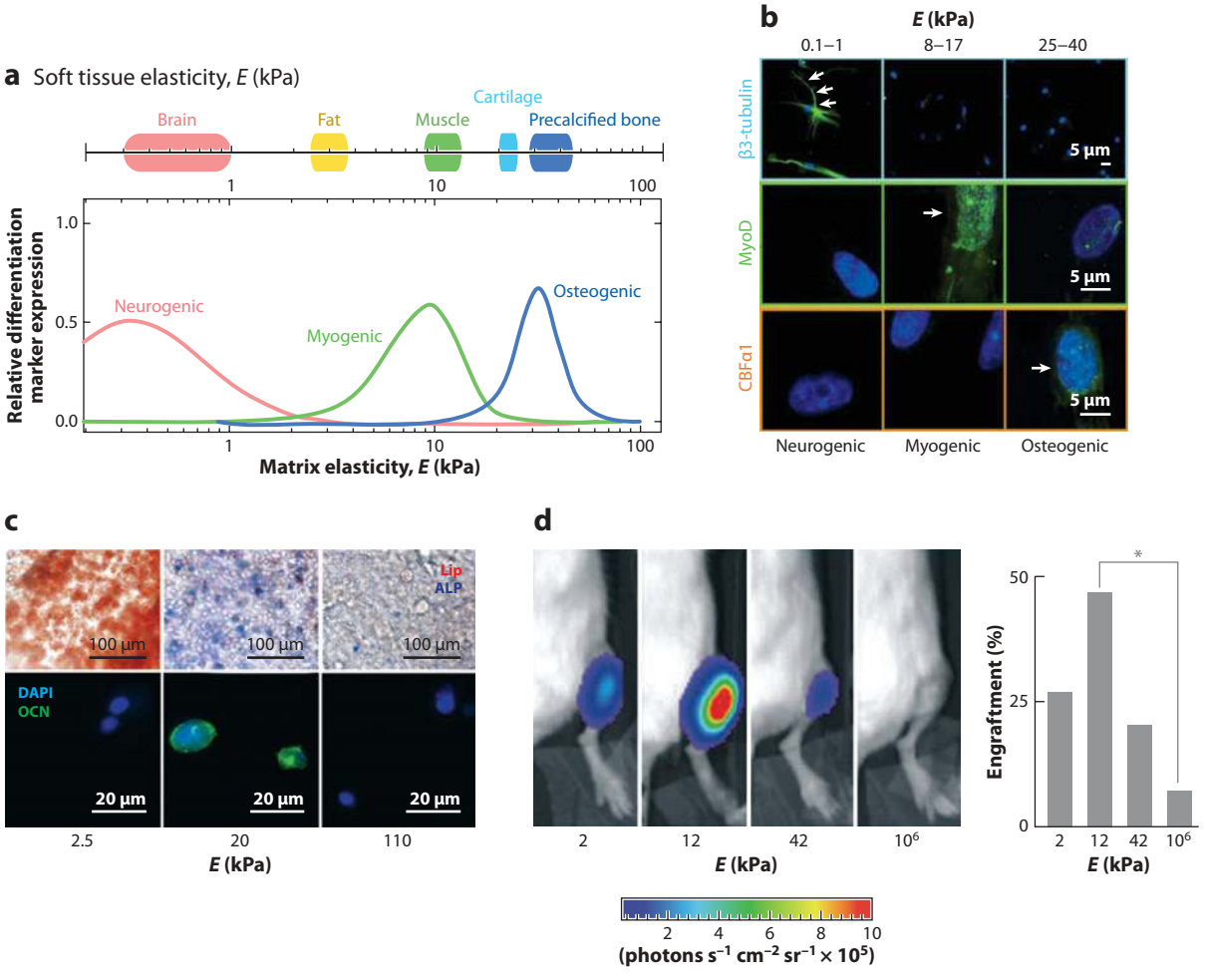
Other types of adult stem cells, including skeletal muscle stem cells (**Figure 4*d***) (43), hematopoietic stem cells (HSCs) (53), and adult neuron stem cells (8, 73, 108), have also been studied for their mechanoresponsive behaviors to matrix mechanics in both 2D and 3D cellular microenvironments. The most definitive experimental evidence to demonstrate mechanosensitivity of pluripotent ESCs to matrix mechanics was shown in a recent study by Chowdhury et al. (20), who reported that mESCs could maintain their pluripotency on soft polyacrylamide gels (~500 Pa) even under long-term culture conditions (at least 15 passages) without exogenous leukemia inhibitory factor (a soluble factor critical for maintenance of pluripotency of mESCs), in sharp contrast to mESCs seeded on the conventional rigid tissue culture plates. Importantly, traction force measurements of these mESCs demonstrated that their cytoskeleton contractility was mechanosensitive and correlated positively with the elastic modulus of the polyacrylamide gels (20), implicating involvement of cytoskeleton contractility in regulating their mechanosensitivity to changes in matrix mechanics.

Collectively, a few common observations can be drawn from the aforementioned studies of the mechanosensitivity of stem cells. All the studies have explicitly or implicitly suggested the involvement of cytoskeleton contractility in regulating the mechanosensitivity of stem cells, suggesting the importance of the force balance along the mechanical axis of the ECM-integrin-cytoskeleton linkage and their regulation by the mechanical signals in the stem cell niche. Moreover, strong evidence suggests that the differentiation potentials of stem cells toward distinct lineages can be maximized if the cells are cultured in the mechanical microenvironment mimicking their tissue elasticity in vivo (**Figure 4*a***). This observation is important for both

functional tissue engineering and developmental biology because it anticipates a major role of dynamic control for matrix mechanics in controlling stem cell function and tissue development. Indeed, dynamic regulation of matrix mechanics has emerged as a critical regulator of differentiation and morphogenesis (22, 88, 144). An emerging hypothesis has further suggested a role for the long-lived cytoskeleton structures as epigenetic memories to determine responses of stem cell shape, function, and fate to changes of matrix mechanics (38).

MECHANOTRANSDUCTION PATHWAYS TO REGULATE STEM CELL FATE

Stem cells can sense and respond to local biophysical signals through integrin-mediated FA signaling, and such signaling can be regulated by the force balance between endogenous cytoskeleton contractility and external mechanical forces transmitted across the cell-ECM adhesions. In this section, we discuss how this force balance across the mechanical continuum of ECM-integrin-cytoskeleton can be further transduced into the intracellular space of stem cells to mediate signaling molecules important for stem cell fate (**Figure 5**).



Integrin Signaling

Ras/MAPK signaling. Stem cells can sense and respond to biophysical signals through integrin-mediated FA signaling. Indeed, forces transmitted through FAs, generated either internally by cytoskeleton contractility or externally by mechanical forces, can trigger both mechanical and biochemical responses in cells. Forces at FAs activate several kinases involved in regulation of cellular functions (111, 116, 133, 137). Perhaps the most important players in this mechanotransduction system are FAK and Src family kinases such as Fyn (45, 72, 100, 132). One major downstream signaling pathway following FAK/Src activation is the Ras-Raf-MEK-ERK pathway (one branch of the MAPK pathway), and the exact molecular mechanism of how integrins regulate MAPK is not yet well defined. Several possible pathways have been proposed, including integrin-FAK-Grb2-SOS-Ras (114) and integrin-Fyn-Shc-Grb2-SOS-Ras (130), as well as through the epidermal growth factor receptor (13). ERK is then translocated to the nucleus to regulate gene expression by activating different transcription factors.

The Ras/MAPK pathway plays a critical role in stem cell fate. For example, MAPK signaling is required for stemness maintenance of both neural stem cells (NSCs) (14) and human epidermal stem cells (147). Interestingly, the FGF/MAPK cascade plays a functional role in promoting differentiation of mESCs, thus inhibition of MAPK signaling can support self-renewal of mESCs (105). In contrast, FGF/MAPK signaling promotes self-renewal of hESCs, indicating that hESCs may have cellular responses to the biophysical signals opposite those of mESCs.

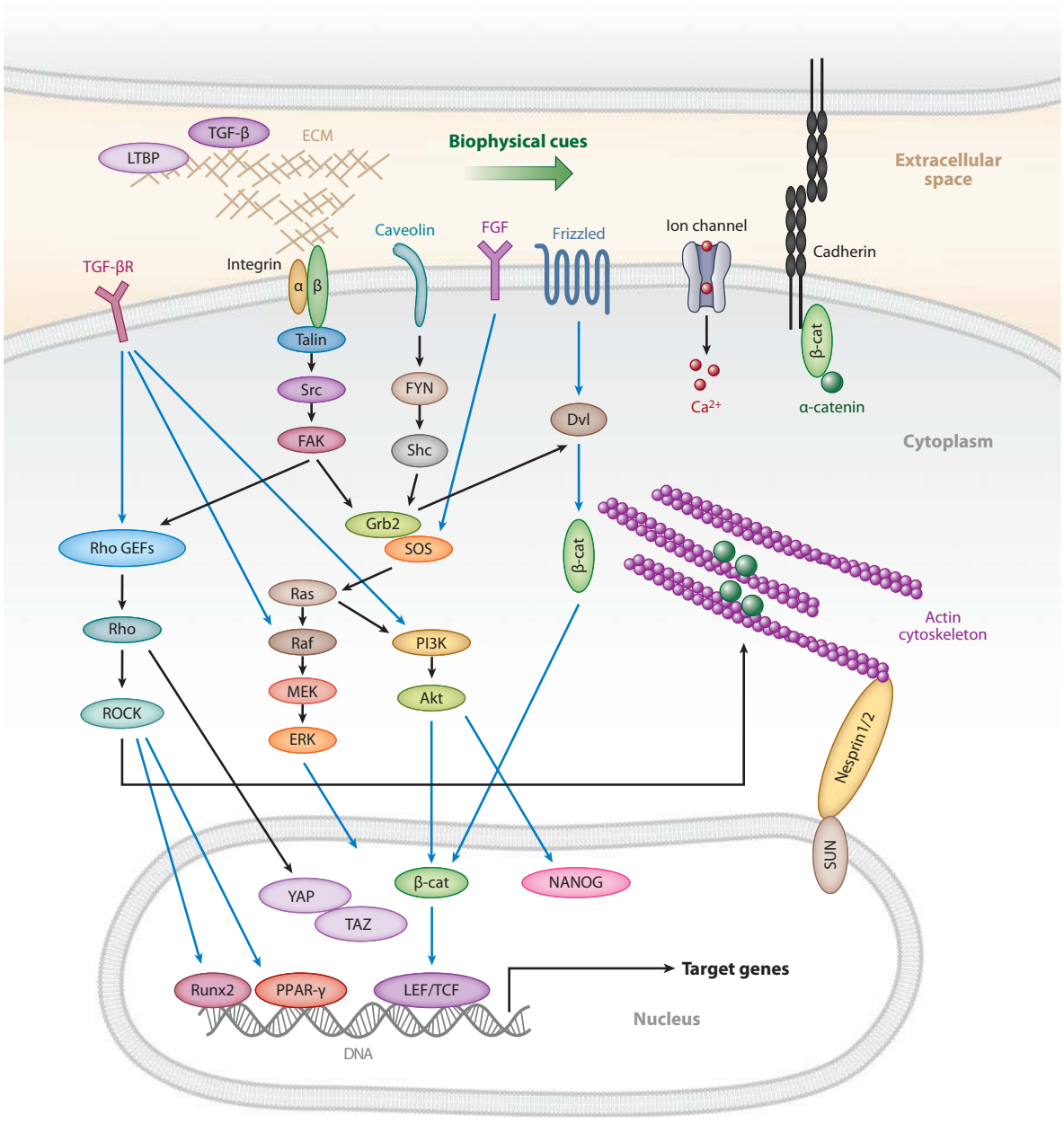
MAPK-mediated stem cell fate is dynamically required during different stages of stem cell differentiation. For instance, activated MAPK signaling is required for the early linage specification of mESCs to adipocytes, whereas the MAPK pathway has to be shut down during their terminal differentiation (11). This observation further suggests that spatial and temporal dynamic modulation of the biophysical signals in the stem cell niche can be necessary for optimizing stem cell behaviors.

Figure 4

Matrix mechanics directs stem cell fate. (*a*) Soft tissue elasticity scale ranging from soft brain, fat, and striated muscle to stiff cartilage and precalcified bone. In contrast, conventional TCPs have a much stiffer elastic modulus ($E \approx 10^6$ kPa). Varying matrix elasticity or rigidity can induce multipotent hMSCs to differentiate into different tissue cell types corresponding to the tissues' relative mechanical elasticity in vivo. The top part of panel *a* is adapted from Reference 30, Copyright © 2009, with permission from the American Association for the Advancement of Science. The bottom part of panel *a* is adapted from Reference 34, Copyright © 2006, with permission from Elsevier. (*b*) Matrix mechanics-dependent differentiation of hMSCs. hMSCs were stained for β3-tubulin, MyoD, and CBFα1 as markers of neurogenic, myogenic, and osteogenic differentiation, respectively. hMSC differentiation correlates to tissue-specific mechanical properties (e.g., soft matrix leads to neural differentiation, whereas stiff matrix leads to osteogenic differentiation). Reprinted from Reference 34, Copyright © 2006, with permission from Elsevier. (*c*) Matrix mechanics regulates mMSC fate in 3D matrix culture. (*Top row*) In situ staining of encapsulated clonally derived mMSCs for ALP activity (*blue*) and Lip (*red*) after 1 week of culture in the presence of combined osteogenic and adipogenic chemical supplements within encapsulating matrices consisting of RGD-modified alginate with varying matrix elasticity as indicated. (*Bottom row*) Immunofluorescence staining for OCN (*green*) and DAPI (*blue*) in cryosectioned alginate matrices of varying matrix elasticity containing mMSCs. Adapted from Reference 55, Copyright © 2010, with permission from Nature Publishing Group. (*d*) Cultured MuSC engraftment is modulated by matrix mechanics. Representative bioluminescence images of recipient mice 1 month after transplantation with 100 GFP/Fluc MuSCs after 7-day culture on substrates of varying stiffness *E*, as indicated. The bar graph (*right*) shows the percentage of mice from each experimental condition that had a bioluminescence value above the engraftment threshold. Asterisk here represents a Fisher's exact test with $P < 0.05$. Adapted from Reference 43, Copyright © 2010, with permission from the American Association for the Advancement of Science.
Abbreviations: TCP, tissue culture plates; hMSC, human mesenchymal stem cell; MyoD, myosin D; mMSC, murine mesenchymal stem cell; ALP, alkaline phosphatase; Lip, lipid droplet accumulation; RGD, arginine-glycine-aspartic acid; OCN, osteocalcin; DAPI, 4′,6-diamidino-2-phenylindole; MuSC, muscle stem cell; GFP, green fluorescent protein; Fluc, firefly luciferase; CBFα1, core-binding factor α1.

PI3K: phosphatidylinositol 3-kinase

PI3K/Akt. Another downstream pathway of Ras is the PI3K (phosphatidylinositol 3-kinase)/Akt pathway, which can also be activated through integrin signaling (18). The PI3K/Akt pathway is critical for the self-renewal and differentiation of both ESCs and somatic stem cells. Paling et al. (99) reported that PI3K signaling was activated by leukemia inhibitory factor and was required to maintain the self-renewal of mESCs, and one possible downstream target of PI3K/Akt signaling is NANOG (121). Other reports have suggested that PI3K was responsible for activating somatic



stem cells, such as HSCs (146) and intestinal stem cells (51), to exit from their quiescent states. There is also signaling cross-talk between the PI3K/Akt pathway and the Wnt signaling pathway, a central pathway that controls the fate decisions of many different stem cells (69).

RhoA/ROCK. By acting through its effector ROCK, RhoA is a key molecular regulator of actin cytoskeleton tension and FA formation (i.e., upstream regulator of integrin). RhoA/ROCK signaling also acts as a downstream target of integrin-mediated signaling through activated FAK (32). RhoA can be activated by different growth factors and cytokines as well as the biophysical signals from the cellular microenvironment. The functional role of RhoA/ROCK-mediated cytoskeleton contractility is well appreciated in the lineage commitments of hMSCs. Activating RhoA promotes osteogenesis of hMSCs by upregulating Runx2 expression, whereas inhibition of RhoA leads to adipogenesis of hMSCs (5, 86). In response to activated RhoA/ROCK signaling, intact actin cytoskeleton structure is required for mechanoresponsive hMSC differentiations. RhoA/ROCK-mediated cytoskeleton contractility can directly regulate certain gene expressions of transcription factors (e.g., PPAR-γ and Sox-9) to influence stem cell differentiation.

Wnt/β-catenin. Wnt/β-catenin signaling can regulate fate decisions of different stem cell types, including ESCs, HSCs, MSCs, and NSCs (10, 26, 84, 91). In the canonical Wnt pathway, the expression and nuclear translocation and accumulation of β-catenin are regulated through Dvl. The role of Wnt/β-catenin signaling in regulating stem cell fates can be complicated. For example, for mESCs, Wnt signaling is necessary for maintaining their pluripotency (7, 112); however, overexpression of β-catenin can also promote neural lineage commitment of mESCs (98). The signaling cross-talk between Wnt and integrin has been identified, and two different models involving integrin-linked kinase and FAK have been proposed. In the first model (95), integrin-linked kinase is suggested to stabilize and/or promote the nuclear accumulation of β-catenin; in the second model (24), Grb2 integrated integrin signaling through FAK with Wnt signaling via Dvl and JNK, a downstream kinase of Grb2, and promoted translocation of β-catenin into the nucleus.

Direct regulation of Wnt signaling by biophysical signals has been demonstrated in osteoblasts. Data show that mechanical loading could regulate Wnt signaling in a time-dependent manner (60). In this study, after a 15-min cyclic stretch, Wnt signaling in human osteoblasts was ultimately downregulated despite an initial increase of β-catenin expression.

TGF-β. TGF-β is a secreted protein that belongs to the TGF-β superfamily. It binds to a latent TGF-β-binding protein that is linked to ECM; therefore, TGF-β is stored in extracellular space (3). The most remarkable role of TGF-β is to inhibit cell proliferation. Given the fact that many

Figure 5

Schematic of signaling cross-talk between the mechanotransductive processes (*black arrows*) and other known soluble factor-mediated signaling pathways regulating the fate decisions of stem cells (*blue arrows*). Abbreviations: TGF-β, transforming growth factor β; LTPB, latent TGF-β-binding protein; TGF-βR, transforming growth factor β receptor; Rho GEFs, Rho guanine nucleotide exchange factors; ROCK, Rho-associated kinase; FAK, focal adhesion kinase; Grb2, growth factor receptor-bound protein 2; SOS, *Son of sevenless*; PI3K, phosphoinositide 3-kinase; FGF, fibroblast growth factor; Dvl, *Dishevelled*; β-cat, β-catenin; YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; Runx2, Runt-related transcription factor 2; PPAR-γ, peroxisome proliferator-activated receptor γ; LEF, lymphoid enhancer factor; Ca^{2+}, calcium ion; ECM, extracellular matrix; Src, Rous sarcoma oncogene cellular homolog; Shc, SH2-containing collagen-related proteins; FYN, a Src family tyrosine-protein kinase; SUN, Sad1p and UNC-84 homology; MEK, MAPK/Erk kinase.

adult stem cells need to be kept in a quiescent state, TGF-β plays important roles in this process. For example, TGF-β can inhibit expansion of NSCs and keep HSCs in their quiescent state (3, 10), and some studies have shown that TGF-β is critical for maintaining the pluripotency of hESCs via Smad2/3 signaling (59, 125). In addition to the canonical pathway via Smad2/3, TGF-β activates multiple major signaling pathways including MAPK, PI3K, and Rho/ROCK (85, 90).

The signaling cross-talk between integrin and TGF-β has been extensively studied. The regulation of TGF-β activation by integrin has been reviewed in detail by Margadant & Sonnenberg (82). Certain types of integrins can directly regulate activation of TGF-β either through cellular traction forces exerted by actin cytoskeleton or through some G-protein-coupled receptors. In addition, integrin can indirectly control the expression of the components in the TGF-β pathway, and it has also been shown that external mechanical forces can activate release of TGF-β from ECM (79, 134). TGF-β can also regulate actin cytoskeleton through the RhoA/ROCK pathway, which has been well recognized in the epithelial to mesenchymal transition process of tumor cells (9). Taken together, forces transmitted through integrins generated either internally by cytoskeleton contractility or externally by mechanical forces can activate TGF-β signaling, which in turn regulates stem cell fate. Several studies have confirmed this important signaling cross-talk between integrin and TGF-β. For example, the pluripotency of hESCs can be improved by directly applying a cyclic mechanical strain or indirectly using stiff substrates to activate latent TGF-β from ECM or fibroblasts as feeder cells (2, 110, 134).

Mechanosensitive Ion Channels

In addition to integrin signaling, mechanosensitive ion channels can also regulate mechanoresponsiveness of stem cells (83). Interestingly, based on the tethered model, mechanosensitive ion channels can be linked with ECM and/or cytoskeleton, and the relative displacement of channels with respect to ECM or cytoskeleton is responsible for the gating of channels (49). Thus, mechanosensitive ion channels can be directly activated by external forces or intracellular cytoskeleton contractility (50, 70, 122).

The major downstream effect of the activation of mechanosensitive ion channels is the changes of the cytoplasmic Ca^{2+} concentration as well as their oscillations (70). Ca^{2+} oscillations have been observed in MSCs and are considered as both an indicator and a regulator for MSC differentiation (64, 123). This Ca^{2+} concentration oscillation is influenced by substrate stiffness (68). Ca^{2+} oscillations have also been found in mESCs, human preadipocytes, and human cardiac progenitor cells (37, 54, 62), indicating that mechanical forces might have the potential to directly regulate the fates of these cell types through modulating calcium signals.

SUMMARY AND OUTLOOK

The molecular mechanisms by which stem cells maintain their self-renewal ability and control their differentiation need to be determined in order for these cells to be used effectively for functional tissue engineering and regenerative medicine. Much of our effort until now has focused on the biochemical components and soluble factors in the stem cell microenvironment that are critical for their self-renewal and differentiation. Yet, recent evidence demonstrates that stem cells are also heavily influenced by coexisting insoluble adhesive, mechanical, and topological cues contained within the dynamic stem cell niche. Experimental evidence has clearly suggested that insoluble biophysical signals, such as cell shape and geometry, external forces and matrix mechanics, and nanotopography can elicit intracellular programs to regulate stem cell fates, likely through the integrin-mediated FA signaling and the force balance across the mechanical continuum of ECM-integrin-cytoskeleton.

The molecular mechanisms for stem cells to sense and respond to different biophysical signals are not yet clear and likely would be specific to cell type and involve different mechanisms working in concert. It also appears that the dominant effect of different biophysical signals on stem cell functions depends on different experimental settings, and that stem cell fate is mediated by the intricate interactions and interdependencies between soluble factors and insoluble biophysical signals in their local cellular microenvironment.

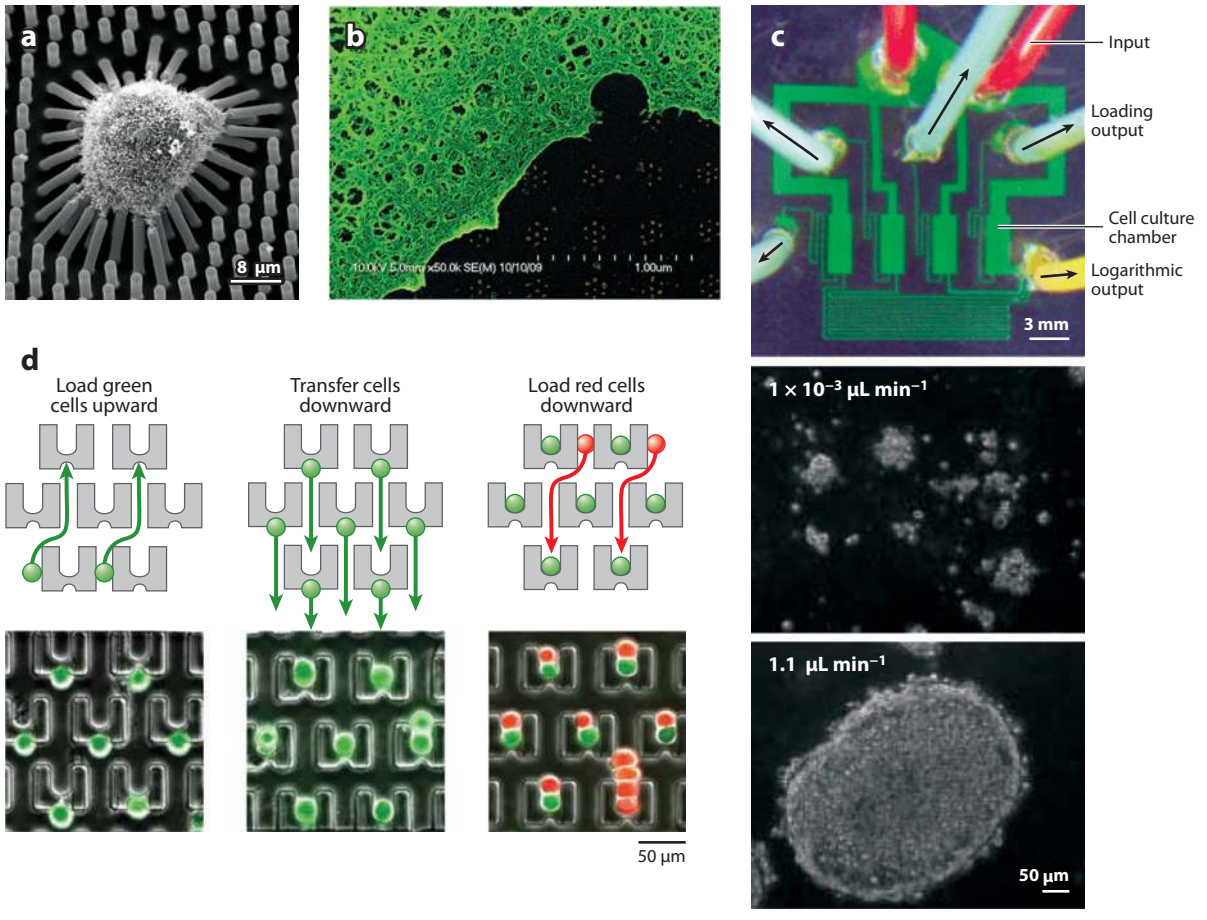


Figure 6

Microtechnology/nanotechnology for constructing synthetic in vitro stem cell niche to regulate stem cell fate. (*a*) SEM of single hMSCs plated on top of microfabricated PDMS microposts. The bending spring constant of the PDMS micropost could switch the differentiation potential of hMSCs between osteogenic and adipogenic fates. Adapted from Reference 40, Copyright © 2010, with permission from Nature Publishing Group. (*b*) SEM image showing single cells spreading on an array of nanodots fabricated using advanced sub-100-nm NIL. These nanostructured surfaces were used to explore how the geometric organization of the binding ligand RGD affects cell adhesion and spreading. Adapted with permission from Reference 115, Copyright © 2011, American Chemical Society. (*c*) Microfluidic arrays for logarithmically perfused mouse ESC culture. The top photograph shows a microfluidic device fabricated using soft lithography with multiple chambers for long-term culture of mouse ESCs. The bottom two brightfield images show colonies of mouse ESCs after 4 days of perfusion at different culture flow rates. Adapted from Reference 67 by permission of the Royal Society of Chemistry. (*d*) Microfabricated cell traps for cell pairing and fusion, by using a three-step cell-loading protocol, as indicated. Adapted from Reference 118, Copyright © 2009, with permission from Nature Publishing Group. Abbreviations: SEM, scanning electron micrograph; hMSC, human mesenchymal stem cell; PDMS, polydimethylsiloxane; RGD, arginine-glycine-aspartic acid; ESC, embryonic stem cell; NIL, nanoimprint lithography.

Moving forward, it is important to recognize that tissue development from stem cells in vivo is a long-term process in which dynamic changes in the chemical and physical environments surrounding the cells abound. How we can generate in vitro stem cell microenvironments to mimic the dynamic nature and complexity of the in vivo stem cell niche is currently a significant challenge. Researchers from different disciplines have devised different bioengineering strategies and microscale/nanoscale tools that can provide good controls of different aspects of the stem cell microenvironment. Some of these techniques have already been mentioned in the examples discussed above, which include microcontact printing, synthetic hydrogels, microfluidics, and microfabrication/nanofabrication (**Figure 6**). These tools, which span different scales, from molecular to cellular to organ levels, have proven to be extremely powerful in allowing stem cell biologists and tissue engineers to identify the extrinsic physical factors and their independent effects on stem cell fates. We envisage that in the future, these tools will be further polished and used in different combinations to allow researchers to generate dynamic and complex synthetic cellular microenvironments, with the molecular, structural, hydrodynamic, and mechanical cues well controlled in conjunction with their spatial and temporal levels and combinations. Given the complexity of the stem cell niche signals, it is also important to utilize high-throughput tools that can help screen different combinations of the environmental signals to elicit the desired stem cell behaviors. Such high-throughput screening assays no doubt can benefit from more in-depth understanding of the molecular mechanisms that regulate stem cell fate.

SUMMARY POINTS

1. Physical signals in the local cellular microenvironment can strongly influence stem cell fate.
2. By controlling cytoskeleton tension, cell shape is a key regulator of stem cell fate.
3. Nanotopographical cues can control stem cell behaviors by modulating the molecular arrangement, dynamic organization, and signaling of the cellular adhesion machinery.
4. External mechanical forces and matrix mechanics can regulate stem cell behaviors through the force balance along the mechanical continuum of the ECM-integrin-cytoskeleton linkage, and their regulation by the mechanical signals in the stem cell niche.
5. The force balance across the ECM-integrin-cytoskeleton linkage can be further transduced into the intracellular space of stem cells to mediate signaling molecules important for stem cell fate, such as those mediated by integrin signaling and mechanosensitive ion channels.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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