

Physical and Engineering Principles in Stem Cell Research

David V. Schaffer

Introduction

Biological research in general is becoming increasingly interdisciplinary, and stem cell research in particular has strong potential to become progressively more so. In this field, there has for example been a growing recognition that, while biochemical signals play critical roles in regulating the behavior and fate decisions of stem cells, biology presents regulatory information to cells not only in the binary absence or presence of a given molecule, but also numerous biophysical aspects of these regulatory cues. These include mechanics, topographical features at multiple size scales, electrostatics, spatiotemporal variation in the presentation of biochemical cues, transport phenomena, and biochemical reaction kinetics. As a result, there are considerable opportunities for physical scientists and engineers to become increasingly involved in stem cell research, not only to gain basic insights into new mechanisms in stem cell biology but to create new technologies to advance this field. Within this report, chapter “High-throughput Screening, Microfluidics, Biosensors, and Real-time Phenotyping” discusses the development of technologies to discover novel signals that regulate stem cell behavior, and chapter “Computational Modeling and Stem Cell Engineering” reviews progress in the development of mathematical models that quantitatively investigate the underlying regulatory mechanisms. The present chapter will review research into how biophysical features of the microenvironment or niche regulate the behavior of a stem cell.

D.V. Schaffer (✉)

Berkeley Chemical and Biomolecular Engineering, University of California,
274 Stanley Hall, Mail Code 3220, Berkeley, CA 94720, USA
e-mail: schaffer@berkeley.edu

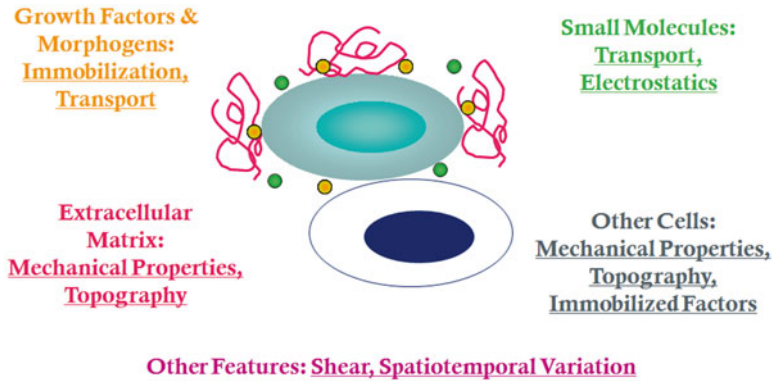


Fig. 1 Schematic of the stem cell niche (Courtesy of the author)

Biochemical and Biophysical Information in the Stem Cell Niche

During development and throughout adulthood, stem cells reside within specialized regions of tissue that continuously present them with regulatory cues, and this repertoire of signals is collectively referred to as the stem cell niche (Schofield 1978; Watt et al. 2000; Scadden 2006). The niche presents a stem cell with considerable molecular information, in the form of soluble molecules; extracellular matrix (ECM) proteins, glycosaminoglycans, and proteoglycans; growth factors and morphogens that may be soluble or immobilized to the ECM; and cues presented from the surface of neighboring cells (Fig. 1). Soluble small molecules, soluble and immobilized proteins, ECM components, and intercellular components collaborate to regulate stem cell behavior. In addition, there are numerous physical and engineering principles that modulate the manner in which these components present information, including mechanical properties, spatial organization, and temporal variation in the presentation of cues, topographical features of the niche on the nanoscale and microscale, mass transport properties, and electrostatics.

Due to efforts in genetics, developmental biology, and cell biology, it is well recognized that biochemical cues within the niche play critical roles in regulating stem cell function. As a prominent example, forward genetics approaches in model organisms are a classical approach to identify novel factors that play roles in organismal development, in many cases via regulating stem cells in developing tissues. For instance, the segment polarity gene *hedgehog* was originally discovered in a random mutagenesis screen in *Drosophila melanogaster* for embryonic lethal phenotypes (Nusslein-Volhard and Wieschaus 1980). The Jessell lab later found that the vertebrate homolog *Sonic hedgehog* (Shh) played a critical role in the differentiation of motor neurons in the developing spinal cord (Roelink et al. 1994), and the same lab subsequently demonstrated that Shh—in combination with other

developmentally important factors—could help guide or instruct the differentiation of mouse embryonic stem cells (mESCs) into motoneurons in culture (Wichterle et al. 2002). In an analogous approach, collections of factors previously discovered via forward genetics and other approaches can be screened for their potential effects on particular stem cell populations. As one such example of a candidate approach, the founding member of the Wnt family of proteins was originally discovered first in *Drosophila* as a gene whose mutation led to the absence of wings (and was thus named wingless (Sharma and Chopra 1976)) and in vertebrates as a gene whose transcriptional activation promotes mammary tumorigenesis (Nusse et al. 1984). Given the importance of Wnt family members subsequently demonstrated for numerous tissues (van Amerongen and Nusse 2009), they have been considered as prominent candidate regulators of stem cell function. In one such important study, Wnt3 was demonstrated to regulate the neuronal differentiation of neural stem cells in the adult brain (Lie et al. 2005).

In addition to clearly demonstrated role of many biochemical cues in regulating stem cell function, as exemplified in the forward genetics and candidate molecule studies cited above, there are numerous biophysical features of the niche that may offer additional regulatory control over stem cells. However, the biophysical properties of a tissue are not monogenic, i.e., they depend on the properties of many molecules and genes. For example, the mechanical properties of a tissue are determined by its constituent materials, including cells and ECM. Analogously, the topography of a tissue depends on the identities of its ECM and cells as well as the history of their assembly, and mass transport properties vary with the tissue interstitial space and potential fluid flow. As a result, these properties do not arise in a straightforward manner in genetic screens, which perhaps contributes to the fact that they have not been as broadly studied in stem cell research as biochemical factors.

However, an emerging theme in the nascent field of stem cell engineering is to use *in vitro* engineered systems—ranging from synthetic materials to microfluidic devices—to systematically vary these biophysical properties, i.e., to provide them with an “x-axis” in a manner that is not currently possible using genetic approaches. While there are inherent challenges with this approach—including demonstrating the *in vivo* relevance of findings, as well as integrating engineering and biology approaches to explore underlying mechanisms—these engineering approaches have broadened the field’s view of the stem cell niche (Saha et al. 2007; Discher et al. 2009; Guilak et al. 2009; Lutolf et al. 2009; Keung et al. 2010).

This chapter discusses the application of engineered microenvironments—or systems that emulate the niche—to vary and thereby investigate the effects of biophysical properties of tissues on stem cell behavior. In addition, while a number of these studies discover new phenomena in the biophysical regulation of stem cell function, there has been increasing progress in understanding mechanisms by which cells respond to these cues. Finally, the application of physical and engineering approaches to create additional technologies to study stem cell function will be discussed, as well as future opportunities for engineers and biologists in the stem cell field.

Mechanoregulation of Stem Cell Function

There are many mechanical properties of tissues that could potentially regulate cell function. The elastic modulus is the linear proportionality constant between the stress applied to a material and its strain or deformation. Though elastic modulus is sometimes used interchangeably with stiffness, the former is an intensive property of the material, whereas the latter extensive property depends on material geometry. In addition, elastic stress-strain relationships can be nonlinear. Furthermore, many tissues are viscoelastic, or have both elasticity and viscosity, i.e., a fluid property describing resistance to deformation by either shear or tensile stress. Like elasticity, viscosity can also be linear or nonlinear, and in all cases these material properties of a tissue can vary in space and time (Humphrey 2003). In principle, a resident cell may be able to sense and respond to any or all of these material properties.

Static Mechanical Properties

Given the complexities of a material's mechanical properties, the field has made strong progress by initially focusing on linear properties, with a strong focus on elastic modulus. In 1997, to study the effects of material stiffness on cell migration, Pelham and Wang developed a linearly elastic, bioactive material—specifically a polyacrylamide (PA) hydrogel coated with the ECM protein collagen—in which the modulus could be varied by changing the proportion of crosslinker during polymerization. Aided by this system, in landmark work Engler and Discher (Engler et al. 2006) demonstrated that the lineage choice of differentiating mesenchymal stem cells (MSCs) is strongly influenced by substrate stiffness, such that cells developed into neuron-like cells on soft PA gels, myoblasts on intermediate stiffnesses, and osteocytes on harder substrates (Fig. 2). They thus proposed that MSCs on different stiffnesses differentiate into cell fates associated with tissues that correspond to those stiffnesses. In work that extended mechanoregulation to another stem cell type, Saha et al. demonstrated that neural stem cells (NSCs) preferentially differentiate into neurons when cultured on soft materials and astrocytes on hard materials (Saha et al. 2008a, b). Additionally, Banerjee et al. found that the effects of stiffness on NSC differentiation extended to cells embedded in three-dimensional (3D) materials (Banerjee et al. 2009). Moreover, the extent of maturation of neurons differentiated from NSCs was enhanced on soft vs. stiff gels (Teixeira et al. 2009).

In addition to differentiation, modulus can influence stem cell self-renewal. For example, it was shown that substrate stiffness strongly impacts the ability of muscle stem cells (also termed satellite cells) to undergo self-renewal in culture. Muscle stem cells were isolated from muscle and grown on soft or stiff substrates composed of a polyethylene glycol hydrogel. Cells grown on the former but not the latter stiffer material were able to expand and, upon implantation into adult muscle,

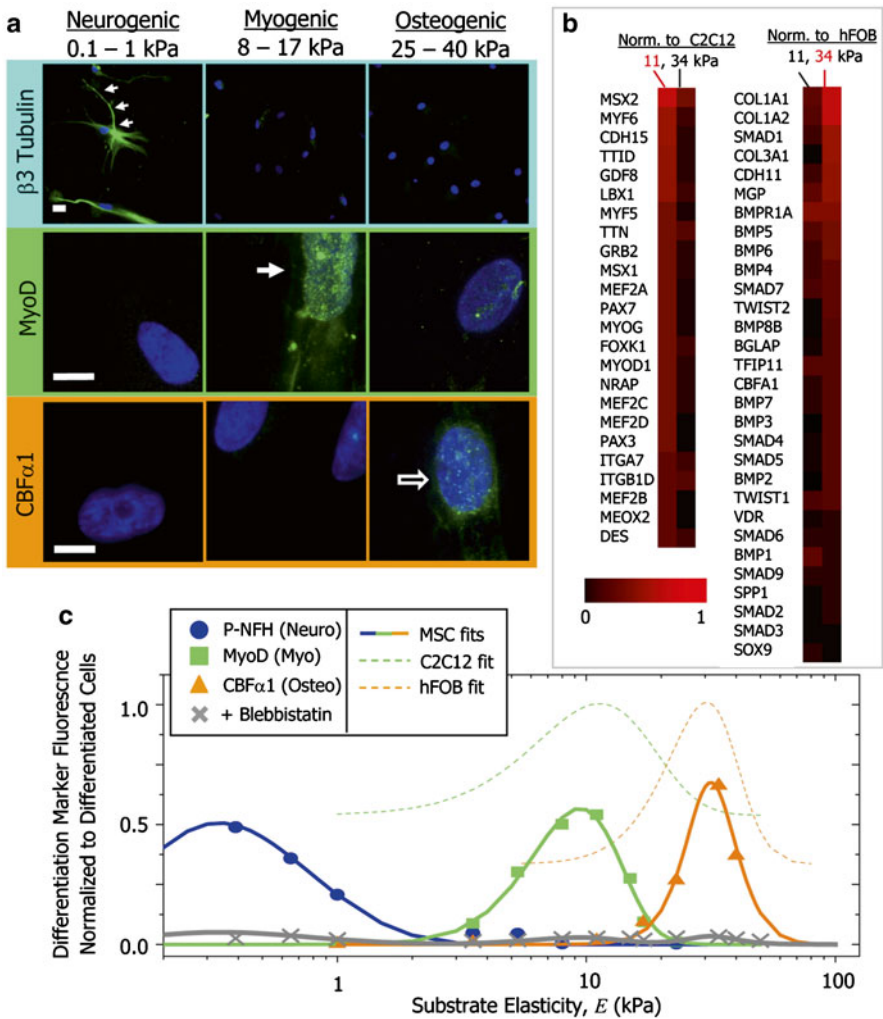


Fig. 2 Substrate stiffness directs mesenchymal stem cell differentiation (From Engler et al. 2006). (a) The neuronal marker β -tubulin III is expressed in MSCs differentiated on soft gels, the muscle transcription factor MyoD1 is expressed on substrates of intermediate elasticity, and the osteoblast factor CBF α 1 is expressed on stiff substrates. (b) Microarray profiles are shown for MSCs differentiated on 11 vs. 34 kPa matrices, showing upregulation of markers indicative of muscle or osteogenic differentiation. (c) Differentiation marker expression as a function of substrate stiffness reveals optimal differentiation into a given lineage at the stiffness characteristic of that lineage

contribute to the tissue (Gilbert et al. 2010). Furthermore, mouse embryonic stem cell self-renewal is promoted on soft substrates, accompanied by downregulation of cell-matrix tractions (Chowdhury et al. 2010). Finally, the development of an innovative high-throughput system for analyzing the effects of stiffness, and other microenvironmental properties, on stem cell function promises to accelerate progress in this area (Gobaa et al. 2011).

Mechanisms for Stiffness Regulation of Stem Cell Fate

At its essence, mechanoregulation of stem cell function requires the cell to convert an extracellular mechanical cue into an intracellular biochemical response (i.e., activation or repression of genes involved in stem cell self-renewal or differentiation). There are numerous mechanisms by which material mechanical properties could influence stem cell behavior. One possibility is that the ECM itself is the “mechanosensor,” as it has been shown that forces involved in cell adhesion can unfold the ECM protein fibronectin and thereby expose additional biochemical information to the cell (Smith et al. 2007). Alternatively, in work showing that MSCs also differentiate in response to material stiffness in 3D, it was shown that the number of bonds between integrins and RGD peptides (i.e., synthetic adhesive ligands containing the arginine-glycine-aspartic acid motif) varied biphasically with stiffness (Huebsch et al. 2010). It was thus proposed that the number of adhesive bonds, which was in turn modulated by cellular reorganization of matrix to cluster the adhesive ligands near integrins, was correlated with downstream cell fate. In more recent work, investigators found that the porosity of polyacrylamide, but not polydimethylsiloxane (PDMS) gels, increased with decreasing modulus (Trappmann et al. 2012). They likewise found that MSC differentiation varied with PA but not with PDMS stiffness, leading them to propose that differences in the number of points or positions within ECM proteins that were crosslinked or anchored to materials of different stiffness was responsible for apparent mechanosensitive cell differentiation. However, this intriguing finding should also be analyzed in light of mechanosensitive NSC and MSC differentiation on hydrogels functionalized with either RGD peptides (Saha et al. 2007; Huebsch et al. 2010) or large ECM proteins (Engler et al. 2006; Keung et al. 2011).

The next question is how cells sense and respond to adhesive bonds. Tension between adhesion receptors and the extracellular matrix is transmitted across the membrane and into the cytoskeleton. These forces are accompanied by the assembly of focal adhesions and biochemical activation of enzymes (e.g., kinases) within this structure. It is clear that the former, specifically actin-myosin contractility, is necessary for stem cell mechanosensitivity. In their original work, Engler and Discher showed that addition of blebbistatin, a myosin II inhibitor, both blocks both cell cortical stiffening and myogenic differentiation as a function of substrate stiffness (Engler et al. 2006). Fu and colleagues, as an innovative alternative method for varying substrate stiffness, generated molded arrays of elastomeric PDMS posts (Fu et al. 2010). By culturing MSCs on posts of variable high height, which thereby require variable magnitudes of cellular force to bend the posts, they again showed that substrate compliance regulates cell fate. Furthermore, they demonstrated that early, transient addition of an inhibitor of Rho kinase, an enzyme that both stabilizes actin filaments and promotes myosin contractility, decreased the extent of osteogenic differentiation 7 days later. Recently, Keung and others showed that NSCs elevate RhoA and Cdc42 (but not Rac1) activity on stiffer substrates and that RhoA and Cdc42 inhibition block the ability of cells to stiffen as a function of substrate stiffness

(Keung et al. 2011). Furthermore, RhoA/Cdc42 activation or inhibition promoted astrocytic or neuronal differentiation respectively *in vitro*, in a manner depending on myosin contractility, and upregulation of RhoA activity in NSCs within the adult brain blocked neuronal differentiation.

These results firmly establish a role for actin-myosin contractility in stem cell mechanoregulation. However, it is unclear how such changes in the cellular cytoskeleton translate into changes in gene expression that drive cell differentiation. An important link was discovered when Dupont and colleagues implicated YAP and TAZ—transcriptional coactivators typically associated with the Hippo signaling pathway—in MSC mechanoregulation. Specifically, they found that these molecules localize to the nucleus in MSCs on stiff but not soft substrates, in a manner dependent on Rac1 and RhoA activity but not Hippo signaling. Furthermore, their knockdown ablates the effects of stiff substrates on MSC differentiation (Dupont et al. 2011). Future efforts will likely elucidate the link between the cytoskeleton and YAP/TAZ, the mechanisms of action for YAP/TAZ in the nucleus, and whether these mechanisms are general to other stem cell types.

Shear Flow

In addition to mechanical interactions with solid phase components of the niche, in some cases stem cells may be exposed to fluid flow and therefore shear forces, particularly within the cardiovascular system. As an early example in this area, Yamamoto et al. found that endothelial progenitor cells, isolated from human blood, responded to laminar flow in adherent culture by increasing their proliferation, enhancing their expression of endothelial markers such as vascular endothelial growth factor (VEGF) receptors, and showed increased tube formation, an *in vitro* readout indicative of vascular formation activity (Yamamoto et al. 2003). In subsequent work, they sorted a fraction of mouse embryonic stem cells (mESCs) that express VEGF receptor 2 (otherwise known as Flk-1) and found that exposing these cells to laminar flow for 24–96 h induced the upregulation of a number of endothelial cell markers in a Flk-1-dependent manner (Yamamoto et al. 2005).

The effects of shear flow on stem cells have also been investigated mechanistically. For example, analogous to the work of Yamamoto, Zeng and others found that shear flow induced the endothelial differentiation of mES cells, and they further explored the mechanisms underlying this process (Zeng et al. 2006). Specifically, shear increased the deacetylation of p53 by histone deacetylase 3 (HDAC3), the activated p53 upregulated expression of the p21, and this cell cycle inhibitor contributed to mESC differentiation. Furthermore, they found that Flk-1 activation by shear, and subsequent activation of the kinase Akt, were required for the HDAC3 activity. Illi and colleagues found, under somewhat similar culture conditions, that shear rapidly altered patterns of histone acetylation in mESCs, and after 24 h of shear exposure, not only endothelial but also cardiovascular markers were upregulated (Illi et al. 2005).

In additional work that is both very creative and practical, investigators explored a potential relationship between mechanical forces and early embryonic development. Naruse and colleagues noted that during passage through the oviduct, embryos are exposed to shear flow, compression, and stretching. In effort to address problems in fertility, they hypothesized that a mechanically active culture system could improve the quality and viability of embryos (Matsuura et al. 2010). The resulting device, a tilting embryonic culture system (TECS) that exposes cells to cyclic shear flow by oscillatory tilting, has enhanced embryo quality for usage in *in vitro* fertilization in recent clinical studies.

Cyclic Strain

In addition to the static mechanical properties of organs and tissues, due to the action of the heart and lungs, many tissues experience cyclic strain with a ~1 Hz frequency. Furthermore, organismal motion made possible by the musculoskeletal system also exposes these and other tissues to strain. Based on these considerations, the effects of cyclic strain on stem cells have been investigated.

In important work, Saha and others applied biaxial cyclic strain to human embryonic stem cells (hESCs) cultured on a deformable elastic substrate and found that strains >10 % inhibited cell differentiation, independent of strain frequency (Saha et al. 2006). They subsequently found that this strain promoted the secretion of transforming growth factor beta (TGF- β), activin, and Nodal into the medium, which in turn contributed to the inhibition of differentiation and promotion of self-renewal via autocrine/paracrine signaling (Saha et al. 2008a, b). In conceptually related work, Shimizu and colleagues applied uniaxial cyclic strain to mESCs cultured on silicone membranes, with 4–12 % strain at 1 Hz for 24 h. Cells aligned perpendicular to the direction of strain, and importantly differentiated into a vascular smooth muscle cell fate in a mechanism dependent on the upregulation of platelet derived growth factor receptor beta (PDGFR β) (Shimizu et al. 2008).

In work that blended cyclic strain with topographical cues (an area discussed greater detail below), Kurpinski and others plated MSCs on elastic substrates patterned with microgrooves to align cell polarity (Kurpinski et al. 2006). When uniaxial strain was applied parallel but not perpendicular to the direction of cell alignment, MSC proliferation and expression of smooth muscle cell markers increased substantively. This work indicates that not only the magnitude and frequency of strain, but its orientation relative to that of the cell, are important.

Dynamic Mechanical Forces During Tissue Morphogenesis

During the process of organismal development, it is appreciated that cells and multicellular structures exert forces on one another in a manner critical for morphogenesis (Ray et al. 2008). Recent innovative investigations have utilized embryonic

stem cells as model systems to address fundamental questions about the role of forces in tissue development. Specifically, the development of the optic cup (a structure that contains the retina and underlying retinal pigment epithelium) initially involves the evagination of the nascent structure to create a “bud,” followed by invagination from the surface of the bud to create a double walled cup (Fig. 3n) where the outer wall becomes the retinal pigment epithelium and the inner wall the neuroretina (Eiraku et al. 2011, 2012). There has been a longstanding debate in the field about the mechanism that enables the invagination to occur, and specifically whether the physical forces necessary for this process are intrinsic to the retinal tissue or require the external action of another structure such as the lens. Sasai and colleagues showed that under certain conditions in mESC aggregates, the evagination and subsequently the invagination occur, implicating an intrinsic or autonomous mechanism (Fig. 3). They proposed that at the onset of the invagination, the ring of cells that would eventually form the rim of the cup exert cytoskeleton-dependent forces to form a wedge shape and by extension create a flattened outer side of the bud. Subsequent rounds of cell division on that flattened side lead to increased surface area and thereby generate compressive forces that bend the layer inward to yield the cup. This highly innovative blend of stem cell and developmental biology yielded insights not only into fundamental mechanisms of tissue development but also offers future translational promise for the ability of stem cells to generate complex tissue structure in culture.

Topographical and Shape Features of the Stem Cell Niche

In addition to providing resident stem cells with a mechanical milieu, niches offer features that can alter the shape of a cell. On the microscale, ECM, neighboring cells, and in some cases mineralized tissue can modulate and even constrain the surface area or volume available to, and therefore the shape of, a cell in a manner important for its function (Paluch and Heisenberg 2009). Likewise, on the nanoscale, ECM proteins often assemble into fibers and other structural features that modulate the topographical features that an adherent cell experiences. Advances in lithography and in materials science have enabled investigators to investigate the effects of these features on stem cell behavior (Kolind et al. 2012).

Cell Shape

In seminal work, which predated investigation of mechanical properties on stem cell differentiation, McBeath et al. used microcontact printing to pattern adhesive islands of different sizes onto a surface (Fig. 4) (McBeath et al. 2004). When MSCs were seeded onto these substrates, it was found that large 10,000 μm^2 islands permitted cell spreading and promoted osteogenic differentiation, whereas small 1,024 μm^2

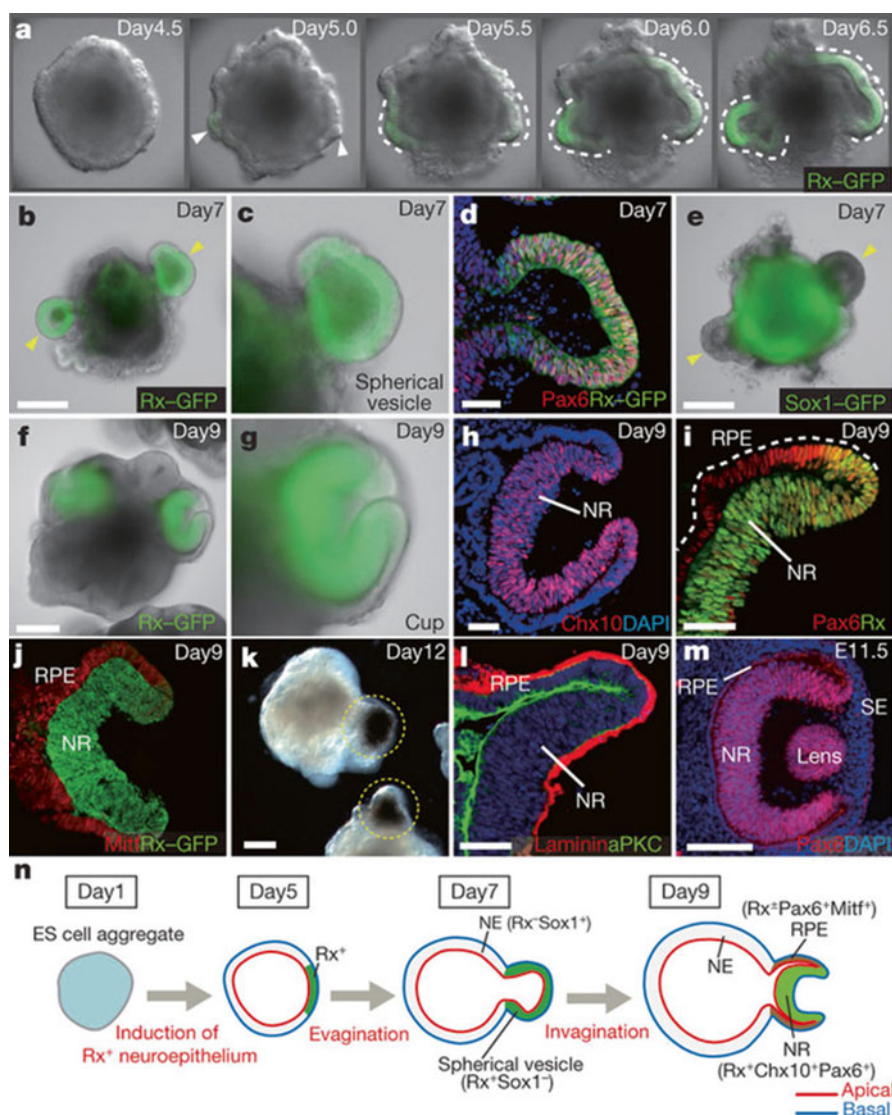


Fig. 3 Development of an optic cup in embryonic stem cell cultures (From Eiraku et al. 2011). (a) Mouse embryonic stem cells expressing GFP under the control of the retina and anterior neural fold homeobox promoter were grown in aggregates. Over 6.5 days, evaginated structures reminiscent of the nascent optic cup were progressively formed. (b, c, f, g) The resulting structure fully budded to form a vesicle, which then invaginated to form a cup. (d, e, h, i) The resulting structures were initially positive for the retinal marker Pax6 but negative for the immature neuroectodermal marker Sox1, and subsequently expressed neuroretinal marker Chx10. (j) The outer shell of the cup, corresponding to retinal pigment epithelium, expressed the marker MITF and (k) subsequently generated pigment. (l) The polarized cell layers expressed the apical marker aPKC and laminin in a surrounding basement membrane. (m) The corresponding structure of the E11.5 mouse eye. (n) A schematic for optic cup self-formation. NE neuroectoderm, NR neuroretina, RPE retinal pigment epithelium, SE surface ectoderm

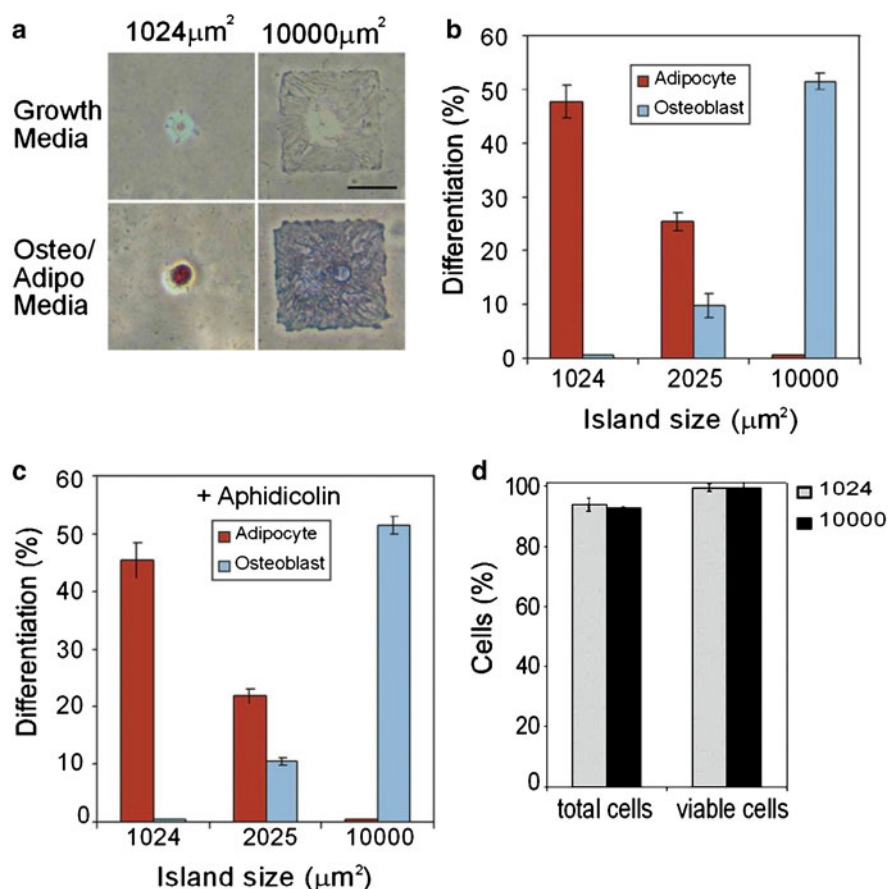


Fig. 4 Cell shape directs mesenchymal stem cell differentiation (From McBeath et al. 2004). (a) Human MSCs were plated onto small (1,024 μm^2) or large (10,000 μm^2) adhesive patterns coated with fibronectin after 1 week in growth or mixed medium. Under mixed differentiation conditions, cells differentiated into adipocytes (red) or osteocytes (blue). (b and c) Proportion of MSC differentiation into adipocytes or osteoblasts after 1 week of culture on 1,024, 2,025, or 10,000 μm^2 islands, either without (b) or with (c) the mitotic inhibitor aphidicolin. (d) Total cells and viable cells within the cultures

islands did not enable substantial cell spreading promoted adipogenic differentiation. Furthermore, cell spreading led to activation of RhoA, and its inhibition promoted to adipocyte differentiation. In subsequent work from this group, which extended the principle to another MSC fate decision, Gao and colleagues showed that upon exposure to TGF- β 3, well spread MSCs underwent smooth muscle cell (SMC) differentiation. In contrast, rounded MSCs differentiated into chondrocytes (Gao et al. 2010). Furthermore, the SMC fate was dependent on Rac1 activation and subsequent N-cadherin expression. These results thus indicate that controlling cell shape, which has an impact on the cytoskeleton, invokes cellular mechanical

mechanisms analogous to those observed for MSCs cultured on different stiffness substrates. In fact, Dupont and others showed that both stiff substrates and microcontact printed islands that enable MSC spreading promoted YAP/TAZ nuclear translocation, which as discussed above plays a key role in mechanosensitive MSC differentiation (Dupont et al. 2011).

The use of materials technologies to control the shape of cell aggregates has also yielded insights into stem cell function. For example, microfabricated polydimethylsiloxane (PDMS) wells surrounded with functionalized protein-resistant, self-assembled monolayers (SAMs) were used to create hESC aggregates of uniform size, which led to higher expression levels of the pluripotency marker Oct-4. In another key study, hESC culture inside microfabricated polyethylene glycol wells yielded embryoid bodies (EBs, an embryonic stem cell aggregate that differentiates in a manner bearing some similarities to that of an early embryo) of various sizes (Karp et al. 2007; Hwang et al. 2009). Higher endothelial cell differentiation was observed in the smaller (150 μm) EBs, due to higher Wnt5a expression, whereas larger (450 μm) EBs enhanced cardiogenesis, as a result of higher Wnt11 expression. Interestingly, another group used microcontact printing of adhesive islands on 2D substrates to control hESC colony size and showed that smaller hESC colonies became more endoderm-biased, whereas larger colonies exhibited greater differentiation into neural lineages (Bauwens et al. 2008).

Topographical Properties

In addition to microenvironmental properties that alter cell shape on the micron scale, topographical cues—such as the organization of the ECM into fibers (Singh et al. 2010)—offer a cell with features that can modulate its shape at the nanometer scale. Such topographical cues are considered to provide features intermediate between a 2D and a 3D microenvironment, and they can be generated synthetically by several techniques, including electrospinning, self-assembly of materials, and lithography based methods.

In early work in this area, coculture of adult NSCs with astrocytes on microgrooves patterned into polystyrene led to significantly higher extents of neuronal differentiation compared to flat surfaces (Recknor et al. 2006). Another study explored the effects of electrospun fibers of polyethersulfone with different dimensions on the behavior of adult NSCs, and they found that fibers of small dimension (283 nm) promoted oligodendrocyte specification, whereas larger fibers (749 nm) increased neuronal differentiation (Christopherson et al. 2009; Fig. 5).

Leong and colleagues investigated the behavior of human MSCs on a 350 nm grating topography and found that cells exhibited smaller and more dynamic focal adhesions, which apparently underlay a faster cell migration along the direction of the grating (Kulangara et al. 2012). They interestingly found that cells specifically on this grating size had lower expression levels of the focal adhesion protein zyxin, which likely contributed to the focal adhesion behavior. Furthermore,

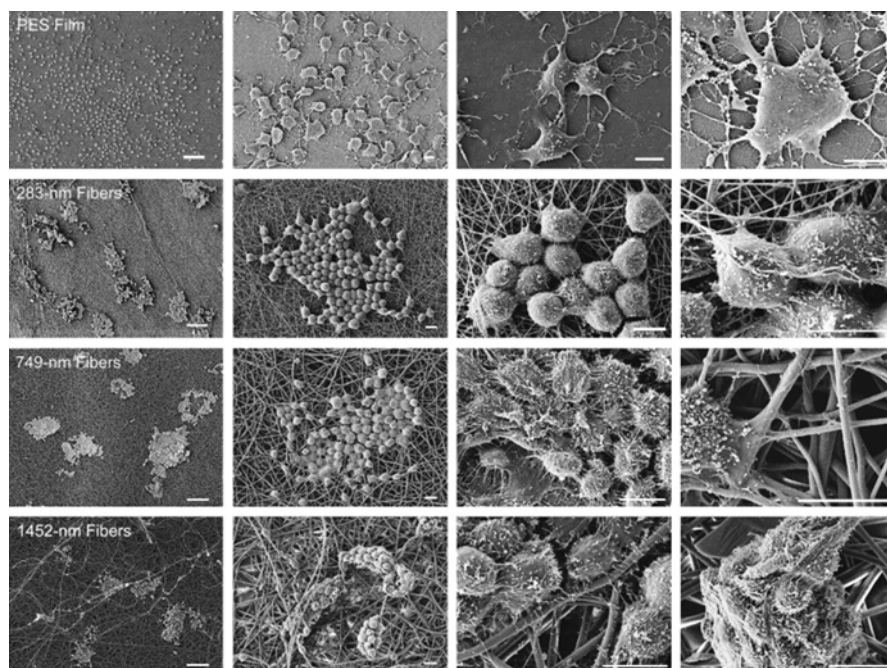


Fig. 5 Substrate topography modulates neural stem cell differentiation (From Christopherson et al. 2009). Adult rat neural stem cells were cultured on flat polyethersulfone (PES) films, or electrospun PES fibers of various dimensions. Scanning electron microscopy images of cultured in serum free medium and FGF-2 for 5 days show strong engagement with the surface topography. Under differentiating conditions, and relative to cultures on tissue culture polystyrene, cells showed a 40 % increase in oligodendrocyte specification on the 283 nm fibers and a 20 % increase in neuronal differentiation on the 749 nm PES fibers

addition of the neuronal inducing factor retinoic acid led to higher extents of neuronal marker expression from MSCs on the 350 nm spaced grating (Yim et al. 2007).

Electric Fields

The role of electrophysiology in the cardiovascular and nervous systems is well appreciated, and several investigators have explored the possibility that electric fields could play a role in regulating the function of stem cells in these tissues. In seminal work in this area, Radisic et al. (2004) seeded neonatal cardiomyocytes onto a collagen sponge and subjected them to a square wave electrical field with 1 Hz frequency, to emulate the natural electrophysiological environment of the heart. Cells became aligned with the direction of the field, exhibited a substantial increase in contractile amplitude, and expressed higher levels of various cardiac protein markers compared to cells that were not electrically stimulated. In another key

study, electric fields designed to mimic neuronal activity enhanced the differentiation of muscle precursor cells (Serena et al. 2008).

The role of electric fields in the maturation and neurite outgrowth of neurons has long been studied (Hinkle et al. 1981), and new materials, for example an electrically conducting polymer, have been shown to enhance neurite outgrowth and neuronal maturation (Schmidt et al. 1997). In addition, the role of electric fields on the behavior of neural stem cells has recently been studied. For example, oscillating electric fields were found to favor NSC survival at a 1 Hz frequency, and to promote astrocytic over neuronal differentiation at 1 Hz (Matos and Cicerone 2010). Under a direct current, adult subependymal neural precursors were found to migrate to the cathode, raising the possibility of guiding neural migration to aid tissue repair (Babona-Pilipos et al. 2011). Also under direct current, adult hippocampal neural progenitor cells experienced lower viability yet a higher proportion of neuronal differentiation (Ariza et al. 2010). Finally, Kabiri and colleagues showed that incorporation of carbon nanotubes into an aligned nanofiber scaffold enhanced the neuronal differentiation of murine embryonic stem cells, which they proposed was due to the resulting increase in the conductivity of the material (Kabiri et al. 2012).

In addition, the effects of electric fields have been investigated on stem cells other than those of the heart and nervous system. For instance, application of direct current to mESC-derived embryoid bodies apparently enhanced endothelial differentiation in a manner dependent on the formation of reactive oxygen species (Sauer et al. 2005). In another example, pulsed electromagnetic field stimulation of human MSC cultures promoted the expression of osteogenic markers over a 28 day period (Tsai et al. 2009).

In summary, the role of electric fields in regulating the function of cardiac, neural, and other cells is being increasingly studied, though not yet to the level of activity of mechanical properties. There may thus be additional opportunities to diversity investigation in this area.

Mass Transfer Influences on Stem Cell Behavior

The transport of mass through tissues can exert strong effects on the local composition of those tissues, including the convection and diffusion of nutrients, oxygen, and signaling molecules. Furthermore, the mass transfer properties of the tissue can modulate the spatial distribution of locally produced molecules. As a result, mass transport phenomena play a role in modulating the behavior of stem cells within their niche, and on a larger scale aid in establishing useful or functional heterogeneity within a tissue.

Gradient Formation and Morphogenesis

As originally proposed by Turing 60 years ago, gradients of signaling factors secreted from signaling centers can aid in tissue patterning and differentiation during development (Turing 1990). The cells undergoing patterning in developing tissues

are often stem cells, and a number of studies have modeled the dynamic effects of morphogen gradients on cell lineage commitment and morphogenesis (Reeves et al. 2006; Saha and Schaffer 2006; Torii 2012).

Such transport limitations can actually pose challenges in stem cell culture systems. In many situations it would be desirable to uniformly differentiate stem cells into a specific cell type for therapeutic application; however, transport limitations of both components from the medium as well as factors produced by the cells themselves, particularly within cellular aggregates such as EBs, can yield highly heterogeneous cultures of differentiated cells. One creative approach to overcome the problem of the diffusion of culture medium components into an embryoid body is to embed the factors, or specifically microspheres for controlled release of such factors, into the EBs (Carpenedo et al. 2009; Bratt-Leal et al. 2011). This approach offers considerable promise for reducing heterogeneity. Additionally, the optimized assembly of stem cells into aggregates can access mechanisms of pattern formation utilized in organismal development, which can thereby yield considerable insights into developmental mechanisms as well as create more complex structures of potential future utility for tissue engineering (Eiraku et al. 2011; Suga et al. 2011; Eiraku and Sasai 2012).

Oxygen

The atmospheric oxygen concentration (20 %) is higher than that in most organs of the body, despite the close proximity ($\sim 100\ \mu\text{m}$) of cells to capillaries in vascularized tissues (Chow et al. 2001). This consideration raises the possibility that stem cells could behave differently in atmospheric vs. niche oxygen levels.

In seminal work in this area, Koller and colleagues found that mononuclear cells isolated from human cord blood and bone marrow proliferated more rapidly and maintained higher frequencies of several colony forming cells when cultured in reduced vs. atmospheric oxygen (Koller et al. 1992). As a key example within the nervous system, Studer and others demonstrated that reduced oxygen (3 %) enhanced the survival and proliferation of neural precursors, as well as enhanced their differentiation into dopaminergic neurons from 18 to 56 % (Studer et al. 2000). Reduced oxygen upregulated the expression of several proteins, including erythropoietin (EPO), and EPO addition to the medium partially recapitulated the effects of reduced oxygen. In another study, culture in 5 % oxygen enhanced clonogenic neural crest stem cell differentiation into a sympathoadrenal lineage (Morrison et al. 2000). The development of small-scale culture systems in which oxygen can be easily and readily controlled would enable additional study.

Development of Novel Technologies to Study Stem Cells

In addition to offering principles to guide the discovery of novel mechanisms for stem cell regulation, the physical sciences and engineering offer technologies that enable new experimental investigations of stem cells. These include the various

innovative material systems discussed above, as well as novel materials technologies developed to manipulate and apply mechanical loads to cells. For example, Ikuta and colleagues at the University of Tokyo have used photo-patterned 3D polymerization of materials to create various devices that can subsequently be interfaced with optical trapping for actuation. With the use of a robotic arm for operator control, they generated a pincer device that can apply defined mechanical loads to cells, for either measurement or mechanical perturbation.

In addition, the ability to genetically manipulate a stem cell is useful both for exploring molecular mechanisms involved in cellular function, as well as in the future for enhancing the therapeutic potential of those cells. Ma and colleagues conjugated magnetic particles to gene delivery vehicles, which enabled magnetically guided gene delivery to cells *in vitro* or *in vivo* (Li et al. 2008). While this technology has not yet been applied to stem cells, it will be promising to do so. Recent developments in protein engineering to create better gene delivery vehicles to stem cells (Asuri et al. 2012), as well as site-specific nucleases to aid in homologous recombination in stem cells (Hockemeyer et al. 2011), are also promising biological approaches for genetically manipulating stem cells.

Another capability that is important for stem cell research, and in particular future translational efforts, is cell separations. Investigators at Lund University have generated a microfluidic device that uses sound waves to separate blood cells based on differences in density, a process termed acoustophoresis (Dykes et al. 2011). In addition, in a dielectrophoretic separation, Miyata and colleagues used alternating electric fields in conjunction with patterned surfaces to achieve cell separations. This process has promise both in separating, for example, differentiated from undifferentiated stem cells, but also in patterning cell deposition on a surface. As a final example, Scadden and colleagues have applied pulsed electric fields to cell mixtures containing hematopoietic stem cells (HSCs) (Eppich et al. 2000). The fields selectively introduce pores into the membranes of larger cells, and in certain parameter ranges the resulting toxicity provides a means to ablate differentiated cells while preserving the smaller HSCs.

Therefore, principles and practices from materials science, physics, electrical engineering, and protein engineering will continue to make new technologies that benefit stem cell research.

Future Directions

Biomaterials Development

As discussed in the Introduction, a number of biophysical properties of the cellular microenvironment are difficult to manipulate and vary genetically, as these properties depend on contributions from more than a single gene. As a result, the systems

described in this chapter have been not only interesting but also critical for assessing the effects of these properties on the function of stem cells. There are considerable opportunities for further innovation, especially in materials development. For example, stem cell lineage commitment is an inherently dynamic process, and elucidating the roles of biophysical cues in regulating the process would benefit from the ability to temporally vary cues. While it is straightforward to dynamically change soluble biochemical cues, and even mechanical inputs such as shear and strain, it is challenging to vary static mechanical properties, topographical cues, and immobilized biochemical cues. However, there has been considerable progress in using, for example, photoresponsive materials where light exposure can alter crosslinking and therefore vary mechanical properties in both space and time (Kloxin et al. 2010; Guvendiren and Burdick 2012). Furthermore, using materials with shape memory enables the dynamic variation of topographical inputs (Le et al. 2011). Finally, the capability to temporally vary local biochemical cues within a material has recently been developed (Kloxin et al. 2009). The future application of such systems to problems in stem cell biology promises novel insights.

In addition, a number of the systems described above enable systematic investigation of the role of an individual microenvironmental property on cell function, yet the niche of course simultaneously exposes cells to many of these properties. The development of advanced systems for multiparameter control of the cellular microenvironment promises to yield insights into how these inputs combinatorially modulate cell function (Gobaa et al. 2011). While there have thus been a number of promising advances in materials engineering, one area that could benefit from additional advances is the study of cell-cell interactions within the niche. Specifically, the ratios and relative positions of various cells within the niche are likely tightly regulated, and investigations of such systems will benefit from new technologies to precisely control the positioning of multiple cell types in culture.

Mechanistic Elucidation

Investigating the mechanisms by which physical and engineering properties of the cellular microenvironment modulate stem cell behavior requires expertise not only in the creation and fabrication of systems to vary these properties but also in the molecular elucidation of cell responses. That is, these studies require expertise in both the physical sciences and engineering and in biology. As described above, in a number of studies that have melded these fields, there has been progress in elucidating mechanisms by which static mechanical properties, shear flow, and topography modulate stem cell function. That said, there are many unknowns in this field that will benefit from collaborations among investigators with complementary expertise. Furthermore, in general the interface between the physical sciences, engineering, and biology represents a major opportunity to train a new generation of scientists capable of highly interdisciplinary research.

Additional Opportunities

In addition to elucidating fundamental roles of biophysical properties on stem cell function, as described above, there are many opportunities to develop novel technologies to benefit both fundamental and translational stem cell research. One critical capability for mechanistic study is the ability to genetically manipulate a stem cell—both to add genetic material and to conduct gene targeting or genome editing. Furthermore, the ability to isolate and investigate specific cell types will benefit from both new affinity agents and novel cell separation modalities. As with basic investigations, each of these areas requires expertise in physical sciences, engineering, and biology.

Global Assessment and Conclusions

The United States is currently a leader in studying the roles of physical sciences and engineering principles in stem cell research. This position to date has benefitted from a broad and deep community of engineers, physical scientists, and materials scientists who have increasingly investigated not only applied but also fundamental questions in the biological sciences. Other countries with pronounced strengths in this area include Japan, Germany, and Switzerland, which have also invested in stem cell biology and engineering research in a manner that is progressively converging.

While the leadership role of the United States in materials science and engineering has clearly benefitted the stem cell engineering field, other countries have recently played an increasing role in the application of physical sciences and engineering to develop new technologies to study stem cells. These include imaging technologies, cell separation technologies, and especially high-throughput “microenvironmental screening” methodologies. Furthermore, both strong government (e.g., Switzerland and Sweden) and private foundation (e.g., Fraunhofer Institutes in Germany and I-STEM in France) support for technology development, application, and commercialization are models that merit deeper study.

In the future, knowledge of biophysical and biochemical regulation of stem cell properties will increasingly be integrated, which will progressively increase our appreciation of the complex means by which the niche orchestrates the various behavioral choices available to resident stem cells. These studies will also provide increasing levels of quantitative data that can be integrated into computational and modeling efforts. Furthermore, the resulting knowledge will aid in the development of cell culture systems for the reproducible, scalable, and economical expansion and differentiation of stem cells for therapeutic application.

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Stem Cell Engineering

A WTEC Global Assessment

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