Mechanical control of stem cell differentiation*

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Abstract

Stem cells interrogate numerous microenvironmental cues, including soluble factors, adhesive contexts, and mechanical signals, in order to mount physiologically relevant differentiation responses. While much is known about how soluble factors and adhesion receptors regulate differential gene expression, the molecular basis for how mechanical signaling controls gene transcription and differentiation programs is only now coming into focus. In this review, we discuss the types of mechanical forces that stem cells experience, and the evidence that applied and cell generated forces regulate stem cell differentiation in vivo and in vitro. In order to understand the mechanistic basis for mechanically-induced differentiation, we explore how mechanical forces are transduced into biochemical signals, that can in turn regulate and synergize with signaling cascades induced by other stimuli. Specifically, we emphasize how applied mechanical forces target the activity and expression or transcription factors and chromatin remodeling enzymes directly involved in gene expression. Furthermore, we highlight recent progress demonstrating that cell-generated forces (intracellular tension) can promote stem cell differentiation in the absence of or in spite of orthogonal signals from soluble factors. Together these findings define an emerging picture of how mechanical forces trigger stem cell differentiation to a specific lineage in complex microenvironments where inducers for multiple cell types are present.

*Edited by Sangeeta Bhatia and Julia Polak. Last revised June 13, 2008. Published October 31, 2008. This chapter should be cited as: Cohen, D.M. and Chen, C.S., Mechanical control of stem cell differentiation (October 31, 2008), StemBook, ed. The Stem Cell Research Community, StemBook, doi/10.3824/stembook.1.26.1, http://www.stembook.org.

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1. Introduction

Stem cells are a driving force in functional tissue engineering due to their self-renewal capacity and pluripotency. Self-renewal enables the extensive ex-vivo (and in vivo) expansion of progenitor cells for a targeted tissue, a key feature for generating sufficient cells to meet the potential demand for tissue replacement. Pluripotency, or the ability to stem cell to differentiate into multiple cell types, allows for the possibility of generating multiple tissues from a single cell source, and even reconstituting complex multicellular interactions required for the function of a single tissue. The multilineage differentiation potential of stem cells is at once an opportunity and also a challenge as differentiation at the wrong time, place, or to an undesired cell type may lead to a pathophysiologic state or a non-functional tissue construct. To avoid such maladaptive responses, stem cells have evolved elaborate circuitry that triggers them to respond to differentiation cues only in an appropriate biological context. While biologists have long appreciated a role for soluble cues (e.g. growth factors and cytokines) in regulating stem cell differentiation, recent evidence demonstrates that the response to these stimuli are strongly modified by adhesive and mechanical cues, and that these microenvironmental factors may be used explicitly to control stem cell differentiation in their own right. Here we describe the evidence for mechanical control of stem cell differentiation and the mechanisms that transduce mechanical signals into differential gene expression.

2. What are mechanical signals?

Mechanical signals are cues that cells can sense as a result of the application of force. The simplest interpretation of how cells react to forces can be derived from the behavior of a classical spring. When forces are applied to springs, two potential responses can occur. First, forces can lead to a change in the spring's length, which is typically reported as a strain (change in length normalized to the original spring length). Second, applied forces can lead to the development of internal tension, often denoted as stress (force divided by the cross-sectional area of the spring). Since primary sites of mechanosensation in cells may experience both strain and stress responses, a useful distinction between types of mechanical signals is between tensile (pulling) forces and compressive forces (those that push on cells/tissues). More detailed consideration of these forces will be given in **Section 3**.

While the behavior of classical springs is a useful starting point for understanding the biological response to mechanical stimuli, the reality is far more complicated due to the contribution of two key factors. First, tissues and cells are not passive materials, but rather have tunable mechanical properties governed by dynamic reorganization of their cytoskeletal networks. On short time scales, the cytoskeleton can fluidize in response to mechanical stretch (Trepat et al., 2007), but cells rapidly transition to an active response in which realignment of the cytoskeleton occurs to counterbalance the applied force (Katsumi et al., 2002; Matthews et al., 2006). In parallel, the actin cytoskeleton contributes to the mechanical forces within the system due to the pulling action of myosin motors (principally non-muscle myosin II) on actin filaments, which result in the production of tensile forces against the extracellular matrix (Pelham and Wang, 1999; Balaban et al., 2001; Tan et al., 2003). Importantly, there is feedback between externally applied forces and myosin II activity such that external mechanical stimuli often activate biochemical signals responsible for increased myosin motor activity and intracellular tension (Wojciak-Stothard and Ridley, 2003; Shiu et al., 2004; Torsoni et al., 2005; Sarasa-Renedo et al., 2006; Zhao et al., 2007). As such, the biological response to exogenous force is governed not only by the initial input force, but also by subsequent alterations in cell-generated forces. In certain contexts, these cell-generated forces may be necessary or sufficient for stem cell differentiation (we will elaborate on this in further detail below).

Second, while mechanosensation begins with a mechanical response (strain, stress, and/or intracellular forces) cells transduce the mechanical stimuli into a biochemical output, otherwise known as mechanochemical signaling or mechanotransduction. Multiple activation mechanisms are simultaneously at play including release of autocrine growth factors (Resnick et al., 1993; Liu et al., 1995; Reusch et al., 1996; Robbins et al., 1997; Cillo et al., 2000; Wu et al., 2001; Zheng et al., 2001; Lindahl et al., 2002), activation of mechanically-sensitive kinases such as Src (Liu et al., 1996; Han et al., 2004; Jiang et al., 2005; Wang et al., 2005; Na et al., 2008), FAK (Li et al., 1997; Smith et al., 1998; Wang et al., 2001; Leucht et al., 2007), and ERK (Yamazaki et al., 1995; Takahashi and Berk, 1996; Jalali et al., 1998; MacKenna et al., 1998; Schmidt et al., 1998; Chen et al., 2000; Iqbal and Zaidi, 2005), and initiation of second messenger signaling (Sadoshima and Izumo, 1997; Liu et al., 1999). Thus mechanical signals have extensive potential to regulate and synergize with classical biochemical signal transduction pathways induced by soluble factors to control stem cell differentiation.

3. What are the sources and functional consequences of applied mechanical forces on stem cells in vivo?

Stem cell differentiation normally plays out in the context of organismal development or as part of the wound healing response. Both development and wound healing contexts are environments where extracellular forces abound, and evidence suggests that these forces instruct the subsequent behavior of stem cells. Typically, tissues may experience compressive forces or pressure, tensile forces (e.g. mechanical loading or stretch), and fluid-applied forces (shear flow). Others have enumerated these forces in thorough detail (Henderson and Carter, 2002; Estes et al., 2004); here we provide just a few illustrative examples where evidence suggests a functional link between forces and differentiation.

Compressive pressure typically arises when cells proliferate and/or deposit large amounts of extracellular matrix in regions where expansion of the tissue is resisted or prevented by rigid external boundaries. Such compressive forces can play key roles in tissue formation by controlling the balance between cell growth versus differentiation. For example, in the developing skeleton, primitive cartilage tissue is usually encapsulated by a rigid perichondrial membrane. Relaxation of this mechanical constraint by transection or removal of the perichondrium results in unregulated growth of the remaining tissue (Rooney and Archer, 1992; Long and Linsenmayer, 1998), likely by allowing chondroprogenitors cells to continue to grow rather than differentiate into cartilage. Furthermore, inappropriate development of compressive pressure can lead to ectopic cartilage formation. In classic tissue explants studies, Glucksmann demonstrated ectopic cartilage formation in developing bones when bone explants were trapped between artificial barriers (Glucksmann, 1939). Similarly, in models of the developing jaw, applied compression to the mandible joint led to ectopic cartilage formation on the jaw bones (Hall, 1967; Hall, 1968) and mechanical compression of the midplatal suture reprogrammed mesenchymal stem cell (MSC)-like cells from an osteogenic to a chondrogenic fate (Saitoh et al., 2000).

Tensional forces are prominent in the skeletal system and arise in response to movement of articulated tissues, such as the flexion of tendons or muscle contractions pulling on bones. It has also been suggested that tensional forces evolve also from the highly directional and asymmetric growth of two articulated tissues, for example the stretching of periosteal tissue anchored to both the bone shaft and the tips of tangentially expanding bone epiphyses (Glucksmann, 1942), or the stretching of musculoskeletal units that bridge rapidly growing skeletal elements (Stewart 1972; Taber 1998). Experimental manipulation of tension can profoundly alter development of the skeletal system. Severe osteogenic defects occur when tension in the periosteal tissue is alleviated by resection of bone epiphyses (Glucksmann, 1942); conversely ectopically applied tensional forces appear to transform cartilaginous tissue into bone (Glucksmann, 1942; Takahashi et al., 1996, 2003). In contrast, loss of muscle-induced tension via experimentally-induced paralysis or limb-tissue transplantation surgeries has more limited effects. These manipulations alter the formation of specific sesamoid bones, such as the plantar tarsal sesamoid and the patella (Drachman and Sokoloff, 1966; Hosseini and Hogg, 1991), and reduce the formation of bony ridges at tendon attachment sites (Murray and Huxley, 1925; Hamburger, 1938; Hamburger and Waugh, 1940).

Another important source of mechanical forces are fluid shear stresses, typically experienced by the developing myocardium (Hove et al., 2003), fetal lung epithelium (Liu and Post, 2000), or kidney (Serluca et al., 2002). In addition to these developmental contexts, fluid shear stress is also experienced in bone, where interstitial fluid flow governs the physiological responses to mechanical loading (Burger and Klein-Nulend, 1999). Shear stress is perhaps best known for its effects on the vascular endothelium (Davies, 1995; Orr et al., 2006). Disruption of hydrodynamic forces by the introduction of beads that block cardiac inflow or outflow produces dramatic cardiac defects in zebrafish, including failure of valve formation, and absence of heart looping and formation of a third chamber (Hove et al., 2003). Although Hove et al. did not establish a direct role for shear flow in regulating endothelial cell differentiation, other studies have demonstrated that changes in shear flow is associated with widescale reprogramming of gene expression in endothelial cells (Dai et al., 2004; Garcia-Cardena et al., 2001), and has inductive roles in determining arterial-venous specification (Le Noble et al., 2004). Moreover, shear flow has been reported to promote endothelial differentiation of mesenchymal stem cells or endothelial progenitors (Wang et al., 2005; Yamamoto et al., 2003, 2005), and cardiac differentiation of embryonic stem cells (Illi et al., 2005).

Collectively, these studies highlight two themes in mechanical regulation of tissue development and cell differentiation by external forces. First, while mechanical stimuli are relevant to a wide variety of tissues/cell types, distinct classes of mechanical forces trigger divergent programs of cell differentiation (c.f. the effects of compressive versus tensional forces on skeletogenesis (Glucksmann, 1939; Glucksmann, 1942)). Second, early differentiation persists even in the absence of external forces; that is, paralytic agents do not prevent the appearance of muscle fibers, although morphology and growth is perturbed (Hall and Herring, 1990); osteoblasts and chondrocytes persist even when limbs

are mechanically unloaded by transplantation (Murray and Huxley, 1925, Fell and Robison, 1929; Hamburger, 1938; Bradley, 1970; Hall, 1972), and endothelial cells form when flow is disrupted (Hove et al., 2003). This is not particularly surprising given the fact that in embryogenesis, differentiated cell types typically appear and segregate prior to coordinated tissue function (such as muscle contraction or blood flow) that might contribute to the generation of substantial forces. Thus the likely mechanism by which applied mechanical forces control stem cells is not through the direct and spontaneous triggering of differentiation per se, but rather, by specification of a particular fate under environmental conditions that can induce several possible cell types. Put another way, mechanical signals act as a molecular switch to bias stem cells towards one lineage over another.

4. How do mechanical forces lead to lineage switching?

Despite the importance of the aforementioned in vivo studies in establishing a link between tissue formation, differentiation, and mechanical stimuli, it is important to acknowledge certain inherent limitations in these experiments, including the inability to measure the change in applied forces or stresses in these experiments, and the likelihood that the mechanical manipulations employed also perturb paracrine signaling and/or adhesive cues. Our understanding of how mechanical signals regulate stem cell differentiation and lineage switching is greatly informed by several elegant studies that directly examine the link between mechanical force, gene expression, and cell differentiation.

4.1. Applied mechanical forces and transcriptional effectors

One potential mechanism by which mechanical forces could alter differentiation is through targeting the transcriptional machinery in the nucleus. Two developmental models in *Drosophila* have identified potential transcriptional mediators of mechanical forces. The first example is Twist, a basic-helix-loop-helix domain protein, associated with regulation of skeletal development (Bialek et al. 2004; El Ghouzzi et al., 1997; Howard et al., 1997). Twist expression is normally restricted to the most ventral (back) cells of the embryo (Farge, 2003). However, when Drosophila embryos were sandwiched between a membrane and a glass coverslip, and a 10% uniaxial mechanical compression applied by lowering the coverslip, Twist expression was induced throughout the embryo (Farge, 2003). Importantly, this ectopic expression of Twist leads to failure of embryonic development, due to disruption of the dorsal-ventral (front-to-back) axis. Moreover, Farge demonstrated that the wild-type pattern of ventral-specific Twist expression during gastrulation results from the compressive pressures generated by expansion of a layer of ectodermal/mesodermal precursors known as the germ band. Although the mechanism by which compression activates the Twist promoter is incompletely understood, the process is rapid (within 8 minutes of stimulation) and involves nuclear translocation of β-catenin (Farge, 2003), a response typically associated with canonical Wnt signaling. The involvement of β-catenin in the mechanical response to compressive pressure is especially intriguing with respect to mechanical regulation of stem cells; while Twist is not intimately associated with stem cell differentiation, Wnt signaling plays a prominent role in specifying sensory neuron fates for neural stem cells (Lee et al., 2004; Hari et al., 2002), and also regulates skeletal differentiation of mesenchymal stem cells (reviewed in Liu et al., 2008). β-catenin, in particular, appears to promote a chondro-to-osteo differentiation switch in progenitor cells/mesenchymal stem cells (Day et al., 2005; Hill et al., 2005). If β-catenin is also responsive to compressive pressure, it may serve as a key regulator of crosstalk between Wnt signaling and mechanical cues in controlling either skeletal patterning or neurogenesis.

In the context of pulling or tensile forces associated with cell motility, the transcriptional target for mechanical regulation appears to be MAL (Somogyi and Rorth, 2004), a myocardin-related transcriptional co-factor for SRF. SRF and its co-factors regulate the expression of smooth muscle proteins, principally structural and contractile proteins associated with the actin cytoskeleton (Cen et al., 2003; Selvaraj and Prywes, 2003; Wang et al., 2001). During *Drosophila* oogenesis, a group of epithelial cells, known as border cells, differentiate into a migratory cluster that traverses the length of the oocyte. The differentiation process is genetically controlled by a C/EBP transcription factor called slbo (Montell et al., 1992). In normal border cell clusters, protrusion at the leading edge of the cluster stretches the front-most cells, and MAL is observed to accumulate in the nuclei of these elongated cells (Somogyi and Rorth, 2004). However, in slbo-null cells, which are genetically deficient for migration, MAL does not undergo nuclear translocation. Importantly, this defect can be rescued, in chimeric border cell clusters, in which the non-migratory mutant cells "piggy-back" on the motile activity of wild-type cells at the front of the cluster. In this case, it appears that the pulling action exerted by the Slbo-expressing cells imparts tensile forces that mechanically trigger nuclear translocation of MAL in the slbo-null cells (Somogyi and Rorth, 2004). The mechanotransduction pathway of stretch to MAL translocation remains an open question, but likely involves force-dependent changes in actin polymerization. Activation of Diaphanous, a member of the formin class of actin nucleator proteins, is sufficient to trigger nuclear translocation of MAL in border cells (Somogyi and Rorth, 2004). Regulation of MAL by actin polymerization through cytosolic sequestration of MAL by G-actin is well documented (Miralles et al., 2003, Sotiropoulos et al., 1999). While

these findings have yet to be implicated in regulation of stem cell differentiation, mechanical signaling to SRF or its co-factors is consistent with the role of applied mechanical stretch in promoting smooth muscle cell differentiation (Kanda and Matsuda, 1994; Reusch et al., 1996; Smith et al., 1997; Yang et al., 2000; Albinsson et al., 2004; Kurpinski et al., 2006).

While the *Drosophila* models suggest that mechanical signals could regulate stem cell differentiation via nuclear translocation of transcription factors, direct evidence exists that applied tensional forces control differentiation of embryonic lung mesenchymal cells through chromatin remodeling (Jakkaraju et al., 2005). This effect is mediated by tension-induced proteins, or TIPs, a set of novel proteins that contain histone acetyltransferase activity, as well as nuclear receptor binding boxes (NRBs mediate recruitment of key transcription factors such as PPARG and SRF). TIP1 expression is specific to lung mesenchymal stem cells undergoing smooth muscle myogenesis, a tension-dependent process (Yang et al., 2000). An applied static 5% axial stretch is sufficient to trigger TIP1 expression and nuclear localization in cultured lung MSCs. Using transient transfection and knockdown of TIPs, Jakkaraju et al. demonstrated that TIP1 is required for stretch-induced myogenesis, and forced TIP1 expression can mimic the ability of stretch to induce myogenic differentiation while suppressing adipogenic genes. Conversely, loss of TIP1 expression or forced overexpression of TIP3 triggers a myogenic-to-adipogenic switch in lung MSCs. Together, these findings suggest that applied stretch regulates a myogenic-adipogenic switch through reciprocal regulation of TIP1 and TIP3. Consequently, TIPs enable stem cells to choose between myogenic and adipogenic fates by controlling positive feedback loops between key differentiation factors for myogenesis (SRF) and adipogenesis (PPARG) and their promoters (Jakkaraju et al., 2005).

It is not yet known how prevalent a role chromatin remodeling will play in mechanical control of stem cell differentiation. But it is noteworthy that a recent study found that application of stretch triggered an adipogenic-to-osteogenic switch in mesenchymal stem cells (C3H10T1/2) through antagonism of PPARG expression and function (David et al., 2007). The fact that applied stretch could downregulate PPARG-mediated gene expression even when PPARG was directly stimulated by the pharmacological agonist, rosiglitazone, argues that the activation state of PPARG is not a direct target of mechanical signaling (David et al., 2007). A likely alternative is mechanical regulation of PPARG-responsive promoters through altered accessibility/recruitment of activated PPARG protein. This stretch-regulated adipogenic-to-osteogenic switch is therefore consistent with mechanically-sensitive chromatin remodeling, and involvement of TIPs. Especially striking is the observation that the application of stretch appears to disrupt the positive feedback loop that normally allows PPARG to promote its own expression in the contexts of both myogenic-adipogenic and osteogenic-adipogenic differentiation switches. Nonetheless given the different time scales for stretch-mediated transcriptional changes in the embryonic lung MSCs (hours) and C3H10T1/2 cells (days), roles for other mechanically-responsive pathways in the stretch-mediated adipogenic-osteogenic switch cannot be excluded.

4.2. Integration of multiple (applied and cell-generated) mechanical forces through cell shape and the actin cytoskeleton

While the aforementioned studies provide the experimental framework necessary to draw a causal link between mechanical force and gene expression, can they also inform our understanding of how cells differentially sense distinct types of applied (e.g., compressive versus tensile) forces? More critically, how do we extend these findings to discover the means by which cells integrate multiple mechanical (and soluble) cues to generate an appropriate differentiation response? One clue would be the existence of a conserved physical response to applied forces in these distinct systems (compressed *Drosophila* embryos, migrating border cells, stretched embryonic lung MSCs). Indeed, applied mechanical forces are associated with stereotypical cell shape changes in all the examples highlighted above. Furthermore, in the case of embryonic mesenchymal stem cells, a change from a rounded to an elongated morphology is sufficient to drive smooth muscle myogenesis, akin to the effect of mechanical stretch (Yang et al., 2000). *This observation suggests that applied forces may regulate cell function through changing cell shape*.

Second, while the molecular players that underlie the transcriptional response to applied force (Twist, MAL, TIPs) are quite divergent, there are intriguing parallels in the regulation of these proteins. Involvement of cytoskeletal components is an emergent theme. β-catenin, a component of the adherens junction, is critical for the mechanical induction of Twist (Farge, 2003). The small GTPase RhoA, which controls stress fiber assembly and the generation of intracellular tension, activates Diaphanous, the upstream regulator of MAL (Somogyi and Rorth, 2004). While no formal relationship between RhoA and TIPs has been shown, one might be inferred, however, based on the parallel observations that myogenic differentiation of embryonic mesenchymal stem cells can be controlled either through RhoA-mediated changes in cell spreading (Beqaj et al., 2002) or stretch-mediated regulation of cell spreading and TIPs (Yang et al., 1999; Yang et al., 2000; Jakkaraju et al., 2004). Moreover, association with the actin cytoskeleton,

directly or indirectly, confers a mechanism for mechano-sensitive cytosolic sequestration of transcriptional effectors. This could account for the shared nuclear translocation response of β-catenin, MAL, and TIPs to applied mechanical forces. *Collectively, these findings suggest that mechanical signals converge on the actin cytoskeleton and the RhoA pathway to regulate stem cell differentiation*.

4.3. Intracellular tension and lineage switching

Do cell shape, the actin cytoskeleton, and the RhoA pathway play important roles in the mechanical control of stem cell differentiation? Several influential studies make a compelling case for these factors as determinants of differentiation, at least in mesenchymal stem cells. Using micropatterned islands of extracellular matrix (fibronectin) to control cell spreading, McBeath et al. have demonstrated that cell shape controls the lineage commitment of MSCs (McBeath et al., 2004). In this system, MSCs can differentiate into either adipocytes or osteoblasts in response to a bipotential differentiation medium that contains inducers for both lineages. However, MSCs confined to small ECM islands (1024 μm^2) selectively underwent adipogenesis, whereas MSCs cells on large ECM islands (10000 μm^2) were biased towards osteogenesis (McBeath et al., 2004). This osteogenic-adipogenic switch in well-spread versus poorly-spread MSCs required the generation of tension through RhoA-dependent acto-myosin contractility. RhoA stimulates tension through its effector, Rho kinase, which indirectly elevates the level of active, phosphorylated myosin light chain (Kimura et al., 1996). Inhibition of tension using either cytochalasin D (an actin depolymerization agent) or Y-27632 (a Rho kinase inhibitor) promoted adipogenesis, mimicking the phenotype of poorly spread cells. 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 Rho kinase (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 cytoskeletal tension (McBeath et al., 2004).

Further evidence that RhoA acts as a differentiation switch is provided by the phenotype of the p190RhoGAP knockout mouse. The normal function of p190RhoGAP is to stimulate GTP hydrolysis and thus inactivation of RhoA. In the absence of p190RhoGAP, RhoA activity is upregulated, and this triggers an adipogenic-to-myogenic switch in vitro and in vivo (Sordella et al., 2003). Interestingly, these authors proposed mutual antagonism between RhoA and insulin/insulin growth factor signaling, whereby insulin-like growth factor 1 inhibits RhoA by activating p190RhoGAP, whereas RhoA inactivates the insulin effector, IRS-1, through ROCK-dependent phosphorylation (Sordella et al., 2002; Sordella et al., 2003). Given the evidence that ROCK inhibits adipogenesis through tension (McBeath et al., 2004), and that Rho-ROCK-myosin signaling regulates SRF activity (Mack et al., 2001; Fan et al., 2007), a role for tension in adipogenic-myogenic switching is also anticipated. Although the mechanism by which RhoA activation can lead either to myogenic or osteogenic differentiation is not yet known, these studies identify RhoA activity as a molecular switch governing the differentiation of MSCs in response to both mechanical (cell spreading, intracellular tension) and soluble (insulin/IGF1) factors.

The role of cell spreading and intracellular tension in controlling lineage switching has come into sharper focus following the important discovery that differentiation of mesenchymal stem cells can be governed by substrate stiffness. In a landmark study, Engler and colleagues demonstrated that plating MSCs on polyacrylamide gels of varying stiffnesses is sufficient to drive expression of neuronal, skeletal muscle, or osteogenic markers in the absence of exogenous soluble cues (Engler et al., 2006). Cell-generated tensional forces exist in equilibrium with the underlying substrate (that is, the substratum develops an equal and opposite counterbalancing force, such that the net forces sum to zero). If only weak counterbalancing forces can be mounted, such as in the case of a soft gel, cellular contractility must undergo a compensatory decrease. Consequently, it is reasonable to attribute stiffness-dependent changes in stem cell differentiation to altered intracellular tension. Indeed, addition of blebbistatin to block intracellular tension generation in MSCs obliterated stiffness-driven differentiation (Engler et al., 2006). Consistent with the hypothesis that cells upregulate intracellular tension as the matrix stiffens (and provides higher resistance forces), MSCs progressively assembled actin stress fibers and focal adhesions (tension-dependent structures) in response to the increasing stiffness of the substrate.

Importantly, these stiffness-mediated cytoskeletal changes are accompanied by dramatic alterations in cell morphology (Engler et al., 2006). While the cell morphology of MSCs on soft, intermediate, and stiff gels is reminiscent of neurons, myoblasts, and osteoblasts, respectively, it is not known whether altered cell spreading is merely a downstream consequence of differentiation, or rather a primary determinant that regulates intracellular tension and

the mechanical signaling required for specifying stem cell fate. Nonetheless, the findings of Engler and colleagues are remarkable in that, unlike other mechanical interventions to date, stiffness appears to have the potential to guide MSCs into multiple fates (rather than the binary switches previously described). Moreover, these findings illustrate that shifts in the cell-ECM force balance have profound effects on stem cell differentiation, and anticipate a major role for dynamic control of matrix stiffness in controlling tissue development. Indeed, dynamic changes in the mechanical properties of three-dimensional matrices are emerging as critical regulators of differentiation (Chun et al., 2006) and morphogenesis (Adams et al., 1990; Moore et al., 2005). As such, designing scaffolds of appropriate matrix stiffness, and understanding how matrix stiffness couples with other mechanical properties of matrices (e.g., topology features and structural anisotropy) to control stem cell behavior will be crucial to functional tissue engineering (Ghosh and Ingber, 2007).

5. Mechanosensors

While cell shape, RhoA signaling, and cytoskeletal tension appear to have major roles in governing the response of stem cells to mechanical forces, it is not yet clear whether these players converge on a single mechanosensitive cellular compartment or molecule, and a mechanotransduction pathway connecting tension to mechanoresponsive transcriptional machinery (see **section 4.1**) remains elusive. Focal adhesions, cellular footholds that anchor matrix-bound integrins to the actin cytoskeleton, are likely to factor into tension-dependent mechanotransduction. Cell-generated traction forces are transmitted to the extracellular matrix at focal adhesions (Balaban et al., 2001; Beningo et al., 2001; Tan et al., 2003), which implies the development of stress within these structures, and these stresses regulate the assembly/disassembly of focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Helfman et al., 1999; Balaban et al., 2001; Riveline et al., 2001; Sniadecki et al., 2007). Second, focal adhesions are molecular signaling hubs implicated in the recruitment and activation of key mechanosensitive kinases such as FAK, Src, and ERK (Miyamoto et al., 1995; Fincham et al., 2000; Wang et al., 2005; Cai et al., 2008), and also in the regulation of nuclear-cytoplasmic shuttling of some transcription factors (Nix and Beckerle, 1997; Petit et al., 2000; Aoto et al., 2002; Shibanuma et al., 2003; Silver et al., 2004). In spite of the potential importance of focal adhesions in mechanotransduction, they are but one of several mechanosensors identified in cells (Ingber, 2006); these include changes in membrane curvature or lipid microdomains (Rizzo et al., 1998; Hamill and Martinac, 2001;), GPCRs (Chachiscilis et al., 2006), mechanosensitive ion channels (Sukharev and Corey, 2004),conformational change of cytoskeletal proteins (Sawada et al., 2006; Johnson et al., 2007), the nuclear lamina or nuclear deformations (Lammerding et al., 2004; Lammerding et al., 2005), and primary cilia (Resnick and Hopfer, 2007). Importantly, no stem-cell specific mechanosensory mechanisms have been proposed, and any number of aforementioned mechanisms may contribute to mechanical control of stem cell differentiation. Nonetheless, in addition to the RhoA/tension/focal adhesion pathway, MAPK signaling and primary cilia probably warrant special mention. MAPK signaling, and ERK in particular, is distinctive in that it can be activated in response to numerous types of applied forces (Iqbal and Zaidi, 2005), sits at a cross-roads between soluble factor and adhesion signaling (like RhoA; Estes et al., 2004; Ross, 2004), and regulates differentiation of stem cells (Jaiswal et al., 2000; Binétruy et al., 2007). Primary cilia are receiving increasing attention following the discovery that these structures play key roles in controlling the Hedgehog signaling pathway (Huangfu and Anderson, 2005; Corbit et al., 2005), one of the major determinants of embryonic patterning (Ingham and McMahon, 2001; Hirokawa et al., 2006). Given the recent identification of primary cilia in cultured embryonic stem cells (Kiprilov et al., 2008), exploring the role of primary cilia in mechanical regulation of differentiation, perhaps by altered Hedgehog signaling, represents an exciting future direction for stem cell research.

6. Conclusions and perspectives

Both externally-applied and cell-generated mechanical forces are pivotal to the differentiation response of stem cells. Largely, the evidence for these effects are focused around mesenchymal stem cells, tensile or compressive strains, and the musculoskeletal system, but compelling evidence exists also in tissues subject to fluid flow, such as the endothelium and the lung. As such, we anticipate that mechanical forces will play an extensive, if not ubiquitous, role in stem cell differentiation. Notably absent from the literature, however, are any studies of how mechanical signals might promote the undifferentiated state or "stemness." Preliminary findings indicate that RhoA/ROCK signaling controls differentiation of embryonic stem cells (Peerani et al., 2007; Watanbe et al., 2007), although a specific role of tension/mechanical force remains wholly unexplored. Given the poor efficiency of forming induced pluripotent cells in vitro (Meissner et al., 2007; Takahashi et al., 2007) and the apparent importance of microenvironmental cues in forming "stem cell niches" (Lensch et al., 2006; Moore and Lemischka, 2006), it is tempting to speculate that the mechanical environment may hold the keys to engineering stemness, and represents an important opportunity and direction for future research.

Also critical to our understanding of stem cells is a better picture of the mechanisms by which mechanical signals are transduced into changes in gene expression. Currently much remains to be addressed, but it is clear that RhoA/ROCK (Sordella et al., 2003; McBeath et al., 2004) and ERK signaling (Iqbal and Zaidi, 2005) are key players in mechanosensation, and these molecules may serve as hubs to mediate cross-talk between mechanical stimuli and soluble factors. Indeed, mechanical forces appear to either enhance or antagonize differentiation signals induced by growth factors and cytokines. Nonetheless, it is also important to acknowledge that in some cases mechanical signals appear to completely bypass the influence of soluble factors by directly altering gene expression (Farge, 2003; McBeath et al., 2004; Jakkaraju et al., 2005; Engler et al., 2006). Moreover, unlike soluble cues, mechanical forces are distinct in their potential for propagating across large distances, and thus may provide an instantaneous conduit for coordinating differentiation events at both the single cell and tissue-wide scales. Given the prominent role of scaffolds in regenerative medicine, scaffold-derived mechanical signals, such as matrix stiffness, must be given careful consideration by tissue engineers for potential effects on stem cell differentiation. Functional tissue engineering, particularly in cases where stem cells must form multiple cell types in situ, will likely hinge on our ability to understand and manipulate the propagation of tissue-scale mechanical signaling.

7. Acknowledgements

Work in C.S.C's lab is supported by grants from the NIH (EB00262, EB001046, HL73305, GM74048), the Army Research Office Multidisciplinary University Research Initiative, and the Penn Center for Musculoskeletal Disorders. D.M.C has also received support from by a Kirschstein National Research Service Award (HL084966).

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