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REVIEW

Understanding the extracellular forces that determine cell fate and maintenance

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ABSTRACT

Stem cells interpret signals from their microenvironment while simultaneously modifying the niche through secreting factors and exerting mechanical forces. Many soluble stem cell cues have been determined over the past century, but in the past decade, our molecular understanding of mechanobiology has advanced to explain how passive and active forces induce similar signaling cascades that drive self-renewal, migration, differentiation or a combination of these outcomes. Improvements in stem cell culture methods, materials and biophysical tools that assess function have improved our understanding of these cascades. Here, we summarize these advances and offer perspective on ongoing challenges.

KEY WORDS: Mechanobiology, Biomechanics, Extracellular matrix, Stiffness, Stem cells

Introduction

Early development is a dynamic process marked by the rapid transformation of a fertilized cell into a three-dimensional (3D) embryo. Directed proliferation, differentiation and migration occur in a highly regulated and sequential order, orchestrated by a suite of soluble and mechanical cues that are present in the cellular microenvironment. The soluble cues that regulate development have been extensively studied, but the roles that physical cues play are less well understood, largely due to the limited availability of mechanical models of differentiation and morphogenesis. As cells pull on their niche (defined here as the cellular environment), e.g. when they migrate, they generate contractions that displace the extracellular matrix (ECM) to which they adhere (Discher et al., 2009; Loganathan et al., 2016). Such cues can be considered 'active' and, by definition, they change over time. Conversely, physical cues can also be passive, by exerting time-independent influence on cell behaviors through matrix stiffness, porosity and topography. For example, differential substrate stiffness has been shown to induce stem cell differentiation into neural, muscle or bone cells (Engler et al., 2006). The importance of physical cues has been demonstrated by the observation that their removal arrests embryogenesis (Behrndt et al., 2012; Martin et al., 2009). Thus, in order to drive development, cues (whether passive or active) must be presented in an appropriate spatial and temporal presentation manner. Moreover, there is an interplay between active and passive physical cues (Discher et al., 2005), and a better understanding of this relationship is necessary to understand how mechanical forces drive development. Regardless of their presentation, physical cues must always exist in equilibrium (Nelson and Bissell, 2006) but can be combined with other inputs, such as chemical gradients. As illustrated in Fig. 1, a cell must integrate all these cues to develop a single response or output – such as proliferation, differentiation, migration or death.

Since the first identification of physical interactions between the niche and cells (Harris et al., 1980), much of the initial focus of mechanobiology research as a field was on understanding stem cell sensitivities to their niche, both active and passive. However, over the past decade, the field has become increasingly influenced by molecular level analyses aimed at understanding how physical cues induce specific intracellular signaling cascades to bring about changes in cellular behavior. Thus, in this Review, we first present an overview of the different kinds of active and passive physical cues that can influence cell behavior. We discuss the methods that can be used to induce such forces, and also make a case for the continued utility of two-dimensional (2D) culture systems (alongside newer, more-complicated 3D systems) for the analysis of biomechanical inputs on cells. We then provide a careful discussion of the signaling mechanisms that respond to these inputs, and of our current understanding of how biophysical signals can be converted to biochemical ones. We conclude with a perspective on the ongoing challenges in medicine for which a force-based approach and improved stem cell differentiation can be of use.

'Inputs' and 'outputs': physical cues and their influence on stem cell behaviors

The past decade has observed a wealth of investigation into the effects of active and passive forces on stem cells using a variety of methods, as described in Table 1 and illustrated in Fig. 2. For illustrative purposes, we have grouped these methods in broad categories including externally applied, biomaterial-induced and cell-induced forces. In the following sections, we describe specific applications of these techniques and methods to investigate the effect of active and passive forces (the 'inputs' to the system) on stem cell responses (and those of their progeny), including the decision to self-renew or differentiate (the 'outputs'). We will also discuss how these responses are modulated by the dimensionality of the niche.

Externally applied forces as inputs that regulate cellular responses

The most obvious developmental process in which forces play a role is morphogenesis. This requires cells and tissues to undergo a number of crucial deformations, such as twisting, bending and stretching against their environment. These deformations are made possible by a number of forces that are produced by cells directly or indirectly. One such example is cardiac looping during heart development, in which cells bend, twist and stretch the straight heart tube while pressure gradients caused by global contraction asymmetrically increase tube stiffness, prompting the tube to bend (Voronov et al., 2004). Though a great deal of information has been gleaned from this and other animal models of development,

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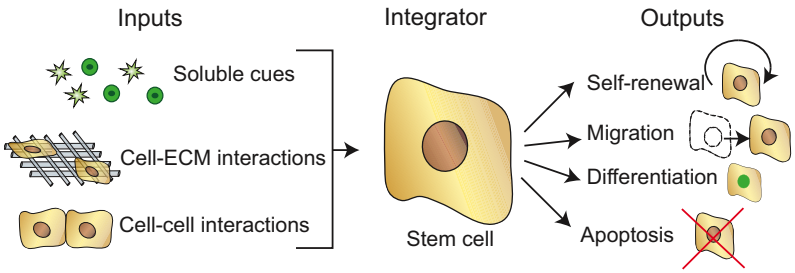

Fig. 1. The stem cell as a mathematical integrator. A stem cell can integrate several input types to result in an output that is the (often amplified) summation of all cues it receives. Representative inputs and outputs are shown for a generic stem cell during development. Such cues can be chemical (e.g. soluble or cell-surface signaling molecules) or physical – involving the generation or modification of intra- or intercellular forces.

researchers have begun developing reductionist techniques – particularly using 2D cultures – to understand how stem cells themselves respond to these inputs and cause subsequent and specific cellular responses or outputs. Here, we consider three main types of externally applied force: twisting, cyclic stretching or strain, and shear – discussing how they can be induced (physiologically and experimentally) and measured, as well as their functional consequences.

Twisting can be both induced and measured by magnetic twisting cytometry (MTC, Fig. 2A), a torque-based deformation method whereby a magnetic field is applied to a bead attached to cell(s) (Wang et al., 2002). The torque experienced by cell(s) can induce changes in behavior, similar to those experienced during development. For example, embedding of paramagnetic beads into embryoid body (EB) cultures and long-term application of force can push embryonic stem cells (ESCs) into contractile fates such as cardiomyocytes or smooth muscle cells (Geuss et al., 2014). Recently, ESCs have been allowed to internalize the paramagnetic particles, and, upon long-term stimulation, this was shown to drive the formation of EBs and subsequent differentiation towards the mesodermal cardiac pathway (Du et al., 2017). Although MTC can be used in 3D systems, it has more commonly been applied in planar culture to observe the response of a cell to a cyclic twisting force. In mouse ESCs, chronic application of MTC induced cell spreading and decreased expression of pluripotency genes, e.g. *Oct3* and *Oct4* (Chowdhury et al., 2010).

As well as its use in applying long-term external force stimulation, MTC has also been used to help measure intrinsic properties, e.g. cell stiffness, via a short-term application of force and subsequent measurement of the deformation of a cell from that force (Wang et al., 2002). Mouse ESCs, when placed on substrates with different stiffness, do not themselves stiffen (Poh et al., 2010), i.e. their deformation does not change over time when a force is briefly applied to the paramagnetic particle. On the other hand, short-term application of force to adult human mesenchymal stem cells (MSCs) shows that they do exhibit a stiffening response when cultured on hydrogels of different stiffness, and this response drives differentiation into osteoblasts versus adipocytes independent of induction method (Ahn et al., 2014).

Cyclic stretching or strain is a second force 'input' to which stem cells respond. Cells are seeded on a deformable membrane and are subjected to periodic strain to mimic the intermittent stretching (Fig. 2B) that occurs *in vivo*, e.g. in the beating heart or blood vessels. Stretch can occur in uni-, bi- or equibi-axial (where cells are stretched in a manner such that they are confined to regions of homogenous strain in both) directions. Unlike MTC and depending on the stem cell type and niche context, cyclic stretching can modulate the balance of self-renewal and differentiation. For example, cyclic strain induces increased proliferation for MSCs but reduces it for adipose-derived stem cells (ASCs) (Lee et al., 2007; Song et al., 2007). As with other actively applied forces, cyclic strain also induces ESC differentiation into cardiomyocytes and vascular smooth muscle cells, improves maturity and aligns cells along the direction of stretch (Gwak et al., 2008; Heo and Lee, 2011). This behavior mirrors *in vivo* behavior, where cyclic strain induced by blood flow contributes to cell alignment along the direction of stretch (Sinha et al., 2016). One problem with this 'input' method is that there are no community-wide standards for stretch duration, intensity or direction of stretching, and we do not know how best to set the parameters to recapitulate *in vivo* conditions; uni-axial stretch is most common and may imitate some vascular conditions, but no clear consensus has emerged.

Such standards are more straightforward for the third type of input discussed here: fluid shear forces applied by fluid flow (Fig. 2C). Hemodynamic forces are essential during development; reductions of these forces induced via deletion of cardiac-specific genes result in embryonic death (Culver and Dickinson, 2010). In development after flow is established, additional transcriptional regulation of many vasoactive endothelial genes, e.g. via KLF2 (Lee et al., 2006) and ephrin B2 (Masumura et al., 2009) among others, occurs. As the embryo develops, higher hemodynamic forces are correlated with further maturation and gene expression (Culver and Dickinson, 2010; Lee et al., 2006). Although significant exploration of the consequences of shear on cell fate has occurred in animal models, equally important analyses have been conducted *in vitro*. For example, shear forces produced by a pulsatile flow bioreactor upregulated endothelial and downregulated smooth muscle cell markers in MSCs (Dong et al., 2009). Recent data in cultured human induced pluripotent stem cells also indicate that initial specification with differentiation media containing VEGF (i.e. inducing endothelial fate) is augmented by shear stress (Sivarapatna et al., 2015). Stem cell models have also proven useful when investigating how shear stress is transduced to regulate transcription, with receptors including FLK1 (Wolfe and Ahsan, 2013) and primary cilia (Hierck et al., 2008) having been implicated. Although further discussion of the interplay of developmental shear stress with other signals is beyond the scope of this Review (for further details see Freund et al., 2012), this is an area in which significant efforts are aimed at recapitulating these combinations *in vitro*.

Understanding these external forces, how to measure them and what their influences are is crucially important, but – as alluded to above – one problem that has plagued mechanobiology has been the limited information in the literature about the amount, timing and location of the forces required by cells to activate appropriate signaling pathways. Some progress has been made with force-sensitive signaling probes, including fluorescent-based tension sensors (Grashoff et al., 2010; Morimatsu et al., 2013) or rupturable gauges (Wang and Ha, 2013), to quantify the amount of force required to activate a biophysically induced signaling cascade. Yet determining the sensitivity of individual stem cell types and lineages to force-activated signaling remains a major challenge for the field. Further challenging these efforts is the likelihood that the

Development (2017) 144, 4261-4270 doi:10.1242/dev.158469

Table 1. Examples of externally applied and biomaterial-induced forces and their resultant output

Method/technique	Cell type	Output	Reference
Biomaterial-induced force			
Stiffness input			
Micropillars (gradient in height)	MSCs	Osteogenic lineages favored short posts; adipogenic lineages favored long posts	Fu et al. (2010); Tan et al. (2003)
	ESCs	Increase in contractility with decreasing pillar length; maintains pluripotency for single cells	Sun et al. (2012a)
PA gels	Fibroblasts and ECs	Cells spread in response to changes in stiffness	Pelham and Wang (1997)
PA gels	MSCs	Directed differentiation towards neurogenic, myogenic or osteogenic lineages using soft, stiffer and comparatively stiff gels	Engler et al. (2006)
MeHA hydrogels	MSCs	Adipogenic and osteogenic lineages were selected based upon time of stiffening and stiffness	Guvendiren and Burdick (2012)
Silicone gels	MSCs	Preference of generation of adipocyte and osteoblast-like cells was stiffness dependent, with adipocyte lineage preferring softer substrates and osteoblast preferring stiffer substrates	Vertelov et al. (2016)
Externally applied force			
Deformation input			
Magnetic twisting cytometry	ESCs	Differentiation into contractile phenotype	Geuss et al. (2014)
	ESCs	Cell spreading	Chowdhury et al. (2010)
Magnetic wires	Fibroblasts	Externally applied magnetic fields caused significantly increased traction forces	Sniadecki et al. (2007)
	SMCs	Contractile reinforcement due to external stimulation	Lin et al. (2012)
Cyclic stretching and surface patterning	MSCs	The combination of stretching and surface micropatterns increased the cardiomyogenic differentiation of MSCs	Qiu et al. (2016)
Cyclic stretching	MSCs	Increased proliferation	Lee and Shin (2007)
	MSCs	Induced differentiation into fibroblast-like cells	Qiu et al. (2016)
	ASCs	Reduced proliferation	Song et al. (2007)
	ESCs	Improved differentiation into cardiomyocytes and vascular smooth muscle cells	Heo and Lee (2011)
Cyclic stretching and bending	MSCs	Resulted in an increase of cardiomyogenic differentiation of MSCs	Yoon et al. (2017)
Mechanical stress and cyclic stretching	ASCs	Reduced proliferation and adipogenic differentiation capacity	Paul et al. (2017)
Fluidic shear forces	MSCs	Upregulation of EC and downregulation of SMC markers	Dong et al. (2009)
Fluidic shear forces and circumferential stretching	MSCs	MSCs were shown to differentiate towards vascular endothelial lineages due to these dynamic mechanical forces	Kim et al. (2016a)
Long-term stretching	ASCs	Resulted in an increase in osteogenesis	Vlaikou et al. (2017)
Compression	MSCs and human dental pulp stem cells	Dynamic compressing of these stem cells aided in the differentiation into odontoblasts	Miyashita et al. (2017)
Biomaterial-induced and cell-induced force			
Cell area and shape input			
Microprinted islands	MSCs	Small islands and low membrane curvature encourage adipogenic differentiation; large islands or high curvature results in osteogenic differentiation	Kilian et al. (2010); Liu et al. (2006); McBeath et al. (2004)
Micropatterns	Adult renal stem cells	Micropatterned substrate induced the commitment of renal stem cells into tubular cells	Sciancalepore et al. (2016)
	Fibroblasts	Switching of fibroblasts into induced neurons is supported by substrate topography	Kulangara et al. (2014)
	Cardiac progenitors	Differentiation of cardiac progenitors into cardiomyocyte-like cells is supported through the use of parallel microgrooves	Morez et al. (2015)
	ESCs and iPSCs	Nanopore patterned substrates promote generation of pancreatic endocrine cells. Authors hypothesized this was due partially to TAZ being downregulated.	Kim et al. (2016b)

ASCs, adipose-derived stem cells; ECs, endothelial cells; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; MeHA, methacrylated hyaluronic acid; MSCs, mesenchymal stem cells; PA, polyacrylamide; SMCs, smooth muscle cells.

method of force application and its directionality (e.g. compressive pushing inward, tensile pulling outward or shear acting on a surface) may influence the response, further complicating our understanding of exactly how a stem cell acts to integrate signals.

Biomaterials mimic passive (and active) cues that regulate stem cell responses

The previous section focused on stem cell responses from applied forces, but there are numerous situations where a stem cell simply responds to its surroundings as it pulls on and 'feels' it. Thus, we will next discuss techniques developed over the past decade to model physical properties of the environment, as well as the outputs regulated by these properties (Fig. 1, right). A crucial development in the late 1990s from Pelham and Wang was the adaptation of polyacrylamide gels, conventionally used for electrophoresis, as a cell culture substrate (Fig. 2D). Using this system, they noted that fibroblasts and endothelial cells spread in a stiffness-dependent manner – stiffer substrates induce greater spreading (Pelham and

Development (2017) 144, 4261-4270 doi:10.1242/dev.158469

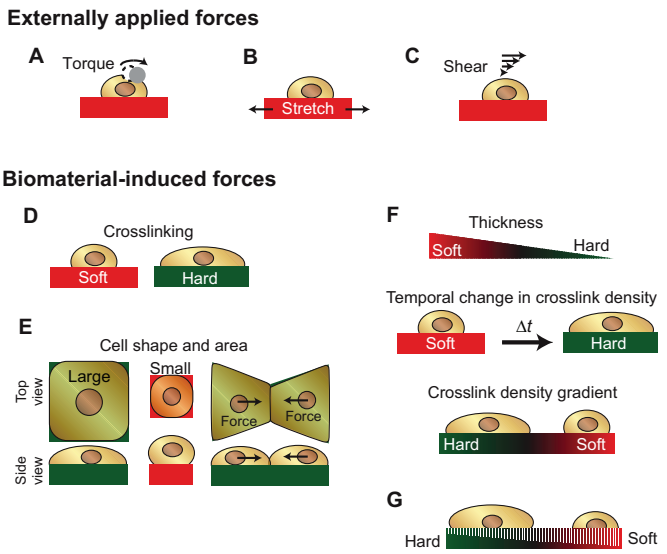

Fig. 2. Externally applied and biomaterial-induced forces. Forces can be applied to cells using multiple experimental techniques (see also Table 1). (A) Magnetic twisting cytometry can locally apply forces to cells via twisting a bead on the surface of the cell. (B) Substrate deformations can be used to modulate cell response by applying forces dynamically through cyclic stretching, and (C) by applying fluidic shear to cell surface. Separately, (D) biomaterials can also be used to apply forces to cells by varying substrate stiffness. (E) The adhesive area to which a cell will attach and spreading can affect the ability of a cell to contract against that surface. When multiple cells are patterned together, their intracellular forces must balance the forces of the adjoining cell, which are transmitted across the cell-cell junction. (F) Biomaterials can also have temporal and spatial gradients. For example, the thickness of the biomaterial can be changed as a function of location. There can also be temporal changes such as dynamic stiffening where the substrate modulus is changed during the culture process. Finally, biomaterials can be fabricated with gradients in crosslinking density changing their presentation as a function of location. (G) Whereas the other methods use continuous surfaces, micropillars of varying height effectively change surface rigidity, i.e. the longer the post, the softer apparent rigidity, to affect a change in cell behavior. Surfaces can still be actively modulated through the addition of magnetic wires within the posts.

Wang, 1997). Subsequent work with bone marrow-derived MSCs showed that matrix stiffness could activate serum-based responses and drive cells towards neurogenic, myogenic or osteogenic lineages when cultured on soft neural-like, firm muscle-like and stiffer bone-like polyacrylamide hydrogels, respectively (Engler et al., 2006), i.e. they express lineage-specific transcription factors. At the time, stiffness-mediated signaling was mainly thought to be regulated by intracellularly generated forces from non-muscle myosins, as increasing contractile forces were required to deform increasingly stiff matrices to the same extent (Legant et al., 2009). Subsequently, stiffness regulation has been observed in ESCs similar to that for MSCs (Evans et al., 2009), where stiffness directed the expression of transcription factors consistent with a single cell fate. Additionally, adipose-derived MSCs that migrated to matrices of muscle stiffness underwent myogenesis and increased fusion rate into myotubes compared with cells on other stiffness. Last, unipotent progenitor cells in muscle cultured on muscle stiffness self-renew, whereas they do not on rigid plastic dishes (Gilbert et al., 2010).

As well as stiffness, substrate geometry – which defines cell area (McBeath et al., 2004) and shape (Kilian et al., 2010) – can modulate stem cell differentiation; small and large microprinted ‘islands’ or low and high membrane curvature encourage adipogenic or osteogenic differentiation of MSCs, respectively (Liu et al., 2006) (Fig. 2E). Similar to MSCs, epidermal stem cells cultured on small micro-printed islands underwent differentiation, whereas cells on large islands did not (Totaro et al., 2017). In either case for cell shape and area, intracellular forces are integrated by the cell to create these responses, even though the forces are in static equilibrium and not dynamically changing. As stem cells rarely exist in isolation, how this equilibrium is maintained across cell pairs has also been explored (Maruthamuthu et al., 2011), revealing that cells will additionally balance forces across their cell-cell junctions.

Taken together, these data reinforce the concept that multiple physical inputs can be modulated by other cues, as implied in Fig. 1. However, what complicates this calculation is its non-linearity; the intracellular forces generated from contracting against ECM of a particular stiffness, or of a specific composition, can be permissive or not for differentiation when presented individually. In combination, however, they are not additive (Kourouklis et al., 2016; Rowlands et al., 2008). Even within a particular cue, e.g. ECM composition, hundreds of unique combinations do not appear additive from their individual counterparts (Flaim et al., 2005; Kourouklis et al., 2016). Thus, one needs to appreciate the complexity of these cues and their influence on force, but even that may not predict how they will be integrated by a stem cell.

Just as cells do not exist in isolation, environmental cues that induce intracellular forces are not static; niche properties change both with space and time (Fig. 2F), and this could affect how a stem cell integrates cues and makes decisions. In culture, stiffness has typically been presented as a single value, but it changes *in vivo* during development and often with disease. One question that arises is at what point is stem cell commitment no longer able to respond to the physical attributes of the niche? Temporal gradients can be induced via sequential ECM crosslinking using biomaterials with single or multiple crosslinking methods (Guvendiren and Burdick, 2012; Young and Engler, 2011). Conversely, materials can have crosslinks degraded to soften ECM or induce stress relaxation (Chaudhuri et al., 2016; Kloxin et al., 2009). Interestingly in all cases, the consequence of the change in stiffness varies according to when it is induced. Thus, for example, during MSC differentiation, changes applied in the first week appear reversible, but those made in subsequent weeks are not (Guvendiren and Burdick, 2012; Young and Engler, 2011). Similarly with neural stem cells, there is a small temporal window in which ECM stiffness maximally affects neurogenic commitment; altering stiffness signaling in this window dramatically impacts neurogenesis (Rammensee et al., 2017), whereas changes at other times have more minor effects.

Tissue stiffness also typically contains spatial gradients, which can be accomplished by changing gel thickness, crosslink density or micropost length in a spatially dependent manner (Hadden et al., 2017; Tse and Engler, 2011; Zaari et al., 2004). Stiffness can vary by six orders of magnitude *in vivo* (Discher et al., 2009) and gradients within tissues can vary by up to three orders of magnitude (Vincent et al., 2013). Most committed cells migrate preferentially to stiffer regions via unbalanced forces created by these gradients, i.e. ‘durotaxis’ (Lo et al., 2000; Vincent et al., 2013). MSCs also exhibit these unbalanced forces but are even more sensitive to gradient slope and range, exhibiting directional migration even when the gradient is at or below natural physiological variation in tissues (Tse and Engler, 2011; Vincent and Engler, 2013). As these cells are uncommitted, it is perhaps surprising that they would exhibit such preference, which also biases their differentiation towards more contractile lineages, e.g. bone. Interestingly, some report similar ‘memory’ of their previous niche post-durotactic

Development (2017) 144, 4261-4270 doi:10.1242/dev.158469

migration, e.g. MSCs that migrated from soft to stiff regions still expressed neuronal markers (Tse and Engler, 2011), which suggests that as with temporal changes, spatial variation can impact stem cell plasticity.

Both ESCs and MSCs use cell-based forces to contract against their niche, but important differences occur between them that give rise to different sensitivities. Common methods to measure these deformations include micropillars (Tan et al., 2003). In the case where posts are fabricated to different heights while maintaining a planar surface, larger posts are more deformable and produce more bending compared with shorter posts (Fig. 2G), creating a gradient similar to those in solid surfaces. MSCs cultured on short rigid posts are well spread with highly organized actin fibers compared with cells on longer more-deformable posts. Furthermore, differential post deformability altered differentiation, as osteogenic fates were favored on short posts and adipogenic fates were favored on long posts (Fu et al., 2010). However, rather than determining cell fate, decreasing pillar length induced increased human ESCs contractility, which makes for a more rigid substrate surface and maintains pluripotency in isolated cells. Furthermore, removal of cell-cell contacts (i.e. only single cells) muted cell-micropost interactions and indicated synergy between the stimuli; thus, the percent of pluripotent cells was reduced regardless of substrate stiffness when cell-cell contact is present (Sun et al., 2012b). These data are consistent with the interpretation that cell-cell and cell-matrix forces balance to maintain cell fate. In an expansion of this application, magnetic wires have been added to these posts to actively stimulate cells via torque induced by a magnetic field moving the micropillars (Sniadecki et al., 2007). Post-stimulation, intracellular forces rearrange and change magnitude (Khademolhosseini et al., 2016; Lin et al., 2012), suggesting the stem cells responses, e.g. fate, could be driven by these externally applied local deformations. Together, these data – particularly the difference in response between MSCs and ESCs – suggest that not only is the cell response to force context specific, it is also stem cell type specific.

The value of 2D systems to observe force-based differentiation

The systems discussed so far have largely been limited to 2D systems, where the role force plays can be more carefully controlled relative to the stem cell differentiation that is observed; indeed, numerous protocols to differentiate stem cells into cardiomyocytes, bone, muscle and fat, and others use 2D systems with defined factors, chemistry and physics. However, development is a complex, 3D process requiring significant intra- and extra-cellular forces to accomplish cell rearrangements. These are very hard to model *in vitro*, although efforts focused on EBs and organoids have, in some cases, proved fruitful. Although simple EBs may not require much force to form their initial structure (Pettinato et al., 2014a), a tremendous amount of deformation occurs during EB maturation, allowing some to form cavities (Li et al., 2002) and contractile chambers (Mathur et al., 2015). Although EB structures introduce increasing complexity that better model certain developmental processes, and while methods can be adopted to measure forces for cells in a more heterogeneous 3D niche (Legant et al., 2010; Mulligan et al., 2017), additional innovation is necessary to properly model the physical cues that regulate development. Yet the problem with 3D systems goes beyond their complexity to how closely they mirror development and the forces therein. For example in 3D systems, although matrix stiffness affects stem cell fate in a similar manner to 2D culture when the cells can contract the material and rearrange crosslinks (Huebsch et al., 2010), it may not be effective in directing differentiation when cells are entrapped in materials by crosslinks that are immobile or non-degradable (Wade and Burdick, 2010).

Despite the clear advantages for 3D systems, their complexity – the fibrillar, heterogeneous and anisotropic structure of 3D structures – challenges reproducibility. A major goal of 2D protocols that exploit externally applied or intracellularly generated forces is to improve stem cell maturation and lineage purity, which may be counterintuitive to the expansion of 3D systems in the field over the past decade (Burridge et al., 2014; Maffioletti et al., 2015; Patsch et al., 2015; Si-Tayeb et al., 2010), but which has clear practical advantages. Thus, our emphasis on these well-defined protocols is consistent with both a drive towards standardization of stem cell protocols and to a demand for more refined biomechanical tools (Dahl et al., 2005; Engler et al., 2007; Yim and Sheetz, 2012; Zhang et al., 2017), global force sensors (Zhou et al., 2015), and molecular strain and tension sensors (Grashoff et al., 2010). In the next section of this Review, we turn our attention to how such tools can be used to understand the cellular effects of physical inputs in a defined and reductionist way.

The cell as an 'integrator': converting biophysical stimuli into biochemical responses

Thus far, we have discussed the kinds of physical inputs that can impact on stem cell behavior. We now consider how these cues are converted from a biophysical metric into a biochemical signal that the cell can interpret and act upon. Several mechanisms have been implicated in this conversion (as summarized in Fig. 3) and include, but are not limited to: (1) actin-myosin associated regulation, (2) focal adhesion (FA) signaling via strain-activated sensors (Holle and Engler, 2011), (3) force-sensitive transcription factor localization (Dupont et al., 2011), (4) stretch-activated channel (SAC)-induced ion changes (Nourse and Pathak, 2017; Ranade et al., 2015) and (5) nuclear-associated protein signaling and chromatin unfolding (Athirasala et al., 2017; Makhija et al., 2016). Once force is converted into a biochemical signal, enzymes such as kinases and phosphatases can respond to changes in protein activity, and/or the transcriptional and translational machinery of the cell can be induced to modulate protein expression. Here, we focus on the four most explored mechanisms: actin-myosin associated regulation, strain-activated FA sensors, force-sensitive transcription factor localization, and nuclear-associated protein signaling and chromatin unfolding, before offering our perspective relating to ongoing challenges that hinder our understanding of and ability to control force-induced signaling. It is important to note that the fifth mechanism, SAC-induced ion changes and its most well-known channel Piezo1, have only recently been described in the context of stem cell mechanotransduction (Heo et al., 2015) and differentiation for lineage-restricted progenitors (Pathak et al., 2014); as such, we will limit ourselves to discussion of the four remaining mechanisms in this section.

Actomyosin-based regulation of cell fate

Actin and myosin II form the classic cytoplasmic contractile apparatus found in muscle and, more generically, in all adherent cells (Fig. 3A): actin forms thin filaments upon which myosin thick filaments bind and slide to create a contractile force. In the fields of stem cell mechanobiology and biomechanics, there has been particular focus on understanding how non-muscle myosin II (NMMII) enables progenitor cells to 'feel' the stiffness, porosity and topography of their environment, and develop intracellular tension (Engler et al., 2006; Kilian et al., 2010; McBeath et al., 2004;

Development (2017) 144, 4261-4270 doi:10.1242/dev.158469

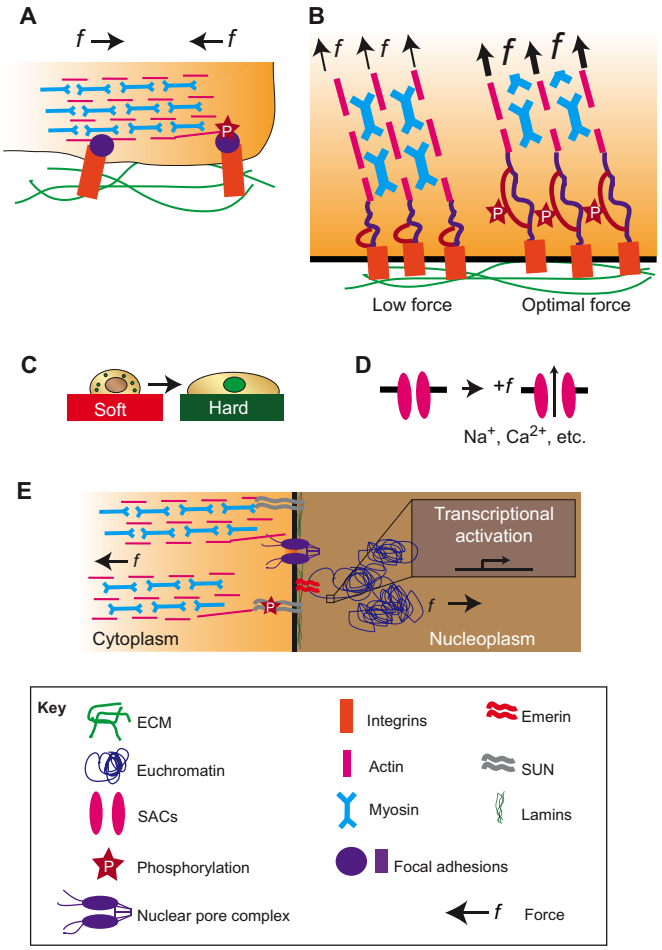

Fig. 3. Integrating and converting biophysical signals. Forces can drive stem cell responses using one or more of multiple mechanisms used to convert these forces into biochemical signals. These include: (A) actin-myosin contraction regulation; (B) focal adhesion (FA)-based signaling mechanisms; (C) force-sensitive transcription factor localization; (D) stretch-activated channels (SAC) causing ion flux changes; and (E) nuclear-associated protein signaling resulting from force transduction into the nucleus via SUN and emerin causing chromatin unfolding. In all cases, these mechanisms result in transcriptional changes, translational changes (not indicated) or protein activity changes in the cytoplasm (not indicated).

McMurray et al., 2015; Wang et al., 2002). These different passive properties of the niche may seem disparate, but inhibition of NMMII actin-binding activity (Guilak et al., 2009) or depolymerization of the structure entirely (Arnsdorf et al., 2009) renders stem cells unable to respond to any of these material effects. Chronic NMMII inhibition can also reduce contractility-related apoptosis in stem cells in culture (Chen et al., 2010; Watanabe et al., 2007). Direct application of external force is also crucial for myosin II-mediated cell responses (Riveline et al., 2001). Although there are direct mechanical connections between the actomyosin contractile apparatus and focal adhesions (Zamir and Geiger, 2001), the intra-cellular forces are distinct, spatially decoupled from each other (Plotnikov et al., 2012) (i.e. one adhesion can be actively transducing a contraction whereas an adjacent one does not), and insensitive to other stimuli that affect maturation of focal adhesion structures (Stricker et al., 2013). Although this and other evidence strongly suggests a role for actomyosin contractions in transducing biophysical signals, the specific molecular details in stem cells regarding how NMMII could convert these contractions into biochemical signal remain unclear. More generally, however, one potential actomyosin transduction candidate is serum response factor (SRF), which is a transcriptional regulator controlled by actomyosin dynamics (Connelly et al., 2010).

‘Molecular strain gauges’ and mechanically activated transcription factors

Focal adhesions are a cluster of proteins physically bound to one another that connect extracellular matrix receptors with the cytoskeleton of the cell, which contains its actomyosin contractile structures. Within these adhesions, transcription factor-binding sites have been identified that, when exposed, could act as molecular switches (Fig. 3B, star) that convert a stem cell into a mature cell based on the passive forces that the cell can exert on its environment. One of the best-studied examples of a focal adhesion tension sensor is vinculin (Holle et al., 2013). In human MSCs, vinculin can act as a strain gauge and control stiffness-mediated differentiation; too little or too much force transduced across the protein can lead to cryptic kinase-binding sites remaining buried or completely unfolding, respectively. Thus, vinculin-mediated signal transduction via kinases is force sensitive. For example, MSCs lacking vinculin remain adherent to their ECM and contractile but fail to differentiate into muscle in stiffness-based differentiation assays due to a lack of mitogen-activated protein kinase (MAPK) binding to a cryptic site on vinculin. Stem cells rescued with mutant forms of vinculin (headless, tailless or lacking the MAPK-binding site) also failed to differentiate into muscle. These data are consistent with observations in other cell types in which partial length vinculin was insufficient for many cell functions (Dumbauld et al., 2013). Beyond vinculin and MAPK signaling, other focal adhesion-based, strain-sensitive regulators that employ a force-dependent, conformational change-based mechanism, i.e. molecular strain gauge, have been identified by high-throughput screening to detect proteins that when deleted would prevent stiffness-based induction of differentiation (Holle et al., 2016). These sensors often will have different set points to regulate differentiation to other lineages. For example, sorbin and SH3 domain-containing protein 1 (SORBS1) was found to regulate stiffness-sensitive osteogenesis in MSCs in the same manner that vinculin governs myogenesis, albeit with a higher stiffness set point – i.e. greater force is required to activate SORBS1 than vinculin. This type of mechanism offers great precision; the sensor is tuned to unfold or change conformation only over a narrow range of tensions. Thus, its ‘set point’ is exceedingly sensitive.

Force-sensitive transcription factor localization

Strain-sensitive proteins in focal adhesions may change their confirmation, but additional force-sensitive switches, including some transcription factors, appear to shuttle information to the nucleus in a similar manner. For example, YAP/TAZ is a robust signaling complex that translocates from the cytoplasm to the nucleus as the niche becomes stiffer or stem cell shape changes for MSCs among other stem cell types (Dupont et al., 2011). Translocation can also occur via changes in cell density, shear stress, in 2D or 3D, and via stretch (Panciera et al., 2017). This regulation of cell fate occurs independently of the Hippo/LATS cascade and upstream of Notch signaling to regulate somatic cell stemness (Totaro et al., 2017). Conversely, TWIST1 acts as a mechanomediator that induces epithelial-to-mesenchymal transition (EMT) on stiff matrices by translocating to the nucleus. (Wei et al., 2015). This pathway appears to function independent of the classic YAP/TAZ ‘switch’ identified in MSCs in the past

Development (2017) 144, 4261-4270 doi:10.1242/dev.158469

decade. In both instances, the exact mechanisms are not entirely clear, but they may involve changes in the phosphorylation of the factor or its chaperones (Shamir et al., 2014; Wei et al., 2015). However, it is important to note that initial stiffness-sensitive transcription factor nuclear translocation may not be sufficient for complete lineage specification; MSCs, which express stiffness-sensitive RUNX2 (Engler et al., 2006), require cell-cell contact for later osteogenesis markers (e.g. alkaline phosphatase and calcium deposition) to be induced. These data demonstrate the interplay that exists between force-sensitive differentiation mechanisms and other inputs (Mao et al., 2016). Regardless, transcription factor localization appears switch-like, centered around a particular set point as with focal adhesion-based sensors, i.e. mechanism in Fig. 3B.

Nuclear-associated-protein signaling and chromatin unfolding

DNA is typically wrapped tightly around histones, which buries some transcription factor-binding sites. Cells can modulate this packing, often by unwinding DNA from histones. Although this association is conventionally thought to involve changes in histone acetylation via histone acetyltransferase and histone deacetylase (HDAC), more recent evidence suggests that intracellular (Makhija et al., 2016) or extracellular forces (Heo et al., 2016; Swift et al., 2013) can deform the nucleus and stretch regions of DNA to change the accessibility of transcription factor-binding sites. Beginning with initial observations that stem cell nuclei become less flexible upon differentiation (Pajerowski et al., 2007) as a result of changes in the nuclear lamina (Swift et al., 2013), there have been tremendous advances in our understanding of how force can convert heterochromatin to transcriptionally active euchromatin (Fig. 3D). Transmission of these forces requires an intricate network of proteins that link the actomyosin contractile apparatus of the cell to the nucleus. That specific machinery includes transnuclear membrane proteins such as SUN and LINK (Jaalouk and Lammerding, 2009), which are anchored to the membrane by the nuclear lamins. A second network of proteins, including emerin (Ungricht and Kutay, 2017), tether DNA to the lamins, thus creating a bridge between the force-generating region of the cell and the chromatin. These structures are exceedingly dynamic, feeding back on themselves as the nucleus is exposed to forces.

As a stem cell differentiates, the chromatin condenses and binding accessibility changes, silencing regions of the DNA that are no longer necessary (Rajapakse et al., 2009). In this context, forces can still activate new regions of transcriptional activity, but without force, such regions may remain silent. If any part of this sensing machinery is depleted or knocked down, that reduces strain-induced rearrangements and blocks stem cell differentiation (Li et al., 2011). Recent experiments in CHO cells have shown that myosin-mediated intracellular forces are sufficient to deform chromatin and extracellular forces applied by MTC can induce transcription of a reporter, e.g. a green fluorescent protein (GFP)-tagged dihydrofolate reductase (DHFR) transgene (Tajik et al., 2016). Intracellular forces can also rearrange chromosome territories, resulting in force-mediated gene regulation (Wang et al., 2017). Conversely, significant biaxial cyclic stretch, e.g. 10% at 0.1 Hz, may actually be counterproductive and transcriptionally silence many genes in stem cells. In such cases, force-mediated recruitment of emerin or associated proteins may drive defective heterochromatin anchoring, leading to the silencing (Le et al., 2016).

Although we have focused this discussion on cellular forces, thus far, biomaterial-induced responses by the cells also can regulate DNA rearrangements. For example, stem cells align with microgrooved patterns, and when the patterns are either compressed or stretched orthogonal to the microgrooves, stem cells show decreased HDAC activity and increased histone acetylation (Li et al., 2011), implying that accessibility changes are the result of external sensing of the physical change. Additional regulation by shear, spreading, stiffness and the nucleus:cytoplasm ratio has also been observed in manners that reflect the topography (Lee et al., 2014). Observations of both the transducing machinery and chromatin accessibility changes in stem cells suggest that higher force-mediated DNA accessibility can enable differentiation, which subsequently makes these rearrangements more difficult as the nucleus stiffens and lineage is maintained.

Independent of the mechanisms described above (along with signaling via mechanosensitive ion channels), the signaling, transcriptional and translational machinery must be able to respond to intra- and intercellular forces to affect what a cell does, i.e. in the case of stem cell differentiation, which lineage-specific cell markers it expresses. Although presented as distinct mechanisms, linkage between these three is very likely. For example, disrupting F-actin or inhibiting contraction reduces nuclear force transduction via LINC and thus transgenic marker transcription. Conversely, activation of endogenous contraction and nuclear transduction initiated force-induced transcription (Tajik et al., 2016); strain-sensitive molecular switches also are sensitive to cytoskeletal disruption (Dupont et al., 2011; Holle et al., 2013). Thus, a more holistic view of Fig. 3 would suggest that mechanisms do not occur in isolation but as a collective. This perspective may be a more accurate representation of what occurs both *in vitro* with stem cells and *in vivo* during development.

Conclusions

Embryonic development and efficient stem cell differentiation are clearly regulated by a variety of stimuli (Fig. 1) which the cell integrates into a singular response. While the inputs and outputs are clear, furthering our understanding of the specific role for force in this process faces several clear hurdles in the near future. First, and despite much effort to push towards more completely differentiated organoids (Kurosawa, 2007; Li et al., 2002; Mathur et al., 2015; Pettinato et al., 2014b; Sargent et al., 2010), the continued reliance on 2D systems is a double-edged sword. It has enabled the development of numerous protocols that yield near pure populations of very committed cells and has provided invaluable insights into the governing mechanisms. However, these efforts may have inhibited a continued push towards 'embryogenesis in a dish' or at least the development of multi-organ systems in a single structure. Efforts to assemble individual systems into a complex network have been extremely successful (Benam et al., 2015; Huh et al., 2010), but may not completely recapitulate the characteristics of a developing embryo. The first reports of self-organized amniogenesis (Shao et al., 2017) may signify a return to 3D systems, but we would caution that this should be performed only in cases where conditions can be completely defined.

A second hurdle to overcome is the need to more closely integrate the work of molecular biologists and biophysical scientists to tackle the problems associated with our incomplete understanding of each biophysical signaling mechanism and how they interact – synergistically or antagonistically – with each other and with parallel and downstream biochemical pathways. *In vivo* tissue-engineering strategies will also benefit from an enhanced understanding of endogenous stem cell signaling mechanisms, their physiology and their force sensitivity. By addressing these

Development (2017) 144, 4261-4270 doi:10.1242/dev.158469

concerns, we believe that the field can continue to advance towards a more complete understanding of how stem cells are regulated by forces and how those forces can be used to our advantage to create better therapeutic targets for drugs and better stem cell-based therapies.

Competing interests

The authors declare no competing or financial interests.

Author contributions

A.K., J.K.P. and A.J.E. conceived of and wrote the manuscript.

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