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Mechanobiology of bone marrow stem cells: From myosin-II forces to compliance of matrix and nucleus in cell forms and fates ☆

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ABSTRACT

Adult stem cells and progenitors are of great interest for their clinical application as well as their potential to reveal deep sensitivities to microenvironmental factors. The bone marrow is a niche for at least two types of stem cells, and the prototype is the hematopoietic stem cell/progenitors (HSC/Ps), which have saved many thousands of patients for several decades now. In bone marrow, HSC/Ps interact functionally with marrow stromal cells that are often referred to as mesenchymal stem cells (MSCs) or derivatives thereof. Myosin and matrix elasticity greatly affect MSC function, and these mechanobiological factors are now being explored with HSC/Ps both *in vitro* and *in vivo*. Also emerging is a role for the nucleus as a mechanically sensitive organelle that is semi-permeable to transcription factors which are modified for nuclear entry by cytoplasmic mechanobiological pathways. Since therapies envisioned with induced pluripotent stem cells and embryonic stem cells generally involve *in vitro* commitment to an adult stem cell or progenitor, a very deep understanding of stem cell mechanobiology is essential to progress with these multi-potent cells.

1. Introduction

Since the first human bone marrow transplantation (BMT) (Thomas et al., 1957) and the revelation of hematopoietic stem cell/progenitors (HSC/Ps) by clonal assay (Becker et al., 1963), adult stem cells of many tissue types have become an intense focus of research. These cells can either integrate in a target tissue or act as vehicles to deliver important signals to a tissue without integration (Daley and Scadden, 2008). More than 50,000 BMT procedures were performed in the year 2006 alone, saving a large percentage of treated patients but not all (Gratwohl et al., 2010). Such clinical advances and limitations have motivated investigators to elucidate mechanisms by which a few or perhaps just a single stem cell can generate an entire, regenerating tissue. Indeed, the demands are great in human hematopoiesis with 10^5–10^6 nucleated blood cells, anucleated red blood cells (RBCs), and platelets produced every second in marrow to enter blood. New approaches for HSC and HSC-derived blood cell transplantation are still needed to generate potent donor cells that engraft efficiently. Factors that regulate proliferation, differentiation, and dispersion of adult stem cells continue to be elaborated, and include physical factors (*e.g.* Fig. 1) that are the focus of this Review. Some of the latest findings and methods for blood cells are likely to prove applicable to other types of adult stem cells and progenitors such as bone-marrow derived mesenchymal stem/stromal cells (MSCs) (Friedenstein et al., 1976). MSCs are also rapidly emerging as clinically relevant (Hare et al., 2012) and have proven to be highly mechanosensitive—as will be reviewed. Such findings have stimulated new mechanical approaches to other stem cells including HSC/Ps. Lessons learned from these adult stem and progenitor cells will no doubt be useful in the understanding and application of the forms and fates of embryonic stem cells and induced pluripotent cells.

The hierarchical nature of blood cell development has been elucidated through advances in the identification and isolation of HSC/Ps *versus* derived lineages. Particularly critical has been the prospective isolation of HSC/Ps by flow cytometry with a specific set of cell surface antigens (Weissman and Shizuru, 2008) combined with limiting dilution transplantations that quantify HSC frequency (Szilvassy et al., 1990). Aided by these methods, a number of soluble growth factors have been identified that support the differentiation of various blood lineages. However,

Abbreviations: 3D, three-dimensional; AFM, atomic force microscopy; Bleb, blebbistatin; BM, bone marrow; BMT, bone marrow transplantation; ECM, extracellular matrix; ESC, embryonic stem cell; G-CSF, granulocyte colony-stimulating factor; HSC/P, hematopoietic stem cell/progenitor; kPa, kilopascal, units of elasticity; MAL/MKL, megakaryocytic acute leukemia protein; MK, megakaryocyte; MLCK, myosin light chain kinase; MSC, mesenchymal stem cell; NMM-II, non-muscle myosin-II; OB, osteoblast; RBC, red blood cell; RLC, regulatory light chains; ROCK, rho-associated protein kinase; SDF-1, stromal derived factor-1; SRF, serum response factor; YAP, yes-associated protein

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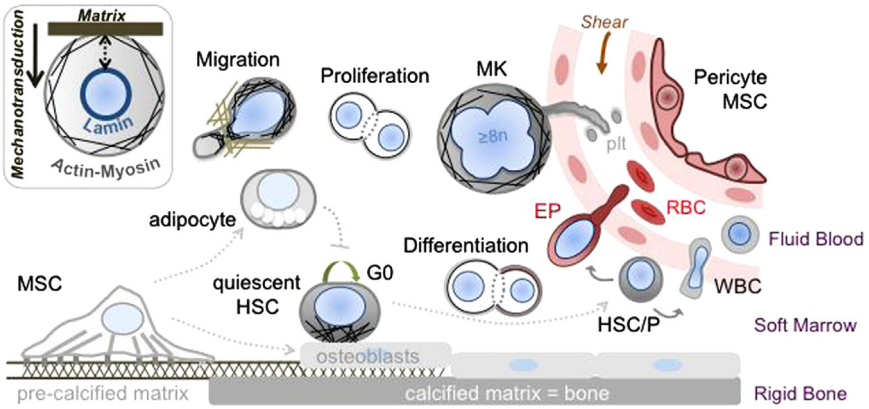

Fig. 1. Myosin, lamin and mechanical factors influence differentiation and trafficking both within and from bone marrow. Bone marrow microenvironments may provide different modes of biophysical regulation, which are principally regulated by actin-myosin at the cell membrane that generates force when cells sense matrix elasticity, followed by force transmission to the nucleus and lamins (Inset). Mesenchymal stem cells (MSCs) in marrow sense matrix elasticity and differentiate into stromal lineages, including osteoblasts and adipocytes. Hematopoietic stem cells (HSCs) are kept in quiescence (G0) near osteoblasts at the endosteum, which is stiffer than the central marrow space. HSC numbers are also suppressed by adipocytes. HSCs traffic towards the vascular niche and undergo either self-renewing expansion (symmetric division) or differentiation (asymmetric division) into progenitors (HSC/Ps) and mature cells. Cells at the vascular niche might also be exposed to shear force, which could affect differentiation. Cells undergo selective trafficking out of the marrow due to physical properties of both cell membrane cortex and nucleus as regulated, respectively, by myosin and lamin. MSCs are additionally present as pericytes and could also regulate hematopoietic cell trafficking. MK, megakaryocyte; EP, erythroid progenitor; plt, platelet; RBC, red blood cell; WBC, white blood cell.

much less is known about physical factors *in vivo* that might govern the self-renewal and function of HSCs. A stem cell "niche" or "microenvironment" was proposed to be a requirement for long-term hematopoiesis from HSCs (Schofield, 1978). Deeper understanding of the niche is likely to foster strategies to direct HSC/Ps *ex vivo* in order to enhance *in vivo* engraftment in patients. Such an effort will also benefit sustained *ex vivo* production of more mature blood cell types, especially RBCs and platelets that are transfused from donors in massive quantities today. While human erythropoietin (Miyake et al., 1977) and thrombopoietin (Kaushansky et al., 1994) have emerged as major factors to, respectively, facilitate erythroid and megakaryocyte (MK) differentiation from HSC/Ps, sustained generation *in vitro* of terminally differentiated cells has remained a major challenge. Indeed, none of the growth factors, cytokines or chemokines has yet proven sufficient to reproduce artificial marrow-like environments conducive to generation of all blood cell-types from HSC/Ps.

Mechanotransduction refers to the conversion of extracellular mechanical inputs to intracellular signals, both biochemical and biophysical (Wang et al., 2009). It is mediated in part by intracellular tension that is sustained by adhesion to matrix or other cells and generated by the actin-myosin-based cytoskeleton (Discher et al., 2005) which ultimately couples to the nucleus (*e.g.* Pajerowski et al., 2007). Given that HSC/Ps, MSCs and derived lineages interact in many ways with their microenvironments, mechanical aspects of microenvironment can in principle regulate stem and progenitor cell functionality. As reviewed here, stem cells generate forces in processes ranging from cell division to migration, while external stresses in stem cell microenvironments that include fluid flows and pressures will impact adhesion and associated signaling as do mechanical factors such as matrix elasticity for MSCs (Discher et al., 2005) and nuclear elasticity of HSC/Ps (*e.g.* Pajerowski et al., 2007; Shin et al., 2011). All of these structures with mechanical functions can in principle impact stem cell maintenance, lineage specification, and trafficking. We will discuss recent progress in the mechanobiology of adult stem cells and progenitors, with particular emphasis on bone marrow derived HSC/Ps and MSCs. Functional roles of actomyosin forces in stem cells are first introduced, followed by both cell-intrinsic and extrinsic biophysical properties. We then discuss the applicability of mechanobiology across different topics as well as the engineering of artificial marrow models. Throughout we try to highlight how insights gained from studies with MSCs can be relevant to the underlying mechanobiology of HSC/Ps and *vice versa*.

2. Actomyosin force regulation in stem cell and progenitor functions

In response to extracellular mechanical processes that range from matrix adhesion to fluid shear, cells generate intracellular forces with actin and myosin. This force generation process contributes to diverse biological functions that are relevant to differentiation, including cytokinesis, cortical tension, and migration. Here, we discuss the molecular basics of mammalian non-muscle myosin II (NMM-II), emphasizing hematopoietic and mesenchymal tissues and stem cells/progenitor contexts.

Actin is a principal filamentous constituent of the cytoskeleton that also translocates (as monomer) into the nucleus. Myosin motor proteins pull on actin filaments by dynamically crosslinking and translating along the filaments driven by the hydrolysis energy of ATP (Pollard and Korn, 1973). Such activities can impact actin monomer pools (Wilson et al., 2010) that can in principle impact serum response factor (SRF), which is a ubiquitous actin regulated transcription factor with mutiple co-factors that shuttle between cytoplasm and nucleus (Olson and Nordheim, 2010). Prototypical muscle myosins are type-II myosins that self-assemble into microns-long bipolar filaments, which register in striations (Brown et al., 2009). Non-muscle cells express isoforms of NMM-II that assemble into smaller mini-filaments that appear far less ordered than in muscle. The three isoforms of the NMM-II heavy chain, A, B and C, are encoded by the genes *MYH9*, *MYH10* and *MYH14*. *MYH9* expression is regulated in part by SRF. NMM-IIA is widely expressed across different tissues, while NMM-IIB is expressed in cardiac and neural tissue, and NMM-IIC appears largely restricted to epithelial cells (Ma et al., 2010). At the molecular level, NMM-IIA has the highest rate of ATP hydrolysis compared to other isoforms (Kim et al., 2005), while NMM-IIB has a higher affinity for ADP, leading to a longer period of binding to actin filaments during force generation (higher duty ratio) than NMM-IIA (Wang et al., 2003). Hematopoietic cells and MSCs express A and B isoforms but not C, and these cells show dynamic expression patterns during differentiation (Engler et al., 2006).

Genetic methods show that NMM-II is essential to diverse biological functions, including embryo survival and cytokinesis,

cortical tension and stiffness generation, and also sensing of matrix and shear stress. NMM-IIA knockout is embryonic lethal to mice at stage E6.5 with defects in visceral endoderm morphogenesis and overall tension of the embryo, which appears flaccid (Conti et al., 2004): essentially, no differentiation occurs even though the cells adhere and divide. NMM-IIB knockout mice survive longer but die at E14.5 due to abnormalities in heart and brain that include more binucleated cells (Takeda et al., 2003; Tullio et al., 1997). NMM-IIC knockout mice remain viable to adulthood with no obvious difference in phenotypes compared to wild-type mice, although the double B, C knockout show impaired cytokinesis compared to single NMM-IIB knockout mice (Ma et al., 2010). Deletion of myosin-II in the ameba Dictyostelium leads to increased distortion of the cell membrane and multinucleation in suspension cultures where cells cannot crawl apart to facilitate division (Merkel et al., 2000). Pharmacological inhibition of NMM-II in adherent cells by blebbistatin (Bleb) likewise tends to increase the number of binucleate cells, indicating that NMM-II contributes to efficient cytokinesis (Straight et al., 2003). Importantly, NMM-II also plays a critical role in matrix sensing: Bleb eliminates the effect of matrix elasticity on lineage specification of MSCs (Engler et al., 2006) and also on maintenance of HSC/P numbers on highly flexible tropoelastin surfaces (Holst et al., 2010), as elaborated upon below.

NMM-II levels vary greatly between cell types and during differentiation, but NMM-II also responds rapidly under physiological settings to intracellular and extracellular forces through changes in its phosphorylation states. NMM-II consists of two heavy chains (230 kDa), two essential light chains (17 kDa) and two regulatory light chains. Myosin heavy chain consists of an ATP-hydrolyzing N-terminal motor or head domain, which directly interacts with F-actin, and a ~150 nm long C-terminal helical tail which forms coiled-coil dimers and can further assemble into myosin filaments (Adelstein et al., 1972). Essential light chains are required for stabilizing the heavy chain (Hernandez et al., 2007). A number of studies (see Matsumura, 2005) have shown that regulatory light chains (RLC) modulate NMM-II activity *via* phosphorylation by numerous kinases, including myosin light chain kinase (MLCK) and Rho-associated protein kinase (ROCK), both of which can phosphorylate the Thr18 and Ser19 RLC residues. In heavy chains such as NMM-IIA, phosphorylation sites exist in the tails (*e.g.* Ser1943) and heads (*e.g.* Tyr277). The tail site has been found in MSCs to be more phosphorylated on soft matrix, where NMM-IIA is down-regulated in terms of expression levels, assembly state, and contributions to traction forces (Raab et al., 2012). Protein kinase C and casein kinase-II phosphorylate the heavy chain but additional kinases are likely relevant (Dulyaninova et al., 2005; Even-Faitelson and Ravid, 2006). Since heavy chains bind F-actin directly, regulation of the heavy chain seems likely to have the most direct impact on NMM-II activity compared to RLC phosphorylation. Indeed, recent mutagenesis studies indicate that NMM-IIA phosphorylation regulates the polyploidization of MKs more dramatically than, for example, inhibitors of MLCK or ROCK (Shin et al., 2011).

Although myosin-II's functions in early HSC/Ps remain unclear, it plays critical roles in differentiated hematopoietic lineages. In lymphoid cells, NMM-IIA is required for cytolytic granule exocytosis in human natural killer cells (Andzelm et al., 2007), endocytosis of stromal derived factor-1 (SDF-1) receptor (Rey et al., 2007), and polarization of endocytic clathrin at the uropod of T-cells (Samaniego et al., 2007). NMM-IIA is also required for immunological synapse stabilization and T-cell receptor microcluster assembly and trafficking (Ilani et al., 2009) as well as T-cell motility in three-dimensions (Jacobelli et al., 2010). NMM-II is required for B-cell receptor-driven antigen presentation (Vascotto et al., 2007). In myeloid cells, dendritic cell migration upon cytokine stimulation (Fernandez et al., 2011) or *via* CD74 (Faure-Andre et al., 2008) requires NMM-II as does CD47-mediated deactivation of macrophage phagocytosis (Tsai and Discher, 2008) and traction force generation by neutrophils in chemotaxis (Shin et al., 2010). In erythroid lineages, actin-mediated forces contribute to enucleation (Koury et al., 1989), and both colony forming unit-erythroid cytokinesis and erythroid enucleation are blocked by NMM-II inhibition, with NMM-IIB but not IIA required for enucleation (Ubukawa et al., 2011). In MK lineages, *MYH9* mutations associate with May–Hegglin anomaly, which is characterized by low platelet count (thrombocytopenia) and giant platelets (Kelley et al., 2000; Seri et al., 2000). Such genetic changes have been recapitulated recently in mice that harbor mutations of *MYH9* and show longer bleeding times and defects in clot retraction (Zhang et al., 2011). While *MYH9* mutations appear to impair platelet function, paradoxically, other previous studies using *in vitro* MKs show increased proplatelet formation upon NMM-II knockout or pharmacological inhibition (Chen et al., 2007; Eckly et al., 2009). Indeed, Bleb promotes platelet-like fragmentation by the fluid stresses that mimic shear stresses in capillaries of the marrow (Shin et al., 2011). Despite these enigmatic issues, actomyosin forces are clearly an attractive target to mechanically modulate hematopoiesis by synergizing with soluble factors in lineage specification and/or function.

3. Trafficking out of the niche: mechanical control *via* cortical and nuclear deformability

For stem cells and the lineages they generate, adhesion, retention, migration, and escape from a niche are all programmatic processes predicated in part on cell mechanics. While some mature blood cells such as RBC must circulate throughout the body for their function, at least some of the immature HSC/Ps must be retained in marrow to sustain hematopoiesis. It has long been appreciated that the deformability of hematopoietic cells changes during differentiation, and so deformability is a likely factor in regulating cell trafficability under mechanically constrained conditions that involve migration across the marrow-blood endothelial barrier and into 2–3 μm diameter capillaries. An early micropipette study showed that a 20-fold increase in aspiration pressure was required to deform myeloid progenitors (immature myeloblasts) compared to highly deformable mature granulocytes (Lichtman, 1970). Myeloblasts also cannot transit through 1–8 μm pores under a chemotatic gradient, whereas mature granulocytes can (Giordano and Lichtman, 1973). While studies of other soft tissue cell types, such as glioma cells have established a critical role for NMM-IIA in migration through capillary-size pores of 3 μm diameter (Beadle et al., 2008), how hematopoietic differentiation leads to changes in the physical deformability of cells and hence affects trafficking has not been deeply investigated. The same challenge applies to MSCs trafficability, although recent studies of MSC migration through soft, 3D collagen gels have revealed a key role for NMM-IIA (*via* heavy chain phosphorylation) in elasticity sensing and NMM-IIB in directional persistence (Raab et al., 2012). More generally, both cell cortex and nucleus likely contribute to overall cell deformability and thereby impact cell motility, niche retention, and trafficking.

3.1. Mechanical properties of membrane cortex and myosin-II contributions

In light of the flow stresses on blood cells, membrane deformability has been characterized for platelets (White et al., 1984), megakaryocytes (Smith et al., 1989), granulocyte and lymphocytes (Dong et al., 1991), and red blood cells at a detailed molecular level (Discher et al., 1994). It is clear from all of these studies that the specialized cytoskeletons are particularly key and part of each cell types program. In contrast to granulocyte differentiation, immature

erythroid progenitors are stiffer than mature RBCs (Waugh et al., 2001), which could be coupled with nuclear condensation during erythropoiesis. Nucleus stiffening could be a driving factor for enucleation to allow egress of RBCs (Lichtman et al., 1989). In contrast to differentiated cells, membrane cortex elasticity remains to be characterized further for early HSC/Ps, but studies with other systems illustrate a common theme that may be relevant to HSC/Ps. In general, primitive stem cells tend to be soft, perhaps due to the immature cytoskeletal architecture; and thus allow cell plasticity to be particularly susceptible to physical changes in external forces in their surroundings. Undifferentiated ESCs are, for example, 10-fold softer than differentiated progeny and possess higher sensitivity to a local cyclic stress that triggers cell spreading and differentiation (Chowdhury et al., 2010). Likewise, primitive MSCs possess a lower cortical elastic modulus compared to differentiated fibroblasts (Titushkin and Cho, 2007).

Since myosin-II regulates cortical stiffness, whether HSC/Ps are softer than differentiated cells is likely to depend on the absolute abundance of myosin-II isoforms during hematopoiesis. Conversely, myosin-II itself is sensitive to external force; for example, myosin-II becomes polarized and assembles in regions of high stress (Reichl et al., 2008). In addition, both NMM-IIA and NMM-IIB are up-regulated in MSCs upon adhesion to stiff matrix compared to soft matrix (Engler et al., 2006). In migration of MSCs from soft to stiff matrix, NMM-IIB becomes polarized rearward, while NMM-IIA remains unpolarized (Raab et al., 2012), indicating that different isoforms have distinct functional roles in cortical deformability, migration and trafficking. In the epithelial-to-mesenchymal transition, cells undergo myosin-II isoform switching from C to B, promoting cell motility and invasion (Beach et al., 2011). This insight has now been extended to the hematopoietic system where isoform switching is seen to regulate differentiation and motility (Shin et al., in preparation).

3.2. *Mechanical properties of the nucleus*

In contrast to MSCs, hematopoietic cells have a small cytoplasmic volume, and so in addition to cell cortex, the nucleus is likely to contribute to overall cell deformability and trafficking. Nuclear deformability is largely understudied across the hematopoietic system. We have begun to approach this issue with two micropipette approaches involving either aspiration of isolated nuclei or aspiration of nuclei in live cells treated with actin polymerization inhibitors (Ivanovska et al., 2010). Our previous studies have indicated that nuclei in human hematopoietic $CD34^+$ cells from marrow were slightly stiffer than nuclei in human ESCs but considerably softer than other differentiated cell nuclei, suggesting that nuclear deformability is developmentally regulated (Pajerowski et al., 2007). The same study further demonstrated that nuclear stiffness is dependent on lamins, which are intermediate filaments that assemble as coiled-coil dimers at the nuclear envelope and associate at least indirectly with chromatin (Shimi et al., 2010). While our initial micropipette study suggested the involvement of lamins and nuclear stiffness in 3D entry into pores, and a recent review speculated that this concept also applies to cancer cell migration and metastasis (Friedl et al., 2011), direct evidence to support the relevance of nuclear deformability in the hematopoietic system is just emerging (Shin et al., in preparation). One notion, for example, is that the polyploid nucleus of an MK is too large and rigid with a complex lamina to allow transmigration into blood, but the consequent marrow anchorage still allows these cells to extend anuclear proplatelet projections into blood that then fragment under shear flow to make platelets as seen recently (Junt et al., 2007). As another example, lamin-A,C is strongly up-regulated upon T-cell activation (Andrade et al., 2003) and this could similarly favor retention of a T-cell at a site of inflammation. Similar to the hypothesis that NMM-II actively regulates deformability of the cell cortex, it appears reasonable to hypothesize that differential lamin expression patterns also regulate nuclear stiffness throughout hematopoiesis, influencing the ability of cells to migrate through and within the dense marrow or tissue and across an endothelium into blood. MSCs also express lamins and their levels can at least affect differentiation (Akter et al., 2009), but any relationship to nuclear mechanics, retention in a niche, or broader trafficking is thus far unclear.

4. Mechanical control of differentiation by forces during cell division

Since cytoskeleton proteins contribute cell division forces through the mitotic spindle and also in the cytokinetic ring of actin and NMM-II (Scholey et al., 2003), it is likely that these forces contribute to lineage specification. In particular, for hematopoietic progenitors and T-cells, proteins segregate asymmetrically during asymmetric division, providing an important basis for self-renewal and differentiation (Chang et al., 2007; Giebel et al., 2006; Ting et al., 2012). However, involvement of cytoskeletal machinery in the asymmetry has not been addressed and clearly needs to be. For example, polarized adhesion of one of the dividing daughter cells to an antigen-presenting cell can asymmetrically segregate a number of proteins such as atypical protein kinase C (Chang et al., 2007). A couple of cytoskeletal components, including Rac GTPase activating protein 1 and Tropomodulin 1, segregate asymmetrically during division, and overexpression of these proteins enhance HSC engraftment *in vivo* (Ting et al., 2012). Pharmacological inhibition of NMM-II has also been found to combine with soft matrix to maximize MK polyploidization (Shin et al., 2011), consistent with the more recent finding that the NMM-IIB isoform specifically regulates MK maturation (Lordier et al., 2012). Distinct roles of NMM-II isoforms in HSC/P differentiation are just beginning to be elucidated (Shin et al., in preparation), and more attention is generally needed to the asymmetric division of MSCs in differentiation processes.

5. Niches and microenvironment mechanical properties

MSCs were originally isolated as the plastic adherent cells from marrow and certainly have characteristics typical of cells that adhere within solid tissues (Engler et al., 2006). HSC/Ps and derived lineages do not adhere to plastic but do thrive in a range of solid-like tissues, including microenvironments in bone marrow (BM: HSC/Ps and different lineages), spleen (B-cells, MK/erythroid lineages, and HSC/Ps) and thymus (T-cells). However, unlike solid tissue cells such as MSCs, many blood cell types are also viable for days to months or longer while suspended in blood, which is fluid of course. Only a few studies have been conducted recently to elucidate roles of cell-extrinsic mechanical factors on hematopoietic functions (*e.g.* fluid suspensions *versus* adhesion to soft or stiff solids), but studies over the past 20 years have helped to identify cell types in niches as well as molecular factors that influence hematopoietic differentiation and trafficking.

The endosteum in BM has been viewed traditionally as a major contributor to the HSC niche. Osteoblasts (OBs) – which MSCs can differentiate to (Engler et al., 2006) – are located at the endosteum and provide necessary cytokines to facilitate myeloid differentiation and B lymphopoiesis from HSC/Ps (Taichman and Emerson, 1994; Zhu et al., 2007). In addition, genetic manipulations show that increased OB numbers lead to increased HSC numbers (Calvi et al., 2003; Zhang et al., 2003). Specific molecular signals emanating from OBs are known to regulate HSC functions and include angiopoietin-1 and Tie-2 which maintain HSC quiescence

(Arai et al., 2004) as well as osteopontin, which suppresses HSC numbers (Nilsson et al., 2005; Stier et al., 2005). The notion that the endosteum is the main HSC niche component has been challenged recently by the discovery that long-term HSCs (LT-HSCs) are frequently located near BM vascular cells (Kiel et al., 2005); the BM vasculature consists of sinusoids, which are thin-walled blood vessels lined by endothelial cells. While it appears difficult to distinguish vascular niches from nearby endosteal niches (Kiel et al., 2005), the ability of HSCs to self-renew and differentiate during fetal development in spleen (Morita et al., 2011) and liver (Taniguchi et al., 1996) indicates that HSC functions can be maintained without the involvement of bone, although the species specific nature of such findings (mouse *versus* man) remains unresolved.

Sinusoidal endothelial cells are required for long-term hematopoietic reconstitution by donor HSCs, since deletion of vascular endothelial growth factor receptor-2 leads to failure in regeneration of the endothelium after sub-lethal irradiation, thereby preventing restoration of hematopoiesis (Hooper et al., 2009). Furthermore, endothelial cells facilitate functional expansion of HSC/Ps upon protein kinase B activation, while mitogen-activated protein kinase activation leads to inhibition of HSC/P expansion and shifts cells towards maintenance and differentiation (Kobayashi et al., 2010). In addition, endothelial cells support myeloid and MK differentiation in culture (Rafii et al., 1995). Given that HSCs are now further divided into dormant and active populations, a recent hypothesis is that dormant HSCs tend to be located at the endosteum, which is devoid of endothelial cells, while active HSCs may be located at the vascular niche, and that the HSCs can be stimulated or stressed to traffic between the niches (Trumpp et al., 2010) – even if these niches are not so anatomically distinct as mentioned. The cell and molecular nature of HSC/P niches has become even more complicated because other regulators have been demonstrated, including SDF-1 abundant reticular stromal niches (Sugiyama et al., 2006), osteoprogenitors nearby BM sinusoids (Sacchetti et al., 2007), BM adipocytes (Naveiras et al., 2009), and nestin-positive pericyte-like cells (Mendez-Ferrer et al., 2010), all of which are either MSCs or derivable from MSCs.

Mechanics of marrow environments at the sub-cellular level can in principle be quantified by methods such as atomic force microscopy (AFM), but studies are thus far limited. Macroscopic measurements of extracted bone marrow suggest an elasticity of ~0.3 kPa (Winer et al., 2009) although removal of marrow can be highly disruptive to structure and mechanics. *In situ* micro-scale measurements with AFM of marrow show the central marrow to be moderately heterogeneous but generally soft ($<$ 0.3 kPa) while bone is invariably rigid ($>$ 1000 kPa) (Shin et al., in preparation). Osteoblasts in culture secrete a thin, endosteal-like extracellular matrix (ECM) measured by AFM to be 20–40 kPa. Remarkably, when MSCs are grown on collagen-I gels in this range of matrix elasticity, osteogenesis predominates, whereas softer gels that mimic muscle (~10 kPa) prime MSCs for a myogenic lineage (Engler et al., 2006). Myosin-II activity has been found to be required for such lineage specification *in vitro*, but controlled *in vivo* studies are needed to thoroughly address molecular mechanisms. As illustration of the important molecular questions that arise, calcifications in the heart have been seen to result from mouse-MSCs that are injected into a myocardial infarct (Breitbach et al., 2007), which remodels to a stiff fibrotic scar (E~35–70 kPa) that is several-fold stiffer than normal myocardium and mechanically more like endosteal-like ECM (Berry et al., 2006).

Consideration of how MSCs differentiate across different elasticities has important functional implications for understanding MSCs as well as HSC/P differentiation. While some of the known cell types that are key to HSC niches are of mesenchymal origin, relative contributions of immature MSCs and mature lineages to HSC functions remains to be quantified in parallel. This could be challenging using gene or promoter specific approaches. For instance, while nestin$^+$ MSCs appear more pericyte-like and distinct from osteochondrial lineages, parathyroid hormone may activate both of them and accelerate differentiation of MSCs into osteoblasts to increase HSC number (Mendez-Ferrer et al., 2010). The col2.3 promoter transgene bearing mice have been widely used to deplete osteoblasts (Visnjic et al., 2001) to study HSC functions (Calvi et al., 2003). However, advances in MSC biology have led to speculation that other mesenchymal lineages – which express and respond to type I collagen (Engler et al., 2006) – could be affected as well. Therefore, controlled approaches to modulate physical properties of ECM *in vitro* and *in vivo* (by direct implantation, see below) could be useful to further characterize MSC and HSC niches.

Studies in other systems have since confirmed that stem cell maintenance and/or differentiation is indeed regulated by tissue microelasticity. Neural stem cells develop into neurons on soft matrices ($<$ 1 kPa), whereas glial cells dominate on stiffer matrices (Saha et al., 2008). Skeletal muscle stem cells cultured on compliant muscle-mimetic hydrogels (~10 kPa) in contrast to rigid plastic dishes are able to maintain stemness and contribute to muscle regeneration *in vivo* (Gilbert et al., 2010). Particularly relevant to the present focus, HSC/P number is maintained (and perhaps increased) on highly flexible tropoelastin surfaces compared to stiff, heavily cross-linked tropoelastin (Holst et al., 2010), and myosin-II activity once again seems critical. Whether a soft, elastic environment generically facilitates stem cell expansion *versus* maintenance or differentiation needs to be studied in greater mechanistic depth, especially in light of a recent finding that functional tumor-initiating cells are enriched, rather than expanded, in soft 3D fibrin gels (Liu et al., 2012). Regardless, the *in vitro* studies reviewed above provide compelling evidence that the mechanics of a niche can have a regulatory role in stem cell fates.

As implied by extramedullary hematopoiesis (*e.g.* liver and spleen), microenvironment factors can also regulate hematopoietic trafficking. A number of soluble factors, including SDF-1 and granulocyte stimulating factor (G-CSF) impact the trafficking of HSC/Ps and mature cells (Mazo et al., 2011), since they influence direct interaction between HSC/Ps and stromal or endothelial cell niches (Dar et al., 2005; Sugiyama et al., 2006), and are exploited clinically for HSC/P mobilization (DiPersio et al., 2009). In addition, receptors for ECM-engaging factors, such as integrins α2, α4/α5/β1, α6 that, respectively, interact with collagen, fibronectin, and laminin, have been implicated in HSC trafficking and self-renewal (Benveniste et al., 2010; Gu et al., 2003; Notta et al., 2011; Papayannopoulou et al., 1998). Consistent with findings for marrow-derived, CD34$^-$ MSCs (Engler et al., 2006), adhesion and motility of human CD34$^+$ cells are enhanced on stiff compared to soft fibronectin matrix (Lee-Thedieck et al., 2012), and cell spreading and morphology depend on the substrate elasticity and dimensionality of collagen hydrogels (Choi and Harley, 2012). While both of these *in vitro* studies reveal matrix elasticity as a potential factor in regulation of the hematopoietic trafficking, the *in vivo* relevance of these results as well as roles of other cell extrinsic mechanical factors require further investigation.

Some insight is perhaps provided by studies of focal adhesion kinase (FAK), which has an important role in mechanotransduction of signals from stiff matrix to most solid tissue cell types (Wang et al., 2001); while the formation of focal adhesions is unexpected for hematopoietic cells due to a lack of stress fibers in cells on stiff matrix, deletion of the FAK gene nonetheless increases MK ploidy and platelet number (Hitchcock et al., 2008). This seems consistent with the phenotype observed with myosin-II inhibition and soft matrix (Shin et al., 2011), although platelet adhesion and spreading are predictably impaired with ablation of FAK. Activation of FAK also associates with B-cell

differentiation and trafficking (Glodek et al., 2003). FAK deletion does not intrinsically impair long-term HSC functions and steady-state hematopoiesis (Lu et al., 2012), but FAK signaling does maintain *primitive* acute leukemia cells (Despeaux et al., 2012). Such genetic approaches to key mechanosensing molecules (*e.g.* myosin-II isoforms) within stem cells in health and disease are likely to provide even more insight in the future.

6. Shear flow physiologically modulates lineage differentiation

Another important extrinsic biophysical factor to consider in differentiation is fluid shear forces, which have been investigated most extensively with endothelial cells (due to direct exposure to blood flow) but also to some extent with osteocytes which experience distinct fluid flows as bone is stressed in daily activity (Weinbaum et al., 1994). Endothelial cells can be differentiated from ESCs upon shear flow *in vitro via* the downstream activation of histone deacetylase 3 (Zeng et al., 2006), and osteoblast differentiation from BM-derived MSCs is facilitated by continuous shear *in vitro* (Stiehler et al., 2009). In addition, controlled shear flow *in vitro* induces differentiation from embryoid-body-derived cells into hematopoietic precursors as indicated by a strong upregulation of the Runx1 transcription factor (Adamo et al., 2009). Zebrafish models were likewise used to show that both chemical and genetic ablation of blood flow severely reduces HSC number (North et al., 2009).

An interplay between intrinsic and extrinsic mechanical factors likely contributes to cell fates. Fluid shear produces forces that are balanced by cell adhesions and the actomyosin cytoskeleton, or else fluid shear can be so strong that it dominates to convect and disperse cells or even fragment them such as shown in platelet biogenesis (Junt et al., 2007). Moreover, an absence of adhesion to external surfaces for many cell types limits cytokinesis, with cells tending to become multi-nucleated in suspension (if they survive) ,whereas adhesion-based traction forces help cells pull apart (Ben-Ze'ev and Raz, 1981). MK polyploidization is thus inhibited on stiff matrix where increased adhesion maximizes the traction forces for cell division even when myosin-II is inhibited (Shin et al., 2011). On the other hand, cell cycle progression can also be inhibited by soft matrix for at least some adherent cells (Klein et al., 2009). Adhesion, matrix elasticity, and external forces thus couple to myosin in processes central to stem cell fates such as cell division—whether it is symmetric, asymmetric, or suppressed.

7. Engineering stem cell niches: *in vivo* material approaches that control mechanics

Ectopic implants of materials with bone marrow cells or fragments have long been known to support hematopoiesis. A cellulose membrane placed in the peritoneum of mice will predictably produce the usual foreign body response with macrophages and other adherent cells recruited to its surface within days, but when such mice are irradiated and donor marrow cells are infused intraperitoneally (not intravenously), hematopoietic colonies form on the cell layer (Seki, 1973). Macrophages contribute to HSC niches (Winkler et al., 2010) and certainly have a key role in erythroid development (Chasis and Mohandas, 2008), but the colonies on cellulose were mostly granulocytic with limited erythroid and no MK or lymphoid lineages. A tubular cellulose membrane pre-coated with stromal cells derived either from the spleen or marrow (and probably including MSCs) is capable of full trilineage reconstitution upon intraperitoneal transplantation of donor marrow cells (Knospe, 1989).

With a better understanding of biomaterials and stem cells, a number of new ectopic transplant models have been proposed recently as useful tools to examine roles of microenvironment in stem cell biology. One study performed subcutaneous transplantation of mouse marrow stromal cells (MSCs) encapsulated in gelatin sponges to form an ossicle, but microvessel density, lineage number, and HSC/P number in the ossicle proves to be far lower than in the femur (Song et al., 2010). Interestingly, adipocyte number is ~30-fold higher in the ossicle than the femur, consistent with a previous observation that marrow adipocytes negatively regulate HSC number and engraftment (Naveiras et al., 2009). In another study, a polyacrylamide-based hydrogel scaffold was seeded with *human* marrow stromal cells, and subcutaneous transplantation of this cell-laden scaffold was found to first induce angiogenesis by recruiting *mouse* endothelial cells, followed by recruitment of endogenous $Lin^-Sca\text{-}1^-c\text{-}Kit^+$ (LSK) progenitor cells (Lee et al., 2012). While factors released from human cells may thus be able to recruit mouse niche cells, the LSK frequency in this model is ~5-fold lower than that in femur and the functionality of the recruited HSC/Ps still needs to be confirmed *in vivo*. The hydrogel scaffold in the cited study has a stiffness about 50-fold higher than that of central marrow (0.3 kPa, see above), and the % engraftment of human marrow blood cells in the scaffold is far lower than that achieved in marrow by intravenous transplantation. Recent efforts with softer ECM-based scaffolds appear to show the *in vivo* formation of a bone organ with better capabilities to recruit and/or maintain HSCs. One study used matrigel that has an elastic modulus of 0.5 kPa (Soofi et al., 2009), and showed that the % engraftment of human blood cells in the extramedullary bone is comparable to that in femur (Chen et al., 2012). Most recently, human MSCs were seeded in collagen-based scaffolds (1–2 kPa elastic modulus) and supplemented with IL-1β to induce endochondral bone formation followed by implantation *in vivo*, demonstrating recruitment of endogenous functional mouse HSCs comparable to femoral HSCs (Scotti et al., 2013). These implanted material models are thus increasingly promising mimics of bone marrow.

While the recent studies highlight useful strategies to engineer stem cell niches, our understanding of how mechanical factors regulate stem cells remains to be leveraged systematically to improve such strategies. Further biomaterial strategies to better mimic hematopoietic microenvironments *in vivo* could be adapted from bone regeneration studies where growth factors are gradually delivered by subcutaneous implantation of scaffolds to generate marrow-containing ectopic bones in a cell-free manner (Alsberg et al., 2002). This type of approach could therefore be revealing when combined with insights into the role of matrix mechanics as well as niche geometries, and/or porosity.

8. Mechanotransduction lessons from bone marrow derived MSCs

Although little work has been done to study mechanotransduction in hematopoietic systems, the mechanistic insights gained from studies with MSCs – or variations thereof – are likely applicable. Cultures of MSCs on collagen-coated polyacrylamide hydrogels of tuned elasticity have provided evidence of lineage specification in terms of cell morphology, nuclear translocation of some key transcription factors (e.g., MyoD, CBFA1), protein expression, and mRNA profiles consistent with neurogenesis on soft brain-like matrix (0.1–1 kPa), muscle on stiffer matrix (8–17 kPa), and bone on the stiffest matrix (25–40 kPa) (Engler et al., 2006). Cell fates to fat and bone can also be affected by constraining cell size and geometry (Fu et al., 2010; McBeath et al., 2004). The cascade of events that lead from mechanical stimulation exterior to the cell to modulation of a presumed transcriptional response within the nucleus is not understood although nuclear entry and enrichment of transcription factors has been clear (Engler et al., 2006).. A direct pathway of mechanotransduction from the

microenvironment seems possible with mechanical linking of ECM through focal adhesions to the cytoskeleton and across the nuclear membrane *via* the complex linking nucleus to cytoskeleton (LINC) and to the nuclear lamina and chromatin. This could bring about changes in physical conformation at any step, particularly those that distinguish active euchromatin from tightly packed, inactive heterochromatin that tends to concentrate near the lamina. An application of force to the surface of HeLa cells *via* magnetic beads can alter chromatin organization and deform the nucleus (Iyer et al., 2012b), which could be important given the spatial location of particular chromosomal territories within the nucleus that broadly regulate transcriptional programs (Iyer et al., 2012a).

Force-induced changes in protein conformation, complex formation and/or post-translational modification can influence cellular or nuclear localization of key molecules (perhaps including translocation from cytoplasm to nucleus) or direct activation or inactivation of transcription factors. Force sensitive mechanisms at focal adhesions are clearly able to regulate actin assembly and the force-generating machinery in cells (Geiger et al., 2009). In human keratinocytes, the assembly state of actin – which couples to matrix stiffness that drives cell spreading – reportedly dictates the cellular location of megakaryocytic acute leukemia protein (MAL or MKL) and its interaction with SRF, thus regulating differentiation (Connelly et al., 2010; Iyer et al., 2012b). The cellular location of transcription factor yes-associated protein (YAP) in MSCs and endothelial cells is also influenced by matrix softness as well as constraints on cell area, both of which favor cytoplasmic YAP, whereas stiff matrix and unconstrained cell area promotes translocation to the nucleus (Dupont et al., 2011). The impact on differentiation requires tension in the actomyosin cytoskeleton (inhibited by Bleb) and the activity of Rho GTPase but is not governed by the Hippo/Large tumor suppressor (LATS) cascade that is normally associated with YAP in pathways controlling organ size (Zhao et al., 2011). Co-factors and/or post-translational modifications that might control this translocation are an active topic of research, with hints of regulation by the nuclear lamina (Swift et al., in preparation).

In the hematopoietic system, deletion of SRF increases HSC/P numbers by 3-fold without long-term modification of cell cycle dynamics, but it severely impairs cell retention in BM partly due to downregulation of integrins and hence of adhesion (Ragu et al., 2010). MAL deletion inhibits MK maturation and proplatelet formation (Gilles et al., 2009), while MAL overexpression increases MK differentiation as long as SRF is expressed normally (Cheng et al., 2009). YAP can be detected in a broad range of tissues, spanning from the very soft (brain) to the very stiff (bone), although protein levels do not appear to be a monotonic function of tissue stiffness (Swift et al., in preparation). YAP is detectable at low levels in long-term HSCs but is barely detectable in other HSC/P or lineages, consistent with the marrow being very soft (see above). Overexpression of YAP does not alter normal hematopoiesis (Jansson and Larsson, 2012), although effects of YAP deletion have not yet been reported. As described earlier, the composition of the nuclear lamina varies dramatically across the hematopoietic lineages, and so one might predict a corresponding modulation of cellular tension and therefore a means of directing activity of transcriptional co-factors such as MAL, SRF, and/or YAP (Shin et al., in preparation).

9. Conclusions

The prototypical hematopoietic stem cell/progenitors has saved many thousands of patients for several decades now. Hematopoietic cells show not only intrinsic differences in mechanical properties but also extrinsic sensitivity to mechanical cues from their niches, both of which could result in a variety of signaling and behavioral responses. Previous studies have revealed the importance of mechanotransduction pathways in stem cells, such as matrix elasticity effects on various transcription co-factors, some of which are being explored with HSC/Ps both *in vitro* and in well-established *in vivo* analyses. Novel therapies envisioned with induced pluripotent stem cells and ESCs generally involve *in vitro* commitment to adult stem cells or progenitors, and so a deep understanding of pathways, such as those reviewed here, in adult stem cell mechanobiology seem broadly important for the many other sub-fields of stem cells. Therefore, adult stem cells and progenitors remain of great interest for their clinical application, and this interest motivates further studies to reveal mechanistic insights into the complex interactions between cells and their physical surroundings.

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