Blood and immune cell engineering: Cytoskeletal contractility and nuclear rheology impact cell lineage and localization

Biophysical regulation of hematopoietic differentiation and trafficking

*Jae-Won Shin and Dennis E. Discher**

Clinical success with human hematopoietic stem cell (HSC) transplantation establishes a paradigm for regenerative therapies with other types of stem cells. However, it remains generally challenging to therapeutically treat tissues after engineering of stem cells in vitro. Recent studies suggest that stem and progenitor cells sense physical features of their niches. Here, we review biophysical contributions to lineage decisions, maturation, and trafficking of blood and immune cells. Polarized cellular contractility and nuclear rheology are separately shown to be functional markers of a hematopoietic hierarchy that predict the ability of a lineage to traffic in and out of the bone marrow niche. These biophysical determinants are regulated by a set of structural molecules, including cytoplasmic myosin-II and nuclear lamins, which themselves are modulated by a diverse range of transcriptional and post-translational mechanisms. Small molecules that target these mechanobiological circuits, along with novel bioengineering methods, could prove broadly useful in programming blood and immune cells for therapies ranging from blood transfusions to immune attack of tumors.

Keywords:
hematopoiesis; lamin; matrix; mechanobiology; myosin

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Abbreviations:
AFM, atomic force microscopy; **BM,** bone marrow; **BMT,** bone marrow transplantation; **FACS,** flow activated cell sorting; **FDA,** Food and Drug Administration; **G-CSF,** granulocyte colony-stimulating factor; **HSC/P,** hematopoietic stem cell/progenitor; **kPa,** kilopascal, units of elasticity; **MK,** megakaryocyte; **mPB,** mobilized peripheral blood; **MSC,** mesenchymal stem cell; **MS-IF,** mass spectrometry calibrated intracellular flow cytometry; **MII,** myosin-II; **PDMS,** poly(dimethylsiloxane); **RBC,** red blood cell; **SDF-1,** stromal derived factor-1; **SRF,** serum response factor; **UCB,** umbilical cord blood; **YAP,** yes-associated protein.

Introduction

Hematopoietic stem cell and progenitor (HSC/P) transplantations have been used to treat many patients over several decades [1]. Such success is now inspiring much broader research into the use of other types of adult stem cells to hopefully treat many more human diseases. Stem cells are able to self-renew while also generating many differentiated cells required for a tissue with high turnover, such as blood ($\sim 10^5$ cells per second). The hierarchical nature of blood cell development has been elucidated through advances in the prospective isolation of HSC/Ps and of different lineages by fluorescent-activated cell sorting (FACS) with a specific set of cell surface antigens [2]. This approach is combined with limiting dilution transplantations in vivo to quantify HSC frequency and multi-lineage differentiation [3]. To sustain healthy tissues for the long term, any cell death or turnover must also be balanced by stem cell self-renewal and lineage differentiation, which are processes that seem optimized in specialized tissue microenvironments called "niches" [4].

Previous studies suggest that the HSC niche in the bone marrow (BM) is formed in part by mesenchymal stem cells or marrow stromal cells (MSCs) and their lineages (e.g. osteo and adipo lineages) that provide a number of key factors to regulate HSC functions [5]. Recent studies suggest that MSCs are pericytes and contribute to BM vasculature, together with endothelial cells [6]. Endothelial progenitors and lineages also play critical roles in HSC self-renewal [7]. While BM has been the major source of HSC/Ps for transplantation, alternate sources have now included mobilized peripheral blood (mPB) and umbilical cord blood (UCB). Injection of granulocyte-colony stimulating factor (G-CSF) disrupts the interaction between HSC/Ps and their niches, mobilizing cells to enter blood from BM. However, some patients do not respond to G-CSF, which has prompted the development of agents against other molecular targets that retain HSC/Ps in BM, such as antagonists of the stromal derived factor-1 (SDF-1) receptor [8]. UCB has been approved by the United States Food and Drug Administration for HSC/P transplantation, and over 20,000 UCB transplantations have been performed since the late 1980s [9]. However, its use remains challenging due to low numbers of HSC/Ps per cord blood unit [10]. Such clinical advances and limitations have motivated the exploration of mechanisms that underlie the balance between stem cell self-renewal, differentiation, and trafficking in and out of the marrow niche. While soluble factors and cell–cell contacts regulate these biological processes, biophysical features inside and outside cells are also important for these processes. As reviewed here, stem cells can intrinsically generate and resist physical forces, while external stresses from the marrow niche, such as shear flow and matrix stiffness, impact adhesion, and associated intracellular signaling. Indeed, matrix stiffness directs lineage differentiation of MSCs, which is regulated by contractile forces generated by myosin-II motors [11]. Here, we review some of the recently described biophysical regulation of these processes in hematopoietic cells ultimately in relation to a small set of structural molecules found in all of the diverse marrow cells.

Structural proteins modulate asymmetric division and nuclear limits on niche trafficking

One evolutionarily conserved mechanism that explains how stem cells can both self-renew and differentiate is asymmetric division via unequal inheritance of cell fate determinants [12]. This is particularly important for stem cells to maintain tissue homeostasis, as a parent stem cell must give rise to one daughter cell that maintains stem cell functions and the other cell that undergoes differentiation. While asymmetric segregation of proteins during division has been largely demonstrated in invertebrate models [13], evidence in mammalian cells is rapidly emerging. In the hematopoietic system, molecules have been identified in HSC/Ps to play roles in either regulating the segregation of known cell fate determinants, such as numb [14, 15], or themselves segregating asymmetrically during division [16–18]. While cell fate decision by asymmetric segregation of proteins could be induced by cell intrinsic positioning of the mitotic spindle [12], adhesive cell extrinsic cues from neighboring cells in the microenvironment have been implicated in directing asymmetric polarization of T- [19] and B-cells [20]. Introduction of microscopy techniques to continuously image single dividing cells with fluorescently tagged molecules [16, 18, 21] has provided more direct evidence for asymmetric division of HSC/Ps. Exactly how and to what extent cell fate determinants are physically segregated during HSC/P division remains to be elaborated further to control the switch between asymmetric and symmetric divisions for clinical purposes.

Cell polarization is inextricably linked to physical forces generated by cytoskeletons. The actin cytoskeleton breaks the symmetry to induce polarized distribution of molecules, while microtubules maintain the stability of polarization [22]. Non-muscle myosin-II (MII) proteins are key motor proteins that generate contractile forces through the sliding of actin polymers (Fig. 1A). MII underlies cell intrinsic cortical tension that both stabilizes the plasma membrane [23] and drives cytokinesis by the coordination of forces between the equatorial constriction ring and poles of dividing cells [24]. While polarized distribution of MII during cytokinesis produces different-sized daughter cells in *Caenorhabditis elegans* [25], its significance in asymmetric division of mammalian stem cells and fate decision is not clear. In addition, the intracellular tension is sustained by adhesion to extracellular matrix, and so MII regulates the ability of stem cells to sense extrinsic physical properties of the matrix, which in turn direct their differentiation [11, 26]. While external stresses can direct asymmetric localization of MII in *Dictyostelium* [27], their significance in driving asymmetric division of mammalian stem cells and cell fate remains unclear. Interestingly, it was discovered more than 40 years ago that as granulocytes in BM differentiate, they become more deformable to traffic through the endothelial barrier and into blood [28]. Whether this is due to changes in MII activities by asymmetric segregation during hematopoiesis was unclear.

In considering physical forces that drive asymmetric differentiation and trafficking, it is also important to note that the largest single organelle in every cell is typically the nucleus, and, hence nuclear mechanics could play rate-limiting roles in these processes. Lamins are intermediate filaments that regulate physical deformability of the nuclei: some differentiated cells have more rigid nuclei than stem cells or progenitors due to higher lamin content [29] (Fig. 1A). Our group recently demonstrated that the lamin levels scale with external stress provided by tissue stiffness and regulate stem cell differentiation [30], suggesting that the intracellular tension generated by MII is likely coupled with physical properties of the nuclei. As lamins are physically connected to specific sites of chromosomes that undergo remodeling during differentiation [31], how nuclear mechanics as controlled by lamins regulate hematopoietic differentiation and trafficking will be an important issue to address along with MII-dependent contractile forces.

In reviewing here some of the recent progress in the mechanobiology of hematopoiesis, our recent work [32–34] is placed in a broader perspective of the field. First we present an integrated view of how biophysical contributions of the contractile cytoskeleton and nuclear intermediate filaments

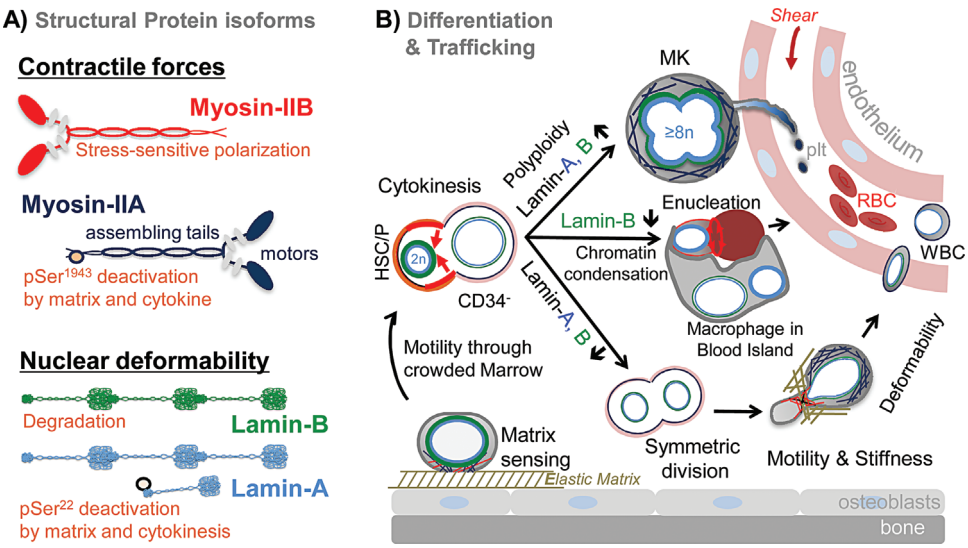

Figure 1. Biophysical determinants regulate fundamental biological processes in the BM microenvironment that lead to blood formation. **A**: Key structural protein isoforms regulate biophysical processes behind hematopoiesis and trafficking. MII is the major motor protein that generates contractile forces and consists of a pair of heavy and regulatory light chains. Lamin is an intermediate filament that confers mechanical properties on the nucleus. Hematopoietic lineages express variable levels of MIIA and B, and lamin-A and B isoforms. These structural proteins are subject to regulation by both transcriptional and post-translational mechanisms. MIIB is asymmetrically segregated during HSC/P division through stress-sensitive polarization. In contrast, MIIA is less stress sensitive, but regulated by heavy-chain phosphorylation at the serine residue 1943 ($pSer^{1943}$), which can be modulated by matrices and cytokines. $pSer^{1943}$ leads to disassembly of MIIA, and, hence reduce MIIA activity. Lamin-B turnover is regulated by degradation, while lamin-A turnover is regulated by pSer22 upon matrix stiffening or cytokinesis. **B**: Biophysical determinants regulate fundamental biological processes that lead to blood formation. Contractile forces generated by MII are important in sensing matrix stiffness, which is heterogeneous in the BM microenvironment. During the cell division process, HSC/Ps ($CD34^+$) undergo asymmetric division to segregate MIIB into one daughter cell. Without MIIB, cells divide symmetrically. The other daughter cell becomes differentiated into three different lineages. Because MKs upregulate both lamin isoforms by endomitosis, and they are too large to traffic through endothelial barriers. Instead, they undergo fragmentation into platelets, which are facilitated by relaxation of contractile forces. Lamin-B is decreased during erythroid differentiation, which leads to chromatin condensation. Condensed nuclei are either too stiff to migrate through the endothelial barrier or phagocytosed by macrophages, leading to enucleated RBCs. Nucleated leukocytes or WBCs can cross the endothelial barrier, as they express low lamins and have active MIIA. MK, megakaryocyte; RBC, red blood cell; WBC, white blood cell; HSC/P, hematopoietic stem cell/progenitor.

are linked to specify hematopoietic lineages and the tissue distributions of cells. We then discuss some of the key methods that have helped to reveal biophysical contributions to lineage decisions, maturation, and trafficking in tissues. Prospects of harnessing such mechanobiological insights for clinical purposes are discussed at the end.

Biophysical determinants regulate hematopoiesis and trafficking

The physiological steps leading to platelet generation exemplify the interplay between intrinsic and extrinsic biophysical factors in hematopoiesis (Fig. 1B). Platelets are shed from megakaryocytes (MKs), which are unique cells that undergo maturation by polyploidization. This process occurs because a weak adhesion to external interfaces limits cytokinesis. Early studies with non-mammalian cells show that adhesion to matrix provides traction forces to pull cells apart [54]. Consistent with this observation, we showed that MK polyploidization is inhibited on stiff matrix where stronger adhesion increases traction forces to drive cell division [33]. In contrast, both soft matrix and inhibition of contractile forces maximize MK maturation. After maturation, MKs release proplatelets into the blood stream. In general, cell adhesion and cytoskeletal forces in marrow are balanced by fluid shear stress in circulation. However, when this becomes imbalanced, fluid shear overrides cellular forces, and proplatelets are then fragmented [55]. This process requires weakening of cortical tension by inhibiting MII [33].

Erythroid progenitors undergo nuclear condensation during differentiation [56]. At the terminal stage of erythroid differentiation, the rigid nuclei are not only too rigid to traffic through the endothelial barrier, but also engulfed by BM macrophages, forming the red blood cell (RBC)-macrophage island [57] (Fig. 1B). Importantly, particle rigidity facilitates phagocytosis by macrophages [58], likely in a MII-dependent manner [59]. MII also regulates the terminal enucleation process [60], likely in a manner analogous to asymmetric division during cytokinesis [61]. These biophysical processes collectively contribute to enucleation of RBCs prior to circulation.

In contrast to MK and erythroid lineages, leukocytes can traffic through the endothelial barrier as nucleated cells (Fig. 1B). This not only requires active forces generated by myosin-IIA (MIIA) during migration [62], but also highly deformable nuclei [34, 63]. As leukocytes play immunological

functions, it is of importance to note that MII also regulates antigen presentation of NK cells to T-cells by forming immunological synapses [64]. In cancer patients, leukocytes can be rigidified by chemotherapeutic treatment, which likely contributes to vascular occlusion [65]. While it has not been measured directly, it is plausible that leukocyte rigidity is attributed to nuclear rigidity as a number of chemotherapeutic agents target DNA. Nuclear rigidity induced by disease or drug treatment will then likely contribute to poor infiltration of immune cells into tumor sites [66], which may lead to tumor resistance. Conversely, rigidity of the microenvironment may impact leukocyte trafficking by stiffening of the extracellular matrix or niche cells [67].

Early hematopoietic differentiation is also subject to regulation by matrix mechanics in combination with other physical factors. HSC/Ps number is maintained or increased on highly flexible compared to stiff cross-linked tropoelastin [26]. Indeed, MII plays an important role in mechanosensing of HSC/Ps. Using MS-IF cytometry, we showed that the isoform switching process of MII during adult hematopoiesis (Fig. 2A) is due to polarization of myosin-IIB (MIIB), which is induced by external stress, including fluid shear, and stiff matrix [32]. External stress can be induced either by directed polarizing cues from microenvironments or by spontaneous intracellular fluctuation [68]. Blood flow and shear stress promote embryonic hematopoiesis [53, 69], but how MII isoforms and contractile forces play a role in this process remains unclear. When the polarization occurs during cell division in adult hematopoiesis, MIIB becomes asymmetrically segregated to a daughter cell that maintains its HSC activity, while the other cell with lower or no MIIB becomes more differentiated. Based on protein quantification, it is likely that total protein level of MIIB in two daughter cells is gradually reduced per division during differentiation (Fig. 2B), indicating that there may be additional mechanisms for protein regulation, such as degradation. In contrast to fluid shear and stiff matrix, soft matrix prevents MIIB polarization. When MIIB becomes downregulated in differentiated cells, MIIA becomes activated by dephosphorylation, which induces its polymerization. As expected, soft matrix suppresses MIIA dephosphorylation. Together, these findings suggest that soft matrix likely maintains early HSC/Ps by suppressing MII isoform switching, while stiff matrix drives asymmetric division. The study also demonstrates that asymmetric segregation of MII not only drives differentiation, but also hierarchically influences the ability of different lineages to traffic through barriers, potentially linking asymmetric division of mammalian stem cells to homeostatic lineage distribution across tissues.

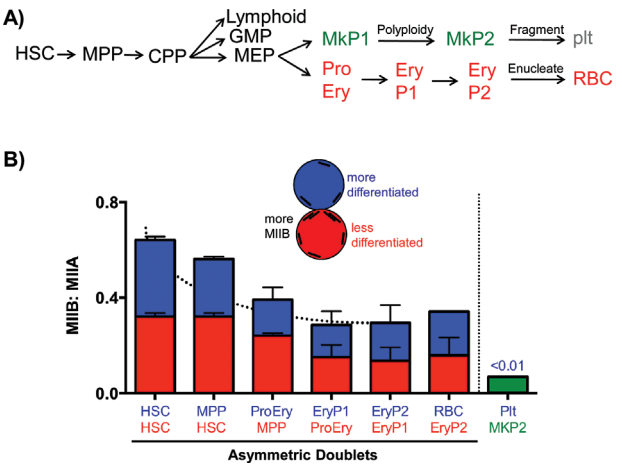

Figure 2. MII expression and partitioning in hematopoiesis. **A**: Hierarchical organization of hematopoiesis. HSC, Hematopoietic Stem Cell; MPP, Multi-Potent Progenitor; CPP, common potent progenitor; MEP, megakaryocyte and erythroid progenitor; MkP1, early megakaryocyte progenitor; MkP2 (DNA 2n or 4n), late megarkaryocyte progenitor (polyploidy, DNA $\geq$8n); Plt, platelet; ProEry, Proerythroblast; EryP1, early erythroid progenitor; EryP2, late erythroid progenitor; RBC, red blood cell; GMP, granulocyte and monocyte progenitor. **B**: Stoichiometry of MIIB to MIIA in pairs of two possible daughter cells shows that MIIB decays over time during asymmetric division toward more differentiated cells. Platelet shows the lowest MIIB to A ratio. Values and error bars ($\pm$SEM, $n \geq 4$ donors) from [30]. The relative absence of MIIB in the MK lineage makes it susceptible to *MYH9* related diseases, while residual MIIB in the erythroid lineage could help maintain normal erythropoiesis.

We also showed that in addition to cytoskeletal compositions, the composition of nuclear lamin isoforms is also changed during differentiation, and specifies hematopoietic lineages [34]. While lamin-A expression scales well with the stiffness at the tissue level [30], it appears to be more nonlinear for hematopoietic lineages at the cellular level, likely because differential nuclear lamin expressions can also trigger other biological functions, such as senescence [70]. Lamin A:B ratios are increased during myeloid and lymphoid differentiation but the total lamin expression is decreased, leading to increased trafficability. In support of this notion, Wong et al. showed that overexpression of lamin-B1 decreases lymphoid and myeloid cell number in circulation [71]. The erythroid lineage undergoes a >30-fold increase in lamin A:B ratios due to upregulation of lamin-A and downregulation of lamin-B, both of which lead to nuclear condensation and marrow retention. Indeed, MK polyploidization accompanies upregulation of both lamin isoforms. Consistent with the observations from non-hematopoietic lineages, rheological experiments with hematopoietic lineages also demonstrate that lamin-A is viscous, while lamin-B is elastic (Fig. 3). The biophysical studies with hematopoietic lineages provide insight on how adhesion, matrix elasticity, and external shear forces couple to intrinsic cytoskeletal and nuclear mechanics in the biological processes central to stem cell self-renewal and fate decision.

Systems mechanobiology describes forces in hematopoietic networks

Integrated analyses of MII and lamin isoforms across different hematopoietic lineages reveal molecular maps that reflect alterations in cellular structures and functions during hematopoiesis. In general, cell structure maps defined by lamin versus MII isoform ratios are delineated by two linear boundaries (Fig. 4A). In the lower boundary, MII B:A ratios are changed by more than two orders of magnitude, while lamin

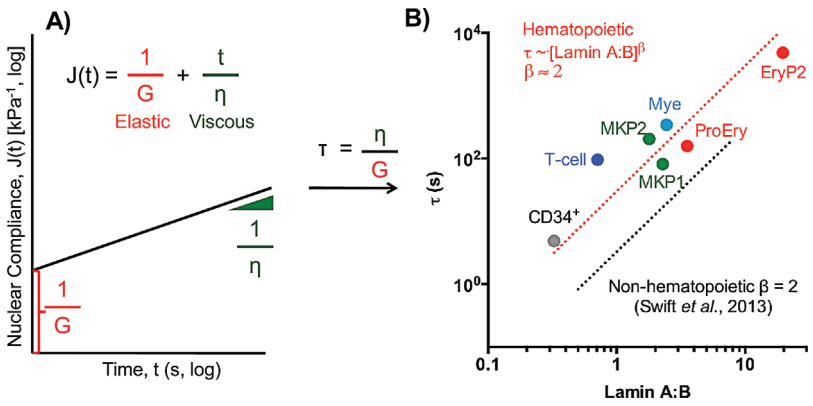

Figure 3. Nuclear rheology of hematopoietic lineages. Nuclei in cytoskeleton-disrupted cells that are aspirated into micropipettes [32] show that the time τ to respond to stress increases with the stoichiometry of A to B-type Lamins. τ is defined by the ratio between viscous (η) and elastic (G) constants, which can be derived by fitting the data from the graph, nuclear compliance ($J(t)$, inverse of stiffness, unit in Pascal^{-1}) versus time (in seconds) (left). τ values can then be plotted against lamin A:B ratios (right). A strong power law fit ($\beta \approx 2$) indicates that lamin-A contributes strongly to viscosity while B-type lamins confer elasticity, such that the ratio of viscosity and elasticity yields τ.

A:B ratios are changed no more than fivefold. In the upper boundary, MII B:A ratios remain relatively unchanged (lass than twofold), while lamin A:B ratios are changed by two orders of magnitude. The former boundary corresponds to lineages that tend to be polarized and egressed upon differentiation (myeloid and lymphoid), while the latter boundary corresponds to lineages that are retained in marrow during differentiation (erythroid and MK), but undergo specialized nuclear remodeling steps, which eventually lead to retention of nuclear mass upon enucleation of RBCs and platelet fragmentation from MKs (Fig. 4B). Lineage trajectory analyses suggest that each lineage shows distinct transition patterns from one boundary to another during differentiation. Lymphoid lineages remain in the lower boundary throughout differentiation, while myeloid lineages go through the "intermediate zone" first, and then back to the lower boundary (Fig. 4C). In contrast, erythroid and MKs make a dramatic transition from the upper boundary to the lower boundary upon terminal differentiation (Fig. 4D). Further insights will be revealed by extending these maps to multi-dimensional plots based on both expression patterns and post-translational modification status of biopolymers and their regulatory proteins.

Recently, we showed that increased MII activity by matrix stiffening suppresses lamin-A phosphorylation, which leads to increased lamin-A polymer assembly and subsequently enhances MIIA transcription, suggesting a feedback loop between cytoskeletal forces and nuclear rheology [72]. Extending this notion, we have constructed a mechano-sensitive circuit model in hematopoiesis based on existing literature and our work (Fig. 5). MII isoforms are regulated by distinct transcription factors. Serum Response Factor (SRF) regulates MIIA expression [73]. Interestingly, conditional knockout of SRF leads to decreased adhesion of HSC/Ps, which increases their expansion but decreased BM engraftment with increased number of circulating HSC/Ps [74]. In contrast to SRF, RUNX1 regulates transcription of both MIIA and MIIB in an inverse way [75], connecting underlying molecular circuits of both isoforms. While the connection between lamin-B and MII remains to be elucidated experimentally, lamin-B transcription is regulated by E2Fs, which are inhibited by phosphorylated retinoblastoma tumor suppressor protein (Rb) [70]. Interestingly, knockout of MIIB leads to increased cyclin D [76], which activates Rb [77]. As cyclin D can also suppress RUNX1 [78], these findings suggest a regulatory loop that connects between MIIB and lamin-B. Yes-associated protein (YAP) becomes localized in the nucleus upon lamin assembly on stiff matrix, and directs osteogenesis [79]. In cancer-associated fibroblasts, YAP regulates the protein expression of myosin light chain 6 and MIIB, but not the gene expression [80]. However, YAP overexpression does not perturb hematopoiesis [81], which could reflect that physiologically relevant matrix stiffness tends to be soft for hematopoietic cells. Together, mechanobiological circuits are beginning to be elucidated to explain how mechanical forces regulate hematopoietic fate decision. Whether well-known lineage specification transcription factors, such as GATA1 and PU.1 are implicated in mechanobiological circuits of hematopoiesis remains to be studied. Another interesting question is whether extensively characterized epigenetic modifications in hematopoiesis are subject to biophysical regulation. A recent study raises this possibility by showing that matrix softness promotes H3K9 demethylation in tumor-repopulating cells [82]. An increased level of methylated H3K9 is associated with ineffective hematopoiesis and transformation to acute myeloid leukemia in mice deficient for Arid4a [83], raising an interesting possibility that matrix stiffening during BM fibrosis could contribute to leukemogenesis through H3K9 methylation.

Tools to probe biophysical regulators of differentiation and trafficking in the hematopoietic system

Methods from the physical sciences and engineering enable investigators to quantitatively probe how mechanical forces influence different aspects of hematopoietic biology. Here, we describe some of these methods with particular emphasis on probing mechanics of blood cells and their microenvironment, which can be used in combination with genetic and molecular approaches to reveal deeper insight into molecular, cellular, and systems mechanobiology of blood.

Micropipette aspiration

Micropipette aspiration has been used for several decades to probe rheological properties of single cells (Fig. 6A). A cell in

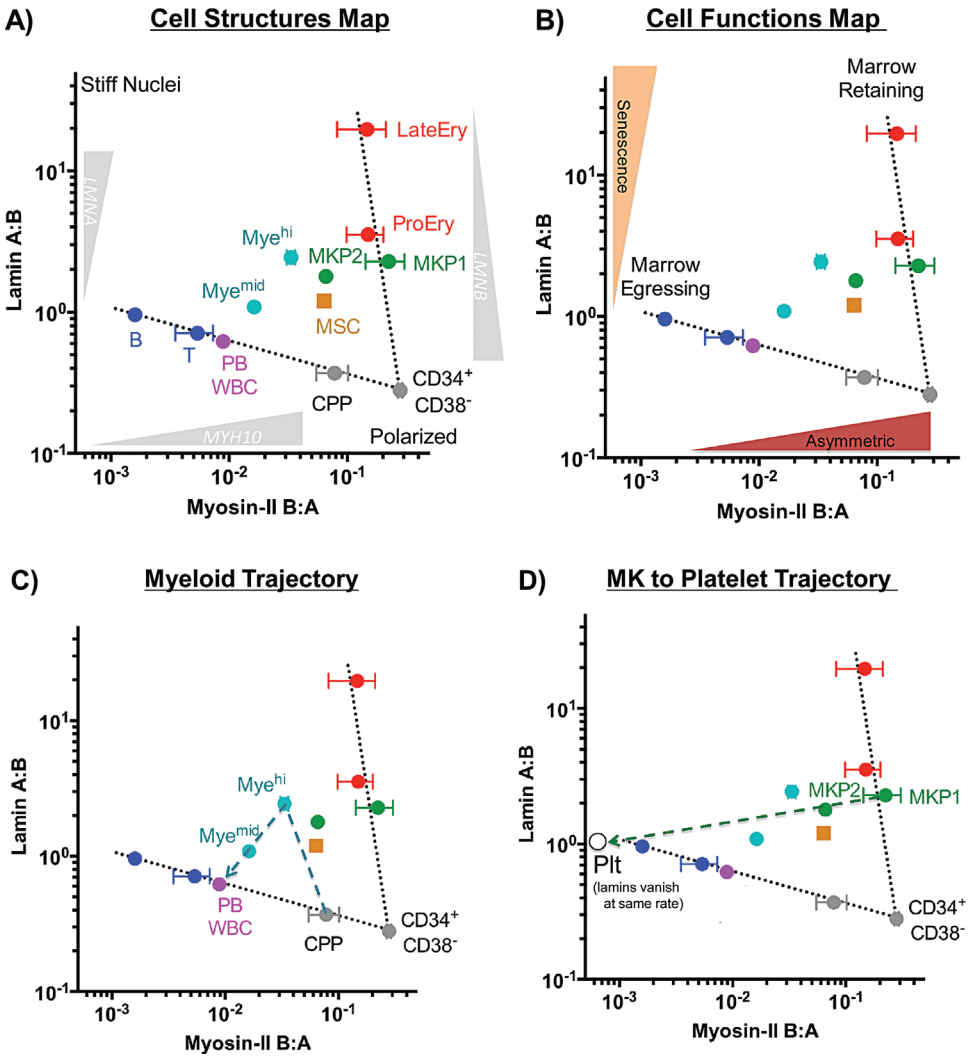

Figure 4. Correlations between polarized contractility and nuclear rheology delineated by MII and lamin isoform ratios. Values and error bars (±SEM, $n \geq 4$ donors) in the lamin A:B (nuclear rheology) versus MII B:A (polarized contractility) graph were derived from [30] and [32]. Two dotted lines indicate the outer boundaries of data points from different lineages. The lower boundary indicates cells that have relatively the constant lamin A:B ratio, but undergo significant downregulation in MIIB via force polarization. The upper boundary indicates cells that maintain the relatively constant MII B:A ratio, while lamin-B is decreased. The data are annotated as follows: **A**: annotations of cell structures showing nuclear rheology (upper left) and polarized contractility (lower right). **B**: Annotations of cell functions showing senescence (upper left) and asymmetric division (lower right). **C**: Developmental trajectory of myeloid differentiation (dashed cyan line), showing deviation from the lower boundary but returning upon myeloid cell trafficking into circulation. **D**: Developmental trajectory of platelet generation (dashed green line), showing transition from the upper boundary to the lower boundary upon platelet fragmentation from megakaryocytes. B, B-cell; T, T-cell; PB WBC, peripheral blood white blood cell; CPP, common potent progenitor; LateEry, late erythroid cell; ProEry, proerythroblast; MKP1, early megakaryocyte progenitor; MKP2, late megakaryocyte progenitor; Mye^{hi}, CD33^{hi} myeloid cell; Mye^{mid}, CD33^{mid} myeloid cell; MSC, mesenchymal stem cell.

physiological saline is positioned against the pipette under the microscope using the micromanipulator to apply a controlled suction pressure that is tuned by the manometer. With the pipette diameter smaller than that of blood cells (<10 μm), the method simulates the migration process where cells have to deform and squeeze through biological pores, including those formed by the extracellular matrix, the interface between two cells, and the capillaries (e.g. neutrophil transmigration). In this case, the aspiration pressure can be varied to form a hemispherical projection and measure the corresponding length. By plotting a pressure versus length graph, the slope can be used to derive Young's modulus (E:kPa), a measure of the cell's stiffness. Some blood cells show increased extension with increased pressure (following the law of Laplace), but beyond the critical pressure when the length of extension becomes equal to that of the radius of the pipette, the cells rush into the micropipette [35]. This "liquid drop-like" behavior is distinct from some non-blood cell types that tend to be more elastic or "solid-like" [36]. Another way to derive useful information on the mechanical behavior of living cells is to aspirate a cell at a constant pressure and record the extension length over time (the creep response). An early analysis with

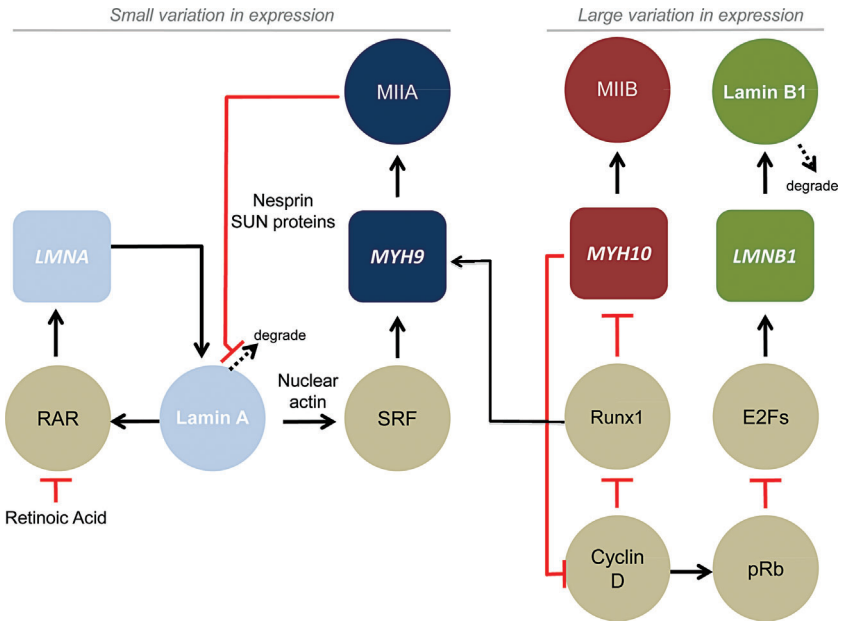

Figure 5. Systems mechanobiology of hematopoiesis. Underlying structural molecular circuits of hematopoiesis reveal potential targets that can be perturbed to manipulate biophysical determinants of hematopoiesis. In general, MIIA and lamin-A expression levels remain relatively constant throughout hematopoietic lineages, while MIIB and lamin-B expression levels change dramatically. MIIA and lamin-A expression levels are tightly regulated by a feedback inhibitory loop to prevent degradation of lamin-A by high MIIA and a feed-forward loop between lamin-A gene and protein. The MIIA and lamin-A circuit is regulated by serum response factor and retinoic acid receptor. MIIB and lamin-B expression levels are regulated post-translationally by asymmetric segregation and degradation, respectively. The potential transcription factors that regulate the underlying circuit between MIIB and lamin-B are highlighted. MIIA, myosin-IIA protein; MYH9, myosin-IIA gene; LMNA, lamin-A gene; RAR, retinoic acid receptor; SRF, serum responsive factor; MIIB, myosin-IIB protein; MYH10, myosin-IIB gene; LMNB1, lamin-B1 gene; pRb, phosphorylated retinoblastoma protein; Runx1, Runt-related transcription factor 1.

neutrophils showed that cells exhibit a transition in viscoelastic response: an instantaneous reversible deformation (elastic) is followed by a gradual, irreversible change in length over time (viscous) [37].

As early as the 1970s, Lichtman and Kearney used micropipette aspiration to show that granulocytes become more deformed as they become mature and to predict that "contracting-relaxing... macromolecules in motile cells" could play key roles in regulating deformability during lineage differentiation [28] – which today would be called "active gels." By pharmacological inhibition of cytoskeleton assembly or activity, contributions of the cytoskeleton can be minimized to reveal the properties of the nucleus – the largest organelle in most hematopoietic cells. Starting in the 1990s, advances in chemical tagging and genetic engineering have also enabled researchers to study how fluorescently tagged macromolecules in cells respond to micropipette aspiration and mobilize at the molecular level under live imaging (e.g. [38]). These macromolecules are generally biopolymers assembled by monomeric proteins, for instance, spectrins in RBCs. This approach has thus opened the door to map out individual biopolymers to specific mechanical behaviors of blood lineages in various biological contexts, including differentiation and trafficking.

Atomic force microscopy (AFM)

The use of an atomic force microscope (AFM) to probe mechanical properties of biological materials is relatively new, considering that the tool was initially developed in the 1980s to probe inorganic materials in the semiconductor industry. An AFM consists of a cantilever with a tip that is pressed into either a cell or a native microenvironment of a tissue (Fig. 6B). The degree to which the cantilever is bent is precisely measured by monitoring the displacement of a laser beam that reflects off of the back of the cantilever. This measurement can then be used to calculate Young's modulus *E*. The advantage of this method is that the mechanics can be characterized at a nanoscale or a single molecule level. For instance, an AFM was used to characterize the mechanics of single fibrin fibers [39] and that of single contracting platelets [40]. It was also used to reveal the heterogeneity of the BM mechanics in different regions, ranging from very soft marrow (0.3 kPa) to stiff pre-calcified bone surface (40 kPa) [32]. Another advantage of an AFM is that nanoscale surface topography of a sample can be measured and imaged by scanning with the tip. Using this approach, the mechanical topography of RBCs was measured at a sub 10 nm resolution [41]. The nanotopography of the tissue microenvironment was measured and shown to be important in directing MSC differentiation [42]. Therefore, an AFM can be used to probe cell intrinsic and extrinsic mechanical properties at a high resolution.

Quantitative mass spectrometry

"Big data" quantitative biology of biopolymer protein compositions provides insight on how mechanical properties of each cell type are defined as molecular circuits. The ability to quantify proteomes across the hematopoietic hierarchy will help determine how protein is partitioned and synthesized in each differentiation step. Two recent methods highlight the combination of mass spectrometry and flow cytometry to generate high-volume proteomic data across blood lineages. The mass cytometry method by the Nolan group uses mass spectrometry to analyze single cells labeled with transition element isotope-tagged antibodies so that multiple markers (30–40) can be probed without a need for fluorescent compensation, which is required for typical multi-color flow cytometry [43]. This method was used to show that some cytokine signaling responses are hierarchically organized during hematopoiesis. As antibodies have

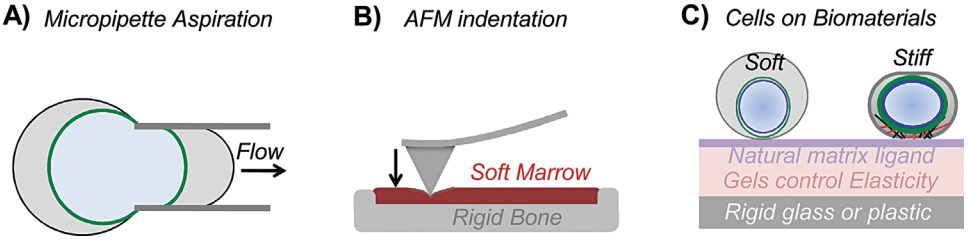

Figure 6. Physical methods and materials combined with molecular manipulations reveal biophysical regulation of hematopoietic differentiation and trafficking. **A**: Micropipette aspiration is used to quantify rheological properties of single cells and nuclei. **B**: AFM indentation measures the softness or rigidity of fresh, intact tissues such as BM or bone. **C**: Biomaterials are used to tune mechanical properties of matrix so that mechanosensing of cells and nuclei can be studied in vitro.

differential sensitivities against their target proteins, it is not possible to quantify the absolute amount of proteins by solely relying on antibodies. This is especially important when a relative contribution of each protein isoform to a biological function needs to be determined. To address this, our group recently developed a label-free mass spectrometry method to quantify absolute ratios of biopolymer isoforms by analyzing the amount ("ion flux") of peptides unique to each isoform [30]. The results from this method with standard cell lines have been calibrated against those from the intracellular flow cytometry method. The conversion factors from this calibration are subsequently applied to any sample of interest to convert the values from flow cytometry to absolute protein expression values. This method, mass spectrometry-calibrated intracellular flow (MS-IF) cytometry, has been used to reveal myosin isoform switching during hematopoiesis [32] and is especially well suited to abundant proteins such as structural proteins.

Biomaterials to modulate extracellular matrix mechanics

Hydrogels have been used to culture cells for several decades. For instance, methylcellulose is derived from cellulose and is widely used to culture cells for colony forming assay to quantify the number of hematopoietic progenitors. Despite this history, the biological significance of the physical properties of hydrogels has become appreciated only recently. Pelham and Wang developed a polyacrylamide hydrogel system (essentially the same as the one used in protein electrophoresis) where mechanical properties can be changed without affecting surface chemistry [44] (Fig. 6C). By using a modified version of this system characterized by AFM, we previously showed that matrix elasticity directs MSC differentiation [11]. We have since used this system to demonstrate that soft matrix maximizes the maturation of megakaryocytes [33] and suppresses HSC polarization during differentiation [32]. Another study used a protein-based tropoelastin gel to show that soft matrix expands HSC/P number [26]. In these studies, hematopoietic cells were cultured on the two-dimensional surface of hydrogels functionalized with matrix molecules. Whether the insights from these studies translate into three-dimensional hydrogels needs to be studied in greater mechanistic depth.

Microfluidics

The development of soft lithography techniques in poly-(dimethylsiloxane) (PDMS) has accelerated the progress of exploring biology at the microscale, as microfluidics devices can be created many times by casting PDMS on a master created by photolithography [45]. As small amounts of fluids can be manipulated under flow, it seems natural that microfluidics is appropriate to study hematopoietic biology. While earlier applications of microfluidics were focused more on genetic, proliferation, and deformability analysis of hematopoietic cells at a single cell level [46–48], more recent studies used it to develop a disease model for microvascular occlusion [49] and to recapitulate hematopoietic microenvironments [50, 51]. While microfluidics is indeed useful to probe or manipulate cells at the microscale for analytical purposes, it remains challenging to scale up blood production using microfluidics alone to match the quantity produced by the human body.

Rheometer

Rheometers have been traditionally used to characterize mechanical properties of natural and synthetic materials, including hydrogels [52]. Generally, a material sample is placed on a plate and a flat or cone shaped geometry is placed on top of the material. The defined force can be applied in oscillation to measure elastic (G') and viscous (G'') moduli of the material, which can be converted to Young's modulus (E): assuming Poisson ratio $= 0.5$, $E = 3 \times G'$. Using this method, various mechanical parameters, including stress versus strain, and stress relaxation can also be measured. In hematology, rheometers have been used to measure blood coagulation [53]. They are widely used to apply defined shear stress to cells in bulk fluid suspension, so that effects of shear stress on biological functions of cells can be measured. By this approach, the roles of shear force on embryonic hematopoiesis were investigated [53].

Conclusions and prospects: Mechanobiology for engineering blood and immune cells

Insights gained from mechanobiology of the hematopoietic system can be potentially useful for clinical applications in

hematology. For instance, controlled shear force can be used to maximize the production of platelets from cultured megakaryocytes in vitro [33, 84]. In addition, it may be useful to culture HSC/Ps in soft gels to maintain or expand their numbers prior to umbilical cord transplantation. Emerging studies demonstrate possibilities to create artificial BM microenvironments in vivo for hematopoietic modulation by subcutaneously implanting normal or genetically manipulated MSCs in hydrogels [85, 86]. To use this strategy, however, it will be necessary to further explore roles of physical forces in the interaction between HSC/Ps and MSCs or endothelial lineages. Small molecules to modulate mechanobiological circuits may be useful to pharmacologically engineer hematopoiesis and immune cell trafficking, such as reversible inhibition of MII by blebbistatin [32, 33] and downregulation of lamin-A by retinoic acid [34]. It will also be interesting to explore FDA approved drugs, such as fasudil (ρ-associated protein kinase inhibitor). Moving beyond normal hematopoiesis, it will be important to investigate roles of physical forces in abnormal hematopoiesis, which will inform novel intervention strategies that target mechanobiology.

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