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Biomechanical force in blood development: extrinsic physical cues drive pro-hematopoietic signaling

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Abstract

The hematopoietic system is dynamic during development and in adulthood, undergoing countless spatial and temporal transitions during the course of one's life. Microenvironmental cues in the many unique hematopoietic niches differ, characterized by distinct soluble molecules, membrane-bound factors, and biophysical features that meet the changing needs of the blood system. Research from the last decade has revealed the importance of substrate elasticity and biomechanical force in determination of stem cell fate. Our understanding of the role of these factors in hematopoiesis is still relatively poor; however, the developmental origin of blood cells from the endothelium promts a model for comparison. Many endothelial mechanical sensors and second messenger systems may also determine hematopoietic stem cell fate, self renewal, and homing behaviors. Further, the intimate contact of hematopoietic cells with mechanosensitive cell types, including osteoblasts, endothelial cells, mesenchymal stem cells, and pericytes, places them in close proximity to paracrine signaling downstream of mechanical signals. The objective of this review is to present an overview of the sensors and intracellular signaling pathways activated by mechanical cues and highlight the role of mechanotransductive pathways in hematopoiesis.

Keywords

hematopoietic stem cells; hemogenic endothelium; biomechanical force; shear stress; mechanotransduction; cellular microenvironment

INTRODUCTION

An extensive body of work has contributed to our appreciation for cellular plasticity in the last two decades. In the context of development and cancer biology, we have learned that a cell can differentiate, dedifferentiate, and even transdifferentiate, resulting in entirely new functions and cellular identities. These capacities are defined by intrinsic properties inherited by developmental origins and by signals from the microenvironment. There is

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increasing need for a better understanding of the private *in vivo* lives of stem cells (Sanchez Alvarado, 2008). In the last decade, it has become apparent that regulatory pathways which define cellular behavior such as cell cycle entry and differentiation can have vastly distinct roles *in vitro* and in stem and progenitor cells of the developing embryo (Chen et al., 2009a; Chong et al., 2009; Wenzel et al., 2011). This highlights the need to study the native stem cell environment and to accurately model niche components that determine stem cell potential.

The blood system, and arguably the skin, gut, and neural system, have contributed the most to our understanding of mammalian stem cell niches, in terms of the many complex cellular interactions and regulatory pathways that determine quiescence, self-renewal, and differentiation. There is a diverse array of specialized cell types that form the niche. Many of these are terminally differentiated and some remain in a relatively plastic state, while contributing essential factors to promote development and/or maintain homeostasis. Over 300 years ago, researchers were fascinated by the fundamentals of cellular mechanics (Pelling and Horton, 2008), but only recently has the field of stem cell biology acknowledged the roles of intracellular tension and extracellular biophysical factors as critical determinants of differentiation (Engler et al., 2006). Important physical properties of the extracellular matrix (ECM) include elasticity, nanotopography, and spatial distribution of adhesive substrates and ligands. Biomechanical forces include mechanical loading or pressure, friction, and stretching. Together, these mechanical cues determine the activity of critical regulatory pathways that modulate differentiation, cell division, cell survival, and motility. The physical environment impacts cell-intrinsic signaling, as well as paracrine signaling that can dictate cellular potential and behavior of nearby neighbors.

Advances in chemical engineering and materials science have broadened our biological understanding of stem cell biology through the use of lithography, including soft lithography and capillary force lithography, microcontact printing, microfluidics, and microassembly. For review of advances in engineering and materials-based approaches, the reader is referred elsewhere (Jakab et al., 2010; Kim et al., 2012; McNamara et al., 2010; Whitesides et al., 2001). With further exploration, this interdisciplinary field has potential to impact not only classically defined areas of regenerative medicine, such as reconstruction of bone and cartilage, but also other cellular therapies used for treatment of disorders and injuries complicated by dysregulation of the immune system, bone marrow failure syndromes, autoimmunity, autoinflammation, and hematological malignancy. In this review, we describe recent advances in our understanding of how biophysical cues and mechanosensory pathways determine blood development and homeostasis.

HEMATOPOIETIC ONTOGENY

The first clues to hematopoietic ontogeny were reported at the turn of the century in several vertebrate species, including man, mouse, and chick (Dantschakoff, 1907; Emmel, 1916; Jordan, 1916; Maximov, 1909; Minot, 1912; Sabin, 1917; Stricht, 1899). In the past 30 years, our understanding of the developmental origins of blood has matured, aided by the study of humans, as well as diverse model systems such as the quail and chicken, zebrafish, mouse, fly, and frog (Ciau-Uitz et al., 2010; Dieterlen-Lievre et al., 2006; Evans et al., 2003; Medvinsky et al., 2011; Orkin and Zon, 2008; Tavian and Peault, 2005). In particular, the accessibility of the chick embryo has permitted interspecies transplantation that has enabled tracing of the regions of the early embryo that contribute to the blood system. Zebrafish have allowed for transparent viewing of blood emergence and migration and have proved a powerful tool for large-scale pharmacological identification of critical regulatory pathways in hematopoiesis. Importantly, the mouse has provided genetic tractability and embryonic stem cell based *in vitro* modeling of blood development.

During embryogenesis, the first hematopoietic cells to populate the vasculature emerge from yolk sac blood islands. “Primitive” blood consists primarily of megakaryocytes, macrophage progenitors, and nucleated erythrocytes that express embryonic and fetal globins (ε and (βH1) and do not contribute to the adult blood system (Silver and Palis, 1997). By embryonic day 9.5 (E9.5), and even E8.0, cells arise with greater multipotency and, when delivered pre- or peri-natally, can contribute to adult hematopoiesis (Weissman et al., 1978; Yoder et al., 1997). The identity of these cells can be characterized by their requirement for the Runxl/AML1/Cbfa2 master regulator of hematopoiesis (Cai et al., 2000; North et al., 1999; Okuda et al., 1996; Samokhvalov et al., 2007; Tanaka et al., 2012). Indeed, lineage tracing experiments have revealed that cells derived from the E6.5 to E7.5 extraembryonic mesoderm of the yolk sac are a source for intraembryonic sites of hematopoiesis that later account for the majority of the downstream descendants in the adult blood system (Samokhvalov et al., 2007; Tanaka et al., 2012). Because of their ability to contribute to adult hematopoiesis via a neonatal intermediate, yolk sac derived cells could be considered akin to pre-hematopoietic stem cells (pre-HSCs) most recently defined as the precursor to the adult-type definitive HSC that arises within the embryo proper (Medvinsky et al., 2011). It is important to note, however, that the origin(s) of the hematopoietic stem cell is still strongly debated and continues to polarize the field of hematopoiesis.

Definitive hematopoietic stem cells (HSCs) replenish the blood supply throughout life, and their precursors are known to arise during embryogenesis at E10.5 in the region of the embryo containing the dorsal aorta, gonads, and mesonephroi (AGM) (Medvinsky and Dzierzak, 1996; Muller et al., 1994). Evidence from both zebrafish and mouse now implicate a subpopulation of endothelial cells with hemogenic potential as a *de novo* source of HSCs that contribute to life-long hematopoiesis (Bertrand et al., 2010; Boisset et al., 2010; Chen et al., 2009b; Eilken et al., 2009; Kissa and Herbomel, 2010; Lam et al., 2010; Lancrin et al., 2009; Zovein et al., 2008). Indeed, endothelial cells and nascent hematopoietic cells share several of the same cell surface markers, and both are derived from an angioblast population that expresses Brachyury and Flk-1 (Huber et al., 2004; Kabrun et al., 1997; Millauer et al., 1993; Shalaby et al., 1997; Shalaby et al., 1995; Yamaguchi et al., 1993). Hemogenic endothelial cells line the dorsal aorta and are enriched near the vitelline artery (Yokomizo and Dzierzak, 2010). In the zebrafish, nascent hematopoietic cells emerge from the lumen of the vessel in a carefully choreographed exit that maintains vessel integrity while allowing entry into the blood stream (Kissa and Herbomel, 2010). On the wall of the dorsal aorta in mice and chick, intra-aortic clusters of varying sizes extend into the blood stream. Labeling of these hematopoietic clusters shows gradual transition from endothelial phenotypes on the vessel wall to hematopoietic at the distal edges of the cellular aggregate (Jaffredo et al., 1998; Yokomizo and Dzierzak, 2010). This endothelial to hematopoietic transition is dependent upon the expression of Runxl, which is thought to mediate suppression of endothelial genes, including VE-Cadherin/ CD144, CD31, and Flk-1/KDR/ VEGFR2, and upregulation of hematopoietic genes, CD41, CD45, Gfi1B, Ikaros, and PU.1 (Iacovino et al., 2011; Mizuochi et al., 2012). Late stage E11.5 AGM regions contain peak numbers of definitive HSCs, as measured by careful assessment of somite stage and transplantation assays for determination of long-term, transferable multipotency of blood lineages (Taylor et al., 2010). Beginning around E11.0, hematopoietic stem and progenitor cells (HSPCs) emigrate from the AGM to the fetal liver for expansion and maturation (Morrison et al., 1995). The placenta is also a significant reservoir for HSCs and blood progenitors around this time (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). After E14.5, HSPC egress begins for colonization of the fetal spleen, thymus, and bone (Ciriza et al., 2013; Godin and Cumano, 2002; Morrison et al., 1995; Samokhvalov et al., 2007). The bone marrow becomes the primary site of hematopoiesis in adult life, and provides a niche suitable for HSC homeostasis and progenitor maturation.

BIOMECHANICAL FORCES IN THE HEMATOPOIETIC NICHE

Blood flow is a critical determinant of arterial lineage specification, vascular remodeling, and hematopoiesis, and begins at E8.25 in the murine embryo (Adamo et al., 2009; Chouinard-Pelletier et al., 2013; Lucitti et al., 2007; North et al., 2009). Three types of hemodynamic forces influence vascular and hematopoietic cells, including shear stress [friction), circumferential strain [stretching], and hydrostatic pressure. We have found that shear stress, specifically, has important implications for hematopoietic signaling (Adamo et al., 2009) and changes during development as a function of blood viscosity, cardiac output (i.e. blood velocity), and vessel dimensions/location. For this reason, entry of erythroblasts into the early circulation has been found to significantly increase blood viscosity and consequently shear stress with important implications for angiogenesis (Chouinard-Pelletier et al., 2013). Blood flow in the murine yolk sac vasculature has been measured at peak values of 1 to 2 dyne/cm2 (highest in the yolk sac at 16 somites) (Chouinard-Pelletier et al., 2013) and in the E10.5 dorsal aorta at 5 dyne/cm2 (Adamo et al., 2009). The fetal liver is also highly vascularized, but patterns of shear stress in this organ at the time of HSPC colonization are poorly understood. Migrating HSPCs survey microsites in the liver prior to settling, akin to the process of rolling and arrest observed in mature leukocytes. Later in development, long after blood production has shifted to the bone marrow, some areas of the mouse aorta can experience instantaneous wall shear stress that exceeds 600 dyne/cm2, an order of magnitude higher than values found in the human (Suo et al., 2007).

Bone provides a number of distinct mechanical environments associated with sinusoids, blood vessels, and mineralized bone matrix. Several of these regions of the bone are complex but well characterized niches comprised of osteocytes, osteoblasts, osteoclasts, endothelial cells, mesenchymal stromal cells, pericytes, nerve cells, as well as mature blood lineages such as macrophages (Bianco, 2011; Ehninger and Trumpp, 2011). HSPCs can experience a wide array of elasticities in the done marrow, notably the range covering adipose ($E_{substrate}$<1KPa, cell memdranes ($E_{substrate}$=1–3KPa), and collagenous bone ($E_{substrate}$=100KPa) (Discher et al., 2009; Engler et al., 2006). Bone is a vascularized organ and must provide passage in and out of the marrow to circulating endothelial progenitors, hematopoietic cells, nutrients, and signaling molecules. Fluid flow is expected to be slow in the vasculature that supplies blood to the medullary cavity of the bone, but fluid movement in the lacunar-canalicular network of the bone can expose osteocytic processes to shear stresses of 6–50 dyne/cm2 associated with mechanical loading and ambulation (Piekarski and Munro, 1977; Price et al., 2011; Weinbaum et al., 1994; Zeng et al., 1994). Not only does this have important implications for mechanical stimulation, this fluid flow around osteocytes is necessary for delivery of nutrients and other signaling molecules, including glucose, lactic acid, nitric oxide, estradiol, testosterone, prostaglandin, adenosine-5'-triphosphate (ATP), vitamin D, and corticosteroids (Price et al., 2011). This suggests that the hematopoietic cells in the endosteum would not be directly exposed to fluid forces of these magnitudes, but could be impacted by paracrine signaling downstream of osteocyte mechanotransduction and biochemical signaling. Either directly or indirectly, endothelial cells and pericytes are also likely to interface with fluid forces and provide signals to HSPCs that tightly regulate cell cycling and egress.

MECHANOSENSORS & SIGNAL TRANSDUCTION

Little is known regarding the mechanisms by which biophysical cues in the microenvironment may affect cellular functions or indeed how the sensing apparatus and transduction of mechanical signals operates in HSCs. The most likely mechanism by which cells monitor substrate elasticity involves two-way interactions with the actin-myosin cytoskeleton, coupled through membrane receptors such as integrins (Even-Ram et al.,

2006). But, as outlined in Table 1, there are numerous cellular components and mechanosensitive molecules that transmit mechanical information into the cell and to the nucleus, including integrins (Chen et al., 1999; Wang et al., 1993), adherens junction proteins (Shay-Salit et al., 2002), G protein-coupled receptors (GPCR) (Chachisvilis et al., 2006; Gudi et al., 1996; Lehoux et al., 2006; Makino et al., 2006), ion channels (Brayden et al., 2008; Olesen et al., 1988; Owsianik et al., 2006), glycocalyx (Weinbaum et al., 2003), myosin motors, cytoskeletal filaments, caveolae (Park et al., 2000; Park et al., 1998; Rizzo et al., 1998b) and other signaling molecules. As depicted in Figure 1, mechanoreceptors are organized on and throughout the cell according to cell type and mechanical environment. In endothelial cells, for example, several membrane-associated structures reside on the apical (luminal) surface, including ion channels (Barakat, 2001; Olesen et al., 1988; Schwarz et al., 1992; Traub et al., 1999), caveolae (Park et al., 2000; Park et al., 1998), and G proteins (Gudi et al., 1998). By contrast, a number of other proteins and molecules are enriched on the basolateral surfaces and throughout the cells such as integrins (Gloe et al., 2002), cell-cell adhesion molecules (Fujiwara et al., 2001), adherens junctions (Shay-Salit et al., 2002), and the cytoskeleton. This arrangement of sensors provides polarity and impacts cell division, shape, function, and motility.

Cell Adhesion Molecules

Cell adhesion molecules play central roles in regulating vascular development, hematopoiesis, hemostasis, and immune and inflammatory response (Ye et al., 2012). The stem cell microenvironment is particularly dependent upon the ligand-binding properties of supportive niche cells to control quiescence and trafficking of stem and progenitor cells (Chen et al., 2013; Ehninger and Trumpp, 2011). In addition to mediating attachment to ECM and/or neighboring cells, some adhesion molecules transmit information bidirectionally across the cell membrane. Some of these include integrins, cadherins, selectins, connexins, CD44, sialomucins, intercellular adhesion molecule 1 (Icaml/CD54), and vascular cell adhesion molecule 1 (Vcaml/CD106) (Imai et al., 1999; Osawa et al., 1996; Simmons et al., 2001; Uchida et al., 1998).

Early support for the notion that anchorage sites might be important for HSC homeostasis came from studies of integrin α4(β1 (VLA-4) (Anderson et al., 1990; Martin et al., 1990; Williams et al., 1991; Zsebo et al., 1990). Integrins are heterodirmeric glycoproteins that consist of α and β peptide chains, and each subunit contains a large extracellular domain, a single-pass transmembrane domain, and a short cytoplasmic tail. Antibodies against the β1 subunit were shown to inhibit reconstitution activity from bone marrow and to efficiently mobilize CD34+ hematopoietic progenitors into the bloodstream (Papayannopoulou and Nakamoto, 1993). Since these first reports, it has become evident that α4β1 integrin is indispensible for definitive hematopoiesis both during embryogenesis and in adulthood, due almost exclusively to its role in adhesion to supportive stroma. It is expressed highly on HSCs and progenitors, and ablation of (31 integrin function genetically or by neutralizing antibodies renders HSPCs unable to engraft the bone marrow or spleen (Craddock et al., 1997; Papayannopoulou et al., 1995; Potocnik et al., 2000; Williams et al., 1991). In embryogenesis, ItgB1-null hematopoietic progenitor cells are specified normally, accumulate in the peripheral blood, but fail to populate the fetal liver (Hirsch et al., 1996; Potocnik et al., 2000). This defect has been characterized as an inability to anchor to the endosteal surface and less so a problem of finding the medullary space, as measured by optical trap and *in vivo* fluorescent imaging of the adult femur (Askenasy et al., 2003). Presumably with similar functions, the α4, α6 and α9 integrins have also been found to be critical for HSC-microenvironment interactions (Grassinger et al., 2009; Qian et al., 2006; Schreiber et al., 2009). Notably, hemogenic endothelium is specifically enriched with a4 integrins (Ogawa et al., 1999), and HSPCs express high levels of α4, α6, α7, α9 and β1

(Grassinger et al., 2009; Potocnik et al., 2000; Schreiber et al., 2009; Voura et al., 1997). Genetic pathways downstream of integrin signaling include Ras/MAPK, phosphatidylinositide 3-kinase (PI3K)/Akt, RhoA/ROCK, Wnt/β-catenin, TGF-β (Sun et al., 2012), but those most relevant to hematopoietic development will depend upon the integrin heterodimers present on the cell surface and the composition of the ECM in the niche. Whereas integrins can bind cell-surface molecules and components of the ECM, such as fibronectin, collagen, and laminin, and provide the cell with information about ECM rigidity, nanotopography, microgeometry, and mechanical stress (Barczyk et al., 2010; Hynes, 2002), cadherins mediate cell-cell contact.

Cadherins were originally identified as cell surface glycoproteins responsible for Ca^{2+}-dependent hemophilic cell-cell adhesion during mouse embryo and chick development (Yoshida and Takeich, 1982) and their function extends to multiple aspects of tissue morphogenesis, including cell recognition and sorting, boundary formation and maintenance, coordinated cell movements, and the induction and maintenance of structural and functional cell and tissue polarity in the three decades since their discovery. In particular, VE-cadherin is important in the hematopoietic population. Fraser et al. were the first to show that the VE-cadherin+ population from different stages of embryonic development in mice generated more hematopoietic cells *ex vivo* relative to the Flk-1+ VE-cadherin- population(Fraser et al., 2002). Moreover, VE-cadherin expressing cells in the human embryonic liver showed outstanding self-renewal, proliferation and differentiation potential, suggesting that VE-cadherin marks a primitive HSC population (Oberlin et al., 2010). The function of N-cadherin in hematopoiesis is still controversial. N-cadherin deficiency in mice causes no detectable defects in HSC maintenance or hematopoiesis(Kiel et al., 2009) but does contribute to the long-term engraftment of HSCs(Hosokawa et al., 2010). Further, disruption of N-cadherin with blocking antibodies significantly diminished colony formation from CD34+ progenitor cells, implicating N-cadherin in the differentiation program of early hematopoietic progenitors (Puch et al., 2001). Another classical cadherin, E-cadherin also has been shown to function during normal erythropoiesis. Inhibition of E-cadherin with anti-human E-cadherin antibodies decreased the differentiation of erythropoietic progenitors into erythroblast, suggesting that E-cadherin is involved in the differentiation and maturation of the erythroid lineage(Armeanu et al., 1995). However, its role during myeloid and lymphoid differentiation has not been studied. Corn et al. reported that E-cadherin was expressed in mature PBMCs and normal lymphoblastoid cell line. These suggest that E-cadherin is involved in multi-lineage hematopoiesis. Moreover, they found E-cadherin was aberrantly methylated in leukemia cell lines(Corn, 2000), indicating that the epigenetic regulation of E-cadherin is important during leukemogenesis.

A Disintegrin And Metalloprotease (ADAM) Family

The ADAM family is a class of proteases that cleave and/or modulate binding affinity of adhesion molecules, chemokines, cytokines, growth factors, and transmembrane receptors (Weber and Saftig, 2012). The proteolytic activity of ADAMs can be triggered by factors such as mechanical and osmotic stress, G protein-coupled receptors (GPCRs), protein kinase C, intracellular calcium, serum factors and growth factors (Huovila et al., 2005). Several members of the ADAM family have been implicated in regulation of cellular adhesion of blood cells. For example, ADAM28 binds α4βl integrin and can modulate lymphocyte emigration (Weber and Saftig, 2012). Another family member, ADAM8, is expressed primarily in cells of the immune system, and was demonstrated to be essential for the release of primitive erythrocytes into the blood stream of the zebrafish embryo (Fourie et al., 2003; Iida et al., 2010). Prior to emergence of primitive erythrocytes, “bloodless” plasma flows through the vasculature. Newly specified erythrocytes first aggregate in and adhere to the lumen of the dorsal aorta and posterior cardinal vein. A unified and sudden release of

erythrocytes from the vessel walls occurs at 27 hours post-fertilization, allowing the blood cells to enter circulation (Iida et al., 2010). Inactivation of ADAM8 or use of metalloprotease inhibitors was found to cause erythroid retention on the vessel walls, stagnation, and presumably subsequent cell death. The trigger for this synchronized detachment is unclear, but the authors speculate that plasma flow (i.e., shear stress) could play a role, as tnnt2 morphants which lack a heartbeat exhibited a similar defect. Further, they suggest the substrate for ADAM8 is likely to be an adhesion molecule, such as CD41 or a related integrin. Alternatively, degradation of the ECM may serve to either remove the matrix as a barrier to cell motility (Werb et al., 1999) or result in the release of biologically active fragments of ECM molecules that regulate motility, proliferation, and cell survival (Larsen et al., 2006). It is interesting to speculate that a similar event could occur later in development during definitive hematopoiesis as HSPCs leave the aorta for the fetal liver and could coincide with downregulation of endothelial-type cell adhesion molecules such as VE-Cadherin/CD144 and CD31 (Mizuochi et al., 2012; Yokomizo and Dzierzak, 2010).

There is also evidence to suggest that ADAMs play a critical role in HSC specification (Gibb et al., 2010; Gibb et al., 2011; Yoda et al., 2011). ADAM10 is required for S2 cleavage of the Notch receptor extracellular domain. Only after removal of the extracellular domain can S3 cleavage occur by the γ-secretase complex, allowing the NICD to translocate to the nucleus and activate Notch target genes. It has also been suggested that ADAMs may be involved in ectodomain shedding of membrane-bound Delta ligand. Interestingly, blood flow is required for activation of Notch in the vasculature. One potential mechanism for Notch activation is direct modulation of ADAM10 by shear stress, though this idea remains untested. Alternatively, the shearing forces caused by blood flow may amplify mechanical forces exerted by ligation to clustered or immobilized Delta ligand thereby exposing the Notch cleavage site to ADAM10 (Gordon et al., 2007). In culture systems, immobilization of Delta on culture substrates or beads is necessary for downstream signaling (Varnum-Finney et al., 2000), T cell differentiation from HSCs (Taqvi et al., 2006), and expansion of hematopoietic cord blood progenitor cells (Delaney et al., 2005). This highlights the importance of spatial presentation of regulatory factors and may be indicative of a role for Notch1 in concert with ADAMs in detection of tension and cytoskeletal rearrangements that occur as a result of blood flow.

Nitric Oxide Signaling

Nitric oxide (NO) is an important endocrine and paracrine signaling molecule that plays a key role in the regulation of vascular tone, angiogenesis, endothelial migration, and hematopoiesis (Adamo et al., 2009; Davies, 1995; Lucitti et al., 2007; Michurina et al., 2004; North et al., 2009). Many studies have demonstrated that hemodynamic flow is an important biophysical cue that regulates nitric oxide synthase (NOS) activity and NO release from vascular endothelium (Fukumura et al., 2001; Ranjan et al., 1995; Uematsu et al., 1995). Indeed, pulsatile flow typical of a normal cardiac cycle has been shown to trigger rapid and sustained NO production (White and Frangos, 2007). There are three NOS genes in the mammalian genome, each of which has distinct modes of regulation and cell type specificities (Alderton et al., 2001). In the hematopoietic lineages, the neuronal isoform (nNOS) is detectable in neutrophils (Chen and Mehta, 1996; Forstermann et al., 1998; Greenberg et al., 1998; Wallerath et al., 1997), whereas the endothelial isoform (eNOS) is found in lymphocytes, megakaryocytes, and platelets (Chen and Mehta, 1996; Sase and Michel, 1995). Inducible NOS (iNOS) is expressed in megakaryocytes, eosinophils, and monocytes (Amin et al., 1995; Wallerath et al., 1997). Additionally, both the nNOS and eNOS isoforms are detectable at high levels in the fetal liver and in the bone marrow stroma (Krasnov et al., 2008).

NO production is coordinated by multiple mechanotransductive elements including ECM, cell-ECM adhesion, cell-cell adhesion complexes, cytoskeleton, and membrane components. Although fluid shear stress is strictly applied to luminal surfaces of endothelial cells, shear forces are transmitted to cell-cell junctions and the ECM. Several studies demonstrate that inhibition of FAK signaling by interfering with autophosphorylation at Tyr-397 impairs flow-induced phosphorylation of Akt (Ser473) and eNOS (Serll79). This not only points to the FA complex and FAK as critical components of the mechanotransduction machinery in endothelium but also reveals a potential mechanism of shear-induced regulation of NO signaling (Koshida et al., 2005). Other flow-based studies have suggested that phosphorylation of eNOS at Serll79 occurs via the PI3K-Akt-eNOS signaling pathway in human umbilical vein endothelial cells (HUVECs) and enhances enzyme activity in a Ca^{2+}-independent manner (Dimmeler et al., 1999; Fisslthaler et al., 2000). Interestingly, shear stress appears to promote packaging of eNOS in caveolar vesicles, and this has been suggested to contribute to the regulated activity of NOS (Rizzo et al., 2003).

Accumulating evidence has shown that NO signaling plays a critical role in definitive hematopoiesis. In particular, NOS activity has been found to regulate quiescence of hematopoietic progentors in the bone marrow and spleen (Krasnov et al., 2008). Despite detectable levels of NOS isoforms in hematopoietic cells, these effects on cell cycling were found to be mediated exclusively by paracrine NO signaling from stromal cells of the hematopoietic niche (Krasnov et al., 2008). Importantly, nNOS is the primary isoform responsible for production of NO in mesenchymal stromal cells of the bone marrow and fetal liver. As HSCs are derived from hemogenic endothelial cells within the dorsal aorta, NO produced locally in endothelial cells could direct HSC emergence and control self renewal. We and others have shown that ectopic NO enhances hematopoiesis and can rescue defects caused by cardiac dysfunction, while inhibition of NO signaling results in significant loss of blood stem and progenitor cells (Adamo et al., 2009; North et al., 2009). NO appears to be absolutely required for maintenance of HSC in the AGM region and is activated downstream of Klf2a in zebrafish (Wang et al., 2011). This upregulation of the Klf2-NO axis is consistent with signaling through PI3K, but this remains to be definitively demonstrated. Further, there is evidence to suggest that NO could lie downstream of Notch and Wnt signaling, based upon the ability of NO donors and antagonists to control HSC numbers in Notch and Wnt mutants (North et al., 2009).

G Protein-Coupled Receptors (GPCR)

The GPCR superfamily is perhaps the largest and most diverse group of membrane proteins, encoded by over 800 genes in the human genome (Fredriksson et al., 2003). GPCRs share in common a seven-pass transmembrane domain that functions by binding a wide spectrum of extracellular signals, including photons, ions, small organic molecules, and proteins (Venkatakrishnan et al., 2013). GPCRs undergo conformational changes upon ligand binding, resulting in coordinated activation of cytosolic signaling networks and cellular response (Venkatakrishnan et al., 2013). GPCRs are diverse in structure and function but can be phylogenetically categorized into 5 primary receptor families: rhodopsin, glutamate, frizzled/taste2, adhesion, and secretin (Fredriksson et al., 2003). GPCRs of particular relevance to hematopoietic development can be found in the rhodopsin family (chemokine C-X-C motif receptor 4 (CXCR4) and sphingosine 1-phosphate receptor (S1PR1/EDG1)), glutamate family (calcium sensing receptor (CaSR)), and frizzled family (Wnt receptors Smoothened (SMOH), Frizzled 4 (FZD4), and FZD9) (Adams et al., 2006; Corrigan et al., 2009; Gering and Patient, 2005; Ranheim et al., 2005; Seitz et al., 2005; Zou et al., 1998). Functions of these receptors in hematopoiesis include regulation of lineage specification and homing.

A number of GPCR modulated by shear stress may protect the self renewal capacity of the HSPC as it prepares to travel to the fetal liver for expansion, such as the EP2 and EP4 $G\alpha_s$ protein-coupled receptors of prostaglandin E2 (PGE2). Stimulation by shear stress has been shown to induce prostaglandin synthesis in arterial endothelial cells (Roller et al., 1993). Importantly, in addition to roles in vasodilation, PGE2 has been found to inhibit macrophage maturation (Zaslona et al., 2012) and promote cell survival and expansion of HSPCs (Goessling et al., 2011; Goessling et al., 2009; North et al., 2007). Upon binding to PGE2, the EP2 and EP4 $G\alpha_s$ protein-coupled receptors trigger an increase in intracellular cAMP levels (Regan et al., 1994) and amplify Wnt signaling activity via PKA-mediated stabilization of β-catenin (Goessling et al., 2009). In human cells, and perhaps at sufficiently high doses in rhesus macaque, PGE2 may regulate HSC cell survival and proliferation by impacting major regulators of HSC development, including RUNX1, LM02, LY6A, HOXA9, CXCR4, FLT3, JAK1, CCR1, and CD8a, HHEX, JUNB, LCK, and TF (Goessling et al., 2011; Goessling et al., 2009). Interestingly, Wnt signaling can also be activated directly downstream of (β1-integrin in an ILK-GSK3b dependent pathway, which further leads to Notch activation (Rallis et al., 2010). Notably, osteoblasts collected from the adult bone marrow activate many of these same pathways that modulate HSPC self renewal and cycling in response to shear stress, including PGE2 production, intracellular Ca^{2+} release, and NOS activity (Reich and Frangos, 1991; Smalt et al., 1997; Williams et al., 1994).

Sphingosine 1-phosphate (S1P) is a sphingolipid messenger molecule produced in response to laminar shear stress which is known to be a potent chemoattractant for HSCs and progenitors in the adult (Massberg et al., 2007; Seitz et al., 2005). The majority of circulating plasma S1P is produced by erythrocytes and endothelial cells through the activity of sphingosine kinases, but is also known to be released by platelets (Pappu et al., 2007; Venkataraman et al., 2008). S1P levels are high in the blood at micromolar concentrations, but are extremely low in tissues due to degradation by S1P lyase (Hanel et al., 2007; Hla et al., 2008; Okajima, 2002; Schwab et al., 2005). Production of S1P and its GPCR S1PR1/EDG1 has been found to be induced in vascular endothelial cells by laminar shear stress typical of arterial vessels and can activate eNOS activity (Hughes et al., 2005; Igarashi et al., 2003; Takada et al., 1997; Tanimoto et al., 2002; Venkataraman et al., 2008). HSPCs express S1P receptors, undergo chemotaxis in response to S1P, and have been found to utilize S1P gradients to emigrate from peripheral tissues to the lymphatic system (Kimura et al., 2004; Massberg et al., 2007). The same signals that provide direction to emigrating HSPC from the bone marrow could also be linked with instructions for homing and adhesion in the fetal liver. S1PR1, via Flk-1/KDR/VEGFR2 and possibly also protein kinase C, phosphorylates the Rac1 GTPase-associated protein CrkII (Endo et al., 2002; Igarashi et al., 2003; Tanimoto et al., 2002). S1P also potently induces yes-activated protein (YAP) dephosphorylation, nuclear localization, and transcriptional activity of cell proliferation related genes such as CTGF and Cyr61 (Miller et al, 2012). As S1P lyase inhibitors have been reported to disrupt CXCR4 antagonist-based mobilization (Ratajczak et al., 2010), it is intriguing to speculate that newly emergent HSCs from the AGM could use the S1P chemokine as a guide for entry into the blood stream and that this might occur in connection with Yap activation and associated cell division.

Genetically engineered mice have implicated stromal derived factor 1-α (SDF1-α] and its GPCR receptor, CXCR4 in HSC homing and engraftment to the marrow (Avigdor et al., 2004; Barker, 1997; Ding et al., 2012; Nagasawa et al., 1996; Papayannopoulou, 2003; Zou et al., 1998). Notably, SDFl-α −/− and CXCR4−/− embryos have greater impairment of myelopoiesis in bone marrow as compared to the fetal liver, suggesting that SDFl-α and CXCR4 are involved in colonization of bone marrow by hematopoietic progenitors during embryogenesis but not as critical for seeding of newly specified HSCs from the AGM [Ma et al., 1998; Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). SDFl-α is

expressed by developing stroma in fetal bones, and its receptor CXCR4 is expressed on HSPCs (Ara et al., 2003). Interestingly, actively signaling CXCR4 is associated with lipid rafts [Lee et al., 2009; Ratajczak et al., 2004). As the CXCR4 receptor is a lipid raft-associated protein, its signaling sensitivity can be modulated by colocalization with other signaling molecules, including Rac1 (del Pozo et al., 2004; Gu et al., 2003). Packaging of CXCR4 together with Racl in lipid rafts facilitates GTP binding and activation of Rac1 (Ratajczak et al., 2010).

Calcium Sensing Receptor (CaSR], an ion-sensing G protein-coupled receptor, functionally couples to integrins and, in conjunction with intracellular calcium release, promotes cellular adhesion and migration in tumor cells (Tharmalingam et al., 2011). Other studies have suggested that extracellular Ca^{2+} sources, such as those found in the bone, can influence HSC lodgement in the endosteal niche via the CaSR (Adams et al., 2006; Lam et al., 2011). Activation of the CaSR on HSCs by extracellular Ca^{2+} allows HSC to adhere to ECM components like collagen I, thus retaining them in close proximity to osteoblasts (Adams et al., 2006; Lam et al., 2011).

Other Mechanosensors

The type III receptor kinase known as Flk-1/KDR/VEGFR2 is renowned as a mesodermal surface marker expressed on cells with hematopoietic potential. It also plays a critical role in mechanosensation in complex with the cell adhesion molecules PECAM and VE-Cadherin (Tzima et al., 2005). Flk-1 initiates a cascade of intracellular signaling events through activation of Src-MAPK, PI3K-Akt, and eNOS downstream of shear stress (Jalali et al., 1998; Jin et al., 2003; Tzima et al., 2005). These signaling pathways can activate integrins and small GTPases (Rho, Rac and Cdc42) to orchestrate cytoskeletal reorganization [Tzima, 2006). There is evidence from embryonic stem cell based-models of hematoendothelial development that shear stress promotes endothelial and hematopoietic specification from the Flk-1+ fraction of differentiated embryonic stem cells (Adamo et al., 2009; Wolfe and Ahsan, 2013; Yamamoto et al., 2005). Consistent with the Flk-1−/− phenotypes in mice, pharmacological treatment with the Flk-1 kinase inhibitor SU1498 blocks differentiation of endothelial cells and their downstream hematopoietic progeny (Shalaby et al., 1997; Shalaby et al., 1995; Wolfe and Ahsan, 2013; Yamamoto et al., 2005). SU1498 inhibits both Flk-1 and ERK kinase activity, implicating PI3K and MAPK signaling in these shear-induced commitment events (Boguslawski et al., 2004).

The relevance of other mechanosensors in the hematopoietic system remains less well described, yet there are hints that ion channels (Brayden et al., 2008; Olesen et al., 1988; Owsianik et al., 2006) and caveolae (Park et al., 2000; Park et al., 1998; Rizzo> et al., 1998b) may regulate intracellular signaling critical for self renewal and survival. Recently, Barbosa et al. reported that ATP was effective at inducing differentiation of hematopoietic cells via activation of Ca^{2+} influx in mice (Barbosa et al., 2011), implicating Ca^{2+} oscillation in the regulation of HSPC quiescence and differentiation. Other studies suggest that Ca^{2+} signaling could be controlled by channels linked to the cytoskeleton or ECM. The increase in intracellular Ca^{2+} is localized to FA complexes associated with β1 integrin, and is detectable milliseconds after mechanical stimulation (Kobayashi and Sokabe, 2010). Ca^{2+} influx can also be altered in response to ECM rigidity. Endothelial cells cultured on stiff substrate show spontaneous Ca^{2+} oscillations of larger amplitude than those cultured on soft substrate (Kobayashi and Sokabe, 2010). Thus, mechanosensitive ion channels can be directly activated by externally applied mechanical stimuli as well as internal cytoskeletal rearrangement. Ca^{2+} influx can also be triggered by stimulation of primary cilia. Nearly all interphase and nondividing cells have a single, nonmotile cilium that protrudes from the cell surface and in some cell types acts as a mechanosensor (Eggenschwiler and Anderson, 2007). In embryonic aortic and cardiac endothelium, these hair-like structures can be

approximately 2 μm in length (Nauli et al., 2008; Poelmann et al., 2008).and serve as low shear sensors. Interestingly, mature hematopoietic cells lack primary cilia (Pazour and Witman, 2003) but, some human lymphoid and myeloid cells have been found to express intraflagellar transport (IFT) 20, an essential molecule in ciliary assembly (Finetti et al., 2009).

Caveolae are specialized vesicular organelles of the plasma membrane that are involved in transmembrane signaling, adhesion, differentiation, endocytosis, and transport of large and small molecules like cholesterol [Anderson, 1998; Okamoto et al., 1998). Caveolae are a type of lipid raft present on most cell types, including endothelial cells, smooth muscle cells, fibroblasts, and adipocytes (Fernandez-Hernando et al., 2010; Rothberg et al., 1992). In immune cells, caveolae appear in cells of the myeloid lineage but not the lymphoid lineage, with the exception of some T cell leukemia cell lines and bovine lymphocytes (Harris et al., 2002a; Harris et al., 2002b). Trafficking of caveolae to the membrane has been found to be induced by laminar shear stress and is dependent both on integrins and the microtubule network. β1 integrin or ILK ablation in mice results in dramatically reduced numbers of caveolae due to impaired transport of caveolin-1-enriched vesicles along microtubules to the plasma membrane (Wickstrom et al., 2010). Recently, Sinha and colleagues showed that flattening of caveolae allow endothelial cells to compensate rapidly for membrane tension changes associated with mechanical stress (Sinha et al., 2011). This instantaneous remodeling of the membrane surface provides the cell with reservoirs of signaling receptors and ligands available for immediate deployment (Freund et al., 2012), as occurs during the NO signaling response to changes in biophysical cues (Feron et al., 1996; Garcia-Cardena et al., 1996; Shaul et al., 1996; Venema et al., 1997). Early work demonstrated in vasculature of the lung that fluid flow and hydrostatic pressure could stimulate protein tyrosine phosphorylation and release of activated eNOS at the luminal endothelial plasma membrane (Rizzo et al., 1998a; Rizzo et al., 1998b). This activity was localized within caveolae and resulted in a Ras/Raf/ERK 1/2 signaling cascade (Rizzo et al., 1998b). In light of strong evidence for regulation of NO signaling by caveolae in the context of shear stress, it will be important to evaluate whether caveolae play a role in modulating NO production within embryonic vasculature of the AGM during HSC specification

CONCLUSIONS

The hematopoietic system is dynamic in adulthood, but perhaps more so during development, in terms of spatial and temporal transitions. Cells migrate, self renew, and differentiate into a complex array of immune cells with very distinct functions and cellular morphologies. Specific developmental history (Dieterlen-Lievre et al., 2006), trophic effects of neighboring tissues (Peeters et al., 2009), and biophysical cues (Adamo et al.,2009; Hoist et al., 2010; North et al., 2009) determine the potential of hematopoietic cells. Indeed, it is a combination of intrinsic and extrinsic factors that must interface to regulate the development of the blood system. The path to producing transplantable HSPCs from pluripotent sources is gaining momentum. Importantly, studies designed to test the capacity of human pluripotent stem cells to support definitive hematopoiesis have demonstrated the value of extensive knowledge of developmental signals critical to achieving this goal (Amabile et al., 2013; Kennedy et al., 2012). Recent research detailing the complexities of the blood system and its biophysical requirements highlight the need for a more complete concept of the niche. Future work aimed at interrogating the roles of elasticity, nanotopography, and hemodynamic force will broaden our understanding of the types of signals, soluble and mechanical, that define the hematopoietic microenvironment and will advance the field toward establishing alternative, high quality sources of hematopoietic cells that can be used for cellular therapies.

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Abbreviations

ADAM	a disintegrin and metalloprotease
AGM	aorta-gonad-mesonephros
CaSR	calcium sensing receptor
CXCR	chemokine C-X-C motif receptor
DAG	diacylglycerol
Dll	Delta-like
ECM	extracellular matrix
FA	focal adhesion
FAK	focal adhesion kinase
Flk-1	Fetal liver kinase-1
FZD	Frizzled
GPCR	G protein-coupled receptor
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cells
HUVEC	human umbilical vein endothelial cells
ILK	integrin linked kinase
IP3	inositol triphosphate
MAPK	mitogen activated protein kinase
NICD	Notch intracellular domain
NOS	nitric oxide synthase
PGE2	prostaglandin E2
PI3K	phosphatidylinositide 3-kinase
PKA	protein kinase A
PKD	Polycystin
PLC	phospholipase C
ROCK	Rho-associated protein kinase
S1P	sphingosine 1-phosphate
S1PR/EDG1	Sphingosine 1-phosphate receptor
SDF1	stromal derived factor 1
TRPV4	Transient receptor potential Vallinoid Type 4

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Highlights

- Mechanical cues in the niche evolve during hematopoietic ontogeny to accommodate changing needs of the blood system
- Vascular forces contribute to several stages of hematopoiesis, including definitive HSC emergence
- Shear stress activates intracellular signaling critical for blood development
- Current methodologies for *in vitro* generation of hematopoietic cells can be improved by a better understanding of regulatory biophysical cues

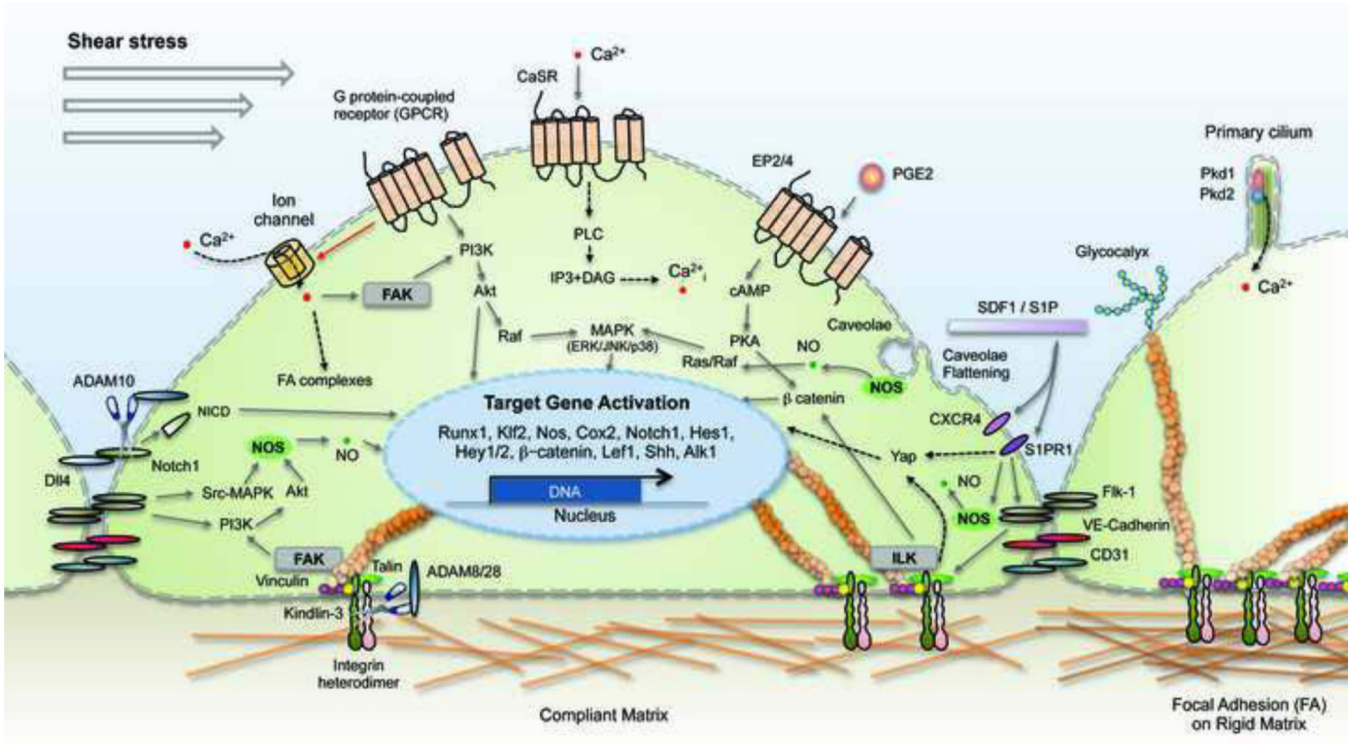

Figure 1. Putative mechanosensors of the hemogenic endothelium

Biomechanical stimuli such as shear stress and substrate rigidity can be sensed by integrins and other cell adhesion molecules, ADAMs, GPCR, ion channels, caveolae, glycocalyx, and primary cilia. Mechanical cues detected by these sensors are converted into biochemical signals that regulate self renewal, differentiation, and homing behaviors. Intracellular signaling mechanisms demonstrated to lie downstream of biophysical cues in hematopoietic or hemogenic endothelial cells are represented by solid lines. Pathways found in other cell types are represented by dashed lines. ADAM, a disintegrin and metalloprotease; CaSR, Calcium Sensing Receptor; DAG, diacylglycerol; FA, focal adhesion; FAK, focal adhesion kinase;Flk-1, fetal liver kinase-1; GPCR, G protein-coupled receptor; ILK, integrin linked kinase; IP3, inositol triphosphate; NICD, Notch intracellular domain; NO, nitric oxide; NOS, nitric oxide synthase; PGE2, prostaglandin E2; Pkd1, polycystin-1; Pkd2, polycystin-2; PKA/ PKC, protein kinase A/ C; PLC, phospholipase C; SIP, sphingosine-1 phosphate; SDF1, stromal derived factor 1.

Table 1

General Mechanosensors and Mechanosensitive Signaling Pathways

Mechanosensors	Functions in hematopoiesis	Interactions	Downstream	References
Cell Adhesion Molecules				
α2β1 Integrin	Homing and homeostasis	Extracellular matrix	p38 MAPK	(Klekotka et al., 2001)
α4β1(VLA-4)	Homeostasis	Fibronectin (CS-1)		(Craddock et al., 1997; Papayannopoulou et al., 1995; Potocnik et al., 2000; Williams et al., 1991)
β1 Integrin	Migration		ILK-GSK3β, Wnt, Notch	(Hirsch et al., 1996; Potocnik et al., 2000; Rallis et al., 2010)
VE-cadherin	Proliferation and maintenance			(Fraser et al., 2002; Oberlin et al., 2010)
N-cadherin	Differentiation			(Hosokawa et al., 2010; Puch et al., 2001)
Flk- 1/KDR/VEGF2	Endothelial and hematoendothelial specification	Cell adhesion Shear stress	Src-MAPK, PI3K-Akt eNOS, integrins, small GTPase	(Jalali et al., 1998; Jin et al., 2003; Tzima et al., 2005)
G-protein coupled receptors (GPCR)				
$G\alpha_{i2}$	Erythropoietin modulation	Fluid shear stress	Ca^{2+} influx, ERK, JNK	(Jo et al., 1997; Miller et al., 1996)
EP2 and EP4 $G\alpha_s$	HSPC self renewal and expansion	PGE2	cAMP, Wnt/β- catenin, intracellular Ca^{2+}, NOS	(Goessling et al., 2009; Reich and Frangos, 1991; Smalt et al., 1997; Williams et al., 1994)
S1PR/EDG1	Chemoattractant for HSCs and progenitors	Fluid shear stress, Flk- 1/KDR/ VEGFR2, protein kinase C	eNOS, Rac GTPase-associated protein CrkII, YAP	(Hughes et al., 2005; Igarashi et al., 2003; Takada et al., 1997; Tanimoto et al., 2002; Venkataraman et al., 2008)
CXCR4	HSC homing and engraftment to the marrow	SDF1-α	Rac1	(del Pozo et al., 2004; Gu et al., 2003; Ratajczak et al., 2010)
CaSR	Cell adhesion and lodgement	Ca2+, Integrins	Integrins	(Adams et al., 2006; Lam et al., 2011; Tharmalingam et al., 2011)
Ion channels				
TRPV4		Membrane stretching, Polycystin-2	Ca^{2+}, NO, endothelial reorientation	(Köttgen et al., 2008; Mendoza et al., 2010;

Mechanosensors	Functions in hematopoiesis	Interactions	Downstream	References
				Thodeti et al., 2009)
PKD1, PKD2		Fluid shear stress	Ca^{2+}, NO, HH signaling	(Abou Alaiwi et al., 2009; Hierck et al., 2008; Nauli et al., 2008)
A Disintegrin				
And Metalloprotease (ADAM) Family				
ADAM28	Lymphocyte emigration	α4β1 integrin	Degradation of ECM	(Weber and Saftig, 2012)
ADAM8	Releasing first erythrocytes into the blood stream	Fluid shear stress	Degradation of ECM	(Fourie et al., 2003; Iida et al., 2010)
ADAM10	HSC specification	Notch, Fluid shear stress	NICD	(Gibb et al., 2010; Gibb et al., 2011; Yoda et al., 2011)
Nitric Oxide (NO) signaling				
NOS	Regulation of vascular tone, angiogenesis, endothelial migration and hematopoiesis	Fluid shear stress, Klf2a, PI3K, Notch, Wnt	NO	(Adamo et al., 2009; Davies, 1995; Lucitti et al., 2007; Michurina et al., 2004; North et al., 2009)
Other sensors				
Glycocalyx	HSPS maintenance, differentiation, homing, mobilization	Fluid shear stress	NO, reorganization of the cytoskeleton	(Florian et al., 2003; Hemmorantaa et al., 2007; Thi et al., 2004)
Caveolae		Integrins, laminar shear stress	Ca^{2+}, NO, Ras/Raf/ ERK1/2	(Drab et al., 2001; Rizzo et al., 1998; Wary et al., 1998)
FAK	Hematopoietic lodgment and lineage development	Fluid shear stress	Akt, eNOS	(Glodek et al., 2007; Koshida et al., 2005)

* Abbreviations: Flk/KDR/VEGF2 (Fetal liver kinase/Kinase insert domain receptor/Vascular endothelial growth factor), S1PR/EDG1 (Sphingosine 1-phosphate receptor), CXCR4 (chemokine C-X-C motif receptor), CaSR (Calcium sensing receptor), TRPV4 (Transient receptor potential Vallinoid Type 4), PKD (Polycystin), NOS (Nitric oxide synthase), FAK (Focal adhesion kinase)