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New Insights Into the Differentiation of Megakaryocytes From Hematopoietic Progenitors

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Abstract—Megakaryocytes are hematopoietic cells, which are responsible for the production of blood platelets. The traditional view of megakaryopoiesis describes the cellular journey from hematopoietic stem cells, through a hierarchical series of progenitor cells, ultimately to a mature megakaryocyte. Once mature, the megakaryocyte then undergoes a terminal maturation process involving multiple rounds of endomitosis and cytoplasmic restructuring to allow platelet formation. However, recent studies have begun to redefine this hierarchy and shed new light on alternative routes by which hematopoietic stem cells are differentiated into megakaryocytes. In particular, the origin of megakaryocytes, including the existence and hierarchy of megakaryocyte progenitors, has been redefined, as new studies are suggesting that hematopoietic stem cells originate as megakaryocyte-primed and can bypass traditional lineage checkpoints. Overall, it is becoming evident that megakaryopoiesis does not only occur as a stepwise process, but is dynamic and adaptive to biological needs. In this review, we will reexamine the canonical dogmas of megakaryopoiesis and provide an updated framework for interpreting the roles of traditional pathways in the context of new megakaryocyte biology.

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Key Words: blood platelets ■ hematopoiesis ■ hematopoietic stem cell ■ megakaryocytes ■ spleen

The role of platelets in both the initiation and propagation of cardiovascular disease has been firmly established over the past 2 decades.[1–3] The importance of platelets in cardiovascular disease pathology is further underscored by the ongoing successful use of antiplatelet agents in reducing myocardial infarction, stroke, and vascular-related death.[4] As such, a large body of work exists examining how platelet function can be modulated in both health (hemostasis) and disease (thrombosis). However, there is surprisingly little known about megakaryocytes, the cells that generate platelets. Megakaryocytes are large (50–100 μm) cells that are quite rare—making up ≈0.05% of the cell population in human bone marrow.[5] After megakaryocytes are terminally differentiated, they undergo a complex maturation process that is required for platelet production. As they mature, megakaryocytes undergo endomitosis, resulting in a polyploid nucleus that is 16N on average, but has been observed up to 128N. In addition, megakaryocytes create an invaginated membrane system that is continuous with the plasma membrane, permeates the cytoplasm, and provides the extra membrane necessary for platelet formation. Once mature, megakaryocytes extend long processes called proplatelets into the blood vessel lumen. This elaborate process is powered by the megakaryocyte cytoskeleton, and more specifically, the sliding and telescoping of microtubules over each other.[6] Once released into the lumen, the shear forces associated with flowing blood help facilitate fragmentation of proplatelets into 1 to 2 μm platelets.[7]

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Megakaryopoiesis is the process by which megakaryocytes are derived from hematopoietic stem cells (HSCs), primarily in the bone marrow, along the myeloid branch of hematopoiesis. HSCs in the bone marrow sit at the top of a hierarchical system and give rise to increasingly restricted progenitors which eventually produce, and continuously replenish, ≈1 trillion mature blood cells per day.[8] During steady-state hematopoiesis, all blood lineages are produced through a series of committed progenitors, the megakaryocyte being derived through the multipotent progenitor (MPP), common myeloid progenitor (CMP), and megakaryocyte-erythroid progenitor (MEP).[9,10] However, recent studies have begun to redefine this hierarchy and shed new light on alternative routes by which HSCs are differentiated into megakaryocytes. In this review, we will focus on new studies that both refine and challenge the field of megakaryopoiesis.

Hematopoiesis and Megakaryopoiesis

Hematopoiesis occurs in a succession of tissues during vertebrate embryonic development, beginning in the yolk sac

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Nonstandard Abbreviations and Acronyms	
CMP	common myeloid progenitor
HSC	hematopoietic stem cell
IGF	insulin-like growth factor
IL	interleukin
LT-HSC	long-term HSC
MEP	megakaryocyte-erythroid progenitor
MPP	multipotent progenitor
TGF	transforming growth factor
TPO	thrombopoietin
VWF	von Willebrand Factor

around embryonic day 7.5 in mice and during the third week of human development.[8,11,12] Following this, HSCs are derived from hemogenic endothelial cells in the aorta-gonad-mesonephros and placenta and migrate to the fetal liver, thymus, spleen, and finally the bone marrow.[8,11] This spatially and temporally varied developmental process gives rise to independent waves of hematopoiesis. Early hematopoiesis in the yolk sac produces blood cells essential for survival, primarily oxygen-carrying erythrocytes, and platelet-producing megakaryocytes.[13] HSCs from the aorta-gonad-mesonephros and placenta are preferentially self-renewing, whereas HSCs in the fetal liver and bone marrow are primed to differentiate into all blood lineages.[14] Bone marrow long-term HSCs (LT-HSCs) are largely quiescent—a state marked by G0 cell cycle and low mitochondrial activity—during steady-state hematopoiesis.[15] However, during times of biological demand such as hemorrhage, HSCs lose quiescence and become mobilized to differentiate. Canonical hematopoiesis occurs through LT-HSCs, short-term HSCs, which are capable of reconstituting all mature lineages but have limited self-renewal capability, and MPPs, a group of nonrenewing lineage-biased progenitor cells (Figure 1). Finally, MPPs differentiate into more committed progenitors such as the common lymphoid progenitor and the CMP, which further branches into the MEP, a shared progenitor between megakaryocytes and erythroblasts, and the granulocyte-macrophage progenitor (Figure 1). These committed progenitors give rise to terminally differentiated mature blood cells.

In recent years, the dogma of hematopoietic hierarchy, which has traditionally been determined by surface markers via flow cytometry, has been challenged. In particular, the origin of megakaryocytes, including the existence and hierarchy of megakaryocyte progenitors, has been redefined. First, the MPP population has been further characterized to include subsets of lineage-biased MPPs.[16,17] Additionally, several groups have shown through various technologies that traditionally defined, self-renewing LT-HSCs are capable of unilaterally differentiating into megakaryocytes.[18–24] Finally, the existence of the MEP population itself has been challenged. While a common progenitor capable of producing erythroblasts and megakaryocytes exists in ex vivo assays, newer technologies have shown that this bipotent progenitor is transient and difficult to detect in native hematopoiesis, and the classification of the MEP may require refinement. Both the canonical pathway and pathways recently discovered to impact megakaryopoiesis are outlined in Figure 1.

Identification of Megakaryocyte-Biased MPPs and HSCs

As platelets are often the first line of defense not only during vascular injury, but also during states of inflammation, infection, and wound healing, it is not surprising that there would be a hematopoietic demand to quickly make megakaryocytes. Thus, rapid differentiation from an HSC to a megakaryocyte is necessary to replenish the platelet pool. More recently, MPPs have been defined as a set of unique multipotent, but lineage-biased, progenitors. Specifically, MPPs exist in multiple states, designated MPP1–4, each having a unique signature and lineage bias.[16] MPP1 is classified as a short-term HSC, MPP3 is largely granulocyte/macrophage biased, and MPP4 is largely lymphoid biased.[17] MPP2 is biased towards the megakaryocyte/erythroid lineage, and it is the only MPP capable of replenishing platelets in a transplant model.[17] This megakaryocyte-biased MPP2 population has more recently been shown to directly differentiate into a megakaryocyte, bypassing the canonical CMP and MEP progenitors.[24] Furthermore, an expanding body of work has uncovered the persistence of a megakaryocyte-biased, self-renewing HSC that, while originally thought to be used under times of hematopoietic stress, is increasingly recognized as a precursor to a large proportion of megakaryocytes during native hematopoiesis.[18–24]

The heterogeneity of HSCs and their self-renewal and repopulating abilities has historically been demonstrated with single-cell HSC transplantation; individual HSCs exhibit varying degrees of quiescence as well as myeloid or lymphoid bias.[25–28] A megakaryocyte-like subset of myeloid-biased HSCs (FLT3 [Fms-related tyrosine kinase] negative), was first recognized due to its increased expression of megakaryocytic transcripts such as the transcription factor GATA1 (GATA-binding protein 1), the TPO (thrombopoietin) receptor Mpl, and the platelet and endothelial aggregation factor VWF (von Willebrand Factor).[29] Further examination of these megakaryocyte-like HSCs defined a subset of HSCs expressing the megakaryocyte-specific integrin CD41.[30] CD41-expressing HSCs have a myeloid-bias, increase with age, and are associated with the megakaryocyte-specific transcription factor, GATA1.[30,31] These observations further led to the question of whether megakaryocyte-like, myeloid-biased HSCs were specifically megakaryocyte and platelet-biased. A seminal study using a VWF-GFP (green fluorescent protein) reporter found that ≈60% of HSCs in the bone marrow are VWF^+ and that these HSCs are strongly biased towards reconstituting megakaryocytes and platelets in a transplant assay.[18] Furthermore, this study revealed that VWF^+ HSCs are hierarchically above VWF^- HSCs, suggesting that HSCs originate as megakaryocyte-primed and loss of megakaryocyte markers is necessary for differentiation into other lineages.[18] Further exploring the heterogeneity of HSCs, Yamamoto et al[19] showed that traditionally defined self-renewing HSCs are capable of differentiating directly into myeloid-biased, megakaryocyte-biased, or megakaryocyte/erythroid-biased HSCs, all of which maintain self-renewal capability. Two studies also identified high c-Kit receptor density on HSCs as a marker self-renewal capability and megakaryocyte bias.[20,21] Complementing these studies, Carrelha et al[22] used sensitive analysis (detection as low as

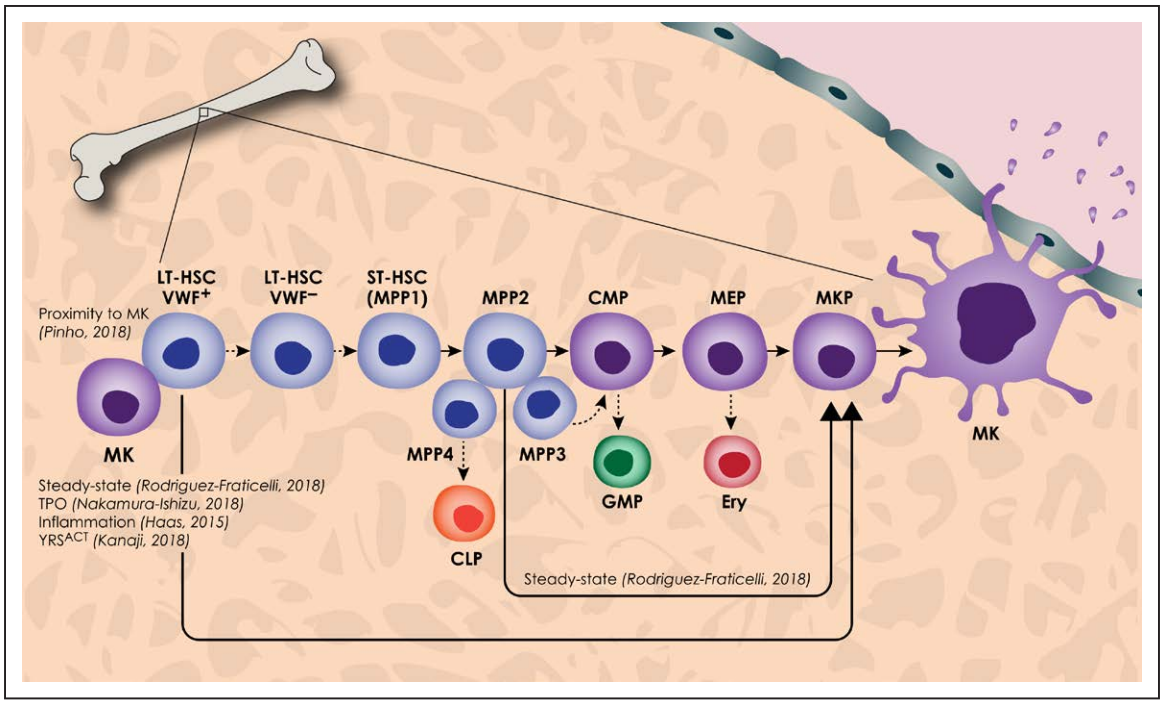

Figure 1. Canonical and new models of hematopoiesis and megakaryopoiesis. Canonical megakaryopoiesis involves differentiation through the long-term hematopoietic stem cell (LT-HSC), short-term HSC (ST-HSC) or multipotent progenitor (MPP)-1, MPP (primarily MPP2), common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), and megakaryocyte progenitor (MKP). The megakaryocyte (MK) then undergoes a maturation process involving an increase in size, increase in DNA content, and extension of proplatelets through sinusoids in the blood vessel into circulation. Also in this canonical hematopoiesis pathway, deemphasized with dashed arrows, VWF$^+$ LT-HSCs differentiate directly into VWF$^-$ LT-HSCs which give rise to all MPPs. MPP4 primarily differentiates into the common lymphoid progenitor (CLP), MPP3 to the granulocyte-macrophage progenitor (GMP), MPP2 to the MEP and erythroblast (Ery). The megakaryocyte-biased pathway is highlighted here, including new findings which propose that VWF$^+$ LT-HSCs are principally biased towards the megakaryocyte lineage through the MPP2 and the MEP. Furthermore, VWF$^+$ LT-HSC and MPP2 populations differentiate directly to MKs, bypassing intermediate progenitors, a phenomenon seen during steady-state, TPO (thrombopoietin) induction, inflammation, and expression of YRS^{ACT}. Additionally, VWF$^+$ LT-HSCs reside in close proximity to, and are influenced by, megakaryocytes in the bone marrow. VWF indicates von Willebrand Factor.

0.01%) of peripheral blood cells after HSC transplantation to define VWF$^+$, megakaryocyte-biased HSCs as unipotent—capable of repopulating platelets but no other blood lineages. Megakaryocyte-biased HSCs have also been observed in human fetal liver, cord blood, and bone marrow cells.[23] Taken together, these data strongly support the existence of a repopulating HSC that is partially or completely biased towards differentiating into megakaryocytes and producing platelets.

One significant caveat to many HSC lineage tracing studies is that they rely on HSC transplant into lethally irradiated mice, which may not fully recapitulate native hematopoiesis. However, a recent article by Rodriguez-Fraticelli et al[24] confirmed the existence of megakaryocyte-biased MPP2 and HSC populations during native hematopoiesis. In this article, megakaryocyte-biased HSCs were observed during native hematopoiesis by tracking inducible random transposon integration, thereby bypassing the need for lethal irradiation and transplant. This study reveals that LT-HSCs and MPP2 populations are both direct sources of megakaryocytes and replenish ≈31% of the native pool. Interestingly, mature megakaryocytes are capable of differentiating directly from the LT-HSC and skipping the MPP2 intermediate. Furthermore, this study did not find megakaryocytes that had a shared lineage tracing with erythroblasts, failing to recognize the existence of an MEP. This study supports the idea that megakaryocyte-biased HSCs are not only strongly biased, but constitute a subpopulation of HSCs primed to directly differentiate into megakaryocytes, bypassing other commitment steps. However, the specific megakaryocyte differentiation pathways taken during normal and pathological hematopoiesis is a question that remains unanswered and will likely be the topic of important research in the coming years.

Redefining the MEP

The new dogma suggesting that megakaryocytes are capable of differentiating directly from a lineage-biased HSC or MPP, and may do so frequently, brings to attention the importance of the traditionally accepted progenitor, the MEP. Rodriguez-Fraticelli et al[24] found no evidence of common lineage between megakaryocytes and erythroblasts using a novel method of interrogating native hematopoiesis. However, flow cytometry, colony-forming unit (CFU) assays, and transplant studies have historically identified progenitors with bipotent megakaryocyte/erythroid potential.[32] Additionally, Yamamoto et al[19] showed in a reporter-based system the existence of repopulating megakaryocyte/erythroid-biased HSCs in addition to megakaryocyte-biased HSCs. These data strongly support the existence of a bipotent progenitor. One possibility for this discrepancy is the inconsistent definition of the MEP. Xavier-Ferrucio et al[32] suggest that the classic flow cytometry gating scheme for mouse MEPs represents a population that is largely erythroid committed (CD34$^-$ Lin$^-$ c-Kit$^+$ Sca$^-$ FcgR$^-$).[9] Thus, phenotyping of the MEP may require further dissection, as a

series of subprogenitors have been defined using the CD150 and CD105 markers—Pre-MK/E ($CD150^+/CD105^-$), Pre-CFU-E ($CD150^+/CD105^+$), and CFU-E ($CD150^-/CD105^+$).[33] A recent study aimed to further refine the MEP gating strategy to include additional markers (lin^- $CD34^+$ $Flt3^-$ $CD45RA^-$ CD38 mid MPL^+ $CD36^-$ $CD41^-$), which reconstitute all 3 megakaryocyte, erythroid, and megakaryocyte/erythroid colonies in a functional assay.[34] As such, it is possible that a progenitor with only megakaryocyte and erythroid potential exists, but may require refined phenotyping, may be too transient to detect in native hematopoiesis such as in the transposon study, or may exist higher up in hematopoiesis as a self-renewing, bipotent HSC.[19,24] Regardless, the origins of embryonic hematopoiesis suggest a common progenitor, as erythrocytes and megakaryocytes are the first terminally differentiated blood cells to be produced during embryonic hematopoiesis. Furthermore, the existence of a common progenitor between the megakaryocyte and erythrocyte is supported by the similar genetic signature and reliance on common transcription factors, such as GATA1.[35,36] It is important to continue to define these various cell types in multiple ways, including more sensitive phenotyping and functional assays, to determine both their fate and potential. Overall, it is becoming evident that hematopoiesis does not only occur as a branched tree hierarchy, but is dynamic and adaptive to biological needs.

Signaling Pathways in Megakaryopoiesis

Megakaryocyte development is regulated at multiple levels by different cytokines, the most critical of which is TPO (Figure 2). TPO, along with its receptor c-mpl, was discovered and cloned in 1994, where it was shown to promote development and maturation of megakaryocytes from their HSC precursors.[37–44] TPO predominately regulates megakaryocyte differentiation from the HSC, and thus all progenitor cells primed to become megakaryocytes, including HSCs, CMPs, and MEPs, express c-mpl.[45] This regulation is the result of a feedback loop; TPO is constitutively produced by the liver and then circulates in plasma, where it is sequestered by circulating platelets in a c-mpl–dependent manner. As such, reduction in platelet counts leads to increased levels of circulating TPO which is free to exert its stimulatory effects on bone marrow HSCs, thus increasing megakaryocyte (and platelet) numbers.[46–48] TPO signaling results in internalization of the c-mpl-TPO receptor-ligand complex and initiation of multiple signal transduction pathways including JAK2 (Janus kinase), STAT3 (signal transducer and activator of transcription)/STAT5, MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinases), and PI3K (phosphoinositide 3-kinase)/AKT (protein kinase B).[49–52] Specifically, TPO induces phosphorylation of JAK2 which phosphorylates downstream targets including activation of the transcription factors STAT3/STAT5.[51] Signaling through these pathways causes downstream activation of megakaryocyte-specific transcription factors and regulation of expression of megakaryocyte-specific genes. Studies in TPO and c-mpl knockout mice show a global decrease in HSCs, with a particularly drastic reduction in megakaryocytes and platelets.[53] However, TPO and c-mpl knockout mice are able to make a small (but sufficient) number of platelets. In addition, humans with a complete loss of functional c-Mpl have a median platelet count of 21×10^9/L or below, suggesting that patients without functional TPO signaling also retain a form of platelet production and that TPO-independent pathways of megakaryopoiesis in mammals exist.[54] Although TPO is a critical regulator of megakaryopoiesis, it does not have an essential role in the final stages of proplatelet formation and release.

TPO has also recently been shown to regulate HSC quiescence and mobilization by stimulation of HSC entry into the cell cycle.[55,56] Considering the newly recognized megakaryocyte-primed HSCs, recent work has sought to reconcile the effect of TPO on HSCs and megakaryocyte bias. Nakamura-Ishizu et al[57] found that TPO stimulation biases HSCs towards megakaryocytes in vivo and may cause differentiation directly to megakaryocytes while bypassing other progenitors. This report found that HSCs, which are normally quiescent and have low mitochondrial activity and number, are mobilized to differentiate into megakaryocytes after TPO stimulation. This process was marked by increased mitochondrial activity, increased cycling, decreased proapoptotic gene expression, and increased expression of the cell surface marker CD9. This gives a possible mechanism explaining HSC mobilization and megakaryocyte-biased differentiation through TPO signaling.

Alternative Signaling Effectors of Megakaryopoiesis

Although residual platelet production persists in the absence of TPO signaling, TPO-independent regulators of megakaryopoiesis remain elusive. However, some alternative pathways have been shown to enhance this process. In fact, the IL (interleukin) family was found to influence megakaryopoiesis before the discovery of TPO; in early studies, injections of IL-1β and IL-6 increased platelet counts in vivo.[58,59] However, these cytokines were ultimately found to be associated with increased plasma TPO, suggesting that ILs upregulate TPO production, rather than directly stimulating megakaryopoiesis. IL-3 also increases megakaryocyte colony size and numbers in vitro, but does not affect megakaryocyte maturation.[60,61] IL-1α is perhaps the only known IL to have a possible TPO-independent phenotype, as it acts on mature megakaryocytes to cause rupture, and thus shedding of proplatelets into the bone marrow.[62] More recently, other cytokines and chemokines not in the IL family have been identified as megakaryocyte-promoting factors. IGF (insulin-like growth factor)-1 promotes $CD34^+$ differentiation towards the megakaryocyte lineage, a process mediated through AKT signaling with the assistance of steroid receptor coactivator 3.[63] In vivo administration of IGF-1 increases platelet counts in both lethally irradiated mice and c-mpl knockout mice, suggesting a TPO-independent phenotype.[63] Another chemokine, CCL5 (C-C motif chemokine ligand 5; RANTES [small inducible cytokine A5]), has also recently been shown to increase megakaryocyte ploidy, proplatelet production, and suppress apoptosis in vitro.[64] Seeing as these cytokines are often upregulated during inflammation, it remains to be seen if they also regulate megakaryopoiesis during normal hematopoiesis and in a TPO-independent manner. Additionally, in the context of recent studies, it is possible that these inflammatory cytokines also bias HSCs towards the megakaryocytic lineage.

Recent studies have identified the Notch pathway as a mediator of megakaryopoiesis. Notch signaling is required for murine megakaryocyte commitment from the HSC and

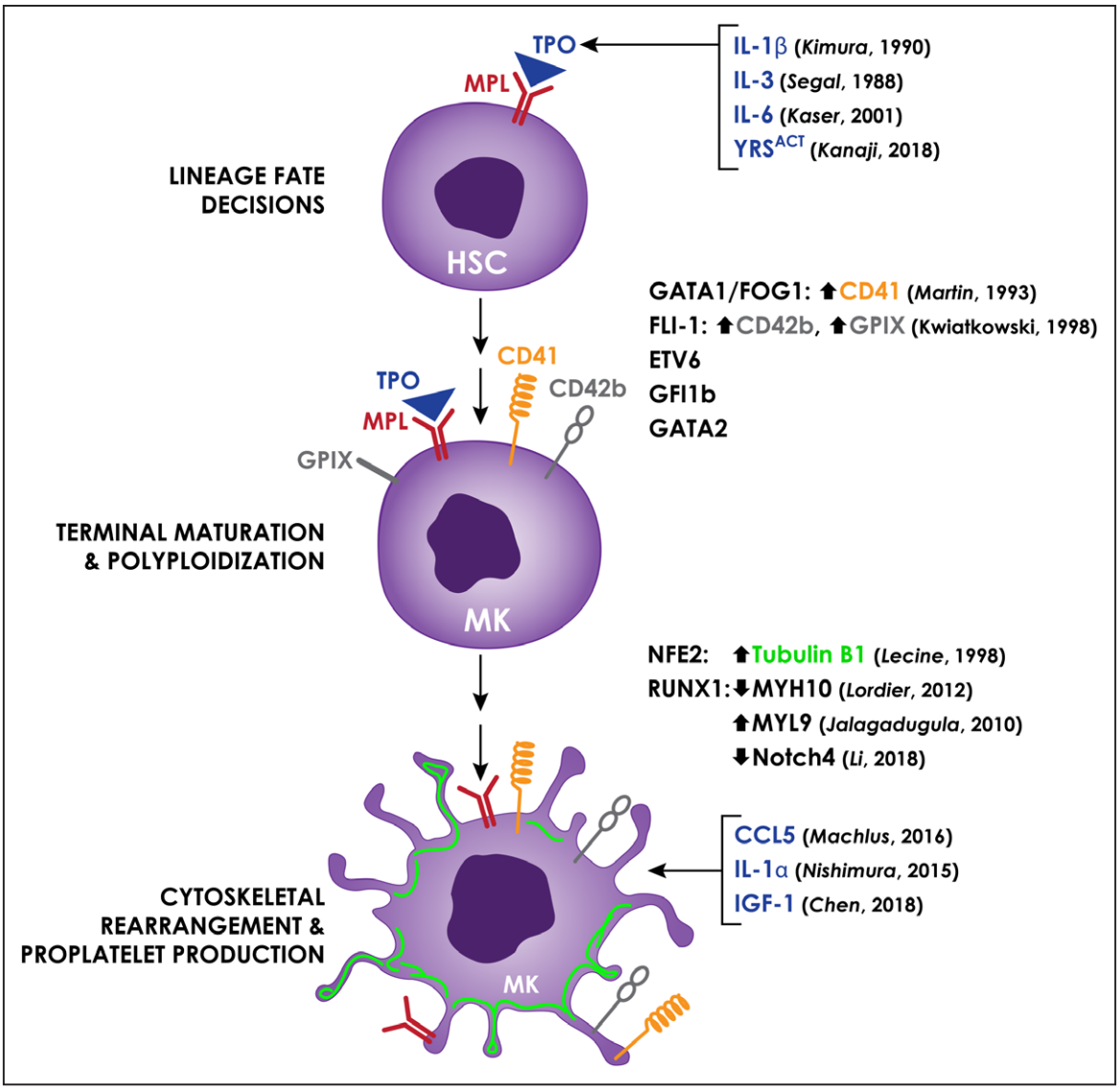

Figure 2. Key cytokines and transcription factors involved in the regulation of megakaryocyte (MK) maturation. Megakaryopoiesis is regulated at multiple stages via cytokines (blue). In the classical pathway, megakaryopoiesis begins with thrombopoietin signaling via its receptor c-mpl (red), which is expressed throughout every differentiation stage; from the hematopoietic stem cell (HSC) through to platelets produced from the mature MK. Various lineage fate decisions lead to the upregulation of transcription factors and consequent surface receptor expression, which are currently used to define a committed, mature MK. Expression of GATA1 (GATA-binding protein 1)/FOG1 and FLI1 lead to expression of CD41 (orange), CD42b (gray), and GPIX (gray), respectively. The expression of RUNX1 and NFE2 (nuclear factor, erythroid 2) are crucial in terminal maturation processes, including polyploidization (upregulation of MYH10 [myosin heavy chain], downregulation of MYH10 and Notch4) and cytoskeletal rearrangement (expression of tubulin B1 [green]). In addition to TPO (thrombopoietin), other cytokines have been found to affect megakaryopoiesis, both in a TPO-dependent (IL [interleukin]-1β, IL-3, IL-6, YRS^{ACT}) and independent fashion (IL-1α, CCL5 [C-C motif chemokine ligand 5], IGF [insulin-like growth factor]-1). ETV indicates ETS variant 6; FLI1, Fli-1 proto-oncogene, ETS transcription factor; FOG1, zinc finger protein, FOG family member 1; GFI1b, growth factor–independent 1B transcriptional repressor; GPIX, glycoprotein IX; MYL9, myosin light chain 9; and RUNX1, runt-related transcription factor 1.

increases CD41$^+$ cells, CMPs, and MEPs, potentially via AKT signaling and consequent suppression of FOXO (forkhead box O).[65,66] However, in human CD34$^+$ cells, Notch activation inhibits terminal megakaryocyte maturation.[67] Furthermore, characterization of mouse MEPs revealed that Notch signaling through recombinant DLL (delta-like canonical notch ligand) skews towards the erythroid lineage and away from the megakaryocyte lineage.[68] Li et al[69] demonstrated that downregulation of receptor Notch4 by RUNX1 (runt-related transcription factor) is required for normal megakaryocyte development in human cells. Specifically, deletion of the RUNX1-binding site in intron 29 of *NOTCH4* causes an increase in Notch4 expression and inhibition of megakaryocyte differentiation. Pharmacological inhibition of Notch signaling enhances human megakaryopoiesis in vitro.[69] Overall, megakaryopoiesis appears to be regulated by Notch signaling at different stages of development, potentially by requiring Notch signaling for development of earlier progenitors, but downregulating Notch for terminal megakaryocyte commitment.

Signaling Affecting HSC Bias

TPO signaling not only directs differentiation of HSCs along the megakaryocyte lineage through committed progenitors, but also skews HSCs towards a direct megakaryocyte bias,

bypassing other progenitors.[22,24] Seeing as the discovery of megakaryocyte-biased HSCs is relatively new, the TPO-independent effectors of HSC bias directly towards megakaryocytes are not known. However, a recent study identified a tyrosyl-tRNA synthetase variant (YRS^{ACT}), an activated version of an enzyme responsible for protein synthesis, as a potential TPO-independent regulator of megakaryocyte differentiation.[70] YRS^{ACT} administration increases platelet counts in acute models of thrombocytopenia as well as in mice deficient in TPO signaling. The TPO-independent effect was attributed to skewing of HSCs towards megakaryopoiesis, as was determined by an increased population of megakaryocytes positive for the Sca cell surface marker, a marker for HSCs. While a direct effect of YRS^{ACT} on HSC bias remains to be seen, these data indicate that TPO-independent mechanisms of megakaryocyte-biased HSC skewing may exist.

Transcription Factors in Megakaryopoiesis

Hematopoietic cell fate and megakaryocyte differentiation are largely coordinated by the temporal expression of various transcription factors. Just as HSCs and megakaryocytes share similar cell surface receptors, they also have similar expression of transcription factors. Transcription factors are involved in fetal HSC specification, adult HSC maintenance and renewal, and megakaryocyte differentiation. For example, SCL (TAL BHLH transcription factor 1, erythroid differentiation factor) and RUNX, 2 transcription factors necessary for megakaryocyte differentiation, are also essential for fetal HSC determination, but are dispensable for adult HSC maintenance.[71,72] Likewise, GATA2 is required for HSC maintenance as well as further downstream during early megakaryopoiesis.[73,74] A conditional knockout mouse of ETV6 (ETS variant), an ETS (ETS proto-oncogene, transcription factor) family transcription factor, also has impaired HSC viability and megakaryocyte differentiation.[75]

Transcription factors regulate differentiation along each lineage commitment decision point from HSC to megakaryocyte. The first step of myeloid commitment, the CMP juncture, requires a balance of expression of antagonistic transcription factors GATA1 and PU.1 (Spi-1 proto-oncogene), which influence hematopoietic skewing towards the MEP and granulocyte-macrophage progenitor, respectively.[76–78] MEP commitment to the megakaryocyte or erythrocyte is coordinated by expression of various transcription factors that must be expressed in a time- and dose-dependent manner. Of these, NFE2 (nuclear factor, erythroid 2), SCL, GFI1B (growth factor–independent transcriptional repressor), GATA1, GATA2, KLF1 (Kruppel-like factor), and ETV6 are all expressed in the MEP, whereas transcription factors EKLF (erythroid Krüppel-like factor) and c-Myb are exclusively expressed in the erythrocyte lineage and FLI1 and RUNX1 are exclusively expressed in the megakaryocyte lineage[79–82] (Figure 2). GATA2 and GATA1, along with its cofactor FOG1, are expressed in an antagonistic manner in the MEP; GATA2 promotes megakaryopoiesis at the expense of erythropoiesis, whereas GATA1 promotes erythropoiesis.[83–86] However, these relationships are not exclusive; GATA1 is largely studied for its requirement in erythropoiesis and activation of globin gene expression but is also essential for megakaryopoiesis and transcription of the gene encoding megakaryocyte-specific receptor glycoprotein IIb.[84,87,88] Also functioning in the MEP to skew towards megakaryocyte fate, RUNX1 actively represses the erythroid-specific transcription factor KLF1, thus allowing a shift in the ratio of increased megakaryocyte-specific FLI1 to KLF1 and promoting megakaryocyte differentiation.[80,82] During later stages of megakaryopoiesis, RUNX1 directly represses and activates the expression of myosins MYH10 (myosin heavy chain) and MYL9 (myosin light chain), respectively, the regulated expression of which are required for megakaryocyte maturation.[89,90] Furthermore, megakaryocyte transcription factor FLI1 (which is negatively regulated by ETV6) regulates expression of megakaryocyte receptors GPIX and GPIbα (glycoprotein Ib platelet subunit alpha; Figure 2).[91,92] Finally, the transcription factor NFE2 is required during the terminal stage of megakaryocyte maturation, proplatelet production. Mice lacking NFE2 have normal proliferation of megakaryocytes, but defective platelet production (Figure 2).[93,94] This is largely due to transcriptional regulation of the megakaryocyte-specific microtubule component β1-tubulin by NFE2.[95,96] Several of these transcription factors, including GATA1, RUNX1, FLI1, GFI1b, and ETV6, are mutated in inherited thrombocytopenias.[35,97–105] In fact, most of these genes were originally discovered by sequencing individuals with congenital thrombocytopenia, underscoring their importance in maintaining megakaryocyte and platelet number.

Canonical transcription factor regulation of megakaryopoiesis has largely been explained by the previously mentioned transcription factors; however, new and more accurate, high-throughput technologies have revealed that these transcription factors are not the sole actors. Genome-wide ChIP-sequencing of GATA1, GATA2, SCL, FLI1, and RUNX1-targeted promoters in human megakaryocytes found that these factors bind promoters simultaneously in multiple combinations.[106] Further complicating this process, many of these transcription factors act as both transcriptional activators or repressors, depending on their association with other factors.[91,107] Thus, it is difficult to predict the effect of upregulating or downregulating a single transcription factor. While many of the essential transcription factors are well known, recent work suggests that the list of transcription factors involved in megakaryopoiesis is much longer than previously thought. A recent large-scale screen of CRISPR/Cas9 in megakaryocytes uncovered several unrecognized transcription factors that influence megakaryopoiesis, including those downstream of the Notch pathway (HES7 [Hes family BHLH transcription factor]), in the NFAT (nuclear factor of activated T cells) family of transcription factors, as well as histone deacetylases.[108] Of particular interest, the prospect that histone modifiers affect megakaryopoiesis suggests a layer of epigenetic regulation, further complicating the process of transcription factor accessibility.

Megakaryocyte-biased HSCs, as defined by high CD41 expression, high c-Kit expression, or VWF expression, are also enriched for megakaryocytic transcription factors such as GATA1, GFI1b, GATA2, and FLI1, suggesting that HSC megakaryocyte bias may occur at the transcriptional level before differentiation.[18,20,30] This is further supported by clonal analysis and single-cell gene expression of megakaryocyte-primed HSCs, which have a similar transcriptional signature

to terminally differentiated megakaryocytes.[24] Interestingly, genome-wide chromatin mapping in hematopoietic cells found that megakaryocytes and HSCs have strongly overlapping active chromatin signatures.[109] These shared open chromatin sites also correspond to megakaryocytic transcription factor binding sites, an overlap that is not seen between megakaryocytes and erythroid progenitors, suggesting that epigenetics controls unilateral differentiation from HSC to megakaryocyte.[109] These new data provide an updated framework in which to view transcriptional control regulating canonical hematopoiesis. In addition, it provides intriguing new ideas supporting the existence of megakaryocyte-biased HSC populations. In the future, it will be interesting to see if researchers will look to transcription factors or epigenetics to help further define the biases inherent in HSC subpopulations.

Interactions Between HSCs, Megakaryocytes, and Bone Marrow Cells

The bone marrow microenvironment regulates HSC number and quiescence, but the exact mechanism is controversial. The interaction between HSCs and the bone marrow was originally thought to be mediated by expression of and signaling through HSC receptors such as Tie2 and c-mpl, the ligands of which (angiopoietin and TPO, respectively) are produced by osteoblasts.[56,110,111] However, subsequent studies found that HSCs are often in close proximity to perivascular cells adjacent to sinusoids in the bone marrow.[112] This relationship was attributed to production of the chemokine CXCL12/SDF1 (C-X-C motif chemokine ligand 12) by perivascular cells, signaling through which regulates HSC quiescence and maintenance.[113] Of note, megakaryocytes are also primarily localized to vascular sinusoids, as it is necessary for proplatelets to extend and release into the vascular space, a process guided by a sphingosine 1-phosphate gradient from the blood.[114] This interaction between the vascular sinusoids and megakaryocytes is also mediated by the chemokine CXCL12/SDF1 and interaction with the CXCR4 (C-X-C motif chemokine receptor) receptor as well as an interaction between podoplanin on perivascular cells and CLEC-2 (C-type lectin domain family member-2) on megakaryocytes.[115–118] Additionally, megakaryocyte maturation and platelet production are accelerated by IL-9, a cytokine produced by bone marrow osteoblasts.[119] More widely and recently studied, however, is the fact that shared interactions with perivascular cells position HSCs and megakaryocytes in close proximity to each other. Whole-mount imaging of bone marrow revealed a physical association of HSCs with megakaryocytes.[120] This physical interaction is thought to be functional due to the fact that megakaryocyte depletion results in proliferation of HSCs and an associated loss in quiescence, an interaction largely attributed to megakaryocyte release of CXCL4 (PF4 [platelet factor 4]).[120] Additional studies have found that megakaryocyte-derived TPO and TGF (transforming growth factor)-β also mediate HSC quiescence.[120–122] In addition to direct release of proteins, recent attention has also turned to the release of microparticles within the bone marrow space as a method of cell-to-cell communication. In fact, Jiang et al[123] revealed that megakaryocyte-derived microparticles bind to HSCs and induce them to differentiate into megakaryocytes, providing a possible mechanism of direct communication between the megakaryocyte and the HSC.

Although evidence suggests that megakaryocytes are essential for HSC maintenance, in vivo imaging has often found that HSC distribution throughout the bone marrow is random, and cell depletion studies showing these effects are not representative of native hematopoiesis.[124] Recent work has sought to parse these data by examining HSC and megakaryocyte niches in the context of new studies showing the heterogeneity of HSCs. In particular, a study by Pinho et al[125] examined significant niche association in the native bone marrow of the 2 subsets of VWF^+ and VWF^- HSCs. This study found that almost all VWF^+ (megakaryocyte-biased) HSCs exist within 5 μm of megakaryocytes, but are not significantly associated with arterioles. Of note, the converse was true for VWF^- HSCs, indicating that spatial positioning of HSCs in relation to megakaryocytes and arterioles may contribute to or be associated with HSC heterogeneity. This study builds on previous findings that megakaryocyte depletion, resulting in loss of PF4, causes expansion of the HSC population, by adding that this expansion is largely specific to VWF^+ HSCs.[120,125] Similarly, PF4 injection selectively reduces the number of VWF^+ HSCs. This study also sought to uncover the role of periarteriolar cells on lineage-biased HSCs by depleting $NG2^+$ periarteriolar cells and tracking the independent effects on VWF^+ and VWF^- HSCs. Periarteriolar cell depletion selectively reduces the number and alters the localization of VWF^- cells, but does not have an effect on VWF^+ cells.[125] These data begin to unify the controversial literature about bone marrow niches and the relationship between HSCs and megakaryocytes by viewing HSCs as a heterogeneous population. Nonetheless, it is well-supported that the bone marrow microenvironment provides a space for cell-cell communication that is essential for functional hematopoiesis and megakaryopoiesis. Interestingly, a recent study revealed that the vascular architecture of the bone marrow is more complex than previously thought[126]; a network of transcellular capillaries traverses perpendicularly through long bones, providing even more vascular area for HSC and megakaryocyte interaction. Thus, as technologies to map and define the bone marrow advance, it is likely that the relationships between HSCs, megakaryocytes, and their cellular neighbors will evolve as well.

Bone Marrow Structure/3-Dimensional Contacts

Although the exact niche distribution of megakaryocytes and HSCs is not completely defined, the 3-dimensional (3D) architecture of the extracellular space in the bone marrow does affect megakaryocyte differentiation and maturation. The bone marrow extracellular environment is composed of fibrillar matrix proteins and glycosaminoglycans which create a low stiffness, elastic, 3D matrix.[127] This environment plays an important role in HSC maintenance and differentiation and is also essential for megakaryocyte maturation and platelet production.[128,129] In general, a less stiff matrix favors platelet production, while increased stiffness favors HSC and megakaryocyte maturation at the expense of proplatelet formation.[130] Recently, megakaryocytes were found to contribute

to the bone marrow microenvironment, in that they can create their own niche that includes fibronectin, type IV collagen, and laminin.[131] Megakaryocytes synthesize these extracellular matrix components through TPO and TGF-β signaling.[132] In addition, megakaryocytes synthesize the lysyl oxidase LOX1 (lysyl oxidase), which is largely responsible for cross-linking collagen I in the bone marrow.[133] LOX1 is expressed in immature megakaryocytes and downregulated as megakaryocytes mature, perhaps speaking to the different stiffness requirements necessary for megakaryocyte maturation versus proplatelet formation.[134] Indeed, this may be one mechanism by which megakaryocytes can alter their microenvironment as they mature, thereby inhibiting the production of proplatelets until the necessary maturation state is achieved. Another important component of the bone marrow extracellular matrix is a diverse number of glycosaminoglycans such as hyaluronic acid, which is depolymerized by the megakaryocyte to modulate its own maturation.[135] A knockout mouse of hyaluronidase-2, the depolymerizing enzyme of hyaluronic acid in megakaryocytes, causes megakaryocytes with a malformed demarcation membrane system and thrombocytopenia.[135]

All of these interactions depend on cellular receptors and subsequent signaling in the megakaryocyte, which are not only involved in creating and degrading the extracellular matrix, but are also essential for sensing the extracellular matrix stiffness. Megakaryocyte integrins such as α2β1 as well as a family of mechanosensing ion channels, Piezo, are essential for environment sensing and proplatelet production.[136–138] These data exemplify the multidimensional relationship between megakaryocytes and the bone marrow, including a reciprocal relationship with both the extracellular environment and the residing HSCs. While researchers have often overlooked the impact of the microenvironment by using cells in culture, recent work has begun to address the importance of the 3D microenvironment and the contribution of its tensegrity, stiffness, and molecular components on megakaryopoiesis. Investigators have replicated the stiffness and various components of the bone marrow in vitro by culturing megakaryocytes in hydrogels, scaffolds, and 3D silk matrices, all of which increase megakaryocyte ploidy and proplatelet production in vitro.[139–142] The success of these culture systems underscores the importance of the continued use of 3D culture systems in the study of megakaryocyte maturation in the future.

New Concepts in Megakaryocyte Biology

Emergency Megakaryopoiesis During Inflammatory Stress

In recent years, several landmark studies have revealed novel insights into the regulation of megakaryopoiesis under stress conditions. It has been well-documented that systemic inflammation can lead to acute thrombocytopenia or thrombocytosis.[143–145] It is therefore not surprising that mechanisms are in place to rapidly respond to inflammation and increase platelet counts. These mechanisms may lay dormant during homeostasis when TPO signaling dominates decisions about megakaryocyte production. Research into the situations in which emergency hematopoiesis is used have described novel pathways towards megakaryopoiesis and focused on HSC bias towards the megakaryocyte lineage. Such new insights have significant implications for the plasticity of the bone marrow to respond to stress and the particular importance of megakaryopoiesis.

Inflammatory signaling accentuates HSC bias towards the megakaryocytic lineage. It was originally observed that the megakaryocyte-biased MPP2 is preferred in regenerative states after bone marrow transplant.[17] Furthermore, in studies conducted by Haas et al,[146] mimicking viral, bacterial, and general acute infection upregulates expression of megakaryocyte proteins from preexisting dormant transcripts. Specifically, megakaryocyte proteins were upregulated when mice were injected with type-1 interferon and lipopolysaccharides, which occurred via signaling through STAT1/mTOR (mechanistic target of rapamycin kinase) and TLR4 (toll-like receptor)/MYD88 (myeloid differentiation primary response gene)/TRIF, respectively. Thrombocytopenia induced by other means, such as platelet-depleting antibodies, do not trigger the same increase in megakaryocyte protein expression. In conjunction with the known effects of other inflammatory cytokines on megakaryopoiesis, these findings may help to explain the thrombocytosis that is present in inflammatory states.

New Insights Into Cell Cycle Regulation of Megakaryopoiesis

Cell cycle regulation and cell fate decisions during hematopoiesis are an area of growing interest. Cell cycle activity is associated with loss of HSC quiescence, pushing progenitors out of dormancy and thus disrupting their self-renewal ability.[147] As previously mentioned, TPO signaling is a potent stimulator of HSC entry into the cell cycle and megakaryocyte bias.[57] Interestingly, the cell cycle is also involved in cell fate determination of nonquiescent, committed progenitor cells. Work from Lu et al[148] shows that differences in cell cycle speed in the MEP may contribute to fate decisions between erythroid and megakaryocyte lineages. This work reveals that MEPs with increased cell cycle speed are pushed towards the erythrocyte lineage, whereas those with decreased cell cycle speed skew towards megakaryocyte specification.[148] Furthermore, the differentiated megakaryocyte undergoes multiple rounds of endomitosis as a part of maturation, a process requiring both coordination and restriction of cell cycle components.[149–153] Ultimately, tight regulation of the cell cycle is required for all stages of megakaryopoiesis, but the overlap of these processes during endomitosis and lineage commitment, as well the extent of this regulation in HSC commitment is just beginning to be appreciated and understood.

Alternative Functions of Megakaryocytes

Megakaryocytes have roles in hematopoiesis that are independent of their function in platelet production, including the ability to behave as immune and inflammatory cells. Megakaryocytes express several surface markers related to immune function, including members of the TLR family (TLR1, 2, 3, 4, and 6), FcγRIIA (humans only), and CD40L (the ligand of CD40 on immune cells).[154,155] Mature megakaryocytes also present antigens via expression of MHC I (major

histocompatibility complex), which is transferred to platelets, thus increasing their ability to cross-present antigens.[156,157] Megakaryocytes also contribute to inflammation by packaging cytokines and chemokines into their α-granules and microparticles, which when released contribute to pathogenesis of proinflammatory conditions such as systemic lupus and inflammatory arthritis.[123,158–160] Another way in which megakaryocytes contribute to immunity is through emperipolesis, a process by which other bone marrow cells, particularly neutrophils, are internalized by mature megakaryocytes while staying morphologically intact.[161] Although the functional significance of this is still unclear, it has been hypothesized that emperipolesis allows the exchange of membrane and other cellular material between the cells. In addition to the role of megakaryocytes in the bone marrow, extramedullary megakaryocytes reside in the lung and may be positioned to allow them to function more effectively as immune effector cells.[162] Combined with recent knowledge that inflammatory and infectious stimuli trigger megakaryocyte-primed HSCs to fast-track differentiation, these data suggest a relationship between cellular phenotype of megakaryocytes and response to inflammation and infection.

Concluding Remarks

The changing dogma of megakaryopoiesis, specifically the differentiation of megakaryocytes directly from biased HSCs, opens new opportunities to investigate megakaryopoiesis more carefully. First, the molecular processes involved in megakaryocyte-biased HSC differentiation have only been superficially investigated. It has been proposed that canonical signaling molecules and transcription factors are involved in this process, but further work is needed to tease apart the triggers of HSC commitment towards a direct megakaryocyte lineage. Furthermore, megakaryocytes should be studied not solely as platelet-producing cells, but also as cells involved in maintaining the bone marrow niche and cell-to-cell communication. It is necessary to determine if megakaryocytes themselves exist as a heterogeneous population with varied roles. Finally, megakaryocytes and megakaryopoiesis have been shown to be dynamic in the setting of inflammation and disease, suggesting that these processes are sensitive to their environment. The changes in megakaryocytes and platelets during disease may exacerbate inflammation by changing the bone marrow environment as well as the molecular signature of platelets. Understanding these changes may help to diagnose and treat inflammatory diseases more effectively. The biological drive for direct differentiation from HSC to megakaryocyte is intuitive due to the body's need to replenish platelet pools during blood loss. However, it is also possible that, given the diverse roles of megakaryocytes, direct differentiation from the HSC is also essential for bone marrow maintenance and immune and inflammatory responses. To this extent, the question remains whether the route of differentiation (canonical or directly from the HSC) results in molecularly and functionally different megakaryocytes poised for different roles. Overall, this is an exciting time in the field of megakaryopoiesis; the changes in dogma are not simply rewriting what is known, but are also helping to clarify previously controversial topics and opening opportunities for new investigation.

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Disclosures

None.

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Highlights

- New advances in interrogating hematopoiesis have revealed the presence of a megakaryocyte-biased hematopoietic stem cell which may differentiate directly into a megakaryocyte.
- Megakaryocyte-biased hematopoietic stem cells express megakaryocyte-specific proteins such as VWF (von Willebrand Factor) and CD41, can be triggered by extracellular signals such as TPO (thrombopoietin), and reside in close proximity to megakaryocytes in the bone marrow.
- Megakaryocyte-biased hematopoietic stem cell differentiation may occur under steady-state conditions, but is also upregulated during times of stress such as inflammation.