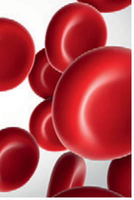

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MEGAKARYOPOIESIS AND PLATELET PRODUCTION

Fetal vs adult megakaryopoiesis

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Fetal and neonatal megakaryocyte progenitors are hyperproliferative compared with adult progenitors and generate a large number of small, low-ploidy megakaryocytes. Historically, these developmental differences have been interpreted as "immaturity." However, more recent studies have demonstrated that the small, low-ploidy fetal and neonatal megakaryocytes have all the characteristics of adult polyploid megakaryocytes, including the presence of granules, a well-developed demarcation membrane system, and proplatelet formation. Thus, rather than immaturity, the features of fetal and neonatal megakaryopoiesis reflect a developmentally unique uncoupling of proliferation, polyploidization, and cytoplasmic maturation, which allows fetuses and neonates to populate their rapidly expanding bone marrow and blood volume. At the molecular level, the features of fetal and neonatal megakaryopoiesis are the result of a complex interplay of developmentally regulated pathways and environmental signals from the different hematopoietic niches. Over the past few years, studies have challenged traditional paradigms about the origin of the megakaryocyte lineage in both fetal and adult life, and the application of single-cell RNA sequencing has led to a better characterization of embryonic, fetal, and adult megakaryocytes. In particular, a growing body of data suggests that at all stages of development, the various functions of megakaryocytes are not fulfilled by the megakaryocyte population as a whole, but rather by distinct megakaryocyte subpopulations with dedicated roles. Finally, recent studies have provided novel insights into the mechanisms underlying developmental disorders of megakaryopoiesis, which either uniquely affect fetuses and neonates or have different clinical presentations in neonatal compared with adult life.

Hematopoietic sites during development

In human and murine development, the first hematopoietic cells emerge in the blood islands of the yolk sac, at embryonic day (E)7.5 in mice and at 23 weeks postconception (WPC) in humans.[1] In this initial wave, primitive erythroid progenitors are found alongside megakaryocyte (MK) progenitors and MK/erythroid progenitors.[2] At E8.5 in the mouse, a second wave of hematopoiesis emerges in the vascular plexus of the yolk sac, in a process known as endothelial to hematopoietic transition, and generates erythromyeloid progenitors. These progenitors seed the liver, where they proliferate and differentiate, but they lack hematopoietic stem cell (HSC) activity.[1] A third and definitive wave of hematopoiesis starts at ~E10.5 in mice, when HSCs are first detected in the dorsal aorta and in the extraembryonic vitelline and umbilical arteries.[3-6] Human HSCs first emerge in the aorta at 33 to 41 days after conception[7] and seed the liver at 7 to 8 weeks' gestation.[8]

MKs are first found in the human yolk sac at 4 WPC,[9] and platelets are found in circulation at 8 to 9 weeks.[10] The migration of HSCs from embryonic hematopoietic sites to the fetal liver (FL) leads to the liver rapidly becoming the main fetal hematopoietic organ.[11] MKs at all maturational stages are found in the liver, although they differ from adult MKs by their significantly smaller size.[12-14] The final transition from hepatic to bone marrow (BM) hematopoiesis starts at 11 to 12 WPC[15,16] and progresses throughout gestation, with the contribution of the liver slowly decreasing. Between 16 and 40 WPC, the percentage of hematopoietic cells in the liver decreases from 50-70% to 25-30%,[17] and by 20 weeks, the BM is the main site of human hematopoiesis.[14,18-20] Thus, even in the most extremely preterm neonates, the BM is the primary hematopoietic site at birth, although hepatic hematopoiesis continues. The exact age at which the liver stops contributing to hematopoiesis is unknown. In mice, in contrast, the main hematopoietic site at birth is the liver, but hematopoiesis transitions to the BM over the first 10 to 14 days of postnatal life.[21] These serial transitions over the course of development allow for different microenvironmental signals to simultaneously promote the expansion of undifferentiated HSCs and the production of the large numbers of differentiated blood cells needed to populate the BM and blood of a rapidly growing fetus.[22]

Human embryonic, fetal, and adult hematopoiesis and megakaryopoiesis

Recent studies have questioned the traditional model of hematopoiesis and have unveiled developmental differences between human fetuses and adults. Until recently, hematopoiesis was

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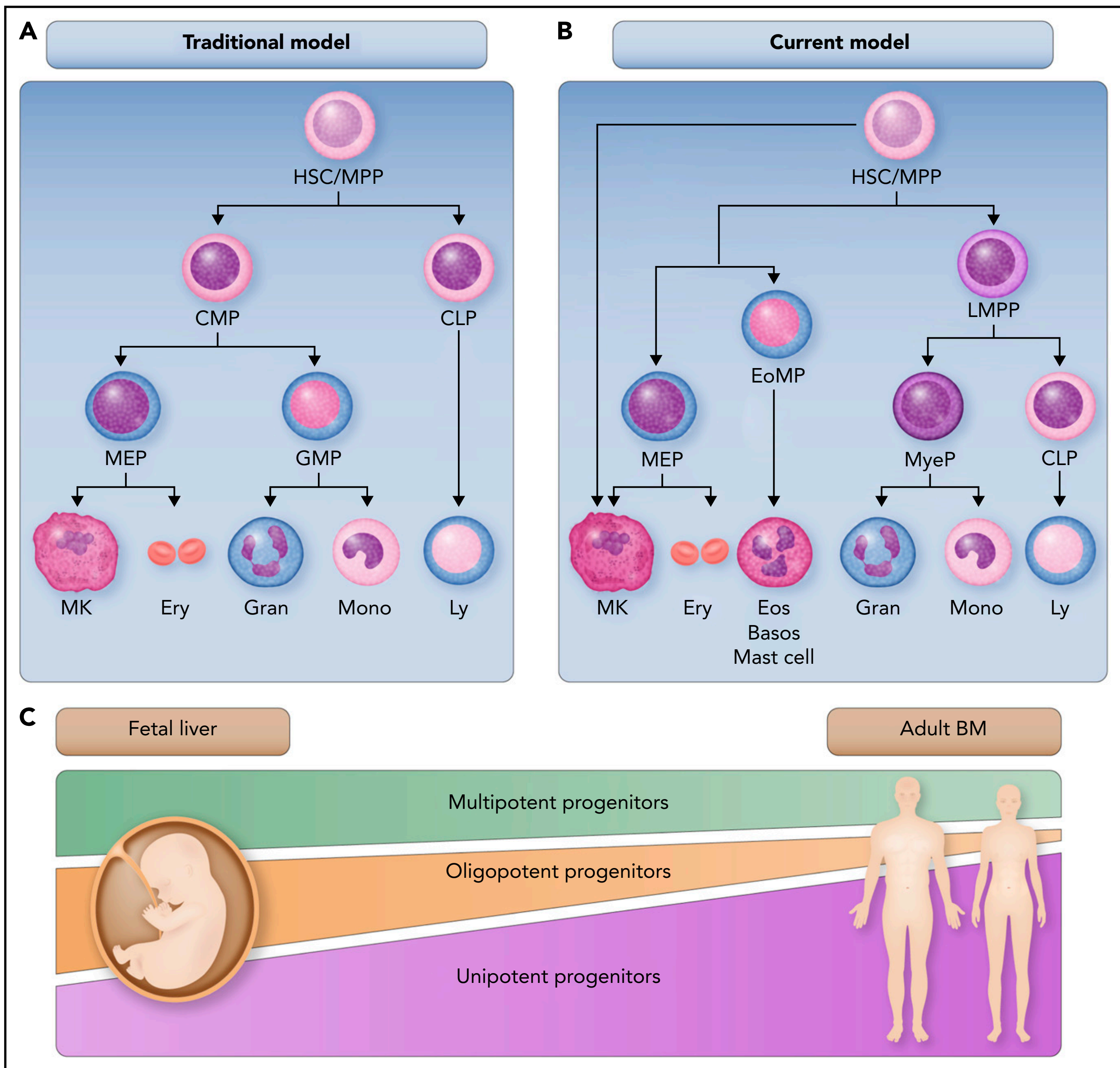

Figure 1. Schematic representation of the traditional and current models of hematopoiesis and developmental changes in progenitor composition. (A) In the traditional model of hematopoiesis, the initial separation segregated myelopoiesis and lymphopoiesis, and MKs emerged exclusively from MEPs. (B) Current literature supports a model in which the initial step separates MK-E/myeloid and lymphomyeloid progenitors, and MKs arise directly from the HSC compartment, as well as from classically defined MEPs (adapted from Psaila and Mead[28]). (C) There is a gradual decrease in the ratio of multipotent to unipotent progenitors in the course of human development. FL CD34$^+$ cells give rise to multipotent, oligopotent, and unipotent progenitors, whereas adult BM CD34$^+$ cells primarily generate multipotent and unipotent progenitors, with a striking paucity of oligopotent progenitors. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; EoMP, eosinophil-basophil-mast cell progenitor; Ery, erythroid cells; GMP, granulocyte-monocyte progenitor; Gran, granulocytes; LMPP, lymphomyeloid progenitors; Ly, lymphocytes; Mono, monocytes; MPP, multipotent progenitor; MyeP, myeloid progenitor. Professional illustration by Somersault 18:24.

conceived as a cellular hierarchy, with self-renewing HSCs at the apex producing stem cell intermediates with less self-renewal potential, culminating in the generation of multipotent progenitors (MPPs). In the traditional model, MPPs gave rise to a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP). CMPs then generated bivalent granulocyte-monocyte progenitors (GMPs) and MK-erythroid progenitors (MEPs), which went on to make granulocytes/monocytes and MKs/erythrocytes, respectively (Figure 1A). This traditional model, in particular the origin of MKs, was challenged by the discovery of platelet-primed HSCs at the top of the hematopoietic hierarchy in the mouse.[23-25]

In recent studies, single-cell approaches have revealed substantial heterogeneity in the HSC compartment in humans, where multipotent but MK/platelet–biased HSCs, similar to those in the mouse, have been found.[26] Furthermore, the first divergence in humans seems to occur in the phenotypic HSC compartment and separates MK-E/myeloid and lymphomyeloid progenitors (LMPPs) (Figure 1B).[27] Although multiple observations support a

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shared trajectory of the MK and E lineages, hematopoiesis is increasingly viewed as a continuum of differentiation that passes through "branch points," rather than a strict sequence of discrete progenitors.[28] Indeed, classically defined MEPs isolated from human adult BM are a heterogeneous population that primarily consists of pure (unilineage) E progenitors (consistent with the predominance of unipotent progenitors in the adult BM),[26] but with a small percentage (~20%) of bipotent cells with capacity for MK and E differentiation and an even smaller percentage of cells fully committed to MK differentiation (identified by CD42 expression).[28,29] Together, the available studies suggest that human MKs arise both directly from the HSC compartment and from MEPs (Figure 1B).[28] Single-cell studies of human FL hematopoietic cells (7-17 WPC) have also identified an MK-erythroid-mast cell progenitor immediately below the HSC/MPP.[30]

HSCs/MPPs also have different intrinsic potential depending on gestational age and hematopoietic site. Within the FL, HSC/MPPs from earlier gestational ages exhibit a strong erythroid lineage bias, with lymphoid and myeloid lineages appearing at later stages.[30] Compared with the FL, HSC/MPPs in the fetal BM are biased toward neutrophil and B-lineage lymphocytes, with neutrophils, eosinophils, and basophils first emerging in the fetal BM.[31] Over the course of human development, there is also significant heterogeneity within the $CD34^+$ cellular compartment, and a progressive reduction in the ratio of multilineage to unilineage progenitors. While $CD34^+$ cells in the FL consist of multipotent progenitors, a large number of oligopotent progenitors, and some unipotent progenitors, adult BM $CD34^+$ cells nearly exclusively give rise to multilineage and unilineage colonies, with a remarkable paucity of oligopotent progenitors (Figure 1C).[26] Importantly, all 3 types of progenitors (multipotent, oligopotent and unipotent) give rise to MKs in the FL, although MKs mostly arise from multipotent progenitors in the adult BM.[26] More recently, a unipotent MK progenitor was identified in the $CD34^+/CD38^+$ cell population in the human BM.[32]

In embryonic hematopoiesis, a subpopulation of hematopoietic progenitor cells expressing thrombospondin 1 (THBS1) and with increased potency for MK differentiation was recently identified in human embryonic stem cell (hESC) cultures.[9] The time at which these MK-primed $THBS1^+$ cells were first detected in hESC cultures is similar to when hESCs generate the hemogenic endothelium,[33] suggesting that a fate decision bias toward MK differentiation could occur as soon as hematopoietic progenitors begin to emerge from hemogenic endothelium. However, it is unknown whether MK-primed $THBS1^+$ endothelial cells are present in human embryos in vivo.[34]

Fetal MK progenitors

Most studies of human fetal MK progenitors have used umbilical cord blood (CB) as a source. MK progenitors are present at much higher concentrations in CB than in adult blood,[35] with a progressive decrease in circulating progenitors over the course of fetal life.[36] This process follows the same developmental pattern in infants born prematurely[37] and is thought to reflect the transition from hepatic to BM megakaryopoiesis.

In semisolid assays, colonies derived from CB MKs contain substantially more MKs than adult blood- or BM-derived MK colonies (Figure 2A).[38] Extremely large MK colonies containing 300 to 500 MKs, exclusively seen in fetal BM and CB cultures, have been thought to represent a more immature fetal MK progenitor not present in the adult BM.[39,40] Consistent with these observations, CB $CD34^+$ cells in liquid cultures produce 10 times more MKs than $CD34^+$ cells from adults.[41]

Embryonic, fetal, and adult MKs

The first platelet-producing cells in the yolk sac of the mouse are not polyploid, but rather are diploid cells that have been named "diploid platelet-forming cells" (DPFCs).[42] DPFCs are first found in the yolk sac at E8.5, and the first platelets are produced at E9.5. Polyploid MKs (8N) first appear in the FL at E11.5 and become progressively more polyploid, as the fetal platelet mass expands.[43] At birth, MKs in the murine liver are still significantly smaller than adult BM MKs and increase in size over the first 2 weeks of life.[21]

Similarly, human yolk sac MKs are mostly diploid, with only 1.7% of MKs having a ploidy ≥8N, compared with ~15% in the FL.[9] Recently, primary MKs from healthy human yolk sacs (4 WPC) and FLs (8 WPC) were characterized using single-cell RNA sequencing (RNA-seq). Comparative analysis of these 2 sources revealed a distinct gene expression signature of human yolk sac MKs, characterized by an enrichment of the "glycolysis/gluconeogenesis" pathways, compared with an enrichment of genes associated with "cell cycle" and "DNA replication" in FL MKs (consistent with their high proliferative rate).[9] These findings suggest that human yolk sac "primitive MKs" may represent the MK equivalent of primitive erythroid cells.[34] Interestingly, yolk sac MKs also express *HBE1*, which encodes the embryonic hemoglobin subunit $\varepsilon 1$, whereas FL MKs express *HBB* (the gene encoding β-globin). This is consistent with other studies showing that fetal and neonatal MKs express erythroid lineage makers, which suggests incomplete separation of the erythroid and megakaryocytic lineages at early developmental stages.[29,44,45]

Phenotypically, MKs from fetuses and neonates are smaller and have lower ploidy levels than MKs from adult sources.[20,46,47] There is a progression to higher ploidy levels in the course of fetal development,[48,49] but the majority of MKs (78%) in the fetal BM at 7 to 8 months' gestation still have a ploidy ≤8N, compared with only 33% of the MKs in adult BM.[49]

Despite their low ploidy, FL MKs have ultrastructural features of mature MKs, such as abundant granules and a normal demarcation system.[48] Similarly, CB-derived MKs are smaller and less polyploid than adult MKs (Figure 2B), but have features of mature MKs when examined by flow cytometry, microscopy, and electron microscopy (Figure 2B-D),[41] in contrast with adult MKs, in which ploidy level and degree of maturation are somewhat coupled.[41] These findings suggest that the features of neonatal MKs are the result of a developmentally unique uncoupling of proliferation, polyploidization, and cytoplasmic maturation, which leads to the production of a high number of small, low-ploidy, but otherwise mature MKs. At the individual MK level, however, the small neonatal MKs produce fewer platelets than the comparatively larger adult MKs.[50]

In regard to the timing of the transition to an adult MK phenotype, a study measuring MK diameters in BM samples from

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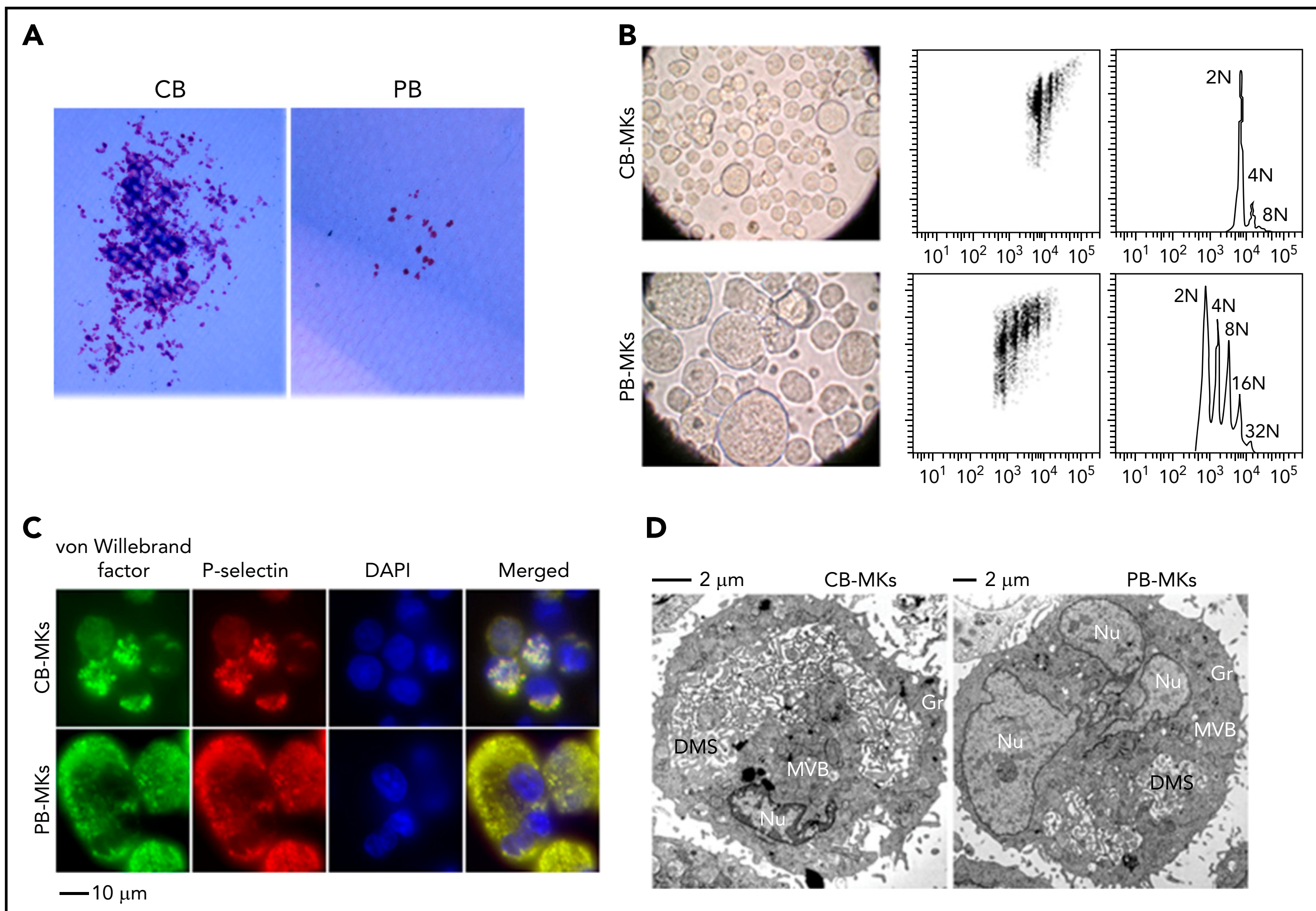

Figure 2. Key features of neonatal megakaryopoiesis. (A) Hematopoietic progenitors from full-term CB or adult PB were cultured in a collagen-based semisolid culture medium (Megacult; StemCell Technologies, Burnaby BC, Canada) in the presence of TPO only. MK colonies generated from CB progenitors were significantly larger than those generated from adult PB. Photomicrographs were taken at a magnification of 200x. (B) $CD34^+$ cells from CB and PB were cultured in a serum-free liquid culture medium with 50 ng/mL of recombinant human TPO as the only growth factor. MKs were evaluated after a 14-day culture period. Representative photomicrographs and ploidy levels by flow cytometry of CB- and PB-MKs demonstrated the smaller size and lower ploidy levels of CB-MKs. Both pictures were taken at a magnification of ×600. (C) Despite their small size, cultured CB-MKs exhibit abundant α-granules containing von Willebrand factor and P-selectin. (D) Flow sorted 2N/4N CB-MKs (left) contain abundant granules (Gr) and a well-developed demarcation membrane system (DMS), consistent with mature MKs and similar to flow-sorted PB MKs with ploidy ≥8N (right). Adapted from Liu et al[28] and Davenport et al.[114]

subjects between the ages of 3 days and 80 years found uniformly small MKs in neonates. Beginning at 2 years of age, separate clusters of smaller and larger MKs were identified, and by 4 years larger (adult-like) MKs predominated.[51]

Mechanisms underlying the developmental differences in megakaryopoiesis

Studies transplanting neonatal liver vs adult BM cells into irradiated adult mice suggested that the phenotypical differences between fetal/neonatal and adult MKs are the result of both cell-intrinsic and environmental factors. Transplanted neonatal MKs were larger and of higher ploidy than nontransplanted neonatal MKs, but were still smaller than transplanted MKs from adult BM cells.[52] Similarly, CB-$CD34^+$ cells achieved higher ploidy when cultured in adult BM stromal cell–conditioned medium compared with those cultured in serum-free medium with thrombopoietin (TPO).[53] These findings suggest that the adult BM microenvironment supports polyploidization, but cell-intrinsic factors determine the smaller size and lower ploidy of neonatal MKs.

Cell-intrinsic mechanisms

The first comprehensive study comparing the transcriptomes of human MKs differentiated from hESCs, FL, full-term CB, and adult peripheral blood (PB) $CD34^+$ cells identified 695 genes differentially regulated along developmental stages. Of those, 253 genes were upregulated through development and were mostly related to increased polyploidization, proplatelet formation, and platelet function. In contrast, the 442 genes that were downregulated during ontogeny were related to the extracellular matrix, membrane components, and interaction of the cells with the environment.[54] In addition, developmental differences were found in the expression of 32 micro-RNAs (miRs), including elevations in miR-9, miR-224, miR-99a, and miR-125b at early developmental stages. Both miR-9 and miR-224 target *CXCR-4* (the SDF-1 receptor), and the elevated miR-9 most likely contributes to the downregulation of *CXCR-4* in fetal MKs.[54-56] Adult BM MKs express comparatively higher levels of CXCR-4, which enables them to migrate in response to an SDF-1a gradient and

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leads to their interaction with BM sinusoidal endothelial cells, promoting MK maturation and TPO-independent megakaryopoiesis.[57] Because of their lower CXCR-4 levels, fetal MKs are less responsive to SDF-1a gradients, a feature that may lack relevance in the FL.[56] The elevated levels of miR-99a in fetal MKs have been proposed to contribute to their hyperproliferative phenotype through downregulation of CTDSPL (a retinoblastoma protein phosphatase that controls RNA polymerase II transcription machinery), resulting in induction of D-type cyclins.[58] Both miR-99a and miR-125b are chromosome 21 miRNAs, and miR-125b in particular is critical for the pathogenesis of Down syndrome (DS) transient myeloproliferative disorder, as described later.

Differences have also been described between the responses of human neonatal and adult MKs to TPO. In response to TPO stimulation, CB MKs display stronger activation of the JAK2 and mTOR pathways compared with adult MKs.[41] When the mTOR pathway is inhibited in adult MKs, there is a decrease in polyploidization, cytoplasmic maturation, and proliferation.[59] In contrast, mTOR inhibition in CB MKs results in decreased proliferation and maturation, but no effect on polyploidization, possibly because of the lower p21 levels in neonatal compared with adult MKs.[60] Thus, the neonatal hyperactivity of the mTOR pathway, combined with the hypoactivity of p21, may contribute to the uncoupling of MK maturation and polyploidization in neonatal MKs.[41] Furthermore, studies in mice lacking the TPO receptor (*c-MPL*$^{-/-}$ mice) have shown that the TPO/c-MPL axis has different functions in the regulation of megakaryopoiesis at different developmental stages (see "Congenital amegakaryocytic thrombocytopenia" below).

Adult MKs, unlike most cells in the body, depend on sustained, high-level activation of P-TEFb (a complex of Cdk9 and cyclin T1), to meet their high transcriptional demands by releasing RNA polymerase II (RNAPII) from proximal stalling.[61,62] In most non-MK cells, a feedback loop maintains P-TEFb sequestered in an inactive state within the 7SK small nuclear ribonucleoprotein (snRNP) complex. In adult MKs, P-TEFb is released from the complex by proteolysis of MePCE and downregulation of LARP7 (2 components of the 7SK snRNP complex). Similar to adults, neonatal MKs proteolyze MePCE and downregulate LARP7, but (unlike in AD MKs) neonatal P-TEFb remains bound to the complex and inactive. This finding was recently explained through the discovery that neonatal MKs contain high levels of a fetal-specific 7SK stabilizing protein, IGF2BP3, which maintains P-TEFb in an inactive state, despite lineage-appropriate changes in the 7SK-stabilizing factors (Figure 3).[45] Knockdown of *IGF2BP3* in neonatal MKs leads to 7SK downregulation, enhanced P-TEFb activation and development of adult MK features, including cellular enlargement, proliferation arrest, polyploidization, and erythroid suppression.[45] The mechanisms by which activated P-TEFb exerts these effects in neonatal MKs remain unknown.

In addition, MKs exhibit developmental differences in pathways that regulate functional differences between fetal/neonatal and adult platelets. One of the most important differences is the low P-selectin levels found in activated neonatal compared with adult platelets.[63,64] Functionally, these low P-selectin levels limit the ability of fetal platelets to bind to neutrophils.[64] In the mouse, the low P-selectin levels in fetal platelets result from significantly higher *Lin28b* and *Hmga2* expression levels in fetal compared with adult MKs. Importantly, fetal hematopoietic progenitors transplanted into adult mice produce platelets with low P-selectin levels, indicating that the developmental regulation of P-selectin is cell intrinsic.[64] To what degree this pathway is present in human neonatal MKs is unclear, because human neonatal platelets have P-selectin levels (determined by western blot analysis) comparable to those of adult platelets.[65] However, activated human neonatal platelets also exhibit decreased surface P-selectin compared with adult platelets,[66,67] because of decreased activation in response to agonists and a degranulation defect.[65,68]

Environmental factors

The interplay between site-specific environmental signals and megakaryopoiesis is complex. In the adult BM, the extracellular matrix (ECM) components are important regulators of megakaryopoiesis and thrombopoiesis. Specifically, type I collagen promotes MK differentiation but inhibits proplatelet formation.[69] In contrast, fibronectin and types III and IV collagens, which are the key components of the pericellular matrix surrounding MKs in the BM,[70] stimulate proplatelet formation.[71,72] Interestingly, MKs themselves synthetize and secrete components of the BM ECM in response to TPO stimulation.[70,73] The stiffness of the ECM, which is largely determined by collagen type and by the secreted enzyme lysyl oxidase, is also an important regulator of MK maturation and platelet production.[74] Some degree of stiffness promotes MK maturation,[75] but a less stiff ECM promotes thrombopoiesis by activating TRPV4, a mechanosensitive ion channel on the MK surface.[74,76] Finally, hyaluronan is a glycosaminoglycan that fills most of the BM interstitial space,[77] and hyaluronan depolymerization by MK hyaluronidase-2 is necessary for normal thrombopoiesis.[78]

Studies comparing the composition of different hematopoietic niches during development have found a >10-fold higher proliferative capacity of FL vs adult BM stromal cells and higher expression of Wnt signaling pathway regulators in FL than in adult BM stroma (which has higher expression of Notch signaling pathway).[79] Similarly, the murine neonatal BM contains higher proportions of primitive mesenchymal stem cell subsets with increased expression of niche cross-talk molecules that support HSC self-renewal (Jagged-1 and CXCL-12) compared with adult BM,[80] consistent with the need of the fetus to expand the HSC pool. Single-cell transcriptomic studies of the human fetal BM have identified significant stromal cell heterogeneity,[31] with 19 stromal cell states that closely correlate with postnatal mouse BM stroma.[81]

Mediators released into the different microenvironments also affect megakaryopoiesis in a developmental stage–specific manner. In the FL, endothelial cells, stromal cells, and hepatocytes produce cytokines and growth factors that promote HSC expansion, including stem cell factor, TPO, insulin-like growth factor 2 (IGF-2), and angiopoietin-like-2 and -3 (Angptl2 and -3).[82] Stromal cell-secreted IGF-2, in particular, activates the IGFR1/mTOR/E2F pathway and increases the expansion of fetal, but not adult, hematopoietic stem cells and MKs.[83-85] Another example is interferon (IFN)-β, which is secreted by osteoblasts and osteoclasts in the BM, but not by cells in the FL. Type 1 IFN signaling is antiproliferative in multiple cell types, and IFN-

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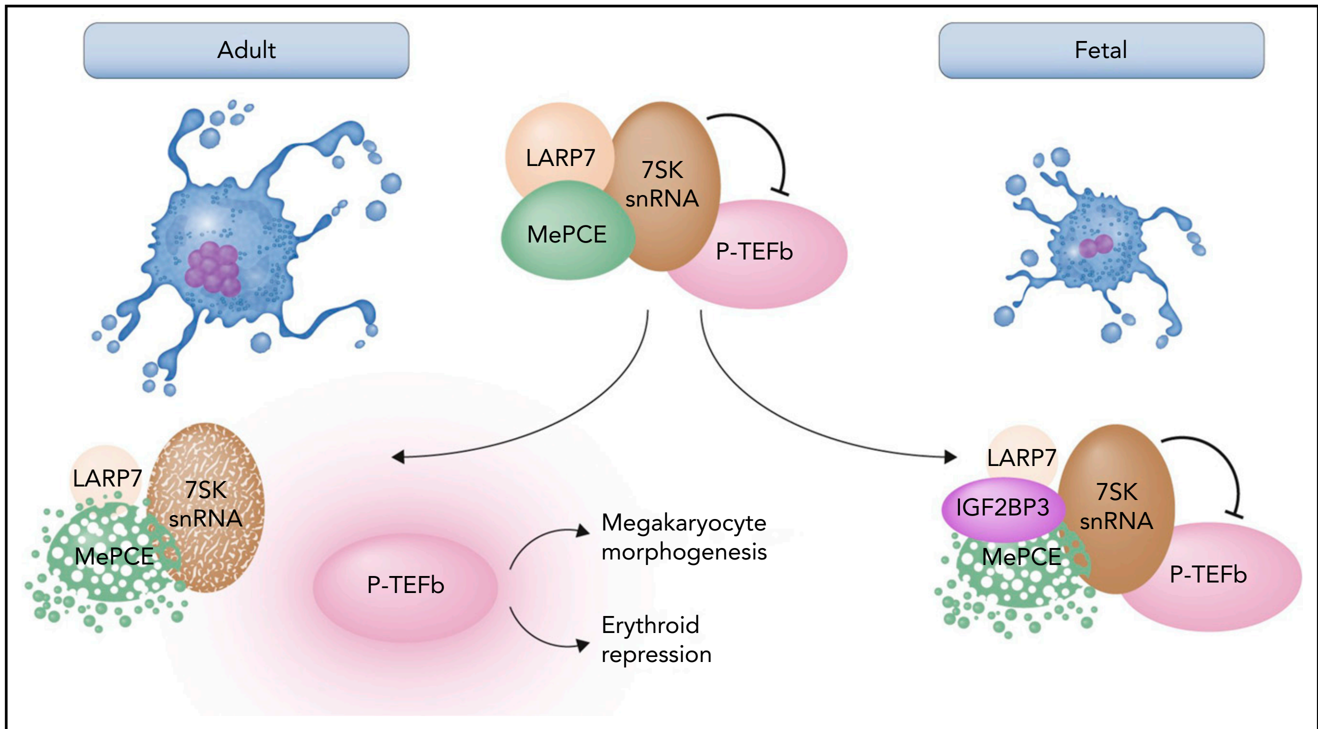

Figure 3. Model of ontogenic regulation of MK morphogenesis. In most cells, most P-TEFb is sequestered in an inactive state within the 7SK small nuclear ribonucleoprotein (snRNP) complex. In adult megakaryopoiesis (left arrow), downregulation of LARP7 and proteolysis of MePCE destabilize 7SK snRNA, leading to unopposed P-TEFb activation. This mode of P-TEFb activation promotes upregulation of MK morphogenesis factors and repression of erythroid markers. In fetal megakaryopoiesis (right arrow), IGF2BP3 stabilizes 7SK snRNA despite the downregulation of LARP7 and proteolysis of MePCE. Persistence of 7SK allows for inhibition of P-TEFb, dampening both the upregulation of MK morphogenesis factors and lineage consolidation via erythroid repression. Professional illustration by Somersault 18:24.

response genes are upregulated in primary murine adult BM MK progenitors, compared with FL MK progenitors.[44] These findings suggest that both the presence of IFN-β and the ability of MK progenitors to respond to it are developmentally regulated.

MK subpopulations

Recent studies using single-cell RNA sequencing have revealed the existence of distinct MK subpopulations at early stages of human development. In a study pooling human yolk sac (4 WPC) and FL (8 WPC) MKs together, 6 transcriptionally different MK subsets were identified.[9] Each one of these MK subpopulations had gene expression patterns that suggested functional specialization, including "platelet production," "niche support," and "immune functions" (Table 1). In the "immune" MK cluster, the most enriched biological processes were phagocytosis, antigen processing and presentation, and macrophage migration.

Four distinct MK subpopulations were also recently identified in the human and murine adult BM.[86] Similar to findings in the human embryo, these MK subpopulations included a "niche" MK cluster, a "platelet generating" cluster, and an "immune" MK cluster (Table 1). Although the functional specificities of the various MK subpopulations were very similar in the human yolk sac and FL and in the adult BM, the transcriptional signatures of these subsets were different in embryos and adults. For example, genes enriched in embryonic "niche" MKs were mainly related to extracellular structure organization, whereas adult "niche" MKs were mostly enriched in genes involved in cell-cell signaling and cytokines. "Immune" MKs in embryos were mostly enriched in genes for phagocytosis, whereas adult "immune" MKs expressed more mature and diverse immune programs, involving both innate and adaptive immunity (Table 1).[86] These observations indicate that the heterogeneity of MKs is present in early embryonic and adult hematopoiesis, but the specific profile and functions of the various subsets may vary over the course of development, perhaps in response to different environmental signals.

In support of the importance of the environment, platelet-producing MKs found in the adult mouse lung have gene expression patterns and functions very similar to those of antigen-presenting cells.[87] The immune phenotype of these lung MKs is very likely driven by the unique environment of the postnatal lung, which includes pathogens and cytokines produced by lung resident cells (ie, interleukin-33 [IL-33]). Consistent with this hypothesis, lower levels of the immune molecules major histocompatibility complex class II (MHC II) and intercellular adhesion molecule-1 (ICAM 1) are found in neonatal compared with adult lung MKs.[87]

Implications of the developmental differences in megakaryopoiesis for disease

DS-associated transient myeloproliferative disorder

DS-associated transient myeloproliferative disorder (DS-TMD) is the classic, most studied developmental MK disorder. It is characterized by the increased proliferation and maturational arrest of erythromegakaryocytic cells at birth, followed by spontaneous resolution within the first few months of postnatal

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Table 1. Genes associated with the different megakaryocyte subsets found in yolk sac and FL and in adult BM

MK Subset	Fetal (YS and FL)*	Adult (BM)
Immature	*FAM162A, SLC25A37, DDIT4, BNIP3, KRT18, P4HB, HSP90B1, GYPB, MT1X, HBA1*	—
Cycling	*HSPE1, RANBP1, FABP5, H2AFZ, HMGN2, RPL22L1, HMGB1, HBD, FCER1A*	*POLA2, POLD2, PRIM1, LIG1*†
Thrombopoiesis and coagulation	*TUBA4A, TUBB1, PPBP, PTCRA, C19orf33, SH3BP5, MYL9, CCL5, ACRBP, HIST1H2AC, MYLK, GFI1B, NFE2, GP1BA*	*TUBB1, MYH9, VWF, GP1BA, GP5, GP6, P2RY1, P2RY12, GP9, ITGB3, ITGA2B*‡
HSC/niche supporting	*RBP1, COL1A1, COLEC11, COL3A1, IGFBP3, ID3, PTN, ID1, ACTC1, VIM, MFAP4, ANGPTL6, SPARC, ANXA2, POSTN, CCBE1, COL6A2*	*ACTN1, FLNA, FGFR3, TSPAN32, ZYX, GJA4, HDGF, CTGF, CMTM2, CMTM5, TPM1, TPM4, MYL9, MYO5C*†
Translational initiation	*SPINK2, IGLL1, C1QTNF4, JCHAIN, HLA-DRA, MPO, PLAC8, CD74, ZFP36L2, LTB*	—
Immune	*MS4A6A, CIQA, C1QC, C1QB, HMOX1, MYL7, ZFP36L1, FCGRT, CCL3, SAT1, CYBB, CTSS, MAFB, SAMHD1, RNASE6, S100A9, C3AR1*	*CHIL1, CHIL3, CTSS, ANXA1, SPI1, CEBPB, CEBPD, CEBPE, IRF5, IRF8*‡

YS, yolk sac.

*From Figure 3B and text in Wang et al.[9]

†From supplemental Figure 5B in Sun et al.[86]

‡From Figure 2D and supplemental Figure 3D in Sun et al.[86]

life.[88] TMD affects 5% to 10% of newborns with DS.[89] The spontaneous resolution that occurs around the time that liver hematopoiesis stops and the megakaryoblast infiltration of the liver both suggest that the abnormal megakaryopoiesis originates in the FL.[90] Two factors must be present for this condition to develop: (1) trisomy 21 (T21), which is associated with increased frequency and clonogenicity of MEPs in the FL[91-93] and (2) a mutation in exon 2 of *GATA-1*, leading to the exclusive production of a short isoform of GATA-1, termed GATA-1 short (GATA-1s), which lacks the N-terminal transactivation domain of GATA-1.[94]

Initial studies in mouse models shed light on the developmental stage-specific manifestations of this disorder. Yolk sac- and FL-MK progenitors from mice engineered to exclusively express GATA1s were significantly hyperproliferative compared with wild-type progenitors, but the same was not seen in adult mice.[95] Differences between fetal and adult MK progenitors in 2 signaling pathways (IGF and interferon) have been identified as likely contributors to the fetal-specific manifestations of GATA1s mutations. First, as described earlier, IGF signaling strongly activates mTOR and the E2F transcriptional network in fetal, but not adult, MK progenitors. Full-length GATA1 restricts the E2F activation in normal fetal MK progenitors, to balance proliferation and differentiation. If full-length GATA1 is absent, however, GATA-1s leaves the overactive fetal IGF signaling "unchecked," leading to the excessive proliferation of MK progenitors.[83] Second, IFN-1–responsive genes are upregulated in murine and human adult BM- compared with FL-derived MK progenitors. Exogenous IFN-α markedly reduces the hyperproliferation of FL MK progenitors from GATA1s mice and neutralization of IFN signaling by genetic or pharmacological means increases MK progenitor proliferation in the BM of adult GATA1s mice,[44] suggesting that upregulated type 1 IFN signaling in the BM may contribute to the spontaneous resolution of DS-TMD.

More recently, studies using CRISPR-Cas9 to introduce GATA-1s mutations in primary human FL, CB, and adult hematopoietic stem and progenitor cells (HSPCs) confirmed the same developmental stage-specific effects of GATA-1s in human cells, with the mutation causing hyperproliferation of fetal but not neonatal or adult MK progenitors.[96] Introducing GATA-1s mutations in human HSPCs isolated from the livers of T21 fetuses (T21-FL), followed by xenotransplantation assays, enabled recapitulation of the combined effects of T21 and GATA-1s on the development of DS-TMD and DS-acute megakaryoblastic leukemia (AMKL). These studies identified GATA-1s and the upregulation of 3 chromosome 21 miRNAs (miR-99a, miR-125b, and miR-155) in FL long-term HSCs as the key factors that contribute to the pathogenesis of TMD.[97] The transcription factor ARID3A was further identified as the main target of miR-125b driving the synergy between T21 and GATA-1s. In normal hematopoiesis, ARID3A functionally cooperates with GATA1 to induce MK differentiation. In T21-FL HSPCs, however, miR-125b represses *ARID3A*, and this deficiency in conjunction with GATA-1s leads to MK hyperproliferation and maturational arrest (Figure 4).[98] Although DS-TMD resolves in 85% to 90% of affected neonates, 20% to 30% of them go on to develop DS-AMKL in the first 5 years of life. It has been widely accepted that the progression to DS-AMKL requires the coexistence of T21, GATA-1s, and additional mutations, most frequently in the cohesin subunit *STAG2*.[99] However, recent studies have demonstrated that T21 is indispensable for the development of DS-TMD, but not for the development of leukemia in cells carrying a GATA-1s mutation in conjunction with a knockout of *STAG2*. Knocking out other cohesin genes, particularly *RAD21* and *NIPBL*, in GATA-1s FL cells also leads to leukemia in xenotransplants. CD117/KIT is a marker of the cells the mediate the propagation of GATA1s-induced preleukemia and is a potential therapeutic target (Figure 4).[97]

Other alterations in T21 FL HSPCs may also contribute to the pathogenesis of DS-TMD and DS-AMKL. DS is associated with

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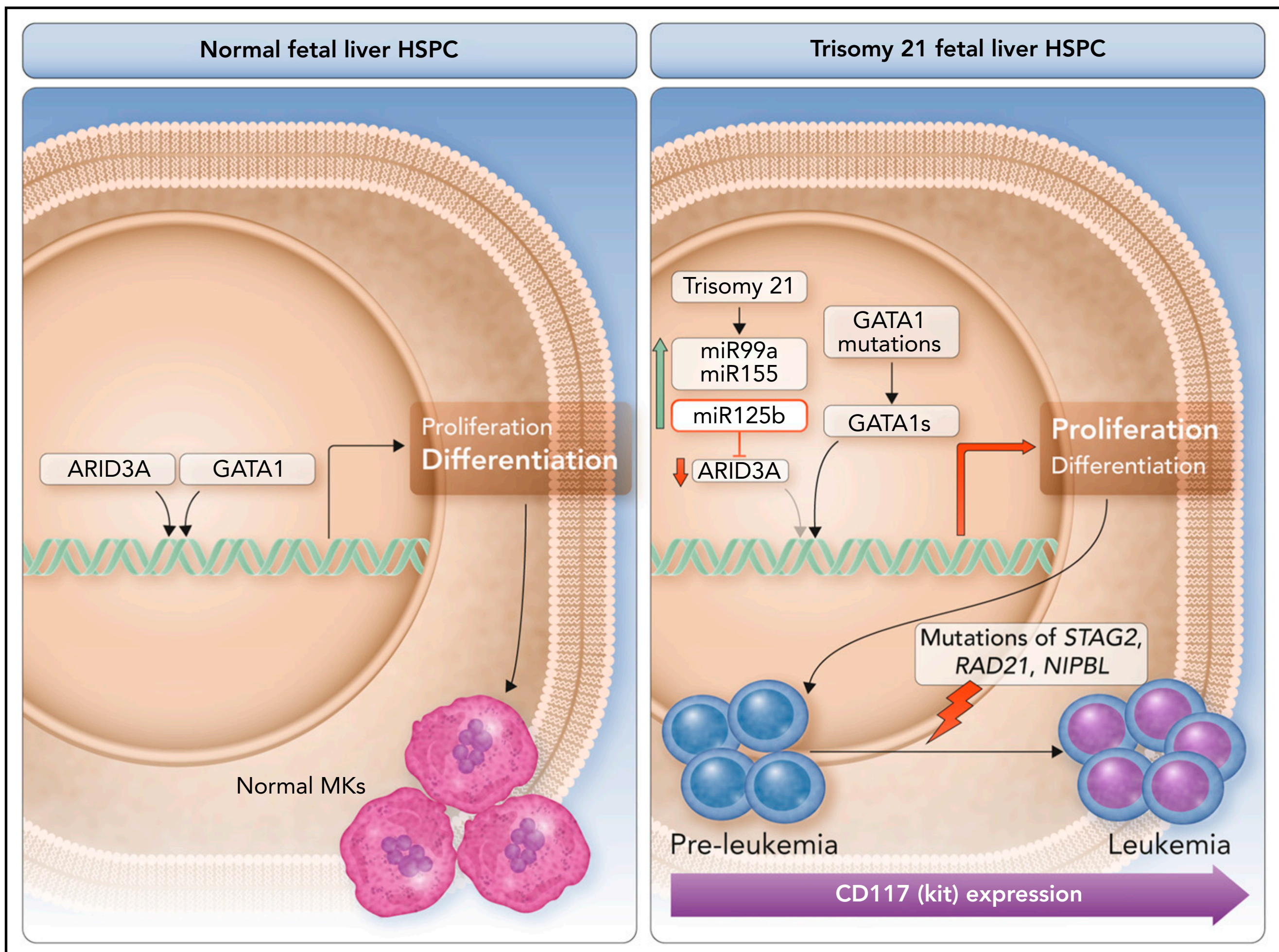

Figure 4. Schematic of the combined effects of trisomy 21 and GATA-1s mutations on the pathogenesis of DS-TMD (preleukemia) and the progression to leukemia. Left panel, in normal FL HSPCs, the transcription factor ARID3A functionally cooperates with full length GATA 1 to induce normal MK proliferation and differentiation. Right panel, in FL HSCs isolated from fetuses with trisomy 21 (T21), 3 chromosome 21 miRNAs are upregulated: miR-99a, miR-155, and miR-125b. The elevated miR125b levels posttranscriptionally repress ARID3A. The combination of low ARID3A levels and GATA-1s in T21 fetuses carrying GATA1s mutations leads to MK hyperproliferation and maturational arrest, which characterize DS-TMD (preleukemia). The progression from preleukemia to leukemia requires additional mutations, most frequently in the cohesin genes *STAG2*, *RAD21*, and *NIPBL*. CD117 (KIT) is a marker of the cells that mediate the propagation of GATA1s-induced preleukemia and GATA1s/STAG2ko-induced leukemia. ARID3A and CD117 (KIT) are potential therapeutic targets for DS-TMD and to avoid the progression to leukemia. Professional illustration by Somersault 18:24.

genome-wide perturbations of gene expression, mediated by epigenetic changes at promoter/enhancer regions of important regulators of hematopoiesis and megakaryopoiesis. The top 2 differentially methylated regions in DS overlap *RUNX1* and *FLI1*,[100] 2 key transcription factors for MK differentiation, and BM MKs from fetuses with DS have lower levels of *FLI1* expression.[31] Multiple inflammatory pathways (ie, TNF and interferon response) are also activated in DS fetal BM stromal cells, suggesting an altered fetal BM stromal environment.[31]

Congenital amegakaryocytic thrombocytopenia

Congenital amegakaryocytic thrombocytopenia (CAMT) is caused by mutations in *c-MPL*, the gene encoding the TPO receptor, and is characterized by severe thrombocytopenia and a near-absence of MKs in the BM. Although this is the classic disease phenotype, CAMT may have different clinical manifestations in neonates, potentially leading to delays in the diagnosis. Neonates with mutations leading to reduced (not absent) c-MPL expression can present with mild thrombocytopenia, which can persist in infancy and childhood before progressing to severe thrombocytopenia and aplastic anemia.[101,102] Because of this, thrombocytopenia is not detected at birth in a quarter of patients with CAMT.[103] A less common presentation of CAMT is that of neonates with severe thrombocytopenia who, on BM examination, have an appropriate number of immature-appearing MKs, suggesting that their thrombocytopenia is caused by mechanisms other than reduced megakaryopoiesis.[104-106]

Although the explanation for this phenotype has not been fully elucidated, studies in *c-MPL*$^{-/-}$ embryos and newborn pups have revealed that TPO and c-MPL regulate different aspects of megakaryopoiesis at various developmental stages. First, *c-MPL*$^{-/-}$ embryos have a normal number of DPFCs and platelets at E10.5, which demonstrates that thrombopoiesis is not regulated by TPO and c-MPL in murine embryos. At E14.5 to E16.5, *c-MPL*$^{-/-}$ fetuses develop thrombocytopenia associated with a block in MK polyploidization (at 8N) but a normal number of MKs.[43] This is the opposite of adult life, when the TPO/c-MPL

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axis primarily regulates the number of MKs and not maturation. *c-MPL*$^{-/-}$ newborn mice have a mixed picture, with a decreased number of liver MKs that have features of abnormal maturation visible by electron microscopy.[63] The presence of MK maturation defects in *c-MPL*$^{-/-}$ fetuses and pups provides a potential explanation for the finding of immature-appearing MKs in the BM of thrombocytopenic neonates who later develop amegakaryopoiesis and aplastic anemia.

Neonatal thrombocytopenia

Thrombocytopenia is common among sick neonates, particularly those born prematurely.[107-109] Although the reasons for this predisposition are multifactorial, evidence suggests that the small size that characterizes neonatal MKs limits the ability of neonates to upregulate platelet production in response to increased demand. Studies in thrombocytopenic human neonates[47] and in newborn mouse models of thrombocytopenia[110] have shown that neonatal MKs (contrary to adults) increase in number but not in size, which significantly limits their ability to increase the MK mass and upregulate platelet production under conditions of increased platelet destruction or thrombopoietic stimulation.[111]

Delayed platelet engraftment after CB transplant

Hematopoietic reconstitution is known to be slower after CB transplant, compared with BM transplant. This delay is particularly pronounced for platelets, with average times to platelet recovery of 60 days for CB vs 29 days for BM grafts.[112] Although a lower number of stem cells in CB grafts most likely contributes to this finding, MKs are significantly smaller (and retain a neonatal phenotype) in children who received transplants of CB cells compared with BM cells.[113] These differences were evident in BM biopsies obtained 1 to 3 months after transplant, suggesting that MKs derived from neonatal HSPCs may not be able to upregulate their size (similar to neonates) for at least a while after transplant. Another factor contributing to the slower platelet recovery may be the lower CXCR-4 levels in CB-derived MKs, which may cause delayed homing of CB progenitors to the BM,[55] although this has not been formally tested.

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Footnote

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