Cell. Mol. Life Sci. 63 (2006) 2460–2476
1420-682X/06/212460-17
DOI 10.1007/s00018-006-6190-8

Cellular and Molecular Life Sciences

Review

Molecular mechanisms of megakaryopoiesis

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Received 26 April 2006; received after revision 9 June 2006; accepted 4 July 2006
Online First 11 August 2006

Abstract. One function of bone marrow megakaryocytes (MKs) is the controlled release of platelets into the circulation. Over the past few years, molecular mechanisms that contribute to MK development and differentiation have begun to be elucidated. This review provides a brief overview of megakaryopoiesis and platelet function, and the importance of selected hematopoietic transcription factors (including GATA-1, FOG, Fli-1, AML1, and NF-E2) and target genes in this biological process. In addition, a discussion of human diseases affecting megakaryopoiesis and mouse models of thrombocytopenia are presented with emphasis on how these systems have and will continue to provide further insights into mechanisms that control the biological functions of the megakaryocytic cell lineage. Ultimately, such knowledge may provide the basis for novel therapeutic approaches for modulation of platelet number and function.

Keywords. Megakaryocyte, transcription, thrombopoiesis, platelet, thrombocytopenia, Ets, GATA-1, Fli-1.

Overview of megakarypoiesis

Megakaryopoiesis is the process by which mature megakaryocytes (MKs) are derived from pluripotent hematopoietic stem cells (HSCs) (Fig. 1). (Additional background can be found in recent reviews [1, 2].) The major function of the MK is to produce platelets (thrombopoiesis), which are critical for hemostasis in the peripheral blood vasculature. During its lifespan, a mature MK can produce up to 10^4 platelets [3]. Each day the human adult produces 1×10^{11} platelets and this number can increase tenfold with demand [4]. The hallmark of the MK is its large diameter (50–100 μm) and its single, multilobulated, polyploid nucleus. In the canonical pathway of hematopoietic lineage development [5–9], the HSC gives rise to two major lineages, the common lymphoid progenitor (CLP) [10] and the common myeloid progenitor (CMP) [7]. The CLP then gives rise to lymphocytes (NK, T and B cells) and the CMP gives rise to both the granulocyte/macrophage progenitor (GMP) and the megakaryocyte/erythroid progenitor (MEP) [11]. However, as shown in Figure 1, recent evidence suggests that the MEP may arise directly from the HSC to give rise to either the erythroid or megakaryocyte lineages without the CMP intermediate [12, 13].

The most primitive MK progenitors are the high proliferative potential-colony-forming unit-megakaryocyte (HPP-CFU-MK) and burst-forming unit-megakaryocyte (BFU-MK). BFU-MK are thought to produce a more differentiated MK progenitor, termed colony-forming unit-megakaryocyte (CFU-MK). CFU-MK are the first in the MK lineage that have been identified by a distinct surface phenotype. Nakorn et al. [14] demonstrated that these cells were the $CD9^+CD41^+Fc\gamma R^{lo}c\text{-}kit^+Sca$-

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1^+IL-7Rα^-Thy1.1$^-$Lin$^-$ population in murine bone marrow and could give rise to almost pure populations of MK and platelets *in vitro* and *in vivo*. CFU-MKs then give rise to immature MKs or megakaryoblasts, a heterogeneous population that undergoes endomitosis to increase in size and ploidy to a DNA content in excess of 16N [15]. In addition to undergoing endomitosis, immature MKs must amass a reservoir of membranes (provided by the demarcation membrane system [16]), granules and cytoskeletal proteins [15]. These cells are transitional cells to the mature MK, which is polyploid and no longer proliferates. Mature MKs then begin the process of shedding their cytoplasm to produce platelets [17]. This is a complex process that requires the formation of cytoskeletal tracks, termed proplatelets [17, 18]. Necessary components such as platelet granules, organelles and ribosomes are transported from the MK body to the ends of the proplatelet where platelet synthesis and release occurs [17]. Platelet release does not occur within the bone marrow, rather proplatelets either extrude into the marrow sinusoids [19] or the entire MK enters into the circulation for release [20]. Platelet formation and release is a terminal process for the mature MK that results in apoptosis and subsequent phagocytosis by macrophages [19, 21–23].

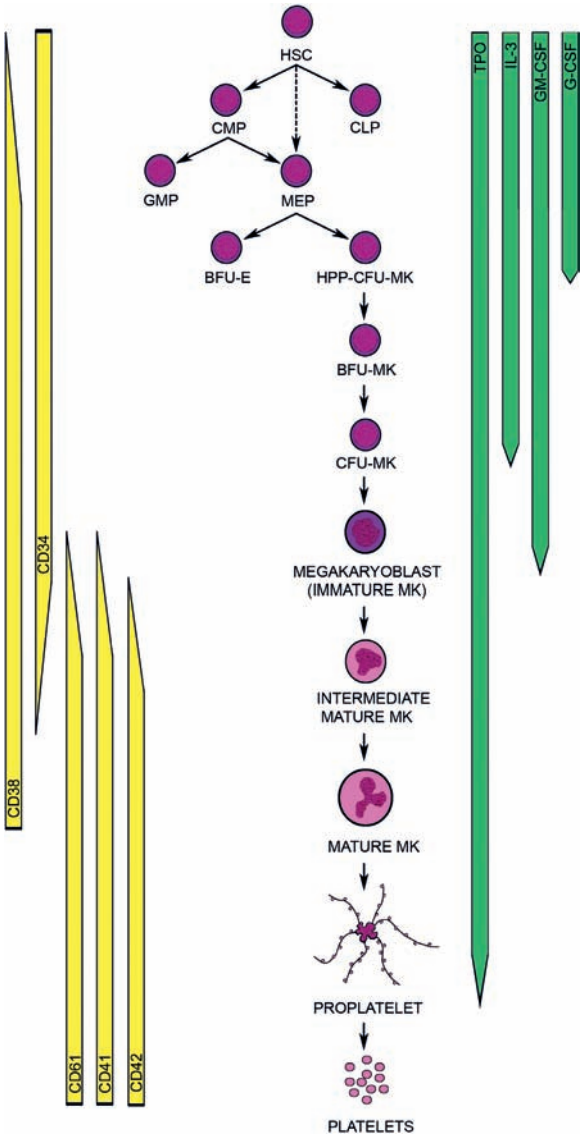

Figure 1. Overview of megakaryopoiesis. Figure depicts the development of mature MKs and platelets from HSCs via both the classical pathway (*i.e.* CMP intermediate) and the recently proposed direct pathway (dashed arrow). Associated surface antigens/markers are shown as yellow arrows to indicate the approximate stage in MK development at which they are expressed. Green arrows to the right represent the role of specific cytokines in megakaryopoiesis. Abbreviations of cell types are as follows: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte/macrophage progenitor; MEP, MK/erythrocyte progenitor; BFU-E, burst forming unit erythroid; HPP-CFU-MK, high proliferative potential colony-forming unit megakaryocyte; BFU-MK, burst-forming unit megakaryocyte; CFU-MK, colony-forming unit megakaryocyte; MK, megakaryocyte. Abbreviations of cytokines are as follows: TPO, thrombopoietin; IL-3, interleukin-3, GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

The maturation of MKs from the HSC is associated with the expression of characteristic CD antigens (Fig. 1). The expression of both CD34 and CD38 are typically used to analyze early megakaryopoiesis. It has been shown that MK progenitors (HPP-CFU-MK, BFU-MK and CFU-MK) and immature MKs express CD34 [24, 25], while more mature MKs are negative for CD34 expression [24]. In contrast, BFU-MK and mixed E/MK progenitors are CD38$^{-/lo}$ [11, 26] and expression of CD38 correlates with MK maturation. With respect to these markers, it must be noted that their expression profile on HSCs differs between human and mouse and with activation state. Studies have shown that both CD34$^+$ and CD34$^-$ populations of human bone marrow cells have the capacity of long-term multilineage engraftment *in vivo* [27]. Likewise, while the majority of HSCs in the adult mouse were shown to be CD34$^-$ [28, 29], murine HSCs can be positive for CD34 when activated [30]. In addition, human HSCs are generally characterized as CD38$^{-/lo}$ [31]; however, in the mouse, HSCs have been shown to be CD38$^+$ [32–34]. Taken together, these studies suggest that the expression profiles of CD34 and CD38 alone cannot define cells of the MK lineage.

The most widely used markers for examining MK differentiation are CD61, CD41 and CD42 (Fig. 1). MK differentiation is characterized by the expression of CD61 (integrin β3) and increased expression of CD41 (integrin αIIb) ([24, 35, 36] and reviewed in [1]), which together form a heterodimeric receptor complex known as glycoprotein (gp) IIb/IIIa [37, 38]. This receptor is present on the surface of cells of the MK lineage from progenitor cells to platelets and their expression levels increase as cells mature [39, 40]. gpIIb/IIIa functions as an adhesive for fibrinogen, fibronectins, vitronectin and von Willebrand factor (vWF) [24, 37]. The expression of CD42

(gpIb, the vWF receptor) is slightly later than that of CD41 [25]; however, expression levels of the two correlate with MK maturity [26]. Thus, $CD34^+CD41^+CD42^+$ and $CD34^-CD41^+CD42^+$ represent more mature MK, while $CD34^+CD41^+CD42^-$ cells represent intermediately mature MK.

While a number of cytokines have been associated with the process of megakaryopoiesis [41], thrombopoietin (TPO) is clearly the major physiological regulator of this process. TPO was identified and cloned as the factor that bound to c-mpl and stimulated megakaryopoiesis to lead to elevated platelet production [42–45]. TPO is expressed primarily in the liver and to a lesser extent in kidneys, bone marrow stromal cells and other organs. TPO has been shown to affect all aspects of platelet formation (reviewed in [46]) (Fig. 1), beginning with the survival and entry into cell cycle of HSCs [47, 48]. During MK development, TPO has been demonstrated to be responsible for stimulating the expression of characteristic cell surface proteins including CD61/41 (gpIIb/IIIa) and CD42 (gpIb) [44, 49] as well as inducing endomitosis [44, 50]. While many of the functions of TPO in MK development are the result of TPO acting in synergy with other cytokines, TPO alone was shown to be the cytokine responsible for the cytoplasmic reorganization and formation of demarcation membranes [51–53], which allows for the release of platelets. Mice deficient in TPO were shown to have reduced numbers of progenitors committed to the MK lineage (~10% of wild-type) as well as reduced MK ploidy [54]. While these studies established that TPO is the major factor promoting the differentiation of HSCs into mature MKs, they also demonstrated that TPO is not critical for the shedding of platelets [54].

Several other cytokines, including interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), have been shown to be important for normal megakaryopoiesis. In contrast to TPO, which plays a role throughout megakaryopoiesis, these cytokines affect megakaryopoiesis during the early stages of MK lineage development (Fig. 1). IL-3 was shown to act on bone marrow progenitor cells through the CFU-MK stage of MK development [55, 56]. Several studies demonstrated that IL-3 acts in synergy with TPO to produce colonies of multiple hematopoietic lineages, including multilineage colonies [47, 57]. With respect to megakaryopoiesis, the effects of GM-CSF were also seen primarily in the BFU-MK and immature MK populations [56]. In synergy with stem cell factor (SCF), GM-CSF was shown to increase proliferation and expansion of both primary bone marrow progenitors and MK cell lines [58]. While the primary affects of G-CSF have been demonstrated for the granulocytic lineage, G-CSF has been shown to have a stimulatory role in bone marrow progenitors of multiple hematopoietic lineages [47, 59]. G-CSF, in combination with TPO, was shown to trigger HSCs into cell cycle and support colony formation [47] as well as stimulate production of HPP-CFU-MK progenitors in myelosuppressed mice [60]. In later-stage MK development, G-CSF alone was shown to suppress MK maturation [61]. Several additional cytokines [IL-1, IL-6, IL-11, IL-12, erythropoietin (EPO), leukemia inhibitory factor (LIF)], as well as chemokines [stromal cell-derived factor-1 (SDF-1) and CXCL12 (PF4)] contribute to megakaryopoiesis [2].

The ultimate function of MKs is to produce platelets. Platelets are the smallest cells (3.6×0.7 μm) in the human blood and in addition to hemostasis and thrombosis, platelets perform important roles in wound healing, inflammation, and tumor metastasis. Several events take place during this regulated process. First, the MK undergo nuclear endomitosis, organelle synthesis, and cytoplasmic expansion and establish a microtubule array. Next, proplatelets are formed and released from the cell, the nucleus is extruded, and individual platelets are released from the proplatelet ends [21]. In healthy humans, 10^{12} platelets flow continuously in the bloodstream [62]. When the wall of a blood vessel is damaged, the exposed collagen binds the vWF, which then serves as the substrate for platelet adhesion, mediated by binding to the gpIb/IX/V complex on the surface of the platelet (Fig. 2). This process usually occurs under high shear conditions. In low shear or static conditions, platelets adhere predominantly to collagen of the endothelium via their surface gpIa/IIa complex. After either of these initiation steps, gpVI also binds to the exposed collagen and acts as a major signaling molecule for platelet activation. As a result, platelets change their shape and express increased gpIIb/IIIa proteins on their surface. Through a fibrinogen bridge, platelets aggregate via this complex. Several

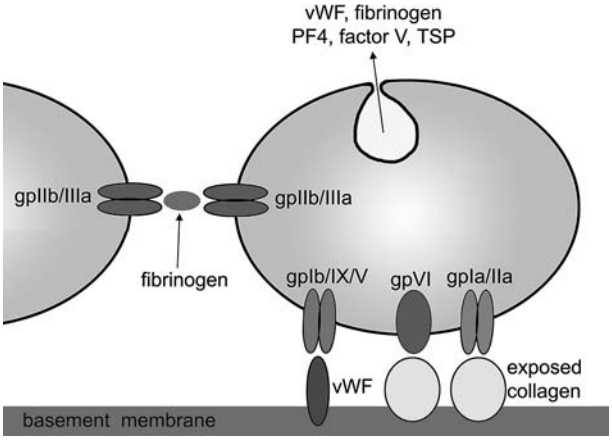

Figure 2. Overview of platelet interactions. When vascular endothelial cells are removed from the basement membrane, platelets can directly bind to the exposed collagen via gpVI or the gpIa/IIa complex, as well as to the vWF. Secretion of specific molecules [*i.e.* thrombospondin (TSP)] promote further platelet formation, and through the gpIIb/IIIa complex these can accumulate on the initial platelet monolayer.

other factors are also recruited to the tethered platelets and, finally, a platelet plug is formed, closing the vessel wall. After activation, platelets release several molecules, including serotonin (vasoconstriction), vWF (adhesion), PDGF, VEGF (mitogenic factors), factor V (coagulation) and protease inhibitors [63].

Role of transcription factors during megakarypoiesis

Several transcription factors have been shown to play an essential role in MK development. Tissue-specific combination of transcriptional regulatory proteins provides a mechanism for proper regulation of relevant target genes in a particular cell type.

Ets genes

The identification of v-Ets related genes from a variety of metazoan species has established the Ets family as one of the largest families of transcriptional regulators, with diverse functions and activities. Each Ets protein contains a conserved winged helix-loop-helix DNA binding (ETS) domain that allows recognition of purine-rich DNA sequences with a core GGA(A/T) consensus, designated EBS (Ets binding sequence) [64–66]. It is clear that Ets proteins often interact *in vivo* with EBS sequences that do not conform to the consensus binding site defined by *in vitro* selection experiments. Binding of Ets proteins to sub-consensus sequences is facilitated by the binding of other transacting factors to cis-elements in proximity to the EBS. Indeed, binding is often mediated by synergistic interaction with transcriptional partners on composite DNA elements. Thus, in addition to binding to DNA, Ets transcription factors participate in protein interactions that affect their functions [67, 68]. Ets factors can function as either positive or negative transcriptional regulators of the expression of genes that are involved in various biological processes, including those that control of cellular proliferation, differentiation, hematopoiesis, apoptosis, metastasis, tissue remodeling, angiogenesis and transformation [69, 70]. While most Ets factors were initially characterized as transcriptional activators or repressors, it has become evident that several Ets factors can function as either activators or repressors, depending upon the specific promoter and cellular context.

Fli-1

Fli-1, a member of the Ets gene family of transcription factors, performs functions critical for normal development and oncogenesis (for a review see [71]). Fli-1 is preferentially expressed in cells of hematopoietic lineages and vascular endothelial cells, and has been shown to transcriptionally activate genes, including the stem cell leukemia gene [72], the anti-apoptotic gene Bcl-2 [73] and several MK-specific genes such as GATA-1 [74, 75], gpIIb [76–78], gpVI [79], gpIX and gpIb [80] and c-mpl [81]. Fli-1 also forms ternary complexes through interaction with SRF to bind to serum response elements of *fos* and Egr-1 promoters [82, 83]. Fli-1 protein interaction with other regulatory proteins modulates their activities [84, 85]. In addition, Fli-1 binding can result in transcriptional repression, dependent on promoter and cell context [86–88].

Fli-1 expression is increased following phorbol ester-induced MK differentiation of K562 cells. Significantly, Fli-1 expression promotes a MK phenotype in K562 cells and increases the expression of MK genes CD41, CD61 [79, 80, 89]. Homozygous loss of Fli-1 in mice was shown to be embryonic lethal and resulted in severe dysmegakaryopoiesis [90, 91]. Accumulated evidence indicates that Fli-1 is as positive regulator of megakaryopoiesis [90–93], while being a negative regulator of erythroid differentiation [73, 94, 95].

Ets-1

Ets-1 is expressed in variety of tissues [96] and has been implicated to play a role in lymphoid development and angiogenesis. Ets-1 mRNA levels increases during phorbol ester induced megakaryocytic differentiation of HEL, Meg01 [76] or K562 [97] cells. Ets-1 levels are elevated and Fli-1 decreased in response to treatment of K562 or HEL pluripotent cells with hemin or arabinofuranoside (Ara-C), agents which induce erythroid differentiation [97, 98]. Furthermore, retrovirus-mediated Ets-1 expression promotes erythroid phenotypes in these cells [98]. Ets-1 has been shown to bind to the PF4 promoter *in vitro* [99]. Ets-1 and GATA-1 showed synergistic activation of the c-mpl and PF4 promoters *in vitro*. Although Ets-1 has been shown to bind to the promoters of gpIIb, c-mpl, gpIX *in vivo*, it appears to be less able to recruit GATA-1 to these megakaryocytic promoters in CMK-5 cells compared with Fli-1 [92]. Consistent with this observation, although Ets-1 binding to the PF4 promoter induces changes in chromatin, it fails to activate MK lineage-specific genes [100]. Thus, the precise role of Ets-1 on regulating MK-specific genes *in vivo* remains unclear, as Ets-1 knockout mice [101–103] do not display any MK defects. Ets-1 does, however, induce increased expression of erythroid markers in the pluripotent erythroleukemic cell lines K562 and HEL [98]. Consistent with a predominate role in erythroid cell rather than MK differentiation, the transcription factor MafB/Kreisler has been shown to negatively modulate Ets-1 function and repress erythroid differentiation [104].

TEL

TEL can act as an inhibitor of Fli-1 transcriptional and biological activity [97, 105, 106]. Overexpression of TEL in K562 cells leads to inhibition of the expression of MK genes and acquisition of erythroid phenotypes [97]. Homozygous loss of TEL leads to embryonic lethality due to yolk sac vascular abnormalities [107]. GATA-1-cre-mediated conditional inactivation of TEL indicates a role of TEL late in MK maturation, and although these mice have increased MK colony-forming cells, they have ~50% decreased platelet counts [108].

Collective results support the notion that multiple Ets family genes expressed in the MK lineage may have specific roles at different stages of development. It is likely that the relative levels of specific Ets factor expression drive MK lineage progression.

GATA-1

GATA-1 is a Cys2/Cys2 zinc finger DNA-binding protein that recognizes and binds the sequence (A/T)GATA(A/G) in the cis-regulatory elements of many lineage-restricted genes [109] (recently reviewed in [110]). Similar to Ets, GATA-1 function is modulated by interaction with other proteins [*e.g.* FOG-1 (discussed below), Sp1, EKLF, Ets (PU.1, Fli-1), p300/CBP]. GATA-1 contains two zinc fingers: The N-terminal finger interacts with FOG-1, while the C-terminal finger is required for DNA binding. GATA-1 is expressed in erythrocytes, MKs, eosinophils and mast cells and has been shown to be essential for maturation of erythroid and megakaryocytic cells [109, 111]. Homozygous null mutation of GATA-1 in mice is embryonic lethal due to anemia [111], while an MK-specific knockdown of these gene results in thrombocytopenia and accumulation of immature MKs [109]. GATA-2 is up-regulated in the absence of GATA-1 [112], and redundancy between GATA-1 and GATA-2 may allow GATA-2 to compensate for GATA-1 deficiency and allow FOG-1-dependent early MK development.

FOG-1

FOG-1 (Friend of GATA-1) is a zinc finger transcription factor, initially discovered as a GATA binding protein, whose erythroid and MK expression pattern coincides with that of GATA-1. While targeted disruption of FOG-1 phenocopies the erythroid defects observed in GATA-1 null mice, unlike GATA-1-deficient mice, FOG-1 mice lack MKs, supporting a critical role in early MK development [113]. FOG-1 has also been shown to bind to GATA-2, which also plays a role in hematopoietic development. Homozygous GATA-2 gene knockout is also embryonic lethal, due to the lack of early hematopoiesis [114].

RUNX1 (AML1)

RUNX1 (AML1, CBFα2, PEBP2αB), a transcription factor expressed in the vasculature and hematopoietic cells, functions as a heterodimeric complex with the core binding factor β subunit (CBFβ). Expression of RUNX1 increases during MK differentiation, and expression is down-regulated during erythroid differentiation, similar to the pattern observed with Fli-1. GATA-1 has also been shown to interact with RUNX1 and RUNX, CBFβ and GATA-1 synergistically activate MK genes (*e.g.* gpIIb) [115]. RUNX1 null embryos die at midgestation with hemorrhage in the central nervous system (similar to that observed for Fli-1 null animals). Mice with conditional RUNX deletion have reduced platelet numbers and small (microMK), hypoploid MKs [116, 117]. Conversely, overexpression in hematopoietic cells leads to megakaryocytic differentiation [118].

NF-E2

Nuclear factor-erythroid 2 (NF-E2) is a bZIP DNA transcription factor expressed in erythroid, megakaryocytic and mast cells. NF-E2 is a heterodimer consisting of the hematopoietic-restricted 45-kDa subunit (p45) and one of three ubiquitously expressed 18-kDa subunits (Maf K, Maf G in MKs). NF-E2 has been shown to regulate transcription of MK genes, including β-tubulin, thromboxane synthase and Rab27b. (For further discussion of the role of putative NF-E2 target genes to MK differentiation, the reader is referred to a recent review [1].) As described above, the terminal phase of MK differentiation, release of platelets from the MK, is dependent upon the formation of proplatelets. Proplatelets are cytoplasmic extensions of the MK and this projection requires microtubuli formation. β1-tubulin, a NF-E2 target gene, is a major component of these microtubuli. Homozygous loss of NF-E2 [119, 120] leads to neonatal lethality due to hemorrhage secondary to a lack of circulating platelets (thrombocytopenia), although these mice have elevated levels of MKs that express PF4 and gpIIb that fail to mature properly. MK progenitors from the NF-E2 null mice showed a reduced response to TPO, indicating that NF-E2 also plays a role in the proliferation of committed MK progenitors [121]. Mice with targeted disruptions of both of the 18-kDA subunits expressed in MK (Maf $K^{-/-}$ Maf $G^{-/-}$) show defective thrombopoiesis (thrombocytopenia) due to impaired proplatelet production, as well as a more severe erythroid phenotype than observed in NF-E2 mice [122].

Transcriptional regulation of megakaryocyte-specific genes

Multiple Ets and GATA-1 binding sites are found within the promoters of MK-specific genes, including gpIX [123], gpIIb [124], c-mpl [81], PF4 [99], gpVI [79], gpV [125] and gpIba [126] (Fig. 3). Often the effects of the transcription factors are synergistic. For example, Ets-1 and GATA-1 synergize at the PF4 [99], gpIIb [76] and c-mpl [81] promoters *in vitro*. GATA-1 and Fli-1 also interact directly *in vitro* and act synergistically to transcriptionally activate the gpIX [80], gpIba [80] and c-mpl promoters [81]. Furthermore, GATA-1 and FOG-1 synergistic activation of the gpIIb promoter is dependent on Fli-1 [77]. In non-MKs both GATA-1 and Ets-1 synergize with MEIS1/PBX2 at the PF4 promoter, which contain tandem MEIS1 binding elements at –219 to –182 [127]. It is worth noting that binding of a single transcription factor is probably insufficient to control gene activation or repression. For example, gene expression and Fli-1 occupancy on the gpIIb promoter *in vivo* in Meg-01 cells, are not correlated. However, transfection/expression of GATA-1 results in gpIIb expression in Meg-01 cells [92].

In summary, the previous studies of the functional role of specific transcription factors in the regulation of most of the MK promoters have been based upon *in vitro* promoter analyses. Recent studies have indicated that such presumptive regulatory sites may not always correspond to sites bound *in vivo*. Transgenic and knockin mice provide experimental systems to define those elements within or region of promoters that direct temporal- and MK-specific expression *in vivo.* For example, a 2-kb genomic fragment that contained the putative 5′-untranslated region of mouse c-mpl gene was found to direct expression of a reporter gene to the MK lineage in both the adult and in the embryo [128]. Using a similar approach, a 245-bp region from the 5′-untranslated region of the human PF4 was demonstrated to be able to direct megakaryocytic specific expression, although expression was integration site specific [129]. As noted above, a promoter knockout that affected expression in MK, while maintaining erythroid lineage expression, was used to define the role for GATA-1 in megakaryopoiesis [109]. Future similar approaches could ultimately be used to mutate specific cis-elements to define the role of specific transcription factors in MK gene expression *in vivo*.

Transcription factors are effectors of intracellular signaling during megakaryopoiesis

As described above, the concerted function of multiple transcription factors play a significant role of proper expression of most megakaryocytic genes. Interestingly, the key final targets of intracellular signaling are transcription factors. Notch signaling has been found to inhibit megakaryopoiesis by suppressing GATA-1 function through Notch-CBF1 transcriptional activation of HES1. HES1 interacts with GATA-1 and results in the dissociation of p300 from GATA-1 bound promoters [130]. GATA-1 also suppresses cell proliferation at several points of c-kit signaling [131]. ERK signaling up-regulates the protein

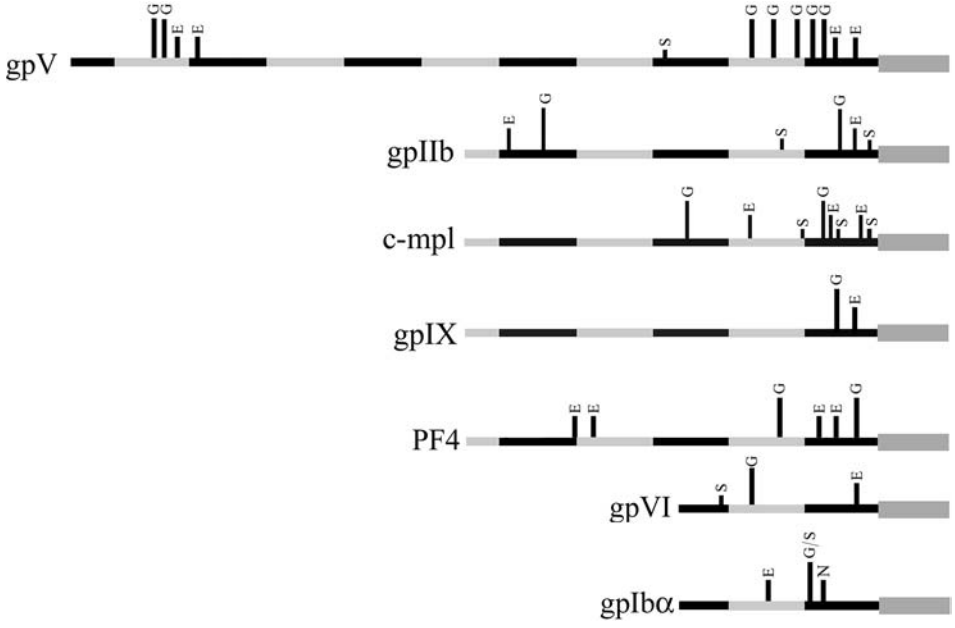

Figure 3. Schematic diagram of promoters of MK marker genes. The wide horizontal gray line represents the 5′ transcribed region of the respective genes. The promoter region is represented by alternating black and gray bars (each bar indicating 100 bp). The vertical bars denote binding sites previously characterized *in vitro* by EMSA and/or by reporter assays. E: Ets; G: GATA; S: Sp1; N: NF-κB.

expression of RUNX1 and CBFβ, and these physically interact and cooperate with GATA-1 [115].

There are numerous studies on the effect of phorbol 12-myristate 13-acetate (PMA) and TPO on MK differentiation (for a review see [2]). A MAPK/ERK inhibitor was shown to inhibit the PMA/TPO-mediated gpIX expression, while a p38/MAPK inhibitor did not have any effect [132]. Furthermore, the Ets site in the gpIX promoter (see above) is necessary for the MAPK/ERK response. Additional intermediate signaling pathways have been implicated in normal and aberrant megakaryopoiesis (*e.g.* PI3K [133], mTOR [134]).

Human conditions associated with altered MK transcription factors or their targets

Fli-1

Individuals affected by Paris-Trousseau Syndrome (PTS) have mild thrombocytopenia with a subpopulation of cells having enlarged α-granules. This syndrome can occur in the context of Jacobsen syndrome, where patients have platelet defects accompanied by mental retardation, cardiac defects, etc. Both of these conditions are associated with deletions on the long arm of the chromosome, including 11q23, where Ets-1 and Fli-1 are located. Thus, these patients have only one copy of these Ets genes due to a heterozygous loss of regions in chromosome 11 and display normal and small, immature, lysing MKs. Lentivirus-mediated Fli-1 expression in $CD34^+$ cells restored normal megakaryopoiesis [135]. It is significant that Fli-1 expression is monoallelic in the intermediately mature $CD41^+CD42^-$ cells, but bi-allelic before and after this stage of megakaryopoiesis [135].

GATA-1

GATA-1 is mutated in patients with two different disorders. GATA-1 mutations have been identified in Down's syndrome patients with transient myeloproliferative disorder (TMD) and acute megakaryoblastic leukemia (DS-AMKL) [136]. These individuals only express a short form of GATA-1 (GATA-1s), which has a different transcriptional regulatory function as shown in animal model studies (see below). Families with hereditary thrombocytopenia have also been characterized as having mutations in the GATA-1 gene. Amino acid substitutions in the N-terminal zinc finger of GATA-1 have been identified in patients with X-linked thrombocytopenia with defects of erythropoiesis. Patients with mutations at amino acids within the N-terminal zinc finger domain (V205, G208, R216, D218; Fig. 4) usually have severe thrombocytopenia with qualitative defects of platelets and MKs, as well as dyserythropoiesis with or without anemia (reviewed in [137]). Four of these GATA-1 mutations (V205M, G208S, D218G, and D218Y) disrupt proper interaction with FOG-1, either being on the FOG-1 interface (V205, G208) or in-between the DNA binding cleft and the FOG-1 interface (D218). The R216Q mutation affects only DNA binding affinity and is associated with X-linked thrombocytopenia with thalassemia (XLTT). Interestingly, the severity of phenotype has been found to be dependent on the specific amino acid substitution. For example, D218G results in thrombocytopenia and mild dyserythropoiesis, while D218Y is more severe, resulting in pronounced anemia and early mortality [138]. The severity of disease in patients with these substitutions is related to the impact on FOG-1 affinity. Similarly, while the Gly208Ser mutation is associated with defective MK development, a G208R substitution was recently reported in a patient with dyserythropoietic anemia and thrombocytopenia [139].

RUNX

A single nucleotide deletion in the RUNX1 gene (Pro218fsTer225) has been identified in patients with

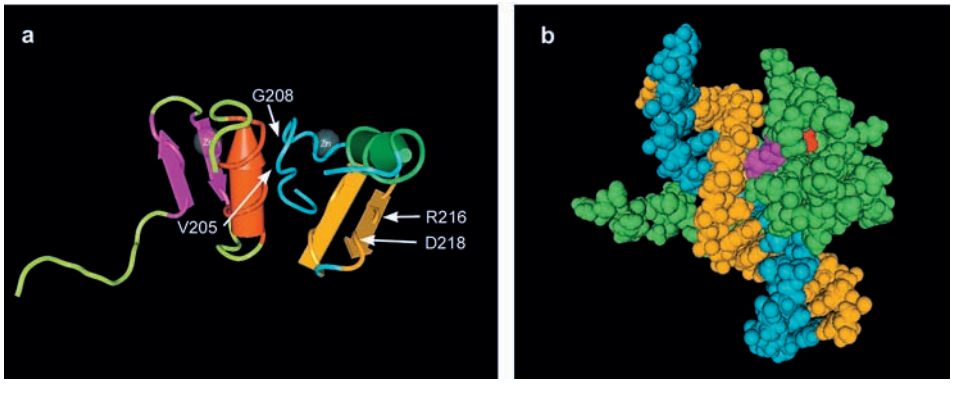

Figure 4. Location of GATA-1 mutations associated with familial blood disorders. (*a*) Structure of the mouse GATA-1 (right) and *Drosophila* FOG-1 (left) complex [185]. (*b*) Chicken GATA-1/DNA complex [186, 187]. GATA-1 is shown in green, R216 highlighted in purple, D218 highlighted in red. Structure coordinates were downloaded from the MMDB database (NCBI) and diagrams were rendered with the Cn3D software [188].

familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) [140, 141]. Affected individuals displayed low Mpl receptor expression and promoter analysis showed several RUNX1 binding motifs in this gene. The leukemic RUNX1-ETO fusion protein represses transcription of RUNX target genes, including GATA-1. Such activity and altered interaction RUNX with GATA-1 and FLi-1 is likely to contribute to the observed thrombocytopenia. Mouse and human HSCs with RUNX-ETO expression have increased capacity for self renewal, concomitant with impaired differentiation.

c-mpl or TPO

Frameshift or nonsense mutations in c-mpl have been found in patients with congenital amegakaryocytic thrombocytopenia (CAMT), who essentially lack bone marrow MKs ([142]; reviewed in [141]). Familial forms of primary or secondary thrombocytosis (elevated platelet count) can be caused by mutations in the TPO receptor (c-mpl) or TPO ligand, which result in overexpression of TPO, constitutive/sustained intracellular signaling, or altered circulating TPO [143].

TPO and other hematopoietic cytokines activate MAPK (mitogen-activated protein kinase), PI3K (phosphoinositol-3-kinase) and JAK-STAT (Janus kinase – signal transducers and activators of transcription) signaling pathways. Raf kinases are well-characterized effectors of MAPK (ERK) signaling, and the MK phenotype of Raf-1 [144] and B-raf$^{-/-}$ [145] further demonstrates the importance of the MAPK signaling pathway. JAK family proteins are important mediators of hematopoietic cytokines. Relative to megakaryopoiesis, an activating mutation (G1849T; Val617Phe) is found about half of patients with essential thrombocythemia (ET) and in 18% (2 of 11) of those with megakaryocytic AML [146].

Surface glycoproteins

Over 100 different mutations in gpIIb/IIIa have been identified in Glanzmann thrombasthenia, which remains the predominant disorder of platelet function [147, 148]. Although these individuals have normal platelet levels, abnormal platelet-specific integrin αIIbβ3 disrupts subunit synthesis, receptor assembly and/or function, preventing αIIbβ3 from binding ligands required for platelet aggregation following vascular injury.

Patients with Bernard Soulier Syndrome (BSS) are characterized by presence of abnormal MKs and platelets [149, 150]. Individuals with BSS have mutations of the gp1b or gpIX genes, which eliminate surface expression of the platelet membrane glycoprotein gpIb-V-IX complex and thus adherence to endothelial cell vWF. DiGeorge syndrome patients present with macrothrombocytopenia and have deletions on chromosome 22q11, resulting in hemizygous gpIb loss. The gpIb also contributes to anchoring platelet membrane to actin cross-linking protein, filamin. The loss of this interaction may also provide a mechanism for the macrothrombocytopenia observed in gpIb knockout mice [151].

Animal models

The generation of transgenic and gene-targeted mice (for review see [152]) has greatly increased our understanding of gene function; however, the phenotype of early mouse models failed to adequately display phenotypes directly related to human diseases. More recently, with increased insights into the molecular mechanisms contributing to the pathogenesis of disease and advancement in genetic engineering to allow temporal and tissue-specific modulation of specific genes, improved models are being generated. Further improvements are likely to be made as mice with alterations of multiple genes are characterized.

Transcription factors

Fli-1

To clarify the physiological role of Fli-1 in hematopoiesis, we [91] and others [90] generated mice with the targeted disruption of Fli-1. The Fli-1 homozygous mutant (Fli-1$^{-/-}$) embryos showed hemorrhage from the dorsal aorta into the lumen of the neural tube and the ventricles of the brain beginning on embryonic day 11.0 (E11.0) and were dead on or before day E12.0. In addition, severe dysmegakaryopoiesis [90, 91] and vascular defects [90] were found. We also noted that livers of the E11.0 Fli-1$^{-/-}$ embryos were pale and contain primarily polychromatophilic and orthochromatic normoblasts [91]. Furthermore, analysis of cultured cells from day 10.0 embryos demonstrated absence of MKs, aberrant red blood cell development, and reduced c-mpl expression [93]. Fetal liver megakaryopoiesis is altered as Fli-1$^{-/-}$ mice show marked decrease in mature:immature MK ratios compared with wild-type (WT) littermates, consistent with a critical role in later stages of MK differentiation. Fli-1$^{-/-}$ bone marrow cells from chimeras created by morula-stage embryo aggregation and rescued from lethality also show decreased levels of c-mpl and GATA-1 mRNA [153]. The Ets protein GABPα has recently been shown to function as a regulator of early megakaryocyte-specific genes [154].

GATA-1

Homozygous deletion of GATA-1 is embryonic lethal due to a failure of primitive and definitive erythropoiesis [155]. Mice with MK-specific knockout of GATA-1 displayed increased proliferation and deficient maturation of MK progenitors, marked reduction in platelet count,

and abnormal platelet morphology and function [109]. Expression of PF4, gpIbα, gpIbβ and c-mpl was also reduced in these animals. Since GATA-1 is located on the X chromosome, GATA-1$^{+/-}$ females have mixed population of wild-type GATA-1 and GATA-1 null cells. These mice showed marked anemia and thrombocytopenia [156]. The authors also noticed accumulation of GATA-1 null MKs in the spleen and also in the hepatic sinuses. Overall, the phenotype of these animals closely resembled the hallmarks of the human myelodysplastic syndrome.

Association of GATA-1 with its cofactor FOG-1 is essential for erythroid and MK development. To assess functions of GATA-1-FOG-1 *in vivo*, transgenic mice harboring a V205G mutation in the GATA-1 gene were generated in a GATA-1 null background [157]. V205G is a FOG binding mutant. High expression of the transgene rescued the embryonic lethality seen in GATA-1 null mice; however, normal level of transgene expression resulted in a much reduced rescue frequency. As discussed above, several GATA-1 mutations are found in human patients that lead to the exclusive expression of GATA-1s. To determine the effect of patient-specific mutations on GATA-1 function, differentiation and gene expression profile between wild-type and GATA-1-deficient MKs were compared. Using homologous recombination, mice were generated that express only this short form of GATA-1 [158]. Interestingly, compared with wild-type GATA-1, the mutated allele failed to down-regulate Myc, Myb, GATA-2 (markers of progenitors proliferation), PU.1 or IKAROS (lineage switch from MEP to GMP). The effect of expression of either GATA-1s or V205G in GATA-1-deficient MKs was also examined. Whereas GATA-1-deficient MKs failed to undergo terminal differentiation and proliferated excessively *in vitro*, GATA-1s-expressing cells displayed proplatelet formation and other features of terminal maturation, but continued to proliferate aberrantly. In contrast, MKs that expressed V205G GATA-1 exhibited reduced proliferation, but failed to undergo maturation. Examination of the expression of MK-specific genes in the various rescued cells correlated with the observed phenotypic differences [159].

FOG-1

FOG-1 is predicted to have nine zinc finger domains, and biochemical studies have demonstrated that four of these are able to interact individually with GATA-1. The function of distinct domains has been examined by assessing the impact of retrovirus-mediated expression of FOG-1 on erythropoiesis and megakaryopoiesis in a FOG-1$^{-/-}$ cell line, similar to the rescue studies described above for GATA-1 [160]. These *in vitro* studies demonstrated that individual domains of FOG-1 influence erythroid *versus* MK differentiation. FOG-1 with mutation (tyrosine to alanine) of all four GATA binding zinc fingers fails to rescue MK and erythroid development. FOG-1 with mutation of three of the four fingers rescued MK and erythroid differentiation, albeit with different efficiencies. These finger mutations and additional deletion analyses indicate that distinct domains differentially affect erythroid *versus* MK maturation.

NF-E2

Mice harboring homozygous null NF-E2 alleles produce MKs; however, these are unable to produce platelets, resulting in absolute thrombocytopenia [120]. These animals had normal PF4 and gpIIb levels in fetal liver, indicating normal early MK maturation. The late maturation affect is independent of TPO. Later studies showed that fetal MK progenitors have reduced proliferation potential *in vitro* [121]. NF-E2 functions as a heterodimer with members of the Maf family. Interestingly, homo- or heterodimers of Maf proteins inhibit expression of NF-E2/Maf target genes, and NF-E2 null mice do not express Rab27b. The gunmetal strain of mice has thrombocytopenia and harbors a mutation in the enzyme RabGGTA1, which targets Rab proteins to distinct membrane compartments. These findings and the similar MKs phenotypes between these mice and NF-E2 mice further support the notion that the NF-E2 target Rab27b is an important mediator of thrombopoiesis. Interestingly, loss of another presumptive NF-E2 target, β1-tubulin, results in reduced platelet numbers [161].

c-Myb

Consistent with a regulatory function for Myb in controlling MK differentiation compared with that of other lineages, mutations in either the DNA binding domain (Ptl3, D152V) or in the leucine zipper domain (Ptl4, D384V) of Myb result in thrombocytosis due to excessive megakaryocytopoiesis, with elevated numbers of MK progenitor cells in the bone marrow and spleen as well as increased numbers of MKs in these organs [162, 163]. A mutation in the transactivation domain in a hypomorphic Myb allele generated by ENU mutagenesis (M303V) resulted in thrombocytosis, megakaryocytosis, anemia, lymphopenia and eosinopenia. This M303V substitution mutation results in 50% transcriptional activity *in vitro*, directly correlated with reduced association with p300.

TPO and c-mpl

Mice with homozygous loss of c-mpl or TPO displayed thrombocytopenia, with ~85% reduction in MK and platelet numbers [164]. c-mpl null mice, however, produce normal level of platelets after 5-FU injection via a TPO-independent mechanism [165]. 5-FU treatment also triggered production of large number of MK progenitors in these animals.

Transgenic mice overexpressing the c-mpl ligand showed a fourfold increase in otherwise normal circulating platelets [166]. MK numbers and MK colony-forming cells in bone marrow and spleen were also elevated. The authors also showed that the c-mpl ligand can also support the maintenance of erythrocyte levels after thromopoietic insult (anti-platelet serum or 5-FU treatment).

Other MK genes

Transgenic mice expressing the SV40 T antigen under the control of the PF4 promoter had low platelet counts and large MKs, and displayed emperipolesis (marrow cells in the MK cytoplasm) and thrombocytopenia [167]. Inactivating one copy of Rb further reduced the platelet level in these mice, which implicates its function in megakaryocytic differentiation. The PF4 promoter was used in a different study to drive MK-specific expression of the c-myc oncogene in an inducible (ER-driven) manner [168]. The affected mice showed frequent immature myeloid cells and increased numbers of MKs characterized by decreased ploidy and size.

Platelets from gpIIb knockout animals failed to bind fibrinogen, did not aggregate and did not react to a fibrin clot, resembling the symptoms of patients with Glanzmann thrombasthenia [169]. Similarly, β3-deficient mice have a Glanzmann thrombasthenia-like phenotype of defective platelets and prolonged bleeding [170].

Mice harboring the homozygous loss of GpIbα displayed the typical characteristics of the human Bernard-Soulier syndrome, including mild thrombocytopenia, giant platelets and bleeding [171]. This phenotype can be rescued by expressing the human GpIbα transgene. Ultrastructural analysis revealed that MKs from affected mice have reduced internal membrane pool [172].

Consistent with the fact that no mutations in the GpV gene have been detected in patients with Bernard-Soulier syndrome, homozygous loss of GpV in mice had no effect on MK development and ultrastructure or on gpIb-IX expression and platelet adhesion [173].

Conclusions and future directions

Megakaryopoiesis is a complex process governed by the concerted functions of many genes. Analysis of each of these genes (especially transcription factors and their selected target genes) individually has begun to provide better knowledge of the process and revealed molecular mechanisms responsible for several human diseases.

The biological function of many of the transcription factors and gene products discussed in this review are controlled at the post-transcriptional and post-translational levels. Ets and GATA family members show alternate splicing, and their mRNAs use alternate translation initiation sites. Ets and GATA-1 function is controlled by phosphorylation-mediated effects on DNA binding, protein-protein interaction, transcriptional activation and subcellular localization (reviewed in [66, 110]). The function of these factors is further regulated by post-translational acetylation (*e.g.* Fli-1, Ets-1, GATA-1) and sumoylation (*e.g.* Ets-1, GATA-1). The impact of these post-transcriptional and post-translational processes on MK proliferation, maturation and differentiation remains to be elucidated.

RNA profiling by serial analysis of gene expression and microarray analysis [174, 175] offers a more detailed analysis of MK differentiation. TPO induced differentiation of $CD34^+$ cells is associated with up-regulation of PF4, annexin A11, s23 and genes in protein synthesis compared with untreated $CD34^+$ cells [174]. Comparing the transcripts of $CD34^+$ $CD38^{lo}$ cells before and after IL-3, IL-6, SCF and TPO induction treatment revealed that hemostasis and platelet-related genes (*i.e.* PF4), adhesion and receptor activity controlling genes (*i.e.* vWF, vWF receptor, gpIIb), genes that play a role in signal transduction [*i.e.* PKC (signaling to gpV, gpIX, gpIIb, gpIb) and Ras/MAPK (enhances GATA-1 activity)] and certain transcription factors (i.e. GATA-1, NF-E2) are up-regulated. As anticipated, genes reported to be associated with lymphoid and myeloid populations were down-regulated, while those important for MK development were not [175]. The identification of expression changes for genes previously shown to be important for MK development provides a rationale to predict that many of the observed changes represent genes with yet to be discovered roles in MK development and function. Similar studies, especially those comparing the transcriptome of patients and healthy individuals will give further insight into MK dysfunction [176]. These explorations will also provide a basis to develop better mouse models for MK-related diseases. Array analysis has the disadvantage of revealing a large number of gene expression changes. Thus, it is critical that a set of selection criteria are developed so that genes for further study can be restricted. While genes demonstrating the greatest differential expression (*e.g.* more than twofold) can be chosen for prioritization, it is important to recognize that the most critical biological changes may not conform to this restriction. The multitude of algorithms being developed is rapidly expanding and such bioinformatic approaches (*e.g.* Gene Ontology Project; GO, www.geneontology.org) allow complex datasets to be examined in a manner to reveal co-regulated network responses in the context of common molecular function, biological process, or cellular component.

Genome-wide chromatin immunoprecipitation analysis will provide an approach to identify those transcription factors and composition of the transcriptional complex present on specific subsets of MK gene transcripts identified by the profiling studies discussed above. As specific

antibodies become available, it will become possible to determine whether specific post-translational modifications affect target gene selection and/or composition of the transcriptional complex.

Micro RNAs (miRNAs) are noncoding 19–25 nucleotide RNA molecules that regulate gene expression by targeting mRNA, inhibiting translation or promoting degradation. Collective data demonstrate that miRNAs participate in many biological processes, including hematopoiesis. A recent study demonstrated that 19 specific miRNAs are down-regulated during MK differentiation. Significantly, some of these may target genes that control megakaryopoiesis (*e.g.* RUNX1, FOG-1, HOXA1). Others are overexpressed in megakaryoblastic leukemia cell lines (*e.g.* RUNX1) [177]. It is possible that down-regulation of specific miRNA could enhance translation of the targeted mRNA and further molecular studies will be required to test the biological significance of these novel observations.

Another area of interest is the mechanisms that regulate protein translation in platelets. Platelets synthesize and release biologically important proteins in the absence of a cell nucleus and transcription [178]. Both constitutive and signal-dependent translation of specific mRNAs are observed in platelets. Signal-dependent translation occurs in response to adherence to different extracellular matrix components or exposure to various soluble agonists.

As mentioned above, after platelet release the MK nucleus is further processed by apoptosis and phagocytosis. Recent studies, however, point out that apoptosis-like events take place during proplatelet formation as well (reviewed in [179]). Overexpression of Bcl-x_L, a known inhibitor of apoptosis, in MKs led to reduced proplatelet formation [180]. Furthermore, in transgenic mice overexpressing Bcl-2, the number of platelets were reduced by half [181]. Caspase-3 and -9 inhibitors blocked proplatelet formation in $CD34^+$-derived MKs, suggesting that these caspases are required for proplatelet formation [19]. However, caspase-3 displayed punctuate cytoplasmic distribution and no DNA fragmentation was seen in these MKs, suggesting that the apoptotic events during proplatelet formation are different from the terminal apoptotic processes. The precise mechanisms and apoptotic pathways that play a role during proplatelet formation are currently unknown. Future studies in these fields will contribute to our understanding of platelet biogenesis and may give some insights into congenital or acquired thrombocytopenia.

Another under-explored area is the identification and characterization of specific protein isoforms that are expressed during megakaryopoiesis. For example, while acetylcholine esterase is a known enzyme and marker for mature MKs, overexpression of a stress-induced truncation variant form (AchE-R) in transgenic mice suggest that AchE-R has thrombopoietic activity [182], which may be mediated by stimulation of progenitor cells [183].

Initial hope for positive therapeutic responses using the major regulator of megakaryopoiesis, TPO, has been tempered by its limited efficacy, due in part to generation of anti-TPO neutralizing antibodies which themselves promoter thrombocytopenia [184]. Thus, understanding the mechanisms of MK development will provide novel insights not only on the pathogenesis of thrombocytopenia described in this review, but also on the pathology and treatment of megakaryocytic leukemia. Such knowledge will also provide additional impact by maintaining proper megakaryopoiesis in cancer patients during and following chemotherapy and other conditions associated with thrombocytopenia (*e.g.* chronic liver disease and AIDS).

Acknowledgements. We apologize to those researchers whose work could not be cited because of space limitations or was only cited indirectly by referring to reviews or more recent publications. This work was supported in part by grants from the NCI [P01 CA78582 (D.K.W.)] and the office of Research and Development, Medical Research Services, Department of Veterans Affairs (A.C.L.).

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