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Megakaryopoiesis: Transcriptional Insights into Megakaryocyte Maturation

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1. ABSTRACT

Platelets are small anucleate cells that travel near the vessel wall during laminar flow. In response to vascular injury, platelets undergo alterations in morphology which allow them to aggregate and cover the injured site. Platelets are produced by megakaryocytes in a process that involves the formation of platelet precursors called proplatelets and subsequent release of these proplatelets into the circulation. By forming a demarcation membrane system within the cytosol, megakaryocytes contain a membrane reservoir which allows for the production of thousands of platelets per mature megakaryocyte. Interestingly, the above process known as megakaryopoiesis is not yet fully understood. However, several groups have contributed evidence to unveil the role of thrombopoietin (TPO), the principal regulator of megakaryopoiesis in vivo. TPO is necessary for megakaryocyte maturation in that TPO deficient mice display greatly reduced megakaryocyte production as well as reduced numbers of mature megakaryocytes. Several transcription factors have also been implicated in megakaryopoiesis including, GATA-1, friend of GATA-1 (FOG-1), nuclear factor-erythroid 2 (NF-E2), and Fli-1. In fact, interactions among some of the transcription factors have been reported to produce synergistic effects. GATA-1 and Fli-1 interactions result in heightened GPIX and GPIb (2 components of von Willebrand Factor (vWF) receptor) expression, while GATA-1, RUNX1 and core-binding factor β interactions result in improved α_{IIb} promoter activity. Mutations in the vWF complex and $\alpha_{II\beta}\beta_3$ have been linked to disorders such as Bernard-Soulier syndrome and Glazmann thrombasthenia respectively. Therefore, a more comprehensive understanding of the transcriptional control of megakaryopoiesis may lead to more effective treatments of platelet-related disorders.

2. INTRODUCTION

In mammals platelets are produced as a result of cytoplasmic fragmentation of a larger cell known as the megakaryocyte. Platelets are anucleate but still possess limited synthetic ability via RNA donated by its parent megakaryocyte as well as the organelles necessary to complete protein synthesis (1). Once released into the bloodstream, platelets travel near the outside of the vessel enabling them to readily respond to a vascular lesion. Platelets remain in the circulation for approximately 10 days and are able to respond to vascular damage as soon as they are produced. Exposure to a site of vascular injury induces a series of signaling cascades that ultimately shift the platelet from an inactive to an active state. In the inactive state platelets maintain a discoid shape and upon activation initiate a series of morphological changes. These alterations in cell shape enable the platelet to cover a much larger area than their inactive structure permits. However, dysregulation of the above processes can lead to several disorders such as Bernard-Soulier syndrome, Glazmann thrombasthenia and Wiskott-Aldrich syndrome. Because platelet function is reliant on megakaryocyte maturation, a better understanding of megakaryopoiesis may aid in treatment of these platelet disorders.

Platelets are spawned from fully mature megakaryocytes. Presently, it is believed that platelets are produced via proplatelets which are defined as long extensions originating from the megakaryocyte which contain many platelet-sized structures (2). It is largely accepted that these proplatelets are formed from a complex, cytoplasmic network contained within the megakaryocyte called the Demarcation Membrane System (DMS). The DMS is believed to form as a result of plasma membrane invaginations which connect within the cytoplasm of the megakaryocyte to produce a membrane reservoir to be used for platelet production (3, 4).

While this process has been documented to occur within the bone marrow, it may also occur in the lung as well as in the circulation. Using electron micrographic techniques, several groups have documented evidence of platelet biogenesis within bone marrow (5-7). Proplatelet extensions appear to emerge from the marrow into the sinusoids where platelets or portions of the proplatelet are shed into the lumen. If portions of the proplatelet are released into the sinusoidal lumen then further maturation must occur in a site other than the bone marrow. The latter stages of platelet biogenesis may occur within the circulation as several investigators have reported proplatelets within the circulation (8-10). To further complicate matters, other investigators have reported platelet production within the lung as megakaryocytes which enter the circulation become trapped in pulmonary capillary beds (11, 12). However, this hypothesis was refuted by Aliberti *et al.* in a study conducted in humans (13). Interestingly, they show no differences in platelet count from radial pulmonary and arterial blood. While controversy exists surrounding platelet biogenesis, it is universally accepted that platelet production in mammals is via megakaryocytes.

Megakaryocytes are large (~60 μM), polyploid cells which, following maturation, produce vast quantities of blood platelets by contributing nearly all of their intracellular contents to that end. One 32N megakaryocyte is capable of producing as many as 3,000 platelets (14). Megakaryocytes are derived from hematopoietic stem cells (HSC) and progression through the megakaryocytic lineage involves development of polyploidy concomitant with an increase in cell size, and expression of certain cell surface markers such as vWF (15), α_{IIb} integrin (16) and β_3 integrin (17). HSC's may follow one of two roads; self-renewal or commitment to a particular cell type. In the case of megkaryocytes, HSC's, which are directed towards differentiation, give rise to colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) (18, 19). CFU-GEMM cells can either become megakaryocyte burst-forming cells, which can produce several hundred megakaryocytes, or a megakaryocyte colony forming cell which produces far fewer megakaryocytes. Subsequent stages include the promegakaryoblast, or stage I megakaryocyte, and the promegakaryocyte, or stage II megakaryocyte.

An extensively studied process necessary for megakaryocyte maturation and eventually platelet production is the development of polyploidy. In a process known as endomitosis, megakaryocytes duplicate their nuclear content and bypass cytokinesis so that the majority of mature megakaryocytes in most species have DNA content of 16N, while some achieve DNA content of 64N and even 128N (20). While the exact mechanisms governing endomitosis have yet to be elucidated, recent experimental evidence allows us to identify several key players. Specifically, GATA-1, nuclear factor – erythroid2 (NF-E2) and the principal regulator of thrombopoiesis, thrombopoietin (TPO), have all been shown to play significant roles in megakaryocyte maturation.

Megakaryopoiesis is executed with the input of several key transcription factors which have the ability to interact with each other, creating a synergistic effect. GATA-1 transcription factor activity is necessary for megakaryocyte differentiation, but without interactions with Fli-1, the GATA-1/FOG-1 complex is unable to induce the expression of the complete vWF receptor complex. Another important transcription factor is RUNX1 and interaction between RUNX1 and GATA-1 is necessary for the expression of the platelet- and megakaryocyte-specific α_{IIb} integrin. GATA-1 may also drive the expression of another important transcription factor, NF-E2. NF-E2 expression has been linked to the expression of other proteins necessary for megakaryocyte maturation like β1-tubulin (21) and Rab27b (22).

TPO was first identified as the ligand for the c-Mpl receptor (23) approximately 15 years ago. Shortly thereafter, TPO was cloned by several different labs (24-28). This spurned a quick expansion of studies surrounding megakaryocyte development and platelet production. Wang *et al.* have recently described the development of megakaryocytes from Rhesus monkey embryonic stem cells treated with TPO (29), presenting a potential method

for studying megakaryocyte maturation at the earliest stages via stimulation with the physiologically relevant TPO. Due to this and many other studies, we now understand that TPO plays a central role in megakaryocyte maturation.

3. SIGNALING PATHWAYS INVOLVED IN MEGAKARYOCYTE MATURATION

3.1. Thrombopoietin

Although the term thrombopoietin was coined several decades ago (30), the polypeptide was not characterized until 1995. TPO is highly conserved in mammals and, although much larger, is more homologous to erythropoietin than any other cytokine (31). Like most proteins found in plasma, it is produced primarily in the liver throughout the lifespan of the individual (32, 33). The full product of the the *TPO* gene is 353 amino acids (34). However, alternative splicing (35) and cleavage via thrombin (36) create splice variants and truncated forms of the polypeptide, respectively. TPO is the ligand for the product of the protooncogene *c-Mpl* which was identified via homology to the murine *v-Mpl* (37) and its sequence similarity to other members of the cytokine receptor superfamily. Gurney *et al.* generated a *c-Mpl* null mouse which displayed an 85% reduction in circulating platelets and megakaryocytes as well as reductions in plasma TPO (38). TPO deficient mice contain less than 10% of total megakaryocytes found in wild-type mice. Additionally, there was a 10-fold reduction of 32N megakaryocytes and a 5-fold reduction of 16N megakaryocytes (39). Clearly TPO is essential to both maintain normal levels of megakaryocytes as well as for complete megakaryocyte maturation. Recent investigation into the signaling initiated via TPO during several stages of megakaryocyte development has enhanced our understanding of the thrombogenic process and illuminated new avenues of exploration.

3.2. TPO-Initiated Signaling in Megakaryocytes

Binding of TPO to c-Mpl (a receptor tyrosine kinase) activates both Janus Kinase 2 (JAK2) and Tyk2 (40). Interestingly, only JAK2 is thought to be required for TPO-induced signaling as Tyk2-deficient cells are capable of initiating TPO-dependent signaling (41). Tyk2, along with JAK2, does appear to regulate TPO receptor localization at the plasma membrane by stimulating recycling and enhancing the stability of the receptor in Ba/F3 cells (42). JAK2 phosphorylation then leads to phosphorylation of the receptor itself at Tyr112, which serves as a docking site for signal transducers and activators of transcription (STAT) 3 and 5 (43). Additionally, adapter proteins such as SHC and SHP2 can bind which leads to the activation of phosphatidylinositol 3-kinase (PI3K) (44).

3.3. Phosphatidylinositol 3-kinase

Cellular signaling involving PI3K has been well studied in many different species and cell types. However there is still much to learn about the role of PI3K in platelets and megakaryocytes. The primary target of PI3K is the serine/threonine kinase Akt (also termed PKB) and phosphorylation at two sites, Ser473 and Thr308, via PI3K is sufficient for Akt activation (45). Once activated, downstream targets of the pro-survival protein Akt include GSK-3β (46), Caspase-9 (47), and BAD (48). Phosphorylation of each enhances cell survival. Additionally, phosphorylation of PI3K via c-Mpl activation has been shown to regulate megakaryocyte binding to fibrinogen. Zauli *et al.* demonstrated that primary megakaryocytes treated with both TPO and wortmannin (an inhibitor of PI3K) displayed a depressed ability to adhere to immobilized fibrinogen (49). PI3K has also been shown to regulate TPO-induced increases in c-myc expression in cell lines and primary megakaryocytes (50). Interestingly, disruption of c-myc regulation in megakaryocytes reduces the number of highly polyploidy cells (51). One target of the PI3K/Akt pathway is mammalian target of rapamycin (mTOR). CD34+ cells treated with rapamycin (inhibitor of mTOR) and TPO showed reduced ploidy and cell size compared to cells treated with TPO alone (52). Concomitant with these changes in cell phenotype, rapamycin treated cells displayed a decrease in cyclin D3 and p21 mRNA as well as protein. Also, rapamycin treatment reduced the amount of proplatelet forming cells, when delivered every 72 hours. Cells cultured for nine days in the presence of TPO and then supplemented with rapamycin were also prevented from forming proplatelets suggesting that mTOR may play a dual role as a regulator of ploidization as well as proplatelet formation. In addition to PI3K activation, TPO also activates Ras through Grb2 and SOS.

Ras activation via TPO initiates activation of the mitogen-activated protein kinase (MAPK) signaling cascade. The MAPK family of serine/threonine protein kinases includes the extracellular signal-regulated kinases (ERK1 and ERK2), and $p38^{MAPK}$ among others. The MAPK's require phosphorylation at two sites (one threonine and one tyrosine) for activation. Activation of these kinases results in transcription of genes that regulate mitosis, meiosis and apoptosis (53).

3.4. Mitogen Activated Protein Kinases

The importance of the MAPK signaling cascade in megakaryocyte differentiation and maturation has been demonstrated in several cell lines as well as in primary CD34+ cells and embryonic stem cells induced to follow the megakaryocytic lineage. Using UT7 cells designed to express a murine c-Mpl, Rouyez and colleagues demonstrated that ERK was quickly phosphorylated with either TPO or PMA treatment (54). Moreover, that activation was maintained for at least 4 days following treatment and reductions in cell growth as well as increases in megakaryocyte specific markers were noted. Interestingly, utilization of a MEK (MAPK activating enzyme) inhibitor revealed that ERK activation was essential for TPO-induced megkarycoyte differentiation, but dispensable for proliferation. Similar experiments were performed by another group using primary cultured CD41+ cells treated with TPO (55). ERK activation was again observed upon TPO treatment however, in contrast to the previous work, ERK activation was only transient suggesting that UT7 cells engineered to express c-Mpl may

have alternate signaling pathways involving MAPK. By then treating CD41+ cells with TPO in the presence of a MEK inhibitor the researchers demonstrated that MAPK signaling may be important for the endomitotic process as the inhibitor reduced the number of highly polyploidy cells in the culture compared to CD41+ cells treated with TPO alone. In embryonic stem cells (ES) transfected to express full length c-Mpl, TPO induced differentiation into all myeloid progenitors and eventually megakaryocytes (56). This technique was then used to introduce mutated forms of the TPO receptor to evaluate which residues are important for downstream signaling. ES cells transfected with a mutated c-Mpl with residues 71-121 of the C-terminal domain deleted failed to achieve the same extent of ploidy as those transfected with full length c-Mpl or cells transfected with a mutated c-Mpl missing residues 71-94. ERK activation was also measured in each of the three cultures and it was determined that those cells devoid of residues 71-121 of the C-terminus of c-Mpl display only weak activation of ERK. Therefore, residues 94-121 of the C-terminus of c-Mpl are required for full ERK activation and subsequent proliferation of megakaryocyte progenitor cells. In addition to signaling mediated by TPO, the MAPK pathway was also shown to influence the effects of the chemokine stromal cell-derived factor 1 (SDF-1).

3.5. Stromal Cell-Derived Factor 1

SDF-1 is an important chemotatic factor for CD34+ cells and mature megakaryocytes (57) as well as for other cells (58). SDF-1 was demonstrated to enhance the growth of cells committed to the megakaryocyte lineage in murine primary cultured bone marrow cells treated with TPO (59). Guerriero *et al.* illustrated increases in polyploidy when human CD34+ cells treated with TPO were supplemented with SDF-1α (60). Furthermore, this phenomenon was enhanced with increasing concentrations of SDF-1α. CD34+ cells treated with TPO or TPO + SDF-1α were also treated with a MEK inhibitor which significantly reduced the number of ploylobated megakaryocytes. Immediate early gene X-1 (IEX-1) plays a role in cell cycle progression and has also been identified as a target for ERK1/2 (61, 62).

3.6. Immediate Early Gene X-1

IEX-1 encodes a protein whose function seems to be cell type specific. In UT7 cells expressing c-Mpl, IEX-1 appears to be involved in ERK activation via TPO in a positive feedback manner (61). IEX-1 was shown to be a transcriptional target of acute myelogenous leukemia 1 (AML1)/Runt-related transcription factor 1 (RUNX1), which is dependant upon phosphorylation via ERK for activation (63, 64). Hamelin *et al.* have recently demonstrated that TPO treatment of primary cultured CD34+ cells results in IEX-1 expression, which can be reversed upon TPO starvation and rescued with subsequent TPO treatment (65). While the exact role of IEX-1 in megakaryocyte development remains unclear, the fact that TPO treatment can so precisely modulate IEX-1 expression suggests that the activity of IEX-1 may be important in TPO-mediated megakaryopoeisis. Recently, a Src family kinase has been identified in the inhibition of TPO-dependent proliferation and may be responsible for reducing the duration of ERK1/2 activtion.

3.7. The Src Family Kinase; Lyn

Src family kinases (Src, Yes, Fgr, Fyn, Lck, Lyn, Blk, Hck) regulate a myriad of cellular effects and one, Lyn, has recently been linked to megakaryopoeisis (66-68). TPO treatment of BaF3 cells engineered to express c-Mpl results in an increase in Lyn activity (67). However, blockage of that activity using a specific Lyn inhibitor as well as a dominant negative Lyn mutant resulted in an increase in proliferation of BaF3 cells following TPO treatment. Lyn kinase inhibition also results in increased phosphorylation of the c-Mpl receptor and a sustained activation of ERK greater than cells treated with TPO alone. Most importantly, $Lyn^{-/-}$ mouse contained megakaryocytes with higher ploidy levels than wild type mice. Additionally, other reports dictate that a $Lyn^{-/-}$ mouse has increased numbers of megakaryocytes, increased megakaryopoeisis, and even increases in Akt phosphorylation following TPO treatment of primary cultured megakaryocytes (66) strongly suggesting that Lyn kinase may play a role, not only in megakaryopoeisis, but also in megakaryocyte survival. A diagram representing TPO related signaling is presented in Figure 1.

4. TRANSCRIPTION FACTORS ASSOCIATED WITH MEGAKARYOPOIESIS

4.1. GATA-1 and Its Cofactor FOG-1

The GATA family of proteins are transcription factors that contain a C-terminal zinc finger for binding the (T/A)GATA(A/G) binding sequence and an N-terminal zinc finger required for stabilization of C-terminal binding in hematopoietic cells. Mutations in the N-terminal zinc finger results in congenital thrombocytopenia or dyserythopoietic anemia. GATA-1, -2, and -3 are involved in hematopoietic development and are commonly found in mast cells, sertolli cells, eosinophils, erythroid cells and megakaryocytes. The protein (GATA-1) is encoded by an X-linked gene (69) and requires the association of its cofactor, friend of GATA-1 (FOG-1) as elimination of FOG-1 results in the absence of megakaryocyte production and hence thrombocytopenia (70, 71). The FOG-1 family of proteins are large muti-type zinc finger polypeptides that are capable of interacting with and altering the activity of GATA proteins. Mutations in FOG-1 confer unfavorable phenotypes just as mutations in GATA.

Mutations in the N-terminal activation domain of GATA-1 are commonly seen in patients with transient myeloproliferative disorder and acute megakaryoblastic leukemia (72-74). These mutations result in the coding of a smaller isoform of GATA-1 called GATA-1s, which has reduced transcriptional activation potential even though it can still interact with FOG-1. Therefore, a normal GATA-1 capable of interacting with its cofactor FOG-1 is essential for megakaryocyte maturation and development (69-71, 75, 76).

A 2001 study by Mehaffey *et al.* revealed that X-linked thrombocytopenia is due to a mutation in GATA-1

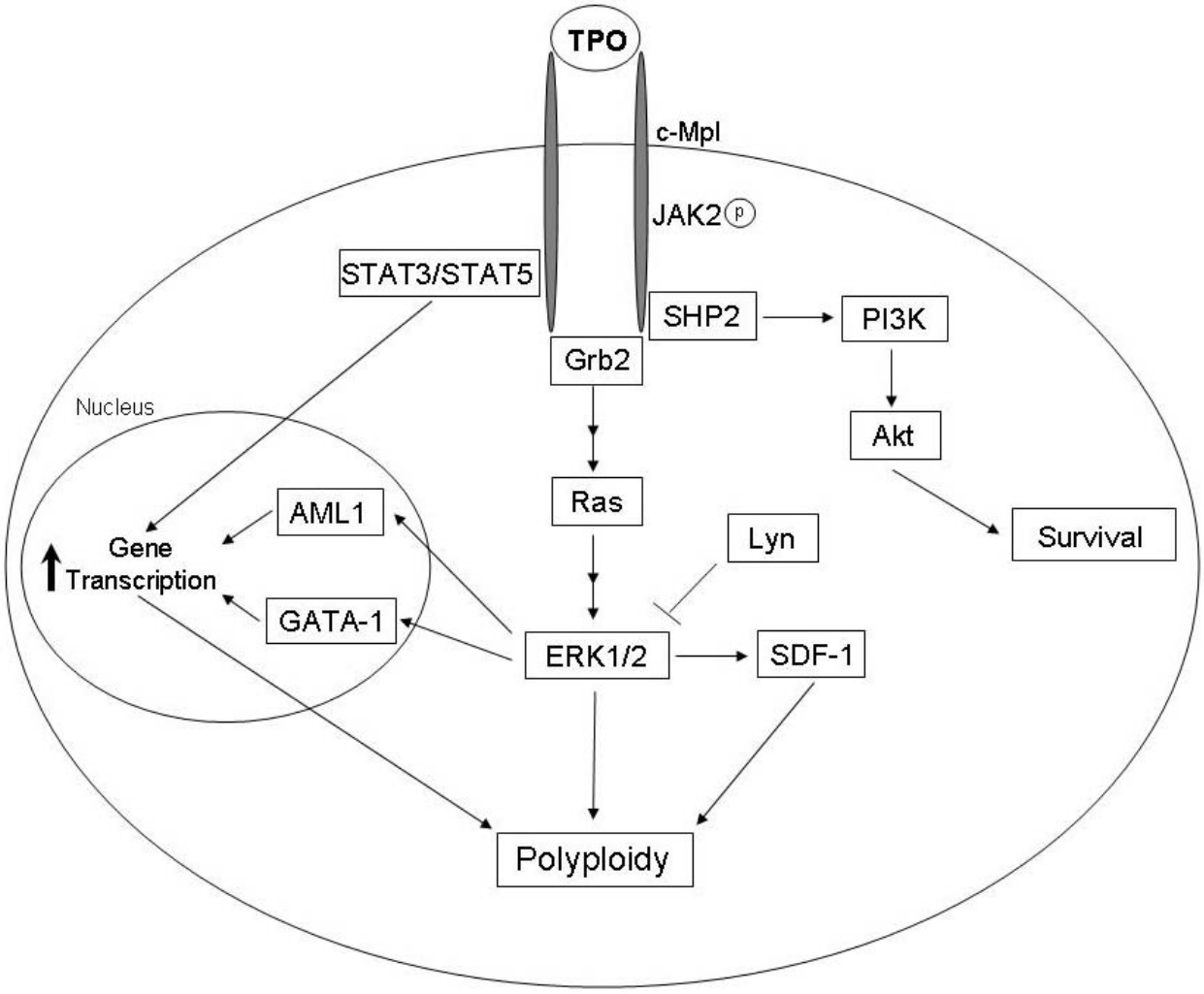

Figure 1. Thrombopoietin signaling during megakaryopoiesis. TPO binds to its receptor, c-Mpl which initiates the phosphorylation of JAK2 and phosphorylation of the receptor at Tyr112. Phosphorylation of Tyr112 provides a docking site for several adaptor proteins. The end result is enhanced cell survival, the development of polyploidy, and transcription of proteins necessary for megakaryocyte maturation.

which inhibits its association with FOG-1 (69). This mutation, which converts glycine 208 to serine, results in abnormal megakaryocyte colony forming units (CFU-MK). These CFU-MKs contained clusters of 3-8 cells which were smaller than normal megakaryocytes and stained strongly for GPIIb/IIIa, as well as large abnormal colonies that stained weakly for GPIIb/IIIa. Additionally, X-linked thrombocytopenia appears to have little effect on erythropoiesis. Using a different GATA-1 mutation which involved substituting glycine 205 for valine, Shimizu *et al.* demonstrated that GATA-1/FOG-1 interactions are necessary during late stage megakaryopoiesis (75). Transgenic mouse deficient in GATA-1 are embryonically lethal due to an inability to initiate erythroid maturity. However, GATA-1 deficient embryos forced to express GATA-1 with a glycine for valine substitution are rescued. This mouse exhibits severe thrombocytopenia without anemia similar to X-linked thrombocytopenia described above. Furthermore, megakaryocytes from these mice are larger and more abundant than megakaryocytes from wild type mice. A more comprehensive analysis of these megakaryocytes revealed an increase in large mononuclear cells and an impaired ability to produce proplatelets. GATA-1 deficient embryos rescued with wild type GATA-1 displayed megakaryocytes similar to wild type megakaryocytes suggesting that an interaction of GATA-1 and FOG-1 is essential for platelet formation. Recently, a role for GATA-1 in the early stages of megakaryocyte and erythrocyte development has been described (77). ES cells from GATA-1 null and wild type mice were treated with TPO and cultured for several months. GATA-1 null ES cells were characterized by hyperproliferation, small size and weak staining for megakaryocytic markers. It was later determined that these cells were bipotent and contained properties of both megakarycyte and erythroid progenitors termed G1ME for GATA-1-megakaryocyte-erythroid. By transfecting these cells with a GATA-1 construct and treating them with a variety of different cytokines, the

investigators were able to induce both megakaryocyte and erythroid development with no crossover of markers between megakaryocytes and erythrocytes. Additionally, the rate of cell division was attenuated in G1ME cells transfected with GATA-1 suggesting that GATA-1 expression inhibits cell division and that this phenomenon is a normal facet of megakaryocyte development. In addition to meditating differentiation toward that of megakaryocytic and erythrocytic lineages GATA-1 also is capable of converting cells of different lineages to that of the megakaryocyte and erythrocyte (78). Clearly, the cell fate decisions mediated by GATA-1 are complex, just as those mediated by FOG-1.

4.2. Friend of GATA-1

It has also been revealed that different domains on FOG-1 regulate erythropoiesis and megakaryopoiesis. This was demonstrated by employing a rescue technique in immortalized hematopoietic progenitor cells from gene-targeted murine embryonic stem cells (70). FOG-1 may serve as a repressor of megakaryocytic differentiation as deletion of the zinc finger region enhanced the ability of FOG-1 to rescue terminal maturation. Also, the amino-terminal domain of FOG-1 seems to be necessary for full megakaryocyte maturation, but not for erythroid differentiation. Therefore, rather than simply serving as a bridge between GATA-1 and DNA, FOG-1 regulates distinct aspects of megakaryocyte and erythrocyte maturation. This raised the possibility that FOG-1 may play a role in megakaryocyte maturation independent of GATA-1. However, results of subsequent experiments suggest that this is not the case and that FOG-1 requires an interaction with GATA-1 to exert its influence on megakaryopoiesis.

Using a gene knock-in mouse technique, Chang *et al.* were able to demonstrate that FOG-1 requires an interaction with GATA-1 or GATA-2 and that disruption of that interaction results in a phenotype similar to a FOG-1 null mouse (71). A knock-in designed to inhibit FOG-1 and GATA-1 interaction was embryonically lethal with erythrocytic maturation halted at an early stage. Yolk sac cells from these embryos did develop megakaryocytes but they were abnormal in that they grew more compactly and formed fewer proplatelet processes. Also, they displayed a disorganized demarcation membrane and a reduced number of platelet-specific granules. In contrast, a knock-in mouse designed to inhibit FOG-1 binding to GATA-2 produced a normal phenotype. A compound knock-in involving both mutations did, however, produce an unusual phenotype with embryomic lethality by E10.5. Yolk sac cells from these embryos failed to produce any megakaryocyte colonies or produce mature erythrocytes like those with a knock-in designed to inhibit FOG-1/GATA-1 interactions. Because yolk sac cells did produce colonies of macrophage and granulocytic cells, the absence of the production of megakaryocytes was not due to the lack of progenitor cells. Given the above evidence, it is fair to conclude that GATA-1 requires FOG-1 to execute its entire program in vivo, but that does not imply that GATA-1 only interacts with FOG-1.

4.3. Other GATA-1 Interactions

Just as GATA-1 interacts with FOG-1 to produce a myriad of cellular effects, GATA-1 also works in concert with other proteins such as Fli-1 and RUNX1 (A.K.A. AML1) to induce further phenotypic alterations. Fli-1 is a member of the Ets family of transcription factors termed for an 85 amino acid region of homology called the Ets domain. Several lines of evidence suggest that Fli-1 is an important transcription factor in megakaryocyte development (79-83). Wang *et al.* have previously reported that a GATA-1/FOG-1 interaction was associated with an increase in α_{IIb} promoter activity and that the α_{IIb} promoter has a functional element that stimulates GATA-1/FOG-1 interactions (84). GATA-1 and FOG-1 interact at an artificial M1α promoter (85) where FOG-1 inhibits GATA-1 activity ~3-fold (86). Only the Ets elements differ between the M1α promoter and the α_{IIb} promoter, suggesting that the Ets elements can regulate the synergy between GATA-1 and FOG-1. This was confirmed when the investigators replaced the Ets element within the M1α promoter with that of the α_{IIb} promoter and the synergistic response observed with GATA-1/FOG-1 interactions was restored. In an effort to determine specifically which proteins bind the Ets site electrophoretic mobility shift assays were used and it was revealed that Fli-1 mediates the transcriptional synergy between GATA-1 and FOG-1. It should also be noted that other proteins have bound to the Ets site but were not revealed. Using a yeast two-hybid system and Fli-1 as bait as well as co-immunoprecipitation, Eisbacher *et al.* revealed that GATA-1 is a partner for Fli-1 (79). Furthermore, the investigators were able to demonstrate a synergistic effect between Fli-1 and GATA-1 as the addition of Fli-1 expression plasmid or GATA-1 expression plasmid modestly induced expression of GPIX and GPIbα, while increasing doses of Fli-1 expression plasmid along with constant GATA-1 plasmid resulted in a 30-fold increase in GPIX and GPIbα promoter activity. GPIX and GPIbα are two of four components of the vWF receptor complex on the platelet membrane. Mutations in this vector complex are often revealed in cases of Bernard-Soulier syndrome, which is characterized by macrothrombocytopenia (87).

4.4. AML1/RUNX1 and Its Interactions Observed in Megakaryopoiesis

The RUNX1 transcription factor is required for megakaryocyte maturation (88, 89) and mutations within RUNX1 are often implicated in leukemia (90, 91). An early marker for commitment to the megakaryocytic lineage is expression of the integrin $\alpha_{IIb}\beta_3$ (16). By monitoring the activity of the α_{IIb} promoter, Elagib *et al.* were able to demonstrate that RUNX1 independently and synergistically with GATA-1 and core-binding factor β (CBFβ) activated α_{IIb} promoter activity and that a dominant-negative RUNX1 inhibited this response (89). Additionally, by co-immunoprecipitation, they were able to demonstrate that GATA-1, RUNX1, and CBFβ exist as a complex and using K562 cells were able to exhibit enhanced α_{IIb} expression with RUNX1 over-expression. GATA-1 expression can direct a cell towards either a megakaryocytic or erythrocytic lineage (78) and RUNX1

expression can direct a cell towards that of the megakaryocytic lineage only (89). Because it has been demonstrated that GATA-1 can complex with other transcription factors besides RUNX1, it is possible that GATA-1 may modulate megakaryopoiesis through these other proteins. Recently, a more comprehensive analysis of alterations in gene expression resulting from a GATA-1 mutation was performed and data from that analysis suggests an even more complex role for GATA-1 in megakaryocyte development.

Using wild type and GATA-1 knockdown megakaryocytes the expression patterns of a host of genes known to influence megakaryopoiesis were illuminated (76). Of the genes affected by GATA-1 knockdown, perhaps the most interesting is α_{IIb} gene, which is in agreement with the above data suggesting that GATA-1 influences the activity of the α_{IIb} promoter (89). Other notable genes downregulated with GATA-1 knockdown are the p45 subunit of nuclear factor erythroid-derived 2 (NF-E2), JAK2, and β1-tubulin. NF-E2 is found only in cells of erythroid and megakaryocytic lineage and a p45 null mouse dies of hemorrhage shortly after birth due to a complete lack of circulating platelets (92). JAK2 is a necessary component of TPO signaling as described above and suggests a role for GATA-1 in cytokine signaling. The downregulation of β1-tubulin could be as a result of the downregulation of NF-E2, since an NF-E2 knockout mouse is nearly devoid of β1-tubulin (21).

4.5. Nuclear Factor Erythroid-Derived 2

NF-E2 is a heterodimer composed of a p45 and a p18 subunit, both of which are members of the leucine zipper family of transcription factors. NF-E2 is expressed in mast cells, erythrocytes, and of course megakaryocytes (93, 94). Small Maf proteins such as MafG or MafF are known to complex with p45 subunit of NF-E2 in primary megakaryocytes and form the p18 subunit (95). NF-E2 knockouts experience severe hemorrhage and very few survive to adulthood (96). Although megakaryocytes are present in NF-E2 null mice, there is a complete lack of circulating platelets suggesting that NF-E2 is required for platelet formation in vivo. Furthermore, blood taken from a mouse deficient in NF-E2 contained small red blood cell fragments and the mouse displayed a hematologic phenotype consistent with splenomegaly and hypochromic anemia highlighting the defective erythropoiesis in this mouse (92, 97). The NF-E2 knockout mouse model has also provided evidence of the proplatelet theory of thrombopoiesis as NF-E2 null megakaryocytes fail to develop proplatelets even in the presence of TPO (98). Recent reports involving NF-E2 have greatly expanded our knowledge of the actions mediated by this transcription factor and are discussed below.

Interleukin-4 (IL-4) and transforming growth factor-β1 (TGF-β1) are negative regulators of megakaryocytopoiesis (99, 100). Using two megkaryoblastic cell lines as well as CD34+ primary human megakaryocytes, Catani *et al.* were able to demonstrate that IL-4, but not TGF-β1, was able to reduce cell proliferation, NF-E2 mRNA or protein expression (100). The investigators have demonstrated that NF-E2 not only regulates proplatelet formation, but also megakaryocytopoiesis. This is the first report utilizing negative regulators of megakaryocytopoiesis. Considering that adminstration of TGF-β1 induces inhibition of both thrombopoiesis (platelet production) and erythropoiesis (99) and that a TGF-β1 knockout mouse displays much higher platelet counts than wild-type controls (101), TGF-β1 clearly plays a significant role in hemostasis. However, that role is not via NF-E2 suggesting that there are multiple cytokines and transcription factors regulating both the enhancement and inhibition of thrombopoiesis.

Rab proteins are ras-related and involved in membrane trafficking and vesicular transport. Of the Rab genes expressed in megakaryocytes, *Rab27B* of the *Rab27* subfamily may play a role in thrombopoiesis (22). The gunmetal mouse model is characterized by severe thrombocytopenia (102) with a gross deficit of Rabα geranylgeranyltransferase, which is necessary for Rab association with the membrane and hence inhibits the function of Rab27B. Phenotypically, the gunmetal mutation mirrors that of the NF-E2 knockout mouse model. Gunmetal mouse megakaryocytes display retarded proplatelet formation with a disorganized demarcation membrane and a paucity of α-granules. Interestingly, the NF-E2 null mouse produces no Rab27B. Using chromatin immunoprecipitation (ChIP) assay Tiwari *et al.* were able to demonstrate that NF-E2 interacts with the *Rab27B* gene locus implying that NF-E2 may directly regulate Rab27B expression (22). Introduction of a dominant-negative Rab27B into wild type megakaryocytes resulted in attenuated proplatelet formation identifying a role for Rab27B in platelet production.

An interesting connection between the NF-E2 null mouse and proplatelet formation was revealed when 3β-hydroxysteroid dehydrogenase (3β-HSD) was identified as a target of NF-E2 (103). There are two isoforms of 3β-HSD expressed in wild-type megakaryocytes (I and VI), but neither are expressed in NF-E2 null megakaryocytes. Interestingly, there is no 3β-HSD expressed in wild type embryonic stem cells suggesting that expression occurs following differentiation to the megakaryocytic lineage. NF-E2 deficient megakaryocytes are also deficient in β1-tubulin, however introduction of β1-tubulin fails to induce proplatelet formation. When β1-tubulin was introduced with 3β-HSD, proplatelet formation was restored. Finally, 17β-estradiol was the only steroid hormone capable of initiating proplatelet formation whereas estrogen receptor antagonists blocked proplatelet formation. These studies highlight the unusual nature of megakaryocyte maturation and platelet formation, a process that includes a variety of cytokines, transcription factors, and even unexpected cell-cell contacts and steroid hormones.

4.6. Other Factors Involved in Megakaryopoiesis

While GATA-1 and NF-E2 transcription factors play a major role in megakaryopoiesis and have been extensively studied in the last several years, they are not the only factors influencing megakaryopoiesis and

thrombopoiesis. Here, we will briefly discuss some other important proteins involved in regulating megakaryopoiesis. Two members of the Ets family of transcription factors, Fli-1 and TEL, are tightly regulated among cells of the erythroid and megakaryocytic lineages and genetic manipulations of each have lead to interesting phenotypes (80, 83, 104-108). Also a member of the Src family of tyrosine kinases Lyn, as well as the adaptor protein Lnk, have been linked to megakaryopoiesis (66, 67, 109) as well as the mammalian target of rapamycin (mTOR) (52). Recent evidence suggests that IEX-1 may regulate the sustained ERK activation typically observed with megakaryocyte differentiation (65), and that BACH1 may repress megakaryocyte-specific transcription (110).

4.7. The Transcription Factor Fli-1

As stated above Fli-1 and GATA-1 are synergistic as Fli-1 can convert FOG-1 from a repressor of GATA-1 into an activator of GATA-1. Fli-1 has two nuclear localization sequences and either sequence is capable of targeting Fli-1 to the nucleus (111). Fli-1 and other Ets family transcription factors have been identified as essential to normal megakaryopoiesis and hemizygous deletions in Fli-1 have been linked to thrombocytopenia (112). In 1996, via mutation and deletion analysis, Deveaux *et al.* revealed that the *c-Mpl* promoter region can be bound by Ets proteins, specifically Fli-1 (104). To examine that functionally, Athanasiou et al treated K562 cells with PMA (which directs differentiation of these cells along the megakaryocytic lineage) and noted an increase in expression of Fli-1 as the cells developed a megakaryocytic phenotype (107). In addition to activating the *c-Mpl* promoter, Fli-1 also activates other promoter regions on genes necessary for development of megakaryocytic features. For instance, Fli-1 can transactivate the *GPIX*, *GP1bα*, and α_{IIb} promoters in 293T cells (80). The platelet-specific collagen receptor *GPVI* gene is upregulated with TPO treatment suggesting that it may play a role in megakaryopoiesis and platelet production and its regulation is via Fli-1 along with GATA-1 and Sp1 (81). Interestingly, overexpression of Fli-1 in K562 cells resulted in increased expression of the GPVI gene. A potential mechanism for Fli-1 transcription and subsequent regulation of Ets-dependent promoters was revealed when K562 cells were treated with IL-6 in the prescence of various signaling protein inhibitors. It was revealed that STAT3 activation is essential for Fli-1 upregulation (105). Since STAT3 is also activated in response to TPO, this provided more evidence for the importance of TPO in megakaryocyte development. A Fli-1 knockout mouse has been developed, however this deletion was embryonically lethal at day E12 (112, 113). It has also been demonstrated that Fli-1 upregulation may play a dual role since overexpression results in megakaryocyte formation and deletion is associated with a lack of functional megakaryocytes, but also an increase in erythrocyte production (107, 114). Therefore, Fli-1 expression appears to be tightly controlled as dysregulation results in over-proliferation of either megakaryocytes or erythrocytes.

4.8. The Cap'n'colar BACH1

Certain erythroid and megakaryocytic genes contain a musculoaponeurotic fibrosarcoma (maf) oncogene recognition element (MARE), which can be bound by transcription factor heterodimers such as the Maf and Cap'n'colar (CNC) family members. One such CNC is BACH1, which is a repressor of transcription. Interestingly, NF-E2 was the first transcription factor revealed to have this configuration with the p45 subunit most closely resembling a CNC and the p18 subunit resembling a maf (94, 115, 116). A BACH1 transgenic mouse directed to express BACH1 at a high level was thrombocytopenic and displayed reduced expression of the p45 target genes β1-tubulin, thromboxane synthase, and 3β-HSD (110) strongly suggesting that BACH1 is a repressor of p45-dependent transcription.

5. SUMMARY

Megakaryopoiesis is a tightly-controlled process whereby extensive changes in morphology, protein expression, and ploidy transform a HSC into a giant cell capable of producing platelets. The primary in vivo regulator of megakaryocyte differentiation is TPO and binding of TPO to its receptor (c-Mpl) initiates a cascade of signaling culminating in altered transcriptional regulation (as outlined in figure 1). One consequence of the interaction of TPO and its receptor c-Mpl is a sustained ERK activation which appears to be essential for proplatelet formation, but not for proliferation. For megakaryocytes to develop "normally" fine regulation of certain transcription factors is essential. Deletions in GATA-1, NF-E2, and Fli-1 all produce severe defects in megakaryopoiesis. Explorations of the precise interactions of these transcription factors revealed that binding of other transcriptional regulators in concert with GATA-1, NF-E2, and Fli-1 will determine the extent of expression of their respective target genes. For instance, RUNX1 interactions with GATA-1 are necessary to activate α_{IIb} production and subsequent cell adhesion to fibrinogen. Additionally, other proteins such as IEX-1 and BACH1 have been introduced to the megakaryopoietic equation suggesting that there is still much to be elucidated. However, recent advances have heightened our understanding and revealed new avenues to explore the mechanisms leading to megakaryocyte maturation. Forthcoming reports regarding the transcriptional control of megakaryopoiesis should begin to intertwine these ideas, hopefully presenting a clear picture of megakaryocyte development.

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