Transcription factor defects causing platelet disorders

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Abstract

Recent years have seen increasing recognition of a subgroup of inherited platelet function disorders which are due to defects in transcription factors that are required to regulate megakaryopoiesis and platelet production. Thus, germline mutations in the genes encoding the haematopoietic transcription factors RUNX1, GATA-1, FLI1, GFI1b and ETV6 have been associated with both quantitative and qualitative platelet abnormalities, and variable bleeding symptoms in the affected patients. Some of the transcription factor defects are also associated with an increased predisposition to haematologic malignancies (RUNX1, ETV6), abnormal erythropoiesis (GATA-1, GFI1b, ETV6) and immune dysfunction (FLI1). The persistence of MYH10 expression in platelets is a surrogate marker for FLI1 and RUNX1 defects. Characterisation of the transcription factor defects that give rise to platelet function disorders, and of the genes that are differentially regulated as a result, are yielding insights into the roles of these genes in platelet formation and function.

Key words

Transcription factor defects, RUNX1, FLI1, GFI1b, ETV6, GATA-1, platelet disorders

1. Introduction

Inherited platelet disorders, accompanied by normal or reduced platelet counts, account for a significant proportion of bleeding diatheses and have been defined historically according to whether the major feature was a quantitative abnormality affecting the platelet count, or a qualitative abnormality affecting platelet function. Over the last 15 years, however, characterisation of the underlying genetic causes of many platelet disorders has allowed better discrimination between these disorders, and classification based on whether the abnormality affects platelet adhesion, platelet receptor signalling, platelet secretion, the platelet cytoskeleton, platelet procoagulant activity and, or platelet production.[1,2] The application of next generation sequencing technology for whole exome or genome analysis is facilitating identification of further underlying genetic defects in patients with previously unexplained platelet disorders.[3] Indeed, this approach has been used in the investigation of an interesting group of platelet disorders caused by defects in transcription factors which are required to regulate megakaryopoiesis, and platelet production, and which can result in both quantitative and qualitative platelet abnormalities.[4] Notably, the number of transcription factor defects identified in patients with platelet function disorders is growing and these appear to account for a significant number of cases.

In this review, the processes involved in megakaryocyte development and platelet production will be outlined briefly before focusing on those transcription factors that are required to regulate megakaryopoiesis and which have been shown to harbour mutations in patients with platelet disorders.

2. Megakaryopoiesis and platelet production

Like other blood cells, megakaryocytes are derived from pluripotent haematopoietic stem cells (HSCs) that reside mainly in the bone marrow and undergo differentiation through discrete steps to give rise to increasingly committed progenitors, including the bipotent megakaryocyte-

erythroid progenitors (MEPs) which are the precursors of cells of both the erythroid and megakaryocytic lineages.[5] Under the regulation of thrombopoietin (TPO), early megakaryocytes undergo a proliferative stage during which progression through the cell cycle is identical to that of other HSCs. This is followed by several rounds of endomitosis where the diploid promegakaryocytes undergo DNA replication without cell division, to accumulate a DNA content of 4N up to 128N in a multilobular nucleus.[6] Following nuclear polyploidization, further maturation of the megakaryocytes is accompanied by formation of an extensive invaginated membrane system, which is continuous with the plasma membrane and permeates the cytoplasm and is thought to act as a membrane reservoir that supports proplatelet formation.[6,7] It is also characterised by the development of alpha (α) and dense (δ) storage granules in the cytosol and by the expression of cell surface receptors that mediate platelet adhesion and aggregation.[8] Thus, megakaryocytes mature into large cells of 150 μM or more in diameter which contain all the necessary cellular machinery for functioning platelets. It is thought that endomitosis occurs during megakaryopoiesis in order to support the protein and membrane synthesis required for production of platelets, through functional amplification of the genome,[9] and indeed there is some evidence for a correlation between the extent of ploidy of megakaryocytes and the number of platelets that they produce.[10]

The precise mechanism by which platelets are derived from megakaryocytes is not fully understood, but the widely accepted model is one in which the megakaryocytes develop multiple, long, branching processes, known as proplatelets, which are decorated along their lengths by platelet-sized, bead-like swellings that are connected by cytoplasmic bridges.[8] The proplatelets extend into the sinusoidal blood vessels of the bone marrow, where the blood flow results in release of their terminal buds into the circulation as platelets. In this way, each megakaryocyte is estimated to release in the region of 10^4 platelets into the circulation,[11] with the average human producing approximately 10^{11} platelets daily.[12]

The formation of proplatelets is a microtubule-driven process, and normal microtubule function is essential to support elongation of the proplatelet branches and accumulation of platelet granules and other cellular components in the proplatelet buds. The importance of microtubules to thrombopoiesis is evidenced by the block in proplatelet production that occurs when megakaryocytes are treated with drugs that disrupt microtubule assembly.[13] Similarly, mutations in the genes encoding tubulin,[14–16] or affecting myosin IIa activity are associated with macrothrombocytopenia.[17,18]

3. Role of platelets in primary haemostasis

Once released into the circulation, platelets have an average lifespan of 9 days and do not interact significantly with the endothelium within the intact vasculature. However, when blood vessel damage leads to exposure of the subendothelial matrix, platelets rapidly accumulate at the site of injury to mediate formation of a fibrin-rich platelet plug that stems bleeding into the surrounding tissue.[19] The initial interactions between platelets and the subendothelium are determined to a large extent by the location of the injury. In areas of low shear, such as in veins and larger arteries, platelet adhesion is mediated primarily by direct interaction of platelet receptors with exposed collagen and other adhesive proteins. In areas of high shear, such as in small arteries and the microvasculature, the platelet glycoprotein (GP) Ib-IX-V receptor complex mediates tethering of platelets to the site of injury through von Willebrand factor (VWF) which is bound to collagen in the extracellular matrix. This interaction allows more stable adhesion of platelets through engagement of their GPVI and integrin $\alpha_2\beta_1$ collagen receptors, triggering intracellular signalling pathways that lead to changes in intracellular free calcium, remodelling of the platelet cytoskeleton, and aggregation of platelets through crosslinking of the activated integrin $\alpha_{IIb}\beta_3$ receptors on different platelets via fibrinogen.[19] Fusion of the intracellular secretory granules of the platelets with the outer platelet membrane and the controlled release of their contents into the local environment also occur mediating further platelet activation and responses with other cells.[20]

4. Transcription factors are key regulators of platelet formation

Transcription factors and their co-activators regulate lineage-specific gene expression during haematopoiesis by binding to cis-regulatory elements located upstream of the promoter of specific target genes to either activate or repress transcription. Knowledge of the key regulatory role of transcription factors in the lineage decisions involved in megakaryopoiesis and platelet production has expanded rapidly in recent years largely as a result of the development of procedures that allow the growth and differentiation of megakaryocytes from HSCs in vitro, and also characterisation of the roles of specific transcription factors by mutating the corresponding genes in mice. More recently, the identification of patients with defects in transcription factor genes that result in disturbances in platelet number and function have provided insights into their role in platelet formation and function. Thus, germline mutations affecting the haematopoietic transcription factors RUNX1, GATA-1, FLI1, GFI1b and ETV6 have been shown to result in platelet dysfunction, thrombocytopenia and variable bleeding symptoms in patients. The roles of these five transcription factors in haematopoiesis will be briefly described (Fig. 1)[21], and the germline defects that have been identified in these genes will be summarised in the remainder of this review.

4.1 RUNX1

4.1.1 Structure and function of RUNX1

Runt-related transcription factor-1, or RUNX1 (also known as core binding factor alpha, CBFα, or acute myeloid leukaemia 1, AML1) is one of three RUNX family members seen in mammals, which binds CBFβ to form the heterodimeric core binding factor (CBF) transcription complex. In humans, heterozygous germline defects in RUNX1 are associated with Familial Platelet Disorder with predisposition to Acute Myeloid Leukaemia (FPD/AML; MIM 601399). The RUNX1 gene, RUNX1, is located at chromosome 21q22.12 and comprises eight exons. Alternative splicing gives rise to at least three RUNX1 variants which differ in size. The largest and least abundant variant, RUNX1c, comprises 480 amino acids and differs from RUNX1b

by having a distinct 27 amino acid N-terminal region. There is no apparent difference in function between RUNX1b and RUNX1c. The smallest variant, RUNX1a, comprises 250 amino acids and has a similar N-terminal region to RUNX1b, but differs in the C-terminal region.[22–24] For ease of reference, amino acid residues in RUNX1 are numbered here according to the RUNX1b sequence (Fig. 2). All three isoforms share a well characterized 128 amino acid Runt domain, which is necessary for binding to CBFβ and also to the consensus sequence 5'-PyGPyGGTPy-3' in DNA.[25] The structure of the Runt domain has been solved and the regions that interact with CBFβ and DNA have been shown not to overlap. The interaction with CBFβ increases DNA binding of RUNX1 by stabilizing the Runt domain in a high affinity DNA binding conformation. The C-terminal regions of RUNX1b and RUNX1c also encompass a transactivation domain which harbours a nuclear matrix-targeting signal and is essential for in vivo function, an inhibitory domain, a proline rich PY motif that allows interaction with WW domain-containing proteins and a C-terminal VWRPY motif that it is thought to be involved in transcription repression.[26]

RUNX1 is critical for definitive haematopoiesis, and knockout of the mouse Runx1 gene is lethal in utero. Conditional deletion of the gene also demonstrated an essential role for RUNX1 in megakaryopoiesis, with megakaryocytes showing a reduction in polyploidization and cytoplasmic development.[27] RUNX1-mediated downregulation of non-muscle myosin IIB heavy chain (MYH10) is required for the switch from mitosis to endomitosis that occurs during megakaryocyte differentiation,[28] and persistence of MYH10 in platelets is a biomarker for FPD/AML, and for thrombocytopenia due to deletion of the transcription factor FLI1.[29] By itself, RUNX1 is not considered to be a strong transcriptional regulator. However, through its synergistic interactions with other transcription factors, it exerts strong transcriptional activation or repression. For example, it co-operates with the transcription factors GATA-1 and FLI-1 to promote megakaryocyte differentiation,[30,31] and with C/EBP factors to activate the macrophage colony-stimulating factor receptor promoter,[32] and several proteins have been identified that interact with motifs in the C terminus of RUNX1 to mediate its activities.[27]

4.1.2 RUNX1 and inherited platelet dysfunction

The autosomal dominant bleeding disorder known as FPD/AML was first described by Dowton et al in a large French-Canadian family. The predominant feature of the disorder was a mild to moderate thrombocytopenia which was also associated with a prolonged bleeding time.[33] Platelets from the affected family members showed reduced aggregation in response to collagen and epinephrine, but aggregated normally in response to ADP and ristocetin. The platelet abnormality was associated with haematologic neoplasms in several members of the family. Subsequent linkage studies in six further families displaying a phenotype similar to that described by Dowton et al, identified a locus on chromosome 21q, and analysis of candidate genes in this region identified nonsense defects or intragenic deletions affecting RUNX1 in affected members of four families, while missense defects predicting amino acid substitution of two conserved amino acids in RUNX1 (p.Arg139Gln and p.Arg174Gln) were identified in the remaining two families.[34] The effects of the RUNX1 defects on megakaryocyte growth were examined by culturing megakaryocytes from peripheral blood and bone marrow from affected members of two of the families, which revealed a reduction in megakaryocyte colony size and formation compared to the controls. Thus haploinsufficiency of RUNX1 was proposed to be the underlying cause of the platelet defect, and to predispose to the acquisition of further somatic mutations that cause leukaemia.[34]

The recognition of FPD/AML increased awareness of the condition and led to an increase in the number of cases reported, allowing better understanding of the clinical expression and molecular pathogenesis of the disorder. Several germline RUNX1 defects have been identified, the majority affecting regions encoding the Runt domain though a nonsense defect occurring outside of the Runt domain has also been identified, p.Y260X.[35] Review of the literature in 2011 revealed the clinical presentation of FPD/AML to be highly variable, patients usually presenting with mild to moderate bleeding symptoms which first appear during childhood, and which are characterised by quantitative and, or, qualitative platelet defects,

and mild thrombocytopenia with normal sized platelets. However, many patients have no bleeding tendency and thrombocytopenia is not always a feature.[36]

4.1.3 Explaining the platelet disorders caused by RUNX1 defects

Several aspects of megakaryopoiesis and platelet function are known to be impaired in FPD/AML. Investigation of the ability of TPO to promote megakaryocyte maturation through its receptor c-Mpl in a patient with a p.Thr219Argfs* RUNX1 defect revealed elevated levels of TPO in the patient, while expression of c-Mpl was reduced and TPO signalling was impaired. Levels of MPL mRNA were also lower in platelets from the patient than in wild-type platelets. The identification of several potential RUNX1 binding sites in the MPL promoter suggested that MPL is a target for RUNX1 and that mutations in RUNX1 are likely to result in a reduction in MPL expression, which would contribute to the thrombocytopenia in affected patients.[37] More recently, further investigation of platelets from carriers of the pThr219Argfs* RUNX1 defect, revealed a decrease in platelet aggregation, impaired $\alpha_{IIb}\beta_3$ activation and reduced α- and δ-granule content. Decreased expression of the transcription factor p45 NF-E2, which is essential for $\alpha_{IIb}\beta_3$ inside-out signalling and for the development of normal granule content, was also demonstrated and the possibility that reduced NFE2 expression could contribute to the functional abnormalities was supported by the finding that RUNX1 could bind the NFE2 promoter and activate its expression in primary megakaryocytes.[38]

Further insights into the mechanisms underlying FPD/AML have been gained from expression profiling of platelets from a patient with FPD/AML and a splice site defect in RUNX1 which revealed 298 genes that were significantly downregulated when compared with wild-type platelets. Of these, MYL9, encoding myosin light chain-9 (MLC) regulatory polypeptide, showed the greatest downregulation, correlating with the reduction in agonist-induced MLC phosphorylation observed in the patient's platelets.[39] MYL9 was subsequently demonstrated to be a transcriptional target of RUNX1, suggesting that the thrombocytopenia associated with RUNX1 defects is at least partly explained by reduced MYL9 expression and the resulting

decrease in MLC phosphorylation.[40] Further studies from the same group showed that in addition to MYL9, several other genes which were downregulated in platelets from the FPD/AML patient were direct targets for transcriptional regulation by RUNX1, contributing to the functional abnormalities observed in the FPD/AML platelets. Thus, the decreased agonist-induced production of 12-hydroxyeicosatetraenoic acid that was observed in the FPD/AML platelets was explained by the downregulation of ALOX12, the gene encoding 12-lipoxygenase.[41] Similarly, a decrease in platelet pleckstrin phosphorylation and protein kinase C-θ (PKC-θ, gene PRKCQ) was explained by the finding that PRKCQ is regulated by RUNX1 in megakaryocytes.[42] Likewise, platelet factor 4 (PF4) was found to be a transcriptional target of RUNX1, explaining the PF4 deficiency associated with RUNX1 haplodeficiency.[43] More recently, RUNX1 was demonstrated to activate PF4 synergistically with the transcription factors CBFβ, ETS-1 and FLI-1 during megakaryocytic differentiation.[44]

Two preliminary studies reported that the genes encoding RAB1B (RAB1B), a low molecular weight GTPase that is essential for vesicle transport between the endoplasmic reticulum and the Golgi apparatus, and Pallidin (PLDN), which is involved in granule vesicle biogenesis, were both downregulated in RUNX1 haplodeficiency, and shown to be direct targets for RUNX1 regulation, providing possible mechanisms for the defective granule biogenesis and secretion observed in RUNX1 deficiency.[45,46]

4.1.4 Association of RUNX1 defects with haematological malignancy

The incidence of myelodysplastic syndrome (MDS) or AML in families with FPD/AML is greater than 40% and the median age of onset is 33 years.[36] The observation that dominant-negative variants of RUNX1 are associated with a higher risk of haematological malignancy than defects causing haploinsufficiency of RUNX1 suggests that RUNX1 dosage contributes to the risk of malignancy.[47] The identification of RUNX1 defects predicting haploinsufficiency of RUNX1 in members of three families diagnosed with a mild bleeding tendency characterised

predominantly by a failure in dense granule secretion, none of whom had a personal or family history of haematological malignancy, would support this observation.[48]

FPD/AML provides a useful model in which to study the alterations in haematopoiesis as a result of a germline RUNX1 defect and the subsequent progression to leukaemia. Comparison of haematopoiesis in two families with FPD/AML, one without AML and a mutation causing haploinsufficiency of RUNX1 (p.R139X) and a second with a strong predisposition to AML and a dominant negative RUNX1 defect (p.R174Q) revealed an increased clonogenic potential of immature progenitor cells bearing both defects. This correlated with markedly reduced expression of the nuclear receptor NR4A3 in CD34+ cells from patients with the p.R139X variant but undetectable NR4A3 expression in cells from patients with the p.R174Q variant. Given that NR4A3 is a transcriptional target of RUNX1, and its deficiency predisposes to AML in mice, it has been suggested that its downregulation can increase the pool of cells that are susceptible to secondary events predisposing to leukaemia.[49] Interestingly, the potential for treatment of FPD/AML was recently demonstrated when the in vitro correction of a p.Y260X RUNX1 defect by gene targeting in induced pluripotent stem cells (iPSCs) derived from two affected patients produced a cell line that exhibited normal megakaryopoiesis.[50]

4.2 GATA-1

4.2.1 Structure and function of GATA-1

GATA binding protein-1, or GATA-1 (previously known as GF1), belongs to the GATA family of transcription factors. It is highly expressed in erythrocytes, megakaryocytes, mast cells and eosinophils, and promotes differentiation by activating lineage specific genes associated with megakaryopoiesis and erythropoiesis, and silencing genes associated with undifferentiated proliferative states.[51] The GATA1 gene lies on the X-chromosome, at position Xp11.23, and comprises 6 exons that encode a 413 amino acid protein which contains two conserved zinc finger domains (Fig. 3). Two splice variants of GATA1 are normally detected in peripheral blood RNA, a full length form, and a shorter GATA1s isoform derived from skipping of exon

2.[52] The C-terminal zinc finger (CF) interacts with DNA through the canonical (A/T)GATA(A/G) consensus sequence and is essential to the function of GATA-1 in regulating megakaryopoiesis and erythropoiesis. GATA DNA binding sites have been identified in several megakaryocyte-specific genes including those encoding GPIbα, GPIbβ, GPIIb, GPIX and GPVI.[53–56] The N-terminal zinc finger (NF) is required to stabilize DNA interactions by binding to non-canonical GATC and palindromic ATC(A/T)GATA(A/G) motifs.[57–59] It also binds to co-regulators, including zinc finger protein, Friend of GATA1 (FOG1; also known as ZFPM1) which is required for activation and repression of the majority of GATA1-regulated genes.[60–62] Through LIM-only protein (LMO2), the NF of GATA1 also binds to the oligomeric TAL1 complex comprised of E2A, LMO2, Ldb1 and TAL1 at sites of transcriptional activation, and LMO2, Ldb1 and TAL1 have all been demonstrated to be essential for megakaryopoiesis and erythropoiesis.[63–67] GATA-1 also possesses an N-terminal transactivation domain which interacts with RUNX1. In addition to expressing full length GATA-1, normal haematopoietic cells also express a shorter isoform termed GATA1s which lacks the N-terminal transactivation domain. The regions of GATA-1 that make contact with DNA, FOG1 and LMO2 have all been defined at a structural level.[64,68,69]

Targeted disruption of murine Gata1 has shown that it is critical for the terminal differentiation of erythroid and megakaryocytic cells, while conditional knockout of Gata1, which results in selective loss of megakaryocyte GATA-1, leads to decreased polyploidization and a lack of cytoplasmic maturation in the megakaryocytes.[70–72] The interaction of GATA-1 with FOG1 is essential for erythropoiesis. This is highlighted by the finding that both FOG1 and GATA-1 null mice demonstrate embryonic lethality due to severe anaemia.[51]

4.2.2 Germline GATA1 defects and platelet dysfunction

The first germline mutation described in GATA1 was a missense defect predicting a p.V205M amino acid substitution in GATA-1, which was identified in two half-brothers with severe thrombocytopenia and pronounced anaemia.[73] Bone marrow from the affected patients

displayed dyserythropoiesis and an abundance of abnormally small, dysplastic megakaryocytes. The platelets also showed dysplastic changes including a paucity of granules. Interestingly, the mother of the two boys had mild thrombocytopenia, most likely as a result of silencing of more wild-type than variant GATA1 alleles during X-chromosome inactivation.[73] Due to the low platelet counts in the patients, it was not possible to perform platelet aggregation studies. The mutation occurs in the NF domain and was demonstrated to cause a reduction in the affinity of GATA-1 for FOG1. Since this report, several other GATA1 defects, primarily affecting amino acids in the NF domain, and all of which decrease the affinity of GATA-1 for FOG1, have been described. The clinical presentations of the patients vary considerably according to the amino acid substituted, and different amino acid substitutions at the same position can lead to considerable variation in phenotypic expression. Thus, while the p.V205M, p.G208R and p.D218Y amino acid substitutions result in severe thrombocytopenia with pronounced anaemia, p.G208S and p.D218G substitutions result in milder clinical phenotypes characterised by moderate thrombocytopenia with minimal or no anaemia.[73–77]

The effects of the p.D218G variant, which was associated with macrothrombocytopenia and mild dyserythropoiesis without anaemia, were studied extensively in platelets from the affected patients.[74] RNA analysis revealed significantly reduced expression of the GATA-1 target genes GP1BB, and GP9, but expression of the Gsα gene, GNAS1, which is not a direct GATA-1 target, but the expression of which is up-regulated in the later stages of megakaryocytic maturation, was also reduced, suggesting incomplete megakaryocyte maturation. The finding that ITGB3 (encoding β_3 integrin) expression was normal would support this suggestion since it is expressed early during megakaryocyte differentiation. Flow cytometry revealed the presence of a population of platelets that showed a wide distribution in size and glycoprotein expression, varying from giant platelets that were up to 5 times the size of normal platelets that showed reduced GPIbα but normal GPIIIa levels to immature platelets that lacked almost all of the membrane glycoprotein receptors tested including GPIbα, GPIbβ, GPIIIa, GPIX and

GPV. The platelets showed an impaired agglutination response to ristocetin that was GPIb dependent, as well as impaired aggregation in response to collagen.[74]

While most of the mutations identified in the NF domain of GATA-1 decrease the affinity of GATA-1 for FOG1, substitution of arginine 216, which lies on the DNA binding face of the NF, with glutamine, resulted in a GATA-1 variant that has normal affinity for GATA sites, but decreased affinity for palindromic sites without disrupting interactions with its co-factor FOG1.[78] The variant, which was associated with a disorder previously referred to as X-linked thrombocytopenia with β-thalassemia was originally found to result in platelets that aggregate normally,[79] though subsequent platelet function studies in a patient with the same variant revealed reduced aggregation in response to collagen, and decreased phosphorylation of GPVI signalling proteins.[80] Although the majority of patients reported with the p.R216Q variant are male, females may have mild to moderate symptoms which are related to the proportion of cells containing the mutated GATA1 allele on the active X chromosome.[81] The p.R216Q variant was also identified as the underlying cause of a mild bleeding disorder in a family with an X-linked form of Gray Platelet Syndrome (GPS) which was characterised by thrombocytopenia and the absence of α-granules from platelets.[82]

A splice site defect in GATA-1 (c.332G>C; p.V74L) that results in synthesis of only the short GATA1s isoform, has been described in seven male members of a Brazilian family affected by X-linked anaemia with or without neutropenia.[83] This suggests that the GATA1s isoform is not sufficient to support normal erythropoiesis, in contrast to the findings in a mouse model in which Gata1 was targeted to introduce alleles that solely generated GATA1s.[84] Whereas somatic mutations that result in the formation of the GATA1s isoform have been found in subjects with Down syndrome acute megakaryoblastic leukaemia, none of the patients with the germline defect had leukaemia. The female carriers of the mutation were unaffected. Platelets from two of the affected male patients showed impaired aggregation in response to

ADP, epinephrine and collagen, and ultrastrutural analysis revealed round platelets with a reduced number of α- and dense granules.[83]

4.3 GFI1b

4.3.1 GFI1b structure and function

The zinc finger containing growth factor independent 1B transcription repressor, or GFI1b, is crucial for normal haematopoiesis and an essential transcriptional regulator of erythroid and megakaryocytic development.[85] Thus, GFI1b controls human erythroid and megakaryocytic differentiation at the megakaryocyte-erythroid progenitor stage, by binding to and repressing expression of type III transforming growth factor receptor gene.[86] Deletion of the Gfi1b gene is embryonic lethal in mice, and the Gfi-1b embryos show delayed maturation of primitive erythrocytes, while the fetal liver contains erythroid and megakaryocytic precursors which have been arrested in their development.[87] GFI1b is highly expressed in HSCs, and its loss in mice leads to significantly increased HSC numbers in bone marrow and peripheral blood, which show decreased expression of CXCR4 and vascular cell adhesion protein-1 (VCAM-1) which are both required to retain HSCs in the endosteal niche. This has led to the suggestion that GFI1b regulates the dormancy of HSCs and their mobilization to peripheral blood.[88]

Located on chromosome 9q34.13, the GFI1B gene comprises 11 exons encoding a 330 amino acid protein that has six C2H2-type zinc fingers at its C-terminus, of which zinc fingers 3 to 5 are essential for binding to their consensus recognition sequence TAAATCAC(A/T)GCA in DNA.[89–91] The N terminal 20 amino acids encompass a SNAG (Snail/Gfi1) domain that is present in other transcriptional repressors and required for nuclear localisation and to mediate interactions with other corepressors (Fig. 4).[92]

4.3.2 GFI1B defects and platelet dysfunction

A small number of germline GFI1B defects have been described in patients with platelet disorders (Fig. 4). The first of these was identified in a four generation Caucasian family which

was first described in 1976, and which displayed an autosomal dominant bleeding disorder of variable severity.[93,94] The disorder was characterised by moderate macrothrombocytopenia and red cell anisopoikilocytosis. In addition, platelets from affected family members displayed an absence of aggregation to collagen, as well as variable responses to other platelet agonists. The patients' platelets showed normal levels of surface glycoproteins, but P-selectin expression and pac-1 binding were reduced following activation with ADP. Linkage analysis and massively parallel sequencing identified the underlying genetic defect as a single nucleotide insertion in GFI1B that introduced a frameshift mutation in the fifth zinc finger of GFI1B (c.880-881insC; p.H294fsX307), which was predicted to result in loss of the invariant histidine residues considered essential to stabilize the zinc ion that is required for correct folding of the DNA binding domain. The mutation was shown to alter the transcriptional activity of GFI1B, and was associated with aberrant expression of the α-granule proteins, P-selectin and fibrinogen as well as membrane glycoproteins, GPIIIa and GPIbα. In addition, there was a reduction in the α-granule content of platelets similar to that observed in platelets from patients with GATA1 defects.[94]

A second GFI1B defect was identified in a family with an autosomal dominant form of GPS.[95] Affected members of the family, which was originally described in 1968, suffered from moderate to severe bleeding tendencies associated with macrothrombocytopenia, and platelets which showed a marked reduction in platelet α-granule content, and a reduction in PF4 and β-thromboglobulin levels.[95,96] Examination of bone marrow from an affected family member revealed an increase in the number of dysplastic megakaryocytes, and emperipolesis was a common feature. Linkage analysis and subsequent sequencing of GFI1B as a candidate gene, led to the identification of a nonsense defect that predicted production of a truncated GFI1B lacking the 44 carboxyterminal amino acids (c.859C>T; p.Gln287*). The mutated GFI1B mRNA was stably expressed in CD34+ progenitor cells from patients and the variant was shown to inhibit the repression activity of wild-type GFI1B in a dominant-negative manner. Immunophenotyping of platelets from several family members revealed reduced GPIbα, CD41

and CD61 as well as strong expression of CD34 on platelets from the patients when compared to control platelets.[95] Interestingly, a preliminary study has suggested that platelet CD34 expression may be a surrogate marker for GFI1B defects since it was not observed in platelets from patients with GATA1, FLI1, or RUNX1 mutations.[97]

The location of defects in GFI1B appears to be clinically relevant. This was highlighted by the preliminary report of a third missense GFI1B defect in the region encoding the first zinc finger domain (c.503G>T p.C168F) which was identified in two unrelated families and characterised by the presence of large platelets with a normal granule content on the blood film, moderate thrombocytopenia and the absence of any platelet aggregation abnormality. However, individuals with the p.C168F defect had significantly milder bleeding symptoms than those with the c.880-881insC defect and the variant also had less impact on transcriptional activity than the c.880-881insC defect.[98]

4.4 ETS transcription factors

Named after its founding member, which was identified in E26 avian retrovirus, the E26-transformation specific (ETS) protein family comprises 29 transcription factors that are expressed in different tissues and regulate gene expression in a diverse array of cellular activities. They all share a common 85 amino acid DNA binding, or ETS, domain that interacts with DNA sequences that are centred on a GGAA/T motif (Fig. 5).[99] The focus here will be on two members of the family, defects in which have been described in patients with platelet disorders.

4.4.1 FLI1

Friend Leukaemia virus integration 1, or FLI1, is expressed at high levels in megakaryocytic progenitors and plays a major role in megakaryopoiesis, regulating the expression of multiple megakaryocyte-specific genes, both early and late in megakaryopoiesis.[100] It seems to exert its effects in concert with a second ETS transcription factor, GA binding protein transcription

factor, α subunit (GABPA), with GABPA preferentially binding to the ETS elements of genes expressed during the early stages of megakaryopoiesis and the ratio of FLI1/GABPA expression levels increasing in the later stages of megakaryocyte development. Thus, studies with Fli-1$^{-/-}$ and GABPα$^{-/-}$ mice have shown that GABPA deficient megakaryocytes have impaired expression of early megakaryocyte-specific genes such as MPL and α_{IIb}, while Fli1$^{-/-}$ deficient megakaryocytes demonstrated reduced expression of genes expressed during the late stages of MK maturation such as GPIbα, GPIX and PF4.[101] The human FLI1 gene is localised on chromosome 11q23.3-24, and comprises nine exons encoding a protein of 452 amino acids, which is expressed primarily in haematopoietic cells and comprises a conserved ETS domain and an N-terminal pointed (PNT) transactivation domain.[102]

Homozygous deletion of Fli1 in mice is embryonic lethal, with the embryos displaying intracranial haemorrhaging, while the heterozygous mice are viable, fertile and appear normal and have a normal tail bleeding time. Examination of megakaryopoiesis from HSCs revealed an increase in the relative number of megakaryocyte progenitor cells in Fli1 -/- mice compared to wild-type and heterozygous mice, and there was an increase in the number of colonies containing undifferentiated megakaryocytes.[103] In humans, transcriptional activation of FLI1 by chromosomal translocation of the genetic region encoding the DNA binding domain of FLI1 to the EWS locus on chromosome 22 leads to Ewings sarcoma.[104]

4.4.2 Chromosomal defects involving FLI1

Partial deletion of chromosome 11 in a region that includes FLI1 results in two syndromes which share many features, Jacobsen syndrome and Paris-Trousseau syndrome.[105–107] These have an estimated prevalence of 1 in one million and the majority are due to de novo deletions that vary in size up to 20 Mb, with the breakpoints occurring in the region of 11q23.3.[108,109] The patients usually have neonatal thrombocytopenia, with an increased number of megakaryoctyes in the bone marrow and dysmorphic facial features, and additional abnormalities affecting the heart, kidney, gastrointestinal tract, genitalia and central nervous

system. Skeletal abnormalities can also occur. The two syndromes differ in severity, with Paris-Trousseau syndrome being characterised primarily by the thrombocytopenia which may be accompanied by other features, while Jacobsen syndrome is more complex. The differences in clinical expression are likely to reflect the size and location of the chromosomal breakpoint in the patients. Paris Trousseau syndrome is characterised by the presence in the circulation of a subpopulation of large platelets containing abnormally large fused α-granules and dysmegakaryocytopoiesis in the bone marrow.[110]

4.4.3 Germline FLI1 defects causing platelet dysfunction

Germline defects in FLI1 have also been described (Fig. 5). Thus, two defects predicting amino acid substitutions in the ETS domain (p.Arg337Trp and p.Tyr343Cys) and associated with reduced transcriptional activity of the protein, were identified in families with a bleeding diathesis which was characterised predominantly by a reduction in dense granule secretion.[48] One of these, the p.Tyr343Cys variant, was also associated with mild thrombocytopenia, alopecia and eczema in affected family members while the p.Arg337Trp variant was associated with alopecia, eczema or psoriasis and recurrent viral infections. The same study identified a 4-bp frameshift deletion (c.992-995del; p.Asn331Thrfs*4) in two members of a third family, who had symptoms of excessive bleeding characterised by a significant reduction in dense granule secretion and thrombocytopenia.[48]

Recently, a homozygous c.970C>T FLI1 defect predicting a p.Arg324Trp substitution in the ETS domain was reported in members of a consanguineous family showing autosomal recessive inheritance of a bleeding disorder that mimicked Paris-Trousseau Syndrome, and was associated with the persistence of MYH10 expression, a recognised biomarker for FLI1 defects.[111] The disorder was characterised by moderate thrombocytopenia, absence of collagen-induced platelet aggregation and the presence of abnormally large α-granules in up to 5% of platelets, but none of the other features of Paris-Trousseau syndrome. The p.Arg324Trp variant was shown to cause a defect in transcription and a reduction in

expression of the FLI1 target genes GP6, GP9 and ITGA2B.[111] The findings of these studies suggest that loss of FLI1 is the likely explanation for the thrombocytopenia associated with Paris-Trousseau syndrome.[48,111]

There has also been a preliminary report of a FLI1 defect resulting in a p.Arg144Gln substitution in the PNT domain of FLI1. This was the first defect to be identified in the pointed domain, and was identified in a patient with mild thrombocytopenia without any bleeding symptoms. Again, the presence of the p.R144Q variant was associated with MYH10 expression in platelets.[112]

4.4.4 ETV6

Ets variant 6 (ETV6), also known as TEL, is widely expressed in all tissues. It was originally cloned in 1994 as the fusion gene resulting from a t(5;12) translocation that occurred in the malignant cells of a patient with chronic myelomonocytic leukaemia. The translocation led to fusion of PDGFRB (encoding platelet-derived growth factor receptor β) located on chromosome 5q31 with a previously unknown gene derived from chromosome 12p12, which was originally named TEL, but later renamed ETV6.[113]

Like GFI1b, ETV6 acts as a transcriptional repressor that requires dimerisation to exert repression, and which is regulated by self-association and auto-inhibition.[114,115] It also modulates the activity of other ETS transcription factors, including FLI1.[116] Knockout studies have shown that deletion of Etv6 is embryonic lethal in mice, and essential for definitive haematopoiesis in the bone marrow.[117,118] Conditional knockout of Etv6 established it as a selective regulator of HSC survival as was evidenced by the loss of HSCs from the adult bone marrow following inactivation of Etv6. Following commitment of the HSCs, absence of Etv6 has little effect apart from the impaired maturation of megakaryocytes.[119]

ETV6 comprises 8 exons and encodes a 452 amino acid protein with three functional domains, a C-terminal ETS domain that binds DNA, a highly conserved N-terminal PNT domain which

mediates homo and heterodimerisation, and is required for nuclear localisation of the protein, and a central regulatory domain (amino acids 127-331) which is located between the PNT and ETS domains of the protein (Fig. 5). The transcriptional repression activity has been mapped to the PNT and central regulatory domains. Repression by the PNT domain is mediated by interaction with the transcriptional repressor lethal(3) malignant brain tumour-like protein 1 (L3MBTL1), a polycomb group protein,[120] while repression by the central inhibitory domain is mediated via corepressor complexes such as SMRT, mSin3A and N-CoR, which recruit histone deacetylases (HDACs) and can be relieved by HDAC inhibition.[121,122]

4.4.5 Germline ETV6 defects and platelet disorders

Since its original description, several somatic alterations involving ETV6 have been described in leukaemia and other haematological malignancies. More recently, there have been three reports of germline defects in ETV6 in patients with autosomal dominant thrombocytopenia and a predisposition to haematologic malignancy (Fig. 5).

Zhang et al[123] identified missense defects in ETV6 in three unrelated families with autosomal dominant thrombocytopenia, and predisposition to a diverse range of haematologic malignancies, including MDS, pre-B cell ALL, multiple myeloma and CMLL.[123] Two of the defects predicted amino acid substitutions in the ETS DNA binding domain. One of these, p.Arg399Cys affects an amino acid that directly contacts with DNA at the ETS binding element GGA(A/T) (the consensus is a 9bp sequence with the core GGA(A/T) motif), while the second, p.Arg369Gln, involved an amino acid that forms a hydrogen bond with Arg414 which is involved in electrostatic interactions with DNA so the substitution by Gln could destabilise the ETS domain, or interfere with the interaction of Arg 414 with DNA. A third missense defect predicted a p.Pro214Leu substitution in the central inhibitory domain. Interestingly, all three defects occurred in regions of ETV6 that are hotspots for somatic mutation in malignancies. The p.Arg369Gln and p.Arg399Cys variants both abrogated binding to DNA, and expression studies in HeLa cells revealed altered subcellular localisation of all three variants, with the

p.Pro214Leu variant mainly confined to the cytoplasm, and the p.Arg369Gln and p.Arg399Cys variants showing reduced nuclear localisation. All three variants also decreased transcriptional repression mediated by wild-type ETV6 in a dose-dependent manner. Proliferation of CD34+ cells expressing the three variants was markedly reduced in all cases, providing an explanation for the thrombocytopenia observed in the patients.[123]

Noetzli et al (2015) described three further unrelated families with ETV6 variants which were associated with autosomal dominant thrombocytopenia, a high erythrocyte MCV, and a predisposition to leukaemia.[124] Two families harboured the previously identified p.Pro214Leu variant, while the third harboured a c.1252A>G transition which caused a frameshift that resulted in exon skipping and partial deletion of the ETS domain (p.Asn385Val fs*7), and production of a truncated protein, which could be detected in overexpression studies, but not in platelets. Interestingly, not all of the mutated RNA was alternatively spliced and a proportion was translated to give rise to a p.Arg418Gly substitution in the ETS domain. Platelets from affected patients appeared normal, though some had elongated α-granules and there were hypolobulated megakaryocytes and abnormal RBC precursors present in the bone marrow. Further characterization of the variants revealed reduced transcription repression activity, and delayed and decreased maturation of megakaryocytes when cultured from CD34+ cells. Like the p.Pro214Leu variant, the p.Arg418Gly variant was concentrated in the cytoplasm of megakaryocytes, and both formed dimers with wild-type ETV6, reducing nuclear translocation of the wild-type protein in a dominant negative manner.[124]

Two further ETV6 variants were identified in two families with thrombocytopenia and ALL. One of these predicted a p.Leu349Pro substitution in the ETS domain, and the second was a 5 base pair deletion causing a frameshift and resulting in production of a truncated protein p.N385fs. Again, both variants displayed a reduced ability to repress expression of genes normally suppressed by ETV6 (PF4 and MMP3), and a failure to promote expression of EGR1

and TRAF1. Both variants were confined to the cytoplasm and were not detected in the nucleus.[125]

Finally, a single base insertion ETV6 has been reported in abstract form. The c.601-602insC; p.L201Pfs*15 defect, which was identified alongside another case of the c.641C>T p.Pro214Leu defect, also demonstrated reduced repressive activity.[126]

Conclusions

The investigation of patients with unexplained inherited platelet disorders has allowed the identification of a subgroup of patients harbouring defects in one of five transcription factors, FLI1, RUNX1, GATA1, GFI1B and ETV6, all of which serve important regulatory roles in megakaryopoiesis and platelet production. These defects usually result in haploinsufficiency, or affect functionally relevant domains of the transcription factors affecting their interactions with co-activators and or target DNA sequences. In most, but not all, cases the transcription factor defects are also associated with mild thrombocytopenia. These disorders vary considerably, making their diagnosis difficult when based solely on the clinical and phenotypic features of the patient. However, the presence of MYH10 in platelets from affected patients is a surrogate marker for FLI1 and RUNX1 defects and CD34 expression may be a marker for GFI1B defects. A history of haematologic malignancy may suggest a RUNX1 or ETV6 defect which will have prognostic implications for the patient, and abnormal red cell features which may be accompanied by anaemia would suggest a GATA1 or a GFI1B defect. Ultimately, the presence of a transcription factor defect should be confirmed by DNA sequencing and any candidate defects should be investigated further to confirm that they are dysfunctional.

Looking to the future, as more patients with unexplained bleeding disorders are investigated by whole exome or genome analysis, we anticipate the identification of further novel defects in the transcription factors that are involved in megakaryopoiesis and thrombopoiesis, and their co-activators. Characterisation of these defects and of the genes that are differentially

expressed as a result, will yield insights into the molecular and cellular mechanisms involved in megakaryopoiesis and in platelet production and function, and has the potential to deliver strategies for treatment of the bleeding symptoms in affected patients. Furthermore, the use of gene editing technologies to correct transcription factor defects in iPSCs offers a potential therapeutic approach for those patients where the defects are associated with haematologic malignancy.

Practice points

- Germline defects in the genes encoding the transcription factors RUNX1, GATA-1, FLI1, GFI1b and ETV6 account for a significant number of patients with previously unexplained platelet function defects.
- Transcription factor defects tend to occur in regions of the transcription factor genes that encode functional domains and affect the interaction of the transcription factor with target DNA sequences and or co-activators.
- Persistence of MYH10 expression in platelets is a useful biomarker for the presence of FLI1 and RUNX1 defects and platelet CD34 expression may be a surrogate marker for GFI1B defects.
- Transcription factor defects in RUNX1 and ETV6 can also be associated with an inherited predisposition to haematological malignancies and may therefore have prognostic implications.

Research agenda

- Investigation of further cases with unexplained inherited platelet disorders is likely to reveal novel defects in other transcription factors that are required for haematopoiesis.
- Identification of the genes that are differentially regulated in patients with platelet disorders as a result of transcription factor defects will yield insights into the molecular

and cellular mechanisms governing platelet production and function, and may identify strategies to allow the in vitro production of platelets for therapeutic use.

- Correction of transcription factor gene defects in induced pluripotent stem cells from affected patients offers a potential treatment for the haematologic malignancies associated with certain transcription factor defects.

Conflict of interest statement

None

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Figure legends

Figure 1.

Relative expression of the transcription factors GATA1, ETV6, FLI1, RUNX1 and GFI1B across eight cell types derived from haematopoietic stem cells (HSC) in the bone marrow. MPP multipotent progenitor; CMP common myeloid progenitor; CLP common lymphoid progenitor; GMP granulocyte-monocyte progenitor; EB erythroblast; MEP megakaryocyte erythrocyte progenitor; MK megakaryocyte. The riverplot was generated using the Blueprint Progenitors dataset and the online tool available at http://blueprint.haem.cam.ac.uk/[21]

Figure 2.

Schematic representation of RUNX1. Different functional domains are highlighted by horizontal black bars and the locations of the PY and VWRPY motifs are also indicated. TD: transactivation domain; ID: inhibitory domain; NLS: nuclear localization signal; NMTS: nuclear matrix targeting signal. The locations of variants identified in patients with platelet defects and referred to in the text are shown. Intronic mutations, predicted to interfere with splicing of the RUNX1 RNA, are shown in italics. Variants shown in boxed were identified in families with no history of haematological malignancy, while the remainder were identified in families with FPD/AML.

Figure 3.

Schematic representation of GATA-1. Different functional domains are highlighted by horizontal black bars and the locations of the PY and VWRPY motifs are also indicated. TD: transactivation domain; NF: N-terminal zinc finger; CF: C-terminal zinc finger; FOG1: Friend of GATA-1; LMO2: LIM-only protein. The locations of variants identified in patients with platelet defects and referred to in the text are shown. The splice site variant in exon 2 that results in synthesis of the GATA-1s isoform which lacks the N terminal 84 amino acids is indicated in italics.

Figure 4.

Schematic representation of GFI1b showing the location of variants identified in patients with platelet function disorders. The domains required for DNA binding and nuclear localisation of GFI1b are indicated by horizontal black bars. ZF: Zinc finger domain; SNAG: Snail/Gfi1 domain.

Figure 5.

Schematic representation of the ETS family members FLI1 (upper panel) and ETV6 (lower panel) showing the domain structure of the proteins and the locations of variants identified in

patients with platelet dysfunction disorders. The domains involved in binding to DNA, and in activation and repression of transcription are indicated by horizontal black bars. PNT: pointed domain; ETS: E26 transformation specific domain.