knockout (TKO) mice on a C57BL6 background. Both Rag2$^{-/-}\gamma c^{-/-}$ double knockout (DKO) and TKO hosts received Thy/Liv grafts under the renal capsule and human fetal liver CD34^{+} cells by intravenous injection. The authors show that TKO-bone marrow, liver, thymus (BLT) hosts have significantly improved multilineage reconstitution and circulation of human immune cells that includes T cells, B cells, and dendritic cell populations. Importantly, circulating mature T cells have a CD4:CD8 ratio comparable to that of normal humans. Increased numbers of human cells with a primitive human hematopoietic stem/progenitor phenotype are also noted in the bone marrow of TKO-BLT vs DKO-BLT controls suggesting that human HS/PC are protected from engulfment by bone marrow macrophages. A further advance is that a significant number of all major human immune cell types are found in lymph nodes and spleens of TKO-BLT mice. Moreover, human T cells and B cells appear to assemble into rudimentary follicular structures in the spleen. There also appears to be significantly improved seeding of the gut lamina propria by human immune cells in TKO-BLT mice (see figure). Importantly, the improved human engraftment comes with little or no xenogeneic GVHD. Xenogeneic GVHD in humanized mice, and the resulting inflammatory milieu, limits the utility of such models. Finally, Lavender et al show that the TKO-BLT mice can be infected with HIV via the rectal administration of virus. Viremia is sustained, and the human T-cell compartment shows depletion of CD4^{+} T cells. Moreover, they document B- and T-cell responses to the HIV pathogen with the latter consisting of responses to peptide, suggesting there is significant human antigen presentation to T cells in the TKO-BLT model. This is a feature whose absence has limited the ability to study vaccination in other humanized models.

The TKO-BLT model represents a significant step forward in the development of immunodeficient models of human hematopoiesis and immunity. Further advances likely await as this model is extended to other immunology, pathogenesis, and stem cell questions. Of particular priority will be the application of the TKO-BLT model to the development and testing of vaccines for HIV and other pathogens. One further improvement the Rag2$^{-/-}\gamma c^{-/-}$CD47$^{-/-}$ model may promote is the analysis of human Peyer's patch generation and the analysis of human lymphoid tissue inducer cell function. Interestingly, Peyer's patch structures containing human immune cells were not noted in TKO-BLT mice. Perhaps this could be induced by introduction of human receptor transgenes onto the Rag2$^{-/-}\gamma c^{-/-}$CD47$^{-/-}$ background that enable murine stromal cells to receive inductive signals from human lymphoid tissue inducer. Such an advance would improve the human mucosal immunity observed in Rag2$^{-/-}\gamma c^{-/-}$CD47$^{-/-}$ mice and its infection by human mucosal pathogens. Perhaps the Rag2$^{-/-}\gamma c^{-/-}$CD47$^{-/-}$ mouse, having lost its appetite for human cells, can now be persuaded to host an even larger array of human hematopoietic, mesenchymal, and neuro-epithelial stem/progenitor cells.

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● ● ● PLATELETS & THROMBOPOIESIS

Comment on Stockley et al, page 4090

Spotlight on *FLI1*, *RUNX1*, and platelet dysfunction

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In this issue of *Blood*, Stockley et al describe mutations in *FLI1* and *RUNX1*, identified by next-generation sequencing (NGS) studies, in 6 of 13 patients with excessive bleeding and impaired platelet dense granule secretion, and highlight transcription factor (TF) mutations as an important mechanism for inherited platelet dysfunction.[1]

In the majority of patients suspected to have an inherited platelet function defect, based on abnormal platelet aggregation and secretion studies, the underlying molecular and genetic mechanisms remain unknown. Platelet aggregation and secretion as monitored in clinical studies are relatively late or "end" responses that follow a sequence of upstream events on platelet activation. Given the complex and redundant platelet pathways, the findings of such studies are generally not predictive with any confidence of the underlying genetic or molecular mechanisms in most patients. Until recently, the focus of studies on the molecular mechanisms has been on postulated candidate proteins and

pathways. This is the major paradigm by which most platelet defects have been identified to date; it is driven by existing knowledge and comes with limitations. At the genetic level, the focus has largely been on delineating mutations in the coding sequence of genes encoding the candidate proteins.

It is in this context that the paper by Stockley et al makes an important contribution. First, it focuses attention on genetic defects in TFs that regulate megakaryocytic and platelet genes as a cause of platelet dysfunction. Second, it highlights the power of newer approaches such as NGS technology in unraveling the genetic abnormalities in patients with platelet function defects.

TFs and the *cis*-regulatory sequences to which they bind are critical players in regulating lineage-specific gene expression. *FLI1*, a member of the ETS (E-twenty-six) family, and *RUNX1*, along with *GATA-1*, *GATA-2*, *TAL/SCL*, *NF-E2*, and others, are key regulators of hematopoietic lineage differentiation, megakaryopoiesis, and platelet production.[2] *FLI1*, *RUNX1*, and *GATA-1* physically interact in a combinatorial manner in regulating megakaryocytic genes.[2] A single TF mutation may alter the expression of numerous genes, affect diverse cellular mechanisms, and in the present context, lead to concurrent defects in platelet number and function.

In their manuscript, Stockley et al[1] report findings in 13 unrelated families from the UK Genotyping and Phenotyping of Platelets study, a multicenter study of patients with suspected platelet function defect. In all, 366 patients have been evaluated in the study. NGS studies were performed in 13 patients who were selected from a group of 56 patients classified as having a secretion defect; they were also selected on the basis of having an affected relative who was available for study. All 13 patients studied had decreased dense granule secretion on platelet activation with multiple agonists and decreased aggregation in response to some agonists. They found *FLI1* or *RUNX1* mutations in 6 families, of whom 5 had thrombocytopenia, leading the authors to suggest that there was an enrichment of these mutations in patients with secretion defects. This spotlight on TF abnormalities is revealing.

The association of *FLI1* and *RUNX1* mutations with thrombocytopenia and platelet dysfunction has been previously documented. It is notable that Stockley et al[1] found *FLI1* mutations in 3 of 13 families studied. Such mutations have been a part of the Paris Trousseau and Jacobsen syndromes, associated with constitutional deletions that include the *FLI1* locus on chromosome 11.[3] These patients are characterized by bone marrow dysmegakaryopoiesis, thrombocytopenia with a subpopulation of platelets with giant α-granules, and anomalies affecting various organs. It is unclear if the patients described by Stockley et al[1] had manifestations besides platelet and skin abnormalities. Moreover, *FLI1* is also a transcriptional regulator of several genes, including *GP6*, *GPIBA*, *GP9*, *ITGA2*, and *c-MPL*. *FLI1* mutations may therefore impact platelet function as well.

Multiple molecular abnormalities associated with alterations in both platelet number and function are documented in patients with inherited *RUNX1* mutations. These patients were initially recognized by an association between autosomal dominant thrombocytopenia, abnormal platelet responses, and an increased predisposition to leukemia, and subsequently linked to a *RUNX1* haplodeficiency.[3,4] The platelet function and biochemical abnormalities reported with *RUNX1* mutations encompass decreased aggregation, secretion, protein phosphorylation (myosin light chain and pleckstrin), production of 12-hydroxyeicosapentaenoic acid and αIIbβ3 activation on platelet activation, dense and α-granule deficiencies, and a selective decrease in 1 protein kinase C isoform (PKC-θ).[5-8] These indicate that multiple aspects of platelet structure and function are compromised. Interestingly, several patients described earlier as having storage pool deficiency (dense or α-granules) have been subsequently shown to harbor *RUNX1* mutations.[5]

Most inherited *RUNX1* mutations have affected the conserved Runt domain.[5] Each TF regulates expression of multiple genes, and consistent with this, platelet expression profiling in a patient with *RUNX1* haplodeficiency revealed downregulation of numerous genes, including *MYL9* (myosin light chain), *ALOX12* (12-lipoxygenase), *PF4* (platelet factor 4), and *PRKCQ* (PKC-θ),[7-10] all directly relevant to platelet biology. *ALOX12*, *PRKCQ*, *PF4*, and *MYL9*[7,8,10] are direct transcriptional targets of *RUNX1*. Furthermore, patients with *RUNX1* haplodeficiency have impaired megakaryopoiesis and decreased platelet thrombopoietin receptors (Mpl).[4,7] Thus, platelet dysfunction in *RUNX1* haplodeficiency is driven by alterations in multiple genes and pathways.

Mutations in hematopoietic TF GATA-1 are also associated with platelet defects. They encompass X-linked macrothrombocytopenia and platelet dysfunction, including impaired responses to collagen and ristocetin (related to abnormalities in GPIbß), diminished platelet GαS, and the gray platelet syndrome.[7]

Stockley et al[1] suggest that mutations in *FLI1* and *RUNX1* may be common in patients with platelet dense granule secretion defects and mild thrombocytopenia. The prevalence of *RUNX1* or *FLI1* mutations in the overall heterogeneous population of patients with abnormal platelet responses remains unknown. The relatively high frequency observed may be a function of patient selection for the NGS studies. Because the primary focus was on patients with impaired dense granule secretion, it would be of interest to know how many had storage pool deficiency, either of dense or α-granules, both reported with *RUNX1* mutations.[5,7]

This study effectively draws attention to the fact that TF abnormalities may be far more common than currently considered in the pathogenesis of inherited platelet function defects, particularly in those with thrombocytopenia. Equally important, it elegantly highlights the ability of an approach utilizing NGS to detect genetic abnormalities in platelet disorders. It would be most interesting to know what hitherto unrecognized platelet genetic defects are uncovered in the larger pool of patients with impaired platelet function and secretion.

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THROMBOSIS & HEMOSTASIS

Comment on McGrath et al, page 4107

Platelet von Willebrand factor: sweet resistance

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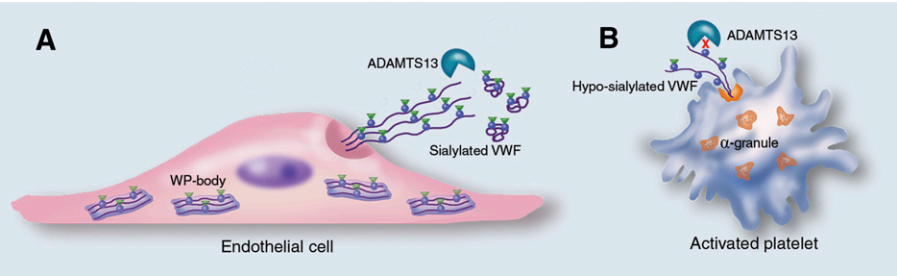
In this issue of *Blood*, McGrath et al show that the terminal glycan structures of platelet von Willebrand factor (VWF) are markedly different compared with such structures present on plasma VWF.[1] Unexpectedly, these differences endow platelet VWF with a specific resistance against proteolysis by the VWF-cleaving protease ADAMTS13, thereby potentially increasing the hemostatic potential of platelet VWF during the formation of platelet-rich thrombi.

Glycosylation is a posttranslational modification that involves more than 50% of the members of the eukaryotic proteome and results in the covalent attachment of carbohydrate structures to the protein backbone. This type of posttranslational modification is a crucial event because it may affect different steps within the lifecycle of a protein—from biosynthesis to function and ultimately to clearance. Impaired glycosylation can be associated with severe clinical manifestations, including neonatal death, psychomotor retardation, cardiomyopathy, and coagulopathy.[2] Whereas glycosylation is relying on an intact machinery to attach the correct glycan structures to the protein, environmental factors such as age, diet, and pathogen infections can also influence this process. In addition, another important determinant of glycosylation is the cellular origin of a protein because the available repertoire of enzymes mediating glycosylation varies per cell type. This may, for instance, affect the antennary (or branches) of the glycans, but also the terminal structures of the carbohydrates, such as the extent of sialylation or the absence or presence of blood group determinants.



Cellular source of VWF determines its susceptibility for ADAMTS13-mediated proteolysis. VWF produced by endothelial cells (A) is stored in the Weibel-Palade (WP) bodies as a glycoprotein containing *N*-linked glycans (blue dots) that are sialylated (green triangles). Upon stimulated release, VWF assembles into long strings that are susceptible to proteolysis by ADAMTS13. Unexpectedly, McGrath et al have now found that VWF stored in the α-granules of platelets (B) contains much less sialic acids on the *N*-linked glycans (blue dots). Moreover, this lack of sialic acids converts VWF into a substrate that is less efficiently proteolyzed by ADAMTS13, providing it with a higher hemostatic potential. Professional illustration by Marie Dauenheimer.

McGrath et al present such an example in which the glycosylation pattern of a protein (in this case, VWF) is dependent on its cellular origin.[1] VWF is a multimeric protein that is pivotal to the recruitment of platelets to the injured vessel wall and subsequent thrombus growth. Its expression is limited to 2 cell types. First, the major part of VWF is produced in endothelial cells (representing approximately 85% of all VWF), where it is either constitutively secreted in plasma or stored in the Weibel-Palade bodies. The remaining VWF protein is synthetized and stored in the α-granules of megakaryocytes and platelets, with little (if any) constitutive secretion from these cells. Consequently, circulating plasma VWF primarily originates from endothelial cells, whereas VWF in α-granules is selectively released upon platelet activation. Based on its presence in the circulation, plasma VWF is thought to be more dominant in the hemostatic process than platelet VWF. Indeed, the contribution of platelet VWF to hemostasis has remained controversial. For instance, platelet VWF is absent in dogs, without any obvious hemorrhagic consequences. However, patient observations and a recent crossover study using wild-type and *Vmf*-deficient mice revealed that platelet VWF can contribute to the hemostatic process as well.[3,4]

Despite having a similar protein backbone, plasma and platelet VWF have been reported to display subtle functional differences.[5] Whereas both sources of VWF bind similarly to collagen, platelet VWF appears more efficient in binding to integrin αIIbβ3 and to