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Clinic, pathogenic mechanisms and drug testing of two inherited thrombocytopenias, *ANKRD26*-related Thrombocytopenia and *MYH9*-related diseases

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ABSTRACT

Inherited thrombocytopenias (ITs) are a heterogeneous group of disorders characterized by low platelet count resulting in impaired hemostasis. Patients can have spontaneous hemorrhages and/or excessive bleedings provoked by hemostatic challenges as trauma or surgery. To date, ITs encompass 32 different rare monogenic disorders caused by mutations of 30 genes. This review will focus on the major discoveries that have been made in the last years on the diagnosis, treatment and molecular mechanisms of *ANKRD26*-Related Thrombocytopenia and *MYH9*-Related Diseases. Furthermore, we will discuss the use of a Thrombopoietin mimetic as a novel approach to treat the thrombocytopenia in these patients. We will propose the use of a new 3D bone marrow model to study the mechanisms of action of these drugs and to test their efficacy and safety in patients. The overall purpose of this review is to point out that important progresses have been made in understanding the pathogenesis of *ANKRD26*-Related Thrombocytopenia and *MYH9*-Related Diseases and new therapeutic approaches have been proposed and tested. Future advancement in this research will rely in the development of more physiological models to study the regulation of human platelet biogenesis, disease mechanisms and specific pharmacological targets.

1. Introduction

Until the end of the last century, we knew a few forms of inherited thrombocytopenias (ITs), all of them diagnosable by clinical aspects and simple laboratory tests. One of the most “ancient” form of non-syndromic IT is Bernard-Soulier syndrome, which was recognized based on the recessive inheritance, the presence of giant platelets in peripheral blood films and the absence of *in vitro* platelet agglutination after ristocetin stimulation due to defective platelet expression of glycoprotein (GP) I alpha (Caen et al., 1976). Another IT known since many decades is gray platelet syndrome, easily recognizable by the “pale” appearance of platelets on blood films due to the absence of alpha granules (Raccuglia, 1971). The prototypical form of syndromic IT is the Wiskott-Aldrich syndrome, easily recognized based on X-linked transmission of deep congenital thrombocytopenia associated with

severe immunodeficiency (Root and Speicher, 1963). All these conditions are characterized by severe bleeding tendency since birth, and their genetic origin is therefore obvious. Since the beginning of this century, the widespread availability of increasingly effective techniques for gene sequencing greatly improved our knowledge of ITs, and we presently know more than 30 ITs due to mutations in more than 30 genes. Of note, 8 “new” ITs have been identified and described in their clinical and molecular aspects since 2015 (Balduini et al., 2017). Analysis of large series of patients with the “new” ITs changed our view of these disorders in that we realized that most patients have mild to moderate thrombocytopenia and, consequently, mild or absent bleeding tendency. But the advancement of knowledge also revealed that many asymptomatic subjects with apparently harmless ITs are at risk of developing additional disorders that seriously endanger their life. This is the case of the ITs deriving from *RUNX1* (Liew and Owen,

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2011), *ANKRD26* (Noris et al., 2011, 2013) and *ETV6* mutations (Noetzi et al., 2015; Zhang et al., 2015), which predispose to hematological malignancies, the disorders caused by mutations in *MPL* (Ballmaier and Germeshausen, 2011) or *THPO* (Seo et al., 2017), which evolve into bone marrow aplasia, and the form caused by *MYH9* mutations, which exposes to the risk of developing kidney failure (Balduini et al., 2011).

In this review we will discuss recent advances in the field of the ITs caused by mutations in *MYH9* (*MYH9* related disease - *MYH9*-RD) and *ANKRD26* (*ANKRD26* related thrombocytopenia - *ANKRD26*-RT). The rationale of this choice is that, at least in our experience, they are the most frequent forms of IT. Moreover, investigation of their pathogenic mechanisms, also in the context of an international collaborative study supported by ERARE, revealed new, interesting aspects of the molecular mechanisms that regulate megakaryopoiesis and platelet biogenesis. Since thrombopoietin (TPO) mimetic have been recently approved for the clinical treatment of some acquired thrombocytopenias, we will discuss the possibility that these molecules are effective also in ITs.

1.1. *ANKRD26*-Related thrombocytopenia

Thrombocytopenia 2 (THC2; OMIM 188000), also named *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT), is a non-syndromic form of inherited thrombocytopenia with autosomal dominant transmission. *ANKRD26*-RT was first reported in two families, one from Italy and the second from North America (Drachman et al., 2000; Savoia et al., 1999). In both cases, the *ANKRD26*-RT locus was mapped through lineage analysis to chromosome 10p11.1-p12 and the possibly causative genes, *MASTL* (Gandhi et al., 2003) and *ACBD5* (Punzo et al., 2010), were identified due to the missense mutations. More than 10 years later, a study performed on eight unrelated families has shown that neither *MASTL* nor *ACBD5* were implicated in the pathogenesis of this disease; conversely, 6 different mutations in the 5'UTR region of gene encoding *ANKRD26* (Ankyrin repeat domain 26) were identified. c.-116C > T, c.-118C > T, c.-125T > G, c.-127A > T, c.-128G > A and c.-134G > A, all of them localized in a small stretch of 19 nucleotides, conserved among primates and bovines (Pippucci et al., 2011). All these mutations were cloned upstream of a luciferase-based reporter and transiently transfected into two cell lines, K562 and DAMI, showing an increased transcriptional activity and suggesting that these mutations affect the *ANKRD26* gene expression level. This discovery led very quickly to the identification of 12 additional families affected by *ANKRD26*-RT (Noris et al., 2011), picturing a more frequent condition than expected. So far, more than 70 patients were reported in literature (Noris et al., 2011) and *ANKRD26*-RT is presently considered one of the most frequent forms of IT. Of note, this condition may be easily misdiagnosed with other disorders. In a series of 78 patients, 30 initially had a diagnosis of ITP, and 16 received undue treatments, splenectomy included (Noris et al., 2011). Moreover, two recently reported patients were misdiagnosed and treated as myelodysplastic syndrome (MDS) (Zaninetti et al., 2017). The diagnostic error occurs because the clinical-laboratory picture of *ANKRD26*-RT is similar to that of both these conditions and misdiagnosis occurs when the inherited origin of the disease is not evident. Currently, the clinical picture of *ANKRD26*-RT is defined for 78 affected members of 21 families; these patients show mild to severe thrombocytopenia, normal platelet size, mild bleeding diathesis, increased levels of serum TPO and dysmegakaryopoiesis in the bone marrow (BM). No defects in platelet function or morphology have been found, but several patients displayed defective GPI α expression on platelet surface and a reduction of platelet α -granules. Overall, these findings suggested that the thrombocytopenia is a consequence of a defect in megakaryocyte (Mk) differentiation and/or maturation. Interestingly, about 10% of patients included in this study developed acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) or chronic myeloid leukemia (CML) (Noris et al., 2011). In 2013, other mutations in the same 5'UTR region were identified (c.-

116C > G, c.-118C > A, c.-119C > A, c.-126T > G, c.-127A > G, c.-127delAT, c.-128G > C) in patients from France, Italy, Japan, US, Netherlands, Canada and Argentina, and 10 new cases of AML, MDS and CML were reported in those patients, while the incidence of lymphoproliferative disorders and nonhematologic cancers was not higher than expected. Together with the previous cases, these results show that *ANKRD26*-RT predispose to myeloid malignancies with incomplete penetrance (Noris et al., 2013). More recently, the c.-140C > G variant was also reported in two studies (Boutroux et al., 2015; Ferrari et al., 2017), enlarging the original stretch of 19 nucleotides to 25. However, this variant is relatively common, its mean allelic frequency ranges from 0.04 to 0.06 (ExAc and gnomAD databases), thus its implication in the disease should be confirmed. The leukemic transformation was reported, in one case, to be associated with *ASXL1* mutation in CMML (Perez Botero et al., 2015). The ultrastructural studies of *ANKRD26*-RT patient Mks and platelets revealed the presence of ubiquitin/proteasome-rich particulate cytoplasmic structures (PaCS). These structures show selective immunoreactivity for the proteasome as well as for polyubiquitinated proteins. They were first detected in gastric epithelium infected with *Helicobacter pylori* and related pre-neoplastic lesions, later in different epithelial neoplasms and in blood neutrophils of patients with the Shwachman-Diamond syndrome. Their absence in unaffected and non-neoplastic cells suggests that they could play a physiologic role in *ANKRD26*-RT, in particular accelerating the apoptosis. However their generation and precise role in *ANKRD26*-RT physiopathology remains to be clarified (Necchi et al., 2013).

ANKRD26 is a member of the POTE gene family, a group of primate specific protein-coding genes, with a mouse homolog (Hahn et al., 2006); it is coding for a 192 kDa protein, highly enriched with spectrin helices and ankyrin repeats, both protein domains with confirmed interactions with cytoskeletal and signaling proteins (Djinovic-Carugo et al., 2002; Mosavi et al., 2004). The disruption of *Ankrd26* in mouse causes hyperphagia, severe obesity, gigantism and diabetes (Bera et al., 2008) with defects in the primary cilia in the regions of the central nervous system that control appetite and energy homeostasis (Acs et al., 2015). Disruption of *ANKRD26* in human could thus explain certain forms of genetic obesity that were described to be linked to the 10p12 locus (Dong et al., 2005). In a knock-out *Ankrd26* mice, a marked adipogenesis was shown to be mediated by an increased activation of the ERK and the mTOR pathways (Fei et al., 2011); the same group described also at least four interacting partners, isolated by two-hybrid assay and IP (Liu et al., 2012). Overall, the *Ankrd26* deregulation could be involved in the modulation of signaling pathways, although there is no explanation of the precise mechanism.

To shed light on the physiopathological mechanism leading to *ANKRD26*-RT, we have recently investigated the megakaryopoiesis of *ANKRD26*-RT patients, focusing on a major signaling pathway that control megakaryopoiesis, TPO/MPL (Bluteau et al., 2014) (Fig. 1). First, we have shown that *ANKRD26* expression in healthy individuals decreases during the megakaryopoiesis, while in *ANKRD26*-RT patients the expression of *ANKRD26* is persistent in mature Mks (CD41⁺/CD42⁺) and platelets. The *in silico* analysis of the 5'UTR region revealed the presence of the binding motifs for the transcription factors RUNX1 (from -125 nt to -114 nt) and FLI1 (from -111 nt to -105 nt), two main regulators of megakaryopoiesis whose genetic alterations cause inherited thrombocytopenia. Indeed, *RUNX1* germline alterations (missense, frameshift mutations, intragenic deletion) are at the origin of familial platelet disorder predisposing to acute myeloid leukemia (FPD/AML) (Song et al., 1999), while *FLI1* monoallelic deletions are found in Jacobsen syndrome or the Paris-Trousseau Thrombocytopenia (Raslova et al., 2004). Recently, some cases of germline point mutations in *FLI1* have been related to inherited thrombocytopenia (Stevenson et al., 2015). *RUNX1* and *FLI1* co-regulate many Mk-specific genes (Tijssen et al., 2011), and as we have shown, they physically bind to the 5'UTR of *ANKRD26*. Interestingly *ANKRD26* gene expression is down-regulated during normal megakaryopoiesis, and the absence of *RUNX1/FLI1*

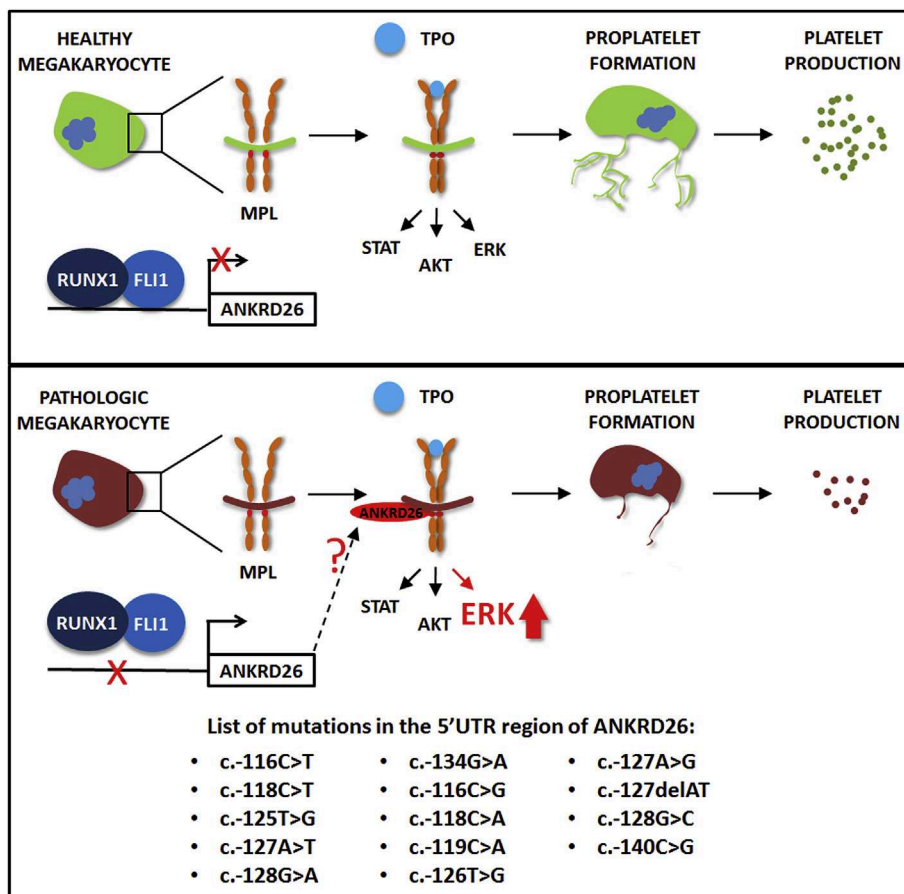


Fig. 1. Schematic summary of mechanisms of thrombocytopenia in ANKRD26-RT. In healthy Mks, TPO promotes thrombopoiesis by activating MPL downstream signaling pathways. In this process RUNX1/FLI1 complex negatively regulates ANKRD26 gene expression. In Mks from ANKRD26-RT, the mutations in 5'UTR of the ANKRD26 gene prevent RUNX1/FLI1 inhibition, leading to its over-expression. It is supposed that in this scenario ANKRD26 accumulates at the inner part of the cell membrane and modifies TPO/MPL signaling, leading to ERK over-activation and consequent decreased proplatelet formation and platelet production.

binding, due to the mutations in the 5'UTR, leads to a persistent expression of ANKRD26, suggesting a repressive activity of the transcriptional complex. The expression of ANKRD26 in patient platelets could therefore be used as a biomarker of ANKRD26-RT. The investigation of *in vitro* megakaryopoiesis has shown no defect in Mk differentiation but a severe defect in proplatelet formation, affecting both the percentage of proplatelet forming Mks and the number of proplatelet branches per Mk.

The fact that ANKRD26 is located in the inner part of cell membrane (Bera et al., 2008) and could act as an adaptor between the receptors and the signaling molecules suggested that ANKRD26 overexpression in Mks could affect the TPO/MPL signaling. Using *in vitro* culture of patient Mks we have demonstrated that the TPO/MPL downstream pathways JAK/STAT, PI3K and MAPK were over-activated, with a more important stimulation of the MAPK cascade (Bluteau et al., 2014). Interestingly, the progressive silencing of MAPK pathway during the normal megakaryopoiesis was shown to be important for proplatelet formation. In accordance to these findings, the use of MAPK inhibitor PD98059 led to an increased proplatelet formation potential in patient Mks. In conclusion, we have demonstrated that the sustained ANKRD26 expression modulates positively TPO/MPL signaling, and the increased MAPK activity leads to the defective proplatelet formation, thus explaining the observed thrombocytopenia (Bluteau et al., 2014). Nevertheless, the precise mechanism linking the ANKRD26 overexpression to the TPO/MPL signaling remains to be investigated. It could not be excluded that ANKRD26 overexpression alters TPO/MPL signaling not only in mature Mks but also in immature progenitors expressing MPL. Indeed, our preliminary results show that the increased ANKRD26 expression leads to the hypersensitivity to TPO and increased proliferation that could contribute to a first step of leukemic transformation (Donada et al., personal observation).

1.2. MYH9-Related Disease

With more than 300 families reported in the literature, MYH9-RD is the most frequent form of IT. It encompasses four dominant entities previously considered as different disorders: May-Hegglin, Sebastian, Epstein and Fechtner syndromes. Diagnosis of May-Hegglin and Sebastian syndromes was based on the presence of congenital macrothrombocytopenia and characteristic leucocyte inclusions, while the diagnosis of Epstein and Fechtner syndromes were given in patients with the additional features of cataracts, hearing loss and/or nephropathy often leading to end-stage renal disease (ESRD).

The disease locus was mapped to 22q12.3-q13.2 chromosome in 1999 (Kunishima et al., 1999) and one year later, mutations in MYH9 gene were identified (Seri et al., 2000) in all the four conditions that are actually named as MYH9-RD (OMIM155100).

MYH9-RD is an autosomal dominant disorder characterized since birth by macrothrombocytopenia with more than 40% of large platelets (> 3.9 µm in diameter). All neutrophils of all patients have in their cytoplasm clumps of MYH9 that can be recognized by immunofluorescence microscopy with antibodies against this protein. Sometimes these inclusion bodies are recognized also in May-Grünwald Giemsa stained blood films and are named Döhle-like bodies. Despite the bleeding episodes are usually rare and mild, or even absent, MYH9-RD is not a trivial disorder because 25% of patients develop a proteinuric nephropathy that in most cases evolves into renal failure (ESRF) and requires dialysis or kidney transplantation. Moreover, near 50% of patients develop sensorineural deafness and 18% presenile cataract. Thus, the clinical course of MYH9-RD is quite variable, ranging from isolated and asymptomatic thrombocytopenia to a complex disorder that severely affects the quality of life.

There are 3 forms of non-muscle myosin II (NMII), NMIIA, NMIIIB and NMIIIC. A single unit of NMII consists of two heavy chains linked to

two regulatory light chains (RLC) that regulate NMII activity and two essential light chains (ELC) that stabilize heavy chain structure. MYH9 is the heavy chain subunit of NMIIA, MYH10 of NMIIIB and MYH14 of NMIIIC. The phosphorylation of key residues on the RLCs induces a conformational change in NMII molecules that promotes the filament assembly and ATPase activity of NMII. NMII thus interacts with the actin filaments in an ATP dependent manner to produce tension and induce sliding of the actin filaments providing therefore mechanical forces necessary for maintenance of cell shape, cell migration (adhesion and polarity) and cytokinesis (Vicente-Manzanares et al., 2009). Only MYH9 and MYH10 are present in Mks and MYH9 alone in platelets (Lordier et al., 2012).

MYH9 is composed of two main domains, N-terminal and tail domains. The N-terminal is composed of the head (HD) or motor domain that interacts with actin and binds ATP and the neck domain that binds myosin light chains. The HD consists of four subdomains, the N-terminal SH3-like motif, the upper and the lower 50 kDa subdomains and the converter subdomain (Sellers, 2000). The C-terminal tail domain (TD) is composed of two regions, a long alpha-helical coiled coil domain involved in dimerization of heavy chains and C-terminal non-helical tailpiece (NHT) playing a role in phosphorylation.

Among the most frequent *MYH9* mutations are those affecting S96 and R702 located in the HD, R1165, D1424, E1841 in the coiled coil and R1933 in the NHT (Balduini et al., 2002). About 35% of cases are sporadic with de novo mutations (Balduini and Savoia, 2012) and two cases have been shown to be due to somatic or germinal mosaicism (Kunishima et al., 2005, 2009). A substitution c.5765 + 2T > A in the donor splice site of intron 40 was also reported. This missense mutation induces a frameshift p.R1922Rfs*43 (Saposnik et al., 2014). Together, about 83 genetic variations have been described so far (Saposnik et al., 2014).

Correlation between clinical phenotype and *MYH9* mutation localization showed that mutations in the motor domain are associated with hearing loss and nephritis while those located in tail domain lead rather to moderate thrombocytopenia with absence of renal disease and cataract development (Pecci et al., 2008b). Recent analysis performed on 255 patients from 11 families defined disease evolution associated to seven different *MYH9* genotypes responsible for 85% of *MYH9*-RD (Pecci et al., 2014). Almost complete penetrance for ESRD and severe thrombocytopenia were associated to mutation at residue R702. ACE-inhibitors or angiotensin receptor blockers were suggested to be effective in treatment of proteinuria and in decreasing progression of kidney damage (Pecci et al., 2008a). After the R702 mutation, the highest risk of developing all the noncongenital manifestations of disease was shown for the p.D1424H substitution. High risk of deafness, but low risk of nephropathy or cataract, were shown for substitutions at R1165 and low risk of noncongenital defects were reported for p.E1841K, p.D1424N substitutions and for C-terminal deletion. In these three cases, a thrombocytopenia is predicted to be the only manifestation of the *MYH9*-RD throughout life.

With some exceptions, the HD mutations impair the ATPase activity whereas those in TD alter molecule assembly (Balduini et al., 2011).

To study the mechanisms leading to a generation of macroplatelets, different mouse models have been developed based on the *myh9* deletion or knock-in with the mutants found in patients. Since *Myh9*^{-/-} genotype causes early embryonic mortality (Conti et al., 2004; Matsushita et al., 2004), major results have been obtained with animals having *Myh9* inactivation restricted to the megakaryocyte lineage. Taking advantage of these models, premature proplatelet formation and macrothrombocytopenia were observed only in homozygous but not heterozygous knock-out mice (Eckly et al., 2009; Léon et al., 2007). The increased proplatelet formation *in vitro* associated to macrothrombocytopenia *in vivo* was first explained by premature release of proplatelets in the BM, however subsequent study using BM explants showed that Mks displayed fewer proplatelets and increased Mk mortality contributing to the thrombocytopenia (Eckly et al., 2010). Knock-

in models expressing R702C, D1424N and E1841K mutations found in human were reported (Zhang et al., 2012). Homozygous expression of a most severe R702C mutant affecting a head domain of *myh9* led to the embryonic death while homozygous D1424N and E1841K and all heterozygous knock-in mice developed macrothrombocytopenia. The heterozygous R702C mice also developed cataract, focal segmental glomerulosclerosis and hearing loss similarly to *MYH9*-RD patients. Clearly, the *Myh9* mutants do not phenocopy a pure haploinsufficiency and rather behave as hypomorphic alleles or dominant negative mutants.

Finally, the *in vitro* studies of Mks derived from *MYH9*-RD patients harboring both head and tail domain mutations showed a decrease in proplatelet formation associated with an abnormal spreading on polylysine, fibronectin and collagen, an abnormal distribution of actomyosin cytoskeleton and a huge increase in contractile forces after adhesion (Chen et al., 2013). Increased cell spreading on collagen was also demonstrated after overexpression of frequently found *MYH9* mutants in a megakaryoblastic Dami cell line (Pecci et al., 2011). Overall, these results demonstrated that *MYH9*-RD is related rather to an excess of actomyosin contractility than to a loss of function. This is in line with the work of Breckenridge showing that tailpiece is involved in modulating the local filament assembly disassembly reaction probably via phosphorylation at S1943 (Breckenridge et al., 2009). Both, myosin II inhibitor blebbistatin and the ROCK inhibitor Y27632 rescued the defect in proplatelet formation. These results provided evidence that *MYH9* mutants induce defect in proplatelet formation through an excess of actomyosin contractility in spreading Mks that could represent a new therapeutic target (Chen et al., 2013).

However, more recently, Spinler et al. proposed that the myosin activity is necessary for the cytofission of the preplatelets on normal sized-platelets. They demonstrated that myosin IIA is inactive in normal Mks and become activated in pre-platelets by dephosphorylation at S1943 under the shear (Spinler et al., 2015). We can hypothesize that in *MYH9*-RD, myosin IIA is slightly activated through Rho pathway leading to the inhibition of proplatelet formation by increased contractility. In contrast, at time of abscission, when it should be activated at higher degree, the mutant may have suboptimal contractile functions resulting in macroplatelet formation.

The pathogenesis of cataracts in *MYH9*-RD is completely unknown, and only limited information has been obtained on the pathogenetic mechanisms of kidney failure and deafness.

Although the development of kidney failure is a major risk for many patients with *MYH9*-RD, a few studies addressed pathogenesis of nephropathy in this condition. As already discussed, we know that some mutations very frequently result in kidney failure, while others almost never affect kidney function (Pecci et al., 2014), but the reasons for these differences are unknown. Morphological and immunomorphological studies of a few kidney biopsies revealed defective NMMHC-IIA distribution within podocytes, segmental effacement of these cell, loss of the interpodocyte slit diaphragm, and a rapidly progressive focal segmental glomerulosclerosis (Balduini et al., 2011). Recent studies of experimental models suggested that NMIIA is essential for maintenance of podocyte cytoskeletal structure and mechanical function (Bondzie et al., 2016), and *MYH9* mutations renders podocytes more susceptible to injury after a damaging stimulus (Cechova et al., 2018).

Also the mechanisms of deafness in *MYH9*-RD are poorly understood. Since NMIIA is expressed in the sensory hair cells of the organ of Corti, it has been suggested that *MYH9* mutations cause hearing loss by disruption of the structural integrity of stereocilia, similarly to what has been observed in experimental models of deafness caused mutations of other members of the myosin family, such as myosins VI, VIIA and XVA (Lalwani et al., 2008).

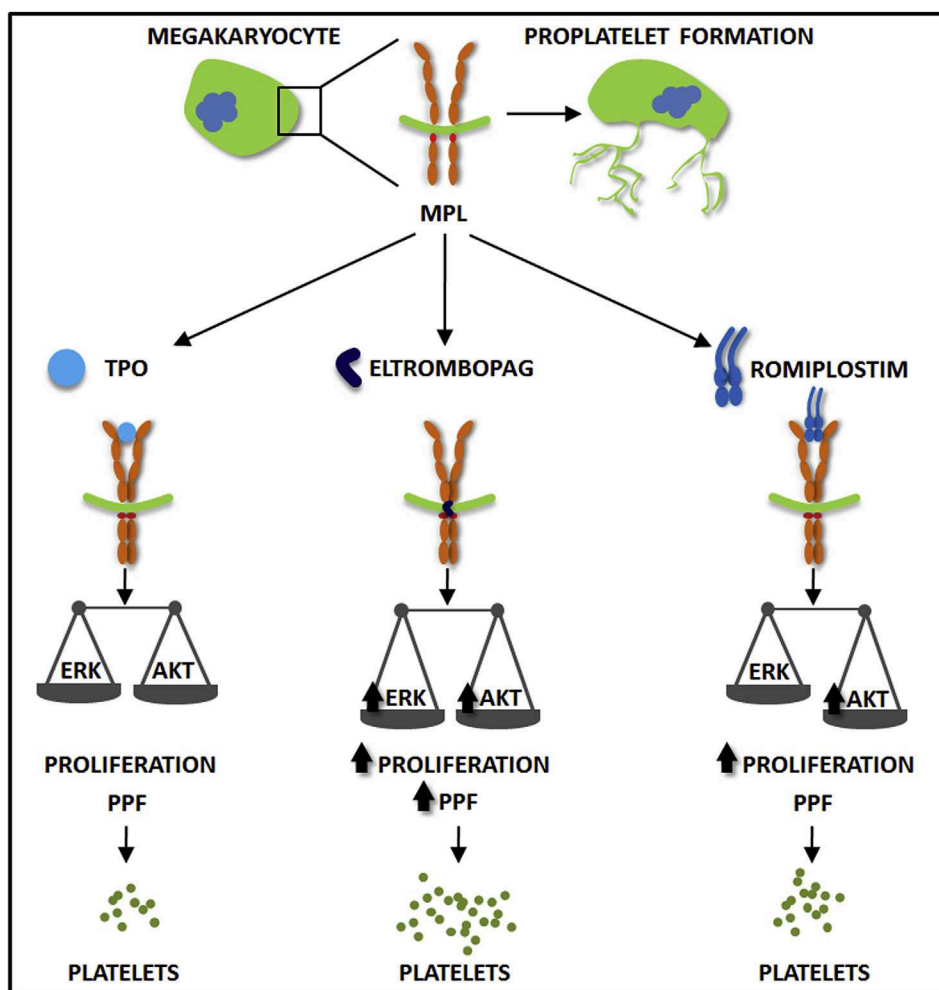


Fig. 2. Schematic summary of different effects of eltrombopag and romiplostim compared to TPO during megakaryopoiesis. Megakaryopoiesis takes place under the activation of c-Mpl, the thrombopoietin receptor. TPO binding to c-Mpl at residues F104, D261 and L265, located at the extracellular domain of the receptor, induces changes in c-Mpl conformation and the activation of downstream signaling pathways. Eltrombopag is a non-peptide TPO mimetic that binds to c-Mpl mainly at residue H499, located at the transmembrane domain of the receptor, and greatly enhances AKT and ERK activation, thus ensuring Mks proliferation and increased proplatelet formation (PPF). At variance, Romiplostim, which competes with TPO for binding to the same sites on c-Mpl, promotes increased AKT but not ERK activation with respect to TPO. This leads to a significant increase in Mk proliferation.

1.3. Thrombopoietin mimetics and inherited thrombocytopenias

Patients with thrombocytopenia are at risk of hemorrhages, either spontaneous or provoked by trauma or other hemostatic challenges. Recently, the opportunity to synthesize *in vitro* molecules able to mimic the physiologic effect of TPO on Mk differentiation and platelet production opened new perspectives for their treatment (Hitchcock and Kaushansky, 2014). Different compounds have been shown to increase platelet count in humans and/or animals, but only two molecules have been so far approved for use in humans: romiplostim and eltrombopag (Fig. 2).

Romiplostim competes with TPO for binding to the TPO receptor (c-Mpl) and activates human and animal megakaryopoiesis through the same mechanisms as TPO. Subcutaneous administration of romiplostim increased platelet counts in patients with idiopathic (autoimmune) thrombocytopenic purpura (ITP) (Bussel et al., 2006) and its use has been approved in this disorder (Thompson, 2008).

Eltrombopag is a TPO nonpeptide mimetic (Erickson-Miller et al., 2009) that has a binding site different from that of TPO (Kuter, 2007) and is likely to act through an alternative signal transduction pathway, as suggested also by the observation that its effect is additive to that of TPO in cell-based assays. It has been approved for treating ITP and for increasing platelet count in thrombocytopenia due to hepatitis C infection (Bussel, 2009; McHutchison et al., 2007).

Currently, only two therapeutic options are approved for increasing platelet counts in ITs: (1) platelet transfusions, which transiently improve thrombocytopenia, but expose to the risks of acute reactions, transmission of infectious diseases, and refractoriness to subsequent

platelet transfusions; (2) allogeneic hematopoietic stem cell (HSC) transplantation, which can definitively cure the disease, but is a high risk procedure (Balduini et al., 2012; Locatelli et al., 2003; Notarangelo et al., 2008). Demonstration that TPO mimetics increase platelet count in acquired thrombocytopenia raised the hypothesis that these drugs can rescue the pathological phenotypes of ITs. However, ITs derive from a variety of intrinsic genetic defects (Balduini et al., 2013), which in some cases predispose patients to leukemic transformation, such as in *ANKRD26-RT* (Melazzini et al., 2016), or development of BM fibrosis, such as Grey Platelet Syndrome (GPS) (Nurden and Nurden, 2007), aspects to take into account for the possible use of TPO mimetics in ITs since it has been hypothesized that they may exert leukemogenic effect and induce BM fibrosis in humans (Cuker et al., 2010; Oshima et al., 2013). Of note, we have recently demonstrated that over-stimulation of TPO receptor leads to Mk release of Transforming Growth Factor (TGF)- β 1 (Abbonante et al., 2016), one of the major BM pro-fibrotic cytokines, which is involved in regulation of platelet formation and can induce synthesis and release of extracellular matrix (ECM) components, typically involved in fibrosis, by both Mks themselves and BM Mesenchymal Stromal Cells (MSCs) (Abbonante et al., 2015, 2016; Badalucco et al., 2013). Therefore, TPO mimetics represent an appealing therapeutic hypothesis for treatment of ITs, but their efficacy and safety may vary depending on the type of mutation. To open new prospective application of TPO mimetics, we searched for the mechanisms of action of these compounds. We demonstrated that increasing doses of romiplostim (100–2000 ng/mL) determines a progressive increase of Mk proliferation with a parallel decrease in Mk ploidy and capacity of extending proplatelets. Most importantly, these differences in Mk function

seemed to be correlated to modulation of AKT phosphorylation (Currao et al., 2013). These results were consistent with previous data, by Leon et al., obtained administering romiplostim to *Myh9*^{-/-} mice (Léon et al., 2012). Mks increased to similar numbers in *Myh9*^{-/-} and wild-type (WT) mice (with an increase in immature Mks), but *Myh9*^{-/-} platelet count response was much less (2.5-fold vs 8-fold increase).

So far, only one clinical trial has been conducted with eltrombopag on a small cohort of IT patients affected by *MYH9*-RD. Short-term treatment demonstrated the ability of this drug to increase platelet count and reduce bleeding tendency in most patients, but the short duration of treatment did not provide any information on the side effects of long-term treatment with this drug (Pecci et al., 2010). On this basis, we believe that eltrombopag has to be considered as a useful alternative to platelet transfusion in preparing *MYH9*-RD patients to elective surgery, but further clinical studies are required before recommending the its long-term administration in the few patients with severe haemorrhagic diathesis. Unfortunately, *in vivo* studies on animals with eltrombopag are prevented because of its specific affinity only for human and chimpanzee c-Mpl (Erickson-Miller et al., 2009), while *in vitro* human culture systems lack to recreate the complex architecture and composition of BM environment (Balduini et al., 2016). Therefore, future advancement in testing the efficacy and safety of eltrombopag strongly depends on the opportunity to adopt bioengineering techniques that accurately reproduce the physiologic features of the human BM niche, including BM cells, soluble and surface-bound signaling factors, ECM components, and blood shear, that together provide an ideal habitat to support thrombopoiesis (Currao et al., 2015; Di Buduo et al., 2016a; Di Buduo et al., 2017a, b; Mendelson and Frenette, 2014). In this regard, we have recently developed a BM model made of silk fibroin, derived from *Bombyx mori* silkworm cocoons, a naturally-derived, biocompatible, non thrombogenic and tunable biomaterial extensively used in cell culture studies, regenerative medicine and biomedical applications (Altman et al., 2003; Meinel et al., 2005; Omenetto and Kaplan, 2010). Our 3-dimensional (3D) BM niche showed that silk vascular tubes, embedded in a silk sponge mimicking the “spongy” marrow, support Mk growth and the release of functional platelets into perfused vascular tube lumen, allowing the study of both physiological and pathological thrombopoiesis (Di Buduo et al., 2015). To investigate eltrombopag's impact on Mk functions, we employed our established *in vitro* model for studying hematopoietic stem cell differentiation combined with our latest 3D silk-based BM tissue model (Di Buduo et al., 2016b). Results demonstrated that eltrombopag favors human Mk differentiation and platelet production in a dose-dependent manner. These effects are accompanied by increased phosphorylation of AKT and ERK1/2 signaling molecules, which have been proven to be crucial in regulating physiologic thrombopoiesis. These data further clarify the different mechanisms of action of eltrombopag when compared to romiplostim, which, as we have shown, induces the proliferation of immature Mks rather than platelet production, due to the unbalanced activation of AKT and ERK1/2 signaling molecules (Di Buduo et al., 2016b).

2. Conclusions

ITs are transforming from mysterious diseases into natural experimental models to better understand the mechanisms of platelet biogenesis. Continuing studies on these disorders is expected not only to further improve knowledge on a complex and interesting biological process, but also to offer for the first time the concrete possibility of improving with a simple medical treatment the quality of life of affected patients.

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