Eltrombopag, a potent stimulator of megakaryopoiesis

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In this issue of *Haematologica*, Di Buduo *et al.* show that eltrombopag induces human megakaryopoiesis *in vitro* and *ex vivo* through an activation of STAT, AKT and ERK pathways that is different from the other thrombomimetic, romiplostim.[1]

Megakaryopoiesis is the process leading to platelet production in the blood from the differentiation of bone marrow progenitors to platelet precursors called megakaryocytes (MKs).[2] This phenomenon involves the commitment of a multipotent hematopoietic stem cell (HSC) towards a MK progenitor. This progenitor undergoes several divisions by classical mitosis, and further maturation involves two unique biological mechanisms: polyploidization through a process called endomitosis, and fragmentation of the MK cytoplasm to produce platelets. This process is highly regulated by numerous transcription factors, including *GATA1/2*, *FOG-1*, *RUNX1*, *FLI1*, *SCL*, *GFI1b*, *NFE2* and *MYB*, and by many extrinsic factors such as cytokines, chemokines and extracellular matrix components. The major cytokine regulating megakaryopoiesis is the thrombopoietin (TPO). TPO binds to the extracellular domain of the type I homodimeric receptor MPL, mainly to two residues, D261 and L265, but also in a site around the residue F104, which is mutated in congenital amegakaryocytic thrombocytopenia.[3,4] TPO binding results in conformational changes of the receptor, leading to the activation of pre-associated JAK2 molecules. Following the transphosphorylation of the receptor, downstream signaling molecules, including STAT, ERK and PI3K, become activated. The expression of MPL and JAK2 gradually increases from the HSC to the MK throughout MK differentiation.[5] Therefore, the TPO/MPL axis not only controls megakaryopoiesis, but also HSC homeostasis. Indeed, *c-mpl*$^{-/-}$ mice present both a defect in HSC function and thrombocytopenia.[6]

Defects in this TPO/MPL/JAK2 axis leads to hematological diseases such as thrombocytopenia or pancytopenia through the inhibition of the megakaryopoiesis process. Alternatively, thrombocytopenic states could occur following chemotherapies and immune or infectious diseases. To overcome thrombocytopenia, several TPO mimetics have been developed including romiplostim (Amgen) and eltrombopag (Novartis). Romiplostim is a TPO mimetic that binds to MPL in a competitive manner, and consists of two identical single-chain subunits composed of human IgG1 Fc linked to a peptide containing two MPL-binding domains.[7] In contrast, eltrombopag is a non-peptide TPO mimetic, which binds to the transmembrane domain of human MPL at critical residue H499, which is not conserved in the mouse Mpl.[8] These two compounds have been approved by the US Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) to raise platelet counts in immune thrombocytopenic purpura (ITP)[9] and in infections caused by the hepatitis C virus.[10] The stimulation of the TPO/MPL axis by TPO mimetics has also been successfully used in inherited MYH9-related thrombocytopenia and in aplastic anemia, in which eltrombopag showed benefits based on multilineage clinical responses.[11,12] The effects of both romiplostim and eltrombopag in the treatment of both myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) have been investigated. The use of eltrombopag in their treatment had very limited clinical benefits; no significant improvements in platelet counts were noted, while a decrease in bleeding episodes was observed.[13] Moreover, no difference in the proportions of malignant blasts was found in the bone marrow and peripheral blood of patients receiving eltrombopag *versus* placebo. Other studies showed that eltrombopag allowed the formation of normal MK colonies and a strong inhibition of leukemic cell proliferation.[14,15] The binding of eltrombopag to MPL is specific to humans and other primates. However, it could affect murine and human leukemic cell proliferation by an MPL-independent pathway through the modulation of intracellular iron content. In aggregate, these studies have shown the importance of TPO mimetics in both the regulation of HSC homeostasis and megakaryopoiesis. Eltrombopag is an oral drug and thus easy to administer, while romiplostim should be injected subcutaneously. However, the mechanism of action of eltrombopag remains incompletely understood, since it could not be studied in preclinical mouse models.

In their work, Di Buduo *et al.* used a classical *in vitro* culture system with human primary cells from cord blood to demonstrate that doses of eltrombopag, ranging from 500-2000 ng/mL, stimulates the MK output at day 13 of culture as well as proplatelet formation by 4-fold, in comparison with 10 ng/mL TPO. The presence of MKs was confirmed by the expression of both surface markers (CD61 and CD42) and transcriptional factors (*RUNX1*, *NFE2*). No difference in the percentage of MKs or in their ploidy was found compared to TPO, similar to the findings of another group.[16] In contrast, in another study, romiplostim was shown to display a different effect than TPO on megakaryopoiesis, stimulating MK proliferation but not maturation, with less polyploid MK and a drastic decrease in proplatelet formation. However, this effect was dose-related and linked to the over-activation of AKT but not of ERK with high romiplostim concentrations, while increased TPO doses over-activated both AKT and ERK pathways[17] (Figure 1). These results indicate two different mechanisms of action for TPO and romiplostim, which bind to MPL at the same site and are expected to activate MPL in the same conformation.

Moreover, Di Buduo *et al.* have also developed a novel and original approach to studying megakaryopoiesis *ex vivo,* using a bioreactor technology based on a 3D silk-based bone marrow system, which reproduces the key steps of megakaryopoiesis and thrombopoiesis. Silk fibroin is a natural biocompatible material with non-thrombogenic features, perfectly adapted for the study of blood cell production *ex vivo*. Indeed,

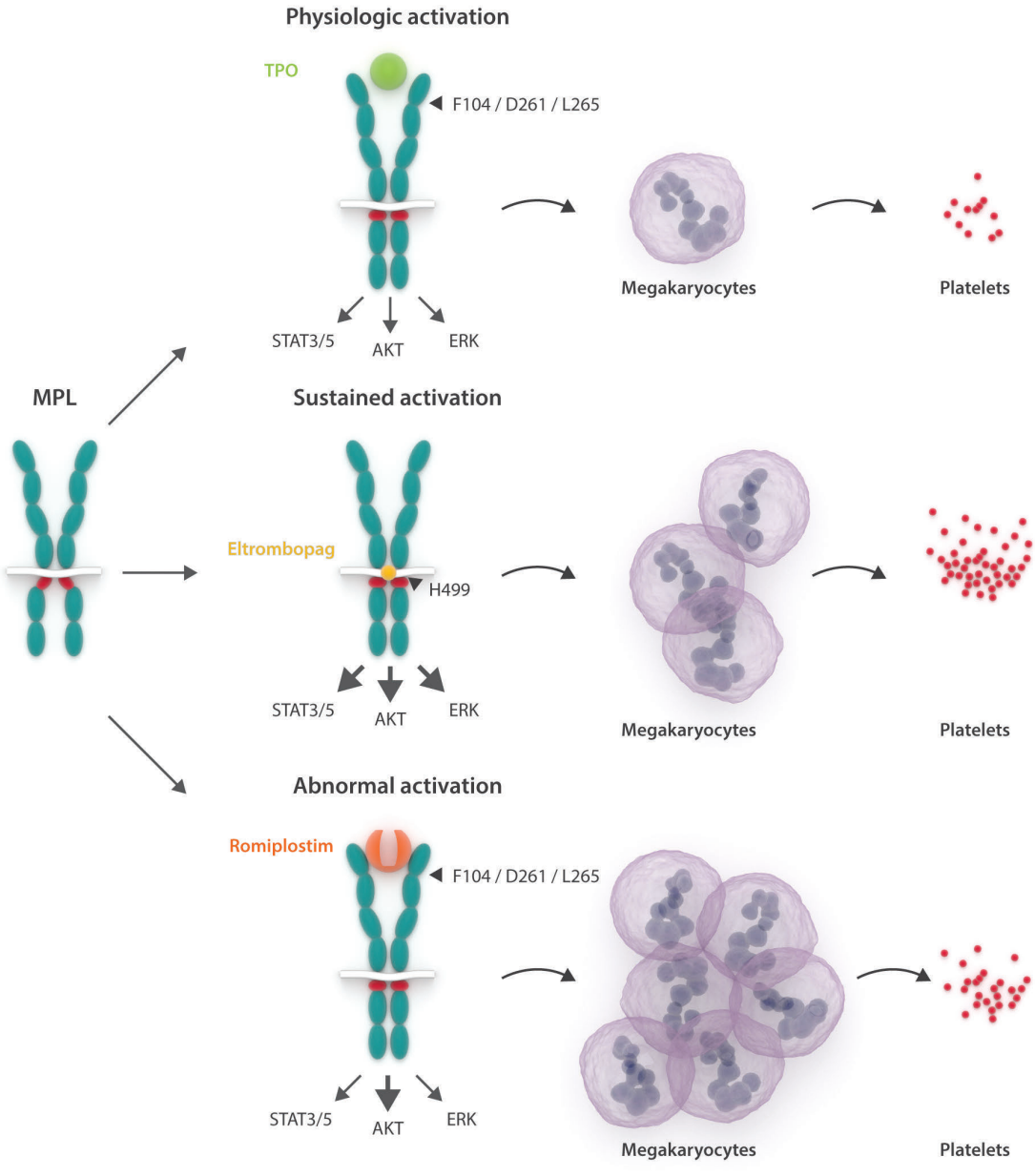

Figure 1: Different effects of eltrombopag and romiplostim compared to thrombopoietin (TPO) during megakaryopoiesis. MPL is a homodimeric receptor important for the megakaryopoiesis process. TPO binding to the extracellular domain of MPL at residues F104, D261 and L265 induces changes in MPL conformation and the activation of STAT3/5, AKT and MAPK signaling pathways. Eltrombopag is a non-peptide TPO mimetic that binds to the intracellular domain of MPL mainly at H499, and greatly enhances the STAT3/5, AKT and MAPK activation at a higher extent than TPO. This leads to megakaryocyte (MK) amplification and overproduction of platelets. Romiplostim is a peptide TPO mimetic that binds to MPL in a similar manner to TPO, but induces a strong activation of AKT compared to STAT and ERK pathways. This leads to a strong MK amplification, which produces a lower platelet yield.

the authors had previously shown that this 3D system provides a tool not only for the study of fundamental biological mechanisms of hematopoiesis, but also for clinical applications.[18] In the study by Di Buduo *et al.*, human CD34$^+$ progenitors from cord blood were seeded in a silk sponge for 13 days, after which MKs were observed based on specific surface markers. Alternatively, silk microtubes embedded in the silk sponge containing MKs was used for the analysis of platelet release, demonstrating that these MKs and the platelets were fully functional, even if the yield was lower than in *in vitro* 2D cultures. Thus, by using this technology, they have also demonstrated that eltrombopag mediates the differentiation of MKs and the release of platelets *ex vivo*.

To elucidate the mechanism of eltrombopag compared to TPO or to romiplostim, the authors investigated the signaling pathways activated in CD34$^+$ progenitors and MKs. They found that eltrombopag induces the phosphorylation of STAT3/5, AKT and ERK pathways, in line with another study.[16] These molecules are involved not only in proliferation and differentiation but also in survival and

anti-apoptotic processes. Interestingly, they found an enhanced signaling in eltrombopag-restimulated MKs or progenitors after cytokine starvation compared to TPO. Moreover, and unexpectedly, they found that MKs or progenitors treated *in vitro* with various doses of eltrombopag also presented a greater dose-dependent activation of these signaling molecules than with TPO (Figure 1). These results may be explained knowing that MPL can assume different intermediate conformations between its inactive and ligand-bound states, in contrast to other receptors such as the erythropoietin receptor. These various conformations activate downstream signaling pathways differently.[19] More particularly, it has recently been shown that eltrombopag activates MPL through the induction of an efficient dimerization of the transmembrane helix of MPL around the H499 residue.[8]

Moreover, AKT and ERK were activated by eltrombopag at a higher extent than by TPO, while romiplostim was previously shown to strongly activate AKT with a mild effect on ERK.[17] While AKT phosphorylation seems to be activated during every megakaryopoiesis step, it has been shown that the ERK pathway is strongly induced at the beginning of megakaryopoiesis but must later be switched off for platelet production.[20] Thus, the question of why the production of proplatelets was increased with eltrombopag, despite an enhanced and sustained activation of ERK, remains elusive. We could hypothesize that: i) there is a fine tuned cooperation between simultaneous activation of AKT and ERK pathways, ii) differential intensity and/or regulation of the signal mediated by eltrombopag (sustained signal over time), and iii) activation of new molecules and signaling pathways by eltrombopag. The way in which eltrombopag activates MPL is analogous to the constitutive activation of MPL induced by MPLS505N and MPLW515K/L/R mutants associated with myeloproliferative neoplasms and hereditary thrombocytosis.[8] Therefore, one can speculate that this non-physiological activation of signaling, which is similar to an oncogenic activation, may be very efficient, but must be carefully evaluated in patients treated long-term with eltrombopag in order to eliminate the risk of developing hematological malignancies.

In conclusion, the data obtained using eltrombopag in the study by Di Buduo *et al.* clearly supports a short period of use of this molecule for increasing platelet counts in thrombocytopenia patients. However, the use of this molecule for long-term treatments will require additional studies, in particular of the non-physiological activation of MPL signaling pathways.

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