Thrombopoiesis

Kenneth Kaushansky

The production of platelets is a complex process that involves hematopoietic stem cells (HSCs), their differentiated progeny, the marrow microenvironment and hematopoietic cytokines. Much has been learned in the 110 years since James Homer Wright postulated that marrow megakaryocytes were responsible for blood platelet production, at a time when platelets were termed the "dust of the blood". In the 1980s a number of in vitro culture systems were developed that could produce megakaryocytes, followed by the identification of several cytokines that could stimulate the process in vitro. However, none of these cytokines produced a substantial thrombocytosis when injected into animals or people, nor were blood levels inversely related to platelet count, the sine qua non of a physiological regulator. A major milestone in our understanding of thrombopoiesis occurred in 1994 when thrombopoietin, the primary regulator of platelet production was cloned and initially characterized. Since that time many of the molecular mechanisms of thrombopoiesis have been identified, including the effects of thrombopoietin on the survival, proliferation, and differentiation of hematopoietic stem and progenitor cells, the development of polyploidy and proplatelet formation, the final fragmentation of megakaryocyte cytoplasm to yield blood platelets, and the regulation of this process. While much progress has been made, several outstanding questions remain, such as the nature of the signals for final platelet formation, the molecular nature of the regulation of marrow stromal thrombopoietin production, and the role of these physiological processes in malignant hematopoiesis.
Semin Hematol 52:4–11.

THE HISTORY OF THROMBOPOIESIS

Carnot, Wright, and others in the early 20th century defined the critical role of platelets in blood coagulation, and their origin from the marrow megakaryocyte based on elegant *camera lucida* images. Based on an evolving understanding of erythropoiesis, particularly the identification of erythropoietin as the humoral regulator of erythrocyte production, Keleman coined the term "thrombopoietin" in 1958 to describe the humoral substance responsible for platelet production.[1]

In the mid-1960s and 1970s, several groups attempted to purify thrombopoietin from the plasma of thrombocytopenic animals; these early efforts were severely handicapped by inconvenient and insensitive assays for the hormone, the in vivo incorporation of radiolabeled methionine into newly formed platelets, and the attempts failed to produce unequivocal proof of the existence of thrombopoietin. In the 1980s a number of in vitro megakaryocyte differentiation assays were developed, facilitating additional purification attempts; however, while some claims were made of its biological activities, attempts to produce a cDNA for thrombopoietin failed.

Occasionally in science, a finding from one field, although in itself important, can have a catalytic and profound effect on a *seemingly* unrelated area of research. The discovery and characterization of the murine myeloproliferative leukemia virus (MPLV) had such an impact on the search for thrombopoietin. MPLV causes an acute myeloproliferative neoplasm in infected mice[2]; in 1990, the responsible oncogene (*v-mpl*) was cloned, and the proto-oncogene (*c-Mpl*) obtained 2 years later.[3,4] Based on the predicted structure of the encoded protein it was immediately evident that *c-Mpl* encodes a member of the hematopoietic cytokine receptor family, which includes the receptors for erythropoietin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, growth hormone, prolactin, and several interleukins (ILs). However, when "c-Mpl receptor" was cloned, the corresponding "c-Mpl ligand" was unknown. Based on the cell from which the receptor was cloned, the bipotent erythroid/megakaryocytic cell line HEL, we, and others, postulated that the c-Mpl ligand might be thrombopoietin.

THE CLONING AND CHARACTERIZATION OF THROMBOPOIETIN

Three distinct approaches yielded cDNA for thrombopoietin. Using an in vitro megakaryocyte-based assay, and

School of Medicine, Stony Brook University, Stony Brook, NY.
Some of the work mentioned in this paper was supported by the National Institutes of Health Grant No. R01 DK49855.
Conflicts of interest: none.
Address correspondence to Kenneth Kaushansky, MD, MACP, Stony Brook University, School of Medicine, Health Sciences Center 4-225, Stony Brook, NY, 11794-8430. E-mail: kenneth.kaushansky@stonybrookmedicine.edu
0037-1963/$ - see front matter

http://dx.doi.org/10.1053/j.seminhematol.2014.10.003

the plasma from 1,100 thrombocytopenic rats, scientists at Kirin Pharmaceuticals employed a 12-step conventional purification scheme to obtain sufficient purified thrombopoietin to obtain amino acid sequence, and then cloned cDNA for rat and then multiple species of thrombopoietin, including the human hormone.[5] Using the *c-Mpl* proto-oncogene product coupled to an affinity matrix, scientists at Genentech obtained sufficient purified porcine Mpl ligand to allow amino acid sequencing and cDNA cloning[6] of ovine and human hormones. In contrast to the biochemical purifications utilized by these groups, an expression cloning strategy using a chemically mutated c-Mpl-bearing cell line was used by Lok and Kaushansky to obtain cDNA for murine and then human thrombopoietin.[7,8] Initial in vitro experiments using the corresponding recombinant proteins demonstrated the effect of thrombopoietin on megakaryocyte maturation, and injections into normal mice resulted in impressive increases in marrow megakaryocytes and peripheral blood platelet counts.[8]

The cloned human thrombopoietin cDNA encodes a polypeptide of 353 amino acids, including the 21 amino acid secretory leader sequence[7]; the mature protein consists of two domains. The amino-terminal 154 residues are homologous to erythropoietin, which like other members of the hematopoietic cytokine family displays a four helix bundle fold,[9] and binds to the c-Mpl receptor. The carboxyl-terminal domain of thrombopoietin bears no resemblance to any known proteins, and acts to prolong the circulatory half-life of the hormone[10]; it also serves as an intramolecular chaperone, aiding in the proper folding of the polypeptide into the mature hormone.[11]

THE BIOLOGICAL ACTIVITIES OF THROMBOPOIETIN

The availability of the recombinant protein allowed the first detailed studies of the biological properties of thrombopoietin. Previous conjecture was that the hormone was solely a megakaryocyte differentiation factor, driving the maturation of megakaryocytes and platelet formation, but had no effect on immature cells of the lineage or other hematopoietic cell types. Initial studies with recombinant thrombopoietin dispelled many of these incorrect assumptions. Thrombopoietin alone is able to stimulate the proliferation of nearly all marrow megakaryocytic progenitor cells (colony-forming unit, megakaryocyte [CFU-MK]) in vitro, and acts in synergy with other hematopoietic cytokines, such as IL-3, IL-11, and stem cell factor (SCF)[12] to promote the growth of CFU-MK. In vitro, thrombopoietin acts to increase megakaryocyte size and expression of lineage-specific megakaryocyte surface proteins, such as glycoprotein (GP)Ib and GPIIb/IIIa.[8,13] Studies of megakaryocyte ultrastructure show increased demarcation membrane and platelet granule formation following culture with thrombopoietin, indicating that the hormone primes megakaryocytes for platelet production.[14] And culture of marrow megakaryocytes in thrombopoietin leads to pronounced polyploidy.[15] However, the final stages of platelet formation and release appear to be thrombopoietin-independent, as withdrawal of the hormone from late-stage megakaryocyte cultures does not eliminate proplatelet formation; in fact, thrombopoietin withdrawal is reported to stimulate it.[16]

Also at odds with the prevailing conventional wisdom on thrombopoietin, in addition to its effects on megakaryocytic progenitors and mature cells, thrombopoietin affects hematopoietic stem cells (HSC) *in vitro*, especially when used in combination with IL-3 or SCF[17,18]. Numerous studies reported the expression of c-Mpl on the surface of HSCs[19,20], indicating the stem cell effects of thrombopoietin are direct. And based on these results, thrombopoietin has been included in many *ex vivo* cytokine cocktails designed to expand HSCs for therapeutic use.[21,22]

More recently, an intriguing paracrine role for thrombopoietin/c-Mpl in maintaining quiescent $Tie2^+$ HSCs at the osteoblastic niche has been identified[23]. Osteoblasts were found to release thrombopoietin, supporting the survival and quiescence of HSCs; inhibition of this interaction reduced the number of HSCs at the osteoblastic niche. Another mechanism by which thrombopoietin affects HSCs is by promoting DNA repair,[24] a finding that could eventually be clinically translated to HSC "protection" from genotypic damage during ionizing radiation or chemotherapy.

In Vivo Thrombopoietic Effects of Hematopoietic Cytokines

Once recombinant thrombopoietin was available purified protein was tested in a range of experimental animals. The initial results were remarkable; within 5 days of daily administration of subnanogram quantities of recombinant murine "c-Mpl ligand", mouse platelet counts were quintupled.[8] These initial experiments made obvious the fact that the c-Mpl ligand obtained by the multiple groups was thrombopoietin, as prior studies of the administration of other megakaryocytic factors (e.g. IL-6, IL-11) would result in, at most, a 50% increase in blood platelet counts. That thrombopoietin is the primary physiological regulator of thrombopoiesis was made clear by genetic studies; the generation of mice engineered to lack either *Thpo* or *c-Mpl* resulted in a 85-90% reduction (although not complete elimination) of platelet counts, and its blood levels were inversely related to platelet count[25,26]. That the *in vitro* HSC effects of thrombopoietin were physiological was then demonstrated when competitive repopulation assays were performed on the *c-Mpl*[27], revealing an approximate 8-fold reduction in HSC activity of marrow cells when compared to normal mouse marrow. Likewise, transplantation of normal HSCs into lethally irradiated normal recipient mice resulted in a 15-20 fold greater increase in post-transplant stem cell expansion compared to transplantation into $Thpo^{-/-}$ recipients.[28] And the final

proof of the critical, non-redundant role of thrombopoietin in HSC biology came from "experiments of nature"; children born with congenital amegakaryocytic thrombocytopenia, who nearly always progress to aplastic anemia (stem cell depletion), almost universally display homozygous, or compound heterozygous inactivating mutations of *c-Mpl*,[29] or far more rarely, *Thpo*.

As noted above, elimination of *c-Mpl* or *Thpo* reduces thrombopoiesis to about 10-15% of normal. A number of investigators have attempted to determine the origins of the remaining platelet production. Given a modest effect of IL-3, GM-CSF, oncostatin-M, IL-6 and IL-11 on megakaryopoiesis *in vitro* and *in vivo*, a number of studies creating double knock-outs of *c-Mpl* and these other cytokines or their receptors have been performed. The combined reductions failed to reduce platelet levels below that seen with *c-Mpl* deficiency alone. Based on the synergy shown between thrombopoietin and erythropoietin in vitro, we also genetically combined *c-Mpl* deficiency and *Epo receptor* deficiency; we also found that the combined elimination failed to further reduce thrombopoiesis over that seen in *c-Mpl* deficient mice. However, the combination of CXCL12 (previously termed stromal derived factor 1) and fibroblast growth factor (FGF)-4, two cytokines that promote megakaryocyte homing to the vascular niche in marrow, were found to restore thrombopoiesis in *c-Mpl* or *Thpo* null mice, unlike IL-6 or IL-11[30]. The investigators proposed that cells or substances in the vascular niche was responsible for the favorable effects of the two cytokines.

Despite significant advances in understanding the role of thrombopoietin in HSCs, progenitors and megakaryocytes, the *physiologically relevant* effects of thrombopoietin and c-Mpl on platelet function remains somewhat elusive. Platelets express c-Mpl and the molecular machinery required for thrombopoietin signal transduction, including; JAK2, STAT3, STAT5, Akt and Ras (see below). Superphysiological amounts of thrombopoietin (>100ng/ml) directly trigger platelet aggregation *in vitro* (reviewed in[31]), whilst more physiological concentrations of the hormone prime platelets for stimulation with other agonists, possibly by increasing activity of Ras[32]. Thrombopoietin also has significant effects on platelet adhesion under flow. Low thrombopoietin concentrations (0.01-1ng/ml) accelerate firm platelet adhesion to von Willebrand factor and prevent de-attachment at higher flow rates, suggesting that thrombopoietin may be important in thrombus formation[33]. However, despite many years of clinical trials, and current clinical use, only a single publication reports an excess of thrombosis in patients treated with thrombopoietin or thrombopoietin receptor agonists, and that was found in patients with severe liver disease undergoing invasive and vascular procedures.[34]

The Thrombopoietic Marrow Microenvironment

Evidence from many sources has established the existence of two distinct anatomical and functional marrow microenvironmental "niches", the osteoblastic niche[35] and the vascular niche.[36] Hematopoietic stem and progenitor cells localize to both, but the functional effects on cells at these distinct locations differ. Megakaryocyte maturation and platelet formation are dependent on cellular migration from the osteoblastic to the vascular niche. At this latter site, once adequately mature, megakaryocytes extend proplatelet processes through or between cells of the sinusoidal endothelial layer and shed platelets into the bloodstream.[37]

Marrow stromal cells are responsible for crafting these local microeenvironments, through their expression of soluble and surface bound cytokines, counter-receptors for integrins and other adhesion molecules on the surface of hematopoietic cells, and through their secretion of extracellular macromolecules. A number of marrow cell types elaborate matrix molecules, including osteoblasts, endothelial cells, fibroblasts, adipocytes, CXCL12-abundant reticular (CAR) cells (which surround sinusoidal endothelial cells), and even hematopoietic cells such as macrophages and megakaryocytes.[38] In specific reference to megakaryocyte development, marrow stromal cells have been shown to secrete thrombopoietin,[39,40] the primary regulator of thrombopoiesis, CXCL12,[41] which is a primary chemokine attracting megakaryocytes and other hematopoietic cells to the marrow microenvironment, and acts to stimulate megakaryocyte growth,[42] to express cell surface-bound SCF,[43,44] which acts in synergy with thrombopoietin to promote megakaryocyte growth,[45] and to express VCAM-1 and fibronectin, which bind to megakaryocyte integrin α4β1 and promote cell growth.[46,47] Different fragments of microenvironmental fibronectin have been shown to differentially support erythroid, or megakaryocytic progenitors[47,48], potentially providing a mechanism for enhancing or suppressing thrombopoiesis depending on the animal's blood cell needs. Platelet-endothelial cell adhesion molecule (PECAM)-1 plays a critical role in megakaryocyte migration, as its genetic elimination blunts cell migration in response to CXCL12.[49] And the interaction of microenvironmental VWF and its megakaryocyte receptor glycoprotein Ib/V/IX appears particularly important for proplatelet formation. In contrast, type I collagen, which localizes to the osteoblastic niche, prevents platelet formation, where megakaryocyte proliferation is favored over maturation into platelets.[50]

Regulation of Thrombopoiesis

While a number of hematopoietic cytokines can stimulate megakaryocyte growth *in vitro* (IL-3, GM-CSF, SCF), and when injected *in vivo* some produce modest increases in blood platelet counts (e.g. IL-6, IL-11), the blood levels of these proteins do not vary with platelet count except for IL-6 (see below on inflammation). In contrast, plasma concentrations of thrombopoietin vary inversely with the platelet count in patients with reduced

platelet production.[51] Multiple organs display RNA transcripts for thrombopoietin, being present at highest levels in the liver in normal animals.[6,7] One model that accounts for the regulation of blood thrombopoietin levels is based on the capacity of platelets to adsorb thrombopoietin from solution, internalize and destroy it[52]; in patients with thrombocytosis, the steady-state level of thrombopoietin production is overwhelmed by platelet-mediated destruction, and so levels are low, and thrombopoiesis is low; in contrast, in thrombocytopenic patients, with little of the hepatic thrombopoietin production removed by platelets, blood levels of the hormone to rise, driving increased thrombopoiesis. Additional support for this model comes from *Thpo*$^{+/-}$ mice;[26] loss of one allele of *Thpo* leads to a 40% reduction in platelet counts.

In addition to this "autoregulation" model, a growing body of evidence suggests other mechanisms regulate thrombopoietin production. At baseline, it is very difficult to detect specific mRNA in marrow stromal cells. However, transcript levels are substantially increased in marrow stromal cells in response to thrombocytopenia.[39,53] The mechanism underlying this response is only beginning to be understood. It is known that platelet derived growth factor (PDGF)-BB and fibroblast growth factor (FGF)-2 stimulate, and platelet factor 4, thrombospondin and transforming growth factor (TGF)-β inhibit thrombopoietin production from cultures of marrow stromal cells;[54] on balance, whole platelet extracts suppresses thrombopoietin production.

In addition to marrow stromal cell thrombopoietin production, a number of inflammatory states (e.g. ulcerative colitis, rheumatoid arthritis) are associated with thrombocytosis, and increased thrombopoietin levels.[55,56] The inflammation-induced increase in thrombopoietin expression is mediated by IL-6, which stimulates hepatocyte thrombopoietin production both *in vitro* and *in vivo*.[57,58] A final new model that helps explain the regulation of blood thrombopoietin levels, and hence thrombopoiesis, is platelet binding to the hepatic Ashwell-Morrell receptor, which triggers enhanced hepatic thrombopoietin production (see: http://www.thsna.org/Presentation_Upload/presentation_uploads/86_57_86_57_Hoffmeister_2014_03_THSNA_CHICAGO.pdf).

The Molecular Mechanisms of Thrombopoiesis

c-Mpl is a member of the type I cytokine receptor family, along with receptors for a number of interleukins, colony stimulating factors, growth hormone and erythropoietin. The receptors of this family are multimeric; either homo- or heterodimeric, or heterotrimeric[59]. The c-Mpl receptor is a homodimer. The binding of cognate ligand to these receptors induces a conformational change in the multimeric receptor, which triggers a number of phosphorylation events, including that of both the cytoplasmic domain of the receptor and its associated proteins. However, type I cytokine receptors, including c-Mpl, lacks intrinsic kinase activity; instead it recruits and directly associates with the cytoplasmic kinase JAK2 to mediate phosphorylation and activation of downstream signaling proteins. JAK2 associates with c-Mpl prior to the receptor being trafficked to the membrane; in fact, the c-Mpl/JAK2 association stabilizes expression of the receptor, increasing the presence of c-Mpl at the membrane.[60]

Extensive work using both c-Mpl-expressing cell lines and primary marrow cells has identified a variety of different intracellular proteins activated and squelched following thrombopoietin engagement of c-Mpl. Due to the relatively close homology between c-Mpl and the erythropoietin receptor, initial studies focused on the JAK/STAT pathway and identified JAK2 and TYK2 as the immediate kinases that bind to c-Mpl and become activated following thrombopoietin binding,[61,62] leading to the activation of the transcription factors STAT3 and STAT5.[63] Thrombopoietin also stimulates the phosphorylation and formation of the Shc-Grb2-SOS adaptor protein complex,[64,65] activates the phosphatases SHIP and SHPTP-2, and both the phosphoinositide-3-kinase (PI3K)/Akt[66,67] and Raf-1/MAP kinase pathways.[68]

The activation of both PI3K and MAPK are instrumental in mediating many effects of thrombopoietin on c-Mpl bearing cells. A number of additional transcription factors are activated in stem cells and megakaryocytes in response to thrombopoietin. The Hox genes were first recognized for their effects on body pattern development, but were subsequently shown important in a number of mature cell settings. HoxB4 and HoxA9 were shown to influence the levels of HSCs.[69,70]

Thrombopoietin induced PI3K and MAPK lead to the synthesis of HoxB4,[71] and the nuclear translocation of HoxA9,[72] helping to explain the effect of the hormone on HSCs. The cytokine can also stimulate expression of hypoxia inducible factor,[73] a transcription factor critical for the expression of vascular endothelial cell growth factor, which also influences stem cell expansion.[74] In addition to these molecular pathways that underlie the favorable effects of the hormone on HSC expansion and/or survival, thrombopoietin also influences stem/progenitor cell lineage fate determination. The relative level of expression of the transcription factor c-Myb influences the lineage choice of bipotent erythroid/megakaryocytic progenitors.[75] By influencing the level of microRNA (miR)-150, which influences the stability of c-Myb mRNA and its translational efficiency, thrombopoietin acts to favor the megakaryocytic lineage.[76]

One of the most obvious effects of thrombopoietin on megakaryocytic progenitors is the induction and advancement of endomitosis, resulting in a highly (32-128N) polyploid cell. Detailed videomicroscopy revealed that megakaryocytes replicate their DNA but abort mitosis in mid anaphase,[77] prior to cellular or nuclear division, and do so over and again resulting in a highly polyploid cell. The small G protein RhoA is expressed by many cells types, and is distributed throughout the cytoplasm during

midphase of the cell cycle. During mitosis, RhoA becomes highly localized to the mitotic spindle, by virtue of it's activation by the guanine nucleotide exchange factors GEF-H1 and ECT2. Endomitotic megakaryocytes express abundant RhoA, but it fails to localize to the mitotic spindle[77,78] as occurs in normal diploid cells. Recent studies have shown that endomitosis is critically dependent on inactivation of GEF-H1 and ECT2, and hence, their inactivation of RhoA.[79] Consistent with this finding, when RhoA was genetically eliminated in megakaryocytes and platelets; the resultant mice displayed enhanced polyploidy.[80] However, a direct link between thrombopoietin signaling and RhoA (in)activation has not yet been established. Obviously, additional studies will be required to fully understand the molecular mechanisms underlying megakaryocyte endomitosis.

Given its importance to hematopoiesis and the growth promoting intracellular signaling pathways it activates, stringent regulatory mechanisms are required to ensure thrombopoietin signaling is tightly controlled. Two main mechanisms exist by which thrombopoietin regulates its own activity; activation of negative regulators, and internalization and degradation of its activated receptor. Of the proteins activated or upregulated in response to thrombopoietin, Lyn, Lnk and suppressors of cytokine signaling (SOCS) have all been identified as mediating important negative feedback mechanisms. Inhibition of the Src family kinase Lyn, enhanced thrombopoietin -mediated ERK1/2 activation and proliferation in c-Mpl bearing cell lines, and promoted megakaryocyte differentiation in bone marrow cells.[81] Moreover, a *Lyn*-deficient mouse exhibits increased megakaryopoiesis and a greater signaling response to thrombopoietin.[82] Overexpression of the adaptor protein Lnk, negatively regulates thrombopoietin-mediated activation of STAT5 and ERK1/2 and inhibits cell growth in cell lines, as well as attenuating megakaryopoiesis when overexpressed in hematopoietic progenitor cells.[83] Furthermore, $Lnk^{-/-}$ mice exhibit greatly increased numbers of bone marrow megakaryocytes and their precursors, and enhanced thrombopoietin-mediated activation of ERK1/2, Akt, STAT3 and STAT5 in megakaryocytes. Increased expression of SOCS proteins dramatically inhibits thrombopoietin signaling by directly binding to and down regulating the c-Mpl receptor and downstream signaling proteins (reviewed in[84]).

In addition to activating negative regulators, thrombopoietin-stimulation results in a rapid internalization and degradation of c-Mpl. The adaptor protein-2 associates with the c-Mpl intracellular motif Y_{591}RRL, driving clathrin coat formation and endocytosis, while an identical intracellular motif Y_{521}RRL then targets the internalized receptor to the lysosome and via two intracellular lysine residues, K_{553} and $K_{573,}$ for ubiquitination and proteasomal degradation.[85,86] siRNA knockdown of the E3 ubiquitin ligase Cbl reduced thrombopoietin-mediated c-Mpl ubiquitination, indicating its role in the process, although ubiquitination was not completely prevented, suggesting other E3 ubiquitin ligases may also be involved.

Remaining Questions

Much has been learned about thrombopoiesis in the 100+ years since the origins of platelets from the marrow megakaryocyte was first postulated, particularly in the 20 years since the cloning and initial characterization of thrombopoietin. However, many questions remain. For example, are there other physiologically relevant "thrombopoietic substances", or cytokines exclusively restricted to promoting the formation and release of platelets from large, highly polyploid megakaryocytes? Do we now know all of the relevant signaling pathways employed by c-Mpl in transducing the myriad of signals sent to a cell by thrombopoietin? What is the molecular link between RhoA inactivation during megakaryocyte endomitosis and thrombopoietin, if any? What are all the clinical roles that thrombopoietin receptor agonists are likely to play, and are they truly thrombogenic in certain circumstances? And while the clear pathogenetic role of c-Mpl and its downstream singling kinase, JAK2 in patients with myeloproliferative neoplasms was not discussed above, does thrombopoietin contribute to mutant c-Mpl or mutant JAK2 mediated disease? Only additional research will definitively address these and other important physiological, pathological and therapeutic questions.

REFERENCES

1. Kelemen E, Cserhati I, Tanos B. Demonstration and some properties of human thrombopoietin in thrombocythemic sera. Acta Haematol (Basel). 1958;20:350-5.
2. Wendling F, Varlet P, Charon M, Tambourin P. A retrovirus complex inducing an acute myeloproliferative leukemia disorder in mice. Virology. 1986;149:242-6.
3. Souyri M, Vigon I, Penciolelli J-F, Tambourin P, Wendling F. A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. Cell. 1990;63:1137-47.
4. Vigon I, Mornon J-P, Cocault L, et al. Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: Identification of a member of the hematopoietic growth factor receptor superfamily. Proc Natl Acad Sci USA. 1992;89:5640-4.
5. Sohma Y, Akahori H, Seki N, et al. Molecular cloning and chromosomal localization of the human thrombopoietin gene. FEBS Letters. 1994;353:57-61.
6. de Sauvage FJ, Hass PE, Spencer SD, et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature. 1994;369:533-8.
7. Lok S, Kaushansky K, Holly RD, et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature. 1994;369:565-8.
8. Kaushansky K, Lok S, Holly RD, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature. 1994;369:568-71.

9. Feese MD, Tamada T, Kato Y, et al. Structure of the receptor-binding domain of human thrombopoietin determined by complexation with a neutralizing antibody fragment. Proc Natl Acad Sci U S A. 2004;101:1816-21.
10. Harker LA, Marzec UM, Hunt P, et al. Dose response effects of pegylated human megakaryocyte growth and development factor on platelet production and function in nonhuman primates. Blood. 1996;88:511-21.
11. Linden HM, Kaushansky K. The glycan domain of thrombopoietin enhances its secretion. Biochemistry. 2000;39:3044-51.
12. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and IL-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. Blood. 1995;85:1719-26.
13. Zeigler FC, de Sauvage F, Widmer HR, et al. In vitro megakaryocytopoietic and thrombopoietic activity of c-mpl ligand (TPO) on purified murine hematopoietic stem cells. Blood. 1994;84:4045-52.
14. Zucker-Franklin D, Kaushansky K. The effect of Thrombopoietin on the development of megakaryocytes and platelets: An ultrastructural analysis. Blood. 1996;88:1632-8.
15. Carow CE, Fox N, Kaushansky K. The Kinetics of Endomitosis in Primary Murine Megakaryocytes. J Cell Physiol. 2001;188:291-303.
16. Choi ES, Hokom MM, Chen JL, et al. The role of megakaryocyte growth and development factor in terminal stages of thrombopoiesis. Br J Haematol. 1996;95:227-33.
17. Ku H, Yonemura Y, Kaushansky K, Ogawa M. Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early-acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. Blood. 1996;87:4544-51.
18. Sitnicka E, Lin N, Priestley GV, et al. The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. Blood. 1996;87:4998-5005.
19. Qian H, Buza-Vidas N, Hyland CD, et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell. 2007;1:671-84.
20. Arai F, Yoshihara H, Hosokawa K, et al. Niche regulation of hematopoietic stem cells in the endosteum. Ann N Y Acad Sci. 2009;1176:36-46.
21. Yagi M, Ritchie KA, Sitnicka E, Storey C, Roth GJ, Bartelmez S. Sustained ex vivo expansion of hematopoietic stem cells mediated by thrombopoietin. Proc Natl Acad Sci U S A. 1999;96:8126-31.
22. Nishino T, Miyaji K, Ishiwata N, et al. Ex vivo expansion of human hematopoietic stem cells by a small-molecule agonist of c-MPL. Exp Hematol. 2009;37:1364-77.
23. Yoshihara H, Arai F, Hosokawa K, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell. 2007;1:685-97.
24. de Laval B, Pawlikowska P, Petit-Cocault L, et al. Thrombopoietin-increased DNA-PK-dependent DNA repair limits hematopoietic stem and progenitor cell mutagenesis in response to DNA damage. Cell Stem Cell. 2013;12:37-48.
25. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. Science. 1994;265:1445-7.
26. de Sauvage FJ, Carver-Moore K, Luoh S-M, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med. 1996;183:651-6.
27. Solar GP, Kerr WG, Zeigler FC, et al. Role of c-mpl in early hematopoiesis. Blood. 1998;92:4-10.
28. Fox NE, Priestley GV, Th Papayannopoulou, Kaushansky K. Thrombopoietin (TPO) expands hematopoietic stem cells (HSCs) in vivo. J Clin Invest. 2002;110:389-94.
29. Ballmaier M, Germeshausen M, Schulze H, et al. c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. Blood. 2001;97:139-46.
30. Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med. 2004;10:64-71.
31. Akkerman JW. Thrombopoietin and platelet function. Semin Thromb Hemost. 2006;32:295-304.
32. van Willigen G, Gorter G, Akkerman JW. Thrombopoietin increases platelet sensitivity to alpha-thrombin via activation of the ERK2-cPLA2 pathway. Thromb Haemost. 2000;83:610-6.
33. Van Os E, Wu YP, Pouwels JG, et al. Thrombopoietin increases platelet adhesion under flow and decreases rolling. Br J Haematol. 2003;121:482-90.
34. Afdhal NH, Giannini EG, Tayyab G, et al. Eltrombopag before procedures in patients with cirrhosis and thrombocytopenia. N Engl J Med. 2012;367:716-24.
35. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425:841-6.
36. Kiel MJ, Morrison SJ. Maintaining hematopoietic stem cells in the vascular niche. Immunity. 2006;25:862-4.
37. Junt T, Schulze H, Chen Z, et al. Dynamic visualization of thrombopoiesis within bone marrow. Science. 2007;317:1767-70.
38. Malara A, Currao M, Gruppi C, et al. Megakaryocytes contribute to the bone marrow-matrix environment by expressing fibronectin, type IV collagen, and laminin. Stem Cells. 2014;32:926-37.
39. McCarty JM, Sprugel KH, Fox NE, Sabath DE, Kaushansky K. Murine thrombopoietin mRNA levels are modulated by platelet count. Blood. 1995;86:3668-75.
40. Guerriero A, Worford L, Holland HK, et al. Thrombopoietin is synthesized by bone marrow stromal cells. Blood. 1997;90:3444-55.
41. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity. 2006;25:977-88.
42. Hodohara K, Fujii N, Yamamoto N, Kaushansky K. Stromal cell derived factor 1 acts synergistically with thrombopoietin to enhance the development of megakaryocytic progenitor cells. Blood. 2000;95:769-75.
43. Miyazawa K, Williams DA, Gotoh A, et al. Membrane-bound Steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form. Blood. 1995;85:641-9.
44. Broudy VC. Stem cell factor and hematopoiesis. Blood. 1997;90:1345-64.
45. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine

megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. Blood. 1995;85:1719-26.
46. Avraham H, Cowley S, Chi SY, Jiang S, Groopman JE. Characterization of adhesive interactions between human endothelial cells and megakaryocytes. J Clin Invest. 1993; 91:2378-84.
47. Fox NE, Kaushansky K. Engagement of integrin α4β1 enhances thrombopoietin induced megakaryopoiesis. Exp Hematol. 2005;33:94-9.
48. Kapur R, Cooper R, Zhang L, Williams DA. Cross-talk between alpha(4)beta(1)/alpha(5)beta(1) and c-Kit results in opposing effect on growth and survival of hematopoietic cells via the activation of focal adhesion kinase, mitogen-activated protein kinase, and Akt signaling pathways. Blood. 2001;97:1975-81.
49. Dhanjal TS, Pendaries C, Ross EA, et al. A novel role for PECAM-1 in megakaryocytokinesis and recovery of platelet counts in thrombocytopenic mice. Blood. 2007;109:4237-44.
50. Balduini A, Pallotta I, Malara A, et al. Adhesive receptors, extracellular proteins and myosin IIA orchestrate proplatelet formation by human megakaryocytes. J Thromb Haemost. 2008;6:1900-7.
51. Nichol J, Hokom M, Hornkohl A, et al. Megakaryocyte growth and development factor: Analysis of in vitro effects on human megakaryopoiesis and endogenous serum levels during chemotherapy induced thrombocytopenia. J Clin Invest. 1995;95:2973-8.
52. Kuter DJ, Rosenberg RD. The reciprocal relationship of thrombopoietin (c-Mpl Ligand) to changes in the platelet mass during busulfan-induced thrombocytopenia in the rabbit. Blood. 1995;85:2720-30.
53. Sungaran R, Markovic B, Chong BH. Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow and spleen using in situ hybridization. Blood. 1997;89:101-7.
54. Sungaran R, Chisholm OT, Markovic B, Khachigian LM, Tanaka Y, Chong BH. The role of platelet alpha-granular proteins in the regulation of thrombopoietin messenger RNA expression in human bone marrow stromal cells. Blood. 2000;95:3094-101.
55. Cerutti A, Custodi P, Duranti M, Noris P, Balduini CL. Thrombopoietin levels in patients with primary and reactive thrombocytosis. Br J Haematol. 1997;99:281-4.
56. Wang JC, Chen C, Novetsky AD, Lichter SM, Ahmed F, Friedberg NM. Blood thrombopoietin levels in clonal thrombocytosis and reactive thrombocytosis. Am J Med. 1998;104:451-5.
57. Wolber EM, Jelkmann W. Interleukin-6 increases thrombopoietin production in human hepatoma cells HepG2 and Hep3B. J Interferon Cytokine Res. 2000;20:499-506.
58. Burmester H, Wolber EM, Freitag P, Fandrey J, Jelkmann W. Thrombopoietin production in wild-type and interleukin-6 knockout mice with acute inflammation. J Interferon Cytokine Res. 2005;25:407-13.
59. Taga T, Kishimoto T. Signaling mechanisms through cytokine receptors that share signal transducing receptor components. Curr Opin Immunol. 1995;7:17-23.
60. Tong W, Sulahian R, Gross AW, Hendon N, Lodish HF, Huang LJ. The membrane-proximal region of the thrombopoietin receptor confers its high surface expression by JAK2-dependent and -independent mechanisms. J Biol Chem. 2006;281:38930-40.
61. Bacon CM, Tortolani PJ, Shimosaka A, Rees RC, Longo DL, O'Shea JJ. Thrombopoietin (TPO) induces tyrosine phosphorylation and activation of STAT5 and STAT3. FEBS Lett. 1995;370:63-8.
62. Drachman J, Griffin JD, Kaushansky K. Stimulation of tyrosine kinase activity by MPL-ligand (thrombopoietin). J Biol. Chem. 1995;270:4979-82.
63. Drachman JD, Sabath DF, Fox NE, Kaushansky K. Thrombopoietin signal transduction in purified murine megakaryocytes. Blood. 1997;89:483-92.
64. Hill RJ, Zozulya S, Lu YL, et al. Differentiation induced by the c-Mpl cytokine receptor is blocked by mutant Shc adapter protein. Cell Growth and Diff. 1996;7:1125-34.
65. Sasaki K, Odai H, Hanazono Y, et al. TPO/c-mpl ligand induces tyrosine phosphorylation of multiple cellular proteins including proto-oncogene products, Vav and c-Cbl, and Ras signaling molecules. Biochem Biophys Res Commun. 1995;216:338-7
66. Sattler M, Salgia R, Durstin MA, Prasad KV, Griffin JD. Thrombopoietin induces activation of phosphatidylinositol-3′ kinase pathway and formation of a complex containing $p85^{P13K}$ and the protooncoprotein $p120^{CBL}$. J Cell Physiol. 1997;171:28-33.
67. Miyakawa Y, Rojnuckarin P, Habib T, Kaushansky K. Thrombopoietin induces PI3K and SHP2 activation through Gab and IRS proteins in BaF3 cells and primary murine megakaryocytes. J Biol Chem. 2001;276: 2494-502.
68. Nagata Y, Todokoro K. Thrombopoietin induces activation of at least two distinct signaling pathways. FEBS Lett. 1995;377:497-501.
69. Sauvageau G, Thorsteinsdottir U, Eaves CJ, et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev. 1995;9:1753-65.
70. Thorsteinsdottir U, Mamo A, Kroon E, et al. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood. 2002;99: 121-9.
71. Kirito K, Fox NE, Kaushansky K. Thrombopoietin stimulates expression of HoxB4: An explanation for the favorable effects of TPO on hematopoietic stem cells. Blood. 2003; 102:3172-8.
72. Kirito K, Fox NE, Kaushansky K. Thrombopoietin (TPO) induces the nuclear translocation of HoxA9 in hematopoietic stem cells (HSC): A potential explanation for the favorable effects of TPO on HSCs. Mol Cell Bio. 2004; 24:6751-62.
73. Kirito K, Fox NE, Kaushansky K. Thrombopoietin enhances expression of vascular endothelial cell growth factor (VEGF) in primitive hematopoietic cells through induction of HIF-1α. Blood. 2005;105:4258-63.
74. Gerber HP, Malik AK, Solar GP, et al. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature. 2002;417:954-8.
75. Mukai HY, Motohashi H, Ohneda O, Suzuki N, Nagano M, Yamamoto M. Transgene insertion in proximity to the c-myb gene disrupts erythroid-megakaryocytic lineage bifurcation. Mol Cell Biol. 2006;26:7953-65.
76. Barroga C, Pham H, Kaushansky K. Thrombopoietin regulates c-myb expression by modulating microRNA (miR)150 expression. Exp Hematol. 2008;36:1585-92.

77. Geddis AE, Kaushansky K. Endomitotic megakaryocytes form a midzone in anaphase but have a deficiency in cleavage furrow formation. Cell Cycle. 2006;5:538-5
78. Lordier L, Jalil A, Aurade F, et al. Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and Rho/Rock signaling. Blood. 2008;112:3164-74.
79. Gao Y, Smith E, Ker E, et al. Role of RhoA-specific guanine exchange factors in regulation of endomitosis in megakaryocytes. Dev Cell. 2012;22:573-84.
80. Suzuki A, Shin JW, Wang Y, et al. RhoA is essential for maintaining normal megakaryocyte ploidy and platelet generation. PLoS One. 2013;8:e69315.
81. Lannutti BJ, Drachman JG. Lyn tyrosine kinase regulates thrombopoietin-induced proliferation of hematopoietic cell lines and primary megakaryocytic progenitors. Blood. 2004; 103:3736-43.
82. Lannutti BJ, Minear J, Blake N, Drachman JG. Increased megakaryocytopoiesis in Lyn-deficient mice. Oncogene. 2006;25:3316-24.
83. Tong W, Lodish HF. Lnk inhibits Tpo-mpl signaling and Tpo-mediated megakaryocytopoiesis. J Exp Med. 2004; 200:569-80.
84. Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol. 2008; 19:414-22.
85. Hitchcock I, Chen M, Fox NE, Kaushansky K. YRRL motifs in the cytoplasmic domain of the thrombopoietin receptor regulate receptor internalization and degradation. Blood. 2008;112:2222-31.
86. Saur SJ, Geddis Sangkhae V, Kaushansky AE, Hitchcock K. IS. Ubiquitination and degradation of the thrombopoietin receptor c-Mpl. Blood. 2010;115:1254-63.