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PLATELET FORMATION

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Abstract

Thrombocytopenia is the underlying cause of a number of major clinical conditions and genetic disorders worldwide. While therapeutic agents that bind and stimulate the thrombopoietin receptor are currently available, the development of drugs that directly stimulate megakaryocytes to generate platelets has lagged behind. To improve the management of thrombocytopenia, we will need to define the cell biological pathways that drive the production of platelets from megakaryocytes. This review integrates the latest research of platelet biogenesis and focuses on the molecular pathways that power and regulate proplatelet production.

Human adults contain nearly one trillion blood platelets in circulation. These small (~2.5 μm), anuclear cells have a highly organized cytoskeleton, unique receptors, and specialized secretory granules. Among their many functions, platelets' most understood role is to respond to blood vessel injury by changing shape, secreting granule contents, and aggregating. These responses, while advantageous for hemostasis, can become undesirable when they cause tissue ischemia or infarction. The terminal differentiation of mammalian megakaryocytes into platelets in the bone marrow and vasculature thus represents a unique problem in hematology with great relevance to human health.

Medical relevance for the study of platelet release

Thrombocytopenia (platelet counts below 150×10^9 per L) is a major clinical problem encountered across a number of conditions, including immune (idiopathic) thrombocytopenic purpura (ITP), myelodysplastic syndromes (MDS), chemotherapy-induced thrombocytopenia, aplastic anemia, human immunodeficiency virus (HIV) infection, and major cardiac surgery, as well as a host of relevant genetic disorders.[1] Qualitative disorders of platelet production form a large group of rare diseases which cover a multitude of genetic defects and commonly present with excessive mucocutaneous bleeding (see article by Geddis in this issue). The magnitude of the problem is not trivial. Platelet transfusions total well over 10 million units per year in the United States, and their steady increase in demand continues to challenge the

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US blood community(see article by McCullough in this issue). There are, however, a number of strategies being developed for the treatment of thrombocytopenia. One such strategy is to mimic the actions of TPO, the primary growth factor that regulates megakaryocyte proliferation and maturation. It has been demonstrated, for example, that TPO mimetics (see article by Kuter in this issue) can increase the platelet count in both healthy volunteers and patients with ITP (see article by Ghanima and Bussel in this issue) and liver disease (see article by Tillmannn and McHutchinson in this issue). However, the therapeutic response to TPO mimetics remains slow due to the long time required for maturation of megakaryocyte progenitors from HSCs before subsequent platelet production can commence. All TPO mimetics must inevitably work within the same time-line; that is, 5 days to increase platelet counts and 12 days to reach maximal effect.

In principle, direct stimulation of megakaryocytes to undergo platelet production would be more rapid, and could complement TPO-based treatments. While the development of agents that induce megakaryocyte conversion into platelets to provide an “auto-transfusion” is ongoing, the main treatment for thrombocytopenia owing to decreased or defective platelet production remains platelet transfusion. Unfortunately, this practice is expensive, time-consuming, wasteful (*eg.* platelet unit outdate), of limited efficacy (*eg.* alloimunization), and carries additional risks (*eg*. sepsis). Thus, the search for novel thrombopoietic agents that directly accelerate platelet production, improve primary hemostasis and, eventually, reduce the need for platelet transfusion, is still warranted. A better understanding of the mechanisms of platelet formation will undoubtedly lead to improved therapies for thrombocytopenia and thrombocytosis.

The proplatelet model of platelet production

Platelets come from larger progenitor cells called megakaryocytes, and platelet production represents the final stage of megakaryocyte development (Fig. 1). The current model of platelet formation recognizes that mature megakaryocytes extend long, branching processes, designated proplatelets, which are comprised of platelet-sized swellings in tandem arrays that are connected by thin cytoplasmic bridges.[2] Proplatelets have been identified both *in vitro* and *in vivo*,[3] and proplatelet-producing megakaryocytes yield platelets that are structurally and functionally similar to blood platelets.[2,4]

In vitro models of platelet production

The discovery of thrombopoietin (TPO) and development of megakaryocyte cultures that reconstitute platelet formation *in vitro* have provided systems to study megakaryocytes in the act of forming platelets. Indeed, megakaryocytes cultured from bone marrow, mouse fetal livers, embryonic stem cells, fetal cord blood, or peripheral blood follow a maturation program that ends in proplatelet and platelet formation. Their use has enabled the dynamic process of forming and elongating proplatelets to be studied in real-time. Indeed, mice lacking distinct hematopoietic transcription factors present with severe thrombocytopenia and fail to produce proplatelets in culture, underscoring the correlation to platelet biogenesis *in vivo*.[5-7]

Methods of *ex vivo* expansion of functional megakaryocytes have been pivotal to our understanding of proplatelet production and platelet release (Table 1). Bone marrow is removed from the femurs and tibiae of 8- to 10-week-old mice and cultured in a medium supplemented with TPO for 4-6 days. Megakaryocytes grow relatively quickly in this culture system and can be isolated by density gradient sedimentation. While the majority of the large megakaryocytes express GPIIbIIIa, GPIbα, and GPV (>90%),[8] few (if any) proceed to extend proplatelets in culture. Conversely, primary megakaryocytes directly isolated from murine bone marrow have been shown to rapidly produce proplatelets on fibrinogen. However, yields are generally low (<14%), and require that mice be given injections of TPO for 4 days prior to bone marrow

aspirate culture to produce sufficient numbers of megakaryocytes for quantitative analysis of proplatelet formation.[9] Primary megakaryocytes can also be obtained from fetal livers recovered aseptically from mice between embryonic days 13 to 15, with optimal megakaryocyte purity on day 13.5.[2] Suspended fetal livers are cultured in a medium supplemented with fetal bovine serum and TPO as previously described, and develop relatively quickly over a span of 3 to 4 days. Large polyploid megakaryocytes readily begin to dominate the culture by Day 3, and can be observed producing proplatelets with yields averaging roughly 60% on Day 5 of culture. While this represents the most desirable culture method to study proplatelet production and platelet release *in vitro*, megakaryocyte yield is generally limited. The murine R1 embryonic stem cell (ESC) line has been used to study integrin signaling,[10] and provides significantly higher yields of large, polyploid megakaryocytes capable of producing proplatelets relative to bone marrow or fetal liver cultures. Nevertheless, it remains unclear why these cultures take longer to mature (~12 days) and yield markedly fewer proplatelets than fetal liver cell cultures in mice.

Megakaryocytes have been derived from human tissues as well, and can be cultured from bone marrow,[11] fetal cord blood,[12] mobilized peripheral blood progenitor cells,[13] and hESC H9 cells.[14] These generally take still longer to mature (14-17 days in culture), and often yield dramatically fewer proplatelets than mouse cell cultures (Table 1). Nevertheless, human systems represent the most clinically relevant model of proplatelet maturation and platelet formation. It should be noted however that the expression of GPIIbIIIa, localization of alpha and dense-granules, morphological characteristics of proplatelet elongation, and transport of molecular constituents from the cell body to proplatelet tips is well conserved across both species. This indicates a conservation of the fundamental mechanisms of megakaryocyte differentiation and proplatelet genesis in murine and human cell lines, and supports the use of mouse models to study proplatelet production and platelet release. These models have provided accelerated insight into the dynamics of thrombopoiesis and the factors that regulate phenotypes of circulating platelets in healthy and diseased states.

The discovery of TPO and development of megakaryocyte cultures that reconstitute proplatelet formation *in vitro* have provided systems to study proplatelets in the act of forming platelets. Nevertheless, many old questions remain unanswered. For instance, what accounts for the discrepancies in size and number between murine and human platelets (Table 2)? Although there does appear to be a conservation of the fundamental mechanisms of megakaryocyte maturation and proplatelet genesis between the two model systems, mouse platelets are always significantly smaller than human platelets (Mean diameters of 1.50 μm *versus* 2.25 μm, respectively). Mouse platelets also have a shorter lifespan (3-4 days) than those of human platelets (8-10 days), and circulate at numbers approximately 4-fold greater. These observations imply differences in the final stages of platelet production.

Mechanics of proplatelet production

Platelet production begins with the erosion of one pole of the megakaryocyte to generate large pseudopodial-like structures that elongate, thin, and branch to yield slender tubular projections of uniform diameter (2-4 μm, Fig. 2). This is a microtubule-driven process[3] which unfolds over a period of 4-10 hrs. Proplatelet shafts become filled with thick bundles of (hundreds of) microtubules that undergo a thinning phase (to ~20) and loop around within the proplatelet to reenter the shaft forming buds at the proplatelet tip.[15] Proplatelet elongations extend outwardly at a steady rate of ~1 μm/min., and generally reach lengths of ~0.5-1 mm.[15] Proplatelet extension is due to a bipolar array of microtubule bundles distributed along the proplatelet shaft that lengthen via continuous polymerization of tubulin at their free plus ends, and dynein-powered sliding of overlapping microtubules at a rate of ~4-5 μm/min.[15] Less is known about the role of actin in this process. Repeated actin-dependent bending and branching that bifurcates

the proplatelet shaft is common, and serves to increase the number of free proplatelet ends from which platelets are thought to be released.[15] Branching occurs when a portion of the proplatelet shaft becomes bent, from which some of the microtubules within the loop separate from the bundle to form a new bulge in the shaft. Elongation/sliding of these microtubules then generates a new daughter proplatelet process.

Microtubule bundles in the proplatelet shaft serve as tracks along which mitochondria and granules move at a rate of 0.1-0.2 μm/min.[16] This process is sequential and bidirectional, culminating at the proplatelet tips where the cargo becomes trapped. The general reduced speed of traffic compared to other cell types, and bidirectional nature of granule/organelle movement suggests that the underlying goal of this transport process is to mix the various granules/organelles within the proplatelet before it is driven to the proplatelet ends—from which platelets are presumably released. Indeed, genetically engineered mice lacking the *Tubb1* gene (β1 tubulin isotype comprising >90% of proplatelet microtubule filaments) are thrombocytopenic. Their platelets are spheroid and contain a marginal band consisting of only 2-3 coilings (versus 8-12 in wild-type platelets).[17] *In vitro* depletion of RanBP10, a novel tubulin binding protein that has been recently demonstrated to concentrate along polymerized microtubules in mature megakaryocytes, causes disruption of microtubule filaments.[18] *In vivo*, RanBP10 deficient mice exhibit disorders in microtubule filament numbers and localization, as well as prolonged bleeding times, and reduced platelet activation.[19] Taken together, these observations suggest a potential role for RanBP10 as a regulator of microtubule organization and stabilization in mature megakaryocytes, and support the function of the microtubule cytoskeleton as a driving force in proplatelet production and platelet formation/release.

In vivo models of proplatelet production

Although the mechanics of proplatelet production have now been studied in great detail, the terminal stages of proplatelet maturation and platelet release remain poorly understood. This is due, in part, to significant limitations in the field, such as the asynchronous maturation of HSCs in culture, and our inability to synchronize proplatelet production. Megakaryocyte cultures always contain a complex mix of HSCs, immature megakaryocytes, proplatelet-producing megakaryocytes, released proplatelets and platelets which can range dramatically in size and shape, and whose relative distribution changes during cell culture. As there are currently no methods available to isolate the multiple intermediate stages in platelet release, most studies to date have focused on the qualitative aspects of megakaryocyte maturation. Many important questions therefore remain concerning platelet formation, such as how and where platelets are actually released, what cytoskeletal forces are involved in this process, whether platelets are released in an intermediate “pre-platelet” form, and what signals regulate each step of megakaryocyte maturation and platelet formation.

Until recently, micrographs of proplatelet producing megakaryocytes *in situ* or *in vitro* images of isolated megakaryocytes in culture have yielded only static snapshots of megakaryocyte maturation and resulted in competing mechanistic models of platelet release. *In vivo* approaches, such as the use of live imaging with multiphoton intravital microscopy by Junt *et al*,[20] have validated the proplatelet model of platelet production. These studies suggest that platelets are formed upon the further fragmentation of cytoplasmic processes extended and released into the sinusoids of bone marrow by extravascularly located megakaryocytes. It should be noted that trans-sinusoidal migration of whole megakaryocytes in these studies was rare, and that the diameter of the bone marrow sinusoid was observed to impose size constraints on released megakaryocyte fragments *in vivo*. Moreover, the presence of proplatelets in the peripheral blood of the CD41-EYFP$^{ki/+}$ mice implies that it is proplatelets, not megakaryocytes, that are directly responsible for individual platelet release. Indeed, almost all megakaryocyte

fragments identified were 10 to 100 times as large as circulating platelets. As many of the shed megakaryocyte fragments were unbranched, and most exceeded platelet dimensions, this may be indicative of an intermediate stage in platelet development. Megakaryocytes routinely released heterogeneous particles with properties resembling immature proplatelets into bone marrow microvessels, suggesting proplatelet morphogenesis continues in peripheral blood to create individual platelets. This process is possibly assisted by intravascular shear forces in pulmonary arterioles and is consistent with observations that proplatelet counts are higher in pre-pulmonary vessels than in post-pulmonary vessels,[21] whereas platelet counts are higher in the latter.

Effect of the bone marrow microenvironment on proplatelet production and platelet release

It has recently become apparent that the bone marrow stroma may also contribute to proplatelet development.[22] Indeed, a number of studies have shown that in addition to providing a suitable surface for attachment, proplatelet production can be regulated by matrix-receptor signaling.[9,23] Extracellular matrix proteins are a major component of the vascular niche, which is known to comprise vitronectin, collagen (type IV), fibrinogen, laminin, fibronectin, and VWF.[19] Vitronectin, collagen,[23] and fibrinogen[9] have all been shown to promote proplatelet formation, and antagonists directed against GPs Ibα, IIb, and IIbIIIa will inhibit this process when added to cultured megakaryocytes.

Although matrix protein substrates in the bone marrow vascular space appear to influence proplatelet production from megakaryocytes, it is unclear whether they are able to regulate the subsequent production of individual platelets from released proplatelet intermediates. GPIb, for example, is complexed to the actin-binding protein filamin, and is known to play an important role in maintaining the normal cytoskeletal architecture of resting platelets.[15,24] Interestingly, Bernard-Soulier syndrome (BSS) presents with large platelets that host genetic abnormalities of the GPIb-IX-V complex, particularly the GPIbα subunit that contains the VWF and thrombin binding sites.[1]

More recently, myosin IIA, the non-muscle myosin heavy chain product of the *MYH9* gene, has been implicated in restraining proplatelet formation in developing megakaryocytes until they are able to achieve terminal maturity in the bone marrow vascular niche.[25,26] In the case of *MYH9*-related disorders, defective myosin IIA could promote premature proplatelet formation in the osteoblastic niche through loss of attenuation, or result in defective proplatelet formation and reduced or abnormal platelet release in the vasculature.[26] It is currently unknown whether the endothelium barrier of the bone marrow sinusoid might also play a role in proplatelet formation or platelet release. These observations suggest that cell surface interactions with elements of the bone marrow vascular and osteoblastic niches may regulate platelet release from megakaryocytes, and warrant further study if the mechanisms underlying platelet production are to be resolved.

Conclusion

Despite recent advances, the mechanistic processes of platelet production are not fully understood and the following questions prevail: (1) What induces platelet release? (2) What are the underlying mechanisms by which this process comes about? (3) How do current models of platelet production correlate with platelet phenotypes in disease states? The final steps of platelet biogenesis are perhaps the most important to resolve, and released proplatelet-enriched systems that can produce *bona fide* platelets in culture may thus prove valuable clinical models by which these questions can now begin to be addressed.

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6 authors should be cited before “et al”.

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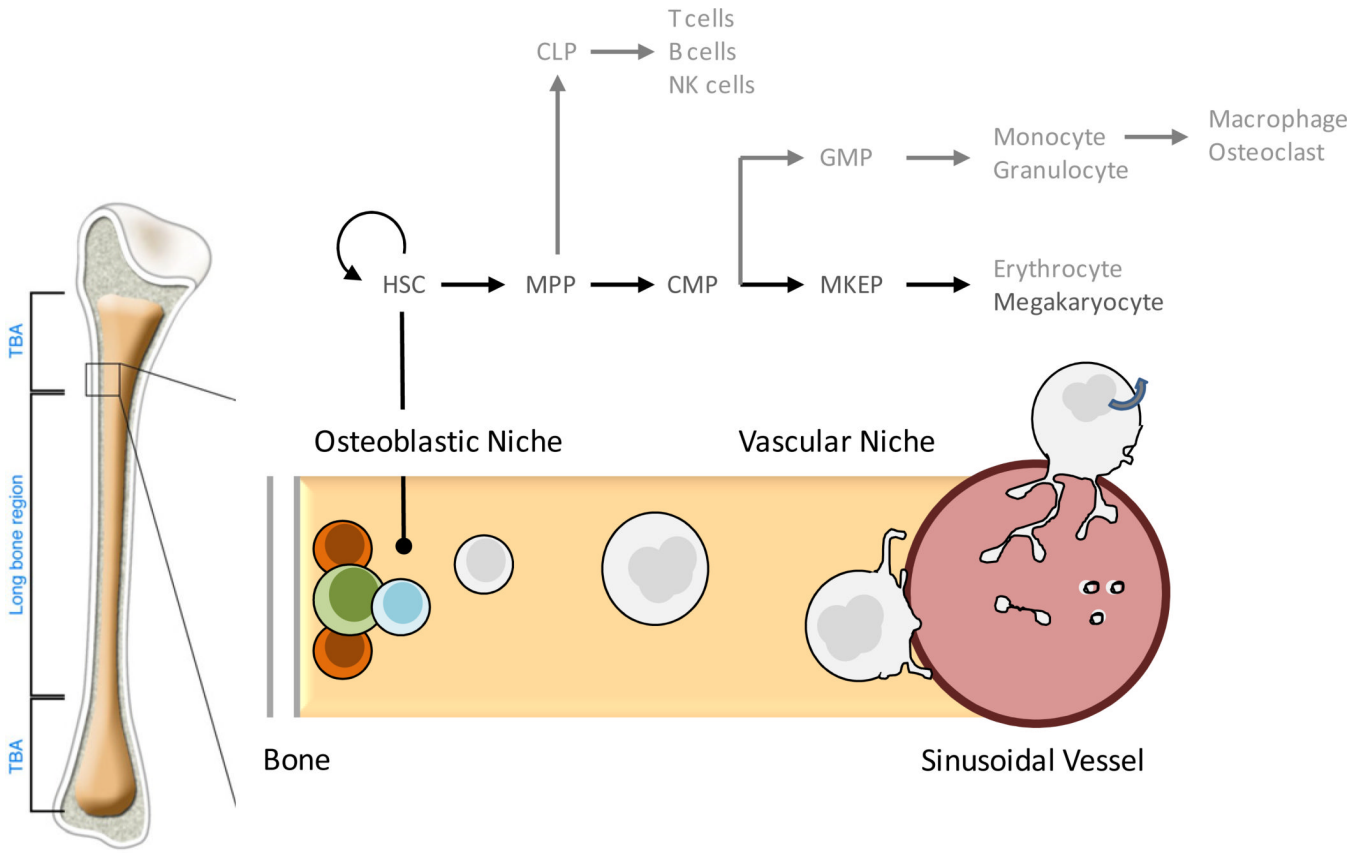
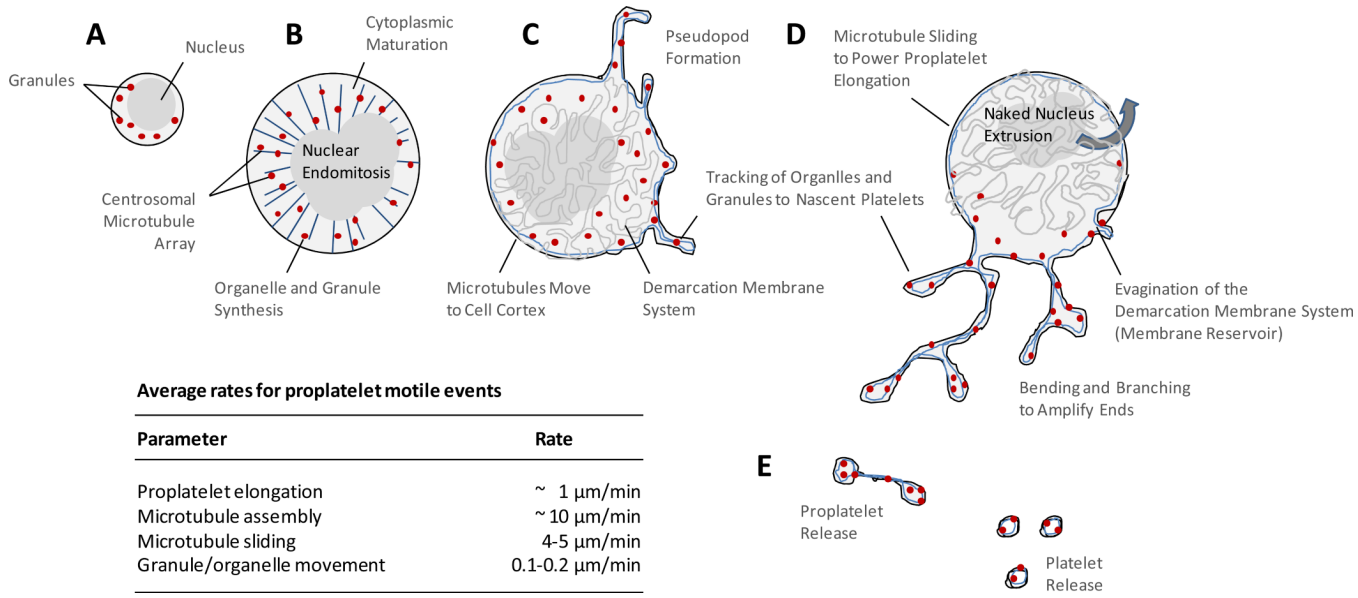


Figure 1. Summary of megakaryocyte maturation and platelet production

Hematopoietic stem cells (HSCs) residing next to the endosteal bone surface produce progenitors that migrate to blood vessels at the center of the bone marrow cavity. Upon each division, a single daughter cell leaves the bone to proliferate and differentiate into various possible lineages of which the megakaryocyte is one. An elaborate intracellular program of nuclear amplification and protein production in maturing megakaryocytes precedes the mechanical extension of proplatelet elongations into the sinusoidal blood vessels of the bone marrow. Released proplatelets continue to mature in the vasculature and ultimately release individual platelets from their tips. CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte/macrophage progenitor; MKEP, megakaryocyte erythroid progenitor; MPP, multipotent progenitor stem cell; TBA, trabecular bone area. Adapted from Yin *et al* 2006.[34]



Average rates for proplatelet motile events

Parameter	Rate
Proplatelet elongation	~ 1 μm/min
Microtubule assembly	~ 10 μm/min
Microtubule sliding	4-5 μm/min
Granule/organelle movement	0.1-0.2 μm/min

Figure 2. Cytoskeletal mechanisms of proplatelet production and platelet release

A systematic series of events occurs as megakaryocytes transition from immature cells (**A**) to released platelets (**E**). (**B**) Immature megakaryocytes will undergo repeated cycles of nuclear endomitosis for the purpose of supporting organelle synthesis, and dramatic cytoplasmic maturation and expansion. (**C**) Prior to the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. A demarcation membrane system that is continuous with the surface membrane of the cell provides a reservoir of membrane for growth of the proplatelet processes. Thick bundles of microtubules fill the shafts and cortex of broad pseudopodia that are subsequently extended by the megakaryocyte. (**D**) Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends. Proplatelet formation continues to expand throughout the cell while bending and branching amplify existing proplatelet ends. (**E**) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. As proplatelets elongate, their microtubule bundles twist, bringing opposing bundles in contact, and allowing them to become zipped together in the proplatelet shaft. This forms loops at the ends of the proplatelets where granules and organelles become trapped. Sliding movements by microtubules in the shaft elongate released proplatelets further, and separate the ends from the shaft, mediating platelet release. Adapted from Hartwig *et al* 2006,[15] and Patel *et al* 2005.

Table 1

Comparison of model systems of megakaryocyte maturation and proplatelet production.

	Mk Development		Proplatelet Yield	
	Human Mouse	Mouse	Human	
Bone Marrow	14 days	3-4 days	10%□	none
Fetal Liver	N/A	3-4 days	-	60%
Embryonic Stem Cell Line		17 days	12 days	none
	none			
Fetal Cord Blood	14 days	N/A	25%§	-
Peripheral Blood	8-11 days	N/A	40%	-
Primary Megakaryocytes from Bone Marrow				
	unknown	none*		
(<0.1% of cell population)				

□40% if perfused on VWF at 1800s^{-1} over 20 min.[27] 30% if cultured in the presence of Mpl-I[28]

§50% if cultured on Matrigel-coated surface, unpublished results[4] 15% if cultured on VWF-coated surface[29] 10% if cultured on fibrinogen-coated surface[29] 5% if cultured on fibronectin-coated surface[29] <5% if cultured on type-III or type-IV collagen-coated surface[29] None, if coated on type-I collage-coated surface[29]

*14% if cultured on fibrinogen-coated surface[9] 4% if cultured on VWF/botrocetin-coated surface[9] <2% if cultured on vitronectin- or laminin-coated surface[9] None, if coated on collagen-coated surface[9]

Table 2
Comparison of megakaryocyte and platelet parameters in the human and murine species

Adapted from Schmitt *et al* 2001,[30] and Tsakiris *et al* 1999.[31]

	Human	Mouse
Platelet Count ($\times 10^9$ per L in blood)	150-400	1000-1500
Platelet Diameter (μm)	2.25	1.50
Platelet Volume (fL)	7.5-10	4-5
Platelet Lifespan (days)	8-10	3-4
Megakaryocyte Diameter (μm)	30-60	20-30
Mk Density in Bone Marrow (Mks/mm^2)	9 ± 2	20
α-Granules per Platelet Section (EM)	5-6□	3-4
Dense Granules per Platelet Section (EM)	1§	0.5

□50-80 α-granules per human platelet[32]

§3-8 dense granules per human platelet[33]