

Anniversary Review Series: Perspectives on the modern exploration of Experimental Hematology

Biomanufacture of human platelets for transfusion: Rationale and approaches

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Platelets for transfusion obtained from volunteer blood donors are a limited resource. Given the increased range of donor restrictions to prevent transmission of disease and the decline in volunteer blood donors, there is a diminishing supply of blood for transfusion. Production of mature blood cells from hematopoietic stem cells via large-scale manufacture is an alternative way of meeting transfusion demands. In this review, we provide a detailed outline of the challenges and opportunities for the biomanufacture of platelets. We describe the scale required for platelet biomanufacture to deliver sufficient cells for transfusion, provide a brief outline of the current understanding of megakaryopoiesis and thrombogenesis, and highlight how the current understanding impacts the design of culture systems and bioreactors for producing platelets. Crown Copyright © 2014 Published by Elsevier Inc. on behalf of ISEH -Society for Hematology and Stem Cells. All rights reserved.

Although blood transfusion therapy has been a part of routine clinical practice since the 1950s, the concept of *ex vivo* production of nascent mature blood cells for therapy is relatively new. It remains unclear who should be credited with first proposing the idea of producing transfusable doses of mature blood cells from hematopoietic stem cells (HSCs). In his manuscript titled “The construction of high efficiency human bone marrow tissue *ex vivo*,” Stephen Emerson appears to be one of the first to do so [1]. In the manuscript, he identified five problems that need to be addressed before manipulation of human bone marrow cultures will yield cells for transfusion. Notably, he predicted that “successful construction of hematopoietic bioreactors will have a tremendous impact on hematopoietic cell biology and on clinical practice,” and “such bioreactors would permit the production of mature blood cells, under controlled conditions, for transfusion therapy.” Now, some 23 years later, his vision may soon be realized.

Significant progress in our understanding of HSC regulation, large-scale production of purified recombinant proteins,

innovative bioengineering, and bioreactor technology, all of which are enabling capabilities, has driven rapid advances in mature blood cell biomanufacture [2,3]. These advances, together with an unlimited supply of HSCs and hematopoietic progenitor cells (HPCs) derived from pluripotent cells as source cells for mature cell generation, have the potential to transform blood transfusion. Significant progress in methods for generation of human red blood cells using *ex vivo* systems [2,4] has led the way, following from the groundbreaking work of Luc Douay and colleagues [5,6]. In comparison, progress toward biomanufacture of transfusable doses of human platelets has lagged. This reflects a gap in understanding the precise mechanism of *in vivo* platelet production and the technical and engineering obstacles that need to be overcome before this process can be replicated *ex vivo*. Two critical observations concerning megakaryopoiesis and thrombogenesis now position the field to strive for this goal: first, cloning of thrombopoietin (TPO) and an appreciation of its critical role in megakaryopoiesis and second, the realization that bone marrow vasculature and endothelial cell-megakaryocyte interactions play an integral role in platelet extension and the final stages of platelet release.

Platelets, the enucleated fragments of megakaryocytes (MKs), are critical in the maintenance of vascular integrity and also contribute to wound healing, angiogenesis, and

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innate immunity. Under normal homeostatic conditions, platelets survive in the human circulation for as many as 7–10 days [7,8]. Their ongoing production is sustained by the well-regulated process of megakaryopoiesis [9,10] and is driven predominately by the hormone TPO [11,12]. In humans, MK development commences with cells that express the cell surface sialomucin CD34 and eventually results in the production of mature functional polyploid MKs that lack CD34 but express a cohort of cell surface glycoprotein receptors that are essential for platelet function. Stages of MK development can be mapped, not only by the obvious increase in cell size, nuclear ploidy and cytoplasmic complexity, but also by the progressive acquisition of the major platelet membrane glycoproteins that are associated with discrete differentiated cell types [13].

Platelet numbers in blood normally range from 150×10^9 and 400×10^9 per liter, and an estimated 1×10^{11} platelets are produced each day in the adult human [14]. Thrombocytopenia and severe thrombocytopenia, defined as platelet counts less than 50×10^9 and 10×10^9 per liter, respectively, increase the risk of spontaneous bleeding [15–17]. In general, thrombocytopenia is caused by either decreased platelet production or increased destruction and consumption. A number of clinical situations, including leukemia, myelodysplasia, autoimmune disease, high dose chemotherapy, and stem cell transplantation, can result in thrombocytopenia. Notably, delayed platelet recovery and extended periods of thrombocytopenia are frequently, if not always, associated with umbilical cord blood (CB) transplantation [18,19]. Independent of the underlying cause, the most effective way to treat thrombocytopenia in the short term is by platelet transfusion, given either prophylactically to prevent bleeding or therapeutically to minimize and control bleeding.

Platelets for transfusion are obtained from volunteer blood donors and as such, are a limited resource. Globally, the blood donation industry is facing an ongoing crisis as the demand for blood products, particularly platelets, frequently outstrips supply. Given the ever-increasing range of donor restrictions to prevent transmission of disease and the decline in volunteer blood donors, there is a diminishing supply of blood for transfusion. As has been suggested by others [20,21], production of mature blood cells from HSCs via large-scale manufacture is an alternative way of providing cells for transfusion.

In this review, we describe the scale required for platelet biomanufacture to deliver sufficient cells for transfusion, provide a brief outline of the current understanding of megakaryopoiesis and thrombogenesis, and highlight how this impacts the design of culture systems and bioreactors for producing MKs and platelets. This is an emerging area of experimental hematopoiesis involving a relatively small number of research groups worldwide. We have attempted to recognize and describe the contributions from these laboratories. We apologize to those groups who have recently

published research on this topic and whose studies have been unintentionally omitted from our review.

The scale and approach for platelet biomanufacture

How many platelets need to be produced by a biomanufacturing system to make it relevant and useful as a source of cells for transfusion? There is no single answer to this question, but it does provide a framework for considering the scale and complexity of the task. Recent reviews discuss in detail the issues of optimal timing and dosing of platelet transfusions [17,22–24]. Over the last 20 years, numerous randomized clinical trials have evaluated various aspects of platelet transfusion therapy and suggest that $3.0\text{--}6.0 \times 10^{11}$ platelets is a clinically useful dose for most adults [25–27]. This translates to approximately $5.0\text{--}10.0 \times 10^9$ platelets/kg bodyweight. In practice, however, the dose of platelets transfused varies greatly, ranging from $1.7\text{--}294.2 \times 10^8$ platelets/kg [27]. Moreover, in many clinical situations, patients require multiple platelet transfusions over many days.

Based on this data and to exemplify our discussion of the topic, we have conservatively defined the platelet production target for a biomanufacturing system as 5.0×10^{11} platelets per day, which represents a single platelet transfusion. An efficient system should, however, be capable of generating multiple transfusion doses over an extended period. As outlined in Figure 1, the biomanufacturing task can be considered a series of intrinsically linked steps that correspond to stages of HSC expansion, MK differentiation and maturation, and finally proplatelet formation and platelet production. One approach utilizes CD34+ cells, obtained from different sources as discussed below; these are stimulated to give rise to committed MK progenitors, megakaryoblasts, and eventually MKs that increase in ploidy and mature before releasing platelets.

A system for platelet biomanufacture should faithfully replicate *in vivo* megakaryopoiesis and thrombogenesis and be optimized to convert one cell type into another so that as many platelets as possible can be generated for every CD34+ cell seeded into the system. Each step of the process represents a unique challenge for a biomanufacturing system. As shown in Figure 2, although generation of a target number of platelets from a given number of CD34+ cells could be achieved via the same cell differentiation pathway, conversion of precursor cells into progeny will depend upon the culture conditions, the combinations of factors that drive these processes, and bioreactor parameters. For example, in Figure 2, scenarios A and B differ in the efficiency of conversion of CD34+ cells into mature MKs and also the number of platelets produced from each mature MK.

Platelet generation *in vivo* is considered to occur by either cytoplasmic fragmentation of intact MKs within the bone marrow (BM) extravascular space [28] or pulmonary circulation [29,30] or via MK proplatelet extensions releasing platelets directly into BM sinusoidal vessels

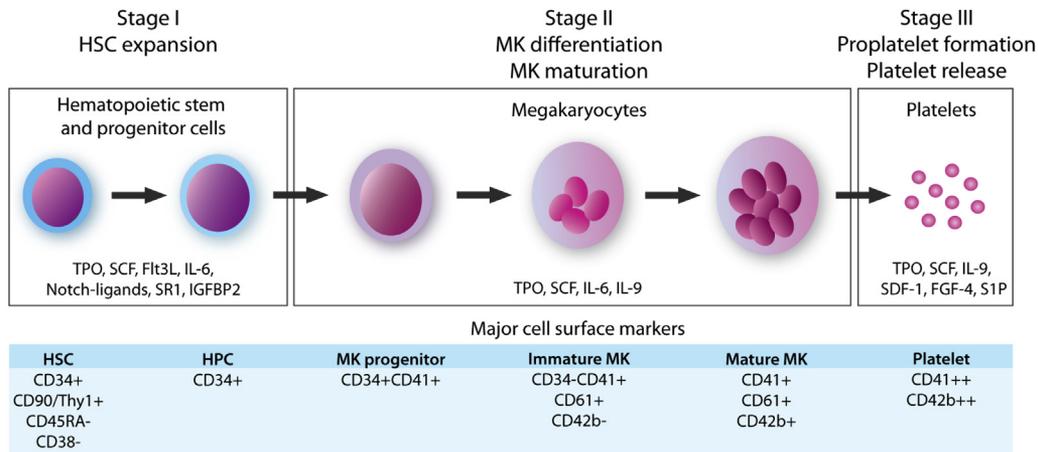


Figure 1. Stages of bioreactor culture and cellular differentiation required for generation of platelets from CD34+ cells. The key cytokines that drive cell differentiation and ultimately platelet production at each stage are indicated. SR1 = Stemregenin-1.

[31–35]. Although there is evidence for cytoplasmic fragmentation occurring in large animals, such as the rat [36] and rabbit, it remains uncertain as to what proportion of platelets within the circulation of humans are generated via this mechanism. The more commonly held view is that the vast majority of platelets are generated in situ within the BM, where maturing MKs of various size make intimate contact with the subluminal surface of BM sinusoidal endothelial cells. Here, they undergo a controlled process of proplatelet and preplatelet formation and subsequent release or shedding of platelets directly into small vessels. Accordingly, we support approaches for replicating this critical cell-cell interaction and construction of an artificial, endothelial cell-like surface upon which mature MKs undergo proplatelet formation and platelet release. These will be a critical feature of next generation platelet bioreactors.

As shown in Figure 2, we predict that between 1×10^9 and 5×10^9 mature, large polyploid MKs need to be generated each day to facilitate a daily production of 5×10^{11} platelets. The optimal conditions for converting CD34+ cells into this

number of large mature MKs should be based on how these cells are produced empirically in vivo. The challenge is to develop ex vivo systems that faithfully replicate the normal extrinsic cues that regulate CD34+ cell proliferation and MK differentiation in vivo. An additional and equally important consideration is the intrinsic potential and regulation of CD34+ cells obtained from different sources or hematopoietic tissues. In this review, we focus on CD34+ cells isolated from umbilical CB. This cellular source is routinely used for clinical transplantation. The growth and differentiation of CB CD34+ cells has been investigated for many years and is relatively well understood.

As depicted in Figure 1, the first stage of megakaryopoiesis involves the expansion of HSC and multipotent progenitors (referred to herein as CD34+ cells for simplicity), and the second stage concerns MK differentiation and maturation. In accordance with how these processes occur in vivo and to achieve optimal outcomes, a platelet bioreactor might comprise at least two compartments that are custom designed for these linked aspects of megakaryopoiesis.

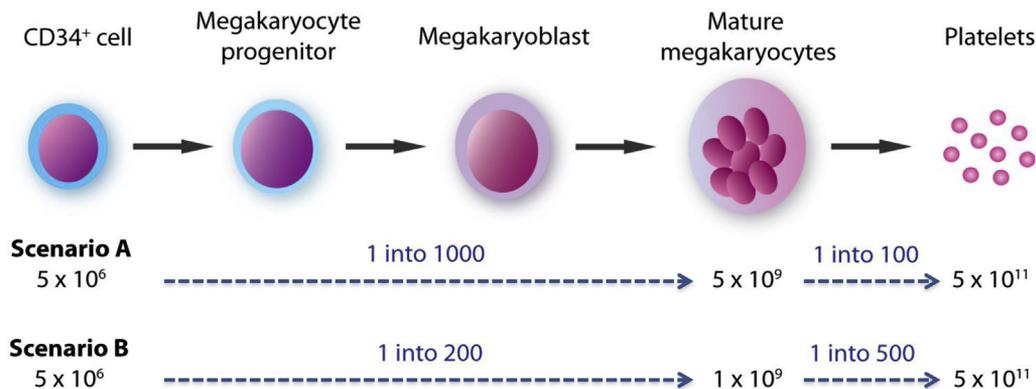


Figure 2. Scenarios for generating 5.0×10^{11} platelets from 5.0×10^6 CD34+ cells. The required conversion of CD34+ cells into mature MKs then into platelets is shown. A starting number of 5×10^6 CD34+ cells reflects a number easily obtained from one cord blood unit.

Expanding the number of CD34+ cells

The rationale for this component of a platelet bioreactor is that increasing the number of HSCs/HPCs with multilineage potential will ultimately increase the number of MKs and platelets produced. Ex vivo expansion of CD34+ cells for improving the outcomes of CB transplantation has been investigated for many years. In brief, these have been informed by an understanding of how HSC proliferation and differentiation is regulated in vivo as well as how purified recombinant hematopoietic cytokines and growth factors direct HSC growth in vitro. Multiple publications, including data from our laboratory [37], clearly demonstrate that recruitment of the most primitive HPCs into division and, thereafter, maximal cell proliferation is dependent on simultaneous stimulation with combinations of early acting synergistic cytokines. It is evident that a minimum combination of stem cell factor (SCF), TPO, and FMS-like tyrosine kinase 3 ligand (Flt3L) [38–42], supplemented with additional factors such as angiopoietin-like 5, insulin-like growth factor binding protein 2 (IGFBP2), insulin-like growth factor 2 (IGF-2) [43,44], and notch ligands [45,46], is a very potent combination for recruitment and subsequent proliferation of quiescent human CB CD34+ cells.

Most recently, with an improved understanding of the molecular basis of HSC regulation by the HSC niche, there has been a focus on culturing HSCs in three-dimensional (3D) microenvironments that faithfully replicate the complex combination of extrinsic cues provided by the niche [47–50]. A key aspect of engineered microenvironments is the ability to present combinations of immobilized ligands to HSCs, not only to enhance proliferation but also to limit differentiation, thus expanding the number of multipotential progenitors. This approach is underpinned by the established importance of HSC regulators that are presented as transmembrane proteins or bound to the extracellular matrix. Notable amongst these membrane bound regulators are SCF, Flt3L, and notch ligands. The severely impaired hematopoiesis evident in *Sl/Sl^d* mice that do not express the membrane bound form of SCF [51] strongly suggests that presentation of this early acting cytokine in an immobilized form is critical for directing HSC fate decisions. Additional evidence supporting the importance for this mode of SCF presentation comes from experiments where immobilized SCF sustains growth of SCF-dependent cell lines [52–54] and HSCs/HPCs [55]; outcomes that in part might be attributed to more persistent phosphorylation and signaling downstream of c-Kit [56]. In addition, stimulation with the immobilized notch ligand Delta1 induces extensive expansion of murine HPCs [57] and also results in expansion of human CB CD34+ cells [45,58]. Likewise, the benefits of HSC coculture with mesenchymal stem cells, endothelial cells, or other BM stromal cells are, in part, attributed to the transmembrane regulators directly binding to their cognate receptors on target HSCs.

Taken together, as proposed by a number of investigators, we believe that optimal expansion of CD34+ cells will be achieved by culturing these cells in an engineered artificial HSC niche that presents combinations of immobilized hematopoietic cytokines and extracellular matrix proteins such as fibronectin [50,59–63]. This concept is easily appreciated, but the efficacy of the approach depends on precise control of the engineered surface. As described by Peerani and colleagues, “niche engineering includes dynamic control over soluble and surface bound cytokines, ECM, cell-cell interactions, mechanical forces and physiochemical cues” [59]. The surface density of immobilized ligands can influence cell responses and is correlated with upregulation of specific target genes that regulate differentiation [58]. Moreover, the surface bound ligands must resist degradation and remain bioactive for the duration of culture, which may be days to weeks.

Other aspects of niche engineering to be considered in HSC expansion include the effect of nanotopography, material surface chemistry, and substrate elasticity. Cord blood CD34+ cells exhibit different growth responses depending on the composition of the polymer material, the dimension of electrospun fibers, and the distance between surface amino groups [64]. Furthermore, elastic substrates, such as tropoelastin, have been demonstrated to enhance not only proliferation of HSC but also the number of nascent HSCs generated in cytokine dependent cultures [65]. We foresee that the next generation bioreactors for large-scale culture of CD34+ cells will comprise polymeric scaffolds that not only provide inductive and supportive chemical and topographical cues but also facilitate immobilization of specific ligands that enhance HSC/HPC expansion.

In accord with our observation that the most potent HSCs reside within the endosteal region of bone marrow [66,67], where there is a relatively lower level of dissolved oxygen [68–72], many groups have recommended expansion of CD34+ under hypoxic conditions [73,74]. There are two important caveats to consider here. First, although there is evidence that quiescence of long-term repopulating HSCs is enhanced [75,76] and, concomitantly, their differentiation is restricted, leading to an increased expansion of HSCs in low (1%–3%) oxygen tension, there are relatively few of these cells (<5%) within the total CD34+ inoculum. In fact, the vast majority of cells within the CB CD34+ population are committed progenitor cells and not sensitive to low oxygen tension. Second and relatedly, CB CD34+ cells normally exist within a neonatal/fetal circulation, where the oxygen tension is higher than that at the endosteal region within bone marrow. Paradoxically, the implication is that survival and proliferation of CB CD34+ cells may be less when cultured at low oxygen tension [77]. A recent systematic investigation of the impact of oxygen tension and growth factor combinations on expansion of CB CD34+ cells demonstrates that culture at 5%

or 10% may be better than 2.5% oxygen [78]. This notion is also supported by the very recent studies from Nombela-Arieta et al., suggesting that HSC hypoxia is not due to extrinsic oxygen tension but is rather an intrinsic property of HSCs, depending on their metabolic status and glycolytic profile [79].

It is also evident that the secreted products of nascent differentiated cells generated within cultures can modulate proliferation and differentiation of HSCs/HPCs [80,81]. Of specific concern are proteins, such as TGF- β , IL-8 and, platelet-derived growth factor (PDGF)-CC, that inhibit CD34+ cell proliferation and MK differentiation [82–84]. One approach for minimizing the inhibitory effect of emerging differentiated cells is continuous removal of these cells by immunomagnetic beads [85,86]. Alternatively, the level of secreted inhibitors during culture should be monitored, and media feeding or perfusion schedules can be adjusted to overcome their growth-limiting effects. Another approach is to directly block the signaling pathway activated by specific secreted proteins; this strategy underpins the utility of the small molecule regulator MK1, which acts through inhibition of the PDGF receptor signaling pathway as described by Boitano et al. [84]. The MK1 example highlights the need to better understand cell-cell interaction networks and how these regulate expansion and differentiation of HSCs. Zandstra and colleagues published a mathematical model to analyze, predict, and interpret the effects of these secreted molecule-mediated networks; the model should be considered a key component of bioreactor control systems [82]. These models need to be underpinned and validated by proteomic and metabolomics data collected throughout the course of CD34+ cell and MK culture.

Optimizing megakaryocyte differentiation and maturation *ex vivo*

Many studies have been performed with small-scale static culture systems to investigate differentiation of CD34+ cells into MKs. Most of these use serum-depleted media, where the influence of and the interactions between specific cytokines and growth factors on MK differentiation and proliferation can be clearly attributed to recombinant proteins/factors rather than serum components. Following cloning and the demonstration that TPO was the main humoral regulator of megakaryopoiesis [12,87–89], numerous groups assessed the contribution of other hematopoietic cytokines on these processes *in vitro* and demonstrated interactions between IL-3, SCF, IL-6, IL-11, and TPO [90–94]. Further studies demonstrated that MKs express chemokine (C-X-C motif) receptor 4 (CXCR-4) and, as expected, respond to its ligand, stromal-derived factor-1 (SDF-1), to mediate MK migration and adhesion to endothelial cells [95–99].

Taken together, these studies demonstrate that a host of individual cytokines, including IL-1, IL-3, IL-6, IL-9 [100], IL-11, SCF, SDF-1, and PDGF-BB [101] synergize or com-

plement TPO in promoting *ex vivo* megakaryopoiesis. The challenge, from the perspective of developing bioreactor technology for generating platelets, is identifying a minimal combination of these cytokines that yields maximal numbers of MK, and platelets from CD34+ cells. One of the most comprehensive and systematic series of studies to investigate *ex vivo* expansion of MKs from CB CD34+ cells has been conducted by the research development group of Hema-Quebec (Quebec, Canada) [102–107]. The group's initial studies focused on the question of what combination and concentrations of early-acting cytokines together with TPO would result in high overall MK progenitor expansion [102]. They demonstrated that low concentrations (<10 ng/mL) of SCF and Flt3L together with IL-6 and TPO for 7 days, followed by culture for a further 7–10 days in higher concentrations of SCF and Flt3L (50 ng/mL), resulted in the largest production of MKs and platelets.

Shortly thereafter, the same group demonstrated that culture of CD34+ cells in this combination of cytokines (TPO, SCF, Flt3L, and IL-6 [TSF6]) at 39°C was 7 times better at producing total MKs (as determined by total CD41a+ cells) than cultures at 37°C [103]. A subsequent publication [105] presented the findings of a more thorough investigation of mild hyperthermia on MK differentiation. The researchers indicated that early, transient culture of CB CD34+ cells at 39°C led to significantly improved MK yields, but did not enhance the proportion of polyploid MKs generated. The molecular basis of this outcome was investigated but remains undefined [105]. There is a suggestion that at least two important MK transcription factors, NF-E2 and Fli-1, are significantly upregulated at 39°C, but it is uncertain whether these are associative or causative events.

A multistep statistical strategy based on Plackett-Burman factorial and central composite designs [108] was used to determine the influence of 13 cytokines on proliferation of MK progenitors and MK maturation [104]. Conducted with CB CD34+ cells, these studies demonstrated, in accord with the findings of others, that in the presence of TPO, MK proliferation was enhanced by SCF, Flt3L, IL-6, IL-9, and erythropoietin (EPO) [90,92,102,109]. They also demonstrated that SCF, IL-6, and IL-9 had a positive effect on MK maturation. In contrast, as might be expected, EPO and IL-8 inhibited MK maturation. Based on these findings, a response surface approach was used to tease out the interactions between these cytokines and to optimize their concentrations when used together for MK maturation. The culmination of this study was development of a two-phase culture system, where CD34+ cells cultured for 7 days in TSF6 followed by a subsequent 7 days in TPO, SCF, IL-6, and IL-9 (TS69) resulted in high MK proliferation and purity. Despite this, it was noted that the yield of platelets per MK remained low, with only 2–10 CD41+CD42+ platelets being generated per CD41+ MK. This may have been attributable to the overall low ploidy

of MKs generated in these culture conditions, where only 5%–15% of MKs was higher than 8N.

A further study with CB CD34+ cells conducted by the same investigators [106] tested the premise that an initial CD34+ cell expansion phase would increase the number of primitive HPCs within the total CD34+ population for subsequent differentiation into MK progenitors and mature MKs. Their strategy involved stimulating division of primitive cells without compromising or biasing their subsequent differentiation [106]. Experiments were performed to investigate two parameters concurrently: the time of expansion culture and the cytokine combination. The experimental design involved culture of cells for different periods, ranging from 3–10 days in expansion cocktails followed by reculture in TS69, a combination previously shown to drive MK development [104]. They demonstrated that the optimal time for the expansion phase was 5 days and that the cytokine combinations inducing the greatest expansion of CD34+ cells and clonogenic precursors did not necessarily result in the best MK yields in the subsequent differentiation phase. Thus, although the combinations TSF6, IL-3, and G-CSF; TSF; and TSF6 were potent for expanding CD34+ cells and progenitors, they did not increase MK yield after 14 days. Furthermore, there was not a linear relationship between the absolute number of CD34+ cells or the proportion of CD34+ cells present at day 5 and the number of MK generated at day 14.

A more detailed analysis of the interaction between the concentrations of SCF, TPO, and Flt3L during the first 6 days of culture led to development of a new optimized MK progenitor cocktail (OMPC) containing SCF at 10 ng/mL, TPO at 35 ng/mL, and Flt3L at 11 ng/mL [107]. When cultured in OMPC for 6 days each, CD34+ cell gave rise to approximately 10 CD41+ MKs. This was a high rate of conversion compared with that achieved over 10 days in TPO and plasma [110] or after 14 days in similar serum-free media with a combination of 7 cytokines (TPO, SCF, Flt3L, IL-3, IL-6, IL-9, and GM-CSF [111]), where 16 CD41+ CD61+ cells were generated.

Given its well-described effects on MKs [95,96,112], a notable aspect of the extensive factorial design experiments conducted by the Hema-Quebec group was the omission of SDF-1. Wang and colleagues demonstrated that SDF-1 increases MK progenitor migration and mature MK adhesion to endothelial cells. Moreover, when used in combination with TPO and SCF, SDF-1 stimulated a modest increase in MK production from CD34+ cells [95]. Similar experiments conducted with peripheral blood CD34+ cells demonstrated that SDF-1 enhances MK ploidy and increases the number of MKs forming proplatelets [112]. An important role for SDF-1 in promoting localization and binding of MK to bone marrow endothelial cells has been demonstrated [98], suggesting that addition of this chemokine to bioreactor systems based on coculture of MKs and marrow endothelial cells would be beneficial.

Similarly, even though fibroblast growth factor 4 (FGF4) alone may not directly regulate MK progenitor proliferation or maturation, it promotes adhesion of MK to endothelial cells and potentially acts in concert with SDF-1 and cell adhesion receptor mediated signaling pathways to drive MK maturation and platelet production [98]. An important consideration in this biology is the role played by heparan sulfate (HS) in facilitating binding of FGF4 to its cognate receptor [113]. In this respect, FGF4 may require a specific HS sulfation pattern to mediate receptor binding, and this may be endothelial cell dependent. Accordingly, it would be desirable to establish MK-endothelial cell cocultures with BM-derived sinusoidal endothelial cells rather than umbilical cord vein endothelial cells, despite the ready availability of the latter.

Although the majority of the studies described previously seek to maximize the number of MKs generated *in vitro*, very few consider the maturational state of these cells and how it impacts subsequent platelet generation. The first phase of MK maturation involves a massive increase in cytoplasmic volume, an increase in the number of alpha and dense granules, and the development of a convoluted network of invaginated membrane referred to as the demarcation membrane system [114,115] or, more recently, as the invaginated membrane system (IMS) [116]. The exact role of the IMS in platelet biogenesis was unclear for many years, but it is now evident that the IMS is continuous with the MK membrane and the source of membrane used within proplatelet extensions and ultimately the platelet membrane [117]. MK maturation also includes formation of a dense tubular network and an open canalicular system for granule release. Accordingly, from the perspective of producing large numbers of functional platelets, it is critical that an *in vitro* bioreactor culture system generates large numbers of MKs, and more importantly, that these cells mature and become loaded with the required organelles and structural elements needed to complete platelet biogenesis. The relationship between MK ploidy, maturational status, and platelet production capacity remains unclear.

However, an early study suggests that proplatelet formation and platelet synthesis is linked to MK ploidy and that MKs must reach a ploidy of 8N to produce platelets [118]. MK polyploidization is also dependent on the origin of the progenitor population; CB CD34+ cells give rise to lower ploidy MKs as compared with BM- or mobilized peripheral blood (mPB)-derived CD34+ cells [91,119–121]. To offset this, CB-derived MKs exhibit a greater proliferative potential than their adult (BM or mPB) counterparts [119,122,123]. Nevertheless, MKs generated from mPB CD34+ cells produce approximately 2-fold more proplatelets than those derived from CB and more platelets per MK [119], outcomes that are attributed to the increased ploidy of the PB-derived MKs. Studies to enhance MK polyploidization have demonstrated that nicotinamide, Rho-Rock

inhibitors, and other cytokines modulators have the ability to enhance MK ploidy [124–126]. However, the ultimate effect of these modulators on platelet formation from individual nascent MKs is controversial. Some report increased proplatelet formation [124], whereas others have demonstrated reduced production. Notably, analysis of many thousands of single CB-derived MKs by live cell imaging revealed that nicotinamide results in significant (sevenfold to thirty-onefold) reduction in proplatelet formation and platelet production [127]. Moreover, a high proportion of di- and polyploid MKs undergo complete proplatelet regression. Utilizing time-lapse live cell imaging, we also have observed the same phenomena during static culture, where large proplatelet extensions are completely resorbed back into the MK within 5–10 min, even in the absence of nicotinamide. Taken together, these observations illustrate that MKs behave in dynamic and unpredictable ways under static *in vitro* culture conditions.

Although there has been a drive to increase the ploidy of *ex vivo* generated MKs, the more critical considerations are promoting normal MK maturation and providing the necessary cues for a high proportion (>80%) of these cells to synchronously form proplatelet extensions. In our experience, formation of proplatelet extensions by human MKs in static culture is asynchronous and infrequent. Novel approaches, such as transfer of mature polyploid MKs to a bioreactor environment customized for proplatelet formation and platelet release, may enhance synchrony and efficiency of this step.

Optimizing *ex vivo* MK proplatelet formation and platelet production

One of the greatest challenges to be addressed before biomanufacture of human platelets becomes feasible is optimizing MK proplatelet formation and platelet shedding from these structures. In our view, approaches for addressing this challenge will come from a more complete understanding of how this occurs *in vivo*. In this respect, it is well known that mature MKs localize to the subluminal side of BM endothelium and either release platelets into the circulation from proplatelet-like structures that extend through endothelial cells or cell junctions [128,129] or escape into the circulation to fragment within the microvasculature of the lung [36]. The general consensus is that the majority of platelets are generated via MK proplatelet formation, and their controlled release occurs in the BM sinusoids from preplatelets [130]. The parasinusoidal location of MKs and the possible importance of this relationship to MK development and thrombogenesis was first described in 1978 [131] and supported by others shortly thereafter [132]. These pioneering electron microscopy studies were conducted with BM and cells removed from bones and as such, only provided a snapshot of a complex dynamic process that most likely unfolds over many minutes.

Although these studies depicted the physical contact between endothelial cells and MKs, they did not hint at the critical functional relationship between these two cell types. In this respect, it is now well accepted that an important codependency exists between MKs and endothelial cells, especially following myelosuppression and BM damage. This relationship is clearly evident when the vascular disrupting agent combretastatin A4 phosphate (CA4P) is administered to either thrombospondin knockout (TSP-DKO; deficient in both TSP1 and TSP2) or wild type mice following 5-fluorouracil (5-FU) treatment [133]. Rather than observing a profound rebound level of BM MK numbers and blood platelet count 13 days after 5-FU, CA4P significantly blunted megakaryopoiesis and platelet recovery. This dramatic change was associated with a reduced and disorganized microvascular network within the BM, strongly implying a mutual interdependence between the BM's sinusoidal vascular compartment and MKs. Megakaryocytes and platelets are known to secrete both pro- and antiangiogenic factors [134], including vascular endothelial growth factor A (VEGF-A), basic fibroblast growth factor (FGF-2), matrix metalloproteinases (MMPs) [135,136], platelet factor-4, and thrombospondin (TSP). TSP inhibits blood vessel formation, as evidenced by the increase in vessels within the BM of TSP-DKO mice [133] and MK production *in vivo*. TSP also effects ploidy of MKs; the TSP-DKO mouse has twice the number of polyploid MKs. Therefore, TSP antagonists may be a useful addition to MK bioreactor cultures.

More recently, elegant studies conducted by the Rafii group have shown that MK progenitors interact with BM sinusoidal endothelial cells in a chemokine dependent manner to promote platelet production independently of TPO [98]. An increase in the level of FGF4 and SDF-1 in TPO-/- mice led to increased platelet production that was not dependent on MK-active cytokines such as IL-6, IL-11, EPO, and granulocyte-colony stimulating factor (G-CSF). The researchers found that SDF-1 and FGF4 directed MK to interact with BM endothelial cells (BMECs) *in vivo* and increased the number of mature polyploid MK at these locations. Notably, when SDF-1 and FGF4 were added to cocultures of MKs and BMECs, a greater proportion of megakaryocytic colony-forming units (CFU-Meg) and large CD42b+ MK were produced. Fibroblast growth factor 4 supported the adhesion of MK to BMECs, enhancing their survival and maturation, whereas SDF-1 enhanced platelet production by promoting adhesion to BMECs via upregulation of vascular cell adhesion molecule 1 (VCAM-1) and most likely other yet to be discovered cell adhesion receptors. Taken together, these observations strongly suggest that, for bioreactor systems based on coculture of MK with BM endothelial cells, the functional status of the endothelial cells, specifically their ability to support adhesion of MKs, is critical for MK development and can be modulated by addition of FGF4 and SDF-1.

To confirm that these events also take place within human BM, it would be necessary to directly visualize MK interacting with endothelial cells and producing platelets *in vivo*. The power of this approach was demonstrated using multiphoton intravital microscopy to visualize platelet generation within the calvarium of CD41-EYFP transgenic mice [34]. MKs displaying considerable morphologic diversity, largely stationary in comparison with other hematopoietic cells, often existed as single cells but occasionally as clusters and were always found in close association with BM sinusoidal vessels. Megakaryocytes were observed to extend proplatelet-like protrusions into small blood vessels, where they were sheared by blood flow to release heterogeneously sized proplatelets directly into the blood. Junt et al. also found that the platelet count in pulmonary veins exiting the lungs was higher than the pulmonary arteries, an observation supporting the view that a proportion of single platelets are generated by blood flow induced shearing of larger proplatelet doublets (or preplatelets) within the pulmonary vascular bed. Given these *in vivo* observations, it is not surprising that MK shedding of platelets is enhanced by hydrodynamic shear stress *in vitro*. Junt et al. demonstrated that simple agitation of MKs grown on the upper chamber of a transwell more than doubles platelet release into the lower well in comparison with static conditions [34]. Therefore, efficient production of platelets *in vitro* would seemingly require the influence of hydrodynamic shear stress akin to that experienced *in vivo*. In this respect, 1.3–4.1 dynes/cm² of shear stress can be generated within the lumen of BM sinusoids [137]. Exposure of MK to higher shear rates accelerates platelet production, events that depend on microtubule assembly, and elongation [138].

The mechanism responsible for triggering the initial protrusion of the MK membrane through or between endothelial cells into the sinusoid lumen remains to be fully defined. We can assume that, due to their unique position at the vascular surface, MKs are likely to be exposed to and interact with blood components. Recent studies in mice identified the lipid sphingosine-1-phosphate (S1P) as an important mediator in this process [35]. Through interaction with its receptor S1pr1 on MKs, S1P present at high levels within the blood triggers the formation and elongation of MK proplatelet extensions and stimulates the subsequent shedding of proplatelets into the blood. This later function occurs under static as well as flow conditions, suggesting that S1P plays a dominant role in the process, and shear stress may not be critical. Recent investigations within our laboratory have confirmed that a small proportion of nascent CD41+ MKs derived from human CB CD34+ cells express S1pr1, and thus S1P could also enhance proplatelet formation and platelet shedding *in vitro*. We suggest that other, yet to be defined guidance signals and molecules may also be involved in these processes. Furthermore, there is a need to determine how lipids and other molecular regulators cooperate with hydrody-

amic shear stress in driving proplatelet extension and platelet shedding. Simple devices, such as those described by us [139], that expose MKs of different ploidy and maturation to controlled flow conditions and gradients will be valuable in dissecting these interactions. The challenge for platelet bioreactors will be translating these conditions from micro devices to large-scale systems.

As stated earlier, there is uncertainty as to how many platelets are, on average, generated by mature MKs *in vivo*, ranging from a few hundred to 5,000 [34,114,140,141]. The generation of nascent platelets by individual MKs *in vitro* is reported to be highly variable, but in general, appears to be much less efficient than generation *in vivo*. Accordingly, a critical aim for those wishing to biomanufacture human platelets is to replicate this process faithfully *in vitro*. Two key objectives concerning thrombogenesis need to be met before this can be achieved: first, efficient generation of MK proplatelet extensions and, second, their complete and efficient conversion into platelets.

If it is assumed that, on average, 500 platelets can be generated from each nascent MK, then 1×10^9 MKs are required to produce 5×10^{11} platelets (a single transfusion dose). If, however, bioreactor culture is only able to support generation of 100 platelets from each mature MK, then 5×10^9 MKs are needed for a transfusion dose. Such mathematical considerations can be used to define cell generation targets at each step of the biomanufacturing process, as depicted in Figure 2.

Bioengineering strategies for generation of MKs and platelets

Culture systems ranging from relatively simple flask or bag-based systems, where cells grow under static 2D, serum-free, cytokine-dependent conditions to more elaborate purpose built bioreactors that provide a 3D aspect in an attempt to mimic megakaryopoiesis and thrombogenesis *in vivo* have been described. A number of groups have conducted studies to generate a mixture of megakaryocytic cells for transplantation [111,142–144]. The rationale for this is that co-infusion of a maturing MK population may contribute to faster platelet recovery after hematopoietic stem cell transplant, an approach most relevant after CB transplantation, where platelet count recovery to safe or normal levels is frequently prolonged 3–6 months. A study where CD34+ cells were seeded into 35-mL Vuelife Teflon (American Fluoroseal Corporation, Gaithersburg, MD) bags at a density of 1.0×10^4 cells/mL and stimulated with a mixture of cytokines (IL-3, SCF, TPO, and Flt3L) demonstrated the feasibility and safety of this approach [145]. Maximal cell expansion occurred at day 11, and MK precursors (CD34+ CD41+) increased 6-fold. The overall median expansion of CD41+ and CD61+ cells was 33-fold and 14-fold, respectively. Although this closed bag culture system seemed effective, there was a large variation in production of CD41+ cells, ranging from two- to

forty-onefold, indicating that inherent variability in the hematopoietic potential of this cellular source may limit its use for this purpose. Furthermore, as the culture system was not designed for generation of platelets, the utility of bags for this purpose cannot be inferred.

Laboratory-scale bioreactors equipped with compartmental hollow fiber capillary membranes have been devised for red cell production [4]. The 2 and 8 mL bioreactors consist of three independent interwoven capillary beds and an integrated perfusion device to facilitate medium recirculation and medium substitution, temperature control, and regulation of air and CO₂ flow rates. Metabolic parameters, including partial pressures of O₂ and CO₂, pH, concentration of glucose and lactate, and lactate dehydrogenase, were constantly monitored during cell culture to assess cell proliferation and determine the time points for passage or harvest. CD34+ cells were cultured for 9 days, and their progeny were then transferred into a second bioreactor for further cell expansion. This system supported significant hematopoietic cell production, particularly of red cells, and might be suitable for larger scale generation of MK.

Notably, there have been relatively few bioreactor systems designed specifically for producing human platelets. One approach used three phases of long-term, serum-free coculture to expand HSC, promote MK differentiation, and generate platelets from CB CD34+ cells [146]. The first phase involved expansion of CD34+ cells in T75 tissue culture flasks with monolayers of telomerase gene-transduced human stromal cells (hTERT stroma) and X-VIVO serum-free media (Lonza, Walkersville, MD, USA) supplemented with a cocktail of SCF, Flt3L, and TPO for a total of 14 days. The second phase cultures involved transfer of unseparated cells generated within the first phase to T75 flasks containing a monolayer of hTERT stroma supplemented with IL-11 in addition to SCF, Flt3L, and TPO, for a further 14 days. The platelet generation phase was performed with 1×10^6 cells transferred from the second phase cultures into six-well tissue culture plates with or without human umbilical vein endothelial cells (HUVECs), SCF, TPO, Flt3L, IL-11, and/or SDF-1 and FGF4. Cells were analyzed after 7 days in the third phase condition. Multiple cytokines, including IL-3, IL-6, IL-11, PDGF, and SDF-1, were added to the second phase cultures and ultimately demonstrated that SCF+TPO+Flt3L+IL-11 was the best combination for generation of CD41+ cells from CD34+ cells. It was also shown that the initial concentration of CD34+ cells in phase 1 cultures affected overall cell and MK production, with 500 CD34+ cells in 10 mL of media being optimal. Based on these findings, it was estimated that 2.1×10^{11} platelets could be generated from a single CB collection containing 5×10^6 CD34+ cells. Thus, a single CD34+ cell could be converted into approximately 40,000 platelets.

As depicted in Figure 2, we suggest that a relevant target for ex vivo manufacture of platelets from CD34+ cell culture

would be to generate 100,000 platelets for each CD34+ cell cultured. This is a challenging target demanding optimization of all stages in the process. The best evidence that this target might be achievable using bioreactor technology comes from the Lasky laboratory in Ohio [147]. A perfusion bioreactor module comprising three woven polyester fabric scaffolds to support cell growth and platelet production was encased between smooth finished fluorinated ethylene gas-permeable membranes. Media were provided above and below the fabric scaffolds, and the entire bioreactor was constructed in polycarbonate. In this configuration, CD34+ cells were seeded onto the top of the surgical polyester fabric (Bard, Murray Hill, NJ), and nonadherent cells, including platelets, generated during culture fell to the bottom chamber and were collected when fresh medium was added. In this design, the fabric scaffold represents an endothelial barrier supporting CD34+ and MK cell growth, and the lower chamber represents the lumen of a blood vessel.

Platelets falling through the porous scaffold were collected once daily, with continuous production of functional platelets for more than 32 days. Media containing IL-6 and IL-11 produced almost fourfold more platelets than media containing a Src kinase inhibitor. Morphologically, a mixture of normal and atypically shaped and sized platelets was seen, which aggregated in response to thrombin and displayed an increase in expression of cell-surface p-selectin (CD62) and CD63. However, activation of platelets without agonists was also reported, implying that cells may spontaneously aggregate during storage or shortly after infusion.

The Lasky group demonstrated the successful ex vivo generation of human platelets sans feeder layer, indicating the significance of having an appropriate 3D microenvironment to replicate in vivo processes [147]. Although their device can manufacture platelets, both design changes and scale up are restricted in the current configuration. Since the devices are modular, it can be argued that they can be connected in parallel to increase platelet production, but each device only supports three 19 mm diameter scaffolds. With each bioreactor producing approximately 1×10^8 platelets from a starting cell number of 6×10^6 CD34+ cells, it is evident that further improvements are required before it could generate a transfusion dose of 5×10^{11} platelets.

It remains uncertain if the polyester scaffold was essential or affected platelet production in this device. Moreover, alternative scaffold materials and polymers were not tested to determine whether the material or its topology and architecture impacted platelet production. In addition, since the device is closed and microscopic examination of cells in culture was not possible, there were no data on the differentiation process or on the number or size of MK produced. Accordingly, platelet generation was described as only a function of the starting CD34+ cell number, and it was not possible to determine how many platelets were produced by each MK, nor the time duration of the process.

Given that platelets were produced over 30 days, it can be speculated that MK differentiation and maturation were asynchronous and continued over the entire timeframe.

Pallotta et al. [148] attempted to reconstruct the BM environment to analyze the migration of MKs within the vascular niche. Poly(dimethylsiloxane) (PDMS) modules were bound to a glass slide and housed three wells, each containing a porous silk microtube with a wall thickness of 50 μm , through which culture media were continuously perfused. The outer surface of the microtubes was coated with growth factors, and a collagen gel was cast in the well between the PDMS wall and the microtube such that a 150 μm gap was left between the collagen gel and the microtube. Megakaryocytes were seeded into this gap, and the infiltration of proplatelet extensions into the microtube was assessed. Migration of MKs toward the microtube was allowed for 16 hours, after which proplatelet extensions were observed to be forming and pushing through the porous microtubes, where they were exposed to flow and shear stress. Notably, only 7% of MKs in contact with the microtube wall exhibited proplatelet extensions, and 200 platelets per MK were produced on average. Although a very low proportion of MK exhibited this behavior, the outcome highlights the utility of such devices for investigating and optimizing MK biology and production of platelets. Moreover, these experiments demonstrate that hollow fibers or even porous sheet-like membranes may be useful endothelial cell mimics and of great utility for large-scale production of platelets.

A combination of a sheetlike porous structure and fluid flow over and under localized MKs has recently been shown to improve the generation of platelets from induced pluripotent stem cell (iPSC)-derived or human embryonic stem cell (hESC)-derived MKs [149]. In a simple bioreactor, MKs were placed on HUVECs cultured as a monolayer on a porous PDMS membrane, then exposed to fluid flow from two directions: perpendicular to the MKs and membrane (i.e., through the membrane) and under the membrane. Studies with a second version of this bioreactor, where MKs were deposited within slits on the scaffold, suggest that the angle between the directions of the two fluid flows was important in enhancing platelet production. Such experiments indicate that further studies need to be conducted to understand the response of MKs to hydrodynamic shear stress and how it can be regulated to optimize proplatelet formation and platelet shedding.

Cell sources: Comparison between CB, mPB, BM, and pluripotent cell sources

Multiple cellular sources are available for platelet biomanufacture, including CD34+ cells isolated from BM, mPB, CB, and pluripotent-derived HSCs/HPCs. It is evident that the intrinsic ability of CD34+ cells from these different sources to proliferate, undergo MK differentiation, and produce platelets is also different [150,151]. Megakaryocytes

derived from BM and mPB CD34+ cells are generally larger and of higher ploidy; they tend to generate more platelets per MK than their CB counterparts. Despite this, CB is an attractive CD34+ source, because a large number of CB units are stored within CB banks worldwide. Moreover, CB units not considered large enough for transplantation could be put aside for platelet production. The other benefit is the ability to generate platelets of specific human leukocyte antigen (HLA) type and ABO blood group by selecting CB with the required immunophenotype. Pooling matched CD34+ cells from multiple CB units could be done to generate HLA/ABO compatible platelets for transfusion into unrelated recipients or patients with alloantibodies.

An alternative option to using CD34+ cells from adult hematopoietic tissues is to obtain equivalent hematopoietic stem and progenitors derived from pluripotent cells such as hESCs, iPSCs, or directly reprogrammed cells. A key advantage for their use is that the undifferentiated pluripotent cells can be propagated in vitro indefinitely, providing an infinite source of HLA typed cells for cell therapy. However, the adoption of this approach is dependent on two important considerations. First, it is essential to be able to efficiently direct mesendoderm differentiation of pluripotent cells so that hematopoietic precursors (CD34+ cells) with functional equivalence (or better) to adult counterparts are generated. Secondly, these progenitors must differentiate into megakaryocytic cells that undergo complete maturation and produce platelets that are functionally equivalent to those produced by adult CD34+ cells. It is evident that MKs and platelets can be generated from hESCs and iPSCs. This was initially achieved by coculture of hESCs with OP9 stromal cells [152] and mouse fetal mesenchymal lines [153]. The latter approach is not readily applicable for clinical products. Subsequent studies demonstrated that MKs and platelets could be generated from multiple hESC lines under serum-free and feeder-free conditions [154]. This approach relied upon generation of a hemangioblast/blast cell as a cell intermediate, claimed to be scalable, and produced platelets with ultrastructural and morphologic characteristics of blood platelets. Moreover, these platelets were activated by thrombin. The hESC-derived platelets were also functional in vivo and were able to incorporate into thrombi within vessels at the sites of laser-induced vascular injury. Advanced Cell Technology has adopted this method of platelet generation for clinical use, although on average, only six platelets are generated from each input hESC MK [154].

More recently, MKs and platelets have been produced from human iPSCs [149,155] and directly reprogrammed cells [156,157]. The detailed studies conducted by Takayama, Eto, and colleagues have clearly demonstrated that the method of iPSC production, specifically the extent and duration of c-MYC expression within MKs, influences the ability of these cells to generate platelets. This is an important reminder that there is still much to be understood

about the impact of epigenetic change and subsequent gene expression associated with induction of pluripotency on the generation of differentiated cell types. As these authors suggest, there is a strong case for selecting iPSCs that exhibit reproducible, efficient MK differentiation and functional platelet production. An alternative approach is establishment of immortalized MK cell lines from human iPSCs, which could potentially provide high quality platelets for transfusion [158]. A further option is platelet generation from induced MKs (iMKs), although as Masuda indicated, the molecular mechanisms underpinning their formation are not well understood [157]. One feature common to hESC, hiPSC, and iMK approaches is the very low conversion of CD41+ MKs into platelets. At best, only 5–10 platelets are formed, which significantly limits the use of these cell sources for sustained biomanufacture of platelets, irrespective of the bioreactor technology used for large-scale culture. A potential risk commonly raised with pluripotent cell-based therapy is the possibility of infusing a nucleated cell with teratoma forming ability. This concern is completely overcome by irradiation of the platelet product before infusion.

A final consideration with the use of pluripotent or iMK cells as a cellular source for biomanufacture of platelets is the function and stability of the platelet product. Despite the recent studies demonstrating that platelets generated from these sources can function *in vivo*, it is essential that platelets generated by any of the different methods, processes, and cellular sources are thoroughly characterized and tested for function. At a minimum, this should include electron microscopy, immunophenotypic analysis, ability to respond to agonists in aggregation assays, storage life, and, ideally, their ability to contribute to thrombi formation *in vivo*. Although, pluripotent cell sources have a major advantage because an unlimited supply of undifferentiated cells can be produced as a starting cellular source for MK differentiation, the efficiency at which these cells go on to produce platelets is significantly less than with BM, mPB, and CB CD34+ cells. For the moment, until further research is conducted with pluripotent cells, we suggest that adult HSC sources are preferred.

Summary and conclusion

The clinical demand for platelets is increasing, while the supply from donors is at best being maintained. In the long term, with a shrinking blood donor base, a new approach for supply of human blood cells for transfusion must be developed. This has driven the recent academic and corporate interests in human blood cell factories: bioreactor systems of sufficient scale and efficiency for production of red cells and platelets. A greater understanding of the molecular regulation of megakaryopoiesis and thrombogenesis and the opportunities provided by pluripotent cells in addition to novel bioengineering platforms and bioreactor technologies has also fueled progress in this area.

We believe that the best systems for platelet biomanufacture will be inspired by biology; they will take into account and replicate cell-cell interactions, biological signaling, hydrodynamics, and the processes that regulate platelet production and fate *in vivo*. Given the need to recapitulate HSC/HPC proliferation, MK differentiation, MK maturation, and the ultimate steps in their conversion to platelets, we suggest that different bioreactor environments will be required for efficient platelet production. In this respect, process optimization should be driven toward maximal conversion of one cell type to another with as little consumption of cytokines, growth factors, media, and other biological agents as possible. Our approach is to develop tailored microenvironments to expand CD34+ cells and potentially in a concomitant manner, increase the number of MK progenitors, then move these cells into a bioreactor for MK maturation, induction of proplatelet extension, and platelet release. A multidisciplinary science approach including, at minimum, cell biology, protein engineering, biomaterials, surface science, bioengineering, computational fluid dynamics, and animal models is required.

Although there has been significant progress in this research field during the last 5 years, we suggest that the key issues that must be addressed to ensure that biomanufacture of platelets becomes a reality include:

1. a better understanding of how megakaryopoiesis and thrombogenesis are regulated *in vivo*;
2. the identification and use of novel small molecules and mimics, such as MK1 [84];
3. development of bioreactor platform technology that can accommodate and control growth of very large numbers of CD34+ cells and MKs ($> 10^{12}$); and
4. devices for optimizing the final conversion of MKs to platelets.

Because many aspects of *in vitro* megakaryopoiesis and thrombogenesis remain to be optimized, we suggest that small-scale research devices and test-bed platforms need to be developed to inform the design of large-scale platelet biomanufacture technology. Such a grand translational science challenge demands investment and input from academic researchers as well as support from the blood transfusion industry, translational funding agencies, and the broader community of biotechnology providers. Platelet biomanufacture is an inspiring challenge with the potential to revolutionize blood transfusion.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this review have been declared.

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