Induction of differentiation of human stem cells ex vivo: Toward large-scale platelet production

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Abstract

Platelet transfusion is one of the most reliable strategies to cure patients suffering from thrombocytopenia or platelet dysfunction. With the increasing demand for transfusion, however, there is an undersupply of donors to provide the platelet source. Thus, scientists have sought to design methods for deriving clinical-scale platelets ex vivo. Although there has been considerable success ex vivo in the generation of transformative platelets produced by human stem cells (SCs), the platelet yields achieved using these strategies have not been adequate for clinical application. In this review, we provide an overview of the developmental process of megakaryocytes and the production of platelets in vivo and ex vivo, recapitulate the key advances in the production of SC-derived platelets using several SC sources, and discuss some strategies that apply three-dimensional bioreactor devices and biochemical factors synergistically to improve the generation of large-scale platelets for use in future biomedical and clinical settings.

Key words: Megakaryopoiesis; Platelet production; Transfusion; Bioreactors; Stem cells

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INTRODUCTION

Platelets are the smallest anucleate cells (2-4 µm in diameter) produced by megakaryocytes (MKs) in bone marrow (BM). They play a pivotal role not only in hemostasis and thrombosis but also in neoangiogenesis, innate immunity and inflammation. Until now, platelet transfusion, which may lead to varying degrees of hemorrhage that is sometimes life-threatening, has remained the most effective way to treat patients suffering from thrombocytopenia and/or platelet dysfunction[6-2]. However, platelets derived from voluntary blood donation pose some challenges, including susceptibility to pathogen contamination, short preservation time, and difficulty satisfying the increasing number of patients requiring platelet transfusion[3]. Therefore, seeking a safe and reliable alternative source of platelets is an effective solution.

In recent years, stem cells (SCs) research has developed rapidly in the field of regenerative medicine, and several research groups have focused on cultured platelet production ex vivo. In addition to hematopoietic SCs (HSCs), human embryonic SCs (hESCs) and human induced pluripotent SCs (iPSCs) have been considered potential sources for generating human MKs[4-6] and platelets[7,8]. The proper use of biochemical stimuli, including growth factors and cytokines, could augment MK maturation and platelet generation during ex vivo stem cell culturing[9,10]. To mimic the physical microenvironment, some bioengineering techniques that promote MK maturation and platelet release have been developed[11,12]. In this review, we will recapitulate the methods that promise to produce sufficient platelets differentiated from SCs ex vivo and some obstacles in this field. We believe that the ex vivo production of platelets is a state-of-the-art technology integrated with the development of unlimited SCs, newly discovered biochemical reagents and three-dimensional (3D) bioreactor systems.

MEGAKARYOPOIESIS AND PLATELET FORMATION

Megakaryopoiesis and thrombopoiesis are elaborate processes that can be divided into several successive stages: The commitment of SCs toward the MK lineage, proliferation of MK progenitors, MK maturation, proplatelet formation and terminal platelet production. In vivo, HSCs located in the osteoblastic niche on the surface of the bone cavity have the ability for self-renewal and multipotent differentiation. They give rise to common lymphoid progenitors and common myeloid progenitors, which differentiate into granulocyte/macrophage progenitors and MKs-erythrocyte progenitors (MEPs). MEPs subsequently commit to the erythrocytic and megakaryocytic lineages, which progressively produce different ages of immature MKs with various proliferative potency. The unique feature of megakaryocytic maturation is endomitosis due to a failure in cytokinesis (not all MKs undergo mitosis). This process is accompanied by an increase in DNA content (up to 128 N) and the following events: Cell enlargement; the emergence of various unique organelles, such as dense bodies and secretory particles; the synthesis and assembly of cytoplasmic proteins; the formation of the demarcation membrane system; and the formation of a membrane reservoir for proplatelets. Upon maturation, MKs migrate to the vascular niche and extend cytoplasmic projections (proplatelets) into the sinusoidal endothelium after cytoskeletal remodeling. Under shear forces produced by the blood stream, proplatelets are released, gradually develop into mature platelets and undergo reassembly and the displacement of microtubules[13-15] (Figure 1).

In vitro, SCs (HSCs, hESCs or hiPSCs) are cultured to increase progenitor cell number before differentiation into MKs. After expansion, SCs differentiate into the megakaryocytic lineage as a result of stimulation with growth factors [e.g., thrombopoietin (THPO) and stem cell factor (SCF)] and MK progenitor expansion with a recombinant human THPO mimic (AMG531)[16]. Immature MKs then undergo the polyploidization process by inducing endomitosis to increase the number of DNA content to 16-128 N. The myosin inhibitor nicotinamide, actin inhibitors and Src inhibitor could be used for MK polyploidization. However, the capacity of MK to undergo polyploidization in culture is significantly lower than in the BM micro-
In vivo, bone marrow is located within the trabecular bones, where the hematopoietic stem cell (HSC) undergo the process of megakaryocyte (MK) differentiation and MK maturation under the control of thrombopoietin. During maturation, MKs migrate to bone marrow sinusoids and form proplatelets. Proplatelets elongate through the vascular endothelium into the vessels, and proplatelet terminal ends are released into the bloodstream by blood shear forces, forming platelets.

In vitro, HSC-derived CD34+ cells or pluripotent stem cell are first expanded in vitro and then differentiate into the megakaryocytic lineage. Once differentiated, immature MKs undergo the polyploidization process via inducing endomitosis to increase the number of DNA content to 16-128 N. During the process of polyploidization, some extended proplatelets are formed and released in high ploidy MKs in culture. To increase the efficiency of platelets, MKs extend proplatelets into the bioreactor, with perfused culture medium mimicking blood flow.

Environment, with a maximum ploidy of 126 N in hESC-derived MKs and 16 N in iPSC-derived cells. During the process of polyploidization, some proplatelets are formed and released in high ploidy MKs in culture. There is a connection between apoptosis and the process of proplatelet formation because the intrinsic apoptosis pathway is necessary for MKs to adequately acquire proplatelets and release platelets. To promote the maturation of MKs and accelerate the release of platelets, scientists have altered the culture conditions, including by treating the cells with chemical substances and mimicking the physical microenvironment (Figure 1).

**STEM CELL SOURCES FOR PLATELET PRODUCTION EX VIVO**

Pluripotent SCs and hematopoietic progenitors are the main sources that have been used to generate MKs and functional platelets. The former, including hESCs and hiPSCs, is considered an unlimited seeding cell source. The latter involves CD34+ (a surface marker usually expressed by hematopoietic stem/progenitor cells) cells from BM, umbilical cord blood (UCB) and peripheral blood (PB). Because they are primary cells, there is a risk of viral contamination. In total, each of these sources has advantages and disadvantages for clinical platelet transfusion.

**HSCs**

CD34+ cells extracted from BM, PB and UCB are simple and feasible sources for platelet production ex vivo. In 1995, Choi et al. isolated CD34+ cells from human PB and cocultured them with serum taken from dogs suffering from aplastic anemia. Upon the generation of MKs, human AB serum (AB-HS) was added to the culture system to promote the production of platelet-like particles. When adenosine diphosphate (ADP) was added, aggregation could be observed. This is the first report of human functional platelet production in vitro. Since then, the application of THPO has accelerated the development of research on platelet generation ex vivo. The scientists found that the addition of different cytokines in the culture medium, except for thrombopoietin, could improve megakaryocytic maturation and platelet production. To some extent, the ultrastructure and physiological functions of derived platelets were similar to those of platelets in vivo. However, because of the inconvenience of obtaining BM samples and because the content of CD34+ cells is low in the medullary cavity, the ability to induce CD34+ cells from BM is greatly
restricted\(^{[22-25]}\). In comparison, cord blood has been widely used because of its extensive sources, convenient collection and high amplification efficiency \textit{ex vivo}. Matsunaga \textit{et al}\(^{[22]}\) reported that when CB CD34\(^+\) cells were cocultured with hTERT stromal cells and interleukin-11 (IL-11), SCF, thrombopoietin and flt-3 ligand (FL) were included in the system, large-scale generation of human platelets could be attained. Using the UCB differentiation system, scientists have investigated various factors that may influence megakaryopoiesis and thrombopoiesis. Employing UCs depends on donation after childbirth, which makes it more difficult to acquire abundant materials to scale platelet production and increases the risks of bacterial/viral contamination\(^{[26]}\).

\textbf{hESCs}

Since the establishment of the hES cell line for the first time by Thomson \textit{et al}\(^{[26]}\) in 1998, scientists have successively attempted to induce hESCs into MKs and platelets. In 2001, Kaufman \textit{et al}\(^{[27]}\) first reported that when cultured iPSCs derived from human epidermal fibroblasts with a mouse BM stromal cells to produce CD34\(^+\) hematopoietic precursor cells, which can give rise to mixed cell clones containing MKs. This was the first report on the differentiation of hESCs into MKs. Then, Gaur \textit{et al}\(^{[4]}\) reported that hESCs could be differentiated into MKs by culturing them with OP-9 stromal cells. After two weeks, CD41a\(’\)CD42b\(’\) (surface marker expressed by MKs) cells were detected in the differentiation system. However, these MKs did not exceed a ploidy of 32 N, and proplatelet formation was only occasionally observed. These results suggested that the MKs were immature and that platelets were not produced in the culture system. In 2008, successful generation of platelets was addressed in a study by Takayama’s group\(^{[30]}\). Through coculture with OP9 or C3H10T1/2 stromal cells and the use of vascular endothelial growth factor (VEGF), hESCs produced many "sac" structures that could provide a suitable microenvironment for hematopoietic progenitor cells. Thrombopoietin, SCF and heparin were then added to the system. After approximately 10 days, platelets that could be activated by ADP or thrombin were produced in the supernatant. Therefore, the efficiency of platelet production in MKs is far less than that under physiological conditions.

In 2011, Lu \textit{et al}\(^{[7]}\) improved the differentiation efficiency of MKs from hESCs by 20-30 times. The breakthrough of the experiment was that the adhesion and aggregation functions of platelets produced \textit{ex vivo} was proven to some extent. More importantly, the cultured platelets were observed to participate in the formation of thrombosis with platelets \textit{in vivo} at the site of damage in arterial blood vessels. However, the application of stromal cells and serum in previous studies reduced the possibility of use in therapies significantly. Subsequently, Pick \textit{et al}\(^{[34]}\) committed hESCs to MKs that fragmented into platelet-like particles using a "spin embryoid body" method in serum-free differentiation medium. However, this method also has disadvantages, namely system instability and limitations to the large-scale production of MKs.

\textbf{hiPSCs}

Compared with hESCs, hiPSCs have several advantages, including the ability to utilize any somatic cell that could develop into MKs and the absence of ethical restrictions. In 2010, Takayama \textit{et al}\(^{[30]}\) first reported that when cultured iPSCs derived from human epidermal fibroblasts with a mouse C3H10T1/2 stromal cell line for 22-26 d, platelets could be released from MKs. It is noteworthy that the platelets were observed to form thrombi at the site of damaged vessels in combination with platelets \textit{ex vivo}. In addition, hiPSCs from subcutaneous adipose tissues\(^{[33]}\), endometrial stromal SCs\(^{[31]}\) and adipose-derived mesenchymal stromal/stem cell line\(^{[30]}\) could differentiate into functional platelets.

In addition to the above-mentioned stromal cell coculture methods and traditional "embryoid body" differentiation methods, some strategies of altering cell fate by transcription factors have also made progress. Ono \textit{et al}\(^{[34]}\) reported that overexpression of the p45NF-E2, Maf G and Maf K genes could convert human and mouse 3T3 fibroblasts into CD41\(^+\) MKs, which can produce platelet-like particles with partial coagulation function. Although the method requires much time and the efficiency is low, it showed that exogenous gene manipulation could directly transform other cell types into MKs. The key was to find the appropriate genes. In 2014, Nakamura \textit{et al}\(^{[34]}\) showed that overexpression of the BM11, bcl-xl and c-myc genes in hiPSCs derived from PB cells could generate stable and immortalized megakaryo-cytic progenitor cell lines (imMKCLs). Once the expression of the three genes was stopped, the imMKCLs gave rise to platelets. Similarly, ectopic expression of the three transcription factors GATA1, FLI1, and TAL1 in hiPSCs achieved the same goal, leading to mature MK production with unprecedented efficiency\(^{[4]}\). Both cell lines could be cryopreserved and expanded upon recovery. Compared to MKs derived from iPSCs, the prominent feature of imMKCLs is the generation of platelets with higher efficiency in less time.
Although some characteristics of the imMKCLs deserve recommendation, the cell lines still face some drawbacks, such as relying on serum and feeder cell culture and virus-mediated genetic reprogramming, which may pose potent risks. Recently, important progress has been made by Liu et al., who developed an efficient system to generate MKs from hiPSCs under feeder-free and xeno-free conditions by adding FDA-approved pharmacological reagents, including romiplostim (Nplate, a THPO analog), oprelvekin (recombinant IL-11), and plas-bumin (human albumin). However, platelet production from iPSCs requires relatively complex and sophisticated methods, and the culture period is long. These results may impede the progress of the industrial-scale generation of cultured platelets and therapeutic applications in regenerative medicine.

REGULATION FACTORS FOR MEGAKARYOCYTIC MATURATION AND PLATELET RELEASE

Megakaryopoiesis and thrombopoiesis are regulated by various effectors in the BM microenvironment, such as stomal cells, cytokines, extracellular matrix and blood flow. Based on theoretical knowledge, researchers have altered the culture conditions, including chemical substances, and the physical microenvironment, which may promote the differentiation and maturation of MKs and accelerate the release of platelets.[14,36,37]

Megakaryocytic maturation

It is generally recognized that the ploidy of MKs is positively correlated with the number of platelets produced. Studies have shown that MKs differentiated from CB, hESCs or hiPSCs have a far lower ploidy level than MKs generated from BM HSCs,[38,39] and the reason has not been fully elucidated. Insights into the signaling pathways associated with megakaryocytic ployploidy may contribute to our understanding and result in the production of more platelets ex vivo through gene manipulation.[40,41]

The Rho/ROCK pathway is a well-known regulator of the actin cytoskeleton. Chang et al.[42] provided evidence that the pathway acts as a negative regulator of proplatelet formation. Overexpression of a spontaneously active RhoA or dominant-negative mutation led to an increase or a decrease in proplatelet formation. The application of the Rho inhibitor Tat-C3 and the ROCK inhibitor Y27362 promoted the proportion of polyploidy MKs and the formation of proplatelets by decreasing myosin light chain 2 (MLC2) phosphorylation. Another study showed that ROCK inhibition drives polyploidization and proplatelet formation in MKs maturation through downregulation of NFE2 and MYC expression.[43]

The tumor suppressor protein P53 plays an important role in regulating the cell cycle and apoptosis.[44] P53 activation inhibits the induction of hematopoietic progenitors and of MKs.[45] Fuhrken et al.[46] found that in the differentiation of the megakaryocytic cell line CHRF-288-11 induced by PMA, reducing the level of P53 improved the proportion of polyploid MKs. In addition, BM HSCs from P53-/- mice can generate 64 N MKs, while the maximum ploidy of wild-type mice is 32 N. Later, Giammona et al.[47] showed niacinamide (NIC) can promote polyploidyization of MKs by inhibition of SIRT1 and/or SIRT2, which belongs to the histone deacetylase Sirtuins protein family. The function of SIRT1 and SIRT2 is to deacetylate the downstream target protein P53. Therefore, the role of NIC in promoting polyploidization may be related to the enhanced transcriptional activity of P53 after acetylation.

Lannutti et al.[48] found that Fyn and Lyn, members of the Src family of protein kinases, were highly expressed during the differentiation of BM CD34+/CD38- cells to MKs. Lyn-deficient mice produced more mature MKs above 8 N in the presence of the Src kinase inhibitor pyrrolopyrimidine 1. In addition, the Src kinase inhibitor SU6656 increased the proportion of polyploid MKs in the differentiation of cell lines UT-7, HEL, and Meg-01 and in patients with myeloid dysplasia syndrome.[49,50]

In addition, some other important molecules, such as DIAPH1 and Gfi1b, also regulate major functions of MK proplatelet formation by controlling the dynamics of the actin and microtubule cytoskeletons.[51,52]

Platelet release

Currently, only a few MKs cultured ex vivo can release platelets. Therefore, it is essential to improve the ability of MKs to produce platelets ex vivo. Factors related to platelet release include the extracellular matrix, blood flow shear force, and MK apoptosis.

There has been evidence that the apoptotic inhibitory proteins Bcl-2 and Bcl-xl are
expressed in the early phase of megakaryopoiesis, but Bcl-2 is absent in neonatal and mature platelets. Moreover, the activity of caspase-3 and caspase-9 are increased in terminally differentiated platelets, suggesting that the maturation of platelets is accompanied by apoptosis\(^\text{[20]}\). However, Josefsson et al\(^\text{[20]}\) found that the platelet numbers in Bax and Bak knockout mice were normal. It seems that platelet production is independent of intrinsic apoptotic pathway activation. In addition, White et al\(^\text{[21]}\) reported that the number and function of platelets in Casp9\(^\text{-/-}\) mice was not affected. Therefore, whether MK apoptosis affects platelet release remains to be investigated.

The extracellular matrix can modulate the production of platelets. Type I collagen in the osteoblast niche may inhibit the formation of platelets by α5β1α4β3. Fibroblast growth factor-4 (FGF-4) enhances the adhesion between MKs and vascular endothelium, contributing to the survival and maturation of MKs. By chemotaxis of stromal cell-derived factor 1 (SDF1), MKs migrate to the vascular niche and release platelets. In addition, von Willebrand factor (vWF) and fibrinogen play a role in promoting the generation of platelets through GPIb-IX-V and αIIbβ3 signaling pathways, respectively\(^\text{[68-71]}\). On the other hand, the podosome could adhere to extracellular matrix substrates and degrade it, which might play a role in proplatelet arm extension or penetration of the basement membrane\(^\text{[69]}\).

Mechanic stress is an important factor in platelet release from mature MKs\(^\text{[65,66]}\). Junt et al\(^\text{[63]}\) observed the process of MKs interplaying with blood vessels to produce platelets in real time by multiphoton intravital microscopy. In addition, confocal and electron microscopy after fixation were used. They found that MK exposure to high shear rates promotes platelet production via GPIb, which depends on on microtubule elongation and assembly. The results provided a theoretical foundation for the application of bioreactors. Dunois et al\(^\text{[62]}\) suggested that high shear rates from blood flow promote platelet production via GPIb, which depends on micropodum and assembly. Recently, Ito et al\(^\text{[60]}\) reported that turbulence activates platelet biogenesis and that turbulent flow promotes platelet release from hiPSC-derived MKs, suggesting turbulence as an important physical regulator in thrombopoiesis.

**Platelet function**

For cultured platelets to be considered for clinical application, they must be very close to donor-derived platelets in terms of quantity and quality. To date, cultured platelets \textit{ex vivo} have fragmented function compared with donors, although ultrastructure and surface markers are similar. The testing of platelet function has mostly relied on the measurement of P-selectin exposure and GPIIb-IIIa activation. In general, a large proportion of cultured platelets are expression of activation markers by agonists stimulating such as ADP or thrombin, while we often observed that a state of pre-activation with P-selectin expression in cultured platelets even if it’s in the absence of any agonist and thus show poor transfusion properties\(^\text{[65,66]}\). In addition, platelets generated \textit{ex vivo} can participate in the formation of thrombosis in the site of damage in blood vessels in NOG mice. However, the degree to which these platelets can play an active role in hemostasis remains to be evaluated.

Ideally, platelets cultured \textit{ex vivo} should be evaluated using a standard platelet aggregation assay that requires a platelet count of at least 100 × 10^9/L, which has not been reported by the research group. Furthermore, the loss of platelet membrane surface glycoproteins such as GPIb is another important factor that interferes with platelet function. The matrix protein metalloprotease inhibitor GM6001 can prevent GPIba protein hydrolysis by ADAMTS17 and improve platelet function\(^\text{[65,66]}\). Based on the current situation, platelets collected \textit{ex vivo} have at least some physiological functions, but avoiding self-activation in culture is a difficult challenge.

**PROGRESS AND FUTURE DIRECTIONS IN PLATELET BIOREACTORS**

Compared with platelets in PB, the platelets produced \textit{in vitro} were more heterogeneous in size and were produced at a lower output. One explanation is the static quality of cell culture, which is unlike the microenvironment \textit{in vivo}. A reasonable approach for large-scale platelet production is to combine bioreactors with chemical factors. Different bioreactors recapitulate different physiological conditions (Table 1), including gas exchange, media perfusion\(^\text{[67]}\), extracellular matrix proteins, scaffold composition and the effects of blood shear stress\(^\text{[65,66]}\).

In 2003, Li et al\(^\text{[72]}\) first discovered that a murine CCE ES cell line could be used to drive hematopoietic cells in a 3D fibrous matrix to direct hematopoietic differentiation using specific cytokines and inhibitors. In 2009, Sullenbarger et al\(^\text{[62]}\) reported that a 3D bioreactor with surgical-grade woven polyester fabric or purpose-built hydrogel
Table 1  Some major advances in bioreactors in platelet formation and platelet release from 2013 to 2018

| Type of bioreactor                           | Principles and methods                                                                 | Designers / users    |
|----------------------------------------------|----------------------------------------------------------------------------------------|----------------------|
| Two-directional flow bioreactor              | The bioreactor consisted of two-directional flow, in which the angle between the directions of the main and pressure flow is 60 degrees | Nakagawa et al[64], 2013 |
| Microfluidic platelet bioreactor             | The bioreactor is based on polydimethylsiloxane bonded to glass slides to construct some upper and lower microfluidic channel | Thon et al[74], 2014 |
| Spinning-membrane filtration device          | Separating in-vitro-derived PLTs and recovering immature MKs and the precursor cells of PLTs by use of spinning-membrane filtration device | Schlinker et al[77], 2015 |
| RCCS bioreactor                              | Shear force, simulated microgravity, and better diffusion of nutrients and oxygen from the RCCS | Yang et al[67], 2016 |
| Innovative bioreactor                        | The bioreactor consisted of a membrane, and using flow through the membrane and shear across the membrane to drive the megakaryocytes to release PLTs | Avanzi et al[61], 2014 |
| Microfluidic device                          | This device consists in a wide array of von Willebrand factor-coated micropillars, allowing them to remain trapped and subjected to hydrodynamic shear | Blin et al[75], 2016 |
| Turbulent flow-based bioreactor              | Stimulation with optimized shear stress and turbulent energy, collaborates with several growth factors for proplatelet formation | Ito et al[69], 2018 |

RCCS: Rotary cell culture system.

scaffolds could facilitate platelet output when coated with THPO with/or fibronectin. Subsequently, Lasky et al[43] designed the bioreactor by optimizing oxygen concentrations and media perfusion to promote platelet output, but they did not consider blood shear stress. Later, Nakagawa et al[64] developed a two-directional flow bioreactor and found that two flows in different directions could promote platelet production by as much as 3.6-fold compared with static cultures. In 2014, Thon et al[74] made a microfluidic platelet bioreactor that attempted to use biomimetic BM and blood vessel microenvironments and supported live imaging for platelet generation. Blin et al[75] built on previous work, reporting a bioreactor consisting of a wide array of vWF-coated micropillars to act as anchors on MKs. MKs were anchored and subjected to shear stress. Elongation of MK cytoplasm and proplatelet formation were observed. However, these devices have mostly focused on the development of proof-of-concept basic research, low throughput, custom-made tools.

Based on the concept that human MKs are partly regulated by the extracellular matrix, scientists began to design bioreactors with different materials to emulate BM physiology. To increase platelet production, Pallotta et al[67,76] applied biocompatible silk microtubes with fibrinogen, type 1 collagen, and SDF1, mimicking the release of platelets in a blood vessel. The MKs extended proplatelets through the micropores of the microtube and released platelets when exposed to low shear stress. Additionally, silk sponges[46], polycarbonate filter membranes[77] or the hyaluronan-based hydrogels[78] were also used to mimic the bioengineered 3D BM environment, while there are major limitations to implementing the material in biomedical devices.

The combination of bioreactors and chemical factors might promote megakaryo/thrombopoiesis. Recently, we used the rotary cell culture system (RCCS), a unique 3D cell culture method, to investigate the potential role in megakaryopoiesis. Our results indicated that RCCS significantly improves the efficiency of platelet generation, which recapitulates some special characteristics, including shear force, simulated microgravity, and better diffusion of nutrients and oxygen. Additionally, we demonstrated that RCCS combined with chemical compounds and growth factors identified via small screening can further increase platelet generation efficiency[64], while there is limitation to lacking the function of automatic change of medium. Analogously, a novel bioreactor with a membrane and three ports of input and output was developed and can produce a high number of platelets from UCB-derived CD34+ cells. Based on in vivo imaging, Ito et al[69] found that turbulence is an important physical regulator of thrombopoiesis. They developed a turbulent flow-based bioreactor (VerMES Bioreactor), which enabled high yield and quality ex vivo biogenesis from imMKCLs. In addition, three novel chemical factors (IGFBP2, MIF, and NRDC) that contribute to the remodeling of mature MKs and shedding of
platelets might represent a potential mechanism to promote proplatelet shedding in the VerMES bioreactor\textsuperscript{10}. Thus, next-generation platelet bioreactor need to be designed with comprehensive consideration fluid dynamics modeling, automation of flow control and avoid the too highly cost of device design and cell culture.

**CONCLUSION**

As the demand for platelet transfusions increases, many countries are competing to improve the efficiency of platelet production \textit{ex vivo}. However, the field of megakaryopoiesis and platelet research still faces several limitations, including (1) The massive number of platelets (100-300 billion platelets) needed for one transfusion; (2) the high economic cost for the entire culture period; and (3) the lower viability of \textit{ex vivo} platelets compared to donor-derived platelets, though they express the proper surface marker for platelet function and participate in the aggregation reaction at the site of damage in a mouse model. Optimizing the key steps of megakaryopoiesis and platelet generation may provide a better understanding of the cellular and molecular mechanisms. Introducing SCs into advanced bioreactors and simultaneous exposure to a subset of chemical compounds may synergistically contribute to the production of a large number of platelets for clinical applications. In addition, before clinical application, the platelet function produced \textit{ex vivo} must be defined in detail and fully verified. Collection, cost-effective and highly controllable strategies and methodologies represent an important step toward large-scale platelet production for future biomedical and clinical applications.

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