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Stem cells, megakaryocytes, and platelets

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Abstract

Purpose of review—Stem cells are an important tool for the study of ex-vivo models of megakaryopoiesis and the production of functional platelets. In this manuscript, we review the optimization of megakaryocyte and platelet differentiation and discuss the mechanistic studies and disease models that have incorporated stem cell technologies.

Recent findings—Mechanisms of cytoskeletal regulation and signal transduction have revealed insights into hierarchical dynamics of hematopoiesis, highlighting the close relationship between hematopoietic stem cells and cells of the megakaryocyte lineage. Platelet disorders have been successfully modeled and genetically corrected, and differentiation strategies have been optimized to the extent that utilizing stem cell-derived platelets for cellular therapy is feasible.

Summary—Studies that utilize stem cells for the efficient derivation of megakaryocytes and platelets have played a role in uncovering novel molecular mechanisms of megakaryopoiesis, modeling and correcting relevant diseases, and differentiating platelets that are functional and scalable for translation into the clinic. Efforts to derive megakaryocytes and platelets from pluripotent stem cells foster the opportunity of a revolutionary cellular therapy for the treatment of multiple platelet-associated diseases.

Keywords

cellular therapy; disease modeling; megakaryopoiesis; platelets; stem cells

INTRODUCTION

Stem cell-derived megakaryocytes and platelets are an important tool for the study of megakaryopoiesis, modeling of related disease, and incorporation into cellular-based therapies. Thrombocytopenia, for example, is a disorder marked by deficiencies in platelet number and function that can be caused by multiple genetic and epigenetic determinants.

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Conflicts of interest

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Thrombocytopenic patients suffer from prolonged bleeding episodes that, in some cases, are only ameliorated by bone marrow transplantation or platelet transfusion. As a result, and in conjunction with limitations surrounding platelet donation and abbreviated shelf-life, in-vitro derived platelets represent a transfusion technology of limitless potential. Particularly with respect to induced pluripotent stem cells (iPSCs), platelets can potentially be generated in a patient-specific manner that avoids concerns related to contamination, immunogenicity, or supply.

This review will outline the most recent efforts to optimize in-vitro megakaryocyte and platelet generation using stem cell technology. We will highlight studies that utilize stem cell modeling to further encapsulate the behavior and regulation of hematopoietic stem cells (HSCs), megakaryocyte progenitors, and terminally differentiated megakaryocytes and platelets. Finally, we will discuss significant strides in the modeling and genetic correction of platelet-related disorders and explore the potential for cellular therapeutics adapted from ex-vivo derived material (Fig. 1 [1▪▪–4▪▪,5▪,6▪▪–9▪▪,10▪,11▪, 12▪▪–14▪▪]).

NOVEL DISCOVERIES FROM STEM CELL MODELS OF MEGAKARYOPOIESIS

Megakaryocyte and platelet derivation from pluripotent stem cells and circulating hematopoietic progenitors has led to important work that sheds light on basic biological questions concerning stem cells of the hematopoietic system and progenitors of the megakaryocyte lineage. These studies are crucial for the optimization of ex-vivo platelet generation in that they offer new molecular targets that can be harnessed to promote the recapitulation of in-vivo thrombopoiesis.

Hematopoietic stem cells and megakaryopoiesis

As efforts mount to optimize megakaryopoiesis *ex vivo*, similarities in the mechanisms of HSC and megakaryocyte maintenance and expansion are of particular interest. Thrombopoietin (TPO), the major regulating cytokine of megakaryopoieisis, was first implicated in HSC homeostasis through studies of knockout mice for the TPO receptor (*mpl*) that identified deficiencies in the capacity of *mpl* null bone marrow to achieve long-term hematopoietic reconstitution in irradiated recipients [15]. Further work proved that TPO/MPL signaling was crucial for the maintenance and expansion of quiescent long-term HSCs [16]. The field continues to expand upon these concepts, providing evidence that megakaryocytes directly regulate HSC homeostasis and challenging what is known about hematopoietic ontogeny. Indeed, a subset of HSCs are now known to express the gene for Von Wille-brand’s factor, a platelet-associated peptide once thought to be restricted to the megakaryocyte lineage. These cells produce greater transcript levels of *C-mpl* and are primed for megakaryocyte lineage commitment [17▪▪]. This population is actively regulated by TPO and may precede all other HSC subsets, a finding that provides greater insight into the role of megakaryocyte-associated cytokines and HSC maintenance. Additionally, recent studies show that transplanted HSCs preferentially home to adjacent megakaryocytes within the endosteal bone marrow niche, in which TPO promotes niche expansion [18▪], and mature megakaryocytes release cytokines to promote HSC proliferation [19▪]. Evidence now

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exists for a myeloid-restricted progenitor that may be a direct descendant of the HSC, completely bypassing the oligopotent progenitor thought to be a crucial intermediary of normal hematopoiesis [20▪]. This population may descend from CD41+ HSCs, recently discovered to be more entrenched and less transient than once thought [21▪]. These discoveries are part of a growing inquisition of hematopoietic hierarchy, and they reveal that progenitors are significantly plastic with respect to lineage commitment [22]. Thus, the megakaryocyte lineage and its associated cytokines may be the primary regulator of HSCs, and platelet-biased HSCs and myeloid progenitors may confer a rapid proliferative response for platelet reconstitution following acute stress.

Cytoskeletal regulation of megakaryocyte-lineage commitment and terminal maturation

Multiple studies are incorporating stem cell technology to study the role of cytoskeletal dynamics in megakaryocyte development. P-TEFb, a kinase classically associated with cytoskeletal regulation, has been found to upregulate a discrete subset of actin-associated cytoskeleton remodeling factors in a Calpain 2-dependent mechanism that is dysregulated in patients with Gata1 mutations associated with megakaryocytic leukemias [23▪]. Actin polymerization, in turn, has been found to influence megakaryocyte transcriptional patterning by promoting a serum response factor (SRF) transcriptional coactivator (MKL1) to translocate to the nucleus in TPO-stimulated primary megakaryocytes [24▪]. Myosin II, the main driver of cytokinesis, has been shown to exist as different isoforms throughout hematopoietic differentiation [25▪]. Myosin IIa (*Myh9*) is expressed throughout the hematopoietic compartment, whereas Myosin IIb (*Myh10*) is lost as progenitors asymmetrically divide. When both isoforms are inhibited in static culture, megakaryocyte ploidy and platelet generation is greatly increased [25▪]. These results are congruous with previous work suggesting that RUNX1, a major specifying transcription factor of megakaryopoiesis, actively regulates transcript level expression of both *Myh9* and *Myh10* [26]. All of these studies incorporate in-vitro differentiation of pluripotent stem cells or hematopoietic progenitors. In doing so, they provide evidence that cytoskeletal dynamics govern and are governed by transcriptional changes that ultimately control complex, highly ordered processes critical to megakaryocyte and platelet development.

Stem cell-based mechanistic studies of RUNX1-mediated megakaryopoiesis

The role of RUNX1 as a master regulator of megakaryocyte fate has been greatly expanded in the past months. RUNX1 has previously been shown to directly activate megakaryocyte-associated genes, including *Itga2b*, *Gp1ba*, and *C-mpl* [27,28]. Currently, mutations in RUNX1 have been implicated in the cause of Familial Platelet Disorder (FPD) and shown to abrogate platelet factor 4 (PF4) expression in an embryonic stem cell (ESC) differentiation system, providing a plausible mechanism for the FPD phenotype [29▪]. A related study shows that RUNX1 reversibly suppresses genes associated with terminal differentiation through its interaction with protein arginine methyltransferase 6 (PRMT6) in megakaryocyte progenitors derived from mobilized peripheral human CD34+ primary cells [30▪▪]. In this work, RUNX1 was found to bind to the *Itga2b* locus in undifferentiated precursors and to initiate histone H3 arginine-2 dimethylation (H3R2me2a) to suppress transcription prior to differentiation. Upon induction of differentiation, RUNX1 binding persists but PRMT6 gradually dissociates and the H3R2me2a mark is lost concomitant with an increase in

histone H3 lys4 trimethylation (H3K4me3) and subsequent transcriptional activation [30▪▪]. These studies highlight the importance of RUNX1 in the active differentiation of megakaryocytes and reveal a temporally regulated, RUNX1-dependent repression of megakaryocyte-specific genes in immature progenitors.

Recent discoveries concerning megakaryocyte signaling pathways

Studies that utilize stem cell differentiation to the megakaryocyte lineage continue to reveal the complexity of megakaryocytes by implicating various signaling pathways in their differentiation and function. Multiple studies have uncovered dysregulated signaling that may reveal novel regulators of megakaryopoiesis. These factors include, but are not limited to, platelet endothelial aggregation receptor-1 (PEAR-1), a stimulator of PI3K/PTEN signaling [31▪], and RAD001, an mTOR inhibitor currently used as a chemotherapeutic and immunosuppressant [32▪]. Wnt3a has also been implicated as a repressor of human megakaryocyte progenitor expansion in an in-vitro iPSC derivation system that causes robust production of CD41/CD235 dual positive progenitors [7▪▪]. This finding is in direct conflict with studies of Wnt3a signaling in the murine system [33], thus highlighting the importance of incorporating human models of hematopoietic differentiation. Our group, incorporating a similar iPSC-based strategy, has discovered a novel role for the aryl hydrocarbon receptor (AHR) in the regulation of in-vitro megakaryopoiesis [8▪▪] that is seemingly independent of its canonical role in environmental carcinogenesis [34]. Precipitated upon findings that implicate the AHR in megakaryocyte polyploidization and platelet function [35], we found that treatment with an endogenous agonist of the AHR significantly expanded megakaryocyte progenitors and promoted long-term viability in static culture. In these studies, AHR repression in the established progenitor pool resulted in terminal maturation as assessed by megakaryocyte polyploidy and platelet production, suggesting a role for the AHR in normal hematopoietic development. The above studies demonstrate that multiple signaling pathways impact normal and abnormal megakaryopoiesis.

Unraveling mechanisms of platelet production using stem cell-based platforms

The culmination of megakaryocyte development is the highly ordered process of cytoplasmic compartmentalization and platelet generation. As evidence mounts to substantiate the pro-platelet theory of thrombopoiesis [36], more work has been dedicated to characterizing the bone marrow microenvironment that serves as the site of pro-platelet extension. A recent work examined the distribution of extracellular matrix components resident to bone marrow sinusoidal endothelium and found that type IV collagen, fibronectin, and laminin predominate [37▪▪]. TPO-directed differentiation of harvested HSCs was enhanced in the presence of these factors as they were found to promote megakaryocyte specification and, separately, increase proplatelet formation in fetal liver-derived megakaryocytes. Additionally, all three of these extracellular matrix components were found to be upregulated in bone marrow resident megakaryocytes in times of acute stress (i.e., thrombocytopenia and bone marrow depletion), suggesting that megakaryocytes contribute to the reconstitution of the vascular niche under extreme conditions. This work is congruent with reports that suggest sinusoidal homing of bone marrow resident megakaryocytes is crucial for both proplatelet release and megakaryocyte maturation [38].

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LATEST INNOVATIONS IN STEM CELL DIFFERENTIATION TO MEGAKARYOCYTES AND PLATELETS

Although previous in-vitro differentiation strategies have been limited by the incorporation of xenogeneic stromal feeder layers and bovine sera [39,40], current work emphasizes the use of chemically defined conditions that promote reproducibility, scalability, and clinical viability. Recently, a three-dimensional embryoid body-based system has been reported which is able to recapitulate megakaryocyte specification and platelet derivation and function without the use of bone marrow-derived cell lines [10▪]. Megakaryocytes were reported to reach ploidy of 16N and generate platelets with intact functionality as assessed by induced P-selectin surface expression. Additionally, platelet induction has been reported from canine iPSC-derived megakaryocytes with some functional relevance *in vitro*, suggesting that the canine system may provide another invaluable model for platelet biogenesis [41▪]. Nonetheless, the major hurdle in ex-vivo platelet derivation, the production of clinically relevant quantities, remains elusive.

Immortalization of megakaryocyte progenitors

An advance for ESC-derived megakaryocytes involved the production of ‘ES-sacs’ consisting of hematopoietic progenitors surrounded by hemogenic endothelium [40]. In the past year, the same group [9▪▪] optimized this strategy through forced expression of immortalization factors, effectively creating immortalized megakaryocyte progenitors (imMKCLs) from ESCs and iPSCs. Drawing upon their previous report of the role of c-MYC in megakaryopoiesis [42], they transduced megakaryocyte progenitors with *Myc* to promote progenitor expansion, along with *Bmi1* to suppress senescence and *Bcl-xl* to prevent apoptosis. imMKCLs were readily expanded and cryopreserved with the ability to thaw for later use, an unprecedented innovation that may be of vital importance for translating this technology to the clinic. Importantly, inducible suppression of the three-immortalization factors promoted terminal megakaryocyte differentiation and the production of agonist-responsive platelets.

Emulation of bone marrow microenvironments

With the growing emphasis on recapitulating the bone marrow niche during in-vitro megakaryopoiesis, technical advances have been made to allow for manipulation of multiple parameters (i.e., pH, pO_2, and shear stress) that may provide optimal conditions for high yields of functional, inactivated platelets. Using human CD34+ peripheral blood cells as starting material, an elegant optimization strategy was published which screened for various cytokine cocktails and other complex culture conditions [11▪]. In this study, it was found that a three-phase culture system that steadily increases pH and pO_2 is optimal for generating polyploid megakaryocytes and promoting platelet generation [11▪]. Additionally, the group that developed the aforementioned imMKCLs posited that megakaryocytes derived from immortalized progenitors could be incorporated into a dynamic culture system that they had reported on earlier in the year [12▪▪]. A bioreactor system was developed which utilizes microfluidic flow, a technique precipitated by studies showing shear stress as an inducer of platelet production *in vivo* [43]. A microfluidic chamber was generated by way

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of a wax mold made porous through salt leaching. This chamber was designed to mimic the porous bone marrow microvasculature to which megakaryocytes home and extend proplatelets into circulation. As such, the authors seeded the porous structure with human umbilical cord vascular endothelial cells and introduced bidirectional flow. The resultant platelet yield was significantly greater than that of static culture, and the derived platelets were agonist responsive as assessed by integrin activation. Taken together, current optimization strategies for high-yield platelet production incorporate immortalized progenitors and recapitulate the bone marrow microenvironment to mimic in-vivo thrombopoiesis.

Clinical barriers for stem cell-derived platelets

With the unprecedented expansion of pluripotent stem cell-derived platelets recently reported in the literature [9▪▪,10▪,12▪▪], it is seemingly a foregone conclusion that these cells will one day be of great clinical importance. Currently, there remain significant issues throughout the field that must be addressed before these cells can be relied upon as a safe alternative to existing treatments. Although this notion has become controversial in recent years, it is typically accepted that adult megakaryocyte ploidy is correlated with terminal maturation and efficient platelet production. While human megakaryocytes can generate nuclear DNA content of 128N [44], ex-vivo differentiation strategies of ESCs and iPSCs cannot reliably produce cells of greater than 32N [39]. To make a platelet yield that can be scaled for clinical transfusion, the platelet number per megakaryocyte must be demonstrably greater than what can currently be achieved, and doubt still exists as to whether the inability to produce high ploidy megakaryocytes *ex vivo* contributes to this deficit. Although it is important that derived platelets display a proper morphology and in-vitro functionality, these cells must also avoid inappropriate activation prior to transplantation, a problem that persists with many differentiation strategies. Platelets are known to be highly susceptible to ex-vivo activation from improperly buffered media, harvesting methods (i.e., centrifugation), and protease activity [45]. Platelet activation that results from long-term storage can lead to decreased recovery and viability in transfused recipients [46]. Thus, stem cell-differentiated platelets must be kept in an inactivated state to be clinically efficacious. Deficiencies in megakaryocyte ploidy and platelet activation must be addressed before derived platelets can be used as cellular therapy.

DISEASE MODELING AND CORRECTION OF PLATELET-RELATED DISEASE

Whereas most cellular therapies require transplantation of nucleate progenitors, platelet transfusion does not introduce genetic material into the recipient. Thus, platelet cultures can be irradiated for purification and safety before transplant, and iPSCs generated specifically for platelet derivation can be genetically manipulated without compromising their proposed therapeutic intent. Disease modeling in the context of platelet disorders becomes particularly beneficial in that patient-specific iPSC generation and subsequent genetic correction could be of immediate clinical benefit.

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Gene therapy for correcting platelet-related disease

Disease modeling and gene correction of Glanzmann thrombasthenia and congenital amegakaryocytic thrombocytopenia (CAMT) were recently reported in the literature [1▪▪, 2▪▪]. CAMT is a disorder caused by mutations in the *C-mpl* gene that lead to loss of TPO/MPL signaling. This disorder is phenotypically similar to *mpl* null mouse models but actually manifests with a more severe pathology of aberrant hematopoiesis and fatal prognosis if not treated by bone marrow transplant [47]. Due to the lack of primary cells, it is extremely challenging to study bone marrow-derived progenitors for a greater insight into the mechanism of action and potential targets of drug therapy. To address this need, iPSCs were created from the dermal fibroblasts of a CAMT patient [1▪▪]. These cells displayed a characteristic inability to differentiate toward the megakaryocyte lineage, and this defect was attributed, in part, to dysregulated expression of FLI1, a megakaryocyte specific transcription factor. Importantly, *C-mpl* retroviral delivery was sufficient to restore megakaryocyte lineage specification of CAMT iPSCs.

Similarly, mononuclear cells from the peripheral blood of two patients with Glanzmann thrombasthenia were transduced to create iPSCs that were subsequently corrected by gene therapy [2▪▪]. Glanzmann thrombasthenia is caused by mutations in integrin αIIbβ3 that lead to problems in platelet aggregation and can cause potentially life-threatening episodes of unsuppressed bleeding. The corrected α*IIb* cDNA was inserted into the 'safe harbor' AAVS1 locus so as to minimize potential endogenous activation and was driven by the *GPIb*α promoter for megakaryocyte-specific expression. Megakaryocyte differentiated cells from the corrected disease lines stained positive for the PAC-1 antibody when treated with platelet agonists, suggesting that αIIbβ3 was present on the cell surface and induced to an activated state. This study is an important first step to elucidating the capacity for gene correction of Glanzmann thrombasthenia in an effort to create iPSC-derived material for syngeneic transplant.

Platelet delivery of disease-ameliorating peptides

As iPSC-derived platelet transfusion becomes a possibility, efforts have increased to use genetically modified platelets as a means for delivery of circulating factors that are deficient in certain diseases. These so-called 'designer platelets' would overexpress factors through lentiviral transduction of ex-vivo derived megakaryocytes and store them in granules for secretion to relevant areas of injury. Hemophilia is an attractive candidate for this intervention as it is caused by a clotting factor deficiency and is directly associated with platelet function. Using a factor IX (FIX) null mouse model of hemophilia B, it was recently demonstrated that platelet promoter-driven FIX (*2bF9*) transduced HSCs could repopulate the vasculature with FIX expressing platelets in *2bF9* null mice following HSC transfusion [3▪▪]. A similar study was conducted with factor VIII (FVIII) expression vectors for possible hemophilia A correction, and this study incorporated FVIII transduced (*2bF8*) CD34+ human cord blood as transplant material in an immunocompromised mouse model with defective FVIII expression [4▪▪]. In this study, human platelet engraftment peaked at roughly 50% but steadily decreased at later time points. Despite this drawback, FVIII expression was significantly greater in engrafted platelets and completely prevented mortality in a tail clip survival test when individuals had greater than 2% platelet chimerism.

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It should be noted that although immunosuppression was necessary in both studies (the former used irradiation, the latter immunocompromised mice), these papers provide proof-of-concept for platelet delivery of clotting factors that, theoretically, can be adapted to patient-specific iPSC technology for syngeneic transplant [5▪].

Gene therapeutic strategies have recently been adapted for the treatment of Hurler syndrome, a multisystemic disorder caused by a deficiency in α-L-iduronidase (IDUA) that leads to an accumulation of glycosaminoglycans (GAGs) [6▪▪]. Using a mouse model of Hurler syndrome, lentiviral transduction of IDUA into HSCs and subsequent transfusion yielded megakaryocytes that preferentially expressed the transgene and produced circulating platelets with high titers of the active enzyme. These platelets efficiently delivered IDUA to the spleen and liver, in which GAG accumulation was ameliorated in comparison to nontransplanted controls. This study provides further evidence that platelet-dependent peptide production can result in targeted therapy for heritable disease.

CONCLUSION

Stem cell modeling has provided researchers with unprecedented tools to study mechanisms of megakaryocyte development and platelet generation. Through this research, ex-vivo platelet derivation has been increasingly optimized for scalable production and clinical viability. Currently, a phase I trial using megakaryocyte progenitors derived from human umbilical cord blood has reported successful engraftment without noticeable symptoms of graft versus host disease (GVHD) [13▪▪]. Additionally, two groups are embarking on clinical trials of iPSC-derived platelet cellular therapy, with one reporting platelets of comparable "size, structure, morphology, organelles and platelet-specific markers" [14▪▪]. These trials prove that ex-vivo derived platelets, and possibly even megakaryocyte progenitors, have the potential to become the standard of care for thrombocytopenic patients due to their relative safety and efficacy. Widespread acceptance of this treatment modality can foster efforts to transfuse platelets generated from transgenic progenitors that cause upregulation of factors deficient in a variety of diseases. For these reasons, stem cells are ideal for advancing basic scientific discoveries and cellular therapies in the context of megakaryopoiesis and platelet production.

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▪▪ of outstanding interest

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KEY POINTS

- Optimized strategies for megakaryocyte and platelet differentiation give researchers a novel in-vitro tool to uncover mechanisms of megakaryocyte development and platelet generation.
- Platelets differentiated from pluripotent stem cells have the potential to become a well tolerated and efficacious cellular therapy that combats the worldwide shortage in platelet donation.
- Induced pluripotent stem cells can be used to model platelet-associated disease and provide proof-of-principle studies that transgenic replacement can lead to amelioration of disease phenotypes.
- Platelets derived from transgenic stem cells can be used as peptide delivery systems in patients with deficiencies in circulating clotting factors

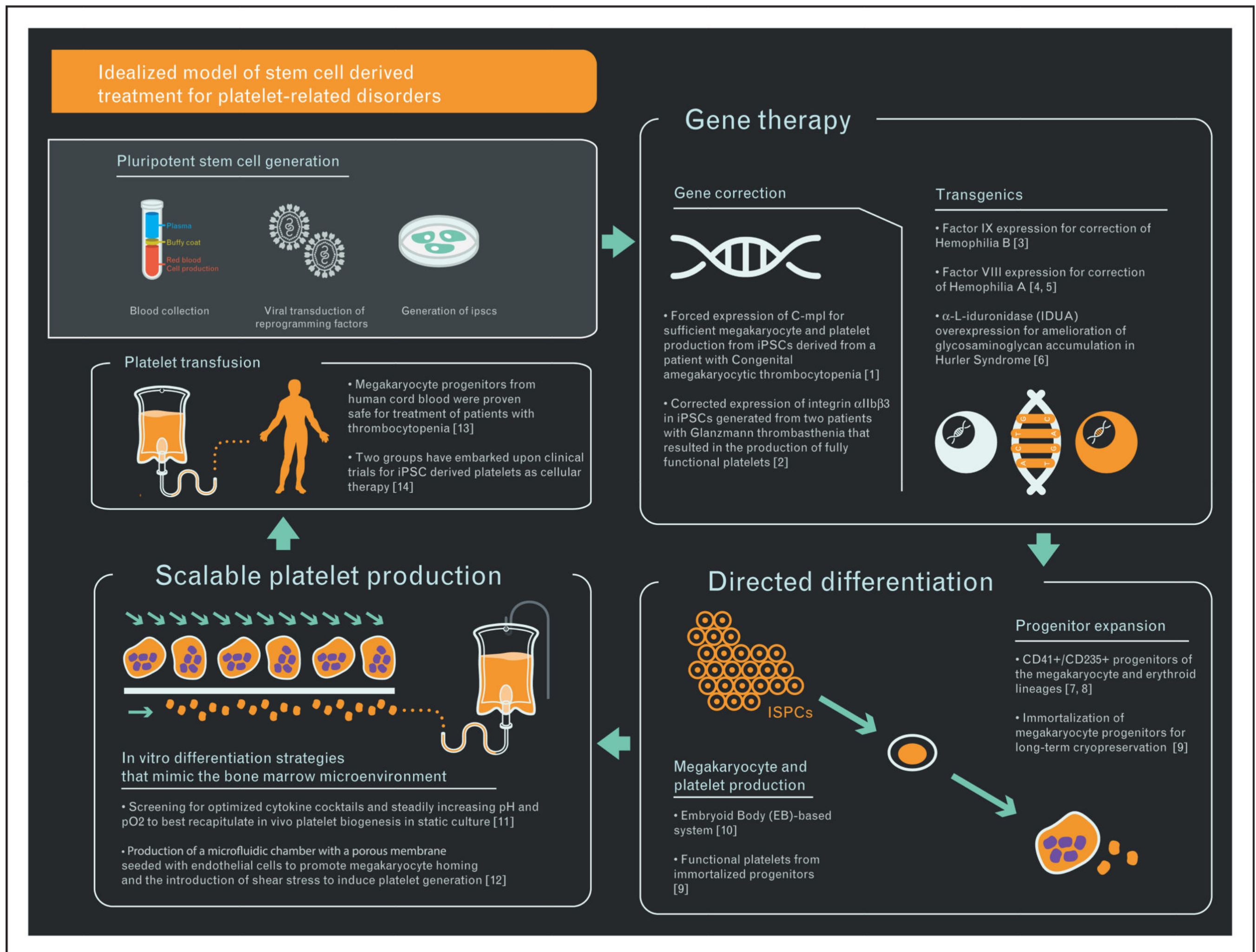

FIGURE 1.

Idealized model of stem cell-derived treatment for platelet-related disorders. Platelets differentiated from iPSCs could be an efficacious cellular treatment derived from patient-specific donor material. iPSCs are made from the peripheral blood of affected individuals. They can then be manipulated by gene therapeutic strategies to correct the disease phenotype by genome editing and expression of the normal disease-specific allele [1▪▪,2▪▪], as well as overexpression of transgenic material [3▪▪,4▪▪,5▪,6▪▪]. Pluripotent cells would then be differentiated into megakaryocyte progenitors [7▪▪,8▪▪] and potentially immortalized via transduction of factors that inhibit apoptosis and senescence [9▪▪]. This would culminate in platelet production, which has been achieved through multiple strategies, including an embryoid body system [10▪] and from immortalized progenitors [9▪▪]. Optimization of static culture conditions [11▪] and adapting platelet differentiation to a bioreactor system [12▪▪] would promote scalable production that could be clinically efficacious. Patients would receive platelet transfusions of iPSC platelets that would be optimized to reduce immune rejection. These clinical efforts are already underway for both transfusion of megakaryocyte progenitors [13▪▪] and platelet transplant [14▪▪]. iPSC, induced pluripotent stem cell.

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