Developments in the production of platelets from stem cells (Review)

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Abstract. Platelets are small pieces of cytoplasm that have become detached from the cytoplasm of mature megakaryocytes (MKs) in the bone marrow. Platelets modulate vascular system integrity and serve important role, particularly in hemostasis. With the rapid development of clinical medicine, the demand for platelet transfusion as a life-saving intervention increases continuously. Stem cell technology appears to be highly promising for transfusion medicine, and the generation of platelets from stem cells would be of great value in the clinical setting. Furthermore, several studies have been undertaken to investigate the potential of producing platelets from stem cells. Initial success has been achieved in terms of the yields and function of platelets generated from stem cells. However, the requirements of clinical practice remain unmet. The aim of the present review was to focus on several sources of stem cells and factors that induce MK differentiation. Updated information on current research into the genetic regulation of megakaryocytopoiesis and platelet generation was summarized. Additionally, advanced strategies of platelet generation were reviewed and the progress made in this field was discussed.

Contents

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1. Introduction

In healthy adults, the mean number of platelets in the blood is maintained at (150-400) x$10^3/\mu$l, with a short life of 7-10 days (1,2). The platelet is an essential blood component that modulates vascular system integrity through coagulation mechanisms, but is also implicated in inflammation and cancer (3,4). Resting platelets are ~3 μm in diameter whilst circulating inside blood vessels (5), and are activated upon contact with the surface of non-vascular intima, such as a surface injury accompanied by exposure of collagen and other agonists (6). The activated platelets then stimulate platelet glycoproteins and release molecules in order to enhance hemostasis (7).

A variety of circumstances, including cancer therapy, trauma, immune disorders, sepsis and inherited platelet defects, may result in thrombocytopenia (8,9). Platelet transfusion efficiently protects patients with severe thrombocytopenia from potentially life-threatening hemorrhage (8). In addition, due to the aging of the population, and the increase in the rate of bone marrow (BM) transplantation (10-12), the demand for platelet transfusion is constantly rising. However, platelet availability is solely donor-dependent at present. The short life of 5 days (13), harsh preservation conditions and high risk of bacterial growth contribute toward the relatively limited supply for clinical use (14,15). Therefore, an increasing number of studies focus on investigating factors that induce megakaryocyte (MK) differentiation and producing platelets from stem cells for clinical application (16,17). With the continuing research, various genetic factors regulating megakaryocytopoiesis and platelet generation are becoming increasingly elucidated and initial success has been reported (13,18).

In addition to the urgent demand of platelets for clinical use, there is a serious problem among patients who require repeated transfusions, as they often develop platelet transfusion refractoriness (PTR) associated with alloimmunization (19). These patients not only face a marked increase in the costs of healthcare, but also have higher morbidity and mortality rates (20). Furthermore, adverse events may occur in transfusion recipients. *Ex vivo* generation of platelets in the laboratory (21) may solve this problem. The findings of recent studies may enable platelet production on a large scale

under specific conditions (22-24). However, the clinical supply remains high.

Achieving highly efficient production of platelets *ex vivo* in the BM is a challenging task. A more detailed understanding of the process from platelet formation to release is urgently required. On this basis, the present review is focused on platelet development from stem cells.

2. Stem cell sources

Various types of stem cell may produce platelets, each with specific advantages and disadvantages in terms of scalability, platelet function and other aspects (25). Therefore, these must be taken into consideration during platelet production. Hematopoietic stem cells (HSCs) are a well-known traditional stem cell source. However, they are not widely used due to their limited numbers. It has been demonstrated that induced pluripotent stem cells (iPSCs), human embryonic stem cells (hESCs) and other stem cell sources, may overcome the shortcomings of HSCs (26), thereby providing a novel approach to large-scale platelet production (Table I). All these types of cell are briefly described in the present review.

HSCs. HSCs, also known as $CD34^+$ cells, are the simplest stem cell source for generating platelets. At present, HSCs are mainly obtained from the BM, umbilical cord blood (UCB) and peripheral blood (27,28).

UCB-derived HSCs typically have a higher ability to proliferate compared with the other two types of cells (29). However, a previous study demonstrated that UCB-derived $CD34^+$ cells were difficult to mature fully, with <10% MKs induced from UCB identified as polyploid after one week of thrombopoietin (TPO) induction (30). Current UCB availability is also limited.

Compared with BM and UCB, peripheral blood is easier to obtain and the procedure is less invasive. An increasing number of studies preferentially use peripheral blood as a source of HSCs (31,32). Following procurement, HSCs may be isolated and further cultured into MKs. However, the main problem with peripheral blood is that HSCs are rare (<0.1% of all nucleated cells in the circulation), resulting in an insufficient yield of MKs (33-35). In addition, cell culture is very slow and labor-intensive for large-scale production (36).

iPSCs. iPSCs are created by artificially inducing non-pluripotent cells to express specific genes (37). Due to the limited availability of HSCs, further studies on iPSCs have been undertaken. Over the past years, iPSCs have shown great potential in biomedical research (38,39).

Feng *et al* (40) created a three-step protocol to generate MKs and functional platelets from iPSCs in a scalable manner within 20 days. In addition, the MK progenitors produced with their method may be stored at low temperatures and proliferate rapidly within a short time. Further analyses demonstrated that iPSC-derived platelets exhibited no major differences with platelets in the circulation. Subsequently, iPSC-derived platelets without major histocompatibility antigen (HLA) were successfully generated by knocking out the β2-microglobulin gene (40). Another study generated a stable HLA-universal iPSC line by silencing the expression of HLA class I up to 82% successfully (41). This HLA-universal iPSC line was able to renew MKs and functional platelets with low immunogenicity. More importantly, iPSC-derived HLA-universal MKs had the ability to escape antibody-dependent cell-mediated cytotoxicity and produce platelets for transfusion. Once the HLA-universal MKs and platelets are efficiently generated, the problem of PTR and limited platelet supply may be resolved.

hESCs. hESCs are primitive pluripotent stem cells derived from a human blastocyst inner cell mass. They can be propagated indefinitely *in vitro*, providing an ideal unlimited source for large-scale production of platelets (2). Several methods for differentiating hESCs into MKs have been developed over time (42). Gaur *et al* (42) demonstrated that hESC-derived $CD41^+/CD42^+$ MKs expressed von Willebrand factor and released functional platelets, but the final number of platelets was small. Subsequently, different culture approaches were described to improve the platelet yield. Lu *et al* (2) achieved 60 MKs per starting hESC with a feeder-free hESC culture approach. These hESC-derived platelets were demonstrated to have the same characteristics of platelets in the blood. Recently, the TPO gene was inserted into the adeno-associated virus integration site 1 locus of the hESC genome (43), creating cell lines stably expressing and secreting TPO. As a result, the production of hESC-derived platelets was increased, and the function of these platelets was comparable with that in the peripheral blood.

Adipose tissue-derived stromal cells (ASCs). With further studies, more strategies for producing platelets have been designed for clinical use (13). ASCs represent an attractive choice for platelet production *in vitro*. As ASCs contain certain essential genes indispensable for MK differentiation and platelet production, they can differentiate without gene transfer (44). Furthermore, ASCs may secrete endogenous TPO that promotes platelet production (45). Tozawa *et al* (46) reported a manufacturing system for platelets from the ASC line (ASCL). In the aforementioned study, ASCs were cultured in MK lineage induction media. At day 8 of culture, the maximum number of ASCL-derived MKs was achieved, and ASCL-derived platelets were obtained, with a peak at day 12 of culture. The inspection results of platelet-related functions are satisfactory.

3. Gene regulation during megakaryopoiesis

MKs are considered to be the progenitors of platelets (47). Multiple extrinsic and intrinsic signaling pathways are involved in megakaryopoiesis, but this process is ultimately under the control of transcription factors (48), including GATA binding protein 1 (GATA-1), friend of GATA-1 (FOG-1), friend leukemia virus integration 1 (FLI1) and runt-related transcription factor 1 (RUNX1).

GATA-1 has been reported to be a key factor during MK differentiation and maturation. GATA-1 can recruit different co-regulators to chromatin in order to participate in the process of megakaryopoiesis (49). Orkin *et al* (50) observed that MKs with GATA-1 knockout required a longer time to mature and exhibited marked hyperproliferation; platelet yield was lower compared with normal platelets *in vivo* as well.

Table I. Different stem cell sources for platelet production.

First author, year	Stem cell resource	Original resource	Difficulty of collection	Advantages	Disadvantages	(Refs.)
Pineault *et al*, 2013; Gertz *et al*, 2018; van den Oudenrijn *et al*, 2000; Nurhayati *et al*, 2015; Six *et al*, 2019; Choi *et al*, 1995; Veljkovic *et al*, 2009; Cohen *et al*, 2013; Pecci *et al*, 2009; Ivetic *et al*, 2016	Hematopoietic stem cell	Cord blood	Hard	Non-invasive acquisition process and relatively short differentiation time	Donation-dependent and poor expansion rate	(27-36)
Orban *et al*, 2015; Sinnecker *et al*, 2013; Heazlewood *et al*, 2017; Feng *et al*, 2014; Borger *et al*, 2016	Induced pluripotent stem cell	Somatic cells	Medium	Wide cell resource, unlimited expansion rate and non-invasive acquisition process	Long differentiation time	(37-41)
Lu *et al*, 2011; Gaur *et al*, 2006; Zhang *et al*, 2018	Human embryonic stem cell	Embryo	Very hard	Unlimited expansion rate	Very limited cell resource, long differentiation time and ethical issues	(2,42,43)
Matsubara *et al*, 2013; Ono-Uruga *et al*, 2016; Tozawa *et al*, 2019	Adipose tissue-derived stromal cell	Adipose tissue	Easy	Simple acquisition, strong regeneration ability and low aging	A group of mixed cells with different characteristics which requires further research	(44-46)

FOG-1 is a multitype zinc finger protein that can interact with GATA-1 (51). According to specific cell and promoter context, FOG-1 can enhance or inhibit the activity of GATA-1, which is important during MK differentiation (52,53).

FLI1 is an E26 transformation-specific proto-oncogene domain transcription factor. Several studies have demonstrated that FLI1 serves an important role in megakaryopoiesis (54-56) and is a key regulator of megakaryopoiesis, working together with GATA-1 (57). Recently, a study investigating the effect of FLI1 during megakaryopoiesis and platelet biology further elucidated this process (58). iPSCs obtained from a patient with Paris-Trousseau syndrome and a control line with FLI1-knockout were cultured. The results revealed that platelets production was decreased in the two cell lines. Overexpression of FLI1 was shown to increase the yield and functionality of platelets (58).

The transcription factor RUNX1 is pivotal in MK development. For example, Okada *et al* (59) demonstrated that the depletion of RUNX1 in UT-7/GM cells led to overexpression of MK markers; however, cell proliferation was decreased at the same time.

The Tribbles Pseudokinase 3 gene (TRIB3) encodes a pleiotropic protein (60) and further study revealed that it is involved in the regulation of cell differentiation. Butcher *et al* (61) built a cellular model system of hematopoietic lineage differentiation *in vitro*. Results from this model demonstrated that TRIB3 acted as a negative modulator during megakaryopoiesis. In primary hematopoietic cell culture, TRIB3-silencing enhanced MK differentiation. By contrast, overexpression of TRIB3 decreased MK differentiation (61).

C3G, also referred to RAPGEF1, is an activator of Rap1 GTPases. It is involved in platelet activation and several other important biological processes (62,63). Ortiz-Rivero *et al* (64) evaluated C3G function in megakaryopoiesis using a transgenic mouse model. The results indicated that BM cells from transgenic C3G mice exhibited increased CD41 and CD61 expression. Overexpression of C3G also increased the number of $CD41^+$ MKs. Subsequently, three different cell lines were cultured, including K562, human erythroleukemia cell line and DAMI, with overexpression or silencing of C3G or GATA-1. It was observed that GATA-1 promoted C3G expression during MK differentiation (64).

4. Promotion of platelet production

Platelet production takes place under specific conditions in the BM microenvironment, with various chemokines, growth factors, calcium, oxygen and adhesive interactions regulating megakaryocytopoiesis and MK migration (48). Several advances have been made to date in the study of promoting platelet production.

Microenvironment of platelet production. The dimensions, hardness, matrix components and other conditions of the BM microenvironment accurately mediate the effects of environmental factors on platelet production. A variety of associated studies have improved our understanding of this process (65-68).

A three-dimensional environment expands the contact area of MKs with the surrounding environment. Platelet production may be promoted through the interaction of proplatelets with the microenvironment (65,66).

Transient receptor potential cation channel subfamily V member 4, sensitive to ion channels, can trigger calcium influx, β1 integrin activation and internalization, and human Akt phosphorylation in order to promote platelet production (67); this process only occurs when MKs adhere to a softer instead of a harder matrix. Experiments demonstrated that lysyl oxidase (LOX) may modulate the stiffness of the BM matrix via collagen crosslinking (68). Therefore, appropriate conditions, including increased LOX levels and a softer matrix, favor platelet production. Prior to blood cell release in the circulation, the interaction between progenitor cells and the vasculature is crucial (68). In order to simulate the vascular network, a custom perfusion chamber containing a multi-channel lyophilized silk sponge was constructed (68), which increased platelet production efficiently.

Previous studies observed *in vivo* flow dynamics and have conducted a series of experiments (24,69). It was reported that turbulent flow was a crucial physical factor for platelet release (24). Based on this result, a novel bioreactor with a flow chamber and multiple pillars was developed, and further experiments produced a higher number of platelets (69).

Different induction factors of platelet production. The factors most widely used to promote platelet production include interleukin-3 (IL-3), IL-6, IL-9, IL-11 and TPO (70-72).

IL-3, IL-6, IL-9 and IL-11 affect TPO-induced MK production indirectly (70-72). Experiments *in vitro* indicated that the addition of mixed cytokines mentioned above could stimulate platelet production (73-75). IL-3 and TPO act synergistically in promoting MK differentiation (73). In the inflammatory state, IL-6 promotes proplatelet formation by increasing the level of TPO (74). Stem cell factor also plays an important role in promoting cell proliferation in the early stage of MK differentiation (73).

TPO is a major regulator of platelet production and is mainly produced by liver cells in serum (75). TPO combines with Mpl to regulate the differentiation, development, maintenance and proliferation of HSCs and MKs (75). The level of free TPO in the plasma increases when the platelet count decreases, stimulating hematopoietic progenitor cells in the BM to differentiate into MK lines to produce more platelets (76).

Eltrombopag (EP) is a second-generation TPO receptor (TPO-R) agonist that promotes the differentiation and proliferation of MKs and platelet generation. It is combined with the c-Mpl transmembrane area of the TPO-R of MKs, leading to the activation of Janus kinase 2 and tyrosine kinase 2, and phosphorylation of related signaling pathways. All these processes induce proliferation and differentiation of MKs and platelet generation (77,78). All these processes induce proliferation and differentiation of MKs, as well as platelet generation (77). EP was first approved by the Food and Drug Administration for clinical use in 2008. Reported results to date have indicated that thrombocytopenia caused by chronic immune thrombocytopenic purpura (78), severe aplastic anemia (79) and chronic infection with the hepatitis C virus (80) notably improved following administration of EP.

Romiplostim is a synthetic polypeptide that activates downstream signals and stimulates platelet production by binding to the TPO-R on MKs (81). A multicenter study of romiplostim for chemotherapy-induced thrombocytopenia was conducted in solid tumors and hematological malignancies; the results demonstrated that 71% of patients responded to romiplostim and weekly dosing was found to be superior to intracycle dosing (82). An analysis of five clinical trials proved that romiplostim self-administration could achieve 95% response without adverse effects (83). Hosokawa *et al* (84) found that high-dose romiplostim was highly effective in patients with AA who were refractory to EP. Furthermore, sequential therapy with EP followed by romiplostim may further improve the prognosis of patients with AA who are refractory to conventional therapy (84).

5. Conclusions

Significant advances have been made in terms of platelet production from stem cells, and this field is progressing steadily. Different stem cell sources display specific characteristics so that the most appropriate source may be selected based on various requirements. Associated studies on gene regulations, production microenvironment conditions and inducing factors may aid in providing more insight. Further progress must be made in platelet production in order to meet the clinical requirements. More accessible stem cell sources, large-scale platelet production *in vitro*, more effective inducing factors and various other problems remain unresolved. However, despite these challenges, the continuous breakthroughs and developments may overcome these obstacles and achieve the final goal, which will hopefully prolong the life span of more patients in need of platelet transfusion.

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Not applicable.

Authors' contributions

JY was a major contributor in writing the manuscript. JL revised the manuscript. YS was involved in writing the manuscript. BC designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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