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Developments in Artificial Platelet and Erythroid Transfusion Products

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Abstract

Platelet and blood transfusions have vital importance to the lives of many patients. Platelet transfusions are a life-saving intervention by reducing risk of bleeding in thrombocytopenic patients. Due to the short shelf life of platelets and their limited availability, researchers have developed various platelet transfusion production technologies. Understanding the cellular and biophysical mechanisms of platelet release is particularly important for development of platelet transfusion products (PTPs) and to translate them to clinical applications in patients requiring platelet infusion. Similarly, due to donor dependence and increased clinical need of blood transfusions, studies on the erythroid transfusion products (ETPs) have recently gained momentum. This led to development of ETP technologies involving differentiation of stem cells to fully functional erythrocytes *in vitro*. During megakaryopoiesis or erythropoiesis, various stimulatory factors, growth factors, transcription factors, and biophysical conditions have been shown to play a crucial role in the formation final blood products. Thus, understanding of the *in vivo* mechanisms of platelet release and erythrocyte maturation is particularly important for mimicking these conditions *in vitro*. This review focuses on latest and up-to-date information about the innovations in PTP and ETP technologies. We also discuss some of the recent fundamental findings that have changed our understanding of *in vivo* platelet release and blood formation.

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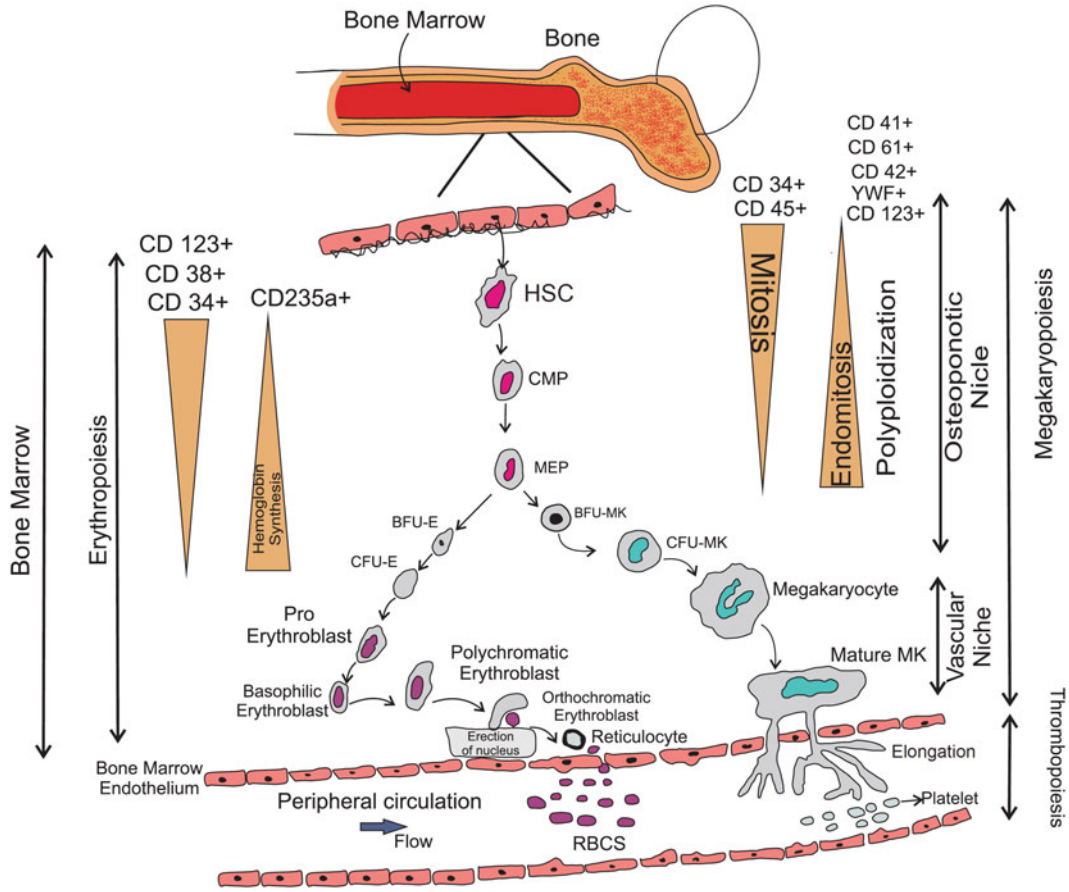
Graphical Abstract



Human bone marrow acts as a source of cells required for erythropoiesis and megakaryopoeiesis. Understanding of molecular mechanism and physiology of these vital and curitial events allowed us to mimic these conditions *ex vivo* and to develop artificial platelet and erythroid transfusion production technologies.

Keywords

Erythroid transfusion products · Erythropoiesis · ETPs · Hematopoietic stem cells · Megakaryocytopoiesis · Megakaryopoeiesis · Platelet transfusion products

Abbreviations

ABI	Aurora B Inhibitor
ADSC	Adipose Derived Stem Cells
BFU-MK	Burst Forming Unit Megakaryocyte
BM	Bone Marrow
CFU-GEMM	Colony Forming Unit – granulocyte, erythrocyte, monocyte, megakaryocyte

CFU-Mk	Colony Forming Unit Megakaryocyte
CMP	Common Myeloid Progenitor
CMPs	Common Myeloid Progenitors
DMS	Demarcation Membrane System
ECM	Extracellular Matrix
ESC	Embryonic Stem Cell
ES-sacs	Embryonic Stem Cell–Derived Sacs
ETPs	Erythroid Transfusion Products
FLI1	Friend Leukemia Integration 1
GATA1	GATA binding protein 1
GPV	Glycoprotein V
HDFs	Human Dermal Fibroblasts
hESCs	Human Embryonic Stem Cells
hiPSCs	Human Induced Pluripotent Stem Cells
HPC	Hematopoietic Progenitor Cells
HSC	Hematopoietic Stem Cell
hTERT	Human telomerase reverse transcriptase
IL-1α	Interleukin 1 alpha
MAPK	Mitogen Activated Protein Kinase
Meis1	Myeloid ectopic viral integration site 1
MEP	Megakaryocyte Erythroid Progenitor
Mk	Megakaryocyte
MKP	Megakaryocyte Progenitor
Mks	Megakaryocytes
NF-E2	Nuclear Factor Erythroid 2
PB	Peripheral Blood
PDGF	Platelet Derived Growth Factor
PI	Phosphatidylinositol
PTPs	Platelet Transfusion Products
RBCs	Red Blood Cells
RRI	Rho Rock inhibitor
RUNX1	Runt related transcription factor 1
SCF	Stem Cell Factor
SDF-1	Stromal-Derived Factor-1
SI	Src Inhibitor
STAT1	Signal Transducer and Activator of Transcription 1
TAL-1	T-cell Acute Lymphocytic Leukemia Protein 1
TGF-β	Transforming Growth Factor-Beta
TPO	Thrombopoietin
UCB	Umbilical Cord Blood
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor

1 Introduction

Platelets are anucleated cytoplasmic discs derived from megacaryocytes that circulate in the blood. They play an important role in hemostasis, immunology, inflammation and cancer (Golebiewska and Poole 2015; Semple et al. 2011; Klinger and Jelkmann 2002; Franco et al. 2015). They involve in wound healing and angiogenesis through the delivery of stored growth factors, such as platelet-derived growth factor (PDGF), *basic fibroblast growth factor* (bFGF), vascular endothelial growth factor (VEGF), platelet factor-4, transforming growth factor-beta 1 (TGF-**β1**), thrombospondin-1 to sites of injury (Huang and Cantor 2009) Thrombopoeitin is produced in the liver at constant rate and controls circulating platelets rate by stimulating bone marrow to produce megakaryocyte. Platelets circulate in the blood for 7–10 days and count is between 140,000 and 440,000/mcl. A number of diseases are associated with platelet deficiency including thrombocytopenia, thrombocythemia, idiopathic thrombocytopenic purpura, myelodysplastic syndromes, chemotherapy-induced thrombocytopenia, aplastic anemia, human immunodeficiency virus infection, and major cardiac surgery (Thon and Italiano 2010). The platelet requirement in the clinic of these patients is increasing day by day. Increasing numbers of donors are needed for platelet transfusions (Ono-Uruga et al. 2016). Donor-dependent platelet availability can be challenging and transfusions can cause issues. In addition, platelets have short life spans of 7–10 days (Lu et al. 2011), donor-dependent platelet supply is limited (Ono-Uruga et al. 2016), and long-term storage of platelets is difficult (Handigund and Cho 2015). Bacterial or viral contamination and the other transfusion-transmitted infections and immunological reactions due to Human leukocyte or platelet antigensare some of the risks that need to be

considered (Ono-Uruga et al. 2016; Cho 2015; Pineault and Boisjoli 2015). Human leukocyte or platelet antigens are also problem for donor-dependent platelet transfusion (Pavenski et al. 2013).

Red blood cells (RBCs) are the most common type of cell found in the blood. RBCs or erythrocytes contain a special protein called hemoglobin, oxygen-binding protein, Which transports oxygen from the lungs to the tissues then returns carbon dioxide from the tissues to the lungs so it can be exhaled. A drastic reduction in the number of blood cells in the body can be associated with thalassemia syndromes, inherited bone marrow failure as well as in the anemia of chronic disease (Zivot et al. 2018). It is important that the infection tests, crossmatch and blood group were determined before transfusion. The need for rare blood groups and the urgency of blood transfusion in emergency cases reveal the challenges of blood transfusion (Sharma et al. 2011).

For all these reasons, studies on the production of non-donor-independent artificial blood elements *in vitro* have been a source of hope for many patients. In this review, current strategies for the development of artificial platelet and erythroid transfusion products will be discussed.

2 Developments in Artificial Platelet Transfusion Production Technologies

2.1 Megakaryopoiesis

The process of megakaryocyte maturation and differentiation in bone marrow is called megakaryopoiesis (Deutsch and Tomer 2013; Smith and Murphy 2014). Hematopoetic stem cells within the bone marrow can differentiate into Megakaryocytes (Mks). Mks are scarce, polyploid, big bone marrow cells able to produce platelets (Huang and Cantor 2009). Mks in the bone marrow are very rare population of cells, which only makes 0.02–0.05% of total nucleated cells (Li and Kuter 2001). Mouse Mks constitute 0.1–0.5% of bone marrow cell (Corash et al. 1989). Mks are associated with bone marrow sinusoidal endothelium, extending cytoplasmic protrusions into the sinusoids to produce platelets.

Megakaryopoiesis process and platelet production is complex and has multiple stages. Firstly, HSC gives rise to the early common myeloid progenitors (CMPs) that can be quantified as the multi-lineage (granulocyte, erythrocyte, Mks and monocyte) colony-forming unit (CFU-GEMM) (Deutsch and Tomer 2006). Erythroid and Mks lineages arise from a common Mk-erythroid progenitor (MEP) derived from the early CMPs (Fig. 1). MEP can develop into the highly proliferative, early MK burst-forming unit (BFU-MK), or the more mature smaller CFU-MK, with the help of cytokines and chemokines, which both express the CD34 antigen (Briddell et al. 1989). On the other hand, MEP can give rise to early and late erythroid progenitors, the BFU-E and CFU-E (Schulze and Shivdasani 2004). The proliferating diploid megakaryoblasts (MKP) lose their capacity to divide, but keep their ability for DNA replication and cytoplasmic maturation (Deutsch and Tomer 2006). This ability of MKP is called *endomitosis* or *endoreduplication*. During endomitosis stage, mature Mks are produced with a ploidy number of $N \geq 8$. Mature Mks build up a unique membrane complex called the Demarcation Membrane System (DMS), as well as various types of granules, including lysosomes, dense granules, and α-granules (Nurhayati et al. 2016; Takayama and Eto 2012). Mature Mks produce long cytoplasmic extensions proplatelets, which release platelets from their tips (Fig. 1).

Megakaryopoeisis process occurs in osteoblastic/endosteal and vascular/endothelial niches of bone marrow (BM). In osteoblastic niche, $CD41^{+}CD34^{+}$ cells arise from HSC and proliferate and differentiate into platelet-producing mature Mks (Machlus and Italiano 2013; Panuganti et al. 2013; Lorenzo et al. 2008). Mature Mks migrate from the osteoblastic niche to the sinusoidal blood vessels of BM, where they extend proplatelets in order to release growing platelets directly into the blood stream (Avecilla et al. 2004). During the megakaryopoiesis

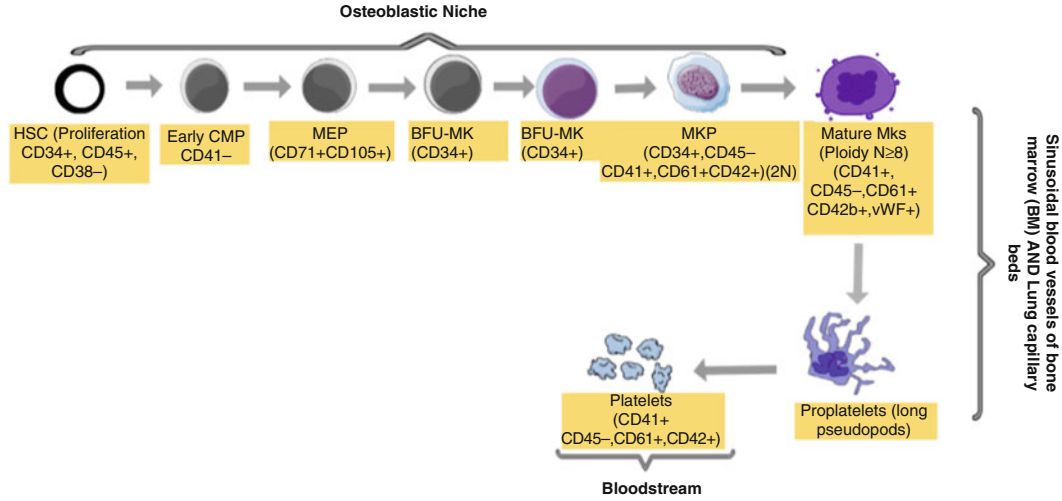


Fig. 1 Overview of cellular differentiation and maturation steps of megakaryopoiesis. CD34 + CD45+ HSC differenciate into CD41 + CD34+ MK in osteoblastic niche. Mature MK migrate to the sinusoidal blood vessels of BM and lung capillary beds where produce platelets into the blood stream. *HSC* Hematopoeitic stem cells, *CMP* Common myeloid progenitor, *Mk* Megakaryocyte, *MKP* Megakaryocyte progenitor, *MEP* Mk-erythroid progenitor, *BFU-MK* Burst forming unit-MK, *CFU-Mk* colony forming unit- Mk

process, before release platelets, mature Mks undergoes endomitosis, granule formation, proplatelet formation, and terminal platelet formation (Machlus and Italiano 2013; Panuganti et al. 2013; Lorenzo et al. 2008). Megakaryopoeisis and thrombopoeisis are very dynamic and efficient system, resulting in the generation of 10^{11} platelets per day 5 (Lambert et al. 2013). Every megakaryocyte can release approximatively 2000 and 5,000 platelets per day. The degree of polyploidization correlates with overall platelet production (Mattia et al. 2002; Ferrer-Marin et al. 2010).

Megakaryocyte differentiation is regulated positively or negatively by transcription factors and cytokines. Various molecular and cellular mechanisms regulate megakaryopoiesis process.

2.2 Stimulating Factors in Platelet Formation

Megakaryopoiesis require cytokines, chemokines and cellular interactions between HSC and bone marrow stromal cells (Avecilla et al. 2004). Megakaryocyte maturity and the level of polyploidization play important roles in the number of platelets released per cell (Mattia et al. 2002) (Fig. 2). The BM microenvironment has a unique protein composition crucial for platelet production. Extracellular matrix (ECM) components are also major factor for regulation of platelet production (Malara et al. 2011; Aguilar et al. 2016; Abbonante et al. 2017). Sinusoidal endothelial cells secrete thrombopoietin (TPO), VEGF and Interleukin 1 alpha (IL-1α) that are important regulator of megakaryopoiesis (Avecilla et al. 2004; Gars and Rafii 2012; Nishimura et al. 2015; Wang and Zheng 2016).

Cytokine TPO has pivotal role in the megakaryocyte development, regulation and maturation. Liver as a TPO producing organ generate TPO at steady level and this available level of TPO is controlled by circulating platelets, which carry on TPO receptors. Therefore, this feedback mechanism driven by TPO, provide the regulation and maintance of the platelets produced by bone marrow megakaryocytes (Yu and Cantor 2012; Lebois et al. 2016; Huang et al. 2016; Bertino et al. 2003; Kuter 2002).

c-Mpl is the surface receptor for TPO. Both megakaryocytes and HSCs have a common

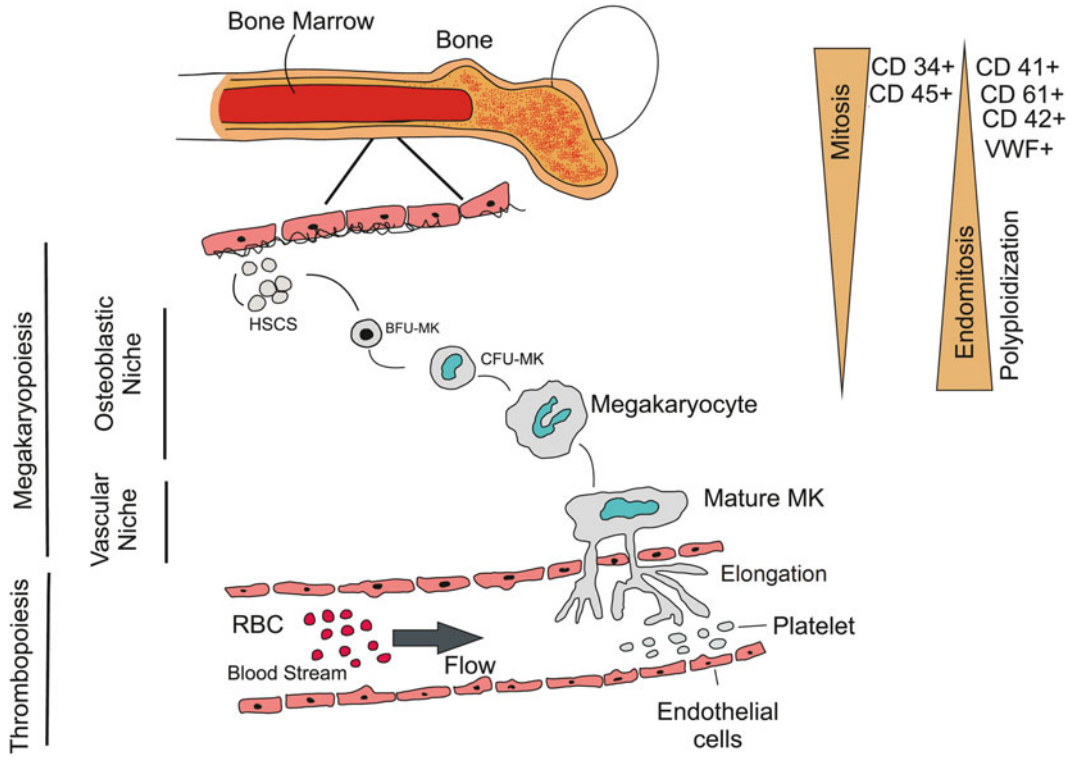


Fig. 2 Platelet release formation journey. Megacaryocyte are derived from HSC, differenciate and proliferate in the bone marrow niches. During proliferation megakaryoblasts lose their mitosis capacity, but keep endomitosis ability. Various stimulating factor regulates megakaryopoiesis process. Mature Mks undergoes endomitosis, granule formation, proplatelet formation, and terminal platelet formation. Thrombopoiesis occurs in vascular system where mature Mks build up the DMS and produce long cytoplasmic extensions proplatelets, which release platelets from their tips in the blood stream. *HSC* Hematopoeitic stem cells, *Mk* Megakaryocyte, *BFU-MK* Burst forming unit-MK, *CFU-Mk* Colony forming unit-Mk, *RBC* red blood cell

feature for the expression c-Mpl receptor on their cell surface. The importance of TPO receptor expression in HSC compartment has been revealed by bone marrow transplant studies taking advantage of the knock-out strategy. Mice lacking TPO receptor c-Mpl displayed deficiencies in long-term repopulating activity in bone marrow (Huang and Cantor 2009). Upon binding TPO to c-Mpl receptor, several downstream signaling pathways are activated including JAK/STAT, phosphatidylinositol (PI) 3-kinase-Akt, ERK1/ERK2 and mitogen-activated protein kinase (MAPK). TPO alone is able to trigger the differentiation of megakaryocyte as well as the HSCs maturation. However, it is not suitable for the production of wide range of megakaryocyte because of their quite low mitotic activity. Besides TPO activated pathways, there are also non-TPO pathways that effect megakaryopoiesis and thrombopoiesis. For instance; Notch signaling, stromal-derived factor-1 (SDF-1)/CXCR4, integrin signaling, src family kinase and platelet factor 4/low-density lipoprotein receptor-related protein 1, have been recently demonstrated both *in vitro* and *in vivo* (Pineault and Boisjoli 2015; Yu and Cantor 2012; Drachman et al. 1999; Gurney et al. 1995; Morita et al. 1996; Pallard et al. 1995; Rouyez et al. 1997; Sasaki et al. 1995; Sattler et al. 1995).

SDF-1 chemokine and its receptor CXCR4 have critical role in the trafficking and homing of hematopoietic precursor cells in the BM. Since

megakoryotic lineages express SDF-1 receptor CXCR4, SDF-1 provides megakaryopoiesis as well as the migration of megakaryocytes progenitors and mature megakaryocytes adhesion to endothelium (Wang et al. 1998). It is also stated that megakaryocyte-active chemokine such as SDF-1 and FGF-4 supports the relocation of progenitors inside the bone marrow microenvironment which is needed for maturation of megakaryocytes and thrombopoiesis (Avecilla et al. 2004; Broxmeyer 2001). Several reports showed that megakaryocytes as HSC-derived bone marrow niche cells modulate HSC quiescence by means of CXCL4 secretion (Wang et al. 1998) or TGF-β-SMAD signaling. However, it is also stated that FGF1 produced by megakaryocytes induces the expansion of HSC under stress condition (Bruns et al. 2014; Zhao et al. 2014).

Besides, Guo et al. demonstrated that c-Myc protein affects the fate of megakaryocyte-erythrocyte progenitors by controlling these progenitors differentiation ability. Mice lacking c-Myc showed increased megakaryocytopoiesis, lower polyploidy and blocked erythrocyte differentiation. Thus, the severe thrombocytosis-anemia-leukopenia syndrome was observed in mice from c-Myc (−/−) (Takayama et al. 2010; Guo et al. 2009). Another study showed that the transient expression of c-Myc plays an important role in both megakaryocyte polyploidization and platelet formation from iPSC (Takayama et al. 2010; Guo et al. 2009).

Since the megakaryocyte-derived platelets are generated from proplatelets through cytoplasmic extension, it is thoughout that the fragmentation of proplatet takes place when they come cross with biomechanical circulatory forces. Shear stress is one of the biomechanical forces known to enhance the formation of proplatet and platelet production inside the sinusoid vessels in bone marrow as well as the blood circulation. Once mature megakaryocytes expose to high shear stress on von Willebrand Factor (vWF), which is adhesive blood glycoprotein, platelet production is assisted through GPlb receptor and this is dependend on microtubules organization. However, ex vivo generation of megakaryocytes in static culture conditions shown to have a low rate of proplatelet formation when they are exposed to shear stress (Dunois-Larde et al. 2009).

Stimulating factors such as interleukin IL-3, IL-6, IL-9, IL-11 and stem cell factor (SCF) are important in megakaryopoiesis (Nurhayati et al. 2016). TPO, high-dose SCF, IL-3, IL-9, and IL-11 treatment are seem to be the favorable compination that supported Mk enlargement. While IL-3 is robustly increase total cell count and IL-9 supports expression of CD41 and CD42b. High-dose of SCF (100 ng/mL) induces Mk production and ploidy (Panuganti et al. 2013).

Several small molecules are shown to enhance Mks production and ploidy (Huang et al. 2016). Src kinase inhibitor (SU6656), Rho-associated kinase inhibitor (Y27632), Aurora B kinase inhibitor (AZD1152) can enhance functional platelet release *in vivo* (Jarocha et al. 2018). During the megakaryocyte polyploidization, endomitosis could be blocked by cytokinesis inhibitors. For this purpose, Zou et al. used four small molecules; Rho-Rock inhibitor (RRI, Y27632), nicotinamide (NIC, vitamin B3), Src inhibitor (SI, Su6656), and Aurora B kinase inhibitor (ABI, ZM447439) as cytokinesis inhibitors in their study. They found that SI (Src inhibitor) and ABI (Aurora B inhibitor) could increase significantly the percentage of polyploidy both in leukemic cells and primary mononuclear cells (Zou et al. 2017). Although ABI induces cell death and apoptosis, it could be used as Mks differentiation supplement. They also found that combination of RRI (Rho-Rock inhibitor) and SI could increase the expression of CD61 and enhance the polyploidy level without a statistically significant cell apoptosis. These indicated that RRI and SI were the suggested small molecule combination for Mk polyploidization (Zou et al. 2017).

Transcription factors such as GATA binding protein 1 (GATA1), RUNt-related transcription factor 1 (RUNX1), Friend leukemia integration 1 (FLI1), T-cell acute lymphocytic leukemia

protein 1 (TAL-1) have crucial role in Mk lineage commitment (Goldfarb 2009; Tijssen and Ghevaert 2013; Moreau et al. 2016). GATA1 plays a fundamental role in megakaryocyte lineage and regulation of polyploidization through its downstream effector stromal-derived factor-1 (STAT1). In addition, RUNX1 and nuclear factor erythroid 2 (NF-E2) genes are critical for terminal megakaryocyte differentiation (Bluteau et al. 2009; Italiano et al. 2007). In their study, Matsubara et al. suggested that p45NF-E2 has a crucial role in the generation of Mks and platelets from OP9 cells (Matsubara et al. 2013).

Myeloid ectopic viral integration site 1 homolog (Meis1) has been implicated as an important regulator of hPSC early hematopoietic differentiation. It was reported that Meis1 deletion disrupts the generation of megakaryocytes *in vivo* (Azcoitia et al. 2005; Carramolino et al. 2010; González-Lázaro et al. 2014; Hisa et al. 2004). Embryonic stem cell (ESC) derived hematopoietic cells study suggested that Meis1 represses erythroid development at the MEP stage for the benefit of megakaryocyte development (Cai et al. 2012).Miller et al. also showed that, Meis1 play important role in the erythroid and megakaryocytic compartments (Miller et al. 2016). In parallel with these studies, Wang et al. demostrated that Meis1 is vital for DMS development and polyploidization during megakaryocyte maturation (Wang et al. 2018).

Studies suggested that microenvironment in the maintanance of HSC pool and their differentiation to megakaryocyte are crucial. Mostafa et al. demonstrated that differential effects of pO2 on Mks progenitor enlargement, differentiation and maturation (Mostafa et al. 2000).Yang et el. also showed that higher pH supports Mk-cell differentiation, maturation, and apoptosis (Yang et al. 2002). Proulx et al. demostrated that cord blood CD34+ cell cultures incubated under mild hyperthermia (i.e. 39 °C) had accelerated and increased megakaryocyte differentiation and maturation over those maintained at 37 °C. Their study showed that mild hyperthermia had little impact on polyploidization and that transient early incubation at 39 °C is sufficient to increase megakaryocyte differentiation (Proulx et al. 2004). In addition, mild hyperthermia could accelerate and enhance Mk differentiation (Pineault et al. 2008). This was not because of cell-secreted factors but could be mediated by the enhanced expression of Mk transcription factors.

2.3 Surface Markers of Mk Differentiation and Platelet Formation

Mk lineage and maturation can be associated with many flow cytometric markers such as CD41 (GPIIb/IIIa), CD42b (GPIb), CD42a, PAC-1, CD62P, vWF, glycoprotein V (GPV) and Annexin V (Nurhayati et al. 2016; Jarocha et al. 2018). While expression of CD34 is gradually decrease during megakaryopoiesis, expression of CD41 (GPIIb/IIIa) increase in megakaryoblast stage. CD41 is a considerable marker for Mks (Nurhayati et al. 2016). In addition, Mori et al. identified CD71 + CD105+ (erythroid progenitor marker) unipotent erythroid progenitors within MEP, confirming that the majority of human MEPs (about 85%) are committed to the erythroid lineage (Mori et al. 2015). Specific markers of Mk maturation are GPV, vWF and CD42b (GPIb) (Nurhayati et al. 2016).

3 Sources of Artificial Platelet Production *In Vitro*

Since the first report by Choi et al., many researchers also have demonstrated human megakaryocytes and platelets could be generated *in vitro* from CD34+ peripheral blood progenitor cell as well as from umbilical cord blood (UCB) (Choi et al. 1995; Tao et al. 1999; Avanzi et al. 2012; Pineault et al. 2013), fetal liver (Ma et al. 2000), peripheral blood (PB) (De Bruyn et al. 2005), human embryonic stem cells (hESCs) (Gaur et al. 2006; Takayama et al. 2008), human induced pluripotent stem cells (hiPSCs) (Gekas and Graf 2010; Takahashi et al. 2007), or BM (Wang and Zheng 2016; Guerriero et al. 1995;

van den Oudenrijn et al. 2000). Although platelet products can be obtained by using these sources, obtained platelet product is not sufficient for clinical use. All these strategies are only proof of principle that platelets may in fact be generated *ex vivo* from stem cells (Avanzi and Mitchell 2014).

In vitro platelet studies have gained momentum in recent years. In these studies, researchers utilized umbilical cord bood, human induced pluripotent stem cells, adipose–derived stem cells (ADSC), human embryonic stem cell, and human CD34+ cells as starting cells for platelet production.

3.1 UCB as Starting Source of Cells for Platelet Production

Despite their similar membrane phenotype, UCB-Mks indicated reduced polyploidization and platelet number compared to PB-Mks. In addition, UCB-Mk has a low 8 N percentage compared to PB-Mks (Mattia et al. 2002). Matsunaga et al. cultured 500 UCB CD34+ cells on telomerase gene-transduced human stromal cells (hTERT stroma) in serum-free medium supplemented with SCF, Flt-3/Flk-2 ligand (FL), and TPO. With a three-stage culture system, they have demostrated that UCB-platelets have similar morphological features with PB-platelets (Matsunaga et al. 2006). De Bruyn et al. cultured UCB with TPO, FL, IL-6, and IL-11 and they obtained a suitable number of immature Mk progenitor cells expressing both CD34 and CD41 antigens (De Bruyn et al. 2005). Studies have shown that UCB-Mks are significantly smaller than those CD34+ PB and BM (Ignatz et al. 2007).

3.2 Use of hiPSCs in Platelet Production

In recent years, because of their availability and low ethical concerns, iPSCs are accepted as the most chosen cell source for *in vitro* platelet generation. A number of research groups have investigated that immortalized iPSC-Mks could be platelet source. Takayama et al. successfully generate platelets from hiPSC clones derived from human dermal fibroblasts (HDFs). The in vitro and in vivo functionality of these platelets had shown in the NOG (nod-scid/IL-2 γc-null) mouse thrombocytopenia model (Takayama et al. 2010; Nakamura et al. 2014). Takayama and colleagues co-cultured undifferentiated hESCs or hiPSCs on either mesenchymal C3H10T1/2 cells or OP-9 stromal cells that support differentiation of hematopoietic niche cells that contains hematopoietic progenitor cells (HPC), which are named "embryonic stem cell-derived sacs" (ES-sacs). They have developed *in vitro* culture system for differentiation of HPC into mature megakaryocytes with the ability to deliver platelets (Takayama and Eto 2012). Nakamura et al. have demonstrated lentivirus-based gene dosage manipulation can be used to immortalize iPSC-megakaryocyte lines. These lines could be grown for extended months and retained megakaryocyte maturation potential when the transgenes Bmi-1 and c-Myc were switched off. Maturing megakaryocytes released platelet with normal function *in vitro* assays and could participate formation of thrombus in a murine model (Nakamura et al. 2014). Feng et al. could managed to generate universal platelets from hiPSCs in serum and animal feeders free conditions (Feng et al. 2014). Other studies supported this view and showed that hiPSCs are potentially source of Mks, which can produce human platelets look alike peripheral blood (Moreau et al. 2016; Nakamura et al. 2014; Feng et al. 2014). The main benefit of hiPSCs will be the possibility to produce patient specific hiPSCs or genetically engineered cells lacking HLA antigens able to generate platelets. In this manner, when recipients are transfused with such transfusion products, they could escape immune response (Hod and Schwartz 2008). For this purpose, Figueiredo and colleagues recently reported that the *ex vivo* production of low expression of human leucocyte antigen (HLA) platelets from adult HSCs resulting in low-immunogenic response. These platelet could be used preferentially in alloimmunized patients or in patients

required frequent platelet transfusions to reduce platelet transfusion refractoriness due to alloimmunization (Figueiredo et al. 2010). On the other hand, few studies showed that iPSC-Mks are tend to be smaller, and have lower ploidy, and release fewer platelets with a short half-life when infused into mice (Bluteau et al. 2013; Potts et al. 2014).

In another study human dermal fibroblasts were transdifferentiated within about 17 days into Mks by the overexpression of *p45NF-E2, Maf G* and *Maf K* genes in the induction medium (Ono et al. 2012). CD41+ polyploid iMks were morphologically similar to BM-Mks and had ability to produce CD41+ platelet-like particles after infusion into immunodeficient mice. Tail-vein blood samples were collected from recipient mice before and after iMks infusion. The rate of human CD41 positive platelet increased in a time-dependent manner.

IPSC-platelet technologies still have several bariers to overcome such as need for robost HSC expansion and output of low number of megakaryocyte progenitors. Thus, several research groups have investigated the molecular method to improve development of iPSC- HSCs.

3.3 ADSC for Platelet Production

Adipose tissues containing abundant of multipotent preadipocytes are able to differentiate to adipocytes, osteoblasts and chondrocytes. Matsubara and her colleagues have been conducted the pioneer study on the generation of megakaryocytes and platelets from human adipocyte precursor cells in vitro (Matsubara et al. 2009). Ono-Uruga et al. showed that adipose tissue-derived stromal cells (ASCs) are able to induce megakaryocytes and platelets differentiation through endogeneous TPO secretion. Thus, ADSCs could be utilized as an alternative cell source for the platelets production and further clinical application (Ono-Uruga et al. 2016).

3.4 hESCs as a Source of Platelets

hESCs are appealing alternative source to achieve megakaryocytes maturation and platelets generation due to their promising well-known features being able to differentiate into all hematopoietic lineages for studying normal hematopoiesis, thrombopoiesis as well as disease conditions.

Eto et al. obtained sufficient amount of large, polyploidy and functional Mks from ES cells when cocultured with OP9 stromal cells suppelemented with TPO, IL-6, and IL-11 cytokines (Eto et al. 2002). Gaur et al. developed a trackable system benefited from genetic approaches to study megakaryocytopoiesis and the function of integrin. This system included coculturing with OP9 stromal cells to differentiate ESC to megakaryocytes. hESC-Mks were able to produce platelet-like particles and sharing similar features in ultrastructure, morphology, and functionality in comparision to blood platelets (Gaur et al. 2006). Additionaly, Takayama et al. established a unique method for culturing cells within the ES-sacs, a structure that provided a suitable microenvironment for hematopoietic progenitor differentiation. In these ES-sacs, hESC cultured with OP-9 cells or C3H01T1/2 stromal cells and further supported with exogeneous VEGF. It is shown that sufficient numbers of mature megakaryocytes could be obtained inside this ES-sacs and they have ability to release platelets which demostrate the activation of integrin alpha IIb beta 3 and extension against to ADP and thrombin (Takayama et al. 2008). Large scale of functional megakaryocyte and platelet production from hESC has been also studied by Lu et al. (Lu et al. 2011). In this study, morphological properties and structural features of hESC-platelets showed similarity with normal blood platelets after analysis with the differential-interference contrast and electron microscopy. Upon thrombin stimulation, hESC-platelets showed functional characteristics such as

microaggregation, enhanced clot formation/retraction, lamellipodia and flopodia formation as well as the linkage between them. hESC derived platelets takes part in the thrombi progression in mice carrying laser-induced vascular injury, which is similarly observed in human blood platelets. These findings indicated that hESC-platelets could be utilized for the platelet transfusion (Lu et al. 2011).

3.5 Human Hematopoietic CD34+ Cells as a Starting Material for Platelet Production

Since megakaryocytes are HSC derived bone marrow niche cells, HSCs are the most widely used cell types for *in vitro* platelet production. Several studies reported the feasible methods to generate functional megakaryocytes and platelets from hematopoietic stem and progenitor cells.

Huang N et al. have isolated human $CD34^+$ cells from CB or BM samples in vitro cultured using StemSpan SFEM media containing cytokines and tested the TGF-β pathway 616,452 inhibitor on the polyploidization and megakaryocyte maturation. It is shown that this small molecule is able to increase polyploidization greater as 64 N. Both CB and adult mobilized PB megakaryocytes size and ploidy were enhanced when treated with this small molecule in a dose-dependent manner. Although attenuated maturation of Mks derived from CB post treatment, platelets production was observed post small molecule treatment in mice transplanting CB CD34+ cells (Huang et al. 2016).

Mattia G et al. compared the ability of CB and PB derived human CD34+ cells to generate Mk and platalets. While CB derived Mks decreased polyploidization and the number of platelet in comparision to PB derived Mks, they both share similar membrane phenotype. Besides, enhanced polyploidization and high number of released platelets were determined compared to CB-Mks (Mattia et al. 2002).

Although they are originated from HSC and other benefits being natural sources, there ara some limitation of platelet production from CD34+ progenitor/stem cells in a large scale such as donor dependency, found in a small quantity and limited capacity of proliferation (Baigger et al. 2017) (Table 1).

4 Recent Breakthroughs in the Biogenesis of Platelet Production

4.1 Megakaryocyte Polyploidization and Associated Platelet Formation

Polyploidy can be occur in plant and animal cells, as a result of mitotic cycle remaining in different levels or doubling of chromosome sets (Winkelmann et al. 1987). Importance of megakaryocyte maturity and the polyploidization (endomitosis) has been shown in the number of platelets released per cell. It is known that megakaryocytes form up to 128 N polyploidy in healthy bone marrow compated to 16 N ploidy *in vitro* cell culture systems. Most of cells stay at the 2 N and 4 N stage after tissue culture (Mattia et al. 2002). Beside cultured megakaryocytes have lower ploidy they are also smaller and less granular than primary megakaryocytes (Takayama et al. 2010). Different strategies have been tested to increase ploidy of *in vitro* grown megakaryocytes.

Mattia G et al. found that there is a relationship between different levels of platelet release and different ploidy levels of megakaryocytes (Mattia et al. 2002). In their study CB-Mks showed reduced polyploidization and platelet number compared with PB-Mks, but they have same membrane phenotype. Most CB-Mks showed a 2 N DNA content (∼80%) and only 2.6% had

Table 1 Megakaryocyte differentiation using different protocols

Cell Source	Serum	Culture Components	Mk/platelet yield	References
UCB CD34+ HSCs	No	StemSpan SFEM, FBS, Pen Strep, SCF, TPO, and Flt-3 ligand, SCF, TPO, IL-3, and 616,452	Greater ploidy	Huang et al. (2016)
UCB CD34+ HSCs Coculture with hTERT stroma	No	SCF, Flt-3/Flk-2 ligand (FL), IL-11 TPO	$1{,}26x10^{11}$–$1{,}68 \times 10^{11}$ platelets	Matsunaga et al. (2006)
UCB CD34+ HSCs	No	TPO, FL, SCF, IL-3, IL-6, IL-11. Growth factors (T, FT, ST, SFT, S3FT, 611 T, F611 T) were added once at the start of the culture.	9.9 ± 1.9 UCB (8 N)	De Bruyn et al. (2005)
iPSCs	No	MK maturation medium (MK-M), STEM Span-ACF + TPO, SCF, IL-6, IL-9 and heparin	2.06×10^9 (MKPs)	Feng et al. (2014)
hESCs	Yes	DMEM high glucose, L-glutamine, penicillin/streptomycin, FBS	$5–20 \times 10^3$	Gaur et al. (2006)
Co-culture with OP9			$1–4 \times 10^4$ (h TPO)	
hESC-MA09 cell lines	No	Stemline II supplemented with TPO, SCF and IL-11	6×10^8	Lu et al. (2011)
CD34+ HSCs	No	StemSpan SFEM, TPO, IL3, IL6, SCF, FMS-related tyrosine kinase 3	1.0×10^6	Ivetic et al. (2016)
CD34+ CD38lo BM HSCs	No	Serum-free X-vivo 10 medium, IL-3, IL-6, SCF, and TPO	5.2 ± 0.2% ≥ 8 N	Shim et al. (2004)
MPB HSCs (Mobilized Peripheral Blood)	No	Serum-free medium, SCF, FL, IL3, IL6, IL11, and TPO	11.4 ± 1.4 MPB (8 N)	De Bruyn et al. (2005)
hADSCs	No	Mk lineage induction (MKLI) medium + human rTPO	62,000 ± 8,400 MKs from 4×10^5 CD71+	Ono-Uruga et al. (2016)
(3 T3-L1 and OP9)				
Adipose tissue	Yes	1)Preadipocyte growth medium-bullet kit	2×10^6 ± 2,500 and 15×10^4 ± 270 Mks and platelets	Matsubara et al. (2009)
		2)TPO media		
		3)DMEM, L-glutamine, Pen/Strep, bovine serum albumin, LDL cholesterol, iron-saturated transferrin, insulin, mercaptoethanol, nucleotide, dNTP, and TPO		

8 N, however 40% of the PB cells had 8 N or more. Platelets were substantially released in PB culture from day 12; at day 14 the CB-Mks were able to release platelets although at a reduced level (∼35%), correlating with their reduced size (Mattia et al. 2002).

During polyploidization of Mks, D-type cyclins are found to be important regulators of G1/S progression (Sherr and Roberts 1999). Sun et al. reported that transgenic mice overexpressing cyclin D1 in Mks displayed higher ploidy than did control mice (Sun et al. 2001). Several researchers used small molecules to promote megakaryocyte polyploidization (Zhang et al. 2004; Lannutti et al. 2005; Giammona et al. 2006). Studies show that hESC-Mks or iPSC- Mks have lower ploidy than in bone marrow, hESC-Mk has maximum ploidy of 126 N and iPSC-Mk has 16 N (Takayama et al. 2010; Giammona et al. 2009). Avanzi et al. found that megakaryocytes treated with a myosin inhibitor both presented a higher level of polyploidization and a higher number of proplatelet formation released in culture (Avanzi and Mitchell 2014).

4.2 Lung as a Major Organ for Platelet Formation

Platelets play key roles in hemoostasis, thrombosis and immune system. There have been recent

and major discoveries on the formation and maturation of platelets in various tissues and organs, however, the exact mechanism has not been completely understood yet. Megakaryocytes are produced in the bone marrow where they are released as proplatelet. Beside this, megakaryocytes were largely located in the lung in a number of early studies (Davis et al. 1997; Levine et al. 1993) but their role and relation to lung microenvironment were unknown. The contribution of lungs to platelet biogenesis were thought to be substantial with approximately half of total platelet production or ten million platelets per hour (Alexander et al. 1996). It was also estimated that each megakaryocyte reaching the lungs produces about 10^4 platelets. Recent studies provided major findings and in vivo quantifiacations which were supporting that the lung may have a major role in platelet formation.

To this end, researchers utilized the ex vivo cultured murine megakaryocytes and infused them into recipient mice. These infused megakaryocytes were shown to locate at pulmonary vasculate, which is the trapped site where they release platalets. They also found that increased platelet numbers approximately 100-fold in recipient mice post infusing ex vivo cultured megakaryocytes. While these released platalets in recipient mice showed slightly comparable half-life to infused platalets, other functional properties such as surface marker expression, size seemed to normal characteristic (Fuentes et al. 2010). In parallel to these, one study conducted on the lung-damaged rats revealed that the level of circulating platelets decreased by this injury. Therefore, this highlihts the pivotal role of lung in the Mk maintance and platelet formation (Machlus and Italiano 2013).

One of the most intriguint studies on the lung as a site for platelet formation in recent years was that of Lefrançais et al. (2017). This study demonstrated the lung as a fundamental site of platelet release from megakaryocytes in mice by video microscopy technology. In addition, Looney (2018) used lung intravital microscopy with fluorescently labeled mouse strains, he observed platelets released from megakaryocytes primarily in lung circulation (Looney and Headley 2018; Looney 2018). In addition, peripheral blood platelet count and bone marrow hematopoietic progenitor regeneration were observed by single lung transplantation to animals lacking thrombocytopenic and hematopoietic progenitors. These studies also showed that lung is reservoir for hematopoietic progenitors. All together, lung appeared as one of the key modulator and terminal production site for platelets.

4.3 Developments in Microfluidic Chips and Bioreactors Designed for Platelet Production

Recently, developments in microfluidic chip and bioreactor desing have gained popularity in the human platelet production. A number of research groups have used different materials to design effective bioreactors and microfluidic systems like platelet on-a-chip.

These systems are based on the mimicking the physiology of bone marrow niche by using bioengineered and biocompatible materials in order to generate platelets in a culture condition. Upon coating stem/progenitor cells within biomaterial, functional Mks make an extension to form proplatelet within perfused culture medium which mimicks the blood flow. The release of platelet in a culture condition can be mediated by flow rate using controlled electronic pump. These systems can be design not only implementing shear stress but also stiffness, surface topography, coculture and extracellular matrix component. Various microfluidic models combined with different biomaterials have been implemented to improve the ex vivo platelet production. This includes but not limited to microfluidic device consisting of micropillars coated with VWF (Blin et al. 2016), polydimethylsiloxane (PDM) bioreactor for platelet on-a-chip system (Thon et al. 2014), bioengineered 3D silk films on PDM (Di Buduo et al. 2015) and bioreactor containing PDM within 2D flow system to yield Mks from hESC or iPCs (Nakagawa et al. 2013).

Sullenbarger et al. developed a woven polyester fabric suited to a 3-dimensional continuous-perfusion bioreactor. With this bioreactor,

they collected platelets for over 30 day of culture (Sullenbarger et al. 2009) and resulted in 300 functional platelets released per starting CD34+ cell after 30 day of culture (Lasky and Sullenbarger 2011). Thon et al. was designed a biomimetic microfluidic bioreactor whice had microchannel size, ECM composition, BM stiffness, endothelial cell contact, and hemodynamic vascular shear stress within a single platform device (Thon et al. 2014). They were isolated hiPSC-Mks and were replaced in designed bioreactor. hiPSC-Mks began producing proplatelets at 6 h post-isolation and reached maximal proplatelet production at 18 h in static culture. After 15 day of culture, Mks with 20–60 mm of diameters were generated, which were ultrastructurally indistinguishable from human blood platelets by electron microscopy. By the end of this study, they were managed to produce functional human platelets for infusion (Thon et al. 2014).

Blin et al. developed microfluidic bioreactor containing wide array of vWF-coated micropillars to produce platelets from cultured megakaryocytes. These micropillars act as anchors on megakaryocytes, letting them to remain trapped in the bioreactor and exposed to hydrodynamic shear. Together effect of anchoring and shear caused the elongation of megakaryocytes and lastly they broke into platelets and proplatelets. Microfluidic device could produce large amount of platelets and their biological characterisation were functional (Blin et al. 2016).

Di Buduo et al. engineered a silk sponge containing 3D structure model closely mimicking the "spongy" marrow in bone and further combined with ECM components and fludic shear rate. This bioreactor setup, which is taking advantages of BM modeling maintained the Mk function and platelet production inside perfused vascular tube lumen (Di Buduo et al. 2015). Due to the functional characteristic of silk protein including weak immunogenicity, non-toxicity and low thrombogenicity, it is favorable to be a substitute of blood vessel. When silk protein was integrated with characteristic ECM constituents, Mks changed their characteristic behavior according to recognition of these proteins. Therefore, silk provides suitable system in order to reconstitute all BM properties that yield the generation of platelet (Di Buduo et al. 2017).

The bone marrow possesses highly dynamic structure harboring different cell types such as hematopoietic stem cells, fibroblast, osteoblast, osteoclast, adipose cells, endothelial cells so on so forth. This heteregenous cell populations in the niche give rise to the special connection and mechanism between bone and blood formation (Morrison and Scadden 2014). It is also indicated in several studies that the important role of vascular microenvironment components in contact with other cell types for the regulation of hematopoiesis (Arai et al. 2004; Butler et al. 2010; Ding et al. 2012; Doan et al. 2013; Kunisaki et al. 2013; Wang and Wagers 2011). In addition to bone marrow endothelial cells constructing the blood vessels network, it contributes the hematopoietic stem cells maintance (Wang and Wagers 2011; Gori et al. 2015; Itkin et al. 2016) as well as the thrombopoiesis. Kotha et al. developed 3D structure mimicking human vascular microenvironment (VME) in order to determine the function of marrow vasculature in the hematopoietic and thrombopoietic mechanistic. It is reported that thrombopoietic VME is constructed with microvessels which contains collagen matrix by seeding differentiated megakaryocytes from HSPCs in culture condition. This system successfully supplies the megakaryocytes migration into the wall of vessel, maturation as well as the platelets release into lumen. This interaction and recent findings correspond to the behavior of megakaryocytes in vivo (Kotha et al. 2018). Furthermore, Nakagawa et al. designed artificial blood vessels mimicking bone marrow in vivo. In this designed biomimetric system, flow culture system is structured with two flows coming from different directions. This provides favourable shear stress and pressure condition to be enforced

on Mks to generate plateletes in vitro from pluripotent stem cells. Moreover, this bioreactor sytem allows real time monitoring of the Mks that are derived from pluripotent stem cells during the time of cell culture (Nakagawa et al. 2013).

5 Artificial Erythroid Transfusion Product Technologies

5.1 Erythropoiesis

The one of the most common cells found in an adult individual are erythrocytes. Human blood contains ~5 × 10^6 erythrocytes per microliter (normal range 4.7 × 10^6–6.1 × 10^6 for males and 4.2 × 10^6–5.4 × 10^6 for females); erythrocytes have an average life span of 120 days. Erythrocytes provide oxygen distribution in our body and are essential in all stages of our life (embryonic, fetal, neonatal, adolescent, and adult). The earliest production of erythroid cells is observed in the yolk sac (Belaoussoff et al. 1998). These early erythroid cells are nucleated and mesodermal origin. Mesodermal cells migrate to yolk sac and have close relationships with endodermal cells. Very early erythropoiesis has been initiated as a result of the interaction of these two cell layers. Erythropoiesis is the name of a process in which red blood cells are produced (Malik et al. 2013; Suzuki et al. 2015). Erythropoiesis covert HSCs eventually into mature red blood cell. Many stages such as cell proliferation, apoptosis, autophagy, and cell differentiation take place in a balanced way during the erythrocyte formation. During erythropoiesis CMPs turn into MEPs. It is rapidly divide in to burst forming unit erythroid (BFU-E), which is known as immature erythroid-restricted progenitors. More mature erythroid progenitors, colony forming unit erythroid (CFU-E) and proerythroblasts (ProE), erythroblasts (EB), reticulocytes (Retic), and mature RBCs are formed respectively (Fig. 3).

5.2 Erythropoiesis Stimulating Factors

Several technologies have been developed for the formation and growth of erythroblasts in liquid

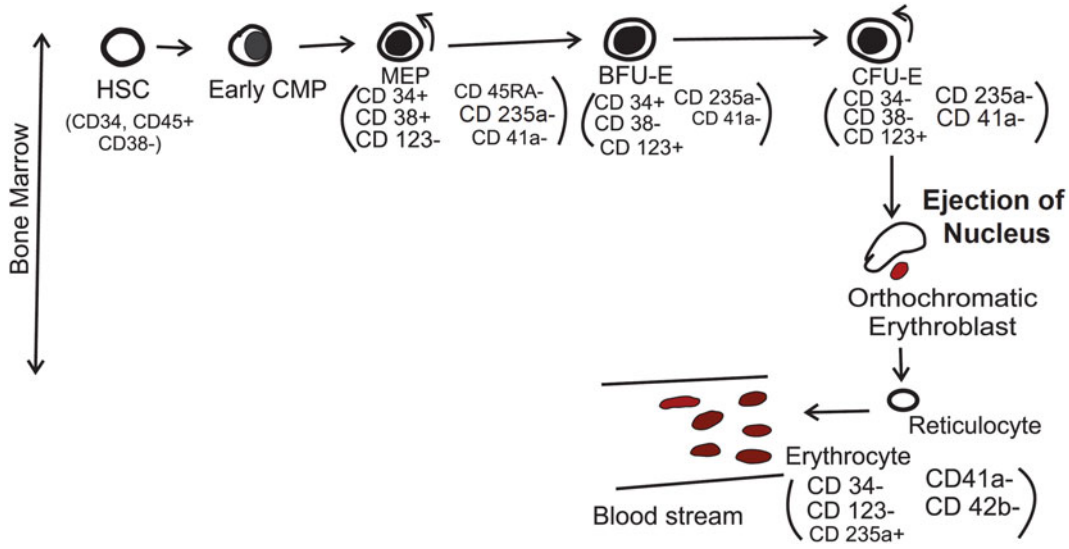


Fig. 3 Overview of biological phases of erythropoiesis. During Eryhtropoeisis HSC differentiate in a mature erythrocyte. Common myeloid progenitors (CMP) differentiate into megakaryocyte erythroid progenitor (MEP), burst forming unit erythroid (BFU-E), colony forming unit erythroid (CFU-E), erythroblasts (EB), reticulocytes and mature erythrocyte

Table 2 Summary of ETP development studies

Cell source	Components	Approach	Analysis/results	References
CD34 + HSCs are	SCF, Flt3L, Tpo, IL3, and HDAC inhibitors VPA, TSA and SAHA	HDAC inhibitors were used for differentiation of erythroid progenitors	NOD/SCID mouse model used to show functionality of erythrocytes	Chaurasia et al. (2011)
CD34 + HSCs	SCF, EPO, IL3, VEGF, IGF-II	Different growth factors and cytokines used for stimulation of enucleation	*In vitro* studies without feeder cells. Enucleation was shown	Miharada et al. (2006)
CD34 + HSCs	Transferin, insulin, hydrokortison, SCF, IL3, EPO	Differentiation to erythrocytes by growth factors and cytokines	Large scale, 100% terminal and functional differentiation to erythrocytes	Giarratana et al. (2005)
CD34 + HSCs	Transferin, ferric sulfate, ferric nitrate, insulin, lipids, hydrocortison, SCF, IGF-I	Differentiation to erythrocytes by growth factors and cytokines	*Ex vivo* human erythrocyte production on a large scale it was performed. *In vitro* and *in vivo* verifications were performed	Neildez-Nguyen et al. (2002)
CD34 + HSCs	SCF, IL3, EPO, Transferin, Insulin, vitamin C, ferrous nitrate, cholesterol,	Both bone marrow and cord blood erythroid transfusion products were developed from HSCs	It has been shown that there are functional erythrocyte differentiation using morphological, phenotypic, and biochemical analysis methods	Jin et al. (2014)
CD34 + HSCs	Transferin, ferric sulfate, ferric nitrate, insulin, SCF, IL3, EPO, hydrokortison	They demonstrated that red blood cell production can be performed *in vitro* using peripheral HSCs	*In vitro* flow cytometry analysis, cell cycle erythrocyte differentiation with analyzes are shown	Boehm et al. (2009)

culture media. The culture conditions used in these techniques varies but stem cell factor (SCF), erythropoietin (Epo), dexamethasone (Dex), and transferrin are commonly present, usually supplemented by insulin or IGF-1 (Table 2) (Dzierzak and Philipsen 2013). The stem cells in the bone marrow are transformed into BFU-E and CFU-E cells, then into the pro-erythroblasts under the influence of cytokines such as IL3, SCF and IGF1. With the help of *Epo*, they are transformed into basophilic erythroid cells. Epo plays an important role in the differentiation of polychromatic and orthochromatic erythroid cells. Transcription factors like PU.1, GATA2, TAL2, GFI-1B, STATs are effective in transforming them into reticulocyte cells. In addition, signal transduction factors such as EpoR, Jak2, and GAB1/2 also are differentially expressed in these stages and play an important role in the formation of mature erythrocyte. Epo, is a hormone that plays a very major role in erythropoiesis (Malik et al. 2013). Epo glycoprotein of 34 kDa interacts with the Epo receptor and regulates the growth, differentiation and viability of erythroid progenitors relative to the low oxygen content. Epo is mainly produced by a hypoxia-induced mechanism from Epo-producing renal cells in the kidney. Therefore, Epo production in any chronic kidney disease decreases and causes complications. In such cases, the recombinant human Epo protein may be used in the treatment of anemia resulting from Epo deficiency. Epo injections reduce the need for blood transfusions and improve the quality of life of patients. However, it was observed that anti-Epo antibodies were formed after continuous and high doses of recombinant Epo injections (Janda et al. 2010). Therefore, it has been predicted that strategies should be developed in the treatment of different anemias. The effect of Epo protein is achieved by binding to Epo receptor (EpoR). Epo knockout mice with EpoR knockout mice show the same anemia phenotype and cause embryonic death with advanced anemia. The two EpoR proteins undergo a conformational change and activate JAK2, and signal

molecules SHP1, SHP2, Grb2, and STAT5 (Suzuki et al. 2015; Ravasi et al. 2010). STAT5 is found to be required during fetal development to for evelevated rate of erythropoietis (Socolovsky et al. 1999).

5.3 Surface Markers in Erythropoiesis

Various cell surface markers have been utilized in the characterization of erythropoiesis. Xi et al. demonstrated the erythroid cells derived from CB HSCs by analyzing the different set of surface marker, which are CD3, CD4, CD8 CD13, CD14, CD19, CD33, CD34, CD38, CD41, CD45, CD71, CD117 and glyccophorin A expression using flow cytometry. CB HSCs cultured over 50 days in a condition supporting erythroid differentiation. During this period, CD71+, CD117+ and GPA+ cell populations were 95%, 90% and 15% at the day of 21, respectively. In addition, cell surface antigens belonging to myelomonocytic, megakaryocytic and lymphatic lienages showed quite low expression level such as 1% for CD14, 2.4% for CD41, 3% for CD4, 2.5% CD8, 0.7% for CD34 and 0.5% for CD3 (Xi et al. 2013).

Some studies have shown that CD71 and Ter119 dual staining work in separating erythroid cells (Suzuki et al. 2015). For example, CD71 proeryroblasts and early basophilic erythroblasts were found to have high expression and decreased expression of cells as erythroid differentiation was observed. In addition, Ter119 is expressed in terminally differentiated erythroblasts. $CD71^{high}Ter119^{high}$ cells (basophilic erythroblasts), $CD71^{high}Ter119^{low}$ cells (proeryroblasts), and $CD71^{low}Ter119^{high}$ cells (orthochromatic erythroblasts) was characterized at different times with flow cytometry (day 1, 4. day, 7th day, 14th day, 21st day) (Suzuki et al. 2015). Proeryroblasts were also characterized by $TER119^{+}CD44^{high}FSC^{high}$. In addition, $TER119^{+}CD44^{low}$ (reticulocytes, erythrocytes) and $TER119^{+}CD44^{high}FSC^{low}$ (erythroblasts) markers were also used.

6 The Importance of HSCs as Erythrocyte Source for *In Vitro* Artifical ETP Development

HSCs are characterized by self-renewal and ability to turn into all types of blood cells. HSCs are responsible for producing billions of mature blood cell lines daily throughout their adult life. HSCs are responsible for producing billions of mature blood cell lines daily throughout their adult life. While these cells form the basis of bone marrow transplantation, they are also promising for ETP studies. The success of HSC transplantation and transfusion products containing artificial erythroid progenitor depends on the presence of human leukocyte antigen (HLA) compatible donors and the ability to reproduce sufficient number of cells *in vitro* for a donor. Even if there are HLA-compatible donors, the lack of the number of HSCs obtained often reduces the success rate of erythroid progenitor *in vitro*. Therefore, an alternative method such as HSCs *in vitro* duplication should be developed to obtain a sufficient number of erythroid transfusion products (Nishino et al. 2011; Zheng et al. 2011; Maung and Horwitz 2019; Sniecinski and Seghatchian 2018; Schuster et al. 2012). It is possible to recognize and isolate human erythroid cells and HSCs surface antigens by flow cytometry. In addition, they can be treated *in vitro* with cytokines such as TPO, FL3, and SCF (Zheng et al. 2011). However, when erythroid growth factors are used, the use of small molecules targeting HSC silence factors is not common. In the studies conducted for the *in vitro* HSC replication, various problems have been encountered due to the loss of self-renewal ability in HSCs, increased differentiation, limited knowledge about HSC regulators (Choi and Harley 2016; Csaszar et al. 2013; Walasek et al. 2012; Knaan-Shanzer et al. 2008). Cytokines such as TPO, FL3, IL3, IL6, IL11, and SCF have been shown to amplify HSCs. Cytokines stimulate dormant (in G_0 phase) long-term HSCs to enter the cell cycle. It is believed that they are doing this by increasing self-renewal factors or by

suppressing cell cycle inhibitors. Interestingly, some inhibitory factors such as p38 have been shown to be increased and activated during *in vitro* replication. To this end, it was shown that use of p38 inhibitor increases the proliferation of HSC *in vitro* (Zou et al. 2012). In addition, Chaurasia et al. (2011) used HDAC inhibitors in combination with cytokine and valproic acid, and demonstrated that CD34 + cells could be expanded *in vitro* and turn into ETPs (Chaurasia et al. 2011; Broxmeyer 2014). Other inhibitors such as StemRegenin 1 (AhR antagonist), Garcinol (non-specific HAT inhibitor), Nicotinamide (SIRT1 inhibitor), and c-Myc inhibitor 10,074-G5 (Aksoz et al. 2018) are also shown to be effective for the growth of human and mouse HSCs (reviewed in (Yucel and Kocabas 2018)). In summary, studies involving loss of function of HSC quiescence genes showed a clear link between normal hematopoiesis, *ex vivo* and *in vivo* HSC proliferation.

7 Conclusions

Approaches to *ex vivo* thrombocyte production are promising technologies to be adapted to clinical practice. Low number of platelets obtained from stem cells, difficulties encountered during maturation, shelf life of platelets are the most important difficulties encountered in ex vivo PTP development. The demostration of lung as a major platelet releasing organ and on-going development of microfluidic chips and bioreactors designed for PTP technologies are the most important advanvements recently in the field. ETP studies have gained importance in recent years due to the increasing need for blood in the clinic and a limited number of healthy donors. The difficulties in PTP and ETP technologies have been partially overcome in the last decade. Making these artificial PTP and ETP technologies accesible to clinical practice will be a source of hope for patients in need of repeated platelets and blood transfusions.

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