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Erythropoiesis and Megakaryopoiesis in a Dish

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Abstract

Erythrocytes and platelets are the major cellular components of blood. Several hereditary diseases affect the production/stability of red blood cells (RBCs) and platelets (Plts) resulting in anemia or bleeding, respectively. Patients with such disorders may require recurrent transfusions, which bear a risk to develop alloantibodies and ultimately may result in transfusion product refractoriness. Cell culture models enable to unravel disease mechanisms, and to screen for alternative therapeutic products. Besides these applications, the ultimate goal is the large-scale production of blood effector cells for transfusion. Cultured RBCs that lack many of the common blood group antigens and Plts-lacking HLA expression would improve transfusion practice. Large numbers of RBCs and Plts can already be generated using hematopoietic stem cells derived from fetal liver, cord blood, peripheral blood, and bone marrow as starting material for cell culture. The recent advances to generate blood cells from induced pluripotent stem cells provide a donor-independent, immortal primary source for cell culture models. This enables us to study developmental switches during erythropoiesis/megakaryopoiesis and provides potential future therapeutic applications. In this review, we will discuss how erythropoiesis and megakaryopoiesis are mimicked in culture systems and how these models relate to the *in vivo* process.

Keywords: transfusion, cell culture model, GMP conditions, defined culture medium, cultured red blood cells, erythropoiesis, megakaryopoiesis, induced pluripotent stem cells, bioreactors, clinical trials

1. Introduction

Blood cells are by far the most abundant cells of which our body is comprised. Red blood cells (RBCs, or erythrocytes) and platelets (Plts, or thrombocytes) circulate in the vascular system, whereas the white blood cells that form our immune system locate both in the vascular system

and in the tissues. RBCs are best known for their function as oxygen transporters and for the clearance of CO₂. Plts exert a crucial function in homeostasis upon vascular damage but they also function during angiogenesis, innate immunity, inflammation, wound healing, cancer, and hemostasis [1, 2]. This chapter focuses on erythropoiesis and megakaryopoiesis. RBCs in the periphery have an average life span of 120 days, constituting approximately 45% of the blood volume. To maintain the population of RBCs, humans generate daily $\sim 2 \times 10^{11}$ reticulocytes [3]. Plts are shed by megakaryocytes (MK) and live approximately 8–9 days in humans, which require a production of $\sim 8.5 \times 10^{10}$ Plts/day [4, 5]. The generation of RBCs and Plts occurs mainly in the bone marrow (BM) in adults, although the lung has also been found to host megakaryocytic progenitors as well as Plts-shedding MKs [6]. A small population of hematopoietic stem cells (HSCs) ensures the life-long generation of blood cells, although the HSCs themselves divide rarely. Mostly, HSCs that divide give rise to one new HSC and a daughter cell that develops to an actively dividing multipotent progenitor (MPP) (Figure 1) [7]. These MPPs undergo specification through reciprocal actions of transcription factors (TF) that enhance or repress expression of lineage-specific TFs and direct the cells to a lineage-specific gene expression program [7]. Erythropoiesis and megakaryopoiesis were long thought to arise from a common progenitor, the megakaryocytic-erythroid progenitor, but recent lineage tracing indicates that MKs can also differentiate directly from HSCs [8–10]. Not only MPPs, also erythroid progenitors (erythroblasts:

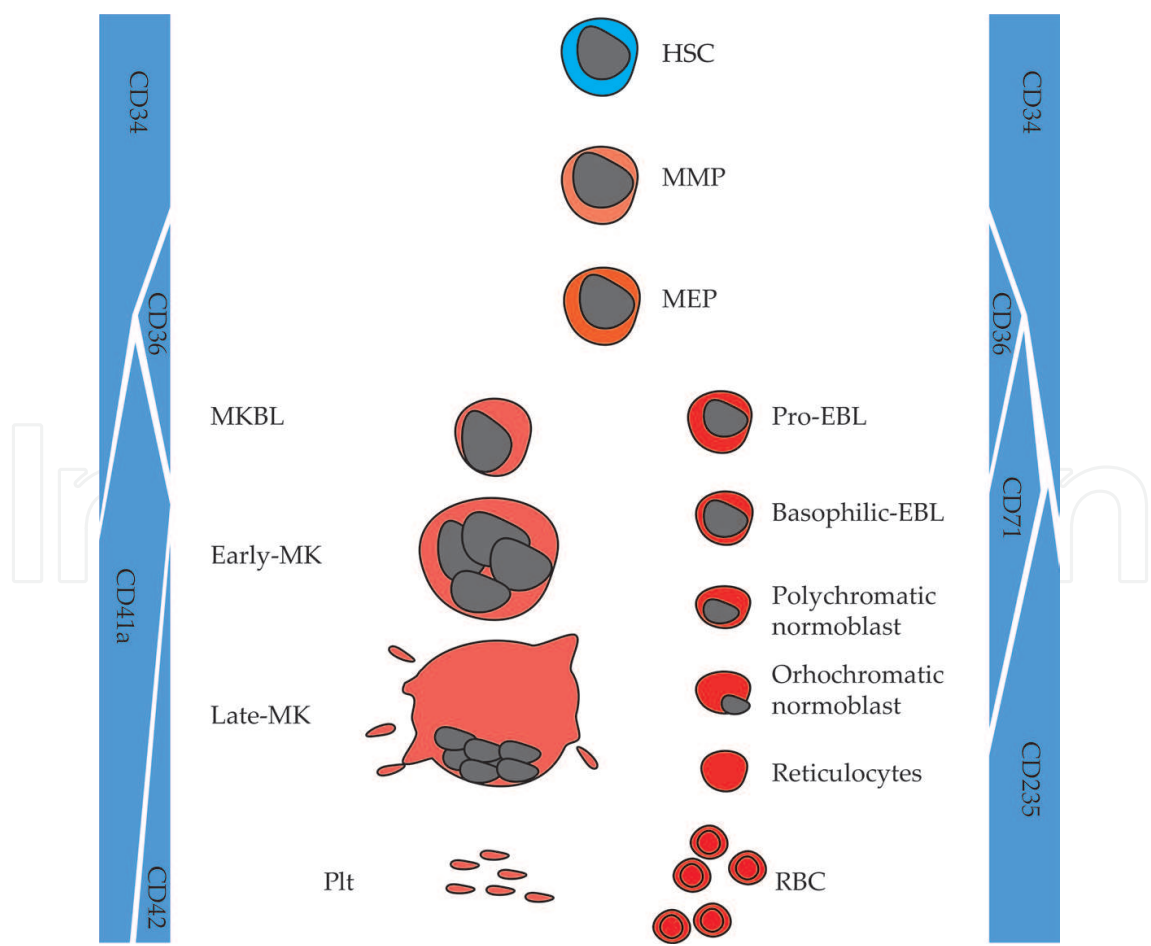


Figure 1. HSC commitment to the erythroid/megakaryocytic lineages with lineage-specific marker expression pattern.

EBLs) and megakaryocytic progenitors (megakaryoblasts: MKBLs) have extensive potential to undergo cell divisions before they commit to the final differentiation program to generate RBCs/MKs. The final differentiation stages of both lineages have unique features. Erythroid progenitors undergo 3–4 additional cell divisions with a short G1 cell cycle phase and without regaining the cell volume (i.e., loss of cell size control) [11–13]. MKBLs, instead, undergo 4–5 cell division cycles without cytokinesis, which results in a single cell with 64–128 genome copies ($N = 64\text{--}128$) [14]. Erythropoiesis and megakaryopoiesis also show spatiotemporal regulation. All blood cell progenitors including erythroid progenitors and MKBLs propagate in close contact with stromal cells that produce membrane-bound factors such as stem cell factor (SCF). Upon terminal differentiation, erythroid progenitors bind to central macrophages that express receptors such as CD163, VCAM1, ICAM4, and CD163 to associate with EBLs [15–19]. Each macrophage binds several progenitors that undergo synchronous differentiation, which ends with phagocytosis of the extruded erythroid nucleus by the macrophage and release of reticulocytes into the circulation. The mature MKs have to interact with the endothelial cells of the vasculature and protrude proplatelets into the capillaries, where shear stress contributes to the shedding of Plts [20].

Whereas steady state erythropoiesis and megakaryopoiesis of adult mammals take place in the BM and lung (MK), distinct anatomic sites of hematopoiesis are employed during development (**Figure 2A**). After gastrulation, in humans, mesodermal precursor cells arise in the primitive streak, migrate to the yolk sac, and develop into blood islands (hemangioblasts), which produce primitive RBCs, primitive MKs, and macrophages [21]. During this process, basic fibroblast growth factor (bFGF) influences the proliferation of the hemangioblast and thereby the production of hematopoietic cells [22]. bFGF is synergistic with vascular endothelial growth factor (VEGF) signaling in this process [23]. The primitive RBCs express embryonic type of hemoglobins (Hbs), retain the size of the early EBLs, and lose their nucleus only after prolonged circulation. Their erythropoietin (EPO)-dependence is unclear at this early stage of development [24, 25]. The primitive MKs are thrombopoietin (TPO) independent, have low ploidy compared to adult MKs, and produce fewer Plts, but contrary to primitive erythroid cells, these cells migrate to the fetal liver, where their polyploidization is TPO dependent [26, 27]. Erythroid-myeloid progenitors (EMPs) arise in the yolk sac from hemogenic endothelium (HE) through endothelial to hematopoietic transition (EHT) and give rise to the first intermediate definitive wave, producing RBCs with fetal type of Hbs, MKs, and other myeloid cells [21, 28]. The EMPs migrate and colonize the developing fetal liver where they transiently produce definitive fetal RBCs and MKs. Permanent definitive hematopoiesis in the fetal liver depends on the “birth” of HSC in the aorta main arteries, and more specifically in the aorta-gonad-mesonephros (AGM) region, where the first $CD34^+$ HSC arises through EHT. These early HSCs are dependent on bone morphogenetic protein 4 (BMP4), VEGF, and bFGF secreted by “feeder cells,” which are located near the endothelial cells undergoing EHT, thereby promoting this transition [23, 29]. These HSCs home to the fetal liver to produce definitive fetal blood cells. From the fetal liver, the HSCs migrate to the final site of hematopoiesis; the BM, where they give rise to adult definitive blood cells. Perinatally, hematopoiesis also occurs in the spleen [30]. RBCs and Plts generated at distinct anatomic sites have distinct characteristics; for example, RBCs express different Hb molecules arising from different sites (**Figure 2B**). Hb consists of two α and two β subunits each bound to an iron-containing heme molecule. The α locus expresses ζ and α protein isoforms, the β locus expresses ϵ , γ ($\gamma 1$ and 2), and β (β and δ) isoforms. Primitive

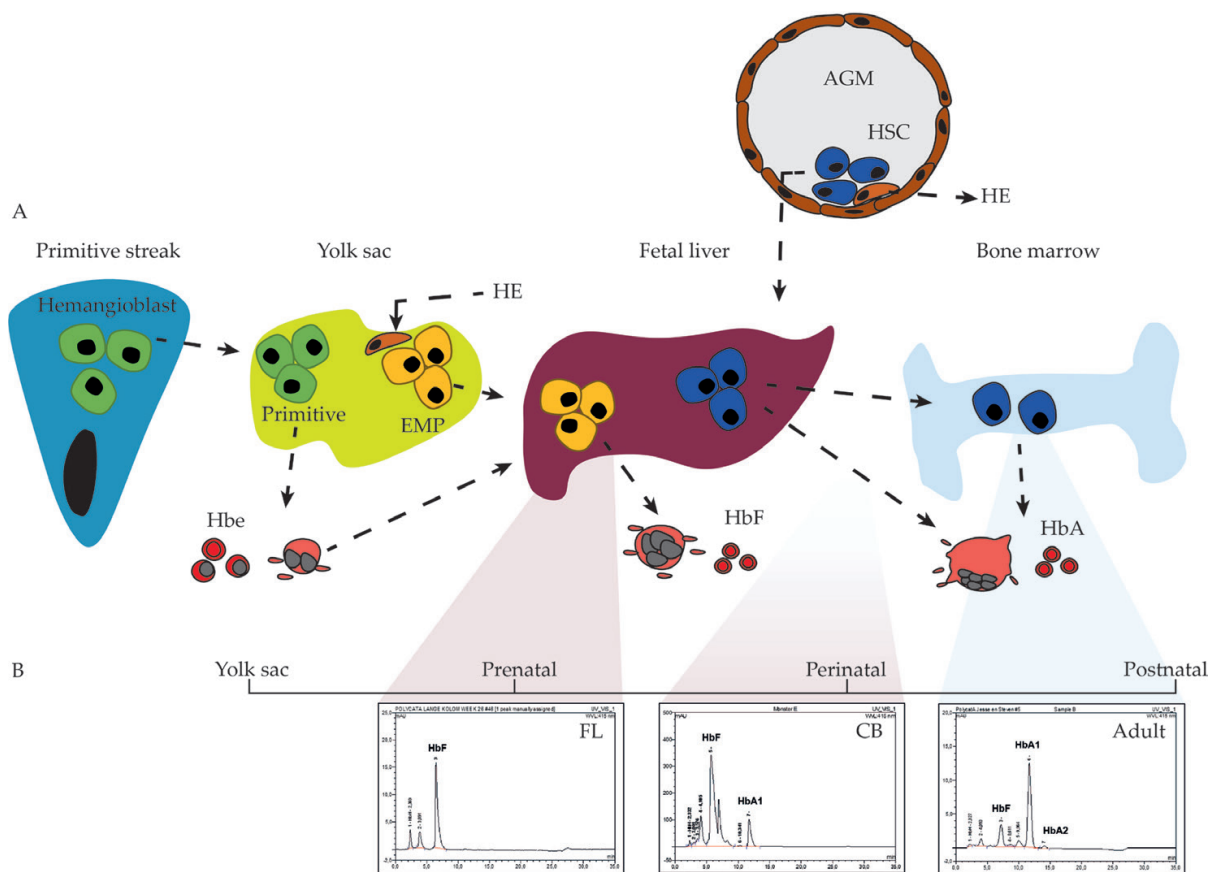


Figure 2. Human erythropoiesis/megakaryopoiesis during development. (A) Schematic depiction of site-specific (yolk sac/fetal liver-AGM/bone marrow) blood production during ontogeny focusing on erythroid and megakaryocytic lineages. (B) Representative HPLC tracks, showing the Hb content of *in vitro*-cultured erythroid cells derived from primary sources originating from distinct anatomical sites. Fetal liver-erythroid cells express fetal Hbs (HbF). Cord blood is obtained at the time of birth when the fetal to adult Hb switch takes place, resulting a mix of HbF and HbA. PBMC/MPB-derived erythroid cells produced by the bone marrow mainly express adult hemoglobins (HbA1, HbA2).

RBCs express Hbe consisting of ζ and ϵ isoforms (Portland 1: $\zeta_2\gamma_2$; Portland 2: $\zeta_2\beta_2$; Gower 1: $\zeta_2\epsilon_2$; Gower 2: $\alpha_2\epsilon_2$); fetal RBCs are characterized by HbF consisting of α and γ isoforms; adult RBCs express HbA consisting of α and β isoforms (HbA1) plus a small amount of HbA2 consisting of α and δ isoforms. Hbs can be used to distinguish RBCs originated from different developmental stages; however, in the megakaryocytic lineages, there is a lack of such markers.

Biochemical and molecular analysis of erythroid/megakaryocytic cells requires large cell numbers. The *in vitro* expansion and differentiation of erythroid and megakaryocytic progenitors from human fetal liver (HFL), cord blood (CB), BM, or peripheral blood enable the production of large cell numbers from distinct ontology and at defined stages of differentiation for basic research, drug testing, disease modeling, or translational purposes (Figure 3). Differentiation of pluripotent stem cell types such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) toward hematopoietic lineages allows to study early blood ontogeny, which is difficult to study *in vivo* as of ethical issues and availability of human material. The knowledge gained by using the abovementioned culture systems is subsequently of great value to control the expansion and differentiation of erythroid/megakaryocytic cells from ESCs/iPSCs leading to donor-independent blood cell production (Figure 3).

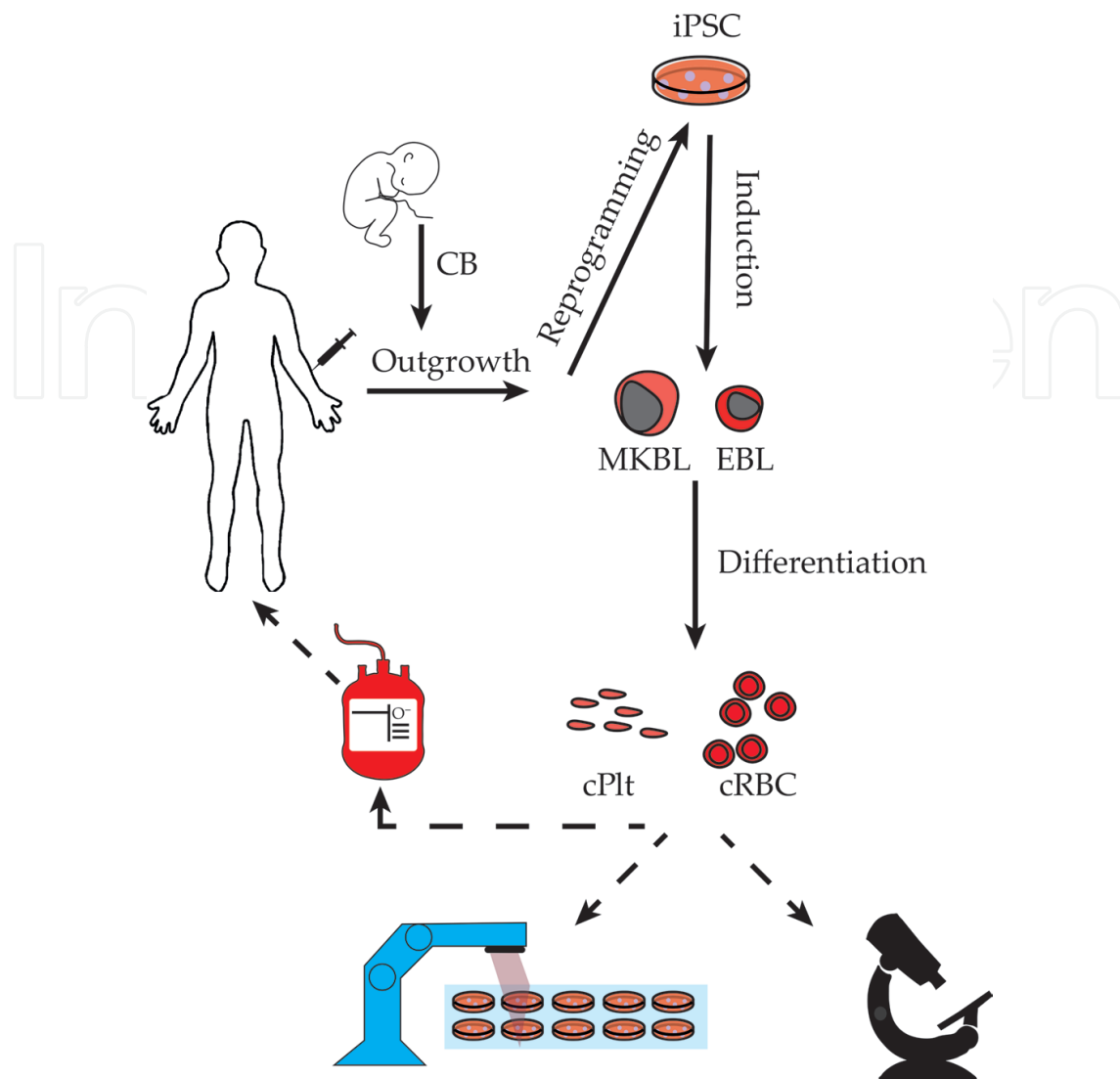


Figure 3. Application of *in vitro*-derived blood products from different primary sources in basic research, drug discovery, and transfusion.

The aim of this chapter is twofold. First, we provide background information of the basic processes of erythropoiesis and megakaryopoiesis that underly the various cell culture models. Second, we provide details, interpret and compare results on current protocols to expand and differentiate erythroid and megakaryocytic progenitors.

2. Erythropoiesis/megakaryopoiesis

2.1. Growth factors and major regulators

2.1.1. Lineage- and stage-specific cytokines

Interleukins (ILs) activate cytokine receptors, which do not have enzymatic activity and recruit Janus Kinases (JAK1, JAK2, JAK3, TYK) to phosphorylate tyrosines in their intracellular tail

that can subsequently recruit signaling molecules. Cytokine receptors are often expressed on a limited set of cell types/differentiation stages. IL-3 and IL-6 are cytokines that promote survival of hematopoietic stem/progenitor cells (HSPCs), but they do not act on EBL-specific stages. IL-9 synergizes with IL-3 to enhance both erythropoiesis and megakaryopoiesis [31, 32]. IL-1 β is an inflammatory cytokine that signals via MyD88 to IRAK and NF- κ B to inhibit cell growth, to induce expression of growth factors and the extracellular matrix, and to cause apoptosis in most tissues. In megakaryopoiesis, it enhances commitment and differentiation [33, 34].

Erythropoietin (EPO) is mainly produced by the kidney (80%) and partially in the liver (10–15%; 30% upon stress erythropoiesis) [35, 36]. EPO binds and activates EPO receptor (EPOR) and functions as a survival factor during erythropoiesis [37, 38]. Deficiency of Epo and/or Epor causes embryonic lethality due to the failure of definitive fetal liver erythropoiesis leading to the lack of mature RBCs [24, 39–41]. Fetal livers of EPO-deficient embryos contain normal numbers of erythroid progenitors that can form colony-forming unit-erythroid (CFU-E) *in vitro* in the presence of exogenous Epo [39]. EPO shows cross-reactivity in human and mouse.

Thrombopoietin (TPO) is the ligand for the MPL receptor. MPL is well conserved between species and TPO showing cross-reactivity between mouse and human. TPO has a direct effect on self-renewal and expansion of HSCs in the BM, but controls megakaryopoiesis as well [42]. MKs and Plts bind TPO and sequester it from the circulation. Upon activation, Plts release TPO into the plasma, thereby stimulating megakaryopoiesis. Thus, the number of Plts and their activation controls TPO levels [43]. TPO is mainly produced by hepatocytes in the human liver. TPO production is increased when hepatocytes bind damaged and aged desialylated Plts via the Ashwell-Morell receptor. In a JAK2/STAT3-dependent manner, this activates TPO transcription and thereby regulates Plt production, HSC renewal, and expansion [42, 44, 45].

2.1.2. Growth factor-binding tyrosine kinase receptors

The tyrosine kinase receptors directly cross-phosphorylate tyrosine residues in their own cytoplasmic tail, and they phosphorylate downstream effector molecules. They are more widely expressed compared to cytokine receptors.

Stem cell factor (SCF) (first described as the *Steel* locus) signals via the mast/stem cell growth factor receptor KIT (CD117; *White* locus). SCF cooperates with other cytokines in order to maintain the viability of HSPCs, and their proliferation/differentiation ability [46–48]. SCF is produced by the stromal cells in the BM as a secreted soluble factor but also as a membrane-bound factor. A specific mutation in SCF (*Steel-Dickie*) disrupting the membrane form of SCF leads to severe anemia, indicating that the membrane-bound form is crucial at least for erythropoiesis [49]. In addition, Kit-deficient mice (*W/W*) suffer from neonatal lethality due to severe anemia [50, 51]. Besides its function on HSPC, SCF is particularly important upon blood loss, to enhance proliferation and delay differentiation of the erythroid and megakaryocytic progenitors [52–54]. In contrast to TPO and EPO, SCF is not interchangeably reactive between mouse and human—mouse SCF activating both, while human does not.

FLT-3-ligand (FL) binds to Fms-like tyrosine kinase 3 (FLT-3 (CD135)) as its receptor. FL is an important growth factor for CD34⁺ HSPC by controlling their proliferation. Underscoring its

function, activating mutations in the FLT-3 receptor are prominent in acute myeloid leukemia [55]. FL, however, has a limited role in terminal MKBL/EBL differentiation because FLT-3 is not expressed on committed cells.

Insulin (Ins) and Insulin-like growth factor-I (IGF-I) bind the Ins receptor (InsR) and IGF-I receptor (IGF1R). Both receptors are very well conserved between species to the extent that Ins even cross-reacts between mammals and birds. The homology between the receptors suggested that Ins may also act through the IGF1R, which is not true. IGF-I has a general cell survival function mediated by PI3K activation [56]. Human erythroid progenitors (pro-EBLs, early basophilic EBLs) express the IGF1R, and not the InsR, which changes during differentiation when the InsR becomes the prominent receptor [57]. InsR signaling is particularly important to control trafficking of GLUT4 glucose transporters to the cell membrane [58]. Late EBLs and mature RBCs depend on glycolysis and EBLs express high levels of glucose transporters [59]. Upregulation of GLUT4, however, is also required for import of glutamine, required for nucleotide synthesis [60]. Because Ins and IGF-I act in physiological concentrations, their effect on *in vitro* cultures is not noticed in the presence of serum or plasma [61, 62].

2.1.3. Ligand-activated TFs

Steroid hormone receptors are the best known nuclear hormone receptors. In addition to these ligand-dependent TFs that bind DNA through a Zinc-finger domain, other ligand-activated TFs exist such as the aryl hydrocarbon receptor (AhR) that binds DNA through a helix-loop-helix domain.

Glucocorticoids bind the glucocorticoid receptor (GR), a nuclear hormone receptor, which translocates to the nucleus upon association with its ligand, where it functions as a transcriptional repressor and activator of gene expression. The GR homodimer binds to glucocorticoid response elements that consists of two inverted repeats. As a heterodimer with for instance STAT5, it only needs a “halfmer” GRE, i.e., a single repeat combined with the STAT5 binding site [63, 64]. The ligand of the GR is glucocorticoid (*in vivo*) produced by the adrenal gland. Several synthetic ligands have been designed to be used as immunosuppressive agents (e.g., dexamethasone—DEX). The GR exerts its immunosuppressive function at pharmaceutical levels of glucocorticoids, and as a monomer that binds and inhibits other TFs such as NF- κ B or FOS/Jun dimers [65]. In contrast, stress erythropoiesis *in vivo*, and expansion of EBL cultures *in vitro*, is induced at physiological levels of glucocorticoids and depends on dimerization of the GR and on the ligand-dependent transcription activation domain [66, 67]. Polymorphisms of the GR may alter the expansion potential of EBLs [68].

3,5,3'-triiodothyronine (T3) binds the α and β thyroid hormone receptors (TR α , TR β). T3 deficiency is associated with anemia, although it is not clear whether this is caused by a direct effect on erythropoiesis [69, 70]. The effect of T3 appears to be highly species and developmental stage specific. T3 has a potent differentiation promoting effect on avian erythropoiesis, but mouse EBLs are only sensitive to T3 during neonatal spleen erythropoiesis [71, 72]. In cultures of human erythroid cells, T3 enhances synchronous differentiation to enucleated reticulocytes [73].

StemRegenin 1 (SR-1) binds and inhibits the AhR, which mediates toxicity of environmental pollutants, by binding to specific DNA enhancer sequences. This receptor can be activated by endogenous or exogenous ligands and contributes to several physiological processes, among which cell migration, apoptosis, and cell growth [74–77]. Importantly, AhR-deficient mice have increased numbers of BM HSCs [78]. The AhR functions in complex with the AhR Nuclear Translocator to induce differentiation by regulating genes directly such as *c-MYC* and *C/EBP*. During hematopoietic differentiation, its targets also include *PU.1*, *β-CATENIN*, *CXCR4*, and *STAT5* [79]. Currently, the antagonist of AhR (SR-1) is used in clinical trials to expand CB-CD34⁺ cells prior to transplantation, thereby reducing the number of CB units needed per transplant from 5 to 1 [80]. Importantly, SR-1 also enhances *in vitro* proplatelet formation [81]. To achieve proper Plts production, MKs have to produce an extensive membrane system; the demarcation membrane system. This requires extensive lipid biosynthesis which can be inhibited by AhR [82]. Thus, blocking the inhibition of AhR with SR-1 may increase the proplatelet formation, through increasing the lipid membrane biosynthesis.

2.1.4. Erythroid-specific regulation

EPO is sufficient for steady-state erythropoiesis, when proliferative signals are mediated through the EPOR-associated RON receptor and EPO-induced differentiation is dependent on STAT5 [83, 84]. Increased erythropoiesis during development and upon blood loss requires the cooperative action of the EPOR and KIT [85]. Activation of KIT prevents differentiation and propagates the long-term proliferation of erythroid progenitors through inhibition of *FOXO3a* and activation of mRNA translation via the PI3K/mTOR pathway [86, 87]. Activation of the GR is required for stress erythropoiesis and to inhibit *in vitro* differentiation. Interestingly, glucocorticoids activate largely the same growth inhibitory genes in EBLs and in immune cells, but growth inhibition is counteracted by EPOR/KIT activation [88]. Even in the presence of serum, glucocorticoids promote selective proliferation of erythroid progenitors by both supporting erythroid proliferation and inhibiting proliferation of other myeloid and lymphoid cells [57].

Erythropoiesis is also regulated by the availability of iron, which is imported into the cell as holotransferrin via the transferrin receptor (TfR; CD71), and by selenium through selenoproteins [89, 90]. *In vivo*, erythropoiesis is dependent on the formation of erythropoietic islands that form around a central macrophage [91, 92]. Whereas CD169 macrophages are essential for erythropoiesis *in vivo*, high level of enucleation can be achieved *in vitro* in the absence of macrophages (van den Akker and von Lindern, manuscript in preparation) [93]. The complete understanding of which macrophage signals control enucleation *in vivo* may further optimize the *in vitro* production of erythrocytes for transfusion purposes.

2.1.5. MK-specific regulation

TPO is the main regulator of megakaryopoiesis, and multiple other factors can work synergistic with it. IL-6 can, in conjunction with TPO, increase hepatic TPO synthesis [94]. This effect is through the shared usage of gp130 and amplification of the same downstream JAK pathways [95]. The direct admission of IL-6, besides its effect on TPO, results in increased polyploidization and subsequently leads to an enhanced Plts production in patients [96]. IL-11 is not constitutively expressed but was shown to be induced in thrombocytopenia patients undergoing

BM transplantation. Exposure of IL-11 on HSPCs directly leads to an inhibition of NF- κ B signaling, which suppresses miR-204-5p, that targets and represses the expression of TPO [97]. Because of this effect on TPO, it has a dual role in MK differentiation, first, in the expansion of HSCs, second, in the terminal differentiation of MKs. In a similar way, IL-9 synergizes with IL-3 as well as with IL-4 and SCF to increase the yield of MKBL [33, 98]. Furthermore, the addition of IL-1 β promotes selective megakaryocytic differentiation. IL-1 β can increase the production of Plts by enhancing the effects of SDF1 and FGF4 that are produced by BM niche cells. IL-1 α was shown to induce MK rupture and is considered as a stress megakaryopoiesis regulator [99].

Inhibition of AhR by SR-1 slows the differentiation program in HSCs, leading to increased expansion, and has a direct effect on MK differentiation, by slowing and/or conditioning cells to have a more synchronized maturation [74, 79, 81]. Through its effect on PU.1, it can influence RUNX1 which is one of the TF-regulating megakaryopoiesis. The introduction of SR-1 into *in vitro* MK cultures results in increased cell size, higher polyploidization, and proplatelet production. In these cultures, a specific MK precursor population is identified that has an increased potential to produce proplatelets [81].

2.2. Cell culture models

2.2.1. Erythroid immortalized cell lines

2.2.1.1. Mouse cell lines

The most widely used immortalized mouse erythroid cell lines are MEL cells, which are EBLs transformed by Friend Leukemia virus. The viral gp55 protein activates the Epor to sustain cell growth, whereas integration of the virus upstream of *PU.1* or Friend leukemia integration 1 (*Fli-1*) induces constitutive high expression and inhibits differentiation. Leukemogenesis and the establishment of cell lines require additional inactivation of tumor suppressor protein p53 [100]. Under conditions that enable transient expansion of primary mouse cultures (serum-free medium supplemented with Epo, SCF, and glucocorticoids), lack of p53 is sufficient to establish immortalized cell lines with full differentiation potential in the absence of SCF and glucocorticoids [13].

2.2.1.2. Human cell lines

Deletion of p53 is not sufficient in human to establish immortalized erythroid cell lines. However, expression of the human papilloma virus E6/E7 proteins in EBLs differentiated from iPSC gave rise to the HiDEP cell line [101]. The E6/E7 proteins inactivate both p53 and retinoblastoma tumor suppressor proteins [102]. They are expressed from a doxycycline (dox)-inducible vector allowing for unlimited growth in the presence of EPO, SCF, and dox, and differentiation in the presence of EPO but without dox and SCF. Lack of p53 does not affect differentiation, but retinoblastoma is required for terminal erythroid differentiation [103–105]. Dox-inducible expression was also used to establish immortalized erythroid cells lines from CB (HUDEP) and from adult EBLs cultured from CD34⁺ HSPC (BEL-A) [106, 107]. The HiDEP, HUDEP, and BEL-A express embryonic, fetal, and adult Hbs, respectively [101, 107].

2.2.2. MK-cell lines

Multiple cell lines have been generated to study megakaryopoiesis with among them; MEG-01 (suspension cells) and DAMI (adherent/suspension cells) both megakaryoblastic leukemia cell lines [108, 109]. These cell lines are mostly positive for MK-specific markers (see in Section 2.3.3) but are not a homogenous population. Although they proliferate in a MKBL-like state with some spontaneous differentiation and limited terminal differentiation, they can be induced to differentiate by the addition of phorbol myristate acetate. Under these conditions, the cells can become polyploid, increase their expression of MK-associated proteins, like von Willebrand factor, and are able to produce proplatelets although with low efficiency. Mechanistic insights were uncovered with these lines; for example, the formation of long, beaded cytoplasmic extensions of MKs that yield platelets upon shear stress. This process was also observed in normal healthy MKs *in vivo*, showing the usefulness of this artificial system to study fundamental processes [110]. Despite the usefulness of these lines, their genetic background (patients with genetic abnormalities) can influence megakaryopoiesis, resulting in incomplete differentiation and potential abnormalities that could possibly be linked to their immortalization process. As such, they are suboptimal models to study megakaryopoiesis and specifically MK polyploidization, synthesis of granules, and proplatelet formation.

2.3. Primary cell culture

2.3.1. Cell source and media

RBCs and MKs can be cultured for research and clinical applications from multiple primary tissue sources including HFL, CB, BM, mobilized peripheral blood (MPB), and peripheral blood mononuclear cells (PBMC). HFL is obtained from abortions on medical indication. HFL-derived erythroid cells express HbF and can be expanded to large numbers and differentiated to hemoglobinized enucleated RBCs. For MK culture, this source is less ideal, mainly because of the harshness of the isolation method. Ethical concerns rule out HFL as a general source for transfusion, but with proper consent allows research into fetal hematopoiesis development. A widely available and ethically accepted source is CB which is commonly used for production of both erythroid cells and MKs. CB is obtained at birth when Hb-switch occurs from HbF to HbA1 (γ to β switch) and both Hb types are expressed in CB-derived cultures. The presence of HSPCs with a fetal hematopoietic program in CB has notable effects in the MK cultures. MKBL expansion is high; MK polyploidization and proplatelet formation are decreased compared to cultures of adult cells. As adult hematopoietic source, either BM or PBMC can be applied. BM is a limited source that can be more difficult to obtain, but does yield large quantities of HSPCs that can differentiate to erythroid and MK lineages. HSPCs can also be isolated from PBMCs, which is less invasive therefore a less limited source. This makes PBMC an ideal source to scale-up RBC production. The HSPC percentage in PBMC is significantly lower compared to BM, which can be enhanced by leukaphoresis and by mobilizing BM HSPCs using G-CSF (10 $\mu\text{g/kg}$) alone or in combination with CXCR4/CXCL12 inhibition [19, 111]. G-CSF alone leads to 5–30% mobilization [15]. HSPC mobilization is

caused by the downregulation of adhesion and chemokine processes and by the loss of BM macrophages [15, 112–115]. The mobilization can cause side effects for the donor, including headaches, fatigue, vomiting, muscle pain, bone pain, thrombocytopenia, citrate toxicity, etc., in which females experience the most adverse events [116]. These MPBs are ideal for RBC/Plt production because of their HSPC richness.

The MKs can be cultured in Cellgro (Corning), and both cell types can be cultured in StemSpan (Stem Cell Technologies) or other Iscove's modified Dulbecco's medium (IMDM)-based media. We generated a completely defined GMP-grade medium called Cell-Quin (Migliaccio et al. with minor modifications) that is highly efficient in expanding and differentiating EBLs/RBCs and MKs/Plts, with the ability to culture other hematopoietic progenitors and blood cell types [117].

2.3.2. Erythroid-specific culture system

Several parameters characterize the differentiation stage of erythroid cells. Expansion of EBL cultures is only possible when they maintain cell size control during their cell cycle, which is achieved by the cooperative action of SCF and glucocorticoids [12, 13, 118]. Terminal differentiation in the presence of EPO involves 3–4 cell divisions during which cells' surface marker expression changes and gets smaller due to loss of cell size control until cell cycle arrest and extrusion of their nuclei, concurrently, accumulating Hb [119]. Thus, surface marker expression pattern, cell size and morphology (enucleation), Hb content, and cumulative cell numbers are a measure of differentiation (**Figure 4A–C**). Morphological features of the cells (nuclei-cytoplasm ratio, hemoglobinization, nuclei condensation, and polarization) are commonly assessed by cytopins coupled with Giemsa/benzidine stainings (**Figure 4A**). The purity of the erythroid population and its distribution over different maturation stages can be assessed by monitoring the progression of various cell surface markers. Commonly used markers are CD36, CD71 (transferrin receptor), CD117, and the erythroid-specific markers band 3 (SLC4A1) and CD235 (glycophorin A). The generally accepted dynamics of these markers during erythroid differentiation: pro-EBLs (immature EBL stage) are characterized by $CD34^{-}/CD36^{+}/CD117^{+}/CD71^{high}/CD235^{low/-}$, while during expansion phase, EBLs gain CD235 expression and become $CD117^{+}/CD71^{+}/CD235^{+}$. In terminal differentiation phase, EBLs remain positive for CD235 and lose their expression of CD117 followed by the gradual loss of CD71, which is associated with reticulocyte formation [120].

The first human erythroid culture systems utilized the knowledge obtained from genetics, e.g., discoveries in the field of cytokines, growth factors, and their receptors. In these first protocols, HSPCs were expanded in the presence of IL-3, SCF plus or minus IL-6. It is followed by a step in which the resulting erythroid progenitors were further expanded and differentiated in the presence of EPO [121]. This protocol was modified, using low EPO concentrations in step 1 (0.5 U/ml) and high concentrations (>3 U/ml) in step 2 [122]. Others used high concentrations of EPO throughout step 1 and step 2 [123, 124]. These two-step protocols are based on the original protocol of Fibach and coworkers who employ IMDM supplemented with serum or plasma [121]. Serum and plasma contain factors that support erythropoiesis in these cultures. The major factor in the serum is transforming growth factor β (TGF β), which is a potent differentiation factor for erythropoiesis [57, 125]. These cultures

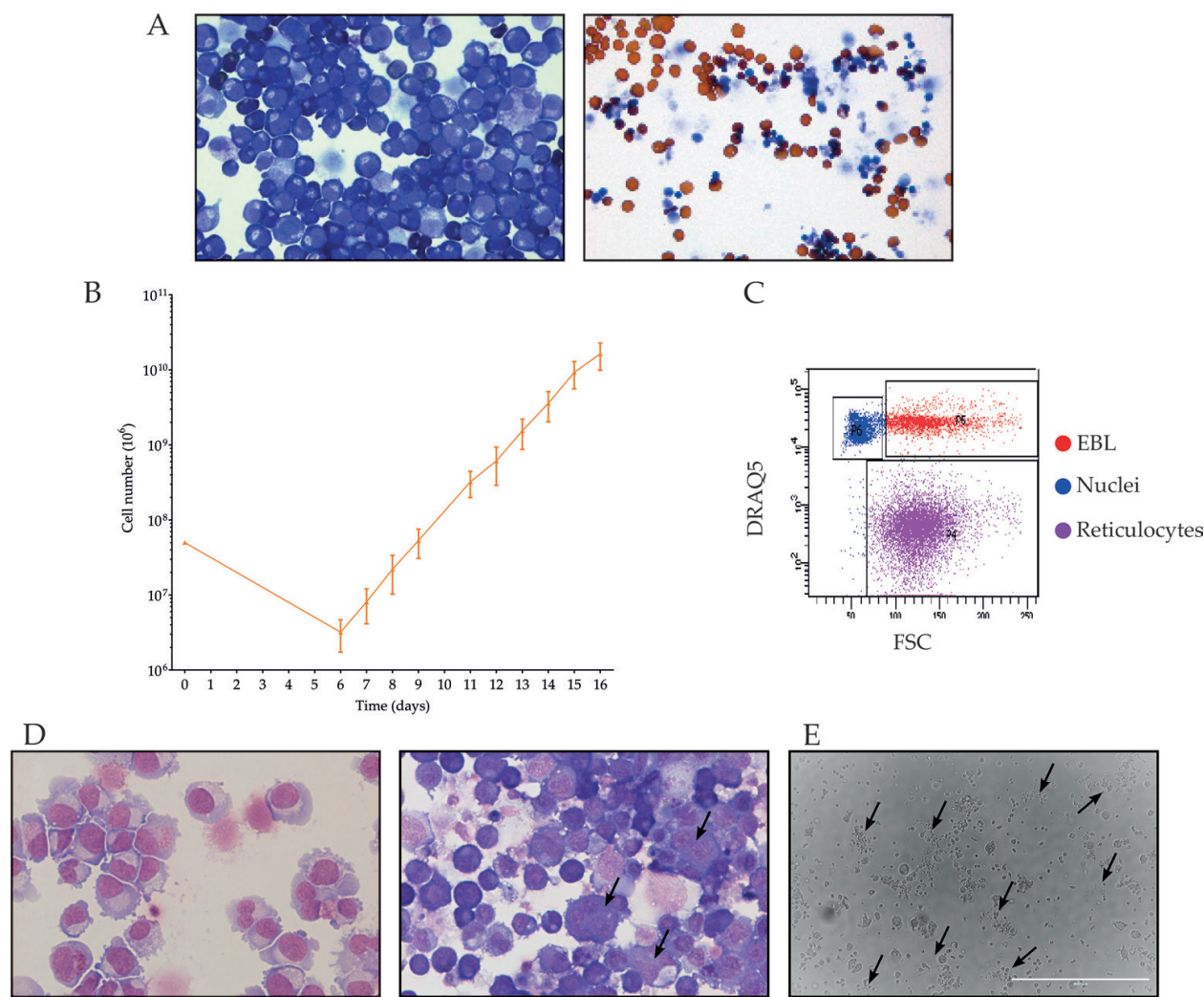


Figure 4. Characteristics of erythroid and megakaryocytic cultures. (A) Erythroid-specific morphology by cytopsin with Giemsa/benzidine staining. Left: pro-EBL, right reticulocytes. (B) Erythroid expansion growth curve from PBMCs ($n = 4$). (C) Flow cytometry of terminal erythroid differentiation, DNA staining by DRAQ5 resulting in three distinct populations: DRAQ5⁺ big cells: nucleated EBLs (red); DRAQ5⁺ small cells: nuclei (blue); DRAQ5⁻ cells: enucleated reticulocytes (purple). (D) MK-specific morphology by cytopsin with MGG-staining. Left: MKBL; right: polyploid MK (arrows). (E) Proplatelet-forming MK (arrows (beads on a string)).

show a high degree of spontaneous differentiation, which is often used to study expansion and differentiation of EBLs carrying a genetic defect. The quality differences between serum batches and the use of different cytokines make these culture protocols difficult to compare. Two major changes increase the yield of these erythroid cultures and enable synchronous differentiation. First, glucocorticoids cooperate with SCF to retain pro-EBLs and early basophilic EBLs in their undifferentiated state [57, 126]. Second, serum-free medium avoids the differentiation promoting effect of TGF β . However, the available serum-free media are sub-optimal and require complementation with lipids [73]. Even better expansion is achieved with a serum-free medium optimized for expansion of EBLs [117]. The differentiation arrest in the presence of glucocorticoids and the absence of serum enables the expansion of a homogeneous early EBLs culture that can undergo up to 20 cell divisions to achieve a million-fold

expansion [73, 119]. Using Cell-Quin, we can obtain 2×10^{10} EBLs within 16 days, starting from 5×10^7 PBMCs (**Figure 4B**). Expansion of EBLs in the presence of serum and in the absence of glucocorticoids irrevocably results in differentiation and transfer of the cells to differentiation conditions. Of note, addition of glucocorticoid agonists in a serum-based culture will still induce spontaneous differentiation due to the presence of TGF β [57]. At any moment during expansion phase, cells can be transferred to differentiation conditions in which the medium is supplemented with EPO, Ins, and low level of plasma/serum [119, 120]. Although expansion of EBL cultures is achieved in serum-free medium, terminal differentiation to enucleated cells requires at least 2% serum or plasma [119, 120]. Using Cell-Quin medium, we currently obtain >90% enucleation, a deformability that corresponds to values between freshly isolated reticulocytes and erythrocytes, and normal oxygen association and dissociation values (van den Akker and von Lindern, manuscript in preparation). We use DRAQ5 staining coupled with flow cytometry analyses to quantify reticulocyte/nuclei/nucleated cell ratio (**Figure 4C**). Flexibility is measured on a ARCA, and oxygen binding by the Hemox analyzer [127, 128].

2.3.3. MK-specific culture system

Commitment of MKBL and differentiation of MKs can be monitored by the expression of cell surface markers and by the morphological features of the cells (**Figure 4D** and **E**). MKBLs are characterized by CD34⁺/CD41a⁺ expression and blast-like morphology. In terminal differentiation, MKs gradually lose their expression of CD34⁺, leading to a subdivision of stages: early MKs are CD34⁺/CD41a⁺/CD42⁺ and late MKs are CD34⁻/CD41a⁺/CD42⁺.

To obtain large numbers of MKBLs, SCF/FL and TPO are used during the first 4–7 days of cultures started from CD34⁺-HSPCs. TPO without SCF and FL allows terminal differentiation to proplatelet-forming cells. To increase the expansion potential, IL-3 can be included only in the initial phase as its prolonged exposure directs the HSPC toward the monocyte/granulocyte lineage. With the addition of IL-6, the MK specification and TPO signaling can be enhanced. With the addition of either IL-1 β , IL-9, or IL-11 during the first phase of CD34⁺ differentiation, MK commitment is enhanced instead of progenitor proliferation. It is important to determine the main goal of an experiment before starting the culture: does the experiment require large numbers of MKBLs, or should MK enrichment be maximal, because a good expansion of MKBL tends to compromise terminal differentiation and vice versa (Hansen and van den Akker, unpublished results). Factors such as IL-1 β and IL-9 increase polyploidization, formation of proplatelets, and Plts shedding. There is some concern about using IL-1 β , because of its proinflammatory nature. Particularly, as it is closely related to IL-1 α , and the increased Plt shedding may cause rupture of MKs [33, 99]. To introduce proplatelet formation, IL-6 can be used in high concentrations (>100 ng/ml), by itself or in combination with TPO. SR-1 influences megakaryopoiesis on an early and late stage of the culture, as described above, having a positive effect on the expansion of HSC and terminal differentiation of MK [80, 81]. During the terminal stages of MK cultures (during proplatelet formation), it becomes increasingly essential to prevent the activation of the MKs and produced proplatelets. The addition of heparin prevents the coagulation of plasma added to the media but cells are still able to clump together, thereby having a negative impact on the differentiation and

proplatelet production. To prevent activation, signaling via the GPIIb/IIIa (ITGA2B) receptor can be blocked with tirofiban hydrochloride monohydrate. Whereas an MK sheds thousands of Plts *in vivo*, shedding large amounts *in vitro* from a single MK has not yet been achieved. *In vivo* of this process requires that proplatelets extrude between the endothelial cells of the blood vessel wall into the capillaries. This increases level of SP1 among others, combined with shear stress of the blood flow is required for the Plts to be released. To mimic this *in vitro*, several specialized bioreactors are being tested (see Section 2.5).

2.4. Erythropoiesis/megakaryopoiesis from iPSC

Pluripotent stem cells offer a novel approach for developmental studies, drug screening/discovery, disease modeling, and regenerative medicine. ESCs originate from the inner cell mass of a blastocyst stage embryo, while iPSCs are somatic cells that are reprogrammed back to this embryonic stage [129–132]. Hematopoietic differentiation of ESC/iPSC cells follows the various stages of blood development from early embryonic stages (**Figure 2A**). This offers a valuable tool to study early human hematopoiesis which is difficult because of ethical issues and tissue availability. Besides, differentiation of iPSCs opens opportunities for large-scale manufacture of blood products with the expectancy of clinical application [133]. Several groups showed the potential of ESCs in blood cell production, the source which was later replaced by iPSCs with similar outcome including our group (**Figure 5**) [134–140]. The published protocols generally include four culture phases: (1) mesoderm induction, (2) hematopoietic/erythroid/megakaryocytic commitment, (3) expansion of the specific cell pool, and (4) terminal maturation. The hematopoietic differentiation phases *in vitro* are directed by stepwise addition of cytokines. This is commonly achieved by BMP4, bFGF, and

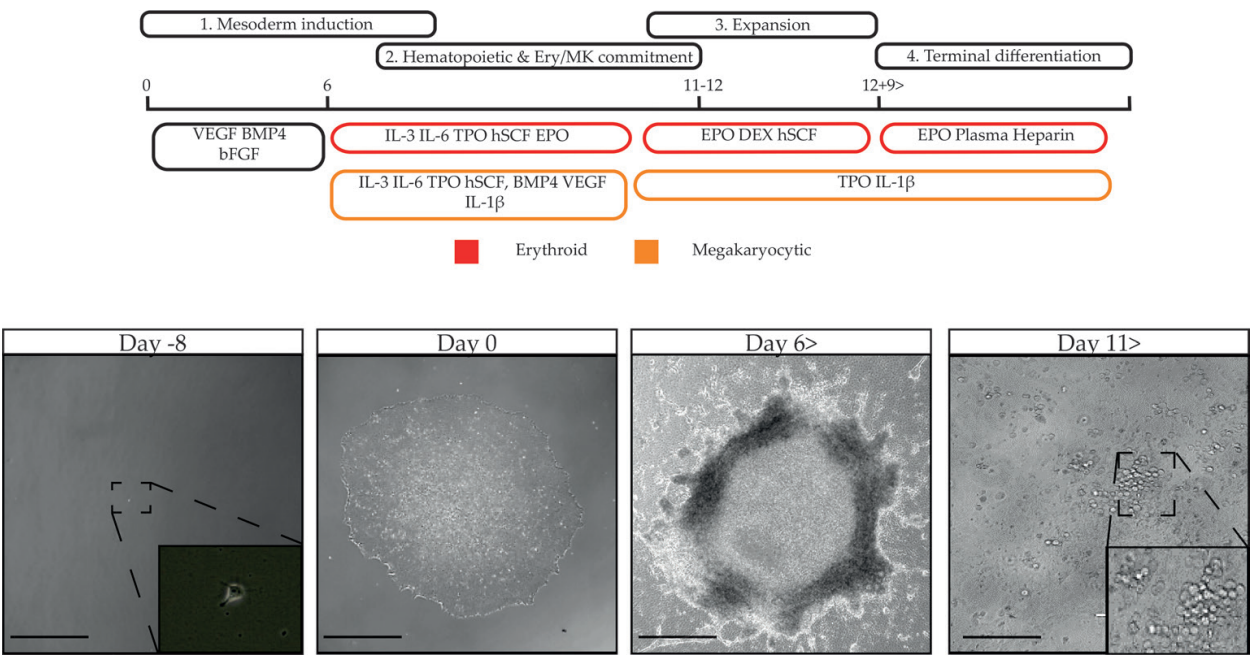


Figure 5. Erythroid/MK differentiation of iPSC according to Hansen et al. showing the different phases of differentiation, with their corresponding growth factor combination and morphological changes [136].

VEGF that drive the cells toward mesoderm, followed by the addition of IL-3, IL-6, SCF, and TPO stimulating hematopoietic specification. Lineage-committed progenitors can be further differentiated toward mature cell types, which is achieved by the combination of medium, growth factors, and hormones. Although most of the differentiation protocols are following the abovementioned scheme, including the listed growth factors, there are multiple technical variations during iPSCs differentiation toward hematopoietic lineages. Two main technical details underlie the major differences in the applied protocols: (i) the induction of differentiation as a 2D monolayer versus 3D embryoid body formation and (ii) the use of coculture with feeder versus feeder-free systems [141–144]. The choice of the differentiation system depends on the application need. 3D systems more closely resemble the *in vivo* process in comparison to 2D systems, offering a tool to study embryogenesis [141, 144–147]. 2D systems, however, are relatively simple, more reproducible, and therefore suitable to scale up production, enabling clinical application [77, 148–150]. The choice of feeder-based or feeder-free differentiation similar to 2D/3D systems also depends on the purpose of the specific research question. Feeder-based coculture systems more resemble the niche, including secretome and cell-cell contact; however, for future clinical application, feeder-free systems are imperative [142, 143, 151, 152]. Protocols can also differ in timing, in the applied media and cytokine cocktails used, which makes comparisons between research groups and methods difficult. Stemline II (SIGMA) is a widely applied base medium during the first and, in some cases, the second phase in feeder-free settings [138, 149, 153]. From the second/third phase onward, the same basic medium are applied that are generally used for other definitive blood cell types such as IMDM with serum/holotransferrin/lipid/Ins supplementation or StemSpan (Stem Cell Technologies) [137, 138, 150, 153]. From the second-step onward, we apply Cell-Quin which in comparison to StemSpan was more efficient in their iPSC-erythroid expansion potential (**Figure 6A**). Some methods still include BMP4, VEGF, and bFGF (or either of them) at this second stage, typically in embryoid body-based system because the 3D structures are less homogenous. Therefore, the transition between phases is not entirely uniform and clear [140, 149, 154, 155]. The few feeder-free 2D systems that have been published mostly do not rely on these three additional factors during commitment phase [148, 149]. FL is also used in erythroid/MK commitment cytokine mixes to improve progenitor expansion [140, 154]. MK commitment/expansion is always based on TPO with or without the addition of multiple other cytokines (e.g., IL-1 β , IL-9, IL-11) [99, 136, 156–158].

As pointed out before, early erythropoiesis/megakaryopoiesis (yolk sac) in humans is not well studied, resulting in a lack of knowledge on the regulatory program at these developmental stages. Therefore, the generally applied cytokines might not ideally mimic the *in vivo* situation or the iPSC-driven hematopoietic program. This could underlie inefficient iPSC to RBC/MK differentiation. For example, EPO is applied in all systems to induce erythropoiesis; however, the role of EPO during primitive wave is not entirely clear. Disruption of *EPO* and/or *EPOR* causes embryonic lethality in mice due to the failure of the definitive fetal liver erythropoiesis with reduced primitive erythropoiesis, suggesting that *EPO* and *EPOR* are already functional in early yolk sac [24, 25, 39–41]. However, others showed that additional *EPO* did not affect heme synthesis in early mouse embryos [159]. Furthermore, Malik et al. found that *EPOR*-null embryos have normal number of primitive, early stage progenitors but subsequently develop anemia with loss of primitive EBL [24]. In line with these findings, the

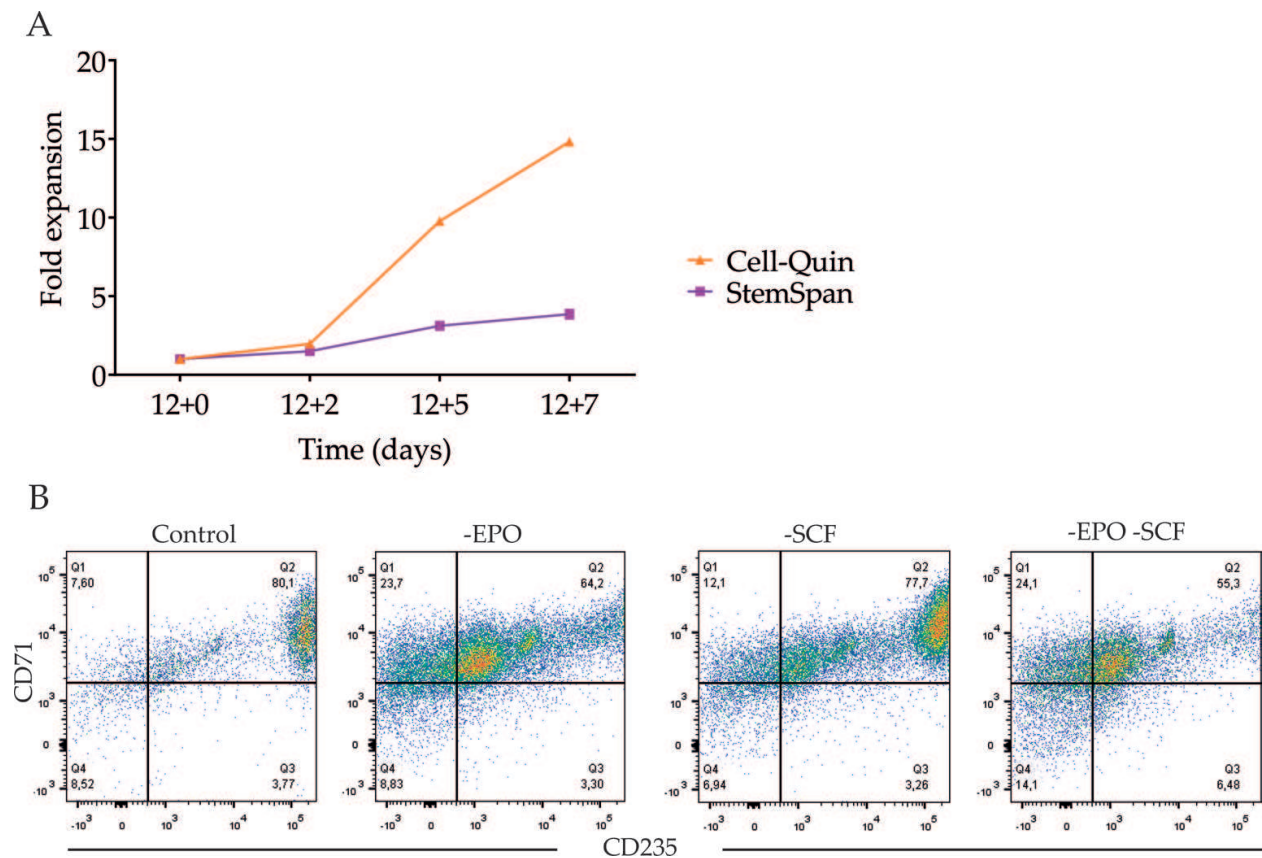


Figure 6. Optimization of iPSC-erythroid differentiation cultures. (A) iPSC-derived erythroid cells arose (D12+0) and expanded in Cell-Quin or StemSpan media. (B) Representative FACS-plots of iPSC-derived erythroid cell (D12 harvest), with or without EPO and SCF from day 6 onward.

same group concluded that EPO signaling is not critical for the survival of human primitive erythroid progenitors, but have a less understood role to promote proliferation and maturation of these cells. Since the role of EPO is controversial in yolk sac erythropoiesis, we tested whether iPSC-erythroid commitment is EPO-dependent. We have tested the requirement of the two most important growth factors for definitive erythropoiesis in various combinations; EPO and SCF (Control), without EPO (-EPO), without SCF (-SCF), and without both cytokine (-EPO and -SCF) [136]. Without EPO, we noticed lower harvest rate/colony number and the loss of CD71/CD235 population. The erythroid commitment was not affected by the deprivation of SCF; however, the addition of SCF together with EPO resulted in a more pure CD71/CD235 population (**Figure 6B**). These data suggest that EPO is required to allow early erythroid commitment while the role of SCF is not entirely clear.

Introduction of erythroid/MK-specific TFs into iPSC-derived hematopoietic cells, often named “forward reprogramming”, is being pursued as an approach to improve differentiation outcome. HOXA9, ERG, RORA, SOX4, and MYB have been introduced into human pluripotent stem cells. Engraftment into NSG mice resulted in erythroid cells, which were more skewed to definitive erythropoiesis (lack of embryonic Hbs, mainly HbF and some HbA, some enucleation) compared to TF-free counterparts [160, 161]. These results suggest the possibility of

more mature erythroid cell production from iPSCs if certain TFs are included; however, the *in vitro* feasibility is not provided presently. To direct the cells more lineage-specific, other lineage instructive TFs may be used. GATA1, FLI-1, and TAL1 (MK-specific TFs) can be over-expressed to direct iPSC to MKs, thereby achieving ~100% MK yield within 15 days [158]. Besides this set of genes, there are other combinations that are used to achieve a similar aim [156, 162].

The technical differences between published differentiation methods are leading to slight discrepancies in marker expression pattern, purity, yield, and stage of development. However, currently all published methods are limited by technical pitfalls, including the production of developmentally immature (nonadult) cell types which may be the cause of low yield and difficulty to terminally differentiate toward functional end stage blood cell types (e.g., low enucleation potential of iPSC-erythroid cells and low efficiency of iPSC-Plt formation).

2.4.1. Marker expression pattern of differentiating iPSC cells

The purity of the iPSC-derived erythroid population, and its distribution over different maturation stages can be assessed by the erythroid-specific markers used for definitive erythroid culture systems (Section 2.3.2); however, their progression differs in some aspects. Based on our differentiation scheme (**Figure 5**), we recognize three maturation stages: (i) an early erythroid population (harvest at day 10–14) is $CD71^{high}/CD235^{high}/CD36^{med/high}$, which is not yet hemoglobinized and displays big nuclei [136]. Furthermore, the cells are negative for CD18 (myeloid lineage marker) confirming specification toward the erythroid lineage; (ii) a 100% pure erythroid population (day 7–9 expansion) is $CD71/CD235/CD36^{med}$ with some spontaneous differentiation, which is recognized by hemoglobinization and condensation of the nuclei; (iii) a mature erythroid population (D7-14 terminal differentiation) gives rise to $CD235^{high}/CD71^{high/med}/CD36^{low}$ cells. However, there is a slight CD71 decrease associated with reticulocyte formation, and iPSC-derived erythroid cells do not become CD71 negative. Morphologically, these cells were somewhat different from their definitive counterparts. Despite hemoglobinization, nuclear condensation, and polarization, we do not observe a decrease of cytoplasm size and the enucleation potential is poor. Technical variations in the published methods (timing, added growth factors) cause notable differences in the erythroid marker expression pattern; therefore, it is hard to compare and/or draw general conclusions. The emergence of $CD71^{+}/CD235^{+}$ population is generally reported with purity discrepancies. For example, Yang et al. [163] reported 80% $CD71/CD235$ purity (with $CD34^{+}/CD43^{+}$ preselection and OP9 coculture), Salvagiotto et al. [148] by a feeder-free monolayer system reached 40% pure population, while Kobari et al. [135] with EB-based induction reached 98–99% comparable to our findings. The pattern of CD36 expression is not entirely clear. Mao et al., for example, used a four-step differentiation scheme, including an AGM coculture induction step, and defined the following gene expression profile: early definitive EBLs derived from $CD235^{+}/CD34^{low}/CD36^{-}$, and they develop to $CD235^{+}/CD34^{-}/CD36^{-/low}$, $CD235^{+}/CD34^{-}/CD36^{-}$ cells in sequence [164]. Others including us found high CD36 expression during the early erythroid stage [136].

The kinetics during differentiation/maturation of MK from iPSC follow the same steps as from definitive CD34⁺ cells, namely MKBL (CD34⁺/CD41a⁺), early MK (CD34⁺/CD41a⁺/CD42⁺), and late MK (CD34⁻/CD41a⁺/CD42⁺). The MKs can undergo some polyploidization albeit not in similar level as *in vivo* or primary CD34⁺ cultures. MKs derived from mouse iPSC can form proplatelets but also in low numbers [157, 165–168]. These Plts can be activated and contribute to clot formation and wound healing when transfused to injured mice, showing that iPSC megakaryopoiesis, although still inefficient, leads to functional Plts. The MK-specific cell surface markers can be also used for iPSC-MKs with the exception of CD41a. This marker is also an early endothelial/hematopoietic marker. Therefore, it is essential to always use it in a combination, for example, with CD42 to confirm the specific MK commitment. Despite this, most groups report MK percentage only based on CD41a expression (~30–80% MK induction) [136, 150, 168]. With our method, we are able to achieve an average purity of 78% (CD41a) that can be easily used in scaleup production [136].

2.4.2. Developmental stage of iPSC-derived erythroid/MK cells

Human ESC/iPSC-derived erythropoiesis/megakaryopoiesis, with the current knowledge, do not reach the adult definitive stage, but give rise to a mixture of primitive and definitive fetal/adult cells. Very little is known about human erythropoiesis/megakaryopoiesis in the early stages, between days 17 and 23 of embryogenesis (yolk-sac, AGM region) due to the fact that abortions are primarily performed at later fetal stages and in addition have serious ethical concerns. Hbs are commonly used to distinguish between developmental waves; however, HbF expressing RBCs both arises from yolk sac and later from fetal liver, and momentarily, there is a lack of markers, which can clearly distinguish these two waves (**Figure 2A** and **B**). The iPSC-derived erythroid cells predominantly express HbF, in addition to embryonic types of Hb. We and others also showed the presence of a small portion of adult Hb [135, 136, 153–155]. From the embryonic type of globins, both the presence of Gower 1 and Gower 2 Hb has been reported, but the ratio greatly differs between methods [135]. The presence of Gower 2 Hb indicates that the cells are capable of the first globin switch (ζ to α) to provide more mature primitive-state RBCs. Interestingly, the presence of adult types of Hb also differs between protocols as some group were able to show HbA1 or the presence of β chain, whereas others, including us, observed mainly HbA2 [135, 155]. It is unknown whether primitive erythropoiesis *in vivo* goes through a stage that corresponds to iPSC-derived erythroid cells (Hb^{mid}, HbF^{high}, HbA^{low/-}) or whether globin synthesis is impaired in these cells. The globins, however, may not be the most accurate markers to define the waves. Altogether, these findings make it difficult to define the state of iPSC-RBCs with respect to their developmental stage. From a technical point of view, these cells are able to produce definitive RBCs; therefore, they potentially can give rise to the required therapeutic product. Better understanding of the underlying regulatory mechanisms such as the site where erythropoiesis/megakaryopoiesis takes place, and Hb switches during development might pave the way for the necessary improvement of the current methods.

Unlike in the erythroid lineages where the expression of stage-specific Hbs can be used to determine the ontogeny phase, this type of readout is not available in the MK lineage. There are, however, intrinsic differences between megakaryocytic cells derived at different sites

during ontogeny. For instance, polyploidy is a measure of ontogeny *in vivo* as yolk sack MK exhibits low ploidy (4–8N), whereas adult MK reach >64N [14, 169, 170]. Besides the lower polyploidization, the number of Plts that are produced follows the same trend from a low number per MK in embryonic/fetal tissues too high numbers of in adult MK, linking polyploidization and MK size to Plts production [170]. However, ploidy levels and Plt production *in vitro* are generally lower compared to their *in vivo* counterparts; therefore, it is not a good marker to access iPSC-MK development stages. The current best approach would be to use data from erythroid cultures and their ontogeny stage/wave and extrapolated this to the MK development because of their close relationship. However, investigation of purified megakaryocytic cultures of defined ontogeny stages through, e.g., RNAseq or mass spectrometry could yield specific makers.

2.4.3. Expansion potential/yield of iPSC-erythroid/MK cells

The final yield of our method is relatively high; however, the comparison with other methods is difficult due to technical discrepancies. There are different ways to calculate the final yield, which also depends on the iPSC maintenance system (single cell vs. clumps) and on the induction system (2D or 3D), resulting various ways to report the final yield. Single cell-seeded iPSC cultures can be normalized both to the initial number of seeded cells or to colony number. Furthermore, the comparison of absolute cell number produced (harvested) between 2D and 3D systems is not entirely realistic because of the different nature of the two cultures. We use single cell-seeding, which allows to calculate the yield/iPSC and yield/colony number to represent differentiation efficiency. In our hands, one iPSC colony can give rise on average 5.6×10^6 erythroid cells after 9 days of expansion. While a single iPSC gives rise to $\sim 8 \times 10^3$ erythroid cells (harvest) and subsequent 9 days expansion results in $\sim 2 \times 10^5$ erythroid cells/iPSC on average [136]. The expansion potential (from harvest day) compared to definitive cell types remains relatively low, in line with other methods irrespective of culture condition (**Figure 4B**). The terminal maturation of iPSC-erythroid cells toward enucleating reticulocytes is inefficient with the existing methods and is currently one of the major hurdles to overcome. In our hands, matured iPSC-erythroid cultures had 30–40% enucleation rate based on their nuclei count; however, the resulting reticulocytes appeared to be instable. Altogether, iPSC-derived erythroid cells are able to expand but for limited time and length, with suboptimal enucleation capacity. Probably, this is also coming from the fact that iPSC-derived erythroid cells, based on their globin expression, do not entirely correspond to a fetal/adult definitive wave.

MK yield from iPSCs is higher than CD34⁺ differentiation (6.9×10^3 cells/iPSC). Unlike the erythroid system in which differentiation can be inhibited for several days by the addition of glucocorticoid analogues, the megakaryocytic system currently lacks such a specific expansion advantage. As a result, iPSC-MK yield and purity is currently low compared to the iPSC-erythroid yield. Even though the yield of MK is low, a small number of MK still could produce significantly large amount of Plts. The *in vivo* production of 2000–8000 Plts/MK is not achieved by far (1–50 Plts/MK in static condition) [4, 5, 81, 168]. Initiatives to increase this yield are needed but will require increasing our knowledge on the later stages of proplatelet formation, which is a current hurdle to overcome in culture conditions and further discussed in the next paragraph devoted to bioreactors (Section 2.5).

2.5. Bioreactors

The production of cultured red blood cells for transfusion purposes has been the holy grail for transfusion medicine. However, a main challenge is the well-described limitation in cell density during the expansion phase [171]. A single transfusion unit contains 2×10^{12} RBCs. Conventional cultivation systems using dishes or flasks can reach up to 10×10^6 cells/mL, meaning that more than 1000 L of culture would be required for the manufacture of a single transfusion unit [172]. Handling of such large volumes is impractical if static culture conditions are maintained. Thus, multiple bioreactor designs have been proposed to improve the volumetric productivity of the process (produced cells/volume of medium). A static culturing system mimicking BM tissue has been proposed, in which cells are grown in a porous scaffold and nutrients are continuously fed through hollow fibers, while used media containing waste from metabolism are removed [173]. This system allows to have continuous replenishment of spent components in the media, while it is possible to re-use some of the most expensive components such as growth factors and transferrin. Also, it separates cells from large shear stress sources. Although an optimal design of this system could lead to the production of transfusion units at competitive costs compared to the price of rare blood units, it would require significant improvements in transfer of nutrients and matured RBCs between the scaffold and the inflow/outflow streams [174]. Mass transfer limitations in diffusion-governed systems can be tackled with agitation. It is relevant to note that conflicting reports have been made on the effect of shear due to agitation in *in vitro* erythropoiesis [172, 175]. Nevertheless, important advances have been made toward culture of EBLs in stirred reactors. Enucleated RBCs were produced in microbioreactors (<20 mL) in which the effect of shear stress on expansion and enucleation of EBLs was evaluated. Gas sparging caused cell death, whereas stirring enhanced enucleation [172]. Expansion can also be performed in shake flasks (40 mL) and stirred tank bioreactors of larger volumes (500 mL) with similar growth kinetics [176]. Hybrid systems combining mechanical agitation and growth of cells in porous materials have also been proposed. This type of systems could improve mass transfer while protecting cells from shear, but harvesting of mature cells from these carriers is still a challenge that must be addressed.

The high sensitivity of MK to shear stress renders culturing and flow cytometry assays challenging. However, it can also be exploited to generate *in vitro* Plts. Multiple techniques can be used to induce the culturing and shedding of Plts like pipetting or the use of flow chambers [157, 165–168]. These approaches mimic more the *in vivo* situation of Plts formation where they are shed in the vasculature, in contrast to static cultures. Plt shedding can be induced by repetitive pipetting of the cultured cells with a hand pipette (p1000) [81]. There is a risk that too much pressure is enforced on the cells, causing them to lyse instead of shed. A more sophisticated method of *in vitro* Plts production is the use of flow chambers, which come in different versions [167, 177]. The most basic one has a linear flow with a surface that is coated to which MKs can attach [165, 167]. These versions were further developed to chambers that include small gaps where MKs are trapped and can extend their cytoplasmic extensions through the gap where they start forming Plts [165, 177]. Another flow chamber setup is the use of pillars in the chamber instead of gaps where the MKs can attach. Coating with a various matrices like fibronectin, von Willebrand factor, or TPO can enhance the efficiency of the Plts generation. In addition, the flow rates that are produced in these chambers can be regulated.

The produced Plts in these systems can be harvested and used for functional test. The aforementioned approaches can also be combined in a way that the culturing and differentiation of iPSC toward MK is performed on coated beads in a stirred spinner flask bioreactor, where subsequently also the Plt production is induced and enhanced, although with a relatively low Plt yield (1 Plt/MK) [168]. Recently, Ito et al. showed that in the vasculature, MKs are not only exposed to shear stress but also to turbulence during Plt sheading, where the process was implemented in their bioreactor [178]. By using their iPSC lines with inducible c-MYC-BMI-1 and BCL-XL expression, they were able to produce a thrombocyte transfusion unit in an 8 L system. This approach proves the possibility of large-scale Plt production but still with a discrepancy between *in vitro* (80–100 Plts) and *in vivo* (2000–8000 Plts) Plt production capacity.

2.6. Clinical trial

The ability to produce large numbers of enucleated, hemoglobinized RBCs opens the perspective of producing cultured red blood cells (cRBC) for transfusion purposes. The feasibility to do so has been demonstrated by the team of Luc Douay who cultured 1 mL of packed cRBC from CD34⁺ HSCs and transfused it to a healthy volunteer, with a cRBCs half-life of 26 days after injection [133, 179]. Donor-derived RBC transfusion is a cornerstone of modern medicine in the treatment of trauma, chronic anemia, and in surgery. The existence of 30 blood group systems, such as the ABO and Rhesus system, generates at least 300 distinct blood group antigens [180]. Recurrent transfusions carry an inherent risk on alloimmunization to non-identical blood group antigens, which complicate further transfusions. Besides, this cellular therapy is dependent on donor availability with a potential risk of blood-borne diseases. *In vitro* derivation of cRBCs allows their thorough characterization, therefore providing access to better matched product. Improved cell culture protocols may eventually enable us to generate cRBCs for transfusion purposes from iPSCs that were selected and/or modified to lack most blood group antigens with the advance of donor-independency offered by iPSCs source.

In a recent publication, Ito et al. [178] were able to generate Plts in transfusion quantities, where the functionality of these Plts was shown in *in vivo* mouse experiments. This work gives a feasible prospect of clinical trials, which still requires the system to be converted to GMP grade [178].

Our culture protocol from human PBMC to RBC

- day 0 expansion: purify PBMCs by Ficoll and seed in StemSpan or Cell-Quin supplemented with 1 ng/ml human IL-3, 2 U/ml EPO, 10 ng/ml hSCF, 10⁻⁶ M DEX at 10 × 10⁶ cells/ml, 37°C, and 5% CO₂.
 - Optional: to remove remaining RBCs after Ficoll purification, the use of RBC lysis buffer is suggested.
- day 2 and 4: replace half of the medium; add all factors to the medium except for IL-3.
- day 5: EBLs appear as large (nongranulated) blasts.
 - Optional: to remove remaining lymphocytes, purify the culture by density centrifugation on a 1.075 g/ml Percoll gradient.

- day 6–20: put the cells daily or every second day to $0.5\text{--}0.7 \times 10^6/\text{ml}$ medium supplemented with EPO, SCF, and DEX (concentration is same as day 0).
- day 0 terminal differentiation: wash the cells twice with PBS and re-seed in medium supplemented with 10 U/ml EPO, 1 mg/ml holotransferrin, 2–5% plasma, 5 U/ml heparin at $2 \times 10^6/\text{ml}$.
- day 9–14: let the cells differentiate with half media change every 2–3 days.

Our culture protocol from CD34+ to MK

- day 0: collect CD34⁺ cells by MACS isolation from CB, BM, PBMC, or MPB and seed in Cellgro or Cell-Quin with 100 ng/ml FL, 50 ng/ml hSCF, 50 ng/ml TPO, 20 ng/ml IL-6 at 1×10^6 cells/ml, 37°C, and 5% CO₂.
 - Re-seed cells when concentration exceeds 2.5×10^6 cells/ml, otherwise keep cells as undisturbed as possible at stable CO₂ levels.
- day 4: cells start to commit to the MK lineage (~10–20%), cells are collected and spun down at 200 g to start terminal differentiation.
- day 0 terminal differentiation: re-seed cells in media (Cellgro or Cell-Quin) supplemented with: 50 ng/ml TPO and 10 ng/ml IL-1 β at 0.5×10^6 cells/ml.
 - During terminal differentiation, the addition of tirofiban hydrochloride monohydrate and heparin is recommended.
 - Pipetting should be kept to minimal.
 - 1 μM of SR-1 can be used to increase polyploidization and Plt production.
- day 11: cells will be committed to the MK lineage (80–100%) and consist of MKBLs and early MKs.
 - Cell collection from these days onward should be performed using 2 mL or larger pipettes and avoid the usage of hand pipettes to circumvent cell lysis and shear stress [157, 166].
 - Centrifugation steps are on 150 g, low ramp, and brake.
 - Flow cytometry techniques on unfixed MKs at this stage will induce granule release and proplatelet formation.
- day 12–16: MK cells will mature to late MKs and start producing proplatelets.
 - Centrifugation steps are 100 g, low ramp, and brake.
 - CB starts proplatelet production earlier than adult sources.
 - Proplatelets can be harvested using Plts isolation protocols.
 - Proplatelets are easily activated, treat them as regular Plts.

3. Conclusions

As described in this chapter, there are multiple protocols to culture RBCs and MKs from a variety of hematopoietic tissues. Depending on the goal (fundamental research, drug screening, or clinical applications), one should consider beforehand which source can be used. For clinical applications, the use of a fully defined unlimited source would be preferred. For this, iPSC (generated with nonintegrating method) hold great potential but differentiation toward RBCs and Plts has to be improved.

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Conflict of interest

None.

Abbreviations

AGM	aorta-gonad-mesonephros
AhR	aryl hydrocarbon receptor
bFGF	basic fibroblast growth factor
BM	bone marrow
BMP4	bone morphogenetic protein 4
CB	cord blood
cRBC	cultured red blood cell
DEX	dexamethasone
dox	doxycycline

EBL	erythroblast
EHT	endothelial to hematopoietic transition
EMP	erythroid-myeloid progenitor
EPO	erythropoietin
EPOR	EPO receptor
ESC	embryonic stem cell
FL	FLT-3 ligand
FLI-1	friend leukemia integration 1
FLT-3	Fms-like tyrosine kinase 3
GR	glucocorticoid receptor
Hb	hemoglobin
HE	hemogenic endothelium
HFL	human fetal liver
HSC	hematopoietic stem cell
HSPC	hematopoietic stem/progenitor cell
IL	interleukin
Ins	insulin
InsR	insulin receptor
iPSC	induced pluripotent stem cell
MK	megakaryocyte
MKBL	megakaryoblast
MPB	mobilized peripheral blood
MPP	multipotent progenitor
PBMC	peripheral blood mononuclear cell
Plt	platelet
RBC	red blood cell
SCF	stem cell factor
SR-1	StemRegenin 1
TF	transcription factor

TPO	thrombopoietin
T3	3,5,3'-triiodothyronine
VEGF	vascular endothelial growth factor

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