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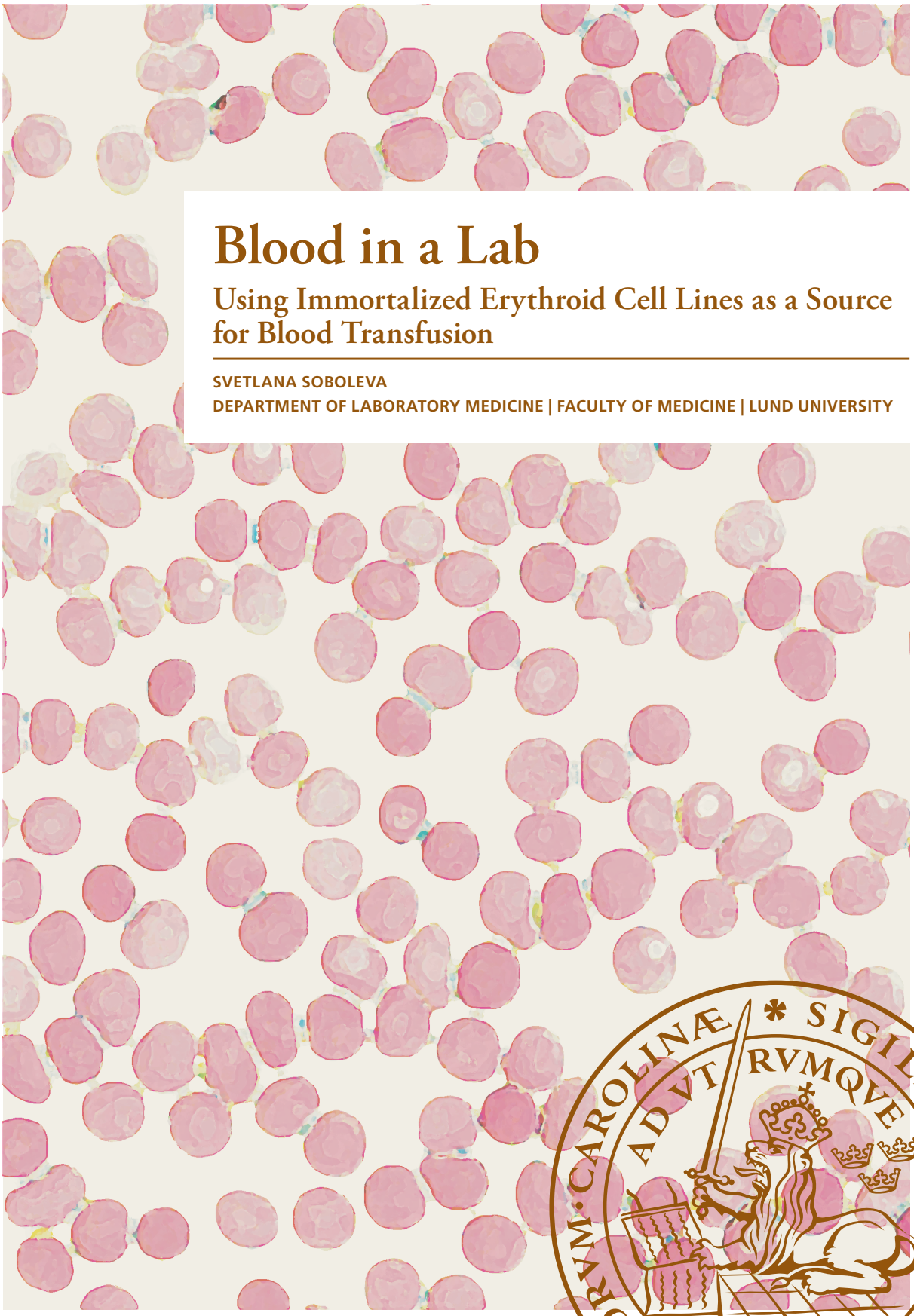

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Using Immortalized Erythroid Cell Lines as a Source for Blood Transfusion

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DOCTORAL DISSERTATION

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Abstract

A significant step towards efficient *ex vivo* red blood cell (RBCs) production. There are already many established methods using various cells as starting materials such as $CD34^+$ hematopoietic stem and progenitor cells (HSPCs), embryonic stem cells (ES), or induced pluripotent stem cells (iPSCs). Immortalized erythroid cell lines offer an alternative up-and-coming starting material in this field. Still, reaching high enucleation efficiency remains a challenging goal and the least-understood step of the RBC production protocol.

In Paper I, we performed chemical compounds screening to induce enucleation of a the human iPS-derived erythroid progenitor cell line (HiDEP). A few compounds belonging to the group of histone deacetylase inhibitors (HDACi) induced enucleation of HiDEP cells. However, they were fragile, possibly contributed to by the main cytoskeletal gene SPTA1's downregulation. Using the CRISPR-activation system, we therefore restored expression of SPTA1 and saw improvement with less cell fragility and increased frequency of enucleated cells. In summary, our protocol can efficiently generate mature erythroid cells from the erythroid cell line HiDEP.

Paper II addressed the need for having inducible human papillomavirus 16 - E6/E7 (HPV16-E6/E7) system in erythroid cell lines. We established Erythroid Line from Lund University (ELLU) by simple overexpression of HPV16-E6/E7. We found that cells could differentiate into mature erythroid cells regardless of maintained HPV16-E6/E7 expression. Additionally, while establishing ELLU, we created ten different clones that, unexpectedly, was hetergenuous for hemoglobin expression and cellular fragility of resulting mature erythrocytes, although the clones originated from one common donor source. In summary, our work proposes a more straightforward method for establishing erythroid cell lines and demonstrates hemoglobin expression heterogeneity, potentially coupled with mature cell cellular properties.

In Paper III, we compared the erythroid potential of CD34+ hematopoietic stem and progenitor cells (HSPCs) isolated from 50 individual umbilical cord blood (UCB) donors. We found that donors gave rise to different frequencies of Glycophorin-A+ cells. Therefore, we grouped donors into two groups, giving high- and low-yields of Glycophorin A+ cells and analyzed their gene expression. This identified donors giving rise to a high frequency of Glycophorin-A+ cellshaving higher expression of genes related to the GPCR signaling pathway. To understand their contributions, we modified the activities by chemical compound modulation of two specific signaling components, and found that one inhibitor resulted in 10 % higher yields of Glycophorin-A+. Our findings preliminarily suggest potential GPCR singling involvement in the efficient development of erythroid cells *in vitro*; however, further studies are required to understand this process better.

Finally, acquired knowledge in this thesis could help design the best conditions and models for *ex vivo* RBC production, which could be a very accessible lifesaving procedure in the future.

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MADE IN SWEDEN

To my family

"I am still learning"

Michelangelo

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Paper I

Soboleva S, Kurita R, Ek F, Åkerstrand H, Silvério-Alves R, Olsson R, Nakamura Y and Miharada K. Identification of potential chemical compounds enhancing generation of enucleated cells from immortalized human erythroid cell lines. 2021 Communications Biology; Vol 4, issue 1, p1-14.

Paper II

Soboleva S, Kurita R, Kajitani N, Åkerstrand H and Miharada K. Establishment of an immortalized human erythroid cell line sustaining differentiation potential without inducible gene expression system. 2022 Human Cell; Vol 35, issue 1, p408-417

Paper III

Soboleva S, Åkerstrand H and Miharada K. Transcriptomic analysis of functional diversity of human umbilical cord blood hematopoietic stem/progenitor cells in erythroid differentiation. 2022 International Journal of Hematology

Papers and manuscript not included in this thesis

Schmiderer L, Subramaniam A, Žemaitis K, Bäckström A, Yudovich D, **Soboleva S**, Galeev R, Prinz C. N, Larsson J, Hjort M. Efficient and nontoxic biomolecule delivery to primary human hematopoietic stem cells using nanostraws. 2020 The Proceedings of the National Academy of Sciences of the United States of America; Vol 117, issue 35, p21267-21273.

Sigurdsson V, Haga Y, Takei H, Okamatsu-Haga C, Mansell E, Koide S, Suzuki M, Radulovic V, van der Garde M, **Soboleva S**, Gåfvels M, Nittono H, Ohara A, Miharada K. Induction of blood circulating bile acids supports recovery from myelosuppressive chemotherapy. 2019 Blood Advances; Vol 4, issue 9, p1183-1843.

Sigurdsson V, Takei H, **Soboleva S,** Radulovic V, Galeev R, Siva K, Leeb-Lundberg L.M. F, Iida T, Nittono H, Miharada K. Bile acids protect expanding hematopoietic stem cells from unfolded protein stress in fetal liver. 2016 Cell Stem Cell; Vol 18, issue 4, p522-532.

Soboleva S, Sudo K, Sabatier P, Zubarev R, Nakamura Y and Miharada K. Identification of novel pathological regulators of erythropoiesis **Manuscript in preparation.**

Abbreviations

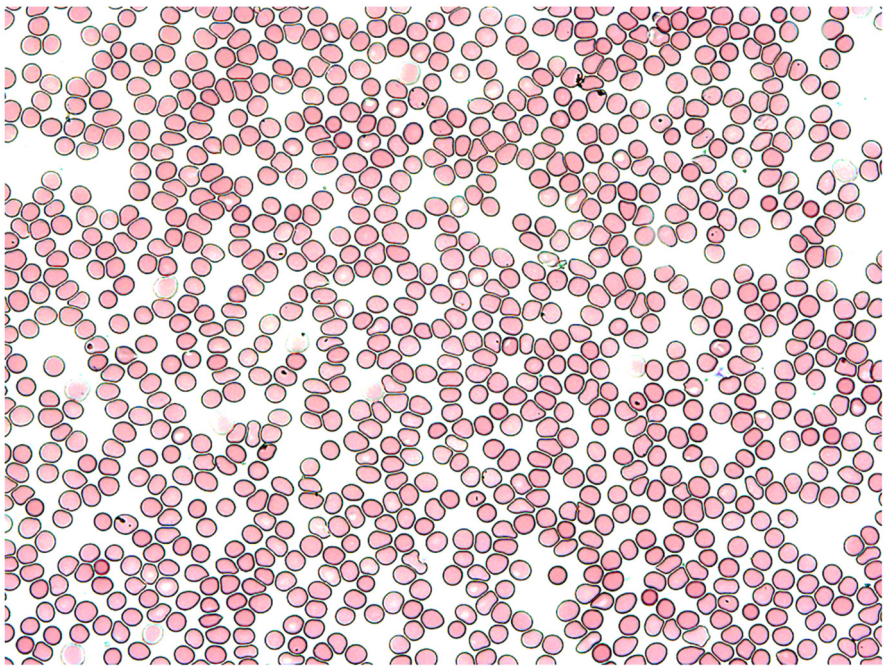
AC	adenylyl cyclase
AGM	aorta-gonad-mesonephros
AKAP	A-kinase-anchoring protein
BCL11A	B-cell lymphoma/leukemia 11A
BFU-E	burst forming unit erythroid
BM	bone marrow
cAMP	cyclic adenosine monophosphate
CFU-E	colony forming unit erythroid
EB	embryoid bodies
ELLU	erythroid line from Lund University
EPO	erythropoietin
ESA	erythropoiesis-stimulating agents
ESC	embryonic stem cell
FACS	fluorescent-activated cell sorting
FL	fetal liver
FS	fluoro-SAHA
GPA	glycophorin A
GPCR	G protein-coupled receptor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HiDEP	human iPS-derived erythroid progenitor
HPV16-E6/E7	human papilloma virus 16 - E6/E7
HSPC	hematopoietic stem and progenitor cell
HSC	hematopoietic stem cell
HUDEP	human umbilical cord blood-derived erythroid progenitor
IBMX	3-Isobutyl-1-methylxanthine
iPSC	induced pluripotent stem cell
KLF1	krüppel like factor 1
mDia2	mammalian Diaphanous-related
MEP	megakaryocyte/erythroid progenitor
MNC	mononuclear cells
PB	peripheral blood
PDE	phosphodiesterase
RBC	red blood cell
SPTA1	spectrin - αI
SPTAN1	spectrin - αII
SPTB	spectrin - βI
SPTBN	spectrin - βII
UCB	umbilical cord blood

Preface

When I first saw healthy human red blood cells under a microscope I felt inspired because I got to know them "in person", whereas regular people know blood as just a red liquid in our body. These cells lacking nuclei looked perfect to me, perhaps because of their simple structure, at least at first sight. It is fascinating that our life and well-being depend on the quality and quantity of our red blood cells. These thoughts and feelings motivated me to learn about red blood cells during my PhD studies. I was fortunate to work on the projects where we focused on red blood cell generation. Our approach was not classic but rather inovative.

What happens in the body *(in vivo)* and in the dish *(in vitro)* could not necessarily be regulated by identical mechanisms. Different environments and starting materials might be reasons for this variation. However, understanding red blood cell development in both settings is essential for finding novel red blood cell regulators, and potentially provide knowledge that could help improve current therapies for anemia. Mass-production of red blood cells in the lab is considered a promising approach for its clinical practice in the future. Although it sounds straightforward, it remains to be an ambitious goal.

I hope the readers of this thesis book will enjoy learning about red blood cells as much as I do.



Blood smear of healthy human red blood cells

Background

Brief historical overview of blood transfusion

Blood transfusion is a lifesaving procedure where the patient's blood is replaced or supplemented by a blood donation. However, patients have restricted access to safe blood transfusions globally making it an urgent need to enable its efficient and continuous availability. Blood production in the lab is an alternative approach to public blood donation, and is one possible solution to the problem of availability.

Blood

Being alive is impossible without this bright, red fluid - blood, which supports our life by transporting oxygen throughout body. Each person has different volumes of blood in the body, depending on their age, body weight, sex and physiological condition, but typically blood represents around 7 - 10 % of body mass (Reviewed in Gutierrez, Reines and Wulf-Gutierrez, 2004). Keeping constant blood volume is critical to maintain sufficient perfusion of all tissues and retain the body's normal function. Blood is a fluid, thicker than water, consisting of the liquid base, called plasma, in which red blood cells, white blood cells, and platelets are suspended. The blood consists of 55 % of the plasma and 45 % of the cells. The plasma is a yellow, slightly murky liquid, mainly consisting of water (92 %) and the rest are essential substances such as proteins, vitamins, nutrients, and electrolytes (Joscilin, Parvathy and Varacallo, 2021). The main functions of the plasma is to transport the blood cells and essential substances throughout the body, to regulate body temperature, to collect metabolic waste from the cells and deliver it to specific organs for recycling, to enable coagulation, and to provide the body with immunological defence (Joscilin, Parvathy and Varacallo, 2021). The cell compartment in the blood is represented by red blood cells (RBCs) (over 99 %), the rest being the immune cells and platelets.

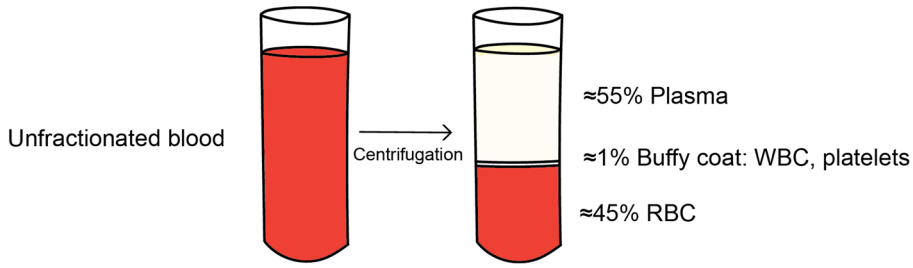


Figure 1. Simplified expamle of the whole blood franctionation into components by centrifugation. Blood can be separated into three components after spinning it down at high speed in the centrifuge.

RBCs is the most abundant cell type in the human body, comprising 84 % of the cells in the body (Sender, Fuchs and Milo, 2016). Each blood cell type has a specific function in the body. RBCs are essential to supply living cells in the body with oxygen (O_2) and nutrients while removing carbon dioxide (CO_2). RBCs are small enough to pass quickly through all sizes of vessels thanks to their ability to adjust their shape. Platelets play a vital role in triggering blood clotting, or coagulation, in order to help prevent serious blood loss when we get injured. White blood cells (WBCs) are a part of immune system that protects our bodies from pathogens and function to maintain homeostasis through interaction and surveillance of foreign material (Dean, 2005).

All mature blood cells originally derive from the same immature cell called hematopoietic stem cell (HSC) and are formed through the process called hematopoiesis. Hematopoiesis is a stepwise process through a hierarchical structure, where the HSC resides on top of the differentiation hierarchy and can differentiate into all the mature blood cells through discrete series of progenitor populations (Seita and Weissman, 2010).

In summary, blood is indispensable to survive because blood components play several essential roles in our body. Performing various analyses of blood could reveal our health conditions. And this simple fact makes blood an important research subject, ranging from aiding in the fight against invading diseases to creating artificial components of this vital fluid.

Blood transfusion through the centuries

It was noticed already from the ancient times that there was a strong correlation between blood and life, because severe blood loss resulted in death. However, it then took many years to understand the blood circulation system and the properties of blood. This knowledge helped to set a foundation for modern blood transfusion applications as used today for clinical purposes. Today, it is common to donate blood, which is stored and then transfused to a receipt in need. We often visit

transfusion services to donate blood almost for granted as a well-established medical procedure; however, it is a relatively recent method and it took hundreds of years to reach the accessibility that we enjoy today.

Before blood transfusion began to be used as a life-saving procedure, bloodletting was a widespread practice for many centuries. In ancient Roman and Greek times, it was believed that removing "bad blood" had a curative meaning. The idea was based on a humoral theory that the human body is filled with four essential ingredients, i.e. blood, phlegm, yellow bile, and black bile. Any illness or difference in personality was a consequence of their disbalance. Thus, different diets, changes in the environment or bloodletting were used to correct the imbalance or cure illness, and was in fact performed for a long time (Reviewed in Giangrande, 2000).

In 1628, English physician William Harvey made one of the crucial discoveries in the blood transfusion history. He was the first to understand and describe the blood circulation system, thereby initiating many speculations about possibilities of prolonging life by blood transfusions and early experimentation with blood infusions using potions and medications (Reviewed in Giangrande, 2000). The first blood transfusion from one animal to another was tried using dogs and was demonstrated in 1666 by Richard Lower (Oxford). Jean Denis (Paris) then transfused blood from different animals (calves and lambs) into humans a year after. However, his experiments were not successful and led to many deaths rather than life rescues. As a result, blood transfusions were considered a criminal act and were not allowed anymore in France and later in other countries (Reviewed in Giangrande, 2000).

All such old blood transfusion attempts were unsuccessful due to a lack of the basic understanding about differences in blood compatibility between inter-species, blood coagulation, and the general absence of efficient equipment for the purpose. Still, it was believed that blood transfusion could treat different unknown illnesses and change personalities, vigour and help regain youth (Reviewed in Giangrande, 2000).

It took almost another 150 years until obstetrician James Blundell (London) in 1818 for the first time speculated that human to human blood transfusion could prolong the life of women suffering from postpartum haemorrhages (Blundell, 1818). During close to the following decade (1818 - 1829) he investigated this hypothesis and documented ten human-to-human blood transfusions. In 1828, Blundell reported to the Lancet journal about the first successful case of human to human blood transfusion. The report described how one of his assistance's blood saved the life of the woman suffering from severe post-partum blood loss (Blundell, 1828). Blundell highlighted in his report that blood has to be transfused to patients only under acute need and encouraged his colleagues to use transfusions to compensate for severe blood loss caused by post-partum haemorrhages. His discoveries and observations represented the start of the blood transfusion era and Blundell is

credited as a father of modern transfusion therapy for his contributions to this field (Welck, Borg and Ellis, 2010).

For another almost 70 years, blood transfusions were performed without recognizing differences in blood between individuals. However, in 1901 Karl Landsteiner (Vienna) observed that the addition of blood of some individuals to others led to agglutination. Later on, he received the Nobel prize in 1930 for classifying blood into the nowadays well-known four blood groups, such as the ABO blood group system (Landsteiner, 1901). In 1939, one of the Landsteiner's trainees, Philip Levin, reported a case study in which mixing a woman's and her husband's blood resulted in agglutination even though they had similar blood groups. This was how Rhesus antibodies in humans were identified, and later recognized as another important blood group system (Levine, Newark and Stetson, 1939). These discoveries inspired other researchers to conduct related studies, which led to identifying several other antigenic systems such as Lutheran, Kell, Duffy and Kidd and others (Callender, Race and Paykoc, 1945), (Coombs, Mourant and Race, 1946), (Cutbush, Mollison and Parkin, 1950), (Fred H. Allen, Diamond and Niedziela, 1951).

Blood storage and blood banks

Along with investigations about blood transfusion, problems related to blood coagulation and storage were recognized. In 1912, a vascular surgeon, Alexis Carrel, received Nobel Prize for development of a method allowing blood transfusion directly from one donor to another by sewing their blood vessels together (Carrel, 1912). However, the most significant limitation of this approach was the need for the donor to be present through the whole surgery which could sometimes take a good deal of time. In 1915, Richard Lewisohn (New York) improved the already promising anticoagulant solution containing sodium citrate, thereby making the first non-direct blood transfusion possible. This solution was highly beneficial for protecting blood from coagulation for some hours in the fridge (Lewisohn, 1916). With time, it was found that dextrose had a better effect on preserving RBCs up to fourteen days (Rous and Turner, 1916); the citrate-phosphate-dextrose solution allowed blood storage up to 28 days (Gibson, Gregory and Button, 1961). The ability to preserve donated blood and the second World War motivated the establishment of the first National Blood Transfusion Services in 1946 and later blood banks. In 1940 Edwin Cohn (Boston) found out the possibility to fractionate plasma proteins therefore there was no need to transfuse whole blood unit to the patient needing only specific blood components to treat illnesses (Cohn *et al.*, 1940), (Blajchman, Shepherd and Perrault, 1979).

Blood transfusion nowadays

Nowadays, blood transfusion is a common procedure which can improve patients' health and save life (Seifried and Mueller, 2011). Even though there is a significant benefit in receiving blood there are also some concerns about blood accessibility and safety (Armstrong, 2008).

World Health Organization (WHO) reported that annually 112.5 million blood donations are collected worldwide, however there is a limited access to safe blood for many patients because of the differences of donation frequencies in various countries (WHO, 2006), (WHO, 2017). Moreover, predictions point toward a projected, global increase in demand of blood supply by 2050, in view of the elderly population increasing (Ali, Auvinen and Rautonen, 2010). Furthermore, unpredictable worldwide pandemics or natural disasters can cause health care crisis as it did during the COVID-19 global pandemic: the healthcare faced problems with a shortage of blood supply and the blood safety in many countries worldwide (Stanworth *et al.*, 2020), (Chang, Yan and Wang, 2020), (Al-Riyamia *et al.*, 2021).

To ensure the beneficial effect for the patient's life, the transfused blood has to be safe. Every country's national health care policy and infrastructure systems have to follow suggested quality requirements to provide safe blood supplies (WHO, 2006). WHO suggests that all donated blood should be screened for infections (HIV, hepatitis B and C, syphilis, malaria) prior to transfusion, however, 35 countries cannot screen all donated blood for one or more infections. Performing tests is not reliable in many countries because of the unstable supply of test kits and its poor quality, shortage of trained personal, or lack of appropriate infrastructures in laboratories (WHO, 2017).

Last but not least, finding compatible donors and recipients is yet another difficulty for safe blood transfusions. Consideration of at least matching ABO and Rhesus blood groups is important to ensure safe blood transfusion. At present, there are more than 40 blood groups identified while new ones are continuously being identified (WHO, 2017), (Daniels and Reid, 2010), (Mitra, Mishra and Rath, 2014), (ISBT, 2021).

Given these important concerns, finding alternatives that could provide limitless, infection free, and personalized sources of blood and its components is of a great value. *Ex vivo* generation of RBCs on a large scale has been thought of as a promising approach to overcome the insufficiency of blood supply. Nowadays, our research has advanced so much that we can accomplish this goal. However, to seriously use *ex vivo* generated blood for transfusions, still optimization of product scalability, safety, quality, and cost are required.

Red blood cells

Over the years, much knowledge has been gained about RBCs, but more detailed understanding is still needed to efficiently produce RBCs in the lab. This section briefly describes typical RBC characteristic features, functions, anatomy, and conditions related to red blood cell disorders.

Characteristic features

Red blood cells, or erythrocytes, are the main cellular component in the blood, and they are devoted to transporting oxygen and carbon dioxide through the body. Lack of the nucleus in the mature RBC enables efficient accumulation of hemoglobin in its cytoplasm. The presence of approximately 270 million hemoglobin molecules per cell contributes to the characteristic red colour of blood (Alessandro *et al.*, 2017). One hemoglobin molecule consists of four heme molecules bound to globin chains. Heme is a ring-like complex, also known as porphyrin. Its unique structure is capable of attaching an atom of iron (4 Fe^{2+}), which sits like a jewel in the centre and is required to bind oxygen to hemoglobin. When iron is in the transition state (Fe^{3+}), it cannot bind oxygen. Therefore, oxygen concentration and the changes in the affinity of iron molecules makes oxygen-binding reversible. One functional adult globin unit ($\alpha_2\beta_2$) consists of four polypeptide chains such as 2 α - like globin peptide chains and 2 β - like globin peptide chains folded around heme (Manning *et al.*, 2020).

Due to the absence of a nucleus, mature RBC possesses the shape of a biconcave-discoid capsule (Schechter, 2008). The size of these cells is approximately ~ 7.5 to 8.7 μm in diameter and ~ 1.7 to 2.2 μm in thickness. A unique feature of RBCs is their capacity for membrane deformation, which repeatedly allows squeezing through narrow capillaries of microvasculature (~3 μm diameter) without breaking the cell membrane and significant changes in membrane surface area (Huisjes *et al.*, 2018).

According to calculations based on human body weight and RBC count per microliter, the total amount of circulating RBC in the 70 kg person equals ~ 2.5 x 10^{13} (25 trillion) cells. The healthy human adult loses ~ 0.8 - 1 % of their total RBCs in a day, which is compensated by approximately ~ 2.5 million RBCs are produced every second. On average, development in the bone marrow takes 14 days and the average lifespan of the mature RBC is around 120 days. Old RBCs are then destroyed by macrophages of splenic and hepatic sinusoids (Palis, 2014), (Fernandez Arias C, 2017).

Erythropoiesis

Erythropoiesis is the process of differentiation and maturation into mature RBCs and occurs in the primitive (embryonic) and definitive (adult) forms. Primitive erythropoiesis is a transient form for a brief period of early-life to support the transition from embryo to fetus. Definitive erythropoiesis then follows to support the transition from fetus to neonates and remains our mode of erythropoiesis for the rest of our life (Reviewed in Baron, Isern and Fraser, 2012), (Palis, 2014). Primitive erythropoiesis occurs in the yolk sac of the early embryo and it is represented by large, nucleated erythroid cells, which mature directly in the circulation. Definitive erythropoiesis, in contrast, primarily occurs in the fetal liver and later in postnatal bone marrow and spleen. Definitive erythroid cells are different from primitive in generating small enucleated cells (Reviewed in Baron, Isern and Fraser, 2012), (Palis, 2014).

Bone marrow is the tissue inside our bones that serves as the primary site for adult blood production from HSCs. Erythropoiesis is the stepwise differentiation process consisting of commitment from HSC and multipotent progenitors of the erythroid lineage; terminal erythroid differentiation, when cells undergo morphological changes and extrude nuclei; followed by reticulocytes maturation step (Palis, 2014). RBC differentiation from erythroblasts to reticulocyte occurs within specific microenvironments, called erythroblasts islands (EBI). The EBI is a multicellular compartment, where macrophages in the center are interacting with erythroid cells at different maturation stages (Al-Drees *et al.*, 2015).

A classical model of hematopoietic hierarchy suggests that HSCs give rise to erythroid cells by undergoing strictly separate intermediate progenitor stages, stimulated by various factors such as cell-cell interactions, intrinsic (e.g., transcription factors), or extrinsic factors (e.g., cytokines, hormones). Additionally, an alternative model of hematopoiesis suggests heterogeneity within the HSC population with respect to their direct lineage commitment potentials, which may further influence lineage commitment into erythropoiesis (Manz *et al.*, 2002), (Doulatov *et al.*, 2010), (Notta *et al.*, 2016). While details are still being investigated, the conventional view states that erythroid cells arise from megakaryocyte/erythroid progenitor (MEP). MEP give rise to the unipotent burst forming unit-erythroid (BFU-E), the earliest erythroid progenitor with a certain self-renewal potential as well as megakaryocyte progenitors (MkP). BFU-E cells continue to mature and become late erythroid progenitors, colony-forming unit erythroid (CFU-E). Growth factor requirements between BFU-E and CFU-E are different. For example, BFU-E are dependent on stem cell factor (SCF) and glucocorticoids (GC) while CFU-E depend on the hormone erythropoietin (EPO), which serves as a differentiation factor to generate pro-erythroblasts (Lodish, Flygare and Chou, 2010). The potential of BFU-E and CFU-E are also different, as the BFU-E give rise to considerably larger erythroid colonies in the semisolid

media. Additionally, BFU-E and CFU-E can also be distinguished by the expression of cell surface markers (Li *et al.*, 2014), however morphologically these cells are not exclusive in comparison to all other blast cells. CFU-Es then mature into relatively larger pro-erythroblasts (diameter between 20 - 25 µm) with the nucleus occupying 75 - 80 % of cytoplasm (Reviewed in Yeo, Lam and Fraser, 2019). It is considered that pro-erythroblasts are the first morphologically recognizable progenitor cell type in the erythroid lineage. Terminal differentiation of pro-erythroblasts into reticulocytes occurs through several morphologically defined cellular forms. Differentiation of pro-erythroblast proceeds into more mature form, called basophilic erythroblast, 18 - 20 µm in size (Reviewed in Yeo, Lam and Fraser, 2019). At this stage chromatin starts to aggregate and start hemoglobin synthesis. Next, basophilic erythroblasts progress further into polychromatic erythroblasts, 10 - 15 µm in size that have more condensed nuclei (Reviewed in Yeo, Lam and Fraser, 2019) and with higher hemoglobin content. This is the last stage of RBC development where cell division can occur. The orthochromatic erythroblasts are the most mature and smallest erythroblasts of all erythroid progenitors. These cells cannot divide, have high hemoglobin concentration and have pyknotic nuclei (irreversible nuclear condensation); therefore, they are considered as the last nucleated cells in the erythroid lineage. After orthochromatic cells extrude their nuclei (enucleate), they become reticulocyte (Reviewed in Nandakumar, Ulirsch and Sankaran, 2016). The newly developed reticulocyte is mechanically less stable than mature RBC and needs to undergo a final maturation process to acquire all the necessary properties. Within 24 - 48 hours, reticulocytes detach from their microenvironment in the bone marrow and leave to the bloodstream. Within 24 - 48 hours, they become mature hemoglobinized RBCs with the essential proteins for retaining the cytoskeletal network after membrane remodelling. During this time, reticulocytes expel remaining cytoplasmic organelles and acquire the distinctive biconcave form (Reyes and Mondor, 1976), (Palis, 2014)(Yeo, Lam and Fraser, 2019).

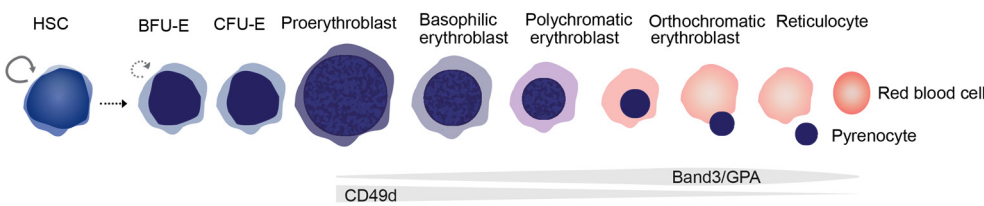


Figure 2. Erythroid cell development. HSCs give rise to erythroid cells which further differentiate through stricktly regulated intermediate distinct stages. Each stage in terminal differentiation can be distinguished with cell surface markers and morphologically such as change in size, nuclear condensation and hemoglobin accumulation.

Changing expression of different cell surface markers during RBC maturation allows us to keep track and distinguish specific cells at different developmental stages using flow cytometry. Band 3 and α4-integrin (CD49d) are two important

markers to monitor RBC development *in vitro* from primary human bone marrow cells. Hu et al. demonstrated that expression of CD49d decreases while expression of band 3 increases during terminal erythroid differentiation, allowing accurate detection of different stages of maturation (Hu *et al.*, 2013). Similarly, glycophorin-A (GPA) is also an erythroid-specific marker that appears on the surface of pro-erythroblasts and continuously increases through erythroid maturation. The combination of GPA and transferrin receptor (CD71) can also be used in similar to CD49d/band 3 (Loken *et al.*, 1987), (Chen *et al.*, 2009), (Ovchynnikova *et al.*, 2018).

Enucleation

Orthochromatic erythroblast extrudes the pyknotic nucleus and develops into reticulocyte through the process termed enucleation, a process that is still not fully understood. Historically there were arguments whether erythroblasts are enucleated either through nuclear extrusion or undergoing a specific form of cell death caused by nuclear fading (karyolysis). Studies using electron microscopy confirmed that it is rather the former, identifying pyknotic nuclear extrusion (Simpson and Kling, 1967), (Awai *et al.*, 1968). Formation of the pyknotic nucleus also happens during cell death (apoptosis), where irreversible activation of nuclear condensation (pyknosis) is regulated by caspases (Zamzami and Kroemer, 1999).

Several studies showed that caspases are not essential for enucleation, but, rather, are important for the early stages of terminal erythroid differentiation (Zermati *et al.*, 2001), (Boehm *et al.*, 2013). However, other groups have demonstrated that caspases are needed for the enucleation and contribute to the nuclear opening prior to enucleation in murine erythroid cells (Zhao, Mei, *et al.*, 2016) and how enzymatic inhibition of caspases in murine cells leads to 50 % reduction of enucleated (Carlile, Smith and Wiedmann, 2004).

It was noticed that while the reticulocyte contains most of the cytoplasm after enucleation, also the pyrenocyte (extruded nucleus) was surrounded by a thin rim of cytoplasm. This simple observation led to the hypothesis about how enucleation happens; this nice separation of reticulocyte and pyrenocyte could indicate that enucleation occurs through a process similar to cytokinesis, which is normally associated with cell division. Thus, reticulocyte and pyrenocyte separation could be thought of as two daughter cells during cell division (Skuletsky and Danone, 1970). Many studies have been made to test this theory. The analogy of cytokinesis to enucleation would imply cell polarization, contractile actomyosin ring assembly (CAR) formation by small GTPase Rho proteins, cleavage furrow ingression and the separation that is coordinated by the vesicle trafficking (Guertin, Trautmann and McCollum, 2002), (Gromley *et al.*, 2005).

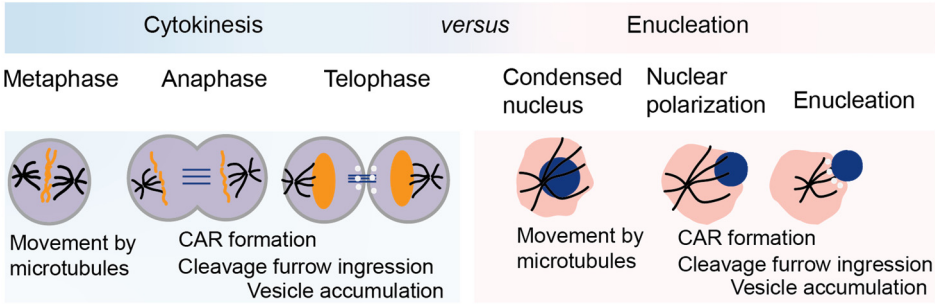


Figure 3. Simplified representation of similarities between cytokinesis and enucleation. Cytokinesis. Microtubules (black) stimulate chromosomes (orange) alignment and separation during cytokinesis. Mitotic spindles (blue lines) coordinate CAR formation, and cleavage furrow ingression occurs. Vesicle (white dots) accumulation occurs during the final cell separation steps, termed abscission. **Enucleation**. During erythroblast enucleation, the condensed nucleus is polarized in the cell and then extruded. During polarization, the nucleus (dark blue) movement is mediated by microtubules (black) activity. Nuclear extrusion occurs through the formation of CAR and cleavage furrow ingression. The separation of the pyrenocyte and reticulocyte is facilitated by accumulating vesicles (white dots).

Some of the similar regulators or typical features of cytokinesis were observed during erythroid cell enucleation. For instance, establishment of cell polarization in erythroblasts is crucial for nuclear extrusion. By using advanced imaging techniques, it was revealed that cell polarization before enucleation is mediated by microtubule-dependent phosphoinositide 3-kinase (PI3K) activity (Wang *et al.*, 2012). Microtubules are thread-like assemblies that provide a platform for various intracellular movements in all eukaryotic cells. Kinesins and dyneins are motor proteins that transport along the microtubules in opposite directions (Rayment, 1996). Studies on human primary cells suggested that enucleation is a dynein dependent process (Kobayashi *et al.*, 2016). In contrast, another study suggested that Trim58 degrades dynein for enucleation to occur by destabilizing microtubules to facilitate nuclear movement and release (Thom *et al.*, 2014). Kinesins are suggested to be involved in nuclear polarization, however additional studies are required to determine it (Thom *et al.*, 2014). Additionally, in similar with cell division, the cleavage furrow formation during enucleation has also been observed (Koury, Koury and Bondurant, 1989). It is believed that non-muscle myosin II by interacting with actin filaments (F-actin) participates in cleavage furrow formation (Ubukawa *et al.*, 2012), and there is also data about non-muscle myosin II involvement in human erythroid cell enucleation. It has been shown that during enucleation there is an accumulation of F-actin between the nucleus and cytoplasm close to the constriction zone (Wickrema *et al.*, 1994). Disruption of F-actin polymerization impairs enucleation in murine cells (Koury, Koury and Bondurant, 1989), (Ji, Jayapal and Lodish, 2008). These studies suggest that actin-mediated forces are important for enucleation. Similar to mitosis, enucleation occurs through the CAR-mediated cleavage through the actions of Rac GTPase and mammalian Diaphanous-related (mDia2) formin. Inhibition of mDia2, a downstream Rac GTPase effector, impairs CAR formation and enucleation of mouse erythroblasts (Ji, Jayapal and Lodish, 2008). It has also been found that vesicle trafficking is

fundamental for the nuclear extrusion, because its disruption blocked enucleation (Keerthivasan *et al.*, 2010).

Thus, these studies on understanding erythroid enucleation have identified several molecules that also facilitates cytokinesis in most cell types. Erythroblast enucleation is occurring through asymmetric cell division initiated by polarized movement of condensed nuclei by microtubules, followed by CAR formation and cleavage furrow ingression resulting in quick pyrenocyte and reticulocyte separation (Ji, Jayapal and Lodish, 2008), (Keerthivasan *et al.*, 2010). Further understanding the mechanism of enucleation *in vivo* and *in vitro* is important to increase the enucleation efficiency during the *ex vivo* RBC production.

Hemoglobin

During human ontogeny, embryonic, fetal, and adult hemoglobins are subsequently expressed at precise times. To compensate variations in oxygen availability during development, embryonic and fetal hemoglobins have stronger oxygen-binding capacity than adult type (Manning *et al.*, 2020). During the normal development, when the expression of one preceeding hemgolobin gene decreases the expression of another gene increases and this process is referred to as a hemoglobin switching. More than 1000 disorders related to hemoglobin synthesis or structure have been identified and characterized (Forget and Franklin Bunn, 2013), emphasizing the need for understanding the regulation of hemoglobin expression in health and disease (Sankaran and Orkin, 2013).

Depending on the stage of life, different types of α-like and β-like genes are expressed, forming typical combinations of embryonic, fetal, and adult types of hemoglobins. In humans, there are three α-like globin genes: *HBZ (ζ), HBA1* and *HBA2 (α)*, located in the α-globin locus on chromosome 16. The α-globin locus is regulated by the major regulatory element named HS-40. The β-globin locus located on chromosome 11 consists of five functional β-like globin genes: *HBE1 (ε)*, *HBG1* ($^{A}\gamma$), and *HBG2* ($^{G}\gamma$), *HBD (δ),* and *HBB (β)*, under the locus control region (LCR) (Schechter, 2008), (Sankaran and Orkin, 2013), (Nandakumar et al., 2016).

During the first weeks of gestation in the yolk sac, primitive erythroid cells synthesize the embryonic type of hemoglobin, such as Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$), Portland I ($\zeta_2\gamma_2$) and Portland II ($\zeta_2\beta_2$). The most common type of embryonic hemoglobin is Gower I. Later, during weeks 8 and until 32, erythrocytes predominantly synthesize fetal hemoglobin termed Hemoglobin F ($\alpha_2\gamma_2$). Shortly after birth, a switch from fetal to adult hemoglobin expression occurs, and Hemoglobin A ($\alpha_2\beta_2$) and Hemoglobin A2 ($\alpha_2\delta_2$) start to be expressed. Hemoglobin A is the most abundant type of hemoglobin in adults and constitutes approximately 97 % of total hemoglobin, while Hemoglobin A2 represents 2 %. Low frequency, around 1 %, of Hemoglobin F is also present in adult blood (Schechter, 2008),

(Sankaran and Orkin, 2013), (Nandakumar et al., 2016). In summary, eight types of hemoglobin tetramers molecules are formed throughout human life: four in the embryo stage, one in fetal, and three in adulthood.

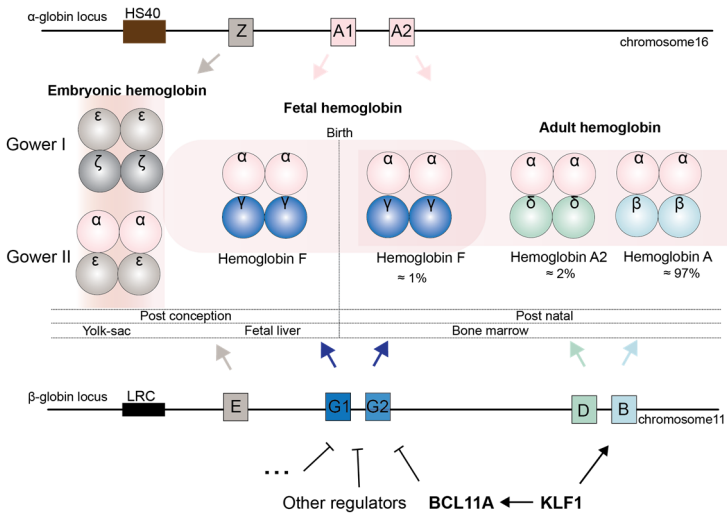


Figure 4. Hemoglobins expresson during human development and life. The top and bottom of this figure represent α- and β-globin loci with specific control regions and expressed genes. In the centre, the expression of the major types of hemoglobins through life are illustrated, such as embryonic, fetal and adult types. Several proteins regulating hemoglobin switching directly or indirectly repressing fetal and activating adult hemoglobins are represented at the bottom, including KLF1, BCL11A and others.

Around the time after birth, fetal γ-globin is silenced and adult β-globin expression is increasing and continues being expressed throughout life. It was found that the GATA1, TAL1, E2A, LMO2, and LDB1 complex interacts with γ- or β-globin promoters to mediate fetal or adult hemoglobins expression (Cavazzana, Antoniani and Miccio, 2017). Fetal to adult hemoglobin switching has been extensively studied because, in both pathological and non-pathological conditions in adults, elevated levels of γ-globin instead of β-globins have been observed. Additionally, reactivation of γ-globin in patients with β-hemoglobinopathies is considered as a therapeutic approach since it inhibits the disease phenotype by ameliorating polymerization of sickle hemoglobin (Cavazzana, Antoniani and Miccio, 2017), (Cavazzana, Antoniani and Miccio, 2017). Interestingly, adult erythroid cells acquire fetal-type globin upon *in vitro* culture (Giarratana *et al.*, 2011). This type of finding implies that globin switching is not unidirectional but reversible, therefore, continuous attempts have been made to reveal the exact mechanism of hemoglobin regulation and switching (Cavazzana, Antoniani and Miccio, 2017).

Genome-wide association studies (GWAS) revealed that *BCL11A* and *KLF1* were associated with fetal globin regulation (Uda *et al.*, 2008), (Lettre et al., 2008), (Sankaran and Orkin, 2013). BCL11A, a transcriptional factor, has been recognized as a major regulator of fetal-to-adult hemoglobin switching. According to some

studies, downregulation of *BCL11A* expression in adult human primary cells is correlated to the induction of γ-globin expression. Conversely, the presence of BCL11A protein is associated with silencing of γ-globin gene expression in adult human erythroid cells (Sankaran *et al.*, 2008). In adult erythropoiesis BCL11A interacts with SOX6, GATA1, FOG1 and also repressor complexes, for instance NuRD, and others to repress the expression of fetal γ-globin expression (Cavazzana, Antoniani and Miccio, 2017).

Another transcription factor, Krueppel-like factor 1, KLF1, is also associated with γ-globin expression. Patients with rare mutations of *KLF1* show increased expression of γ-globins and anemia (Sankaran and Orkin, 2013). Another study of adult human erythroid cells also revealed that *KLF1* knockdown led to low expression of *BCL11A* and high γ-globin/β-globin genes expression ratios. Based on these results, it was proposed that *KLF1* directly activates β-globin expression and indirectly through *BCL11A* repressing γ-globin gene expression, therefore, playing an important role in globin gene switching (Zhou *et al.*, 2010). Another study reported that KLF1 directly drives expression of transcriptional repressor ZBTB7A in human and murine erythroid cells. By recruiting NuRD repressor complex, ZBTB7A represses fetal globin expression independently of BCL11A (Norton *et al.*, 2017). This demonstrates that the mechanism of KLF1 action is still not completely understood and has to be more studied.

In addition to BCL11A, the Lin28b-let7 pathways is also involved in the silencing of γ-globin expression (Cavazzana, Antoniani and Miccio, 2017).

RBC Membrane

Mechanical properties of erythrocytes are essential for their cellular physiology. RBC membrane composition is vital to maintain cell shape, flexibility and deformability, and abnormalities in membrane arrangement lead to early removal from the circulation and various disorders. RBC membrane disorders can be caused by abnormalities in vertical or horizontal interactions of membrane proteins (Oliveira and Saldanha, 2010). Hereditary spherocytosis, hereditary elliptocytosis and sickle cell disease represent such disorders caused by defects in skeletal proteins in the cell membrane (Da Costa *et al.*, 2013).

RBC membrane is formed of a lipid bilayer, membrane proteins and skeletal proteins. The lipid bilayer consists of one outer and inner leaflet, which are composed of phospholipids and separated by a cholesterol molecule. The inner and the outer monolayer RBC membrane are formed by an asymmetrically distribution of four different phospholipids. Phosphatidylcholine (PC) and sphingomyelin (SM) belong to the outer monolayer, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) belong to the inner monolayer. It is believed that the asymmetric location of phospholipids is not spontaneous but regulated by

functions of ATP-dependent enzymes such as flippases, floppases, and scramblases (Zwaal and Schroit, 1997), (Hankins *et al.*, 2016). The proper asymmetrical distribution of phospholipids in the cell membrane could play a significant role in cellular events during RBC life. For example, PS translocation from the inner to the outer monolayer indicates cell senescence, and this signal is recognized and induces phagocytosis by macrophages. Some phospholipids interact with cytoskeletal proteins, both maintaining cell membrane integrity and signal transduction (Wood, Gibson and Tait, 1996), (Manno, Takakuwa and Mohandas, 2001).

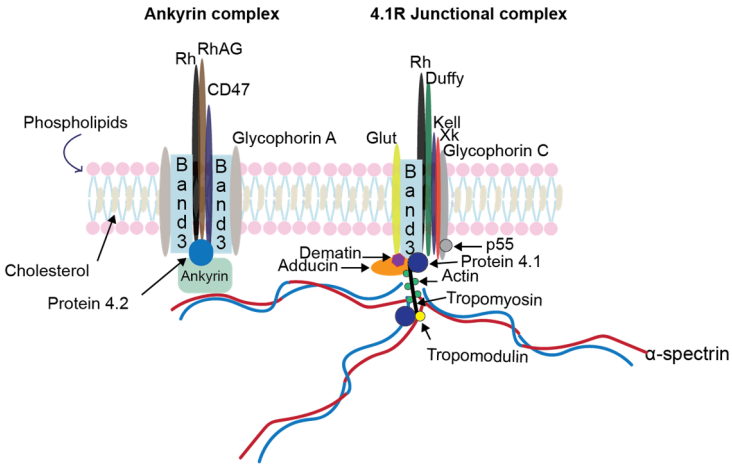


Figure 5. The RBC membrane. Detailed illustration of RBC membrane. The RBC membrane is formed by the lipid bilayer with the membrane and skeletal proteins. The lipid bilayer comprises phospholipids and cholesterol, and all vertical proteins crossing the lipid bilayer represent membrane proteins. Membrane skeletal proteins laminate the inner membrane surface and are attached to the lipid bilayer through the multiprotein complexes such as the Ankyrin complex and the 4.1R junctional complex.

To date, more than 50 transmembrane proteins on RBC have been characterized and linked to various functions of the cell. Membrane proteins are integrated into the cell membrane and form vertical connections with the cytoskeleton (Mohandas and Gallagher, 2008). Membrane proteins have multiple cellular functions, for example, as transporters and channels, as receptors and adhesion molecules, and as structural proteins linking red cell membrane (Oliveira and Saldanha, 2010).

One-fourth of total membrane protein abundance is represented by Band 3. Band 3 is an anion transporter responsible for passaging ($HCO3^-$) and chloride (Cl^-). It plays a significant role as a membrane anchor for many membrane proteins and connects them to the skeletal proteins through different complexes (Mohandas and Gallagher, 2008). In the Ankyrin complex, Band 3 binds to ankyrin, protein 4.2, glycophorin A, RhAG, and CD47. In the 4.1 R junctional complex, Band 3 connects with skeletal proteins through protein 4.1R glycophorin C and protein p55, Duffy, XK, and Rh proteins (Mohandas and Gallagher, 2008). These interactions are essential to support the structural integrity of the membrane. Additionally, Band 3 can bind to

hemoglobin and glycolytic enzymes inside the cell (Mohandas and Gallagher, 2008), (Oliveira and Saldanha, 2010), (Chu *et al.*, 2016), (Campanella, Chu and Low, 2005).

Glycophorins are another representative of RBC membrane proteins and one of the first transmembrane proteins characterized (Theodore and Wallach, 1971). It is believed to play an essential role in preventing RBC aggregation (Oliveira and Saldanha, 2010). The presence of different carbohydrates on glycophorins regulates the negative charge of the cell membrane, which is essential for the reduction of cell-cell interaction (Oliveira and Saldanha, 2010). Glycophorin A (GPA) is the major constituent of the glycophorin family. There is evidence that GPA and Band 3 are tightly connected, and Band 3 is essential for GPA synthesis and stability, so they present approximately similar RBC membrane amounts (Daniels, 2007), (Oliveira and Saldanha, 2010). Additionally, extracellular domains of both Band 3 and GPA are highly polymorphic and represent different blood groups.

The RBC membrane skeletal proteins or cytoskeleton are not inserted into the lipid bilayer but laminate the inner membrane surface. It strengthen the lipid bilayer, thereby contributing to the RBC membrane's durability and flexibility to survive in circulation (Lux, 2016). They form a horizontal lattice in the cytoplasmic surface of the lipid bilayer, and to them belong α - spectrin, β - spectrin, actin filaments and protein 4.1R, ankyrin, protein 4.2, tropomodulin, adducin, p55, and dematin (Lux, 2016).

Spectrins are the most abundant peripheral membrane proteins in the erythrocyte cytoskeleton, and their expression begin already at the pro-erythroblast stage and gradually increase during RBC development. It is essential for keeping the structure and shape of the cell (Hanspal *et al.*, 1992), (Chen *et al.*, 2009). Spectrins are the main component of the cytoskeleton and are a heterodimer of 2 chains: α and β - spectrins. One structural part formed by spectrins is composed of two chains. Each chain is a heterodimer constituted of one αI - spectrin and one βI - spectrin, joined by the N-terminus of the α - spectrin subunit the C-terminus of β - spectrin. Together, two of these antiparallel heterodimer chains form a functional heterotetramer of spectrin. The α - spectrin and β - spectrin are built of numbers of spectrin repeat units (Lux, 2016), (Barbarino *et al.*, 2021). Spectrin heterodimers form flexible scaffold by arranging into hexagonal networks anchored to each other and the lipid bilayer through the Ankyrin and 4.1R junctional proteins complexes (Hanspal *et al.*, 1992), (Chen *et al.*, 2009), (Mohandas and Gallagher, 2008), (Barbarino *et al.*, 2021).

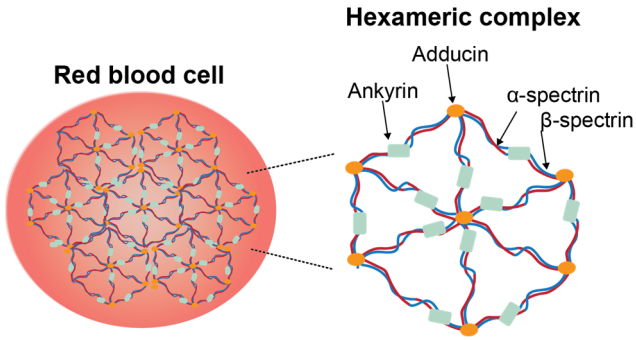


Figure 6. The RBC membrane. A. Enlarged schematic representation of the RBC and its cytoskeleton netwok, consisting of hexameric subunits, forming cytoskeleton. Spectrins are a significant component of the cytoskeleton of the RBC membrane. α- and -β spectrins form spectrin fibrils connected to the membrane and each other via two major complexes containing adducin and ankyrin. These interactions provide flexibility, elasticity, stability and shape to the membrane of RBCs.

Spectrin αI was first identified by Marchesi and Steers in 1967, and at that time, it was considered that only mammalian erythroid cells possess them (Hiller and Weber, 1977), (Pinder, Phethean and Gratzer, 1978), (Marchesi and Steers, 1967). However, later on, it was realized to be expressed in metazoan cells. Nowadays mammalian cells are known to express 7 different genes of spectrins, including two α spectrin genes (αI and αII). Interestingly, erythrocytes only express αI and not αII and it is though that the absence of nucleus in mature mammalian erythrocytes has selected for the advent of α I. It is also known that the affinity of αIβI binding is much weaker (100 times) than αIIβII. This feature might be required to allow RBC membrane deformability by gaining elasticity by rapidly detaching and attaching α and β chains, named “make and break” (Bignone and Baines, 2003), (Reviewed in Baines, 2009). Recently, this statement has been proved by showing that membrane skeleton remodelling, in the presence of dynamic tetramers $\alpha I_2\beta I_2$, is important to support properties of the functional erythrocyte membrane in mice (Hale *et al.*, 2021). Defects in αIβI-spectrin can cause abnormal cell shape, membrane fragility, reduced membrane deformability, leading to hereditary hemolytic anemia (Delaunay, 2002), (Perrotta, Gallagher and Mohandas, 2008).

Spectrins are also crucial for maintaining cellular structure and polarity by providing flexibility and strength to erythroid and other cell types. For example it has been shown that α-fodrin is involved in cardiac and brain development by stabilizing spectrin network, regulating ion channels (Lubbers *et al.*, 2019), (Stankewich *et al.*, 2011), also regulation of cancer and apoptosis (Sreeja *et al.*, 2020). Interestingly, αII - spectrin express a proteolytically sensitive site, which is recognized by caspases 2, 3 and 7 and calpains in a highly regulated manner (Jänicke *et al.*, 1998).

In summary, disruptions at any stage of RBC development have a significant negative impact on their physiology and function, therefore ultimately on our health. These simple-looking cells develop under strict and well-organized conditions.

After nuclear extrusion, it takes additional hours to develop fully. RBCs emerge early in development and adjust to our needs based on oxygen availability by changing the type of the hemoglobin and doing it in a precisely regulated way. Finally, the RBC membrane is a vital part of its functions, providing mechanical strength throughout their life. As we can see, erythropoiesis is not a straightforward process to be imitated in a lab and to do, so it requires a lot of effort and time to understand its finer details.

Definition and causes of anemia

When the number of RBC and/or hemoglobin concentration is lower than what is considered healthy then this condition is called anemia (Chaparro and Suchdev, 2019). Symptoms include fatigue, difficulty concentrating, pale skin, and shortness of breath. In severe cases, anemia can be a life-threatening disorder (Kassebaum *et al.*, 2016). Worldwide, anemia affects almost one-third of the population of which children, pregnant women, and older people are the most vulnerable (Chaparro and Suchdev, 2019), (Kassebaum *et al.*, 2016), (Kassebaum *et al.*, 2015), (WHO, 2022). The word "anemia" derives from the ancient Greek word *anaimia,* which means "lack of blood." Any process that disrupts the balance between RBC production and results in a loss of RBC numbers causes anemia. Decreased production (ineffective erythropoiesis), increased loss of RBCs (bleeding), and increased destruction (hemolysis) of RBCs are the leading causes. Nutritional insufficiencies include iron, vitamin A and B_{12}, folate, but also various inflammations or genetic disorders can cause ineffective erythropoiesis. Iron deficiency anemia, thalassemia, congenital erythroblastopenia and hematological malignancy to name a few. Hemorrhages, red cell membrane disorders, sickle cell disease, immune hemolytic anemia, and malaria represent types of anemia that can be caused by increased RBCs loss and increased destruction of RBCs (Chaparro and Suchdev, 2019), (Pearson and Kalinyak, 2005), (Freeman, Rai and Morando, 2021).

General treatment of anemia

Usually, the treatment for anemia depends on understanding its etiology and pathophysiology. For instance, if the cause of anemia is associated with iron deficiency, then oral or intravenous iron administration is the first-choice treatment. The most prevalent type of anemia globally is iron deficiency anemia (WHO, 2022).

Erythropoietin (EPO) is a hormone produced by specialized renal interstitial fibroblasts called renal Epo-producing (REP) cells in the kidneys, and respond to the low blood oxygen levels. EPO acts directly on erythropoietin receptors (EPOR) expressed on particular erythroid progenitors in the bone marrow which promotes

cellular proliferation, differentiation and survival by initiating a series of signalling events (Schoener and Borger, 2021), (Jelkmann, 2013), (John *et al.*, 2012), (Freeman, Rai and Morando, 2021). Patients suffering, for instance, from chronic kidney disease (CKD) often have anemia as a complication due to the insufficient EPO production by REP cells (Hayat, Haria and Salifu, 2008). Recombinant human erythropoietin (rHuEPO) was generated and became available in clinical practice in the 1980s (Ng *et al.*, 2003). Administration of rHuEPO successfully compensates the deficiency of endogenous EPO production. By efficiently stimulating erythropoiesis, rHuEPO has revolutionized anemia treatment by having more beneficial effects on improving quality of life than adverse or dangerous side effects (Jelkmann, 2013). Generally, rHuEPO therapy has efficiently improved anemia treatment by avoiding risky blood transfusion therapies; therefore, rHuEPO has been considered the first-choice therapy for anemia treatment (Ng *et al.*, 2003).

With time, pharmaceutical companies have manufactured Erythropoiesis-stimulating agents (ESA), which, similarly to rHuEOP, can act on EPOR but with shorter or longer half-lives. ESAs with a longer half-life would require less frequent administration. There is many available ESAs. Epoetin - α and Epoetin - β belong to the first generation of ESA (short half-life); the second generation Darbopoetin (long half-life); and there is also the third generation ESA with continuous EPO receptor activator (Hayat, Haria and Salifu, 2008), (Ng *et al.*, 2003). HSC transplantations and transfusions of specific blood cell components such as RBCs are alternative and curative therapies that have been used for many blood disorders, including anemia (Lucarelli *et al.*, 2012), (İnce, 2017).

Common problems and challenges with the treatment of anemia

Various strategies have been developed for anemia treatment, however they have some limitations and risks. Treating anemia with iron can be very effective, but also with high risks. Sometimes iron supplementation can simply be ineffective if there is also a deficiency of micronutrients interacting with iron (Wessling-Resnick, 2017). Repeated iron administration can accelerate pathogens that use iron for their growth, replication and, spreading in the body. Failure to recognize these infections would results in increased infections rates and even mortality (Canziani *et al.*, 2001). Also, repeated iron administration can cause diseases induced by iron overload in the tissues or organs. For example, acquired forms of hemochromatosis is one of them and can lead to liver fibrosis or cirrhosis, cardiac problems and pancreatic dysfunction. Repeated blood transfusions can cause it too (Wessling-Resnick, 2017). Excess iron can also damage regulatory pathways of proteins or DNA , tissues or organs functions or development by promoting the formation of toxic hydroxyl radicals (Thomas *et al.*, 2009) therefore this therapy has to be carefully dosed and monitored.

RHuEPO and ESA treatment are also associated with risks of developing hypertension and thrombosis by impairing the balance between vasodilating and vasoconstrictor factors or promoting platelets production and reactivity, respectively (Ribeiro *et al.*, 2013). According to statistics, 10 % of patients are irresponsive or develop resistance to rHuEPO or ESA treatment, which is related to a poor prognosis for surviving (Ribeiro *et al.*, 2013). Another drawback of rHuEPO or ESA treatment is that it is not tolerated well by all individuals especially with comorbid conditions. For instance, there are concerns about rHuEPO treatment in patients with cancer because of its potential effect on cancer progression (Ribeiro *et al.*, 2013), (Maiese, Chong and Shang, 2008). Additionally, different pharmacological potencies of stimulation of EPOR by ESA can also harm non-erythroid cells expressing this receptor. Furthermore, there is no clear conclusive information explaining benefits and risks between short and long lasting ESAs (Sakaguchi *et al.*, 2019).

Despite the life-saving effects of blood transfusion, this practice has a list of limitations. There is a high risk to be infected with contaminated blood with known and unknown pathogens. Also, to avoid severe hemolytic reactions, ABO and RhD antigens of donated blood must be compatible with receivers' blood; however, not all countries in the world can afford to test it regularly. Additionally, there are more than 40 other known blood groups. Some patients need to rely on frequent donations of blood types that are rare. These blood types can also be in demand creating a life-threatening situation for the patient (Pandey, Das and Chaudhary, 2014), (Higgins, 2000), (Yazdanbakhsh, Ware and Noizat-Pirenne, 2012). There is also a shortage of blood donors in many countries globally, especially in low- and middle-income countries (WHO, 2022).

These concerns about current treatments clearly describe the necessity of unravelling novel regulators of erythropoiesis to better understand erythropoiesis because it could improve current anemia treatment. Therefore, my research focus during the PhD studies was to discover novel alternative approaches that could be applied to develop a potential cure for anemia, in particular blood transfusion. Efficient production of RBCs in the lab under strict quality control would be beneficial for blood transfusion therapy.

Stem cells build (red) blood cells

Definition

Mature blood cells have various but relatively short life spans, and are thus continuously replaced to maintain the healthy body throughout life. All blood cells originate from the common cell origin, hematopoietic stem cell (HSC). HSCs give

rise to restricted progenitors, which further extensively expand and give rise to mature blood cells (Orkin, 2000). The lifelong and continuous process of blood formation from HSC is called hematopoiesis. The word hematopoiesis derives from two Greek words, haima "blood" and poiēsis "to produce something".

HSCs are an undifferentiated blood cells that have the ability to self-renew and differentiate into multiple blood cell type upon differentiation through lineage-restricted progenitor stages. Self-renewal is the process by which stem cells divide to give rise to one or two own copies upon cell division, and is thus required to keep the stem cell pool throughout the life of an organism. The gold standard to characterize functional HSC is transplantations in which a pool of cells is transplanted into recipient to evaluate long-term reconstitution and multi-lineage potential (Osawa, Hanada, Hamada and Hiromitsu, 1996), (Eaves, 2015), (Doulatov *et al.*, 2012). During differentiation, HSCs, through multiple intermediate progenitor stages, give rise to mature blood cells in response to various intrinsic and extrinsic signals. These simultaneous abilities for self-renewal and multi-lineage potential make HSCs a powerful cell type that constantly renews the hematopoietic pool (Dick *et al.*, 1985), (Lemischka, Raulet and Mulligan, 1986).

HSC emergence and its sources

Studying human HSC emergence is considerably hard because of practical and ethical restrictions, therefore a significant proportion of gained knowledge is based on the studies done in mice. Hematopoiesis occurs in several overlapping stages and at multiple sites of the developing embryo and fetus. Hematopoiesis begins by forming blood cells in the yolk sac around 3 to 6 weeks of human embryo gestation, however the lineage potential is predominantly restricted to erythroid progenitor cell (Palis and Yoder, 2001). The first HSCs emerge during early embryonic development in the aorta-gonad-mesonephros (AGM) region at 27 days of embryo development (Juliena, Omara and Tavian, 2016). These cells travel via circulation and can be found in the yolk sac and placenta. Around 40 days of embryo development, almost no HSCs can be found in the AGM region because they relocated to the primary residence of fetal hematopoiesis - the fetal liver (FL) (Juliena, Omara and Tavian, 2016). This transition happens through the placenta. From week 6 to 22 of gestation, located in the fetal liver, definitive HSCs expand in numbers to build up the blood system in the growing fetus (Palis and Yoder, 2001). Towards the end of pregnancy or soon after birth, HSCs again change their residence site and recolonize in the bone marrow (BM) (Mikkola and Orkin, 2006). Adult human HSCs reside in a particular microenvironment of the bone marrow, referred to as a "niche" (Schofield, 1978), (Lander *et al.*, 2012), (Crane, Jeffery and Morrison, 2017). This specific microenvironment accommodates hematopoietic and non-hematopoietic cells. Cells such as mesenchymal, endothelial, osteoblasts, and vasculature tissue belong to the non-hematopoietic compartment of the niche, which

contributes to maintaining HSCs integrity, longevity and functions (Lymperi, Ferraro, and Scadden, 2008).

During pregnancy the placenta plays essential roles for maternal and fetal physiology and it is filled with maternal and fetal blood. The placenta connects to the fetus through the umbilical cord (UC) and after birth is cut and discarded. Nowadays UC blood (UCB) is an easily accessible source for neonatal human HSCs (Broxmeyer *et al.*, 1989). Due to its accessibility and non-invasive collection method, it has become the central source of human HSCs used in current transplantation therapies and research. Adult human HSCs can be collected from the bone marrow (BM) or peripheral blood (PB); however, the procedure can require unpleasant medical interventions (Hequet, 2015).

Due to HSC features they are in high demand for therapies. Transplantation therapies using bone marrow (BM) or UCB transplantation from donor to the matching patient has been a life-saving procedure used in the clinic (Thomas *et al.*, 1978), (Morgan *et al.*, 2017).

Representative cell surface markers

The HSC population is a rare cell type and it remains challenging to prospectively isolate. For instance, morphological observations using light microscopic analysis is not informative enough to distinguish true HSCs as they do not possess any special morphological features compared to many other hematopoietic cells. Instead, flow cytometry technology has been utilized to identify HSCs from the bulk cells using cell surface expression of proteins and targeted antibody-based labelling with fluorochromes. This approach has identified combinations of various cell surface markers that represent many types of hematopoietic progenitors, including HSCs, especially through studies of murine hematopoiesis. However, finding markers to isolate human HSCs naturally has been more challenging due to their rare frequency and limited availability for research.

Mouse HSCs are undifferentiated cells thus they do not express any cell surface markers identifying mature cell, referred to as "lineage negative" (Lin^-), but do express tyrosine kinase receptor (c-kit) and stem cell antigen 1 (sca-1) markers, termed as $c\text{-}kit^+sca\text{-}1^+$ (Okada *et al.*, 1991), (Ikuta and Weissman, 1992), (Spangrude, Heimfeld and Weissman, 1991). Lin^- $c\text{-}kit^+sca^-1^+$ cell population, so called LSK population, contains HSCs but also other early stages of multi-potent progenitors. To further enrich for HSCs, a combination of $CD34^-$ and $Flt3^+$ was found to be useful to isolate murine HSCs (Zeigler *et al.*, 1994), (Osawa, Hanada, Hamada and Nakauchi, 1996). Alternatively, a combination of markers from signaling lymphocytic activation molecule (SLAM) family $CD150^+$ and $CD48^-$ markers are also used (Kiel *et al.*, 2005).

Interestingly, murine HSCs do not express CD34, however expression of CD34 is associated with human HSCs and hematopoietic stem and progenitor cells (HSPCs), (Civin *et al.*, 1984). Over years additional cell surface markers have been discovered and correlated to the HSCs features. For instance, within $CD34^+$ cell population, there is a population of cells expressing CD38 representing primitive progenitors, therefore it should be excluded for strict HSC isolation. Next, within $CD34^+CD38^-$ population cells not expressing CD45RA, but expressing CD90 would further enrich human HSCs (Majeti, Park and Weissman, 2007). One more recent finding suggests that cells expressing an additional marker CD49f contributes to achieving the highest purity of HSCs and their functionality was proved by transplantation experiments. Combination of these cell surface markers $CD34^+CD38^-CD90^+CD45RA^-CD49f^+$ allows enrichment of human HSC with a 5 - 10% purity (Notta *et al.*, 2011). The Endothelial Protein C Receptor (EPCR) is another promising candidate for enrichment of HSCs (Fares *et al.*, 2017).

Numerous studies have been performed focusing on HSC phenotypic and functional characterizations. Transplantation assays revealed functional diversity of HSC, particularly in multilineage differentiation capacities during bone marrow reconstitution. For instance, some HSCs reconstitute different ratios of lymphoid and myeloid lineages (Jordan and Lemischka, 1990). The concept of cell heterogeneity has been extensively studied and some studies have defined different HSCs subsets exhibiting differentiation bias (Reviewed in (Crisan and Dzierzak, 2016), (Ema, Morita and Suda, 2014)), (Miharada *et al.*, 2011), (Radulovic *et al.*, 2019). The origin of HSC heterogeneity has been extensively studied and discussed and it is proposed that it can be driven by different extrinsic and intrinsic factors such as transcription factors, mutations, metabolic activities, distribution of cell components during cell division, niche microenvironment and interactions with various cells in the niche etc (Jacobsen and Nerlov, 2019). Therefore, in Paper III we compared $CD34^+$ cells, isolated from different UCB donor's potential to give rise to erythroid cells.

Ex vivo RBC production using various materials

Significant progress in better understanding hematopoiesis allows us to generate different blood components in the lab, including RBCs. *Ex vivo* generated RBC has potential for clinical applications. For instance, efficient generation of RBC could contribute to the development of the cells with therapeutic properties, e.g. drug delivery. At the same time, RBC produced on a large scale could be used for transfusion purposes to avoid blood transmitted infections or produce on-demand blood for patients with rare blood groups (Stanworth *et al.*, 2022). Some of the current culture techniques allow *ex vivo* RBC generation; however, these methods are either not efficient, too costly or need to be scaled up to meet needs for

transfusion therapies. Below I am briefly reviewing the advantages and disadvantages of the currently available cell sources for RBC generation such as hematopoietic stem and progenitor cells (HSPC), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSC) and immortalized erythroid cell lines.

$CD34^+$cells from adult blood or cord blood

In 1989 Fibach et al. reported the successful establishment of a liquid culture system that supports differentiation of peripheral blood mononuclear cells to erythroid cells. However, this approach resulted in a heterogeneous cell population since both erythroid and myeloid cells were present in the same cell mixture and erythroid progenitors did not mature into enucleated cells (Fibach *et al.*, 1989). In 2002 Douay's group succeeded in producing human erythroid cells from UBC $CD34^+$ cells at a large scale. Generated cells possessed functional hemoglobin and could undergo terminal differentiation *in vitro*, however with only 4 % enucleation efficiency (Neildez-Nguyen *et al.*, 2002). Three years later, Douay's group reported a new method achieving up to 95 % - 99 % of enucleation using $CD34^+$ cells from diverse origins such as UCB, BM, PB (Giarratana *et al.*, 2005). In 2011, the same group demonstrated proof of principle for clinical feasibility of *in vitro* produced RBC. Giarratana et al. showed that *in vitro* generated RBC from PB $CD34^+$ are functional, can survive during storage and then be transfused. They also demonstrated, for the first time, that *ex vivo* generated RBC cells survived in the human circulation system for as long as native RBC (Giarratana *et al.*, 2011). From these initial successes came the commitment to finding efficient sources and protocols before using generated RBCs for therapeutic purposes. For instance, $CD34^+$ cells isolated from PB or UCB, can give rise to similar frequencies of enucleated cells. However, UCB derived $CD34^+$ cells are more proliferative and give rise to 5 - 10 times more RBCs than from the PB. Currently, $CD34^+$ cells are considered as the best source for RBC production (Giarratana *et al.*, 2011). Still, more studies are needed to confirm safety and functionality of generated RBC from UCB $CD34^+$, by performing allogenic transfusions to multiple recipients from multiple UCB donors (Giarratana *et al.*, 2011) (Reviewed in Severn and Toye, 2018). Historically, high enucleation efficiency was achieved by culturing in erythroid differentiation media with feeder cells or bovine serum. However, to meet clinical requirements, protocols should be fast and not contain ingredients of animal origin. These groups were the first to produce efficient numbers of RBC by replacing stromal cells with serum, 100 % and 77, 5 %, respectively (Miharada et al., 2006), (Leberbauer *et al.*, 2005). The length of the protocol developed by Miharada et al. (20 days) was three times faster than by Leberbauer et al (60 days). With time, many technologies have now been developed that allow further improvement of differentiation media compositions and employing other strategies for differentiation conditions in a larger scale. Timmis et al. demonstrated the potential of wave-type bioreactors for large-scale RBC production for the first time.

Their developed high-yield culture conditions could generate 500 units of transfusable RBC from one cord blood unit (Timmins *et al.*, 2011). Because of the use of bovine serum albumin (BSA), their RBCs were not, however, generated under good manufacturing practice (GMP) to be suitable for clinical grade. The use of other types of bioreactors, such as rapid expansion bioreactor (G-Rex), was tested. Produced under GMP conditions, RBCs with 90 % enucleation efficiency were generated from mononuclear cells (MNC) from PB; however, this method's scalability was not comparable to Timmis et al. and needs to be further addressed (Heshusius *et al.*, 2019) Also, three-dimensional (3D) aggregate culture systems shows great potential for expansion and efficiently maturation of $CD34^+$ cells from UCB towards RBCs (Lee *et al.*, 2015), (Allenby *et al.*, 2019). In summary, $CD34^+$ cells from adult blood or umbilical cord blood is an excellent source for RBC generation; however, scalability and efficient protocols still needs to be improved.

Embryonic and induced pluripotent stem cells

To overcome problems with the numbers of starting material, human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been considered as another valuable starting materials for generating RBCs *in vitro*. These cells can be indefinitely maintained in culture and give rise to any cell type in the body. Additionally, iPSCs are attractive to generate patient-specific RBC for autologous transfusions, thereby avoiding immune rejections. Moreover, iPSCs create an opportunity to solve ethical problems related to the usage of ESCs in many countries. Currently, there are many approaches to generate RBCs using human ESCs as a starting material. In general, those protocols could be divided into two types. One type utilizes ESCs and iPSC co-culturing on stromal cell lines to induce hematopoietic differentiation (Kaufman *et al.*, 2001), (Olivier *et al.*, 2006), (Lu *et al.*, 2008). The other approach focuses on, first, generating embryoid bodies (EBs), that then are differentiated into erythroid cells (Chang *et al.*, 2006), (Oliver *et al.*, 2016). Many groups have also published protocols for RBC generation using iPSCs, with and without feeder cells. These methods succeeded to obtain functional, mature erythroid cells, however with low enucleation efficiency reaching 2 - 15 % rate (Lapillonne *et al.*, 2010), (Wang *et al.*, 2016). A recent study reported further protocol optimization and reaching considerably higher enucleation efficiency, however, the length of the protocol still needs to be improved (Olivier *et al.*, 2019). Thus, the cost and complexity of these methods remain a significant limitation of this technology for future clinical use. Additionally, methods for RBC production using ESC or iPSC are facing problems related to poor enucleation and insufficient hemoglobin synthesis (Lim *et al.*, 2021).

Immortalized erythroid cell lines

Another promising source for large-scale production of functional RBC is immortalized erythroid cell lines. In 1965, Hayflick reported that human diploid cells have limited lifetime during *in vitro* cultures termed as culture senescence causing irreversible cell growth arrest (Hayflick limit) (Hayflick, 1965). The concept of the Hayflick limit is that many cell types can divide a particular number of times before they cannot divide anymore. Overcoming cell growth arrest and allowing continuous cell proliferation would require inducing immortalization by overexpressing oncogenes (Hayflick, 1965), (Hubbard and Ozer, 1999). Because of the robust cell growth, cell lines are normally easy to culture (Shieh, 2015).

Erythroid cell lines can be established by artificially manipulating their proliferation at, for example, the early erythroid progenitor cell stage, making differentiation time and protocols more efficient than using $CD34^+$ cells, ESC, or iPSC. Several attempts to establish mouse and human immortalized erythroid cell lines have been made. In 2008, Hiroyama et al. established the first immortalized erythroid cell lines from mouse ESCs (Mouse ES cell Derived-Erythroid Progenitor: MEDEP). These cell lines can differentiate into mature, functional, enucleated RBCs. Mice transplanted with MEDEP recovered from severe acute anemia, while non-treated mice died, indicating that MEDEP had the potential to give rise to functional RBCs (Hiroyama *et al.*, 2008). In 2010, Wong et al. established first immortalized erythroid cell line from human $CD36^+$ erythroid progenitor cells using human papillomavirus 16 gene-E6/E7 (HPV16-E6/E7), expressing primarily γ-globin type of hemoglobin and with inefficient differentiation potential (Wong *et al.*, 2010). Later, Kurita et al. established human immortalized erythroid progenitor cell lines derived from $CD34^+$ cells isolated from human umbilical cord blood (HUDEP) and from human iPS cells (HiDEP). These cell lines were generated by the inducible ectopic expression of *HPV16-E6/E7*, which is turned off to allow cell differentiation. HUDEP/HiDEP keep infinite growth capacity, express erythroid-specific cell surface markers, and possess the potential to produce functional fetal or embryonic type hemoglobin, however, with low enucleation efficiency and a high rate of cell death after the induction of differentiation (Kurita *et al.*, 2013). In addition, other groups developed methods establishing human immortalized erythroid cell lines using different approaches. Huang et al. established the immortalized cell lines from UCB cells by transducing with three genetic factors (Sox-2, c-Myc and shRNA against TP53 (Huang *et al.*, 2014). Hirose et al. generated immortalized erythroid cell lines from human iPS cells by overexpressing *c-Myc* and *BCL-xL* (Hirose *et al.*, 2013). However, the enucleation efficiencies of these cell lines were also very low. In 2017, Trakarnsanga et al. reported an establishment of an immortalized human erythroid cell line (Bristol Erythroid Line Adult; BEL-A) derived from adult bone marrow $CD34^+$ cells by the inducible ectopic expression of *HPV16-E6/E7* (Trakarnsanga *et al.*, 2017). BEL-A enucleation efficiency reaches up to 30 %. Bagchi et al. used another cell source peripheral blood mononuclear (PBM) cells to establish cell line

with 20 % enucleation efficiency. Their immortalization approach is similar to the previously published Kurita et al. and Trakarnsanga et al. (Bagchi *et al.*, 2021).

As mentioned above, erythroid cell lines are immortalized by the inducible HPV16-E6/E7 system to terminate HPV16-E6/E7 expression when cells need to undergo differentiation. However, the need of discontinuing *HPV16-E6/E7* expression for inducing their differentiation has never been tested. Therefore, we established another cell line (Erythroid Line from Lund University; ELLU) by simply overexpressing the *HPV16-E6/E7*. ELLU can proliferate indefinitely and give rise to enucleated cells without the need for terminating *HPV16-E6/E7* expression. Interestingly, we also discovered cells heterogeneity in terms of hemoglobin expression; even though these cells were derived from one common source ($CD34^+$ cells from adult BM) we found clones expressing embryonic, fetal and adult types of hemoglobin (Soboleva *et al.*, 2021).

It is predicted that hemoglobin type of *in vitro* generated RBCs would reflect on their origin, with embryonic or fetal type from UCB, ESC, and iPSC and adult type from adult donor sources such as PB or BM. However, interestingly, expression of fetal hemoglobin is commonly observed from *in vitro* generated RBCs derived from the adult origin (Giarratana *et al.*, 2011). One of the explanations is that a high cell proliferation rate induced by growth factors, for instance, SCF, can cause the appearance of γ-globin (Bhanu *et al.*, 2004), (Giarratana *et al.*, 2011). One study addressed this problem with fetal hemoglobin expression and demonstrated the ability to robustly induce β-globin expression in erythroid cells generated from UCB or iPSCs by simultaneous overexpression of *KLF1* and *BCL11*A (Trakarnsanga *et al.*, 2014). However, overexpression of each gene individually led to a very modest elevation of β-globin expression. Thus, *KLF1* and *BCL11A* gene expression has to be above the threshold levels to secure sufficient adult hemoglobin induction in cells with fetal or embryonic type hemoglobins (Trakarnsanga *et al.*, 2014).

Immortalization with HPV16-E6/E7

Primary cells stop dividing after a certain number of divisions (Hayflick limit). Genetic manipulations are therefore required to enforce primary cells to indefinitely proliferate by escaping normal senescence and become immortalized cell lines. Currently, there are numbers of approaches that can induce efficient immortalization of cells such as simian virus 40 large T antigen, epstein-barr virus (EBV), adenovirus E1A, human T cell leukemia virus, oncogenes, mutant p53 gene and papillomaviruses E6 and E7 (Katakura, Alam and Shirahata, 1998). Papillomaviruses are small double-stranded DNA viruses that infect mucosal and cutaneous epithelia and induce cellular proliferation. More than 100 human papillomaviruses (HPV) have been identified, of which the majority have been implicated in cervical cancers while a few are rarely found in cancers. Therefore, human papillomaviruses can be divided into “low-” and “high-risk” HPVs

(Tommasino, 2014). High-risk HPV-16 and HPV-18 account for 70 % of cervical cancer cases, of which HPV-16 causes more than 50 %, and due to their high cancerogenic activity, these viruses have been most studied. For this reason, there are available vaccines to prevent humans from HPV infections (Arbyn *et al.*, 2012).

The major oncoproteins driving HPV carcinogenesis are E6 and E7. Their continuous expression promotes cell cycling by causing the inactivation of p53 and pRb by E6 and E7, respectively (Scheffner *et al.*, 1990), (Dyson *et al.*, 1988). P53 is a transcription factor activating genes involved in apoptosis and DNA damage repair, and therefore inhibition of it can lead to immortalization of the cells. E6 and E7 are known to elicit their function through multiple ways. E6 proteins and the E6-associated protein (E6AP) can cause ubiquitination of p53 protein, leading to its degradation. Also, E6 proteins can negatively regulate transcription activity by directly binding to P53 protein. Moreover, E6 protein can inhibit various regulators of p53, for instance, so-called histone acetyltransferases, p300/CBP, which both positively or negatively regulate p53 transactivation. E7 protein has a binding site for the transcription factor E2F leading to destabilisation of pRb. The pRb is a tumour suppressor protein, which plays a role in regulating cell cycle progression. Usually in quiescent cells, pRb is hypo-phosphorylated and associated with E2F. When cells start exiting a quiescent state, the phosphorylation of pRb happens which releases E2F. Unbound and functional E2F promotes the transcription of genes responsible for cell cycle progression. E7 mimicks this mechanism, as the binding of E7 with pRb leads to the release of E2F and a stimulation of cell cycle progression. Both E6 and E7 play essential role in occurrence in cancer (zur Hausen, 2000). E6 and E7 are transcribed polycistronically from a single promoter allowing virus to co-ordinately regulate expression of both proteins in the infected cells. Transcripts of E6 and E7 can undergo alternative RNA splicing to produce different isoforms; however, its importance is not entirely understood. For example, it has been identified several E6 splice transcripts (termed E6*) such as E6*I through E6*VI and E6 ^ E7, E6* ^ E7*I, E6*I-E7*II and amounts of them can differ (Olmedo-Nieva *et al.*, 2018). Interestingly, E6*I can enhance the translation of E7 protein, while unspliced E6 is responsible for full-length E6 expression (Tang *et al.*, 2006).

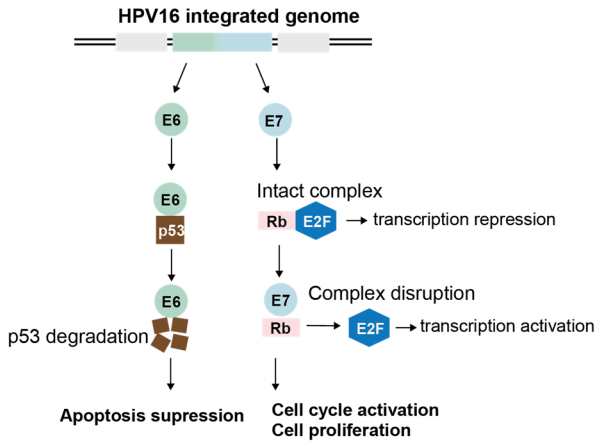


Figure 7.Cell immortalization with HPV16-E6/E7 oncoproteins. Continuous expression of E6/E7 promotes cell cycle progression. E6 by degrading p53 supresses cell apoptosis. E7 activates cell cycle progression by binding to the retinoblastoma protein.

Benefits of erythroid cell lines

Using $CD34^+$ from UCB is considered the best source for *ex vivo* RBC generation, at least in terms of achieving high enucleation efficiency. Still, PB $CD34^+$ and BM $CD34^+$ cells can also efficiently enucleate. PB $CD34^+$ and BM $CD34^+$ cells have lower expansion capacity than UCB $CD34^+$, suggesting limitations of $CD34^+$ cells as a starting material (Giarratana *et al.*, 2005), (Zhang, 2007), (Timmins *et al.*, 2011). A standard therapeutic dose of RBC for an adult comprises approximately 2 x 10^{12} RBC. Current techniques for the large scale RBC production using bioreactors can provide the range of 1 x 10^4 - 2.9 x 10^5 RBCs, leaving significant room for improvement of final yields to be used for transfusions (Severn and Toye, 2018), (Pellegrin, Severn and Toye, 2021).

Ex vivo produced RBCs could benefit patients who require repeated or life-long transfusions. Patients can be continuously supplied with stocks of generated blood on demand. For safe transfusions, produced blood must be genetically matched between donor and recipient. To meet this demand regularly is challenging. Specifically, when UCB $CD34^+$ are used for allogenic transfusions because this cell source is "one-time-only usage". Using PB $CD34^+$ or BM $CD34^+$ for autologous transfusions is inconvenient, since isolation of these cell sources is more expensive and complicated than isolation of UCB $CD34^+$ (Di Buduo *et al.*, 2021). Additionally, protocols using $CD34^+$ cells cannot guarantee consistency with yields because different donors of $CD34^+$ UCB do not give rise to similar RBC yields (Pellegrin, Severn and Toye, 2021), (Di Buduo *et al.*, 2021), (Soboleva, Åkerstrand and Miharada, 2022). This natural donor variation in the starting material in differentiation potential represents another challenge and suggests the need for

limitless stable sources to ensure unlimited continuous productions of RBCs (Migliaccioa, Whitsetta and Migliaccio, 2009).

Thus, immortalized erythroid cell lines are valuable cell sources due to their unlimited proliferation capacity, pre-committment to erythroid lineage, and simple maintenance and differentiation conditions. Numbers of various erythroid cell lines have been generated in terms of hemoglobin content, depending on the source of the origin (UCB, iPS, PB, BM, MNC) (Kurita *et al.*, 2013), (Trakarnsanga *et al.*, 2017), (Soboleva *et al.*, 2021), (Bagchi *et al.*, 2021). Further extensive studies and protocol optimizations for establishing/culturing erythroid cell lines make it possible to develop time and cost-efficient differentiation protocols for efficient production of mature RBC in a large scale, in a short time, and at an affordable price (Pellegrin, Severn and Toye, 2021).

Unlimited starting cell numbers is a tremendous advantage for *ex vivo* RBC production; therefore, immortalized erythroid cell lines have excellent potential because of their homogeneity, simple culture conditions and proliferation speed.

Histone deacetylases

Chemical compound screening is an unbiased approach to find regulators controlling enucleation. Multiple histone deacetylases inhibitors have been identified in Paper I enhancing frequency of enucleated cells in immortalized erythroid cell line HiDEP.

The fundamental structural unit of chromatin consists of the nucleosome. Each nucleosome consists of the wrapped DNA around eight core histone proteins, two copies of H2A, H2B, H3, H4 (Luger *et al.*, 1997). Functioning beyond structural support, the interaction between histones and DNA regulates gene expression in eukaryotic cells. The addition of acetyl groups to histone tails by enzyme histone acetyltransferase (HAT) make the DNA accessible for transcription factors to begin gene transcription. Conversely, the active removal of the acetyl groups by histone deacetylases (HDACs) minimizes access to transcription factors and a resulting decreased gene expression. The balanced activity of the HATs and the HDACs are considered to play essential roles in maintaining cellular homeostasis.

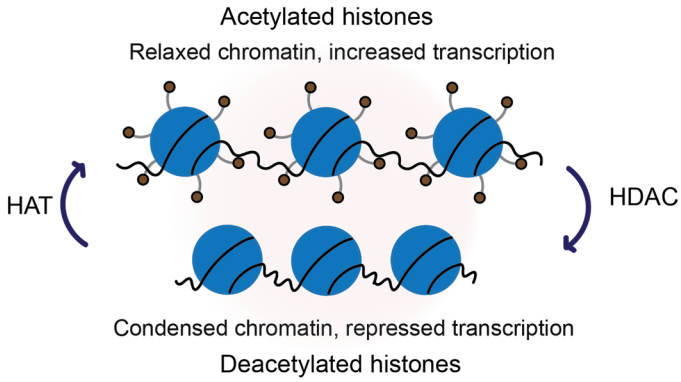


Figure 8. Histone acetyltransferase (HATs) and histone deacetylase (HDACs) regulate acetylation. HATs are a group of enzymes that acetylate histone proteins, and histones acetylation is associated with the activation of gene transcription. HDACs, decrease transcription activity by removing the acetyl groups from lysine residues in the tail regions of histones.

Interestingly, HDACs remove acetyl groups also from non-histone proteins. There are hundreds of proteins which are regulated by acetylation, including HATs and HDACs themselves, cytoskeletal proteins, transcription factors, regulators of DNA repair, recombination and replication, chaperones, ribosomal proteins etc (Glozak *et al.*, 2005), (Reviewed in Roche and Bertrand, 2016). Therefore, HDACs regulate the functions of many proteins, protein-protein interactions, and cellular signalling networks.

To date, the HDAC family consists of 18 isoforms which are subdivided into four separate classes. Class I, II, and IV, are zinc-dependent enzymes, while class III is a nicotinamide adenine dinucleotide (NAD^+)-dependent enzyme (Reviewed in (Yang and Seto, 2007), (Park and Kim, 2020). Almost all Class I and Class IV members are localized to the nucleus, while HDACs from Class II can shuttle between nucleus and cytoplasm, and Class III have several different locations such as cytoplasm nucleus and mitochondria (Reviewed in Michan and Sinclair, 2007), (De Ruijter *et al.*, 2003).

Even though our understanding of HDACs and their substrates are continuously expanding, it is still challenging to correlate specific HDAC activities with distinct cellular effects. One reason is that HDACs have low substrate specificity, meaning that one HDAC can act on several substrates. Additionally, many HDACs act by forming multiprotein complexes, making it difficult to separate individual functions of each member in the complex (Glozak *et al.*, 2005), (Michan and Sinclair, 2007).

HDAC inhibitors

Disruption of the equilibrium in the acetylation dynamics influences gene expression, which can result in various pathological conditions. Several reports

indicate increased levels of the HDACs in specific cancers (Halkidou *et al.*, 2004) (Krusche *et al.*, 2005) (Stojanovic *et al.*, 2016) (Li *et al.*, 2016) as well as other diseases (Benedetti, Conte and Altucci, 2015). These findings opened for investigation and discoveries of naturally occurring and development of synthetic HDAC inhibitors (HDACi) (Newkirk, Bowers and Williams, 2009). Several *in vitro* studies reported that increased acetylation induced by HDACi treatment can activate various cell responses in cancer cells, for instance cell differentiation, apoptosis, cell cycle arrest, and cytotoxicity (Martínez-Iglesias *et al.*, 2008). Hence, it is believed that inhibition of HDACs is a significant therapeutic approach. Many HDACi have been developed and are involved in more than 500 clinical trials (Gryder, Sodji and Oyelere, 2012), some are already approved as anti-cancer drugs (Cappellacci *et al.*, 2018). Those HDACi can be categorized to several groups according to their mechanism of the action. Pan-HDACi are nonspecific HDACi, which act on multiple isoforms of zinc-dependent HDACs. In contrast the class-selective HDACi affect particular classes of HDACs. Finally, there are also isoform-selective HDACi (Bieliauskas and Pflum, 2008)**.** Due to the high probability of interfering with their cellular functions, HDACi treatment can cause unpredictable and undesirable consequences and severe side effects. Thus, current knowledge about HDACs and their diverse functions suggests that HDACi mechanism of action is not completely understood and therefore it is of interest to develop more precise and selective inhibitors for different HDACs in different pathologies (Minucci and Pelicci, 2006), (Bertrand, 2010), (Gryder, Sodji and Oyelere, 2012).

HDACs in erythropoiesis

Although there are a number of studies on the role of HDAC in erythropoiesis, the exact role is still not completely understood. Several reports have shown that inhibition of the activity of the HDAC1/HDAC2 induces expression of a fetal γ-globin in sickle cell disease (SCD). The γ-globin in SCD inhibits disease phenotype by ameliorating polymerization of sickle hemoglobin, thus it is clinically beneficial (Bradner *et al.*, 2010), (Esrick *et al.*, 2015), (Shearstone *et al.*, 2016). Another study described divergent functions of HDACs in erythropoiesis, and found a positive contribution to EPO mediated signalling pathway while negatively regulate IL-3 pathway during erythroid development from $CD34^+$ cells (Yamamura *et al.*, 2006).

HDACs in terminal erythropoiesis

To date there are a number of studies on the role of HDACs in terminal erythropoiesis. In 2009, Popova et al. studied last step of the erythroid differentiation on Friend virus-infected murine spleen erythroblast (Popova et al., 2009). They checked expression of different HDACs and found that *HDAC5* was elevated while other HDACs expression was decreased (*HDAC1 - 4, 10*) or stayed

at similar levels (*HDAC 6* and *8*). In this study, authors report that *HDAC5* is possibly involved in terminal erythroid maturation. They also highlighted that histone deacetylation mediates chromatin condensation and aids in nuclear extrusion. When they treated the cells with an HDACi (Trichostatin A) they found that chromatin condensation and nuclear extrusion to be inhibited. Peng Ji et al reported a critical need for HDAC2 in terminal differentiation of mouse fetal liver cells (Ji *et al.*, 2010). To imitate the effect of *HDAC2* knock-down they used two HDACi, valproic acid (VPA, Specific for Class I) and TSA (a pan-HDAC inhibitor) and found, similarly to *HDAC2* knock-down, a block in enucleation. They proposed that HDAC2 is mediating chromatin condensation and enucleation. Additionally, they also tested *HDAC5* but found that knock down of *HDAC5* did not have any effect on terminal erythroid maturation. Another recent study on *in vitro* differentiation of human $CD34^+$ cells towards erythroid lineage revealed HDAC5 as an important regulator of terminal differentiation, as its knockdown leads to increased apoptosis, reduced chromatin condensation and impaired enucleation (Wang *et al.*, 2021). In another study, HDAC6 was identified as an important regulator of enucleation in mouse fetal erythroblasts (Li *et al.*, 2017). This study demonstrated, that inhibition and knockdown of *HDAC6* by Tubacin or shRNA, results in blocking of CAR formation leading to disruption of both enucleation and cytokinesis. Additionally, it has been suggested that mDia2 is an important regulator of enucleation and is a substrate of HDAC6 (Ji, Jayapal and Lodish, 2008). Indeed, overexpression of unacetylated *mDia2* rescues the negative effect on enucleation under absence of HDAC6. In summary, it has been demonstrated that various HDACs play important roles in terminal erythroid differentiation; however, these findings are still controversial and inconclusive.

GPCR in erythropoiesis

General overview

G protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane receptors found in eukaryotes, and they are involved in various critical cellular responses and physiological functions. Various disorders such as Alzheimer's disease, nephrogenic diabetes insipidus, fertility disorders, carcinomas, to name a few, are all related to GPCRs (Gilman, 1984), (Schöneberg *et al.*, 2004), (Zhao, Deng, *et al.*, 2016).

GPCRs are seven-pass transmembrane proteins that bind to G proteins. The GPCR signaling pathway consists of the cyclic adenosine monophosphate (cAMP) and phosphatidylinositol signal pathways. cAMP is a second messenger molecule playing essential roles in many cellular responses, for instance, cell growth and

specialization, protein expression, gene transcription (Yan *et al.*, 2016). In the cAMP signal pathway, activated GPCR and G proteins trigger the activity of the enzyme adenylyl cyclase (AC) that hydrolyses ATP into pyrophosphate (PPi) and cAMP (Sassone-Corsi, 2012).

AC and phosphodiesterase (PDE) regulate intracellular levels of cAMP. PDE degrades the phosphodiester bond in the cAMP producing 5'-AMP. The PDE family consist of 11 members (Lugnier, 2006). The main effectors of cAMPs are the protein kinase A (PKA), an exchange protein directly activated by cAMP (EPAC) and cyclic-nucleotide-gated ion channels.

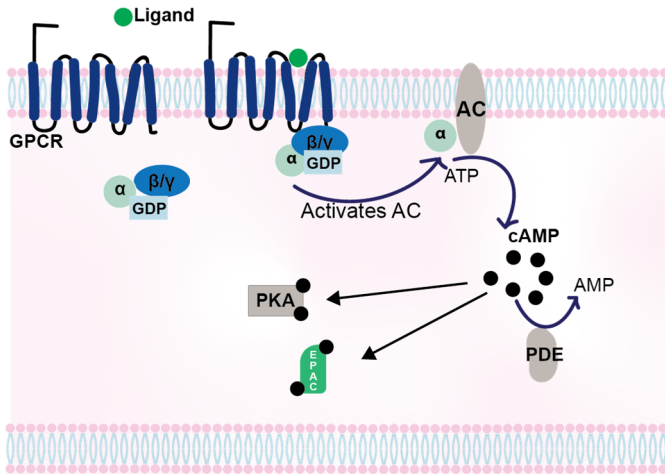


Figure 9. GPCR signaling pathway. Ligand binding activates GPCR, which triggers adenylyl cyclase (AC) activity. The AC is an enzyme hydrolysing ATPs into cAMP molecules. The cAMP molecule is a vital intracellular second messenger regulating many physiological processes, and PKA and EPAC are two main effectors sites of cAMP. The intracellular levels of cAMP can be regulated by the phosphodiesterase (PDE) enzyme activity, which can rapidly degrade cAMP.

Various ligands of GPCR and other regulators or pathways can positively and negatively regulate AC and PDE activities (Kopperud *et al.*, 2003), including calcium signaling, subunits of other G proteins, inositol lipids, receptor tyrosine kinases or A-kinase anchoring proteins (AKAPs), as well as chemical compounds. AKAP is a large and diverse family of functionally related proteins which coordinate a range of signaling events in the cell. To date, there are over 50 AKAPs identified in a range of species (Wong and Scott, 2004). In Paper III, RNA-sequencing analysis identified how $CD34^+$ cells from UCB donors expressing *AKAP9* gave higher frequencies of GPA^+ cells than donors with lower or no *AKAP9* expression. Based on literature, AKAP9 has multiple binding sites, for instance, AC, PKA, PDE, however functions are unknown.

The family of AC consists of 9 AC isoforms with various biochemical properties. Notably, forskolin is a drug that can induce cAMP levels by stimulating specific ACs (Ishikawa and Homcy, 1997). IBMX is a compound inhibiting PDE activity,

thereby inhibiting cAMP breakdown in the cells. IBMX is called a non-specific inhibitor; however, some studies revealed its insensitivity to PDE8 and PDE9 families (Lavan, Lakey and Houslay, 1989).

PKA is a cAMP dependent kinase that mediates reversible protein phosphorylation. Inactivated PKA consists of two catalytic subunits (C) and regulatory (R) subunit dimer with four different isoforms (RIα, RIβ, RIIα, RIIβ), binding cAMP. PKA activity is regulated through multiple mechanisms including interactions with AKAPs. To study the interactions of different AKAPs with PKA several PKA-AKAP complex disruptors have been generated, including STAD2 (Bendzunas *et al.*, 2018).

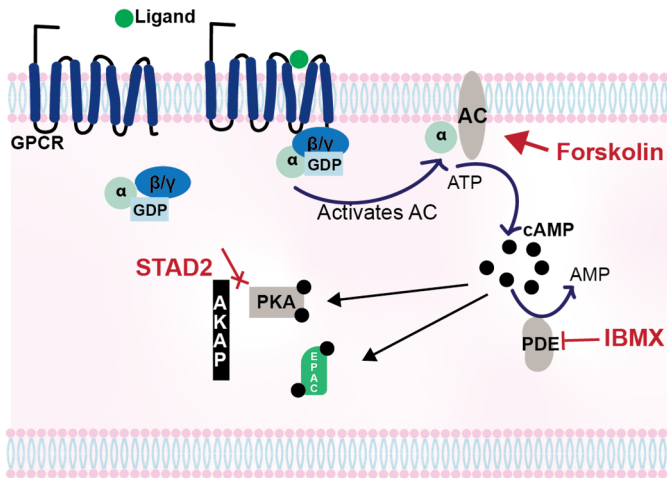


Figure 10. Activities of enzymes from GPCR signaling pathway can be modulated by different chemical compounds. Forskolin activates AC, IBMX inhibits the activity of PDE, and STAD2 disrupts the interaction of the PKA and AKAP complex. Chemical compounds treatment allows testing different enzymes' contribution to the pathway of interest.

GPCR signalling pathway in erythropoiesis

Understanding the role of GPCR related genes in erythropoiesis has been extensively studied. One study suggested the need for a cAMP-dependent signaling pathway. Mouse erythroleukemia cells, SKT6, could similarly spontaneously differentiate after forskolin or IBMX treatment. Inhibition of AC inhibited cell differentiation. This study did not reveal a detailed mechanism but suggested that the cAMPs contribute to the erythroid differentiation, possibly through PKA activation (Kuramochi *et al.*, 1990). Some PDE inhibitors can treat sickle cell disease by inducing γ-globin levels and lowering RBC sickling (McArthur *et al.*, 2020). There is also information that PDE inhibitors can increase membrane stability in mature RBC (Muravyov *et al.*, 2010), (Adderley *et al.*, 2011).

The importance of AKAPs in erythropoiesis have also been noticed. In one study, transplantation of murine hematopoietic stem and progenitor cells with overexpressed miR-669m failed to give rise to erythroid cells. *In silico* study predicted that miR-669m could impair terminal erythropoiesis by inhibiting *AKAP7* expression. Gene expression analysis confirmed *AKAP7* upregulation in erythroblasts and its need for terminal maturation (Kotaki *et al.*, 2020). However, the mechanism of potential AKAP interactions with other regulators is unknown. Studies on human, mice, and zebrafish erythroid cells found that AKAP10 could regulate heme biosynthesis. *AKAP10* expression increases during erythroid maturation, and it recruits PKA to the outer membrane of mitochondria contributing to heme biosynthesis. Knockout of *AKAP10* in maturing erythroid cells led to reduced levels of PKA and a deficit in hemoglobinization (Chung *et al.*, 2017). AKAPs' implications and functions in different stages of erythropoiesis are not entirely understood.

GPCR activated signalling pathway is broad, and the involvement of particular regulators in different stages of erythropoiesis is not well understood. Therefore, it was intriguing to find in Paper III that during HSCs differentiation towards erythroid lineage, forskolin and IBMX treatment resulted in different frequencies of GPA^+ cells. It suggested the cAMP-dependent mechanism but different needs of AC and PDE. More studies are needed to elucidate their role in each step of erythroid development.

Scope of the thesis

The *ex vivo* manufactured RBC is a promising replacement for the well-established donor-dependent blood transfusion therapies. However, manufacturing of RBCs still has several significant hurdles to overcome, including current low differentiation/maturation efficiency and technical challenges necessary for scale-up. In this thesis, I aimed to address these problems through three particular projects.

Immortalized erythroid cell lines are considered promising cell materials for producing unlimited numbers of enucleated RBCs due to their abundance; however, under current protocol these cells do not maturate efficiently, in particular enucleation that is one of the most critical steps. To address this issue, we took a robust approach, chemical compound screening. We believe that finding specific compounds inducing enucleation would be beneficial in establishing efficient protocols for *in vitro* RBC generation. First, it could be a fast and easy way to generate mature RBC. Second, it would be a great tool to study the mechanism of erythroid maturation, especially enucleation, in cell lines once we identify such chemical compounds.

Immortalized cell lines are a promising material; however, their characteristics are quite diverse and there is no agreement on which cell source is the best option. Therefore, establishing different types of immortalized erythroid cell lines and discovering parameters that correlate with their differentiation/proliferation capacity would contribute to selecting the better one among others.

Improving commitment and/or early differentiation efficiency of hematopoietic stem and progenitor cells (HSPC) to erythroid cells could boost final erythroid output, both in primary cells and immortalized erythroid cell lines. Since it has been noticed that HSPC derived from different cord blood donors have a diverse potential to give rise to mature RBCs, comparing gene expression patterns of HSPC from different donors in order to identify genes potentially contributing to such functional heterogeneity would improve the final yield of *in vitro* RBC production.

Summaries of results

Paper I: Identification of potential chemical compounds enhancing generation of enucleated cells from immortalized human erythroid cell lines

Aims:

1. To identify chemical compounds triggering enucleation of immortalized human erythroid cell lines.

2. To explore critical pathways regulating enucleation.

Summary:

For efficient *ex vivo* manufacturing of red blood cells (RBCs), immortalized erythroid cell lines are a promising alternative source, however their maturation is not efficient at present and the viability of generated cells remains problematic. Chemical compound screening is a comprehensive approach to identify drugs triggering a desirable cellular response. In Paper I we aimed to identify chemical compounds inducing enucleation in a human erythroid cell line. We screened more than 3,300 chemical compounds by utilizing an imaging-based high-throughput system. Among several classes of tested chemical compounds, we found that multiple HDACi could enhance the generation of enucleated cells upon treatment. However, we also observed that the generated enucleated cells had fragile cell membranes. Gene expression analysis revealed significant down-regulation of erythroid-specific alfa-spectrin (*SPTA1*) gene expression and instead up-regulation of non-erythroid-specific alfa-spectrin (*SPTAN1*). We speculated that this might underlie the increased frequency of fragile enucleated cells. We restored *SPTA1* expression using clustered regularly interspaced short palindromic repeat activation (CRISPRa) and noticed significant improvements in viability and frequencies of generated enucleated cells using HDACi. Our investigations propose a potential strategy for generating less fragile enucleated cells quickly and efficiently.

Paper II: Establishment of an immortalized human erythroid cell line sustaining differentiation potential without inducible gene expression system

Aims:

1. To establish an immortalized cell line with a simple HPV16-E6/E7 overexpression system.

2. To explore if the cell line sustains differentiation potential with continuous HPV16-E6/E7 expression.

Summary:

Various immortalized erythroid cell lines have been established by ectopic expression of *HPV16-E6/E7* genes. E6 and E7 proteins induce immortalization through transactivating pRB and degradation of p53 proteins. To induce differentiation, expression of these genes is shut down by using a gene induction system such as the Tet-On system. However, the necessity of terminating *HPV16-E6/E7* for efficient differentiation has not been verified. In Paper II, we report the establishment of a new immortalized erythroid cell line, called Erythroid Line from Lund University (ELLU), derived from healthy human bone marrow $CD34^+$ cells. In ELLU cells, we omitted the Tet-On system and instead used continuous expression of HPV16-E6/E7 using a simple expression system. We established more than 10 different ELLU clones derived from the same source. Interestingly, we found that ELLU can differentiate to more mature cells, including enucleated cells, without terminating *HPV16-E6/E7*. We also noticed that ELLU clones have a considerable variation in their erythroid traits and properties such as expression of adult hemoglobin, speed of differentiation, and quality of enucleated cells. Our findings introduce an alternative method to establish immortalized cell lines that are capable to differentiate to mature RBC. Additionally, we also demonstrated the functional diversity of established clones that are potentially correlated to hemoglobin expression.

Paper III: Transcriptomic analysis of functional diversity of human umbilical cord blood hematopoietic stem/progenitor cells in erythroid differentiation

Aims:

1. To compare erythroid potential of $CD34^+$ cells isolated from 50 UCB donors.

2. To identify key genes whose expression level correlate with their erythroid potential.

Summary:

Although HSCs are considered multi-potent and capable of producing all mature blood lineages, several reports identify diversity of HSC capacity for differentiation potential towards specific lineages. Erythroid differentiation potential is not an exception, as our previous studies have noticed a wide range of erythroid production levels of UCB HSC. In Paper III, we investigated the potential connection between gene expression profiles and *in vitro* erythroid differentiation potential among UCB donors. To do this, we isolated $CD34^+$ from 50 UCB donors and functionally characterized them by differentiating them into the erythroid lineage. We measured their erythroid potential by quantifying the frequency of Glycophorin-A^+ (GPA^+) cells after 6 days culture. This analysis found that although frequencies of HSPC within the plated $CD34^+$ population were similar, the frequency of GPA^+ cells produced varied. By comparing gene expression profiles using RNA-seq of donors giving high respective low yields, we identified a positive association of genes related to GPCR signaling with GPA^+ output. We demonstrated the functional implication of GPCR signaling pathway by chemically manipulating two main enzymes in this pathway and observed that inhibition of PDE led to 10 times higher yield of GPA^+ cells than activation AC. These findings indicate importance of GPCR signaling pathway in the degree of erythroid differentiation.

Discussions and future perspectives

Considering future use of *ex vivo* produced RBC for clinical applications, the efficiency of RBC production should be holistically assessed, not only based on the final output (e.g., yield) but also its ease and cost of the methods, as well as quality of the produced cells. Erythropoiesis is a complex process. Lab-produced blood sounds futuristic but is possible even today, with cells resembling the characteristic and functions of natural RBC (Giarratana *et al.*, 2011). Efficient enucleation is an important parameter, but also starting numbers or yield of the final product has to be considered if the aim is to use cells for the transfusion's purposes in the end. Therefore, immortalized erythroid cell lines due to their ability to proliferate unlimitedly should be considered as a significant cell source to obtain the required numbers of RBC. To achieve their use, induction of enucleation has to be optimized though.

In Paper I, our chemical compound screening successfully identified a group of HDACi inducing enucleation in the HiDEP cell line. However, this finding was unexpected because numerous studies proposed that inhibition or deficiency of some HDACs disrupts erythropoiesis and enucleation. For example, it has been demonstrated that HDAC2 and HDAC6 are necessary for enucleation of murine erythroid cells (Ji *et al.*, 2010), (Li *et al.*, 2017), although the importance of HDAC5 for terminal maturation in murine erythropoiesis is controversial (Popova *et al.*, 2009)(Ji *et al.*, 2010). Additionally in humans, one study indicates the absolute need for HDAC5 for enucleation (Wang *et al.*, 2021). Based on our study, we cannot conclude which particular HDAC is essential for inducing the observed enucleation in HiDEP by M344 and Fluoro-SAHA. First, because little information is known about Fluoro-SAHA and M344 mechanism of action in general, and Fluoro-SAHA in particular. It is hypothesized that M344 inhibits HDACs from Class I and Class IIb and that it is a good inducer of cell differentiatiation of mouse erythroleukemic (MEL) cell line (Volmar *et al.*, 2017)(Jung *et al.*, 1999). Fluoro-SAHA's mechanism of action is still incompletely understood. Second, we found that, as an immediate response to HDACi treatment, activities of HDAC1, HDAC2 and HDAC3 were lowered while HDAC5 and HDAC6 was unaffected. However, whether and how the HDAC activity directly affects enucleation is unclear at present, as there is a significant time gap between the quick response to HDACi and the execution of enucleation that usually happens later than 24 hours after the treatment. Further studies using both this and similar erythroid cell lines will be

required to answer this question. For example, inducing natural differentiation of both primary erythroid cells and immortalized erythroid cell lines with or without chemical compounds and then comparing gene expressions or activities of HDACs would help elucidate specific genes in cell lines involved in enucleation. This information would provide new insights into understanding the role of HDACs in erythropoiesis using different model systems. If we found overlapping genes from human *in vitro* and published data from *in vivo* murine systems, this would indicate that these regulators are conserved, essential for erythroid maturation.

It was also unexpected that HDACi treatment led to significant *SPTAN1* upregulation. It is unclear why *SPTAN1* is so highly up-regulated upon the HDACi treatment, possibly to compensate for severe reduction of *SPTA1*. In fact, not only *SPTA1* but also other erythroid-specific cell surface molecules were down-regulated. Additionally, *SPTB* expression was not drastically decreased, possibly SPTB and SPTAN1 form an alternative complex to maintain the membrane in non-erythroid cells but is incapable of maintaining membrane integrity of erythroid cells. Additionally, we hypothesized that expression of SPTAN1 might make a target for cleavage by caspases, particularly caspase 3, which was reported essential for erythroid maturation in some studies. In support of this hypothesis, inhibition of the caspase activity during the HDACi treatment significantly improved cellular viability but at the same time enucleation was blocked. We succeeded in improving the viability of enucleated cells by *SPTA1* overexpression, to a certain degree. These findings indicate that the abnormal conversion of α-spectrin is a significant, but not the sole reason for the cellular fragility upon HDACi treatment. Future studies exploring the potential role of SPTAN1 in erythropoiesis might also be important, as SPTAN1 is reported to have different functions in the non-erythroid cells other than being a part of the cell membrane (Sreeja *et al.*, 2020). SPTAN1 localizes to the cytosol and is a part of different signal transduction mechanisms, including apoptosis (Sreeja *et al.*, 2020). However, our gene expression analyses did not find any genes involved in apoptosis, we therefore don't know whether its role involves non-apoptotic cell death or other types of cellular reactions such as clearance of different organelles. It is noteworthy that together with normal enucleation, we often observed some rupture-like events happening in the HDACi treated cells. It is of interest what exactly is formed by the rupture event and via what cellular reaction. Possibly the resulting small particles are extracellular vesicles. Could these smaller size enucleated cells function like RBCs? The functionality of produced cells needs to be tested both *in vivo* and *in vitro.*

During the maturation process, primary erythroid cells differentiation is coupled to cell divisions during which erythroid cell shrinkage occurs. Cyclin D3 is a cyclin which regulates these specialized cell divisions (Keerthivasan, Wickrema and Crispino, 2011), (Sankaran *et al.*, 2012). Induction of continuous cell growth, or immortalization, is clearly an irregular feature to the non-erythroid cell. When these cells are transformed and start proliferating continuously, they usually stop

differentiating into mature cells. The ectopic expression of *HPV16-E6/E7* is used as a common tool for establishing immortalized erythroid cell lines. After immortalization, cell lines sustain key erythroid features, such as hemoglobin synthesis and morphologic changes during differentiation and enucleation. The expression of *HPV16-E6/E7* is usually terminated when inducing, because every cell division in terminal erythroid differentiation is coupled to maturation (Keerthivasan, Wickrema and Crispino, 2011), (Sankaran *et al.*, 2012). Previously established cell lines, HiDEP, HUDEP, BEL-A, could differentiate and enucleate in this condition, however considerable amount of dead cells were observed, especially from HiDEP and HUDEP (Kurita *et al.*, 2013), (Trakarnsanga *et al.*, 2017). By establishing ELLU we investigated the necessity of shutting down the *HPV16-E6/E7* for differentiation of immortalized human erythroid cell lines and found that those cells were able to differentiate into mature erythroid cells even with continuous expression of *HPV16-E6/E7*. Overexpression of these genes could also have a "cancerous" effect, as E6 gives anti-apoptotic potential through p53 degradation and E7 enforces proliferation by inactivating Rb, and as the result cells would not be able to differentiate (Alberts, Johnson and Lewis, 2002). However, we observed that ELLU cells could differentiate at a similar efficiency to HiDEP/HUDEP. Alternatively, there is a possibility that E6/E7 expression induces expression of key driver genes for differentiation and enucleation. We have not properly demonstrated the mechanism explaining differentiation of ELLU with this immortalization method. It would be important to carefully evaluate the benefit and risks of continuous *HPV16-E6/E7* expression in the differentiation potential of the immortalized cell lines by establishing two types of immortalized cell lines, with or without the shutting-down system, from the same donor.

Two particular ELLU clones (#104 and #116) were able to differentiate in 6 days, which is noticeably faster compared to HiDEP, HUDEP, and BEL-A, however higher cell death frequency was also observed. Of note, these clones have longer size E6 gene integration in the genome while other clones have integration of both longer and shorter sizes of the E6 gene. This truncated E6 is known to affect rather E7 protein levels (Tang *et al.*, 2006), which potentially impacts on proliferation speed of the cells. However, potential roles of the truncated E6 gene remain to be conclusively determined, so that more detailed molecular analyses are needed.

When we established ELLU, it was striking to find considerable heterogeneity in hemoglobin expression considering all clones originated from the same donor. Increase of fetal hemoglobin expression *in vitro* culture is a common feature, however reasons to that are not clear. When characterizing ELLU, we looked at the expression of two primary regulators of hemoglobin switching, *BCL11A* and *KLF1*, and found that they are similarly expressed. Possibly there are other regulators of globin switching independent of BCL11A and KLF1, since one recent study reported that non-POU domain-containing octamer-binding protein (NoNO) plays an essential role in silencing γ -globin expression. In this study, it was demonstrated

that depletion of NoNO in K562, HUDEP-2, and primary erythroid progenitor cells resulted in significant fetal globin expression (Li *et al.*, 2021). Therefore, it would be informative to check the expression of NoNO and other genes such as *SOX6, FOG-1* or repressor complexes, for instance NuRD (Sankaran, Xu and Orkin, 2010). Moreover, performing comprehensive gene expression analysis to compare ELLU clones (and other erythroid cell lines) expressing different hemoglobin would be an informative approach to discover novel genes underlying the hemoglobin patterns of immortalized erythroid cell lines.

Beyond developing high-efficient differentiation (including enucleation) methods, a following important step would be establishing low-cost *in vitro* culture/differentiation conditions towards the future large-scale production. In addition, it would be crucial to try constructing 3D culture conditions or bioreactors (Bayley *et al.*, 2018). Recent publications suggest the promising potential of bioreactors for efficient RBC production using human umbilical cord blood $CD34^+$ (Timmins *et al.*, 2011) and adult peripheral blood mononuclear cells (Gallego-Murillo *et al.*, 2022). In addition, *in vitro* large-scale platelet production from immortalized human megakaryocyte cell lines using a bioreactor system has become realistic (Ito *et al.*, 2018). Thus, developing culture systems combining with bioengineering platforms which is optimal for cell lines is an essential part of the large-scale *ex vivo* RBC production method.

Another serious concern of *ex vivo* manufactured RBC is the fragility of produced cells compared to native cells (Pellegrin, Severn and Toye, 2021). Since RBC need particular flexibility in their shape (deformability) in order to circulate and flow through capillary vessels, membrane integrity of produced RBC is as important as their oxygen carrying potential. Thus, understanding the reason for the cell membrane fragility is critical. While we tried to address the reasons for cell fragility in Paper I and Paper II, we did not discuss about the potential factors contained in the differentiation media, which cause the cell membrane disruption. After HDACi treatment, enucleated cells are very fragile. Under native conditions, newly enucleated reticulocytes need additional time to remodel the cell membrane and acquire proper cell membrane features. Chemical treatment of HiDEP induces the maturation process, but might not provide the appropriate environment for newly generated reticulocytes, which could be the reason of their fragility. It would be crucial to test treating HiDEP and ELLU in other media with more supplements other than standard erythroid differentiation media that were optimized for primary erythroid cells.

In Paper III, we found that $CD34^+$ cells from different umbilical cord blood had various levels of erythroid differentiation potential despite similar frequencies of HSPCs. In this study, only two samples were female, therefore unbalancing the analysis. RNA-seq comparing high and low GPA yield from donors identified GPCR-related genes as a separating factor. *AKAP9* is one of the candidate genes, having an ability to bind to some enzymes from GPCR related pathways such as

AC, PDE and PKA. Based on these facts, we hypothesized if modifying any of this pathway regulators chemically would affect erythropoiesis. We found that destruction of PKA-AKAP complex and AC activation, did not have a strong effect on erythropoiesis, however inhibition of PDE did. Further detailed investigations are required to confirm that inhibition of PDE is required and regulated by AKAP9. It would be informative to measure the activity of AC when PDE is inhibited in order to clarify whether their expression and/or activities are synchronized. To explore precise roles of identified genes, especially *AKAP9,* it would be needed to perform knock-down/knock-out experiments using RNA interference (RNAi) or CRISPR/Cas9 technology, as well as overexpression experiments. Targeting other AKAP genes would further reveal their contributions in different stages of erythroid maturation, since several studies have reported potential implications of different AKAPs in erythropoiesis (Kotaki *et al.*, 2020), (Chung *et al.*, 2017). Yet, there is no detailed studies investigating their mechanism of action.

Since donor $CD34^+$ cells contained similar frequencies of HSPC populations, we used total $CD34^+$ cell frequency for the functional evaluation and gene expression analysis. However, to better understand the functional heterogeneity of HSC in erythroid potential, it would be informative to compare gene expression profiles and epigenetic profiles of more purified cells by sorting HSC population or single-cell analysis. Additionally, obtaining more information about the presence of other specific progenitor cells dominating within the $CD34^+$ population would be required. This type of analysis could help answering if such type of subpopulation(s) has an impact on erythroid output.

Concluding remarks

In summary, the work presented in this thesis describes novel approaches to discover novel regulatory mechanisms in erythroid differentiation/maturation both in primary cells and immortalized cell lines, which significantly contributes to developing robust methods for more efficient *ex vivo* production of RBCs from immortalized erythroid cell lines. Below are three main conclusions of presented works:

1. Identified potential chemical compounds inducing generation of enucleated cells from immortalized erythroid cell line.
2. Established a new immortalized human erythroid cell line sustaining differentiation potential under continuous *HPV16-E6/E7* expression.
3. Investigation into heterogeneity of $CD34^+$ cells from distinct donors for the potential to generate erythroid cells and potential genes involved in this.

We envision that immortalized erythroid cell lines are the promising tool for efficient *ex vivo* RBCs production, yet many technical challenges are to be solved. Our work is a significant contribution to the field, as our unique approaches have successfully identified powerful tool to study immortalized cell line-specific molecular regulations and uncovered previously unknown blockade in their differentiation path. We believe these findings will contribute to establish efficient and robust protocols for *ex vivo* generation of RBCs for future application in transfusion therapies.

Popular scientific summary

Blood transfusion is a lifesaving procedure where the patient's blood is replaced or supplemented by a blood donation. Usually, blood transfusions are needed when a person suffers from anemia (lack of blood) due to illness, or in acute situations where there is a life-threatening blood loss. However, patients have restricted access to safe blood transfusions globally making it an urgent need to enable its efficient and continuous availability. Blood production in the lab is an alternative approach to public blood donation, and is one possible solution to the problem of availability. This source of red blood cells is the main focus of my thesis.

The most abundant cell type in the blood is a red blood cell (erythrocyte), and is used for transfusion therapy. Recently, scientists succeeded to generate functional erythrocytes in the lab, using hematopoietic stem cells (HSCs) as a starting material. For many cell types, including erythrocytes, scientists have found specific conditions for their development. However, HSCs are limited and therefore unsuitable to generate the large number of erythrocytes required to cure anemia (referred to as poor scalability). Moreover, it has also been noticed that HSCs from different donors give rise to different numbers of mature erythrocytes, further hampering their potential as a source of lab produced blood under proper conditions which would make the output variable over time. Recently, scientists addressed the problem of scalability by "immortalizing" cells that are capable of producing a theoretically unlimited number of erythrocytes. These immortalized erythroid cells serve as a source of unlimited erythrocyte production and is independent from frequent HSCs donations. Immortalizing cells are suitable for solving scalability problems, but immortalized cells are not efficiently producing erythrocyte. One problem is their inefficient enucleation, which is a part of the final maturation where the developing erythrocyte spits out its cell nucleus. Possibly the perfect immortalization strategy has to be found to make enucleated erythrocyte production in the lab more efficient.

The goal of my PhD studies was to establish methods allowing the generation of enucleated cells using immortalized developing erythrocytes. In my first project, I tested more than 3,300 chemical compounds and found chemical compounds enhancing the generation of enucleated cells. I also found that these chemical compounds produced fragile erythrocytes that did not function properly. To get a clue into why, we looked at gene expression after chemical compounds treatment and found that one gene *SPTA1* was down-regulated. This gene is related to the cell

membrane and could be a part of the reason why our erythrocytes were fragile. To test this, we overexpressed *SPTA1* which improved the quality and frequency of enucleated cells after chemical compound treatment. This approach allows rapid and efficiently RBC production in the lab.

In my second PhD Project, I established a new erythroid cell line as a potential candidate source for efficient erythrocyte generation using a unique immortalization strategy. Established erythroid cell lines by other scientists give rise to mature RBC if the genes responsible for immortalization is off. However, nobody ever asked why they had to be turned off. So, I created a new cell line that always had the expression of this gene; even then, cells were undergoing development into mature RBCs. From this cell line, I isolated ten different clones, which all originated from one adult HSC donor and found that different clones gave rise to different types of RBCs with adult, fetal, or embryonic features. This different outcome from a single source is an unexpected and exciting finding, and further future studies are needed to understand why this variation happened.

In my third Project, I investigated the molecular heterogeneity in HSC underlying their potential to become erythrocytes. We compared the erythroid potential of $CD34^+$HSC isolated from 50 different umbilical cord blood (UCB) donors and discovered that donors gave rise to various frequencies of erythrocytes despite their similar frequency of HSC at the start, indicating an inherent difference in their capacity for erythrocyte development. We then compared the gene expression of $CD34^+$ cells and found that genes involved in the GPCR signaling pathway were significantly up-regulated in the donors giving high-erythroid output. We chemically manipulated two necessary enzymes of this pathway and found an effect on the erythrocyte development. Our findings suggest that GPCR signaling is potentially involved in the different erythroid potential of individual UCB donors, but further investigations are required to understand this in more detail.

Efficient erythrocyte production in the lab is a solution to a safe blood transfusion worldwide. Finding the best material and creating fast, affordable methods for the efficient generation of erythrocytes are needed. Therefore, developing proper materials and studying how erythrocytes mature is essential knowledge that will be applied to produce functional erythrocytes in the lab.

Populärvetenskaplig sammanfatning

Blodtransfusion är det livräddande ingreppet när en patients blod ersätts helt eller delvis av en donators. Vanligtvis används blodtransfusioner när en person lider av anemi (blodbrist) såsom till följd av en medicinsk åkomma eller vid akuta situationer när en stor mängd blod har gått förlorad. Även fast blodtransfusioner är en grundförutsättning för god medicinsk vård så råder det en global blodbrist och vi har en lång väg kvar till allmän tillgång till säkra och alltjämt tillgängliga blodtransfusioner. Som ett alternativ till allmänhetens donation av sitt eget blod skulle en kontinuerlig tillgång till blod från labbet kunna odlas fram, och därmed säkerställa en teoretisk oändlig källa av blod. Denna källa till röda blodceller är det huvudsakliga fokus för min avhandling.

Röda blodceller (även kallade ”erytrocyter") är den mest förekommande celltypen i vårat blod, och ges till patienter vid blodtransfusion. Nyligen så lyckades forskare framställa erytrocyter i labbet genom att använda sig av hematopoietiska stamceller (förkortat ”HSC”) som ett ursprungsmaterial. Genom att använda sig av kända signaleringsmolekylär kunde dessa HSC stimuleras till att bli erytrocyter. Tillgången till HSC är dock mycket begränsad då det endast finns ett fåtal i varje persons kropp, och därför är de olämpliga för storskalig, labb-baserad produktion av blod. Dessutom tycks det finnas en underliggande variation i förmågan till att bilda erytrocyter från enskilda donatorers HSC, vilket skulle medföra en okontrollerad variation i tillgången av erytrocyter. Man säger att HSC har en dålig förmåga till uppskalning. I ett försök att nå god förmåga till uppskalning så har forskare istället skapat celler som kan förnyas ett teoretiskt oändligt antal gånger och med möjligheten att ge tillgång till en oändlig mängd av erytrocyter. Därmed skulle begränsningen med HSC kunna kringgås och uppskalningsförmågan förbättras avsevärt. En nackdel med den här källan till erytrocyter är att de inte är effektiva i att ”enukleara”, vilket är ett av de slutgiltiga stadierna i bildandet av erytrocyter och innebär att cellkärnan helt enkelt avlägsnas från cellen. Detta tillkortakommande måste överkommas innan vi kan ha effektiv produktion av erytrocyter i labbet.

Målet för mina doktorandsstudier var att etablera metoder för att generera enuklerade erytrocyter från celler som har oändligt liv. I mitt första projekt så testade jag mer än 3,300 kemiska substrat och fann därigenom ett antal som förbättrade förmågan till att ge enuklerade erytrocyterna. Vi noterade dock att de färdiga cellerna var ömtåliga och inte var helt välfungerande. För att försöka förstå och

motverka detta så undersökte vi den underliggande genetiska faktorn i dessa celler, och fann att en gen vid namn ”*SPTA1*” var nedreglerad. Denna gen är involverad i cellmembranet som omger alla celler, och skulle kunna vara en underliggande anledning till varför cellerna var ömtåliga. För att testa detta så återförde vi på konstgjord väg *SPTA1* in i cellerna och fann då att kvalitén och frekvensen av enuklerade erytrocyter ökade. Detta tillvägagångssätt är därför ett steg på vägen mot snabb och effektiv produktion av erytrocyter i labbet.

I mitt andra doktorandsprojekt etablerade jag en ny oändlig källa till celler i labbet som kan ge uppkomst till erytrocyter. Vi använde oss av en unik strategi för att uppnå detta, då andra odödliga cellkällor stänger av uttrycket av vissa gener för att generera erytrocyter. Men varför dessa gener måste stängas av är okänt, så jag skapade en helt ny typ av cell där vi inte stänger av generna; och fann att de fortfarande kunde generera erytrocyter. Från dessa celler isolerade jag tio stycken unika kloner, som alla hade skapats från en och samma donators HSC men som nu i formen av oändligt levande celler började producera olika typer av erytrocyter, med karaktärsdrag från vuxnas, fosters, och embryonala erytrocyter. Att få dessa olika resultat från kloner som genererats från en och samma donator var oväntat och intressant, och ytterligare studier kommer behövas för att förstå varför olika kloner fick sin olika prägel.

I mitt tredje projekt undersökte jag den underliggande molekylära orsaken bakom variationen hos normala HSC förmåga för att bilda erytrocyter. Vi isolerade och jämförde förmågan från 50 olika navelsträngsblod-donationer, och återfann den förväntade variation hos olika HSC att bilda erytrocyter. Vi jämförde därefter genuttrycket och fann att signalering via en viss typ av signaleringsmolekyler (som kallas ”GPCR”) var signifikant högre i donatorer som producerade fler erytrocyter. Ifall vi påverkade den här typen av signalering via kemiska substanser fick vi också en påverkan av mängden erytrocyter. Våra insikter indikerar därför att denna typ av signalering är en underliggande orsak till variation hos olika donatorers olika förmåga till att bilda erytrocyter, men ytterligare undersökningar kommer krävas för att förstå helhetsbilden bättre.

Effektiv produktion av erytrocyter i labbet utgör en lösning för att tillgodose kontinuerliga och säkra blodtransfusioner, och dess tillgänglighet över hela världen. Att finna det främsta materialet som möjliggör snabb och kostnadseffektiv produktion av erytrocyter utgör ett alltjämt skriande behov, och mitt doktorandsarbete bidrar till att utveckla lämpliga material för detta samt studerar underliggande faktorer som skulle kunna användas för att producera funktionella erytrocyter i labbet.

Mokslinio darbo santrauka

Kraujo perpylimas, tai gyvybes gelbėjanti procedūra, kai naudojant kraujo donorų kraują paciento kraujas yra pakeičiamas arba papildomas. Dažniausiai kraujo perpylimo procedūra reikalinga, kada asmuo kenčia nuo anemijos (kraujo trūkumas, mažakraujystė) dėl ligos, ar sunkiose situacijose, kai yra gyvybei pavojingas kraujo netekimas. Deja, pacientų prieiga prie saugaus kraujo perpylimo globaliai yra ribota, būtent dėl to labai svarbu užtikrinti veiksmingą ir nuolatinį šios procedūros prieinamumą. Kraujo gamyba laboratorijose tai alternatyva kraujo donorystei ir vienas iš galimų problemos dėl prieinamumo sprendimo būdų. Šis raudonųjų kraujo kūnelių gavimo būdas yra pagrindinis mano disertacijos akcentas.

Gausiausias ląstelės tipas mūsų kraujyje yra raudonasis kraujo kūnelis (arba eritrocitas) ir jis naudojamas kraujo perpylimo gydyme. Neseniai mokslininkams pavyko sukurti funkcionuojančius eritrocitus laboratorijoje, naudojant hematopoetines kamienines ląsteles (trumpai HSCs) kaip pradinę medžiagą. Daugeliui ląstelių, įskaitant eritrocitus, mokslininkai surado specifines sąlygas jų vystymui. Tačiau HSC yra ribotas kiekis, todėl netinka norint pagaminti didelį kiekį eritrocitų, kas yra būtina gydant anemiją (nurodyta kaip prastas masto keitimas). Be to, pastebėta, kad HSC iš skirtingų donorų duoda skirtingą subrendusių eritrocitų skaičių, o tai dar labiau sumažina jų, kaip potencialių, tinkamomis sąlygomis laboratorijoje pagaminto kraujo, šaltinių, dėl ko išeiga laikui bėgant kinta. Neseniai mokslininkai sprendė masto problemą „ įamžindami „ ląsteles, kurios teoriškai gali gaminti neribotą eritrocitų kiekį. Šios įamžintos eritoidinės ląstelės yra kaip šaltinis neribotai eritrocitų gamybai ir nepriklauso nuo HSC donorystės dažnumo. Ląstelių įamžinimas tinkamas masto problemos sprendimui, bet tokios ląstelės neefektyviai gamina eritrocitus. Viena iš problemų yra neefektyvi jų enukleacija, kuri yra galutinio brendimo dalis, kai besivystantis eritrocitas išspjauna savo ląstelės branduolį. Turbūt reikia atrasti tobulą įamžinimo strategiją, kad bebranduolių eritrocitų gamyba laboratorijoje būtų veiksmingesnė.

Mano doktorantūros studijų tikslas buvo sukurti metodus, kurie leidžia generuoti bebranduolių ląsteles naudojant besivystančius įamžintus eritrocitus. Pirmame projekte aš išbandžiau daugiau negu 3,300 cheminių junginių ir radau cheminių junginių, kurie pagerina bebranduolių ląstelių susidarymą (generaciją). Taip pat pastebėjau, kad šie cheminiai junginiai gamino silpnus eritrocitus, kurie funkcionavo netinkamai. Norėdami suprasti kodėl, pažvelgėme į genų ekspresiją po apdorojimo cheminiais junginiais ir nustatėme, kad genas *SPTA1* buvo žemyn

reguliojamas. Šis genas yra susijęs su ląstelės membrana ir gali būti dalis priežasties, kodėl mūsų eritrocitai buvo silpni. Norėdami tai išbandyti mes smarkiai padidinome *SPTA1* kiekį, kuris pagerino bebranduolių ląstelių kiekį ir dažnį po apdorojimo cheminiais junginiais. Šis metodas leidžia greitai ir efektyviai gaminti RBC laboratorijoje.

Savo antrajame doktorantūros projekte sukūriau naują eritroidinių ląstelių liniją, kaip potencialų šaltinį efektyviam eritrocitų generavimui naudojant unikalią įamžinimo strategiją. Kitų mokslininkų sukurtos eritroidinių ląstelių linijos padidina RBC brandumą jei genai, kurie atsakingi už įamžinimą yra atjungti. Tačiau niekas niekada neklausė, kodėl gi jie turi būti atjungti. Taigi, aš sukūriau naują ląstelių liniją, kuri visą laiką turėjo šio geno ekspresiją; netgi tada ląstelės vystėsi į brandžius eritrocitus (RBC). Iš šios ląstelių linijos išskyriau dešimt skirtingų klonų, kurie buvo sukurti iš vieno suaugusio HSC donoro ir išsiaiškinau, kad skirtingi klonai sukėlė skirtingų tipų RBC su suaugusiojo, vaisiaus ir embriono turinčiomis savybėmis. Toks skirtingas rezultatas iš vieno išteklio yra netikėtas ir jaudinantis atradimas, norint supranti, kodėl toks kitimas įvyko reikia atlikti tolimesnius tyrimus.

Trečiajame savo projekte ištyriau HSC molekulinį heterogeniškumą, kuris pagrindžia jų potencialą tapti eritrocitais. Mes palyginome $CD34^{+}$HSC, išskirtų iš 50 skirtingų virkštelės kraujo (UCB) donorų, eritroidinį potencialą ir išsiaiškinome, kad donorai sukėlė įvairų eritrocitų dažnį, nepaisant panašaus HSC dažnio pradžioje, o tai parodo būdingą skirtumą jų gebėjime vystytis eritrocitams. Tada palyginome $CD34^{+}$ ląstelių genų ekspresiją ir nustatėme, kad GPCR signalizacijos kelyje dalyvaujantys genai buvo žymiai praturtinti donoruose, duodančių didelę eritroidinę išvestį. Mes chemiškai manipuliavome du būtinus šio kelio fermentus ir nustatėme poveikį eritrocitų vystymuisi. Mūsų išvados rodo, kad GPCR signalizacija gali būti potencialiai susijusi su skirtingu atskirų UCB donorų eritroidiniu potencialu, bet norint tai suprasti detaliau būtini tolimesni tyrimai.

Veiksminga eritrocitų gamyba laboratorijose yra saugaus kraujo perpylimo visame pasaulyje problemos sprendimas. Yra būtina rasti geriausią medžiagą ir sukurti greitus, įperkamus metodus efektyviai eritrocitų gamybai. Todėl tinkamų medžiagų kūrimas ir eritrocitų brendimo tyrimai yra esminės žinios, kurios bus taikomos gaminant funkcionuojančius eritrocitus laboratorijoje.

Резюме научной работы

Переливание крови — это спасительная для жизни процедура, при которой кровь пациента заменяется или дополняется донорской кровью. Процедура переливания крови обычно необходима, когда человек страдает анемией (малокровием) вследствие болезни или в сложных ситуациях, когда возникает опасная для жизни кровопотеря. Однако доступ пациентов к безопасному переливанию крови во всем мире ограничен, поэтому так важно обеспечить эффективную и постоянную доступность этой процедуры. Производство крови в лабораториях — альтернатива донорству крови и одно из возможных решений проблемы доступности. Этот метод получения эритроцитов является основным направлением моей диссертации.

Наиболее распространенным типом клеток в нашей крови являются красные кровяные тельца (эритроциты), и они используются при трансфузионной терапии. Недавно исследователи смогли создать функционирующие эритроциты в лаборатории, используя гемопоэтические стволовые клетки (ГСК) в качестве исходного материала. Для многих клеток, в том числе и для эритроцитов, исследователи нашли специфические условия для их развития. Однако количество ГСК ограничено, и поэтому они не подходят для воспроизводства большого количества эритроцитов, необходимого при лечении анемии (так называемая плохая масштабируемость). Кроме того, было замечено, что ГСК от разных доноров дают разное количество зрелых эритроцитов, что еще больше ограничивает их потенциал в качестве источников крови, полученной в соответствующих условиях в лаборатории, что может привести к изменению количества получаемых эритроцитов с течением времени. Недавно учёные обратились к проблеме масштабируемости, использовав иммортализованные («бессмертные») линии клеток, которые теоретически могут производить неограниченное количество эритроцитов. Эти иммортализованные эритроцитные клетки служат источником неограниченного воспроизводства эритроцитов и не зависят от частоты донорства ГСК. Иммортализованные клетки подходят для решения проблемы масштабируемости, но такие клетки неэффективно производят эритроциты. Одной из проблем является их неэффективная энуклеация, являющаяся частью окончательного созревания, когда развивающийся эритроцит избавляется от своего клеточного ядра. Возможно, идеальной стратегией иммортализации может стать способ более эффективного производства безъядерных эритроцитов в лаборатории.

Целью моих научных исследований было создание методов, позволяющих генерировать энуклеированные клетки с использованием иммортализованных развивающихся эритроцитов. В своем первом проекте я протестировала более 3,300 химических соединений и обнаружила химические соединения, усиливающие образование энуклеированных клеток. Я также обнаружила, что эти химические соединения производят хрупкие эритроциты, которые не функционируют должным образом. Чтобы понять, почему, мы посмотрели на экспрессию генов после обработки химическими соединениями и обнаружили, что один ген *SPTA1* подавляется. Этот ген связан с клеточной мембраной и может быть одной из причин хрупкости наших эритроцитов. Чтобы проверить это, мы сверхэкспрессировали *SPTA1*, что улучшило качество и частоту энуклеированных клеток после обработки химическими соединениями. Такой подход позволяет быстро и эффективно производить эритроциты в лаборатории.

В моем втором докторском проекте я создала новую эритроидную клеточную линию в качестве потенциального источника для эффективного образования эритроцитов с использованием уникальной стратегии иммортализации. Созданные другими учеными эритроидные клеточные линии служат источником зрелых эритроцитов если гены, ответственные за иммортализацию, выключены. Однако никто никогда не спрашивал, почему их нужно выключать. Итак, я создала новую клеточную линию, которая всегда имела экспрессию этого гена, даже тогда клетки развивались в зрелые эритроциты. Из этой клеточной линии я выделила десять различных клонов, которые все произошли от одного взрослого донора ГСК, и обнаружила, что разные клоны служили источниками разных типов эритроцитов со взрослыми, фетальными или эмбриональными особенностями. Такой различный результат из одного источника является неожиданным и захватывающим открытием, и необходимы дальнейшие исследования, чтобы понять, почему произошло это изменение.

В моем третьем проекте я исследовала молекулярную гетерогенность ГСК, лежащую в основе их способности превращаться в эритроциты. Мы сравнили эритроидный потенциал $CD34^+$ ГСК, выделенный от 50 различных доноров пуповинных стволовых клеток (UCB), и обнаружили, что доноры давали разлиную частоту эритроцитов, несмотря на их одинаковую частоту ГСК в начале, что указывает на врожденную разницу в их способности к развитию эритроцитов. Затем мы сравнили экспрессию генов $CD34^+$ клеток и обнаружили, что гены, участвующие в сигнальном пути GPCR, были значительно активизированы у доноров, дающих высокий выход эритроидов. Мы химически манипулировали два необходимых фермента этого пути и обнаружили их влияние на развитие эритроцитов. Наши результаты показывают, что передача сигналов GPCR потенциально связана с различными эритроидными потенциалами отдельных доноров UCB, но необходимы дальнейшие исследования, чтобы понять это более подробно.

Эффективное производство эритроцитов в лаборатории является решением проблемы безопасного переливания крови во всем мире. Необходим поиск наилучшего материала и создание быстрых и доступных методов для эффективного получения эритроцитов. Таким образом, разработка надлежащих материалов и изучение процесса созревания эритроцитов является важным знанием, которое будет применяться для получения функциональных эритроцитов в лаборатории.

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