

UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
ACADEMIC STUDY IN BIOTECHNOLOGY

Kristina MARTON

**DIFFERENTIATION OF THE HAEMATOPOIETIC
STEM CELLS FROM UMBILICAL CORD BLOOD
INTO MEGAKARYOCYTES WITH
THROMBOPOIETIN *in vitro***

GRADUATION THESIS

University studies

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**DIFERENCIACIJA KRVOTVORNIH MATIČNIH CELIC IZ
POP KOVNIČNE KRVI V MEGAKARIOCITE *in vitro* S POMOČJO
TROMBOPOIETINA**

DIPLOMSKO DELO
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This thesis work is a completion of university studies in biotechnology. The work was carried out in the laboratory of Sanquin Blood Supply Foundation, Leiden (The Netherlands).

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The results of this thesis are a result of the candidate's own work. Signed agree to the publication of her thesis on the website of the Digital Library of the Biotechnical Faculty. I declare that the work that I submitted in electronic form is identical to the printed version.

Kristina MARTON

Diplomsko delo je nastalo v okviru univerzitetnega študija biotehnologije na Biotehniški fakulteti Univerze v Ljubljani. Eksperimentalni del naloge je bil opravljen v raziskovalnem laboratoriju zavoda Sanquin Blood Supply Foundation v Leidnu, na Nizozemskem, v sodelovanju z Zavodom RS za transfuzijsko medicino v Ljubljani.

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Diplomsko delo je rezultat lastnega raziskovalnega dela. Podpisana se strinjam z objavo svoje naloge v polnem tekstu na spletni strani Digitalne knjižnice Biotehniške fakultete. Izjavljam, da je naloga, ki sem jo oddala v elektronski obliki, identična tiskani verziji.

Kristina MARTON

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 AB Haematopoietic stem cell (HSC) transplantations are more and more common. Their source is usually the bone marrow but this has many disadvantages such as delayed availability and risk for the donors. HSC isolated from cord blood are a better call but unfortunately, they have a huge disadvantage, in one sample, there are not enough HSCs, which can result in delayed engraftment. Many methods were developed to improve engraftment. In our study, we examined one of them, the *ex vivo* expansion, more precisely, we were interested in the responses of isolated HSC from cord blood on thrombopoietin (TPO), as only growth factor, which *in vivo* leads megakariopoiesis. Our results suggest that the cells are responding to TPO after 10 days of culture with differentiation towards megakaryocytic cell lineage, three populations appeared: a population which still expressed CD34, a population that did not express any lineage markers and a population expressing megakaryocyte markers. Most of the cultured cells were divided after 10 days and lost their CD34 marker. We observed some undivided cells as well, which expressed megakaryocytic markers. The other observed response was a $13,9 \pm 7,5$ fold expansion. The long-term repopulating capacity is essential for transplants, which we confirmed with CAFC assay. This ability is believed to be due to the CD34⁺CD38⁻ population, which differentiated faster and expanded more compared to CD34⁺CD38⁺. Therefore, *ex vivo* expansion of HSC isolated from cord blood seem as a promising approach in improving engraftment.

KLJUČNA DOKUMENTACIJSKA INFORMACIJA

| | |
|----|---|
| ŠD | Dn |
| DK | UDK 606:61(043.2)=111 |
| KG | matične celice/popkovnična kri/trombopoietin/krvotvorne matične celice/ <i>ex vivo</i> pomnoževanje/izboljšanje vsadka |
| AV | MARTON, Kristina |
| SA | KNEŽEVIĆ, Miomir (mentor)/ROŽMAN, Primož (somentor)/VAN HENSBERGEN, Yvette (somentor) |
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| TD | Diplomsko delo (univerzitetni študij) |
| OP | XIV, 99 str., 11 pregl., 40 sl., 161 vir. |
| IJ | en |
| JI | en/sl |
| AI | <p>Transplantacije krvotvornih matičnih celice (KMC) so vedno bolj pogoste. Njihov vir predstavlja po navadi kostni mozeg, ki je zaradi dolge dobe čakanja na njihovo razpoložljivost in rizika za darovalca manj primerne. Boljše lastnosti imajo KMC, pridobljene iz popkovnične krvi, vendar imajo veliko slabost: nizko število na enoto vzorca, ki je odgovorno za slabo vgradnjo matičnih celic. Za izboljšanje vraščanja KMC je bilo razvitih veliko različnih pristopov. V naši raziskavi smo preverjali eno izmed teh, to je <i>ex vivo</i> ekspanzija KMC. Bolj natančno nas je zanimalo, kako se KMC, izolirane iz popkovnične krvi, odzovejo na gojenje s trombopoietinom (TPO) kot edinim rastnim faktorjem, odgovornim za megakariopoiezo <i>in vivo</i>. Ugotovili smo, da se KMC, izolirane iz popkovnične krvi, odzivajo na TPO po 10 dnevem gojenju z diferenciacijo proti megakariocitni celični liniji, kjer dobimo tri populacije: celice, ki so ohranile svoj CD34 površinski označevalce, celice, ki niso izražale nobenega linijskega površinskega oznčevalca, in celice, ki so izražale površinske označevalce, ki so značilni za megakariocitno celično linijo. Večina celic se je po 10 dnevem gojenju delila in izgubila površinski označevalce CD34. Opazili smo tudi take celice, ki se niso delile, so pa pridobile površinske označevalce, značilne za mekagariocitno celično linijo. Kot odziv na TPO smo opazili tudi $13,9 \pm 7,5$ kratno ekspanzijo. Ohranjanje dolgoročne repopulacije je seveda ključnega pomena pri transplantatih, kar smo potrdili s CAFC testom. To sposobnost pripisujemo populaciji CD34⁺CD38⁻, ki se hitreje diferencira in večkrat deli v primerjavi z CD34⁺CD38⁺. Zatorej <i>ex vivo</i> ekspanzija KMC, izoliranih iz popkovnične krvi, s trombopoietinom obeta dobre rezultate za izboljšanje vraščanja KMC.</p> |

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ABBREVIATIONS AND SYMBOLS

ADP – adenosine diphosphate
ASC – adult stem cell
Bala HSC – balanced haematopoietic stem cell
BFU-Mk – megakaryocyte burst forming unit
BM – bone marrow
cADPR – cyclic ADP-ribose
CAFC assay – cobblestone area-forming cell assay
CD – cluster of differentiation
CFU-Mk – megakaryocyte colony forming unit
CFU-S – spleen colony forming unit
CLP – common lymphoid progenitor
CMP – common myeloid progenitor
CPDA-1 – citrate phosphate dextrose adenine-1
CXCL-12 – chemokine (C-X-C motif) ligand 12
DMSO – dimethylsulfoxide
DNA – deoxyribonucleic acid
DPPIV – dipeptidylpeptidase IV
EDTA – ethylenediaminetetraacetic acid disodium salt solution
ESC – embryonic stem cell
FACS – fluorescence-activated cell sorting
FBS – fetal bovine serum
G-CSF – granulocyte colony-stimulating factor
GvHD – graft-versus-host disease
HLA – human leukocyte antigen
HSC – haematopoietic stem cell
IL – interleukin
IMDM – Iscove's modified Dulbecco's medium
ITGA2B – integrin alpha chain 2b
ITGB3 – integrin beta chain 3
KMC – krvotvorne matične celice
LT-HSC – long term haematopoietic stem cell
Ly-bi HSC – lymphoid-biased haematopoietic stem cell
MACS – magnetic activated cell separation
MEP – myeloid/erythroid progenitor
MK – megakaryocyte
MkP – megakaryocyte progenitor
MPP – multipotent progenitor
My-bi HSC – myeloid-biased haematopoietic stem cell
NAD⁺ – nicotinamide adenine dinucleotide

NOD/SCID – nonobese diabetic-severe combined immunodeficient

PBS – phosphate buffered saline

Pen/Strep – penicillin/streptomycin solution

Pro-MK – pro-megakaryocyte

PTH – parathyroid hormone

PTPRC – protein tyrosine phosphatase, receptor type C

RIC – reduced intensity conditioning

SC – stem cell

SC – stem cell

SCF – stem cell factor

SDF-1/CXCR-4 – stromal cell-derived factor-1/C-X-C chemokine receptor type 4

SLAM – signaling lymphocyte activation molecule

ST-HSC – short term haematopoietic stem cell

TEPA – tetraethylenepentamine

TNC – total nucleated cell

TPO – thrombopoietin

UCB – umbilical cord blood

1 INTRODUCTION

The number of hematopoietic stem cell (HSC) transplantations is rising due to their essential role in treating leukaemia, cancer, anaemia and other diseases (Broxmeyer, 2010). The classical source of HSCs is bone marrow or mobilized peripheral blood. Lately, HSCs from umbilical cord blood (UCB) are being used for HSC transplantations. Bone marrow or peripheral blood derived HSCs have some disadvantages such as: high human leukocyte antigen (HLA) match requirement, risk for the donor, risk of transmitting diseases and delayed availability. Therefore, HSCs from UCB are a better call: the availability is 25-36 days earlier, the HLA matching requirement is four out of six loci; the frequency of HSCs is higher (Rocha et al., 2010).

Despite of advantages UCB transplants have, they also show some disadvantages; all correlates with a lower content of HSCs in an average cord blood unit (around 100 ml) as compared to an average 500 ml unit of bone marrow or peripheral blood isolated HSCs. For instance, the disadvantages are delayed engraftment of neutrophils, T-cells, and in particular platelets (Schipper et al., 2003). In brief, in one unit of UCB there are not enough HSC populations for an adult bone marrow (BM), therefore platelet transfusions are necessary. Different approaches were developed to improve platelet engraftment such as *ex vivo* expansion (Rocha et al., 2010), the use of blocking or stimulating specific peptides in the BM of the donor (Campbell et al., 2007, cited by Rocha et al., 2010), injection of the cord blood HSCs directly into the bone marrow of the patient's long bones (Frassoni et al., 2008, cited by Rocha et al., 2010), double unit cord blood transplantations (Barker et al., 2005, cited by et al., 2010), coinfusion with a haploidentical T cell-depleted graft (Fernandez et al., 2003; Bautista et al., 2009), coinfusion with multipotent mesenchymal stromal cells (Macmillan et al. 2009) or non-myeloablative conditioning of the patient (Del Toro et al., 2004). The results of these studies are still under evaluation.

Ex vivo expansion of HSCs from UCB into megakaryocyte lineage, i.e. into platelet precursors with thrombopoietin (TPO) is one of the possibilities to enhance post transplant platelet recovery. The biggest concern with *ex vivo* expanded cells is the risk of exhaustion of the stem cells (SCs), which can result in loss of long-term engraftment capacity (Boiron et al., 2006). Van Hensbergen's his colleagues' findings showed an accelerated platelet recovery after transplantation of *in vitro* expanded HSCs with TPO in a non-obese diabetic-severe combined immunodeficient (NOD/SCID) mouse model (van Hensbergen et al., 2006). Recent studies have shown that specific subpopulations that develop during the *ex vivo* expansion of CD34⁺ cells (CD, cluster of differentiation) with TPO (CD34⁺CD61⁻ (CD34⁺) and CD34⁻CD61⁻ (Lin⁻)) contribute for an accelerated platelet recovery and to the long-term engraftment of HSCs in the BM (Schipper et al., 2012). The long-term engraftment is believed to be due to the CD34⁺CD38⁻ cell population, which is considered to contain primitive and MPPs (Sutherland et al., 1989; Hao et al., 1995; Ishikawa et al.,

2003). During *ex vivo* expansion a large fraction of the HSCs differentiates towards pro-megakaryocytes (MK) ($CD34^-CD61^+$, $CD61^+$ cells). These cells, however, play no role in either platelet recovery or engraftment in the BM, which is only facilitated by HSCs (Shipper et al., 2012). In brief, Schipper's and others results indicate that *ex vivo* expansion focused on partial differentiation towards megakaryocyte progenitor cells improves platelet recovery (Bruno et al., 2004; van Hensbergen et al., 2006; Mattia et al., 2008).

Before further clinical trials are performed with this method, we should be able to predict the reactivity of the cord blood $CD34^+$ cells to multiply and differentiate with TPO into the megakaryocytic line as well as to guarantee the preservation of long-term stem cell capacity of *ex vivo* expanded grafts.

1.1 PURPOSE OF OUR STUDY

The main purpose of our research is to determine the properties of TPO cultured HSCs isolated from UCB as a possible strategy to enhance expansion in order to achieve better engraftment and rapid platelet production, in order to predict the reactivity of the cord blood stem cells to TPO and the preservation of long term stem cell capacity of *ex vivo* expanded grafts.

1.1.1 Aims of our study

The aim of our research is to evaluate the ability of isolated HSC from UCB to expand *in vitro* in the presence of TPO into the megakaryocyte lines, as well as to explore the capacity of these cells to preserve the long time repopulation activity.

1.1.2 Design of the study

1. Experiment: Determination of the populations after 10 days of TPO culture

TPO should lead the differentiation of HSCs towards megakaryocytes. The umbilical cord blood HSCs will be isolated with ficoll density separation and magnetic activated cell separation (MACS) columns. The purity of cultured $CD34^+$ cells will have to be higher than 80%. After 10 days of TPO culture, we will follow the differentiation with fluorescent antibodies.

2. Experiment: Responsiveness of UCB isolated HSCs to the TPO medium

HSCs should not equally respond to TPO. To be sure of that, we need to have an insight in cell division. For this purpose, we will use a dye that binds on the membrane and its intensity splits into half after cell division. This dye will be PKH26. We will look at the

PHK26 intensity before culturing and after 10 days of culture with TPO with a flow cytometer to determine the percentage of divided and non-divided cells. This and the previous experiment are going to be carried out simultaneously in order not to waste isolated material.

3. Experiment: Determination of the populations in a 15 day TPO culture and UCB isolated HSCs responsiveness to TPO

In order to follow differentiation and cell divisions, we will start a 15-day, day to day analysis. We will follow the cell divisions with PKH26 label and the differentiation with fluorescent antibodies.

4. Experiment: Preservation of the long term repopulation capacity – CAFC assay

We will observe the long-term engraftment capacity in a CAFC assay, in a five-week culture with a feeder layer. The scoring will be carried out under inverted microscope.

5. Experiment: Different expansion of CD34⁺CD38⁻ and CD34⁺CD38⁺ populations

The more primitive HSCs population are usually the CD34⁺CD38⁻ cells (Ishikawa et al., 2002) and they *in vitro* usually expand much more than the CD34⁺CD38⁺ HSCs (Schipper et al., 1998). After sorting the CD34⁺ cells to CD38⁺ and CD38⁻ with a cell sorter, and later with culturing separately, the differences in expansion should be measurable.

1.1.3 Hypothesis

H₁⁰: The 10 day (and 15 day) TPO culture of purified CD34⁺ cells will represent in two populations; a) a population is responding to the TPO; b) a population that is not responding to TPO. The responding population is due to the responsiveness of a population of highly proliferating primitive HSCs and other megakaryocyte progenitors to TPO (Schipper et al., 2012).

H₂⁰: The responding population is reacting to TPO with differentiation towards megakaryocytes with three populations: 1) a population, which is expressing the megakaryocyte markers (CD34⁻CD61⁺; CD61⁺) 2) a population, which is not expressing any markers (CD34⁻CD61⁻; Lin⁻), 3) a population, which still expresses the CD34 marker (CD34⁺CD61⁻; rCD34⁺).

H₃⁰: There is a difference in expansion between more primitive hematopoietic stem cells and progenitors (CD34⁺CD38⁻) or more mature haematopoietic stem cells and progenitors

(CD34⁺CD38⁺), the first will divide more times and the differentiation will be progressing more.

H₄⁰: The CD34⁺ cells will keep their long-term repopulating capacity after 10 day of culture with TPO in a CAFC assay.

1.1.4 Expected results

1. Experiment: Determination of the populations after 10 days of TPO culture

TPO should lead the differentiation of HSCs towards megakaryocytes. After 10 days of culture with TPO, 71,2% of all cells should be cells expressing megakaryocytes markers (CD61/CD9), 19,2% of the cells should be Lin⁻ (not expressing any typical cell markers) and 9,4% of cells should remain CD34 positive cells (stem cells) (Hensbergen et al., 2006).

2. Experiment: Responsiveness of UCB isolated HSCs to the TPO medium

HSCs should not equally respond to TPO. Just small percentage of the cells after 10 days of TPO culture should remain CD34⁺ and undivided.

3. Experiment: Determination of the populations in a 15 day TPO culture and UCB isolated HSCs responsiveness to TPO

The isolated HSCs should lead the differentiation towards megakaryocytes. In a 15-day culture, the differentiation should progress more.

4. Experiment: Preservation of the long term repopulation capacity – CAFC assay

After the week four and five of CAFC assay, we should be able to see the emerging clusters of small, tightly packed cells within a stromal layer, i.e. the LT-HSCs (Breems et al., 1994).

5. Experiment: Different expansion of CD34⁺CD38⁻ and CD34⁺CD38⁺ populations

HSCs should not equally respond to TPO. The HSCs and progenitors that are more primitive (CD34⁺CD38⁻) should respond more: they should expand more and differentiate more compared to the more mature HSCs and progenitors (CD34⁺CD38⁺).

2 PUBLICATIONS REVIEW

2.1 STEM CELLS

Scientists in the 19th century were occupied for a long time with two main questions in the context of embryology; 1) how is the continuity of the germ-plasm, a zone in the cytoplasm of the egg cells, which contains determinants that will develop into the germ cell lineage and after mitotic divisions give rise to the embryo, possible and 2) what is the origin of the hematopoietic system (Ramalho-Santos and Willenbring, 2007). Today, we know that the answer is hidden in the stem cells (SCs).

2.1.1 The history of the term "stem cell"

Ernst Haeckel was the first to use the term Stammzelle (stem cell) in 1868. The term itself did not relate to haematopoiesis. He drew a number of phylogenetic trees (Stammbäume) to represent the evolution of organisms from the common ancestor. In this context, he used the term "Stammzelle" to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved during evolution (Haeckel, 1868, cited by Ramalho-Santos and Willenbring, 2007). Later he suggested this term also for the fertilized egg, which gives rise to all cells of the organism (Haeckel, 1874, cited by Ramalho-Santos and Willenbring, 2007). Weismann and Boveri also used the term. Weismann proposed that the germplasm, which contains genetic determinants and transmits them one generation to the next, segregates in early embryonic development to specialized cells (germ cells) that are distinct from the rest of the body cells (somatic cells) enabling the continuity of the germplasm (Weismann, 1885, cited by Ramalho-Santos and Willenbring, 2007). Boveri was inspired by this theory and he tried to find the germ cells in the nematode *Ascaris*. In the embryo of this nematode, the chromatin pattern displays unusually: the cells, which will later give rise to somatic cells, lose some chromatin after early mitosis. The chromatin level maintains to be the same only in the germ cells. Boveri proposed that they transmit the intact genetic material to the next generation (Boveri, 1887 and 1892, cited by Ramalho-Santos and Willenbring, 2007) and that cells from the fertilized egg before they commit to the germ cells should be called Stammzelle (stem cells) and with this he took Haeckel's definition one step further (Boveri, 1892, cited by Ramalho-Santos and Willenbring, 2007). In the same year, Häcker observed in a study of development of the crustacean *Cyclops* that the cells, which he also called Stamm Zelle, underwent asymmetric cell division: one of the daughter cells gave rise to the mesodermal cells and the other to germ cells (Häcker, 1892, cited by Ramalho-Santos and Willenbring, 2007). However, in this study the term Stanzelle referred to what we know today as the germline lineage, i.e. primordial germ cells and germline stem cells. In 1896 Edmund B. Wilson spread the term stem cell in English by reviewing Häcker's and Boveri's work in

the book "The Cell in Development and Inheritance" (Wilson, 1896, cited by Ramalho-Santos and Willenbring, 2007).

In the context of haematopoiesis, they started to use the term Stammzelle around the same time. It was the time when the researchers of the hematopoietic system were split into two camps. It was due to Paul Ehrlich's staining techniques, which enabled the identification of different white blood cell lineages. One side did not believe in the existence of a common hematopoietic progenitor stem cell (of erythrocytes, granulocytes, and lymphocytes), but the other side did. The first person to use the term Stammzelle in the context of haematopoiesis was Pappenheim in 1896, although Maximov is usually credited with coining the term in 1909 (Pappenheim, 1896, cited by Ramalho-Santos and Willenbring, 2007; Maximov, 1909, cited by Ramalho-Santos and Willenbring, 2007). Pappenheim's stem cell referred to a precursor cell capable of giving rise to both red and white blood cells (Pappenheim, 1896, cited by Ramalho-Santos and Willenbring, 2007). The existence of the definitive common haematopoietic stem cell was proved quite late, in the 1960s, when Till and McCulloch proved the theory by injecting bone marrow cells into irradiated mice. In the spleen of those mice, visible nodules were observed in proportion to the number of bone marrow cells injected. Later they proved that each nodule grew from a single cell today known as the haematopoietic stem cell (HSC) (Till et al., 1961 and 1964; Becker et al., 1963).

2.1.2 What are stem cells?

Stem cells are a special population of cells that are able to both self renew and give rise to differentiated cells (Till and McCulloch, 1980; Morrison and Shah, 1997). In other words, they are able to self-replicate and generate multiple types of cells. Beside these properties they have many other important ones: ability to undergo asymmetric cell division (Beckmann et al., 2007), they exist in a mytotically quiescent form, so usually they are not dividing (just as a response to certain factors), and clonally regenerate all cells in the tissue type in which they exist (Hall and Watt, 1989; Potten and Loeffler, 1990). Asymmetric cell division, which means that each stem cell gives rise to one daughter cell with a stem cell fate and one which will differentiate, manages both self renewal and differentiation within single cell division (Betschinger and Knoblich, 2004). The only problem in this strategy occurs when the stem cell expansion in number is needed. Consequently, there are also symmetric stem cell divisions (Morrison and Kimble, 2006). In addition, SCs provide an opportunity to investigate embryonic development mechanisms. Due to their proliferation and differentiation capacities, they have a great potential for cell therapies (Daley et al., 2003).

Stem cells are present in embryonic, fetal and adult tissues. In adults, they maintain the homeostasis of tissues, also after injuries we can find them in every tissue type. After the initial studies of Till and McCulloch, the field of SC research increased exponentially. Since the differentiation potential of different stem cell population is not equal, a system of nomenclature had to evolve. There have been two classification groups developed: classification by origin and by type.

2.1.3 Stem cell classification

2.1.3.1 Classification by stem cell origin

One of the most commonly used classification of stem cells is by their origin or location. Depending on their residency, there are two categories: the embryonic stem cells (ESCs) and the adult stem cells (ASCs) or tissue specific stem cells (Can, 2008). Embryonic stem cells, as their name suggests, can be found in embryos. They are derived from the inner cell mass of the preimplantation blastocyst (Thomson et al., 1998, cited by Can, 2008). Since they can also be harvested from human embryos, they raised many debates concerning ethical questions. In the blastocysts they play an essential role as they are pluripotent, undifferentiated cells, which give rise to all cell types of the body (Wobus and Boheler, 2005).

However, adult stem cells or tissue specific stem cells are present in developmental stages beyond embryo or can repair damage after injuries by giving rise to progeny restricted to the tissue of origin. In general we distinguish more sources of tissue specific stem cells: 1) endodermal origin: pulmonary epithelial SCs, gastrointestinal tract SCs, pancreatic SCs, hepatic oval cells, mammary and prostatic gland SCs, ovarian and testicular SCs; 2) mesodermal origin: haematopoietic SCs, mesenchymal stroma SCs, cardiac SCs, satellite cells of muscle; and 3) ectodermal origin: neural SCs, skin SCs and ocular SCs. The tissue specific stem cells can be derived either from a fetus or a postnatal individual (Can, 2008). Umbilical cord blood stem cells also belong into this classification group.

2.1.3.2 Classification by the type of stem cells

The second classification of stem cells is based on their differentiation potential as totipotent, pluripotent, multipotent and unipotent. Totipotent stem cells can give rise to a completely new individual, if appropriate maternal support is provided. They can form both embryonic and extraembryonic tissues. For example, totipotent cells are all cells from the zygote to morula staged embryo (Can, 2008). Embryonic stem cells are pluripotent, as they cannot form extraembryonic tissues, but they are able to differentiate towards germ and somatic cells. They can become any cell types from the three germ layers, the endoderm, ectoderm and mesoderm.

Tissue specific stem cells can be multipotent, oligopotent or unipotent. The multipotent stem cells are able to differentiate towards all kinds of cells of a single tissue type, in other words, they can only differentiate to certain, specific cell type of the same embryonic layer (endoderm, ectoderm, mezoderm). However, it was reported that many adult tissues might contain greater potency than previously thought. The adult stem cells unexpectedly differentiated into other cell type, different from their tissue origin. With other words, they show a considerable degree of plasticity: they showed dedifferentiation, redifferentiation and transdifferentiation (Toma et al., 2001, cited by Can, 2008), probably because the cell type fate after differentiation is determined with niches, with the microenvironment (Sujata and Chaudhuri, 2008). Namely that is why a neural stem cell can differentiate towards haematopoietic cells, if it is in a certain environment. The best-characterized multipotent cells are the haematopoietic stem cells. They give rise to all cell types of the blood system (Till et al., 1961, 1964, 1980; Weissman et al., 2001). Tissue specific stem cells have been isolated from many other organs, including nervous system, epidermis, intestine, liver, lung and others, but they are basically known as mentioned above (in 2.1.3.1) (Can, 2008).

Oligopotent progenitors can produce more than one type of mature cells, thus unipotent stem cells can give rise to just one type of mature cell (Can, 2008).

2.1.4 Stem cells and their niches

The idea of niches comes from Schofield in 1978, when he made a hypothesis that the physiologically limited microenvironment supports the stem cells (Schofield, 1978, cited by Sujata and Chaudhuri, 2008). It is now believed that the niche regulates tissue generation, maintenance and repair with help of several signalling cascades (Scadden, 2006, cited by Sujata and Chaudhuri, 2008; Fuchs et al., 2004, cited by Sujata and Chaudhuri, 2008). In addition, the niches keep the balanced response of the stem cell to the need of the organism (Scadden, 2006, cited by Sujata and Chaudhuri.). Furthermore, the microenvironment influences the stem cells via direct cell-cell interactions or by releasing soluble factors (Calvi et al., 2003, cited by Li and Xie; 2005; Zhang et al., 2003, cited by Li and Xie).

2.2 HAEMATOPOIESIS

2.2.1 Haematopoiesis – the stem cell concept

Haematopoiesis is the process of cell development, more precisely, cell production, multiplication and specialization of blood cells. It is enabled by one of the tissue-specific stem cells, the HSC. In 1997, Morrison and colleagues wondered if the self-renewal for our whole lifetime is also true for HSCs (Morrison and Shah, 1997). Probably it was due to the

fact that most of the HSCs are in G0 phase, it is a phase, when the cell is in a quiescent state and it doesn't divide (Rossi et al., 2007, cited by Seita and Weissman, 2010). It was an interesting conundrum: how is the production of mature blood cells possible since there are one million mature blood cells produced in every second (Ogawa, 1993, cited by Seita and Weissman, 2010). In addition, the mammalian blood system contains more than eight mature cell types: red blood cells, platelets (megakaryocytes), other myeloid cells (granulocytes, monocytes, and macrophages), mast cells, T- and B- lymphocytes, natural killers, and dendritic cells. How are they produced? Today, the mystery is solved, and we know that respectively they all come from one cell. We have an idea how the haematopoiesis is regulated, the stem cell concept is sophisticated and explored in more details (Seita and Weissman, 2010).

The stem cell concept in haematopoiesis was built on Till and McCulloch's studies (Till et al., 1961 and 1964) when they found that some cells in the bone marrow of mice could form myeloerythroid colonies in the spleens of irradiated mice transplants, in which a subset of these cells could self-renew. Many other researchers added their part as well. For instance, Sprangrude and colleagues using flow cytometry and monoclonal antibody cell surface determinants showed three multipotent populations: long-term stem cells, short-term stem cells and multipotent progenitor (MPP) cells. Long-term stem cells are capable of long-term multilineage engraftment, and they can become any cell type of the blood system (Sprangrude et al., 1988; Sutherland et al., 1989; Hao et al., 1995; Ishikawa et al., 2003). The short engraftment is due to the "short term" (ST) HSCs. They kept the ability to give rise to any other cell in the blood system but their self-renewal capacity is smaller, they can repopulate the bone marrow just for a limited period of time (McKenzie et al., 2005). By definition, the MPPs are capable of giving rise to cells from multiple, but a limited number of lineages of the same germ layer, but they no longer have self-renewal capacity (Schöler, 2007).

2.2.2 Hierarchical structure of the haematopoietic system

The haematopoietic system has a hierarchical structure as shown in Figure 1. As written above, the differentiation starts from a long term (LT) HSC, which gives rise to short term (ST) HSCs, a HSC with lower self-renewal capacity (Sprangrude et al., 1988). The ST-HSCs give rise to MPPs, which after differentiation no longer possess self-renewal ability yet keep differentiation potential and spleen colony forming unit ability (CFU-S) (Morrison and Weissman, 1994, cited by Seita and Weissman, 2010). Further, MPPs differentiate to two oligopotent progenitors: (1) the common myeloid progenitor (CMP) (Akashi et al., 2000, cited by Seita and Weissman, 2010) and (2) the common lymphoid progenitor (CLP) (Kondo et al., 1997, cited by Seita and Weissman, 2010). Collectively, these two oligopotent progenitors differentiate to all lineage restricted progenitors: megakaryocyte progenitors (containing megakaryocyte burst forming unit (BFU-Mk) and

megakaryocyte colony forming unit (CFU-Mk) ability), erythrocyte progenitors, granulocyte progenitors, macrophage/monocyte progenitors, dendritic cell progenitors, B-cell progenitors, T-cell progenitors, natural killer cell progenitors and later to lineage effector cells of the haematopoietic system: natural killer cells, dendritic cells, mchrophages/monocytes, granulocytes, erythrocyte, platelets as shown in Figure 1.

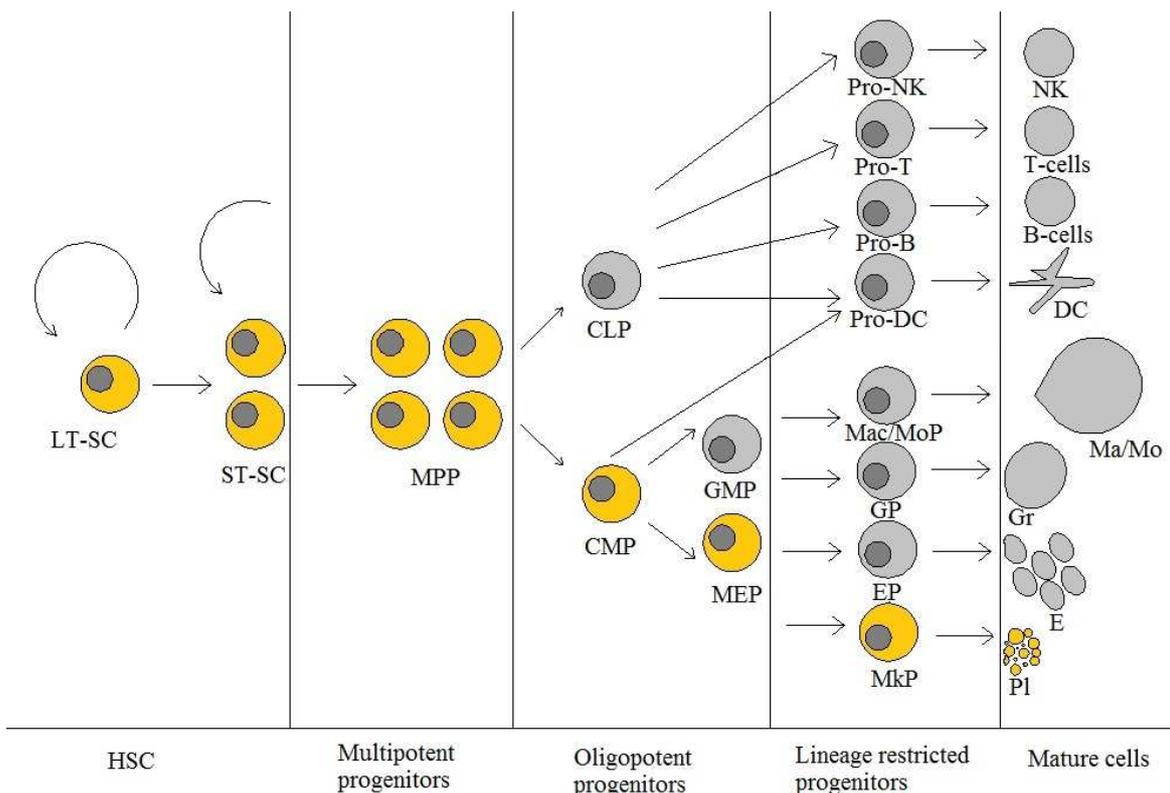


Figure 1: Stem cell hierarchy concept: differentiation from the long term haematopoietic stem cell (LT-HSC) towards mature blood cells: ST-SC, short term stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/machrophage progenitor; MkP, megakaryocyte progenitor; EP, erythrocyte progenitor; GP, granulocyte progenitor; Mac/MoP, macrophage/monocyte progenitor; Pro-DC, dendritic cell progenitor; Pro-B, B-cell progenitor; Pro-T, T-cell progenitor; Pro-NK, natural killer progenitor; NK, natural killer cells; DC, dendritic cells; Ma/Mo, mchrophage/monocyte; Gr, granulocyte; E, erythrocyte; Pl, platelets (Seita and Weissman, 2010)

The HSCs were thought to be homogeneous population for many years. They were thought to be born with same differentiation and proliferation capacity. However, with functional tests it cleared out that we can distinguish three subpopulations of HSCs by their differentiation capacity: myeloid-biased (My-bi), balanced (Bala) and lymphoid-biased (Ly-bi) HSCs. The My-bi HSCs produce few CLPs but normal levels of myeloid progenitors. Whereas, the Ly-bi HSCs generate normal levels of lymphoid but only low levels of myeloid progenitors. The Bala HSCs generate the same ratio of lymphoid and myeloid cells as usually in the blood. It seems that HSCs have reprogrammed

differentiation and self-renewal behaviour, as every daughter cell of a HSC has same life span and differentiation capacity, depending on the age of the HSCs. In mice as well as in human, Ly-bi HSCs are lost with ageing and My-bi increase. Moreover, in mice the aged cells have less self-renewal capacity and homeless (Dykstra et al., 2011).

2.2.3 Regulation of HSC state

HSCs must keep their self-renew capacity and must remain undifferentiated in the body, unless there are other needs. To maintain those properties and to control differentiation, self-renewal capacity, and expansion, the cells are regulated with epigenetic modifications, with their microenvironment and with other growth factors (Oh and Humphries, 2012).

Studies have shown that the gene expression is different after changes in chromatin structures are detectable due to epigenetic modifications (Reik et al., 2001, cited by Oh and Humphries, 2012). This kind of change influences the accessibility of binding sites of transcription factors by histone modifications and therefore it influences the expression of genes (Jaenisch and Bird, 2003, cited by Oh and Humphries, 2012). The modification of histones comprise in acetylation, methylation, phosphorylation, sumoylation and ubiquitylation. Usually, hyperacetylation of histones is connected with opening the chromatin, consequently gene expression is enabled, whereas histone deacetylation results in chromatin condensation, as a result gene expression is inhibited. In addition, methylation can influence gene expression as well. If the DNA is methylated in promoter regions, the expression will be silenced (Rice et al., 2007, cited by Oh and Humphries, 2012). Moreover, the DNA methylation is believed to be involved in the regulation of lineage-specific differentiation as well as in aging-associated changes of haematopoietic progenitors (Ji et al., 2010, cited by Oh and Humphries, 2012).

The undifferentiated haematopoietic cells exhibit less condensed chromatin structures and display a higher rate of histone acetylation, point to dynamic chromatin structures with higher turn-over of chromatin structures than differentiated cells, whereas the mature cells have a very stable chromatin (Chung et al., 2009, cited by Oh and Humphries, 2012). Moreover, the undifferentiated cells have a greater potential flexibility to epigenetic modifications of chromatin structures (Oh and Humphries, 2012). In summary, maintaining the chromatin into a less dense state promotes higher maintenance of undifferentiated status and self-renewal of HSCs (Chung et al., 2009, cited by Oh and Humphries, 2012).

The differentiation and self-renewal capacity is regulated with the HSC's microenvironment, i.e. with their niche as well. Current results suggest that there are two types of niches in the bone marrow (BM): 1) an osteoblastic niche in the trabecular endosteum; and 2) a vascular niche in sinusoidal perivascular areas of the BM (Sacchetti et

al., 2007, cited by Oh and Humphries, 2012). Rather than comprising a static compartment niches are a dynamic environment, and consequently they support rapid increase in haematopoietic cell production depending on the physiological requirements (Avecilla et al., 2004, cited by Chang et al., 2007). Osteoblasts play an important role in HSC regulations as they are secreting/expressing some molecules and factors that regulate maturation and proliferation, such as angiopoietin-1, N-cadherin, Jagged-1 and interleukin-10 (IL-10). In addition, many potential regulators were identified, but notable ones among them is the crosstalk between Jagged-1/Notch-1 and SDF-1/CXCR4 signaling axis (Oh and Humphries, 2012). Moreover, some other growth factors, such as thrombopoietin, which affects the HSCs *in vivo* so they start to differentiate towards platelets (Archimbaud et al., 1996; Vandhan-Raj et al., 1996; Molineux et al., 1996).

Angiopoietin-1 is one of the factors osteoblasts secrete. They control the HSCs quiescence with activating the Tie-2/angiopoietin-1 signaling pathway (Arai et al., 2004). The second molecule, expressed both on the osteoblasts and HSCs is the N-cadherin, a cell adhesion molecule, which induces the adhesion and homing of HSCs into the niche (Calvi et al., 2003, cited by Li and Xie; Zhang et al., 2003, cited by Li and Xie). Further studies showed that N-cadherin expression is down-regulated by reactive oxygen species (oxidative stress), resulting in the release of HSC from the niche (Hosokawa et al., 2007). In addition, results suggest that the IL-10 an immune modulating cytokine produced by osteoblasts stimulates self-renewal of the HSCs (Kang et al., 2007). Furthermore, Jagged-1, a soluble factor generated by osteoblasts, influences HSCs through the Notch receptor (Watt and Hogan, 2000; Calvi et al., 2003, cited by Li and Xie; Zhang et al., 2003, cited by Li and Xie).

Expression of constitutively active Notch-1 receptors on HSCs promotes their self-renewal that retains the potential to generate both lymphoid and myeloid cells *in vitro*. The Notch-1 receptor is activated with ligands Delta-1, Jagged-1 or Jagged-2 (Varnum-Finney et al., 1998). It was discovered that the parathyroid hormone (PTH) induces expression of Jagged-1 and therefore it activates the Notch-1 receptor and the HSC numbers increase (Calvi et al., 2003, cited by Li and Xie). In addition, the Wnt/ β -catenin affects the Notch-1 ligands similarly; it stimulates the HSCs (Kim et al., 2009, cited by Oh and Humphries, 2012). However, osteopontin, a glykoprotein cytokine, regulates the Notch-1 regulatory axis with deactivation and therefore with decreasing in HSC number (Stier et al., 2005, cited by Oh and Humphries, 2012).

As written before, the SDF-1/CXCR-4 axis plays an essential role in regulation of the haematopoietic system; it functions in the homing of HSCs in the marrow microenvironment. SDF-1 (also known as CXC ligand 12 (CXCL-12)) is a cytokine, belonging to the chemokine family, which leads the homing of HSCs, and CXCR-4 is SDF-1's receptor, found on HSCs. SDF-1 is produced by the stromal cells in the bone marrow, where the concentration is higher. When HSCs are infused into a patient, they

respond to the SDF-1 chemotactic gradient of the bone marrow. The SCs transmigrate through the basal membrane in a methylprednisolone-dependent manner, and home in their niche, where they can survive, expand, proliferate, and engraft. However, SDF-1 has an inactivator, the CD26/Dipeptidylpeptidase IV (DPP-IV), an enzyme, which binds to a CXCR-4 receptor of the SDF-1. This means that if the SDF-1 is inactivated with CD26/DPP-IV, the homing of the HSCs into the niche is inhibited as well (Campbell et al., 2007, cited by Rocha et al., 2010). In addition, the SDF-1 cytokine is downregulated with G-CSF and with the sympathetic nerve system, and consequently the HSCs are released from the niche (Oh and Humphries, 2012).

Intrinsic molecules, such as Nf2, Rb, FANCB, BAR- γ , Bis or Sbd regulate niche activities, they represent upregulation. However, they influence niche activities in a yet poorly defined manner but modulate haematopoietic activity in a stroma-dependent manner (Oh and Humphries, 2012).

2.2.4 Characterization of HSCs with help of expressed molecules

The characteristics of different HSCs and blood cell progenitors are determined based on their function, which is drafted in their definition. Functional tests, for instance *in vitro* determination of the colony-forming capacity, are of course time consuming that is why immunophenotype characterization was started (Ivanović, 2010). For this shortcut, we have to have monoclonal antibodies to label specific molecules (labeled with fluorescent molecules), expressed on a specific cell type; a flow cytometer, which detects the signals; and a computer-software to analyze the measurements.

Usually, one type of cells in the same differentiation stage expresses same molecules on its surface as well, but not always. For instance, the HSCs and the CD34 (CD, cluster of differentiation) marker are a good example of that. It was shown that although almost all the HSCs express the CD34 marker *in vitro*, this population is heterogeneous (Silvestri et al., 1992). The most of the CD34⁺ cells (90-99%) coexpresses the CD38 antigen as well. Although, only 1-10% of CD34 positive cells that do not express the CD38 marker have both lymphoid and myeloid differentiation potential (Baum et al., 1992; Huang and Terstappen, 1994; Hao et al., 1998; Miller et al., 1999). In addition, CD34⁺CD38⁻ cells not CD34⁺CD38⁺ cells, are highly enriched for long-term colony-initiating cells (Hao et al., 1996, Petzer et al., 1996) and contain severe combined immunodeficient (SCID) human repopulating cells (Baum et al., 1992) and nonobese diabetic-severe combined immunodeficient (NOD/SCID) mouse repopulating cells (Larochelle et al., 1996, Bhatia et al., 1997) *in vivo* with read-out in secondary NOD/SCID transplants. Further clarification of the human HSC phenotype occurred when the observation was made that single Lin⁻CD34⁺CD90⁺ and not Lin⁻CD34⁺CD90⁻ cells generated lymphoid and myeloid progeny in both cell culture colony assay and in SCID-human mice (Baum et al., 1992). In addition,

virtually all $\text{Lin}^- \text{CD34}^+ \text{CD90}^+$ cells reside in the CD38^- fraction. All of these data combined support the idea that HSCs are contained in the $\text{Lin}^- \text{CD34}^+ \text{CD38}^- \text{CD90}^+$ population (Chao et al, 2008, cited by Ivanović, 2010). However, Engelhardt and colleagues observed that half of the CD34^+ population does not exhibit either progenitor or stem cell functional properties. Some SCs even do not express CD34 marker in a steady state (Engelhardt et al., 2002, cited by Ivanović, 2010). Therefore, we have to be aware of the fact that the CD34^+ cells are not obligatory SCs as well. We cannot use the term SC when speaking about CD34^+ cells without functional tests (Ivanović, 2010). However, in one part of our study we could not test the functional characteristics due to lack of time, therefore we trusted and used the most typically expressed immunophenotypes for cells along haematopoietic differentiation as seen in Table 1.

Table 1: Cell types during differentiation to megakaryocytes and their phenotype (Yang et al., 2005; Ishikawa et al., 2003; Majeti et al., 2007; Chang 2007; Notta et al., 2011)

| Cell type | Phenotype |
|--|---|
| LT-HSC – long term haematopoietic stem cell | $\text{Lin}^{-/\text{low}}, \text{c-kit}^{\text{high}}, \text{CD34}^{+/-}, \text{CD38}^-, \text{CD90}^+, \text{CD45RA}^-, \text{Flt3}^-, \text{c-Mpl}^+, \text{CD150}^+, \text{CD133}^+$ |
| ST-HSC – short term haematopoietic stem cell | $\text{Lin}^{-/\text{low}}, \text{c-kit}^{\text{high}}, \text{CD34}^+, \text{CD38}^+, \text{CD90}^{\text{low}}, \text{CD45RA}^-, \text{Flt3}^-, \text{c-Mpl}^+, \text{CD150}^+, \text{CD133}^+$ |
| MPP – multipotent progenitor | $\text{Lin}^{-/\text{low}}, \text{c-kit}^{\text{high}}, \text{CD34}^+, \text{CD38}^-, \text{CD90}^-, \text{CD45RA}^-, \text{Flt3}^+, \text{c-Mpl}^+, \text{CD150}^+, \text{CD133}^+$ |
| CMP – common myeloid progenitor | $\text{Lin}^{-/\text{low}}, \text{c-kit}^+, \text{CD34}^+, \text{CD38}^+, \text{CD90}^-, \text{CD45RA}^-, \text{Flt3}^+, \text{c-Mpl}^+, \text{CD150}^+, \text{CD133}^+, \text{IL-3R}\alpha^{\text{low}}$ |
| MEP – megakaryocyte/erythrocyte progenitor | $\text{Lin}^{-/\text{low}}, \text{c-kit}^+, \text{CD34}^-, \text{CD38}^+, \text{CD90}^-, \text{CD45RA}^-, \text{Flt3}^-, \text{c-Mpl}^+, \text{CD150}^+, \text{CD133}^+, \text{IL-3R}\alpha^-$ |
| MkP – megakaryocyte progenitors | $\text{CD9}^+, \text{CD41}^+, \text{CD61}^+, \text{c-kit}^+, \text{CD34}^{\text{low}}, \text{CD38}^+, \text{CD90}^-, \text{CD45RA}^-, \text{Flt3}^-, \text{c-Mpl}^+, \text{CD150}^+, \text{CD133}^+$ |

The megakaryocyte stem cells and their progeny to some level do not express any lineage specific or differentiated cell markers. Those cells we simply designate as Lin^- cells. The markers of differentiation of a megakaryocyte lineage are CD9 , CD41 and CD61 . The *CD9* gene encodes the CD9 protein, which is a transmembrane protein of four hydrophobic domains. It mediates signal transduction, modulates cell adhesion and migration, triggers platelet activation and aggregation (*CD9 molecule*, 2012). The *CD41* gene encodes the protein integrin alpha chain 2b (ITGA2B) also known as the platelet glycoprotein IIb of IIb/IIIa complex, which participates in haematopoietic cell lineage differentiation. Integrins are in general cell-surface proteins, composed of an alpha and a beta chain and are known to participate in cell adhesion as well as cell-surface-mediated signaling (ITGA2B ..., 2012). The *CD61* encodes an integrin protein as well, the so-called integrin beta chain 3 (ITGB3) and it is found along the ITGA2B protein (ITGB3 ..., 2012). The

CD9, CD41 and the CD61 markers are all expressed on megakaryocytes, the CD9 appears earlier in time, it is more typical for less mature megakaryocytes, the CD41 and CD61 appear simultaneously (Le Naour et al., 1997).

The markers that are usually connected with SCs are c-kit and CD34. C-kit (CD117 or tyrosine-protein kinase) is a cytokine receptor expressed on the surface of HSCs as well as on other cell types encoded by gene *KIT*. It plays a role in cell survival, proliferation and differentiation (KIT ..., 2012). The *CD34* gene encodes a cell surface glycoprotein, which functions as a cell-cell adhesion factor. It mediates the stem cell to bone marrow attachment. It is expressed in early haematopoietic and vascular-associated tissue (CD34 molecule, 2012).

Usually, the CD38 protein also known as cyclic ADP ribose hydrolase, along with CD34 marker can help us distinguish between LT-HSCs and ST-HSCs. It is a glycoprotein found on the surface of many immune cells, it catalyzes the synthesis and hydrolysis of cyclic ADP-ribose (cADPR) from NAD^+ to ADP-ribose. Moreover, it plays an important role in cell adhesion, signal transduction and calcium signaling (CD38 molecule, 2012).

CD90, also known as Thy-1, is a N-glycosylated surface protein with a single V-like immunoglobulin domain. It can be used as a marker for variety of SCs and for the axonal processes of mature neurons. Moreover, it plays a role in cell-cell and cell-matrix interactions, apoptosis, metastasis, inflammation, fibrosis, etc. (Ades et al., 1980).

The CD45RA, also known as protein tyrosine phosphatase, receptor type C (PTPRC), encoded protein is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signalling molecules that regulate cell growth, differentiation, mitotic cycle, and oncogenic transformation. The CD45RA protein is specifically expressed in haematopoietic cells, on T cells (PTPRC ..., 2012).

The Flt3 protein, also known as FLK2, STK1 or CD135, is a class III receptor tyrosine kinase that regulates haematopoiesis. The receptor consists of an extracellular domain composed of five immunoglobulin-like domains, one transmembrane region, and a cytoplasmic kinase domain split into two parts by a kinase-insert domain. The receptor is activated by binding of the Flt3 ligand to the extracellular domain, which induces homodimer formation in the plasma membrane leading to autophosphorylation of the receptor. The activated receptor kinase subsequently phosphorylates and activates multiple cytoplasmic effector molecules in pathways involved in apoptosis, proliferation, and differentiation of haematopoietic cells in bone marrow. Mutations that result in the constitutive activation of this receptor result in acute myeloid leukaemia and acute lymphoblastic leukaemia (FLT3 ..., 2012).

The c-Mpl protein, the myeloproliferative leukaemia virus oncogene, also known as CD110, is a receptor for thrombopoietin therefore it plays an important role in haematopoiesis and platelet formation (MPL ..., 2012).

The CD150 protein also known as signaling lymphocyte activation molecule (SLAM) is important while subpopulations of haematopoietic stem cells expressing CD150 or not expressing can contribute to long-term multilineage reconstitution, it allows us to distinguish between My-bi HSCs and Ly-bi HSCs, as the My-bi HSCs express higher levels of CD150 (Challen et al., 2010; Beerman et al., 2010; Morita et al., 2010). These two subpopulations are functionally distinct, with respect to lineage output as proliferative status, the My-bi HSCs produce more myeloid progenitors and the Ly-bi HSCs more lymphoid progenitors (Weksberg et al., 2008).

The CD133, also known as prominin-1, is usually expressed on HSCs and haematopoietic progenitor cells (Weigman et al., 1997; Yin et al., 1997). Later it was discovered that the most primitive HSCs analyzable *in vitro*, are highly enriched for CD133⁺ cells (Matsumoto et al., 2000). In addition, CD133⁺ cells in the quiescent phase of the cell cycle have a phenotype consistent with HSCs and have high repopulating activity (Boxall et al., 2009; Drake et al., 2011). The CD133⁺ cells appear to be ancestral to CD34⁺ cells, as CD34⁺ cells can be generated *in vitro* from CD133⁺CD34⁻ cells (Summers et al., 2004). Bissels et al. report that this differences between populations are due to the differentially expressed miRNAs, which are involved in inhibition of differentiation, prevention of apoptosis and cytoskeletal remodeling (Bissels et al., 2011)

2.2.5 Megakaryopoiesis – development of thrombocytes

Mature blood cells are short-lived and must be constantly replaced. A small number of multipotent HSCs are the source of this regenerative process. Megakaryopoiesis is the process by which an undifferentiated haematopoietic progenitor cell undergoes terminal differentiation to yield the highly differentiated, polyploid megakaryocytes and later platelets. The whole process takes approximately ten days, after the differentiation a platelet's life span is also around ten days. That cell's main function is the formation of blood clots in response to vascular injury. The differentiation is regulated by extracellular signals, such as cytokines and growth factors and their receptors (stem cell factor (SCF), Flt3 ligand, IL-3, thrombopoietin, angiopoietin-1, etc.). It takes place in the bone marrow (Tavassoli, 1980, cited by Kaushansky, 2008). In this way the platelet number is maintained constant between $140-400 \times 10^9/L$ of peripheral blood.

Platelets were first observed in 1841 by Addison as "extremely minute granules in blood" (Addison, 1841, cited by Kaushansky, 2008) and were called platelets by Bizzozero, who observed their "increased stickiness after a vessel wall is damaged" (Bizzozero, 1882, cited

by Kaushansky, 2008). In 1890, Howell coined the term megakaryopoiesis and in 1906, James Homer Wright suggested that the platelets are driven from the cytoplasm of megakaryocytes (Wright, 1906, cited by Kaushansky, 2008). This is how the basic elements of megakaryopoiesis were established.

The differentiation towards megakaryocytes and other haematopoietic cell lineages starts with a LT-HSC, which by definition should be a pluripotent stem cell (Golde, 1991). This LT-HSC give rise to ST-HSCs, which differentiates towards committed stem cells: to multipotent progenitors (MPP), myeloid/erythroid progenitor (MEP) and after to the megakaryocyte progenitors (MkP), as shown in Figure 2. The MkP cells undergo 1-8 cell divisions and are removed after 5 days from platelet production (Metcalf et al., 1975; Williams and Jackson, 1978). After that they are giving rise to promegakaryoblasts. A megakaryocyte becomes mature in a process called endomitosis, which involves repeated rounds of DNA replication without cell division (Ravid et al., 2002, cited by Raslova et al., 2003). With this phenomenon megakaryocytes can accumulate DNA content approximately up to 64N, which results in a greatly increased size and protein production (Raslova et al., 2003). The changes are associated with the development of long extensions and cytoplasm demarcation, from which platelets are eventually shed, leaving the megakaryocyte nuclei behind (Cramer et al., 1997). The long extension cells are called proplatelets. In 2007, Junt et al. stated that proplatelets are extended into the blood vessel by the megakaryocytes in the mouse bone marrow and the shear stress of the blood flow causes in the fragmentation of these proplatelets to release platelets (Junt et al., 2007). The main cytokine, which is leading the differentiation towards megakaryocytes is thrombopoietin (TPO) (Kelemen et al., 1958, cited by Kaushansky, 2008).

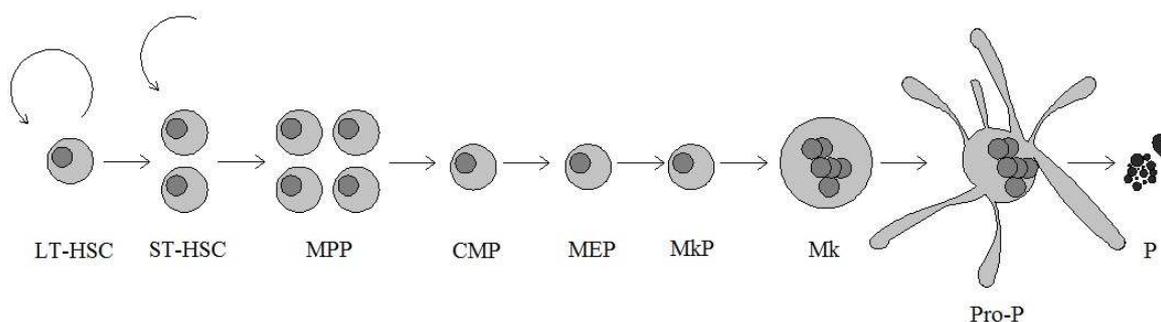


Figure 2: Megakaryopoiesis: LT-HSC, long-term haematopoietic stem cell; ST-HSC, short-term haematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte/erythrocyte progenitor; MkP, megakaryocyte progenitor; Mk, megakaryocyte, Pro-P, proplatelet; P, platelet

2.2.6 Effects of thrombopoietin on haematopoietic stem cells

The life span of blood cells is limited. That is why the process of cell formation and balance keeping is extremely important for a healthy organism. The main actors in homeostasis maintenance are different circulating cytokine signals as well as paracrine cytokine signals from the bone marrow niche, such as SCF, Flt3 ligand, IL-3, thrombopoietin, angiopoietin-1 and erythropoietin. Thrombopoietin is the primary growth factor in the process of platelet formation (Kelemen et al., 1958, cited by Kaushansky, 2008). *In vivo* it results in an increase of megakaryocytes and their progenitors in the bone marrow and in increased number of platelets in the blood (Archimbaud et al., 1996; Vandhan-Raj et al., 1996; Molineux et al., 1996). *In vitro* it leads the differentiation from haematopoietic progenitors towards megakaryocytes. It has an important role in the survival and expansion of HSCs (Fox et al., 2002). In humans, the insufficient level of thrombopoietin causes thrombocytopenia, a disease, which results in a disability to form blood clots, therefore it causes bleeding (Kato et al., 1998).

Research activities on thrombopoietin topic started in the 1950s, when Kelemen stated that TPO regulates the number of platelets in the blood. Until the 1990's, there were just few publications, because of the lack or expensiveness of assays for TPO detection and due to the failure to purify and clone TPO (McDonald, 1992). However, the conundrum of TPO started to get an end with an unrelated discovery. In 1986 a breakthrough was made with the characterization of the murine myeloproliferative leukemia virus, which causes an acute myeloproliferative syndrome in infected mice, in other words it has a capability of immortalizing bone marrow haematopoietic cells from different lineages (Wendling et al., 1986). Four years later, the responsible oncogene, *v-mpl*, was cloned (Souyri et al., 1990), and two years later, its protooncogene, *c-Mpl*, was obtained (Vigon et al., 1992). It was shown that *v-mpl* gene was a member of the haematopoietic cytokine receptor family (Cosman, 1993). First, it was cloned from human erythroleukemia cells, a leukemia cell line that also shows characteristics of megakaryocyte differentiation (Long et al., 1990). In addition, c-Mpl receptor is expressed on megakaryocytes and their progeny as well as on their precursors (Vigon et al., 1992). Moreover, when *c-Mpl* expression was eliminated in HSCs, the megakaryocyte production stopped (Methia et al., 1993). After those findings, five groups cloned the ligand of c-Mpl receptor, the Mpl ligand, which is in fact thrombopoietin (Lok et al., 1994).

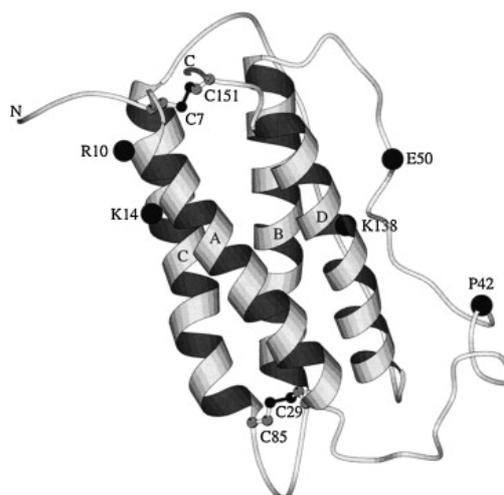


Figure 3: The 3D predicted model of NH₂-terminal domain of thrombopoietin, showing the functionally important residues (Tarasova et al., 2011: 237)

The human *TPO* gene sequence is 6,2 kb long and contains seven exons and five introns (Chang et al., 1995, cited by Kato et al., 1998). Interestingly, in addition, there is just a single copy of the *TPO* in human genome indicated by the Southern blot analysis (Gurney et al., 1995, cited by Kato et al., 1998). The encoded human TPO includes 353 amino acids; however, the entire protein is not directly essential for biological activity, similar to other truncated TPO molecules isolated from animals. The c-Mpl, the TPO receptor, binds the amino (NH₂) terminal half of the molecule (shown in Figure 3), which encloses an erythropoietin-like domain and biological activity. It is build up from two domains separated by a pair of arginine residues that may be a proteolytic cleavage site: the amino terminal region exhibiting sequence homology to erythropoietin and the carboxy terminal region containing multiple potential N-linked glycosylation sites (Kato et al., 1998).

Thrombopoietin is produced in liver and kidney (Nomura et al., 1997, cited by Kato et al., 1998; Sungaran et al., 1997, cited by Kato et al., 1998). It can also be found in spleen and in the hippocampus of the brain (Sungaran et al., 1997; cited by Kato et al., 1998) This cytokine is known to be the main regulator of the differentiation towards platelets, in maintaining the pool of transplantable HSCs, moreover establishing the responsiveness of primitive cells to itself and effecting expansion (Schipper, et al., 1998).

Thrombopoietin appeared to be a good single growth factor for expansion of megakaryocytic lines from the umbilical cord blood in liquid cultures. Schipper and colleagues showed that it has a good differentiation and expansion effect on immature HSCs isolated from UCB. They also observed that the megakaryocytes had at most four times ploidy, but not more (Schipper et al., 1998). In addition, transplantation of thrombopoietin expanded cells from the bone marrow not only induced platelet recovery but also the reconstitution of erythrocytes in mice (Fibbe et al., 1995). Furthermore, Mattia et al. showed that TPO does not threaten the maintenance of the progenitor cell population

when cord blood stem cells are cultured for 7 days with thrombopoietin. Because of this feature it is also ideal for clinical application with clinical grade, serum-free culture (Mattia et al., 2008). Furthermore, when stem cells are cultured with thrombopoietin in a concentration range of 10-100 ng/ml, it does not affect the long-term engraftment capability (Duchez et al., 2011).

2.3 UMBILICAL CORD BLOOD

The umbilical cord blood (UCB) is the fetal blood, derived from placenta, which can be yielded from umbilical vessel after birth giving. The average blood volume is approximately 70-150 ml (McGukin et al., 2007). Since this blood is a part of fetal tissues, it displays all fetal characteristics. In addition, it contains HSCs, which can be used to treat haematopoietic and genetic disorders. Generally, there is on huge disadvantage of UCB: there are not enough SCs in one unit of umbilical cord blood to treat an adult patient; however, it contains significantly higher number of early and committed progenitor cells when compared to adult peripheral blood or BM-derived HSCs (Broxmeyer et al., 1990; Cairo and Wagner, 1997).

2.3.1 Haematopoietic transplantations

The first HSC transplantations were cells isolated from bone marrow, the transplantations were allogenic and they aimed to reconstitute the haematopoietic system of humans irradiated in nuclear accident (Mathe et al., 1959, cited by Ivanović, 2010).

Once umbilical cord blood was considered a biological waste, but today it is used as a precious source of HSCs (Gluckman, 2009). The first groups report about the use of UCB HSCs was of Ende in 1972 (Ende and Ende, 1972, cited by Ali and Bahbahani, 2010). Since the first cord blood transplantation in 1988 (Gluckman et al., 1989; Broxmeyer, 2009), when an HLA-matched female sibling donor gave the UCB HSCs to treat her brother, related and unrelated stem cell transplantations are frequently used. Usually they are an alternative source of bone marrow derived HSCs for the patients lacking an HLA-matched donor. Recent studies reported about success of therapies using HSCs from UCB in leukaemia and cancer (Broxmeyer, 2010), Fanconi anaemia (Gluckman et al., 2007, cited by Broxmeyer, 2010), severe plastic anaemia (Yoshimi et al., 2008, cited by Broxmeyer, 2010), myelodysplastic syndrome (Parikh et al., 2009, cited by Broxmeyer, 2010) and other disorders (Prasad et al., 2008, cited by Broxmeyer, 2010). As previously noted, in an average unit of CB, there are not enough HSCs to treat an adult patient. Consequently, the platelet recovery is delayed, which can lead to transplant failure. However, from 2005 on, UCB HSC transplants are carried out in half of the cases to adults (Sullivan, 2008).

Cord blood derived stem cells are often used because of many advantages they have, such as (1) rapid availability in median 25-36 days earlier than unrelated bone marrow graft, (2) lower HLA matching requirement (four out of six HLA-loci needs to be identical), (3) absence of risk for the donor, (4) lower risk of transmitting diseases, such as cytomegalovirus and Epstein-Barr virus, (5) decreased incidence of acute graft-versus-host disease (GvHD) (Rocha et al., 2010), (6) higher frequency of primitive haematopoietic progenitor cells (Holyoake et al., 1999) and (7) longer survival in culture (Tanavde et al., 2002). In comparison with the bone marrow or the mobilized peripheral blood (derived with help of cytokines), the umbilical cord blood transplants contain more primitive progenitor cells and early committed progenitors (Holyoake et al., 1999). However, the biggest concern about UCB transplants is due to a low number of HSCs in a average UCB unit (around 100 ml) as compared to an average 500 ml unit of BM-derived HSC units, which results in delayed engraftment in adults, in particular platelets (Schipper et al., 2003).

The main requirement for unrelated cord blood transplantations was the establishment of public UCB banks, which can store UCB units with defined HLA type. The number of such banks increased in the past two decades, allowing high numbers of transplantations and different HLA typed UCB units to adequately serve ethnically diverse donors worldwide (Broxmeyer, 2010). The first blood banks were established in New York, Milan and Düsseldorf. Dr. Pablo Rubinstein of the New York Blood Center and his colleagues established the first protocols for collecting, processing and freezing UCB samples (Rubinstein et al., 1995). Today we are able to keep them viable for at least 23, 5 years, but possibly much longer (Broxmeyer et al., 2011). Currently, more than 500.000 stored UCB units are available worldwide (Bone marrow donors worldwide, 2012)

The success of the transplantation correlates with higher number of cells infused and younger age of the patient (Uchida et al., 1998; Michel et al., 2003). For adults the number of transplanted cells was shown to be optimal in concentration $\geq 3,7 \times 10^7$ total nucleated cell (TNC)/kg (Gluckman et al., 1997, cited by Rocha and Weissman, 2010), although Laughling et al. and Rocha et al. reported success from lower numbers of cells infused, such as $2,3 \times 10^7$ TNC/kg (Laughlin et al., 2004; Rocha et al., 2004). We have to mention that the TNC count includes polymorphonuclear cells, monocytes and lymphocytes, which do not provide haematopoietic reconstitution after transplantation. Indeed, in most papers, the TNC correlates with engraftment outcome, kinetics and total cell number in the graft (Smith and Sweetenham, 1995). This correlation results from the fact that the concentration of HSCs after standard therapeutic protocols is more or less constant (Ivanović, 2010). The HLA mismatch between the donor and the recipient is allowed at most 2 loci out of six (HLA-A, -B, -C, -DRB, -DQB, -DPB) (Kurtzberg et al., 1996; Rubinstein et al., 1998).

In addition, the first rate parameters in clinical transplantation in haematology is the CD34 positive cell dosage. If this cell population would contain constant proportion of stem and progenitor cells, the usage of it would not be questionable. Experiences show that the percentage of HSCs in a product varies with respect to the source, pathology, treatment, processing procedure, the *ex vivo* treatment of the graft and so on. Therefore, an universal dose of CD34⁺ cells can't be defined (Ivanović, 2010).

For the bone marrow transplants, the cells in the BM unit remain unmanipulated due to their sufficient number of HSCs and progenitor cells (Rogers and Casper, 2004). Due to the small number of stem cells in one UCB product, expansion with growth factors is sometimes indicated (Schipper et al., 1998; Siddiqui et al., 2011). On the other hand, Salles et al. discovered that pooling the cord blood samples and raising the number of cells transplanted results in a rapid platelet production, without *in vitro* expansion (Salles et al., 2009).

The outcome of UCB and other forms of transplants is very important. To determine the effectiveness of the procedure, various parameters are in use, such as: transplant related mortality, overall survival of the transplanted cells, period until engraftment in days, etc. The engraftment is usually defined as a period in days needed for blood cell count to reach certain level (for instance $50 \times 10^9/L$ platelets or neutrophils or $20 \times 10^9/L$ platelets). However, in adult patients the greatest concern is represented by platelet recovery, which is not satisfactory yet. Therefore the patients can bleed out or die because of different complications, or it can lead to a longer hospital stay (Rocha et al., 2000, cited by Rocha and Broxmeyer, 2010). Consequently, this became the main question of our research: how can we increase the success of platelet recovery. Until now, the best platelet recovery was reached in 48 days ($50 \times 10^9/L$) by Takahashi and colleagues in 2004 (Takahashi et al., 2004).

2.3.2 Strategies to optimize cord blood transplantation

As mentioned before, there usually are not enough HSCs in one unit of UCB; therefore, the engraftment is delayed, in particular in platelets. A good engraftment requires that donor HSCs migrate to the BM, repopulate it and start to self renew, proliferate, survive and differentiate. Towards this end, the stem cell niches are really important, because they are leading all processes listed above. Because UCB HSCs have many advantages over the BM derived ones, many approaches to improve their engraftment have been investigated (McKenna and Brunstein, 2011), as shown in Table 2. In general there are four different approaches to improve platelet recovery after UCB transplantations: (1) to enhance the collection yield of CB cells, (2) to enhance the homing capacity of HSCs (Christopherson et al., 2004, cited by Rocha et al., 2010); (3) to enhance the *ex vivo* or *in vivo* expansion of

these cells (Rocha and Broxmeyer, 2010) and (4) non-mieloablative conditioning of the patient (Del Toro et al., 2004).

Table 2: The problems which occur during UCB transplantation and the strategies for their solution (Rožman et al., submitted for publication)

| Problem | Strategy for the solution |
|---------------------|--|
| Delayed engraftment | <ul style="list-style-type: none"> • double or multiple UCB transplantation • expansion of CD34+ progenitor cells • non-mieloablative conditioning of the patient • synchronous infusion of UCB and haploidentical HSC from the peripheral blood • transplantation of UCB plus MSC • intra-bone transplantation |
| Infections | <ul style="list-style-type: none"> • aggressive and early therapies • potential use pathogene-specific T-cells |
| Relapse | <ul style="list-style-type: none"> • transplantation of 2 UCB units in acute leukaemia patients • adoptive immunotherapy: <ul style="list-style-type: none"> - use of UCB-derived T-cells expressing the hymeric antigen receptors as malignancy targets of B-cells - use of UBC-derived and <i>ex-vivo</i> expanded NK-cells - use of NK-cellular therapy as adjunct to the triple UCB transplants - DLI (»donor lymphocyte infusion«) from haploidentical donors as an addition to the haploidentical UCB transplantation |

2.3.2.1 Strategies to optimize collection yield

To improve the transplantation, as many cells as possible should be obtained from the cord itself (Broxmeyer et al., 2009). For instance, perfusion of placental vessels can give twice as much SCs than just removing the blood from the cord (Broxmeyer et al., 2009). On the other hand, the disadvantage of this technique is that it is not practical and requires lot of well-trained personnel (Rocha and Broxmeyer, 2010).

2.3.2.2 Strategies to optimize homing capacity

There are many approaches to achieve better homing and engrafting capacity of the infused cells. As mentioned before, the SDF-1/CXCR4 axis plays an essential role in the *in vitro* chemotaxis and *in vivo* homing of HSCs in the bone marrow microenvironment. The SDF-1 cytokine leads the homing of HSCs via its receptor CXCR4. HSCs respond to the SDF-1 chemotactic gradient of the bone marrow, produced by the stromal cells. When the HSCs are infused to a patient, they transmigrates trough the basal membrane and home in their niche, where they can survive, expand, proliferate, and engraft. However, the SDF-1 can

be inhibited with the CD26/Dipeptidylpeptidase IV (DPPIV). It is an enzyme which binds instead of SDF-1 to CXCR4 receptor. Therefore, when the SDF-1 is inactivated, the homing of the HSCs is inhibited as well. The type of the cell plays a significant role, which will engraft. For a successful, long-term engraftment it should be the LT-HSCs. Furthermore, the CD26/DPPIV activity can be inhibited with small peptides such as Diprotin A or Val-Pyr (Campbell et al., 2007, cited by Rocha et al., 2010). However, the responsiveness of HSCs to an SDF-1 gradient can also be enhanced by several other factors, such as C3 complement cleavage fragments, fibronectin, fibrinogen and hyaluronic acid (Reca et al., 2003).

There are also some other approaches to enhance homing capacity. Firstly, the number of total nucleated cells needed for transplantation is lower when adding certain graft versus host disease prophylaxis substances such as fludarabine (Nabhan et al., 2008). Conversely, the use of methotrexate as a graft versus host disease prophylaxis results in delayed engraftment and increased risk for graft failure (Locatelli et al., 2003).

Secondly, the lack of enough stem cells from one cord product for an adult patient brought doctors to the idea to use two UCB units. This is how the double cord transplantation started to be used for adult patients. Both of them should be partially HLA matched. Data showed that usually just one of them is engrafting, the non-engrafted has a booster effect. This kind of transplants has significantly better results than single cord transplantations (Barker et al., 2005, cited by Rocha et al., 2010; Verneris et al., 2009).

Thirdly, in order to avoid using double cords due to the lack of high number of stem cells, the direct intrabone marrow injection of UCB isolated cells into the patient's long bones was introduced. With this approach, the necessary cell dosage is smaller, it is between 1, 5 and 2, 5×10^7 TNC/kg. It seems that this approach significantly reduces the problem of delayed platelet recovery observed after cord blood injected intravenously (Frassoni et al., 2008, cited by Rocha et al., 2010).

Finally, the homing can be improved by cotransplantation with other types of cells. One of the possibilities is cotransplantation with highly purified CD34⁺ cells from haploidentical family donors, so called third-party donor cells (Fernandez et al., 2003). These can be also T-cell depleted (Bautista et al., 2009). The other investigated possibility is to use haploidentical parental multipotent mesenchymal stromal cells (Macmillan et al., 2009).

2.3.2.3 Strategies to enhance expansion

The number of stem cells infused is, apart from the HLA matching, one of the most important parameters of engraftment success. We can infuse an increased number of SCs with *ex vivo* expansions, or by adding cytokines or growth factors into the medium we can

accomplish differentiation. To achieve the latter, there are several different ways: with Notch ligands, cytokines and chopper chelators (TEPA) (Delaney et al., 2010a). However, the biggest concern with *ex vivo* expansion is the risk of exhaustion of HSC's long-term engraftment capacity (Schipper et al., 2012).

Firstly, on human HSCs the *Notch 1* gene was found to be expressed. It was cleared that the haematopoietic stem cells have all four Notch receptors (Notch-1, 2, 3, 4) (Kojika and Griffin, 2001), on which Delta-1, Jagged-1 and Jagged-2 ligands can bind. Furthermore, it was shown that when ligands bind to those receptors, the differentiation is inhibited, but the self renew is enhanced. This fact led to the idea to use these Notch ligands in *ex vivo* cultures to expand undifferentiated cells with success (Delaney et al., 2005; Hoffmeister et al., 2007). Lately, Delaney and colleagues demonstrated rapid although temporary engraftment of *ex vivo* expanded CD34⁺ UCB cells with Notch ligands in patients. This was due to exhaustion of HSC's long-term engraftment capacity (Delaney et al., 2010b).

Secondly, many cytokines have been used in *ex vivo* expansions, such as erythropoietin, thrombopoietin (TPO), colony-stimulating factors (G-SCF, GM-SCF), Flt3 ligand, SCF, IL-6, IL-10, etc. (Mattia et al., 2008; McNiece and Bridgel, 2001). All of them are giving promising results alone as well as in combinations. As their result, the SCs and progenitors expand and differentiate during culture.

For instance, Jo-Anna Reems and colleagues tried to deal with the problem of platelet lacking with an idea to generate platelets from SC *in vitro*. They had some success, they decided that the best choice were SCs and progenitors from UCB (enriched for CD34⁺ cells). With cord blood serum they could achieve expansion in a medium with TPO, IL-6, SCF and fsm-like tyrosine-kinase 3 ligand. In a three step culture strategy, they gained approximately 20 units of platelet concentrates from a single UCB unit. However, they have not reached their aim yet: to establish an industrial production of platelets, but it seems to be just a matter of time (Reems, 2011).

Schipper and colleagues on the other hand tried to solve this problem from another point of view. They and others proved that partial differentiation towards platelets *in vitro*, improves platelet recovery (Bruno et al., 2004; Hensbergen et al., 2006; Mattia et al., 2008), and therefore they tried to improve the engraftment with culturing CD34⁺ UCB cells with TPO, resulting in differentiated and expanded megakaryocytic lineage cells (Schipper et al., 2012). The delay in platelet production in a NOD/SCID mouse after transplantation of the cultured cells was shorter, without losing engraftment and multilineage engraftment in the BM (Hensbergen, 2006). Mattia et al. showed similar results; just the cells were cultured with a cocktail of cytokines (Mattia et al., 2008). After 10 days of culturing the CD34⁺ cells with TPO, there are three populations generated: a huge population of CD34⁻CD61⁺ cells (CD61⁺ cells), a substantial number of CD34⁻CD61⁻

cells (Lin^- cells) and a small amount of $\text{CD34}^+\text{CD61}^-$ cells, the remaining CD34^+ cells (rCD34^+). It cleared out that the engraftment is due to the rCD34^+ and Lin^- cells after culture and the cells, which expressed CD61, a marker usually expressed on megakaryocytes, haven't contribute to engraftment (Schipper et al., 2012). In brief, Schipper et al. showed that not only the number of obtained cells after expansion is crucial for the outcome of the transplantation but also the composition of the expanded population. Their results are promising but need further investigation as well (Schipper et al., 2012). However, the approach should be due to the results and goals we would like to achieve after transplantation (short-term engraftment, long term engraftment).

Thirdly, chopper chelators are involved in the regulation of haematopoietic progenitor cell proliferation and differentiation. For instance, Cu chelators, like tetraethylenepentamine (TEPA), cause delayed differentiation at concentrations that moderately lower cellular Cu. Moreover, external addition of Cu salts or ceruloplasmins results in accelerated cell differentiation (Peled et al., 2002). Peled and colleagues showed that a 3-week treatment with TEPA results in enrichment of a progenitor cell subpopulation derived from the cord blood and in enhanced capacity to repopulate NOD/SCID mice (Peled et al., 2004).

2.3.2.4 Non-myeloablative conditioning of the patient

Usually, patients, who receive HSC transplants, receive chemotherapy and irradiation as basic treatment. Consequently, the cancer cells and beside them the immune system and bone marrow cells get destroyed too. Therefore the immune system cannot reject the allogenic transplant, it engrafts without problems (Rožman et al., submitted for publication). This procedure is called myeloablative conditioning, but is very aggressive and has many side effects. This is the reason that many of the patients do not tolerate it. For them, usually the reduced intensity conditioning (RIC) is in use, the so-called non-myeloablative conditioning. This approach is useful for patients with certain nonmalignant diseases or chronic malignant diseases, which were treated with chemotherapy before. After this procedure, the GvHD occurrence is comparative to non-related bone marrow transplants, the mortality before 100 days after transplantation is relatively low, and relapses are more frequent after acute leukaemia as after myeloablative treatments. The RIC method is successful in treatment with children (Del Toro et al., 2004).

3 MATERIALS AND METHODS

3.1 MATERIALS

The umbilical cord blood was collected from the umbilical vein from 10 newborns, more than 36 weeks of gestation, with a signed approval by the mother. The procedure protocol was approved by the LUMC Medical Ethical Committee.

The NIH3T3 feeder cells were a kind gift from IHB, LUMC.

All of the used chemicals (Table 3), cell culture vessels (Table 4), medias (Table 5) and instruments (Table 6) are given in following tables.

Table 3: Used chemicals

| Abbreviation | Whole name | Manufacturer | Code |
|--------------------------------------|---|---|-------------|
| Albumin 20% | Albumin 20% | Sanquin Blood Bank | H163NED |
| PKH26 | PKH26 Red fluorescent Cell Linker Kit for General Cell Membrane Labelling | Sigma-Aldrich | PKH26GL |
| IMDM | Iscove's mod. Dulbecco's medium | Gibco, Invitrogen corporation | 21980 |
| TPO | Thrombopoietin | kind gift from IHB/LUMC | |
| PBS | Phosphate buffered saline | Pharmacy LUMC/ Chempropack BV | |
| DNase | DNase | Calbiochem | |
| Alcohol 70% + 5% isopropylalcohol | Alcohol 70% + 5% isopropylalcohol | Fresenius Kabi Nederland BV | |
| Pen/Strep | Penicillin/Streptomycin solutions | Gibco, Invitrogen corporation | 15140 |
| Heparine natrium | Heparine natrium | Klinische Farmacie en Toxicologie LUMC | 908932 |
| Ficoll-Amidotrizoate | Ficoll-Amidotrizoate | Klinische Farmacie en Toxicologie LUMC | 902861 |
| EDTA | Ethylenediaminetetraacetic acid disodium salt solution, 0.5 M | Sigma-Aldrich | 139-33-3 |
| FcR blocking reagent | FcR blocking reagent, human | MACS Milteny Biotec | 130-059-901 |

to be continued

continuation

| Abbreviation | Whole name | Manufacturer | Code |
|---------------------------|---|--|-------------|
| CD34 MicroBead Kit, human | CD34 MicroBead Kit, human | MACS Milteny Biotec | 130-046-703 |
| DMSO | Dimethylsulfoxide | Klinische Farmacie en Toxicologie LUMC | 925322 |
| CD45-FITC | anti-CD45-FITC | Beckman Coulter | A07782 |
| CD34-PE | anti-CD34-PE | Beckman Coulter | A07776 |
| CD34-PC5 | anti-CD34-PC5 | Beckman Coulter | A07777 |
| CD9-FITC | anti-CD9-FITC | Beckman Coulter | IM1755U |
| CD61-PC7 | anti-CD61-PC7 | Beckman Coulter | PN IM3716 |
| CD38-FITC | anti-CD38-FITC | Beckman Coulter | A07778 |
| Lysing solution | IO Test 3 lysing solution | Beckman Coulter | A07799 |
| 7-AAD viability dye | 7-AAD viability dye | Beckman Coulter | A07704 |
| Trypsin | Trypsin | Invitrogen | |
| Gelatine | Gelatine | Sigma-Aldrich | |
| ALDH Assay Kit | ALDHFLUOR Assay Kit | Stemcell Technologies | 1700 |
| FBS | Fetal Bovine Serum | Gibco, Invitrogen corporation | 26140 |
| HS | Horse serum | Invitrogen | 16050122 |
| L-Glutamine | L-Glutamine | Sigma-Aldrich | |
| Hydrocortisone | Hydrocortisone | Sigma-Aldrich | H2882 |
| □-mercapto-ethanol | □-mercapto-ethanol | Sigma-Aldrich | |
| Transferin | Human transferin saturated with FeCl ₃ *H ₂ O | Sigma-Aldrich | T-2036 |
| DMEM | Dulbecco's modified eagle medium | Gibco, Invitrogen corporation | 32430 |
| H ₂ O | distilled water | Fresenius Kabi | |

Table 4: Laboratory vessels used

| Laboratory vessels | Manufacturer | Code |
|-----------------------------------|----------------------------|-------------|
| 15 ml tubes | Cellstar, Greiner bio one | 188261 |
| 50 ml Leucosep® tubes with filter | Cellstar, Greiner bio one | 227261 |
| 4 ml FACS-tubes | Grenier | 2523749 |
| 15 ml FACS- tubes | Falcon | |
| 24-well plate | Grenier bio one | 662160 |
| 50 ml tube | Cellstar, Greiner bio one | 227261 |
| 96-well plate, flat bottom | Cellstar, Greiner bio one | 655180 |
| Clip lid tube | Greiner bio one | 115163 |
| 3 cm Petri dish | Greiner bio one | 627160 |
| 250 ml flasks | Greiner bio one | 658175 |
| Mini-MAC columns | Milteny Biotec | 130-042-201 |
| Pre-filter | Milteny Biotec | 130-041-407 |
| Mr. Frosty | Nalgene | |
| Blood bags with 25 ml CPDA-1 | Composelect Fresenius Kabi | |

Table 5: Used medias

| Medium name | Composition |
|--|---|
| MEGACULT - Megakaryocyte culture media | 40 ml IMDM 10 ml Citrate plasma 0,5 ml Pen/Strep 1,25 ml Heparin-sodium 0,5 mg/mL human transferin saturated with FeCl ₃ *H ₂ O 850 µl Human serum albumin 0,05 mM β-mercapto-ethanol 0,165 mg/ml CaCl ₂ solution |
| NIH3 media | 89% DMEM 10% inactivated FCS 1% Pen/Strep |
| CAFC media | 88,86% IMDM 3,16% inactivated FCS 3,16% inactivated HS 1,27% glutamine 3,36% Pen/Strep 0,16% Hydrocortisone 0,01% β-mercapto-ethanol |
| Running buffer | 500 ml PBS 2 mM EDTA 0,5% Albumine 20% |
| Thawing media | 92,5% Basic medium for thawing 5% Albumin 2,5% Heparin |
| Basic media for thawing | 96,5% IMDM 1% Pen/Strep 2,5% Heparin |
| Wash media for freezing | 98% IMDM 2% (v/v) Albumin 20% |
| Freezing media | 60% IMDM 2% (v/v) Albumin 20% 20% DMSO |
| Gelatine | 99% Gelatine 1% Pen/Strep |

Table 6: Instruments used

| Name of equipment | Manufacturer |
|--|-------------------------------|
| Pipettes 0,1-2 µl; 2-20 µl; 20-200 µl; 100-1000 µl | Eppendorf |
| Pipette boy | Jencons |
| Refrigerator | |
| Freezer | |
| Aspiration pump | |
| Liquid nitrogen freezer | |
| Multichannel pipette 100-200 µl | Eppendorf |
| Laminar flow chamber interflow | Interflow |
| Inverted Microscope Axiovert 25 CFL | Zeiss |
| Avtoclave MLS 3020U | Sanyo |
| Balance, BL 3100 | Sartorius |
| Water bath incubator PN 2616300 | Hach |
| Incubator | Thermo Scientific |
| Centrifuge Rotina 420R | Hettich |
| Vortex, MS2 minishaker | Omnilabo |
| Flow cytometer CYTOMICS FC 500 | Beckman Coulter |
| FACS sorter, Aria | Becton, Dickinson and Company |
| Magnetic mixer MS-H-S | Scilogex |
| pH meter CB70P | sympHony |
| COULTER Ac T | Beckman Coulter |

3.2 METHODS FOR CD34⁺ ISOLATION FROM UMBILICAL CORD BLOOD

Working with cord blood requires aseptic work conditions, so every reagent had to be sterile, all the work was taking place in a laminar flow chamber (Interflow), except for the flow cytometer measurements; everything that came in the laminar flow chamber had to be sprayed with 70% alcohol.

3.2.1 Collection of umbilical cord blood

The umbilical cord blood was collected from the umbilical vein from 10 newborns, more than 36 weeks of gestation, with a signed approval by the mother. The procedure protocol was approved by the LUMC Medical Ethical Committee. Blood was collected into Macopharma collection bags with gravity drainage. The bags contained 25 ml citrate phosphate dextrose adenine-1 (CPDA-1) preservative solution. Before processing, the blood was stored at 4°C not for more than 48 hours.

The traceability of the samples and information connected with it has an essential role. Therefore, we made some measurements before processing the cord blood: weight of the bag, blood volume and CD34⁺ cell concentration to determine the success of the isolation. We stored 4 ml of plasma from the mother blood of each sample, so we could run some toxicity tests if someone got injured and exposed to the blood.

3.2.2 Mononuclear cell isolation

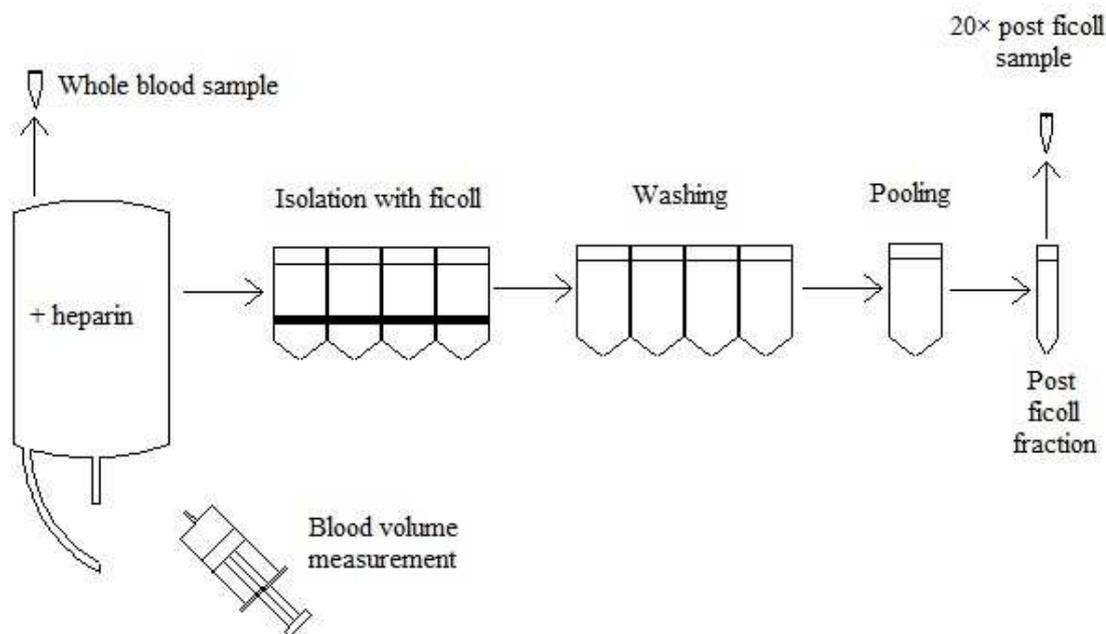


Figure 4: Mononuclear cell isolation

The mononucleated cells are the cells with one nucleus. They consist of monocytes, lymphocytes, usually contain some remaining platelets and of course haematopoietic stem cells. The isolation of mononucleated cells was performed with use of sterile ficoll density gradient (1,079 g/cm³) and 50 ml leucosep tubes with filter, as shown in Figure 4. The ficoll has higher density as the buffy coat, which contains mononucleated cells that is why the mononucleated cells are above the filter, whereas the red erythrocytes and the granulocytes stay under it, as shown in Figure 5.

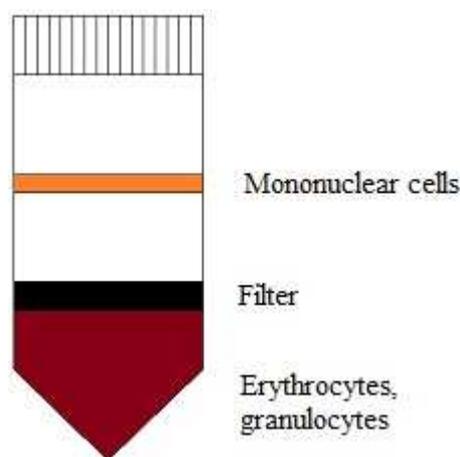


Figure 5: A tube with ficoll and whole blood after spinning

To 50 ml leucosep tubes with filter, we added 16 ml ficoll (4°C). We prepared one leucosep tube for 15-20 ml UCB. In order to bring the ficoll under the filter, we span the tubes for 1 minute on 350 G, built 9, brake 3 at 4°C. In between, we put the adaptor on the blood bag and added heparine with a small syringe. We calculated the volume of the heparine as follows:

$$V_{\text{heparine}} = \frac{m_{\text{bag+blood}} - m_{\text{bag}}}{40} \quad \dots$$

(1)

With the same syringe we took 200 µl sample for ACT counting (it is a blood analyzer, which counts the number of erythrocytes, platelets, white blood cells and other parameters; we counted the white blood cells with it – the HSCs are counted as white blood cells; COULTER AcT, Beckman Coulter) and flow cytometry analysis (CYTOMICS FC 500, Beckman Coulter). With a 50 ml syringe we measured the volume of the blood and added 15-20 ml of it to a leucosep tube with ficoll under the filter. Until 50 ml we filled the volume with PBS with 2 mM EDTA. In order to get rid of part of red blood cells and granulocytes, we span the tubes on 1000 G, built 2, brake 0 at 4°C for 20 minutes (Centrifuge Rotina 420R, Hettich). The upper part we put in new 50 ml tubes and rinsed the old ones with PBS with 2 mM EDTA. In every new 50 ml tube we filled the volume up to 50 ml with PBS with 2 mM EDTA. In order to wash the monocytes, we span the cells on 350 G, built 9, brake 3 at 4°C for 10 minutes. The cells were pulled together after removing the supernatant and resuspending the pellet in one 50 ml tube. The volume of the cells was reduced with spinning on 200 G, built 9, brake 0 at 4°C for 10 minutes. After removing the supernatant and resuspending the pellet, we took 10 µl sample and diluted it 20 times for cell counting on the ACT and flow cytometry analysis. This was the post ficoll fraction.

On the flow cytometer we measured the purity with determining the CD34⁺/CD45⁺ rate, which was measured in the whole blood and post ficoll fraction. To 50 µl sample we added 5 µl of CD34-PE and 5 µl CD45-FITC and incubated it for 15 minutes in dark on room temperature. In the whole blood, we had to get rid of the red blood cells with adding 1000 ml 10 times diluted lysing buffer with distilled water for ten minutes. To determine the concentration we used same volume of beads as sample (50 µl), with known concentration and determined the viability by adding 10 µl 7AAD viability marker.

3.2.3 CD34⁺ isolation from the post ficoll fraction

The CD34⁺ cells were isolated with magnetic cell separation using magnetic beads (CD34 MicroBead Kit, human, MACS Milteny Biotec) for CD34⁺ cells, as shown in Figure 6. First we calibrated the cell concentration to 10⁸/300 µl with spinning (350 G, built 9, brake 3, 10 min, 20°C). For the labelling, we added 100 µl Fc blocker and 100 µl CD34⁺ magnetic microbeads to 300 µl cell suspension. The incubation lasted for 30 minutes in dark on 4°C. The unattached beads were washed away with filling the 15 ml tube up to 14 ml with running buffer and spinning it on 350 G, brake 3, built 9, at 4°C for 10 minutes. After the spinning we removed the supernatant and added running buffer up to 500 µl for 10⁸ cells. For the purification we used separation columns, MS if the cell number was lower than 2×10⁸ or LS if the cell number was higher than 2×10⁸. The separation was carried out following the instructions, which were given by the manufacturer (http://www.miltenyibiotec.com/download/datasheets_en/929/MiltenyiBiotec_DataSheet_CD34-MicroBead-Kit,-human_130-046-702.pdf).

On the flow cytometer (CYTOMICS FC 500, Beckman Coulter) we measured the purity of the CD34⁺ cells with determining the CD34⁺/CD45⁺ rate, which was measured in the CD34⁺ and CD34⁻ fractions. To 50 µl sample we added 5 µl of CD34-PE and 5 µl CD45-FITC and incubated it for 15 minutes in dark on room temperature. To know the concentration we used same volume of beads as sample (50 µl) with known concentration. The viability was determined by adding 10 µl 7AAD viability marker. In order to have enough sample, we added 350 µl PBS, than we measured the sample.

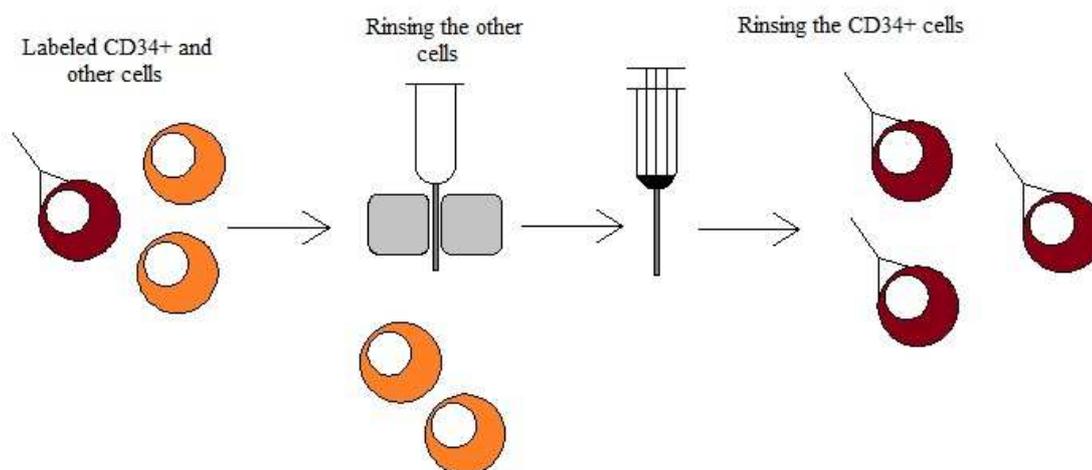


Figure 6: The magnetic separation

3.2.4 Sample freezing

The whole CD34⁺ isolation takes almost a whole day to finish, so the samples had to be stored somehow. Sometimes we had to freeze post ficoll fractions too, since the UCBs arrived later. The samples were stored on -150°C.

The freezing procedure took place on ice, every reagent had to be ice cold, because DMSO protects the cells under 4°C, but above it, the cells get destroyed. Therefore, we cooled the cell suspension and the labelled vials in the refrigerator for at least 15 min. The highest cell count in a vial could be 50×10^6 cells, so we calculated the number of vials needed. One vial is provided for 1000 µl cell suspension; half of it was the cell suspension and the other half the freezing medium with DMSO:

$$V_{cell\ suspension} = V_{freezing\ medium} = \frac{\text{number of vials} \cdot 1000\mu\text{l}}{2} \quad \dots (2)$$

The volume of the cell suspension was reached with adding missing volume of wash medium to the stored fraction (in a 15ml or 50 ml tube); the freezing medium was added in precisely 5 minutes to the cell suspension (in a 15 ml or 50 ml tube). We divided the suspension in the vials (1000µl/vial) and put them in a cooled Mr. Frosty on -80°C for two hours. The Mr. Frosty is filled with isopropyl alcohol, which has a recommended and controlled rate of cooling, 1°C/1 min. After this time we put the frozen cells on -150°C.

3.3 DETERMINATION OF THE POPULATIONS AFTER 10 DAYS OF TPO CULTURE AND UCB ISOLATED HSCS RESPONSIVENESS TO TPO

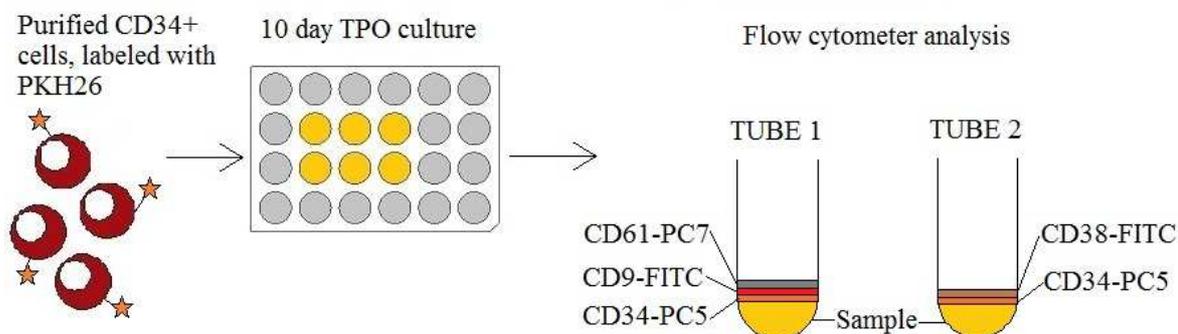


Figure 7: The first (determination of the populations after 10 days of TPO culture) and second (UCB isolated HSCs responsiveness to TPO) experiment

3.3.1 Sample thawing

Usually we had to freeze the samples, unless the isolated HSCs were purified a day before the experiments were planned. Therefore, we had to thaw the cells following the next protocol:

The cells were frozen in DMSO, which protects the cells below 4°C, but above it, the cells get destroyed. Consequently, we prepared boxes with ice to keep the samples on right temperatures while moving them. However, in order to thaw them, we prepared the water baths (PN 2616300, Hach) on 37°C and heated the medias on this temperature as well. The samples were in freezers on -150°C, so we moved them (on ice) to the water bath, and put them there for a minute and half. To dilute the DMSO we followed the procedure in Table 7.

Table 7: The thawing procedure

| Time (min) | Procedure |
|------------|--|
| 0 | We transferred the cells to the 50 ml tube |
| 1 | The vials were rinsed with 200 µl thawing medium/vial and we transferred the cell suspension to the 50 ml tube |
| 2 | We added 200 µl thawing medium/vial |
| 3 | We added 200 µl thawing medium/vial |
| 4 | We added 200 µl thawing medium/vial |
| 5 | We added 1600 µl thawing medium/vial |
| 6 | We added 1600 µl thawing medium/vial |
| 7 | We added 1600 µl basic medium/vial |
| 8 | We added 1600 µl basic medium/vial |
| 9 | We added 1600 µl basic medium/vial |

In order to wash the DMSO away from cells, we span the cells on 350 G, built 9, brake 3 at 4°C for 10 minutes. The supernatant was removed and the pellet resuspended in 100 µl DNase. We added 1-2 ml PBS and left the cells to rest at least for one hour at room temperature. After resting, we span the cells again on 350 G, built 9, brake 3 at 20°C for 10 minutes, removed the supernatant and resuspended the pellet, determined the volume and took 20 µl sample for ACT cell counting.

3.3.2 PKH26 labelling

The first two experiments (determination of the populations after 10 days of TPO culture and UCB isolated HSCs responsiveness to TPO) were carried out together in order not to waste isolated material. Both required cell expansion with TPO (kind gift from IHB/LUMC), we just used different detectors and compensation protocols on the flow cytometer. For the second experiment, i.e. UCB isolated HSCs responsiveness to TPO; we had to have insight in cell division. For this purpose we used a dye, which binds on the membrane and its intensity splits into half after every cell division. This dye was PKH26 (PKH26 Red fluorescent Cell Linker Kit for General Cell Membrane Labelling, Sigma-Aldrich). We looked at the PKH26 intensity before culturing and after 10 days of culture with TPO with a flow cytometer to determine the percentage of divided and non-divided cells.

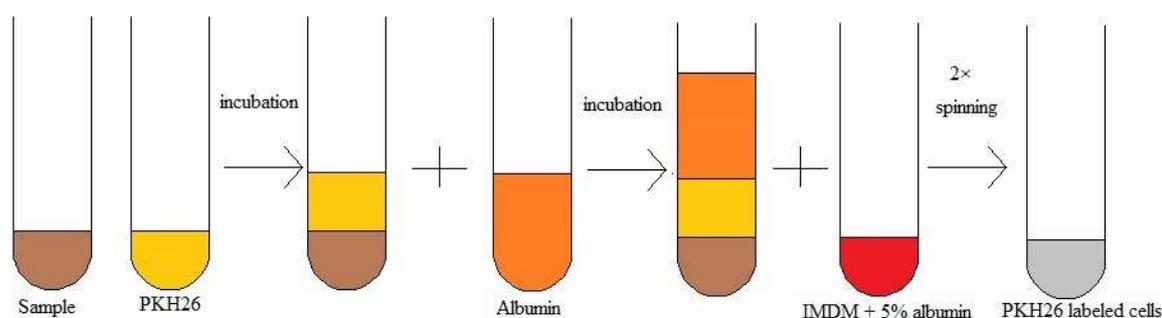


Figure 8: PKH26 labelling

The procedure of PKH26 labelling is shown in Figure 8. Firstly, we made a concentration of $1,0 \times 10^6$ cells/ml in Diluent C. The PKH26 label had to be diluted in Diluent C buffer as well, in a 16 µM concentration. The volume of the cell suspension and the label had to be the same. Secondly, we mixed these two suspensions and incubated it on room temperature in dark for 5 minutes. With albumin (Albumin 20%, Sanquin Blood Bank) we stopped the reaction, therefore we added the same amount of albumin, as the cells and PKH26 label together. The incubation was on room temperature in dark for one minute. After the incubation we had to add 5 ml IMDM (Gibco, Invitrogen corporation) with 5% albumin and span the suspension on 350 G, built 9, brake 3 at 20°C for 10 minutes (removed the

supernatant and resuspended the pellet), to wash the unattached PKH26 dye away. This step we did twice.

3.3.3 10 day TPO culture

As mentioned before, we expanded the PKH26 labelled cells with TPO both for the first experiment (determination of the populations after 10 days of TPO culture) and second experiment (UCB isolated HSCs responsiveness on TPO) as well, to have clearer insight, see Figure 7. The cell expansion was achieved in a standard culture (37°C in a 5% CO₂ humidified atmosphere) with 50 ng/ml thrombopoietin in megacult medium for 10 days. The cells were growing in a 24-well plate, 1000 µl cell suspension in one well, 0, 1·10⁶ cells/well. Into the empty wells we put sterile PBS (Pharmacy LUMC), as shown in Figure 9, and wrapped the plates with film, to keep the humidity. We refreshed the cells on day 7 with megacult and 50 ng/ml TPO, and on day 9 just with megacult. If the expansion was very high (the cells were visible on the bottom of the well with a naked eye, grew almost the whole possible area), we diluted the cell suspension. We added 1000 µl of pure megacult or a mix with TPO (depending which day) to the wells with cell suspension and removed the water from an equal number of wells. To achieve a homogeneous fluid, we resuspended the cell suspension and its additions in each well and split the volume in two wells. If the expansion was not high, we removed half of the well volume (500 µl of old medium, before resuspending the well content) and added 500 µl new medium (with or without TPO, depending on the day), and resuspended it.

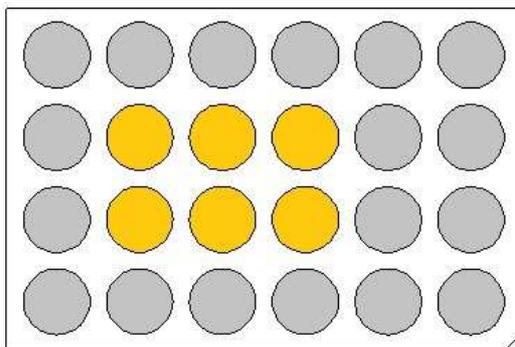


Figure 9: 24-well plate, the orange wells containing cell suspensions, the gray ones are filled with sterile PBS

3.3.4 Cell harvesting after TPO culture

At day 10 we harvested the cells. First, we pooled the resuspended cells together in a 15 ml or 50 ml tube and measured the volume. With a pipette we took 20 µl of the sample to determine the cell concentration on the ACT and calculated the cell number to set the expansion rate.

$$\text{expansion rate} = \frac{V_{\text{harvested cells}} \cdot C_{\text{harvested cells}}}{n_{\text{cultured cells}}} \quad \dots (3)$$

In order to harvest all the cells, we rinsed the well with sterile PBS. To reduce the volume, we spun the cells at 350 G, built 9, brake 3, at 37°C for 10 minutes, removed the supernatant, resuspended the pellet and determined the volume. In 30 µl sample we determined the cell concentration on the ACT and analyzed the populations on the flow cytometer on different markers of interest.

3.3.5 Flow cytometry measurements

After 10 days of culture we checked the populations' morphology, determined the markers they express, and their proportions. As we were investigating the responsiveness on TPO, which leads the differentiation towards megakaryocytes, we used stem cells and megakaryocyte antibodies, such as anti-CD34-PC5 (usually expressed on HSCs), anti-CD61-PC7 (usually expressed on megakaryocytes), anti-CD9-FITC (usually expressed on megakaryocytes) and anti-CD38-FITC (usually not expressed on primitive HSCs). The measurements were made on the flow cytometer (CYTOMICS FC 500, Beckman Coulter).

Table 8: The fluorescent dyes coupled CD antibodies in two tubes for flow cytometer measurement

| TUBE 1 | TUBE 2 |
|----------------|---------------|
| anti-CD34-PC5 | anti-CD9-FITC |
| anti-CD38-FITC | anti-CD34-PC5 |
| | anti-CD61-PC7 |

We marked two tubes: tube 1 and tube 2. To the first tube we added 50 µl diluted sample and 5 µl anti-CD34-PC5 and 5 µl anti-CD38-FITC fluorescent dyes coupled antibodies and incubated it for 15 minutes in dark on room temperature. To the second tube we added 50 µl diluted sample and 5 µl anti-CD34-PC5, 5 µl anti-CD61-PC7 and 5 µl anti-CD9-FITC (incubated in dark on room temperature as well), as shown in Table 8. In order to increase the sample volume, we added 350 µl PBS. We measured the sample with flow cytometer, to determine the proportions of different cells.

3.3.6 Determination of the populations after 10 days of TPO culture

To determine the populations after 10 days of culture with TPO, we used the flow cytometer, as described in 3.3.5. The percentages we were interested in were calculated by the computer software CXP analysis, version 2.0. The average percentage of CD34⁺CD61⁻, CD34⁻CD61⁻ and CD34⁻CD61⁺ population in nine different cords was calculated as follows:

$$\mu = \text{Average} = \frac{1}{N} \sum_{i=1}^N x_i \quad \dots (4)$$

, where the N was the total number of cords, and x_i was the percentage of specific marker in the specific cord blood sample. The standard deviation was calculated as follows:

$$\sigma = \text{standard deviation} = \sqrt{\frac{1}{N} \sum_{i=1}^N (x_i - \mu)^2} \quad \dots (5)$$

The average percentages of different populations were calculated with the mean and the standard deviations, as described above. To calculate the statistical differences, when comparing the results from the literature with ours and when calculating the similarity between the CD34/CD61 and CD34/CD9 marker combinations, we used a non-paired alternate two sided t-test assuming unequal standard deviations were considered significant when $p < 0,1$ or $p < 0,05$.

3.3.7 Expansion rate

In order to find out how many times the cells divide, we have to determine the expansion rate. With the ACT we measured the cell concentration (c) after 10 days of TPO culture and determined the volume (V) as written in 3.6.1. From this, we could easily calculate the cell number (N):

$$N_{\text{after 10 days of TPO culture}} = c \cdot V \quad \dots (6)$$

In each well, we cultured $0,1 \cdot 10^6$ cells, so the expansion rate was calculated as follows:

$$\text{expansion rate} = \frac{N_{\text{after 10 days of TPO culture}}}{\text{number of wells} \cdot 0,1 \cdot 10^6} \quad \dots (7)$$

As we had more samples, we calculated the average and the standard deviation as in 3.3.5.

3.3.8 UCB isolated HSCs responsiveness to TPO

To determine the responsiveness of the isolated HSCs from UCB to TPO, we labelled the cells with PKH26 fluorescent dye. With the flow cytometer, we measured the CD9, CD34, CD38 and CD61 markers, and the PKH26 intensity as well. We measured the intensity on the first day and at day 10, after harvesting; therefore we could compare it after and before culturing. We used the CXP analysis; version 2.0 computer software to put a gate around the cells on day one, consequently two populations appeared; the non-divided and the divided cells. It was possible to do this with all markers separately, so we could conclude how various cell populations were dividing. The average and standard deviations were calculated as described in 3.3.6. The statistical differences in average percentages of divided and undivided cells in CD34/CD61 and CD34/CD9 marker combinations were calculated with non-paired alternate two sided t-test (assuming unequal standard deviations; $p < 0,05$).

3.3.8.1 Gating strategies for determining the expansion of cell labelled with PKH26

To know where to put a cut-off point for expanded and unexpanded cells, we used the day 1 data from the cells, labelled with PKH26. First, we gated the cells and looked at the cells in FLT2 detector, where the PKH26 intensity could be measured, as can be seen in Figure 10a and b.

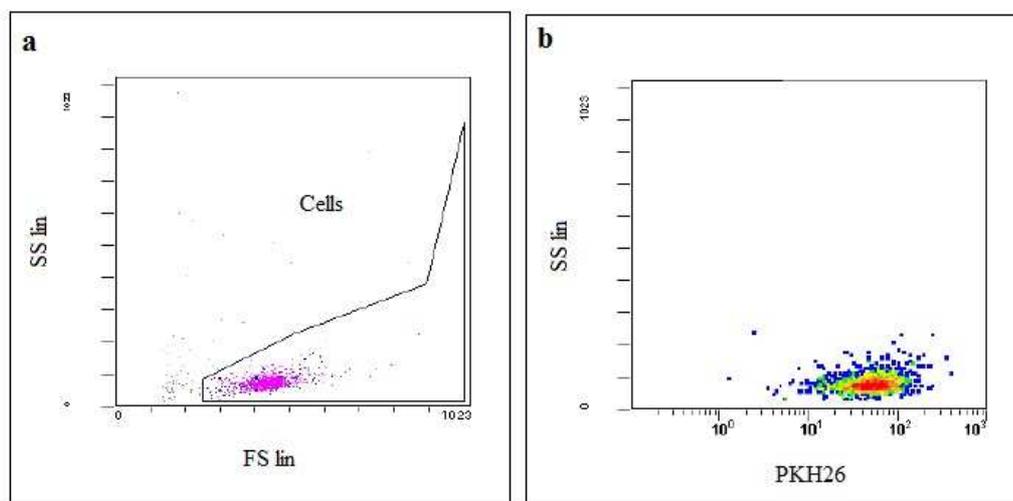


Figure 10: Gating procedure at labelled sample UCB 11-075 before culturing: **a-** we put a gate around cells (SS and FS scatter); **b-** looked at the PKH26 intensity (SS and FLT2)

The cells from day 1 were of course unexpanded cells (we started to count the days from day 1, not day 0). During cultivation, the populations started to move to the left, so for the cut off point, we put a gate as on day 1 results, as shown in Figure 11 a:

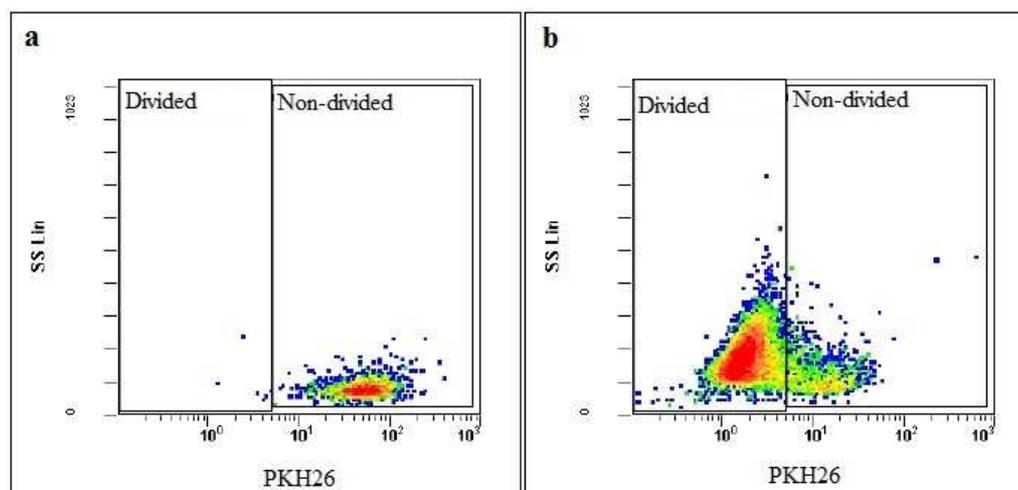


Figure 11: Gating procedure by determining the responsiveness to TPO: **a-** measured PKH26 intensity in labelled sample (cells from UCB 11-075) before culturing – the gate for divided cells has been put on the left side of the cell population; **b-** measured PKH26 intensity in labelled sample (cells from UCB 11-075) after 10 days of culture - we can see the divided population in the left box

If we put data from the same cord from different days in this plot (and keep the gates), we can learn which ones are the expanded and unexpanded cells after culturing with TPO (shown in Figure 11b).

3.4 DETERMINATION OF THE POPULATIONS IN A 15 DAY TPO CULTURE AND UCB ISOLATED HSCS RESPONSIVENESS TO TPO

We repeated the same procedures as in the 10-day cultivation for 15 days on all samples (we put five UCB products in culture). First, we had to thaw them as described in 3.3.1. In order to have an insight in cell division, the PKH26 labelling followed as in 3.3.2. The TPO culture was carried out as 3.3.3, except it lasted longer, so the media refreshing took place more often, dependent on the cell number. Refreshing the culture after day 9 was completed just with megacult media. Every day a little sample was taken (50 μ l sample/well in a 15 ml tube) for flow cytometer analysis, as described in 3.3.5. While the volume was often too high, we span the sample on 350 G, built 9, brake 3 at 20°C for 10 min. After spinning, we removed the supernatant and resuspended the pellet.

The calculations of averages and standard deviations were carried out as described in 3.3.6.

3.5 LONG TERM STEM CELL CAPACITY PRESERVATIONS – CAFC ASSAY

The golden standard to determine long term stem cell capacity is *in vivo* transplantation in mice (Szilvassy et al., 1990). However, this procedure is quite time and money consuming that is why other *in vitro* techniques were developed. One of them is the cobblestone area-forming cell (CAFC) assay. It is a tool, where a feeder layer is used, under which the haematopoietic LT-SCs are migrating. With refreshing the culture every week, we remove other cells, which are not long term (are not migrated), since we keep the culture for five weeks. After week four and five, we should be able to see clusters of small, tightly packed cells within a stromal layer (Breems et al., 1994). To have an overall picture of the steps carried out, see Figure 12.

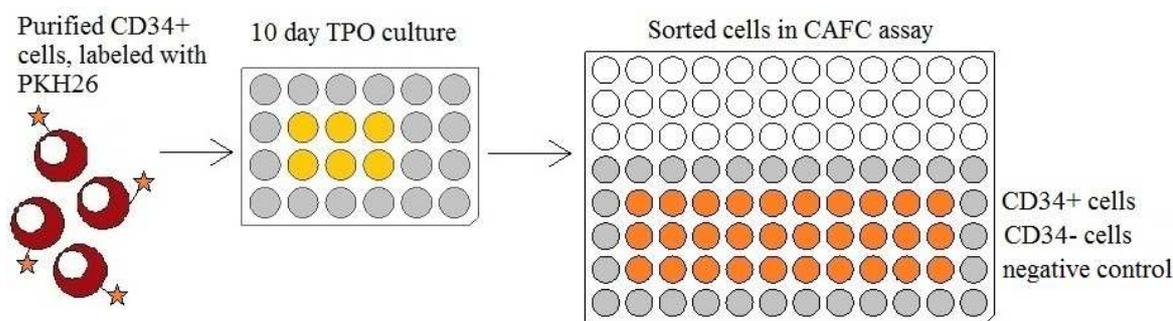


Figure 12: The CAFC assay in steps (the orange wells are cells in media; the gray wells are filled with sterile PBS)

The CAFC cells look like the marked cells on Figure 13:

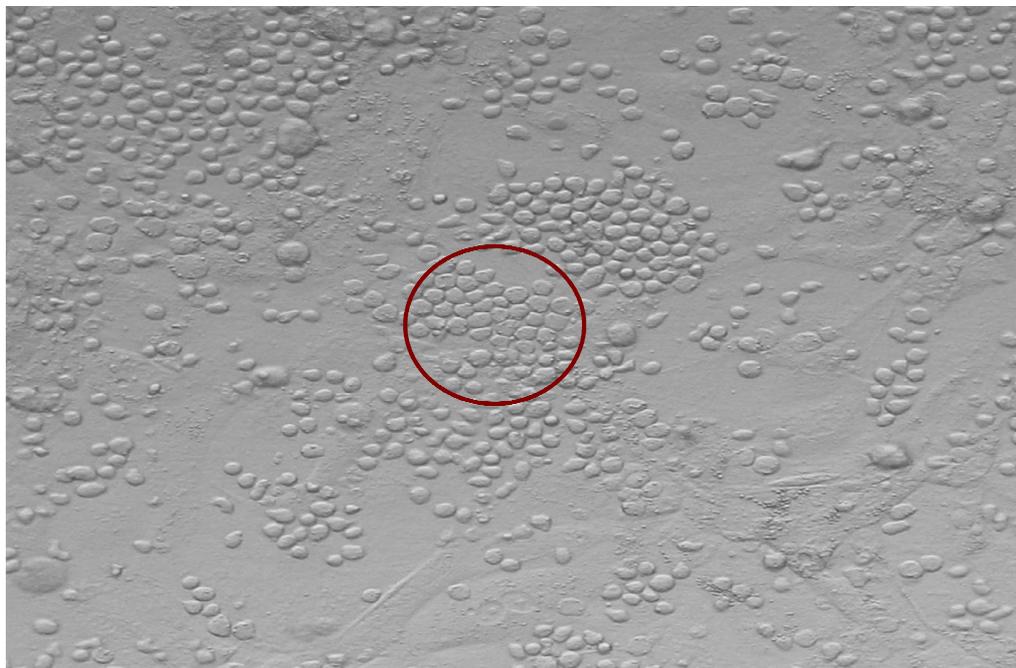


Figure 13: The CAFC cells (Manon K. Slot, 2010)

To carry out a CAFC assay, first, we had to have purified CD34⁺ cells, usually frozen; therefore, we had to thaw them as described in 3.3.1. Secondly, the PKH26 labelling followed as described in 3.3.2. The TPO culture is described in 3.3.3.

3.5.1 Feeder cell culturing for CAFC culture

To carry out a CAFC assay, we had to culture a feeder cell layer, which is attaching to the plastic, one day before the experiment. The long term stem cells have the ability to migrate under the feeder cells and the other cells does not; therefore, we remove the other cells with refreshing the media.

Following the protocol, the NIH3 media and trypsin was put on 37°C (incubator). The flat bottom 96 well plate had to be coated with gelatine; therefore, we pipetted 200 µl/well of gelatine and left it on 37°C for at least 15 min. The feeder cells were in a flask, from where we removed the old medium with a pipette and rinsed the flask with 10 ml PBS. To make the cells floating in the medium again, instead of attaching on the plastic, we used 1, 5 ml trypsin for 2 minutes on 37°C. The trypsin action was stopped with 1, 5 ml NIH3 medium. The cell suspension was collected in a new tube, the volume and the cell concentration was determined with pipette boy and ACT. We made a 27, 2×10⁴/ml cell concentration with NIH3 medium for 200µl/well. The cells were deactivated with irradiation for 40 s, on 500 Rad. After irradiation we removed the gelatine from the 96 well plate and put 200 µl/well

27, 2×10^4 /ml irradiated cell suspension in each well. The flat bottomed 96-well was put in the incubator (Thermo Scientific) for overnight (37°C, 5% CO₂) to achieve confluence (all the surface was occupied with cells).

3.5.2 Flow cytometry analysis

We harvested the cells after 10 days of TPO culture. After harvesting we did the same measurements on the flow cytometer as described in 3.3.5.

3.5.3 FACS sorting for CD34⁺ after 10 days of culturing with thrombopoietin

In order to determine the long-term stem cell capacity in the CD34⁺ cell population after culturing, we sorted the 10-day TPO cultured cells. We were interested in the remaining CD34⁺ cells after culture and CD34⁻ cells, so we sorted on a FACS sorter (Aria, Becton, Dickinson and Company), following the next protocol:

First we span the harvested cells with 3 ml PBS in the clip lid tubes, removed the supernatant until 500 µl and resuspended the pellet. The cells were coloured with CD34-PC5 fluorescent dye (aseptic, 5 µl for 10⁶ cells) and incubated for 15 min in dark at room temperature. We filtered the cell suspension before sorting with a 50 µm filter. The FACS sorter is firstly measuring different cell types for compensation protocol, which is made new every time. For the compensation measurement we had to have (shown in Table 9):

Table 9: Material needed for FACS compensation:

| | |
|--------|---|
| Tube 1 | Plain cells (post ficoll fraction) |
| Tube 2 | PKH26 labelled cells |
| Tube 3 | Labelled cells with a PC5 dye (doesn't matter which kind of antibodies) |

Based on the measurements of compensation in tubes 1-3, the gates around CD34 positive and PKH26 positive cells were put. The gating strategy was to gate first the cells (FS, SS), and after it the CD34⁺/PKH26⁺. The cells were sorted into CD34⁺ fraction and CD34⁻ (the rest), which were captured into two tubes with a click lid with 500 µl 50% FCS and 50% PBS.

3.5.4 Starting a CAFC culture

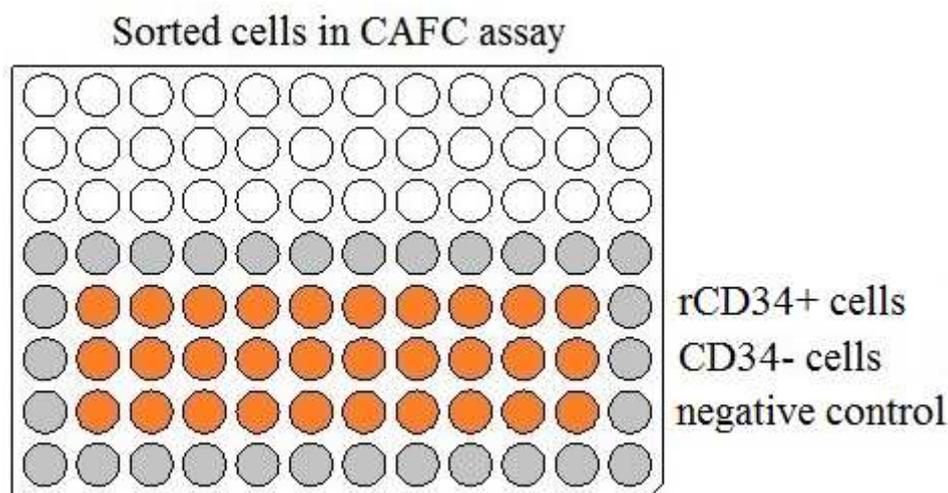


Figure 14 The scheme of CAFC cultured cells: the sorted remaining CD34⁺ (rCD34⁺) and CD34⁻ cells after 10 days of TPO culture in a 96 well plate and the negative control (just feeder cells and CAFC media); in the orange wells are the cells in medium and feeder cells; in the grey wells is the sterile PBS

After sorting from 3.5.3 we prepared from the CD34 positive and negative fractions a $0,025 \times 10^6$ /ml cell concentration in CAFC media. First we removed the NIH3 medium from the feeder cells and added 200 μ l sorted cell suspension/well, as it is seen in Figure 14: In the first ten wells we cultured 200 μ l of rCD34⁺ cells, in the second 10 wells the CD34⁻ cells and the third line was our negative control, where we put just the CAFC media on the feeder layer.

3.5.5 Scoring the CAFC assay results

The flat bottomed 96 well plates were investigated under the inverted microscope on week 4 and 5. If we saw a cluster of tightly packed cells as shown in Figure 12, we marked that well as a positive well. Each positive well represented long term repopulating capacity of 10% cells in the fraction (as we put 10 wells from one fraction in culture).

3.6 DIFFERENCE IN EXPANSION OF $CD34^+CD38^-$ AND $CD34^+CD38^+$ POPULATIONS

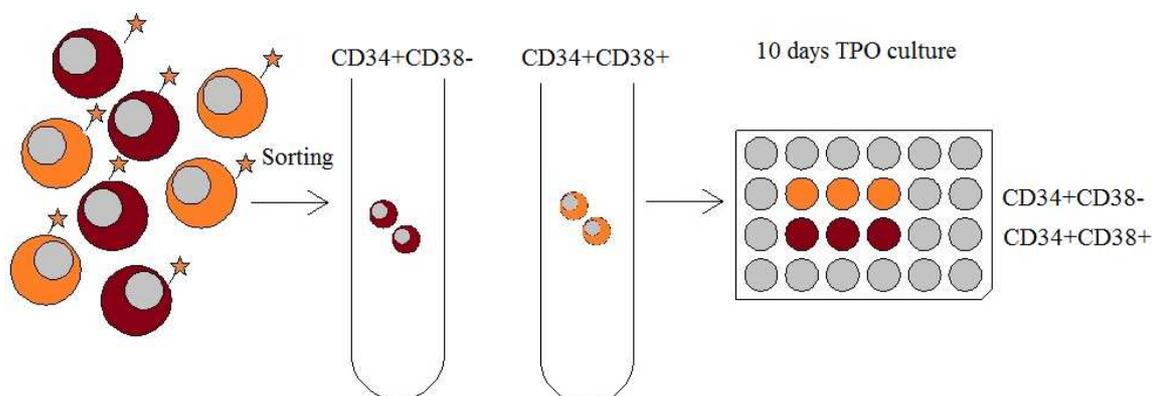


Figure 15: The scheme of the procedures: we sorted the isolated $CD34^+$ cells on $CD34^+CD38^-$ and $CD34^+CD38^+$ populations and cultured them separately in a 24 well plate

Because the $CD34^+CD38^-$ population is believed to contain primitive and multi-potent progenitor cells, there should also be a difference in differentiation pattern while thrombopoietin culturing. In Figure 15 we can see the main steps of this experiment. The samples were fresh, so thawing was not necessary.

To be able to see the cell divisions, the PKH26 labelling was used, as described in 3.3.2.

We sorted the freshly isolated cell. We were interested in the $CD34^+/CD38^+$ and $CD34^+/CD38^-$ cells, so we sorted on a FACS sorter, following the next protocol:

First, we span the cells with 3 ml PBS in the tubes with lid, removed the supernatant until 500 μ l and resuspended the pellet. The cell were coloured with anti- $CD34$ -PC5 and anti- $CD38$ -FITC fluorescent labels (aseptic, 5 μ l for 10^6 cells) and incubated for 15 min in dark at room temperature. After incubation, we filtered the cell suspension before sorting with a 50 μ m filter. For the compensation for the FACS, we had to have (as seen in Table 10):

Table 10: Materials needed for FACS sorting

| | |
|--------|---|
| Tube 1 | Plain cells (doesn't matter if post ficoll fraction or isolated cells) |
| Tube 2 | Labelled cells with a FITC dye |
| Tube 3 | Labelled cells with a PC5 dye (doesn't matter which kind of antibodies) |

Based on this measurement, the gates were put. The gating strategy was to gate first the cells (FS, SS), later the CD34⁺ (CD34-PC5) and at last the CD38⁺ and CD38⁻ (CD38-FITC). The cells were sorted to CD34⁺/CD38⁺ and CD34⁺/CD38⁻ (the rest) fractions, which were captured in to two tubes with a click lid with 500 µl 50% FCS and 50% PBS.

To see the difference in expansion, we had to carry out a TPO culture as described in 3.3.3. The cells were harvested as described in 3.3.4 and analyzed as in 3.3.5. The expansion rate was calculated as in 3.3.7 and because we had more samples, we calculated the average and the standard deviation as in 3.3.5.

4 RESULTS

4.1 EXPANSION

We put 9 different UCB samples in culture in average with $87,3\% \pm 9,0\%$ purity. The expansion rate was in average $13,9 \pm 7,5$ cell divisions after ten days of TPO culture.

4.2 CD34/CD61 MARKER COMBINATION

4.2.1 Determination of the population after 10 and 15 days of TPO culture

We cultivated nine different UCB samples to determine the formation of different populations after 10 and 15 days of TPO culture. On day 1, 10 and 15 following results were obtained (Figure 16): on day one (on the day, we put the cell in culture) we put in culture in average $99,4\% \pm 0,7\%$ of $CD34^+CD61^-$ ($CD34^+$) population and $0,6\% \pm 0,7\%$ of $CD34^-CD61^-$ (Lin^-) population. We did not notice other populations. After 10 days of TPO culture, we observed in average $6,5\% \pm 4,9\%$ of remaining $CD34^+CD61^-$ ($rCD34^+$) population, $18,1\% \pm 12,1\%$ of $CD34^-CD61^-$ (Lin^-) population, $69,2\% \pm 13\%$ $CD34^-CD61^+$ ($CD61^+$) population and $6,3 \pm 7,1\%$ of $CD34^+CD61^+$ population. On day 15, we observed in average $1,0\% \pm 0,5\%$ of $rCD34^+$ population, $4,6\% \pm 2,2\%$ of Lin^- population, $90,1\% \pm 4,4\%$ of $CD61^+$ population and $4,3\% \pm 4,2\%$ of $CD34^+CD61^+$.

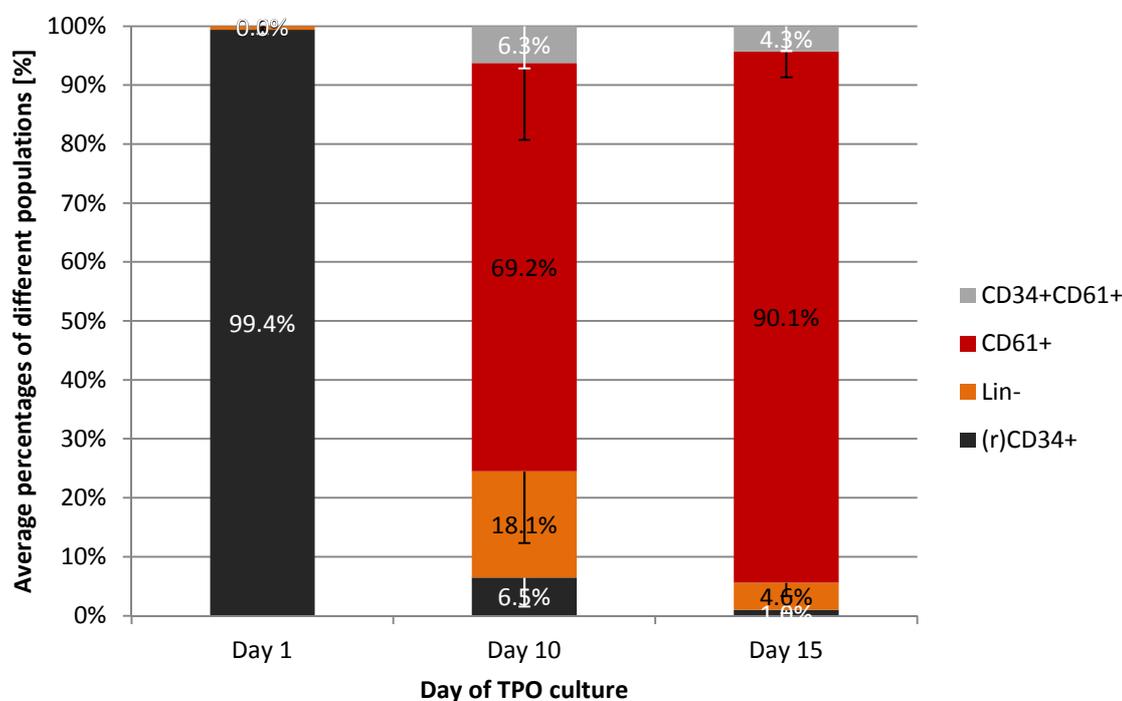


Figure 16: The average percentages with their standard deviation of different populations ((r) $CD34^+$, $CD34^+CD61^+$, $CD61^+$, Lin^-) for day 1, 10 and 15

4.2.2 UCB isolated HSCs responsiveness to TPO

The isolated HSCs respond to TPO with cell divisions and differentiation. We cultivated nine different UCB samples to determine the response of the isolated HSCs after 10 and 15 days of TPO culture. On day 1, 10 and 15 following results were obtained (Figure 17): on day one (on the day, we put the cell in culture) we put in culture in average $100\% \pm 0\%$ of non divided $CD34^+CD61^-$ population and $99,4\% \pm 2,3\%$ of non divided $CD34^-CD61^-$ population. Other populations we didn't notice. After 10 days of TPO culture, we observed in average $91,1\% \pm 5,6\%$ of divided and $9,2\% \pm 6,3\%$ of non divided $CD34^+CD61^-$ population, $75,8\% \pm 13,5\%$ of divided and $24,5\% \pm 13,7\%$ of non divided $CD34^-CD61^-$ population and $94,0\% \pm 3,8\%$ of divided and $6,0\% \pm 4,2\%$ of non divided $CD34^-CD61^+$ population. On day 15, we observed in average $95,9\% \pm 2,5\%$ of divided and $4,1\% \pm 2,5\%$ of non divided $CD34^+CD61^-$ population, $90,5\% \pm 7,3\%$ of divided and $9,8\% \pm 7,8\%$ of non divided $CD34^-CD61^-$ population and $88,4\% \pm 5,8\%$ of divided and $11,3\% \pm 7,0\%$ of non divided $CD34^-CD61^+$ population.

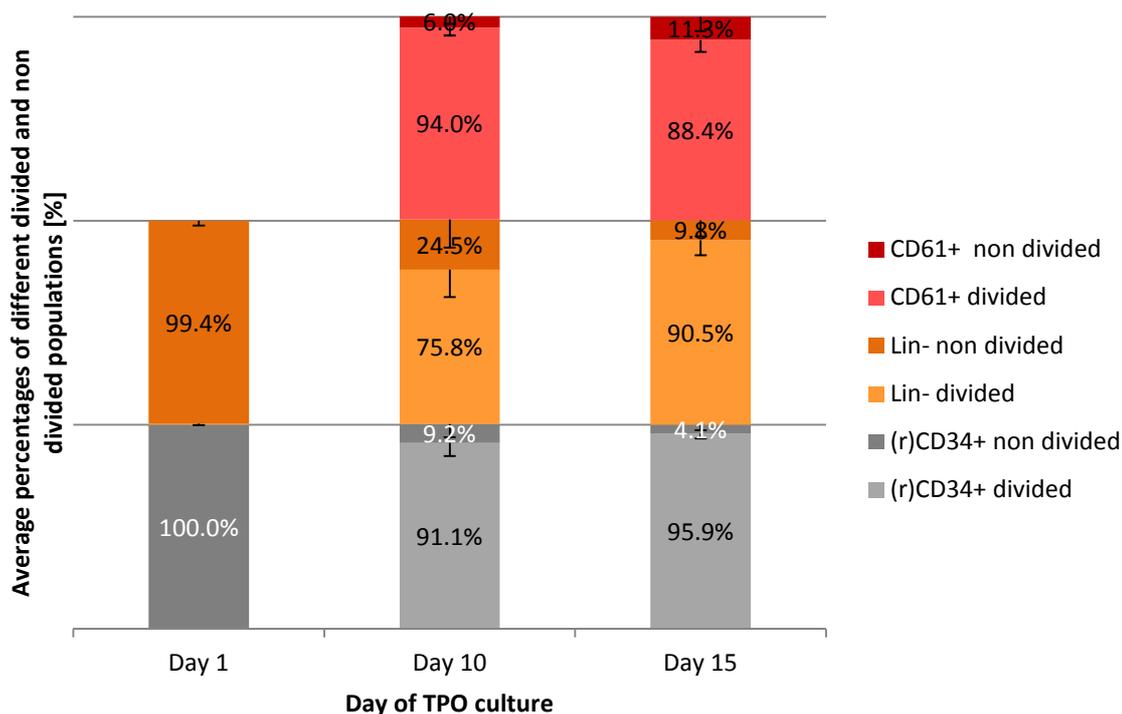


Figure 17 The percentages with their standard deviations of different divided and non divided populations ((r) $CD34^+$, Lin^- and $CD61^+$) on day 1, 10 and 15

4.2.3 Marker expression through time (15 day culture)

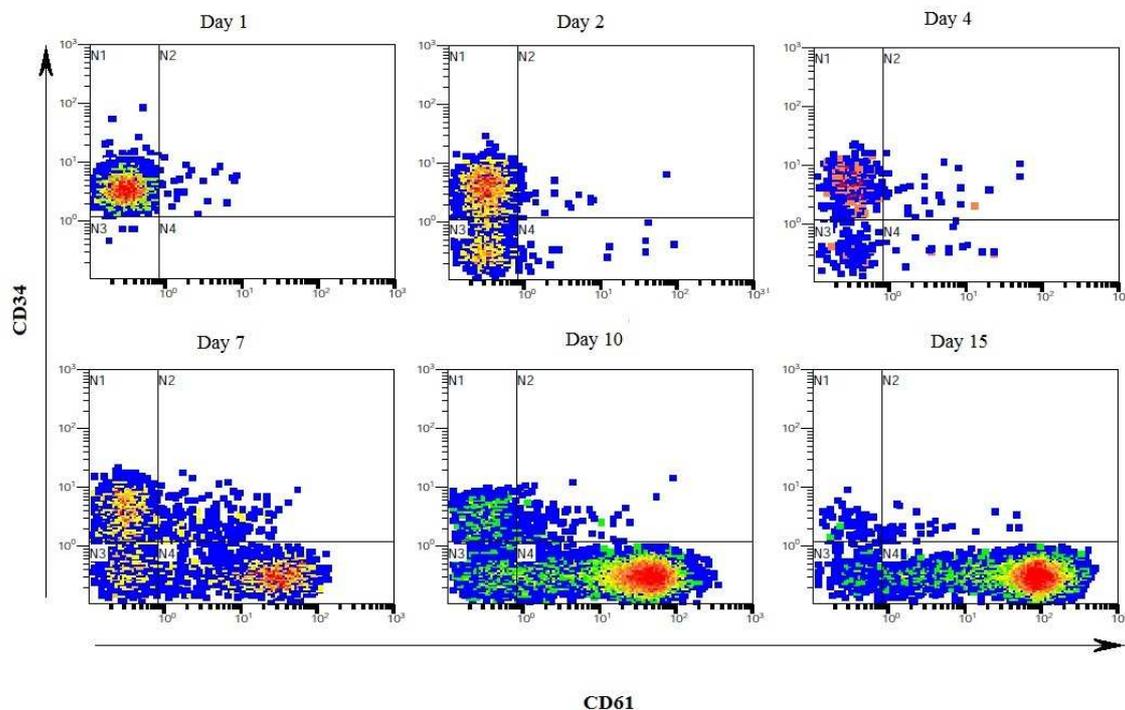


Figure 18: The most typical (density) plots from 15 day culture – we can see how the marker expression (CD34/CD61) is changing through time (day 1, 2, 4, 7, 10, 15)

As we can see in Figure 18, the expression of CD34 and CD61 is changing through time. From the isolated CD34⁺ cells, the differentiation is starting with losing the CD34 marker at day two, and after days 5, 6 or 7, with gaining the CD61 marker. After day 12 there usually are not many cells expressing CD34 marker, and after day 12 or 13, we can see two populations in the CD34⁻CD61⁺ population.

On Figure 19 we can see how the average percentages of the four appearing populations (rCD34⁺, Lin⁻, CD61⁺, CD34⁺CD61⁺) are changing through time. The rCD34⁺ population is dropping during the TPO culture, but still remains in small percentages. The Lin⁻ population reaches its peak in days 3 to 6. The average percentages of CD61⁺ population are higher with every day. The small population of CD34⁺CD61⁺ cells reaches its peak at days 6 to 9.

The trend of the average percentages of divided cells is clearly dropping through time for all different populations (shown in Figure 20).

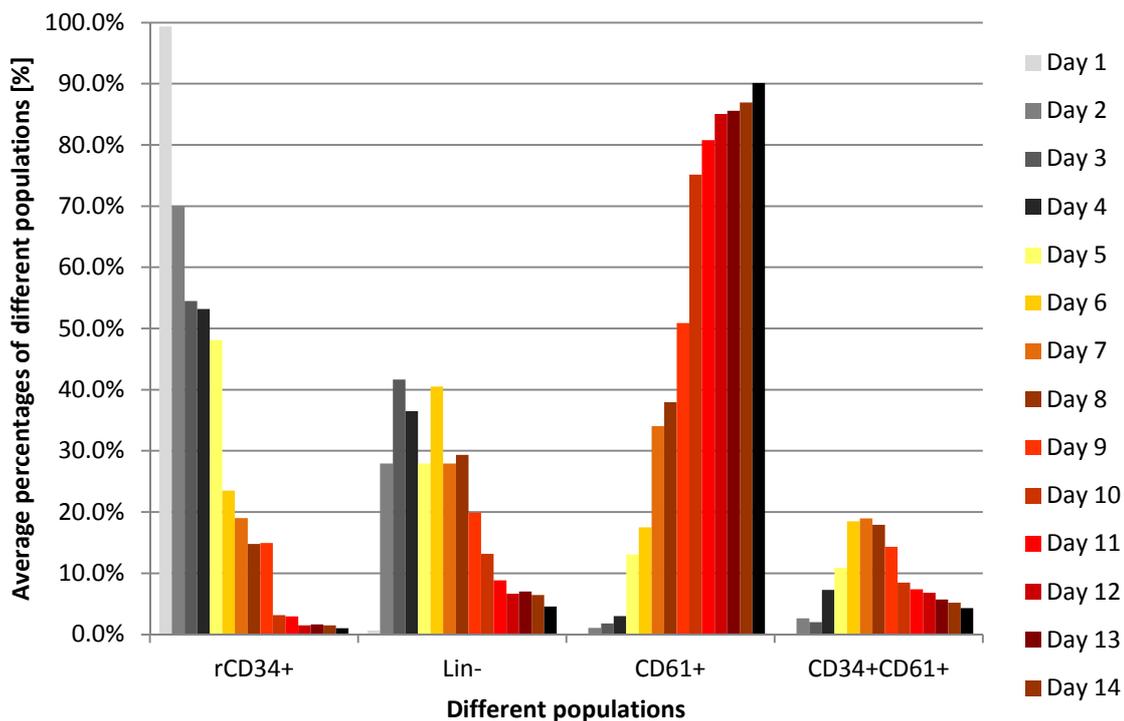


Figure 19: The average percentages of different population (rCD34⁺, Lin⁻, CD61⁺, CD34⁺CD61⁺) for 15 days of culture

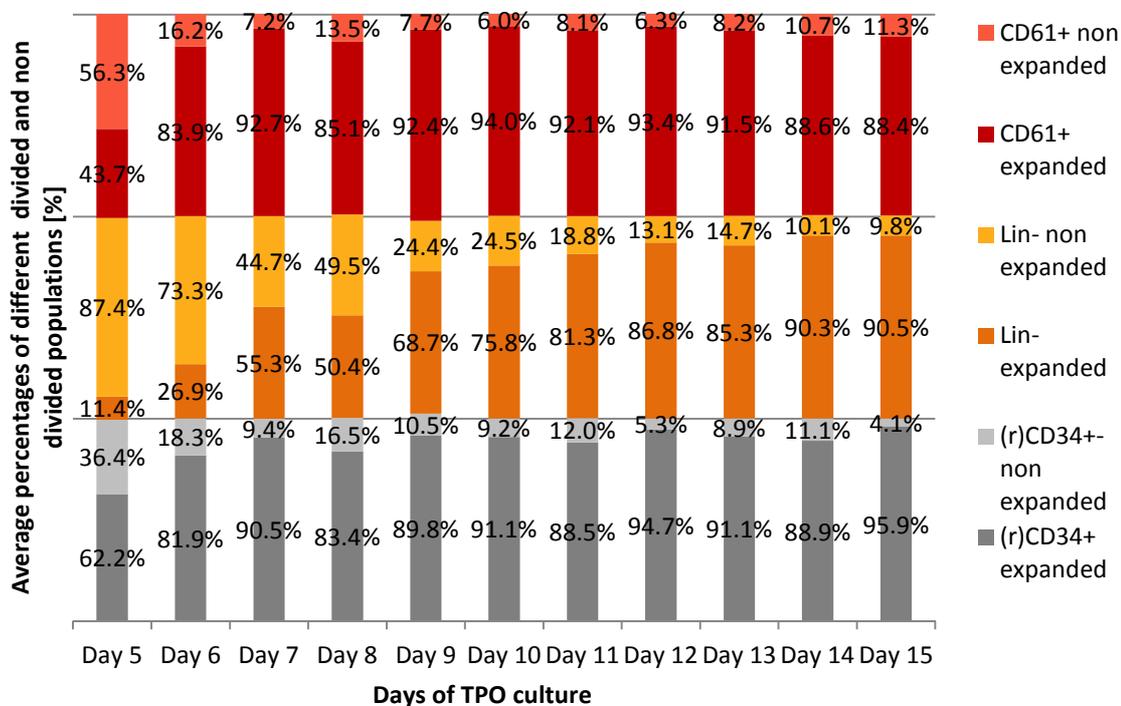


Figure 20: The average percentages of different divided and not divided populations (rCD34⁺, Lin⁻, CD61⁺, CD34⁺CD61⁺) for the last few days of 15 day TPO culture

4.3 CD34/CD9 MARKER COMBINATION

The CD9 and the CD61 markers are both expressed on megakaryocytes, the CD9 appears earlier in time, it is more typical for less mature megakaryocytes (Le Naour et al., 1997).

4.3.1 Determination of the population after 10 and 15 days of TPO culture

For the CD34/CD9 marker combination data from 5 UCB samples were obtained. After 10 and 15 days of TPO culture, four different cell populations were observed: CD34⁺CD9⁻ (rCD34⁺), CD34⁺CD9⁺, CD34⁻CD9⁻ (Lin⁻) and CD34⁻CD9⁺ (CD9⁺) populations. On day 1, 10 and 15 following results were obtained (Figure 21): on day one (on the day, we put the cell in culture) we put in culture in average 99,7% ± 0,5% of CD34⁺ population and 0,4% ± 0,5% of Lin⁻ population. Other populations we didn't notice. After 10 days of TPO culture, we observed in average 5,1% ± 2,7% of rCD34⁺ population, 19,5% ± 8,4% of Lin⁻ population, 67,6% ± 6,1% CD9⁺ population and 7,9 ± 7,2% of CD34⁺CD9⁺ population. On day 15, we observed in average 1,7% ± 1,0% of rCD34⁺ population, 8,2% ± 3,2% of Lin⁻ population, 86,0% ± 4,0% of CD9⁺ population and 4,1% ± 3,7% of CD34⁺CD9⁺.

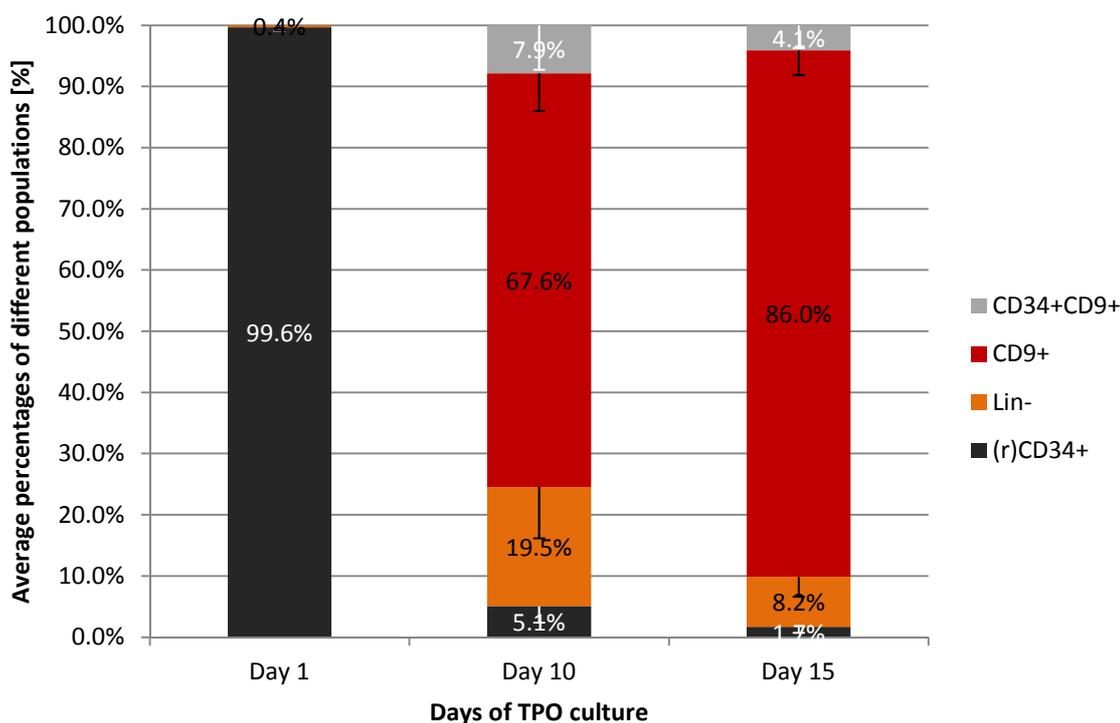


Figure 21: The average percentages with their standard deviation of different populations ((r)CD34⁺, CD34⁺CD9⁺, CD9⁺, Lin⁻) for day 1, 10 and 15

4.3.2 UCB isolated HSCs responsiveness to TPO

The isolated HSCs respond to TPO with cell divisions and differentiation. We obtained data from five different UCB samples to determine the response of the isolated HSCs after 10 and 15 days of TPO culture. On day 1, 10 and 15 following results were obtained (Figure 22): on day one (on the day, we put the cell in culture) we put in culture in average $100\% \pm 0\%$ of non divided $CD34^+$ population and $99,5\% \pm 3,1\%$ of non divided Lin^- population. Other populations we didn't notice. After 10 days of TPO culture, we observed in average $88,8\% \pm 6,3\%$ of divided and $11,2\% \pm 6,3\%$ of non divided $rCD34^+$ population, $68,2\% \pm 12,4\%$ of divided and $31,9\% \pm 12,3\%$ of non divided Lin^- population and $91,5\% \pm 7,5\%$ of divided and $8,3\% \pm 7,8\%$ of non divided $CD9^+$ population. On day 15, we observed in average $90,1\% \pm 11,4\%$ of divided and $9,5\% \pm 10,9\%$ of non divided $rCD34^+$

population, $87,9\% \pm 8,2\%$ of divided and $11,9\% \pm 8,0\%$ of non divided Lin^- population and $89,3\% \pm 8,8\%$ of divided and $11,0\% \pm 9,2\%$ of non divided $CD9^+$ population.

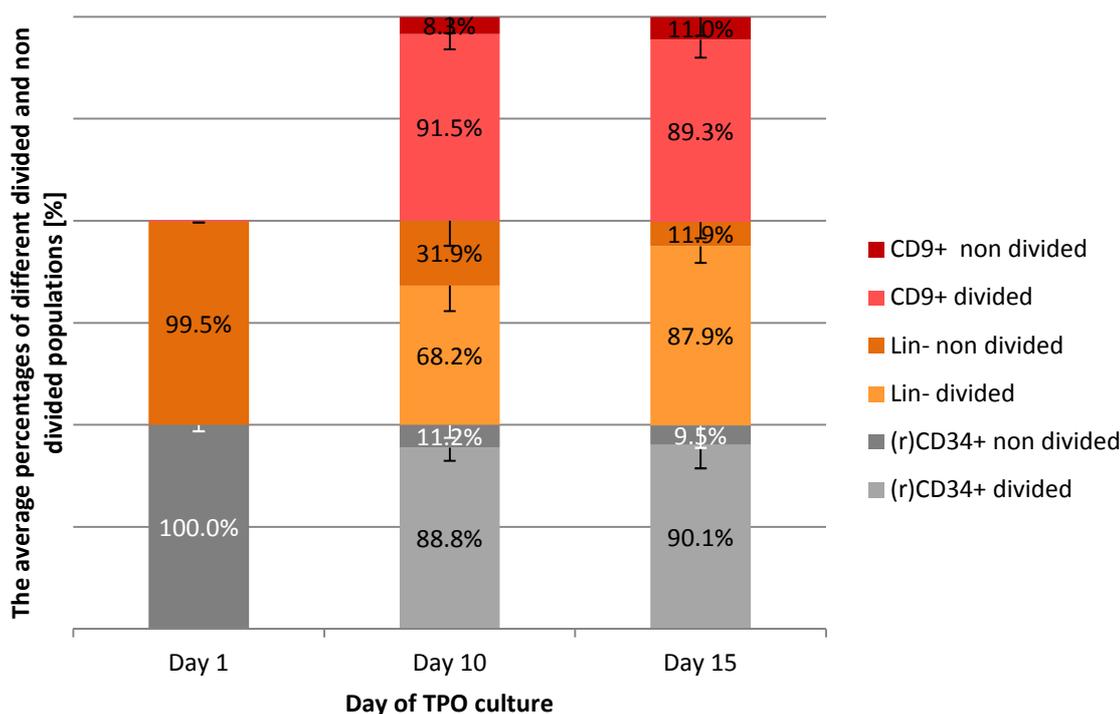


Figure 22: The average percentages and their standard deviations of different divided and non divided populations ((r) $CD34^+$, Lin^- and $CD9^+$) on day 1, 10 and 15

4.3.3 Marker expression through time (15 day culture)

As we can see in Figure 23, the expression of CD34 and CD9 is changing through time. From the isolated CD34⁺ cells, the differentiation is starting with losing the CD34 marker at day two, and after day 5, with gaining the CD9 marker. After day 11 there usually are not many cells expressing CD34 marker, and after day 7, we can see two populations in the CD34⁻CD9⁺ population.

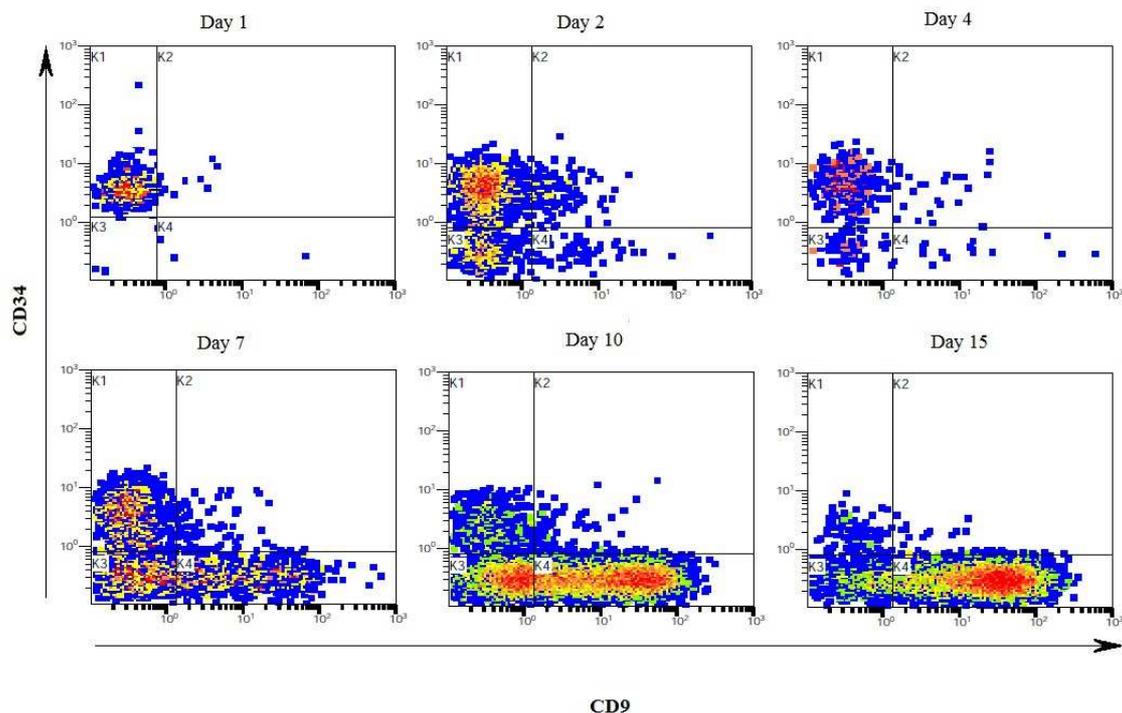


Figure 23: The most typical (density) plots from 15 day culture – we can see how the marker expression (CD34/CD9) is changing through time (day 1, 2, 4, 7, 10, 15)

The results from the average percentages of the four appearing populations (rCD34⁺, Lin⁻, CD9⁺, CD34⁺CD9⁺) are supporting the results from the most typical plots (see Figure 24). The rCD34⁺ population is dropping by time and the average percentage of CD9⁺ population is higher and higher every following day. The Lin⁻ population is peaking on day 5 to 9 and the small CD34⁺CD9⁺ population reaches its highest percentage on day 8.

As we can see in Figure 25, the trend of the average percentages of non-divided populations is dropping as well.

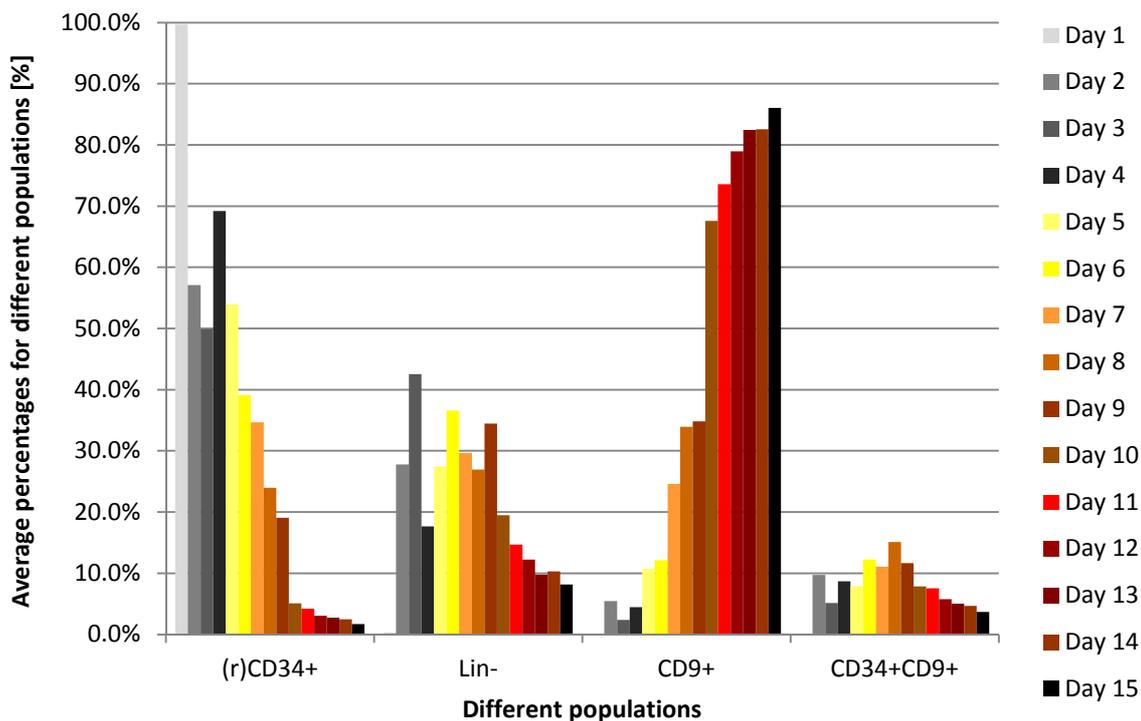


Figure 24: The average percentages of different population ((r)CD34⁺, Lin⁻, CD9⁺, CD34⁺CD9⁺) for 15 days TPO culture

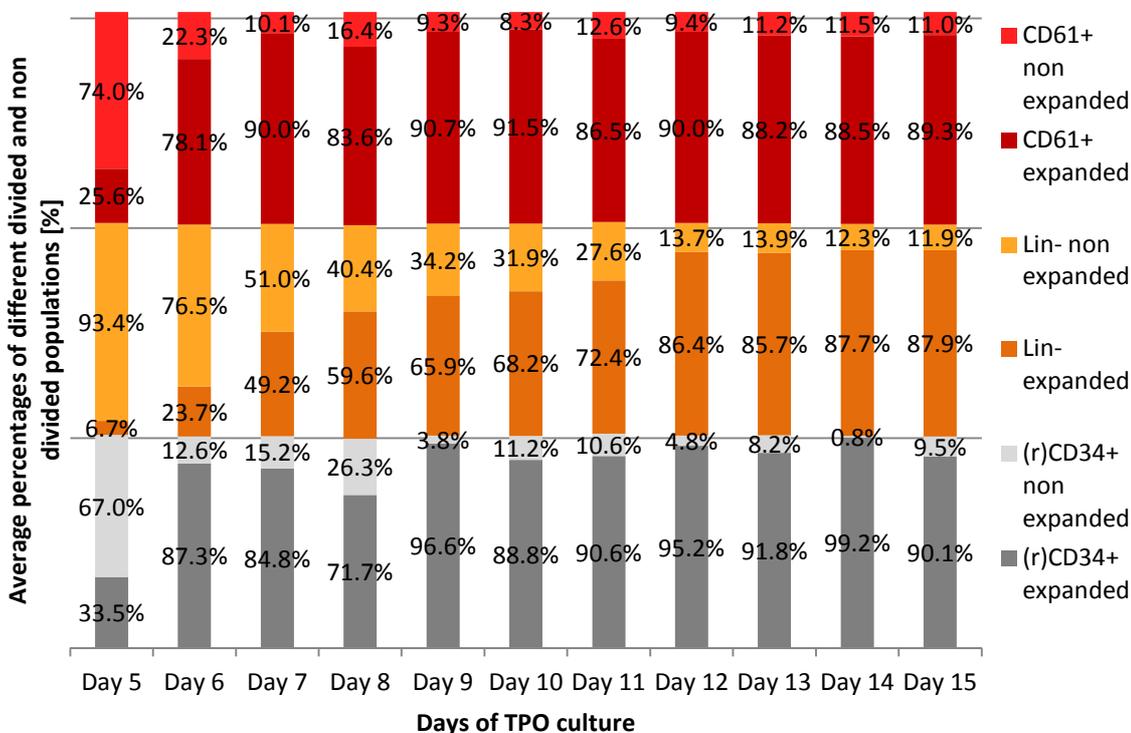


Figure 25: The average percentages of different divided and not divided populations ((r)CD34⁺, Lin⁻, CD9⁺ and CD34⁺CD9⁺) for the last few days of 15 day TPO culture

4.4 CD34/CD38 MARKER COMBINATION

4.4.1 Determination of the population after 10 and 15 days of TPO culture

For CD34/CD38 marker combination we obtained data from five UCB samples. After expansion, we observed four different populations: CD34⁺CD38⁻, CD34⁺CD38⁺, CD34⁻CD38⁻ and CD34⁻CD38⁺. On day 1, 10 and 15 following results were obtained (Figure 27): on day one (on the day, we put the cell in culture) we put in culture in average 85,3% ± 3,2% of CD34⁺CD38⁻ population, 10,0% ± 0,0% of CD34⁻CD38⁻ population, 1,3% ± 0,0% of CD34⁻CD38⁺ population and 3,7% ± 7,4% of CD34⁺CD38⁺ population. After 10 days of TPO culture, we observed in average 6,0% ± 4,1% of CD34⁺CD38⁻ population, 34,5% ± 16,5% of CD34⁻CD38⁻ population, 39,3% ± 16,1% CD34⁻CD38⁺ population and 20,3 ± 14,9% of CD34⁺CD38⁺ population. On day 15, we observed in average 2,7% ± 1,5% of CD34⁺CD38⁻ population, 34,4% ± 16,2% of CD34⁻CD38⁻ population, 45,8% ± 12,9% of CD34⁻CD38⁺ population and 17,8% ± 13,4% of CD34⁺CD38⁺.

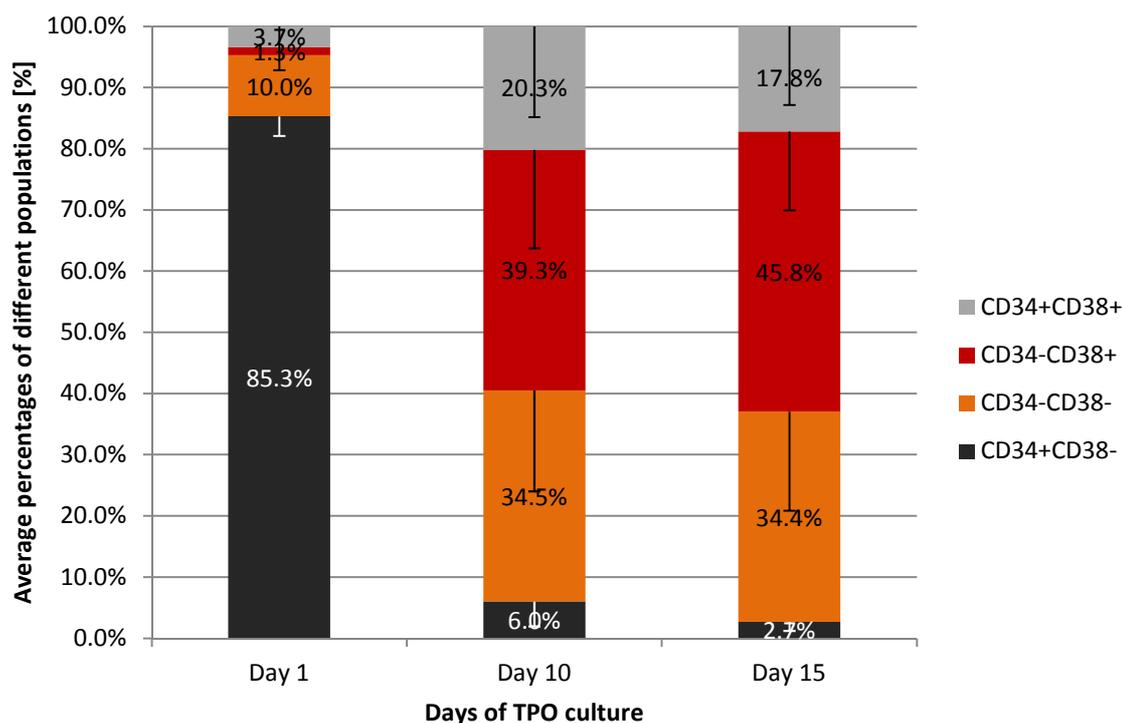


Figure 26: The average percentages and their standard deviations of different populations (CD34⁺CD38⁻, CD34⁻CD38⁻, CD34⁻CD38⁺ and CD34⁺CD38⁺) for day 1, 10 and 15

4.4.2 UCB isolated HSCs responsiveness to TPO

The isolated HSCs respond to TPO with cell divisions and differentiation. We obtained data from five different UCB samples to determine the response of the isolated HSCs after 10 and 15 days of TPO culture. On day 1, 10 and 15 following results were obtained (Figure 28): on day one (on the day, we put the cell in culture) we put in culture in average $99,8\% \pm 0,1\%$ of non divided $CD34^+CD38^-$ population, $92,2\% \pm 10,7\%$ of non divided $CD34^-CD38^-$ population and $99,9\% \pm 0,2\%$ of non divided $CD34^+CD38^+$ population. After 10 days of TPO culture, we observed in average $73,5\% \pm 26,2\%$ of divided and $26,5\% \pm 26,2\%$ of non divided $CD34^+CD38^-$ population, $83,9\% \pm 14,6\%$ of divided and $16,0\% \pm 14,9\%$ of non divided $CD34^-CD38^-$ population, $63,1\% \pm 22,9\%$ of divided and $37,6\% \pm 23,6\%$ of non divided $CD34^-CD38^+$ population and $64,1\% \pm 27,4\%$ of divided and $36,7\% \pm 27,8\%$ non divided $CD34^+CD38^+$ population. On day 15, we observed in average $91,0\% \pm 9,6\%$ of divided and $8,6\% \pm 9,6\%$ of non divided $CD34^+CD38^-$ population, $96,5\% \pm 2,8\%$ of divided and $3,4\% \pm 2,7\%$ of non divided $CD34^-CD38^-$ population, $83,8\% \pm 9,1\%$ of divided and $15,8\% \pm 8,9\%$ of non divided $CD34^-CD38^+$ population and $76,7\% \pm 1,5\%$ of divided and $23,7\% \pm 2,0\%$ of non divided $CD34^+CD38^+$ population.

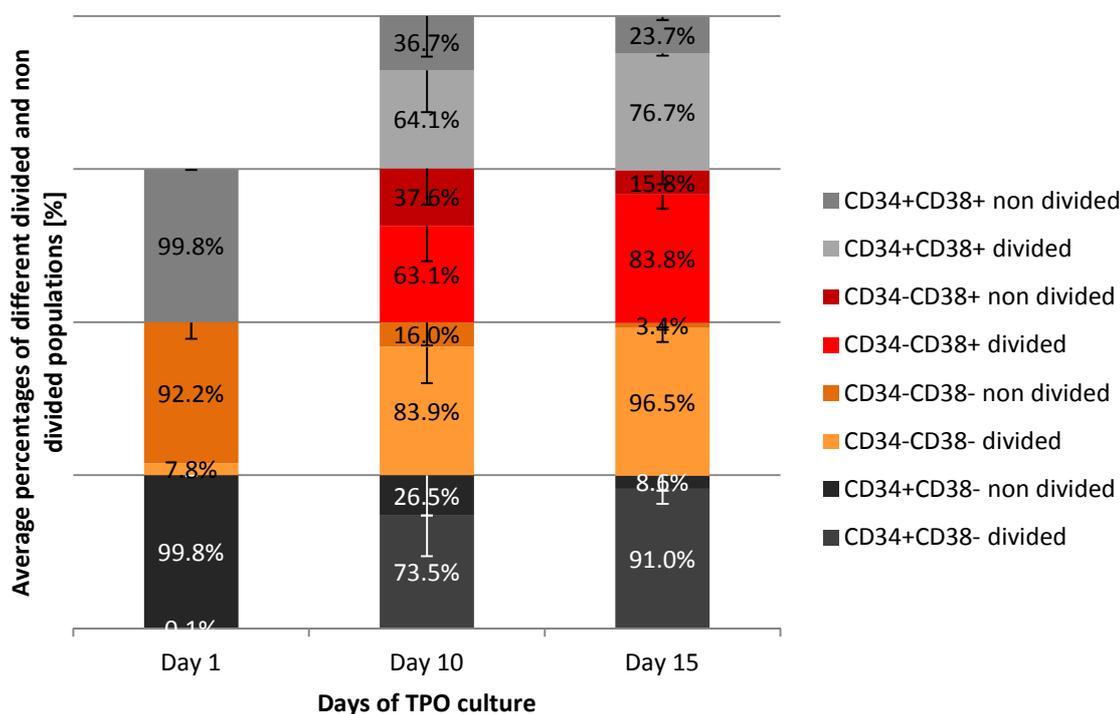


Figure 27: The percentages and their standard deviations of different divided and non divided populations ($CD34^+CD38^-$, $CD34^-CD38^-$, $CD34^-CD38^+$ and $CD34^+CD38^+$) on day 1, 10 and 15

4.4.3 Marker expression through time (15 day culture)

As we can see in Figure 29, the expression of CD34 and CD38 is changing through time. From the isolated CD34⁺ cells, the differentiation is starting with losing the CD34 marker at day two and the CD34⁺CD38⁻ population was dropping through time, on days 4-5 all four populations emerged (CD34⁺CD38⁻, CD34⁻CD38⁻, CD34⁻CD38⁺, CD34⁺CD38⁺), after the 11th day, the CD34⁺CD38^{+/-} populations started to disappear and from day 8 on, the CD34⁻CD38⁻ population started to strengthen. After day 13 there usually aren't many cells expressing CD34 marker.

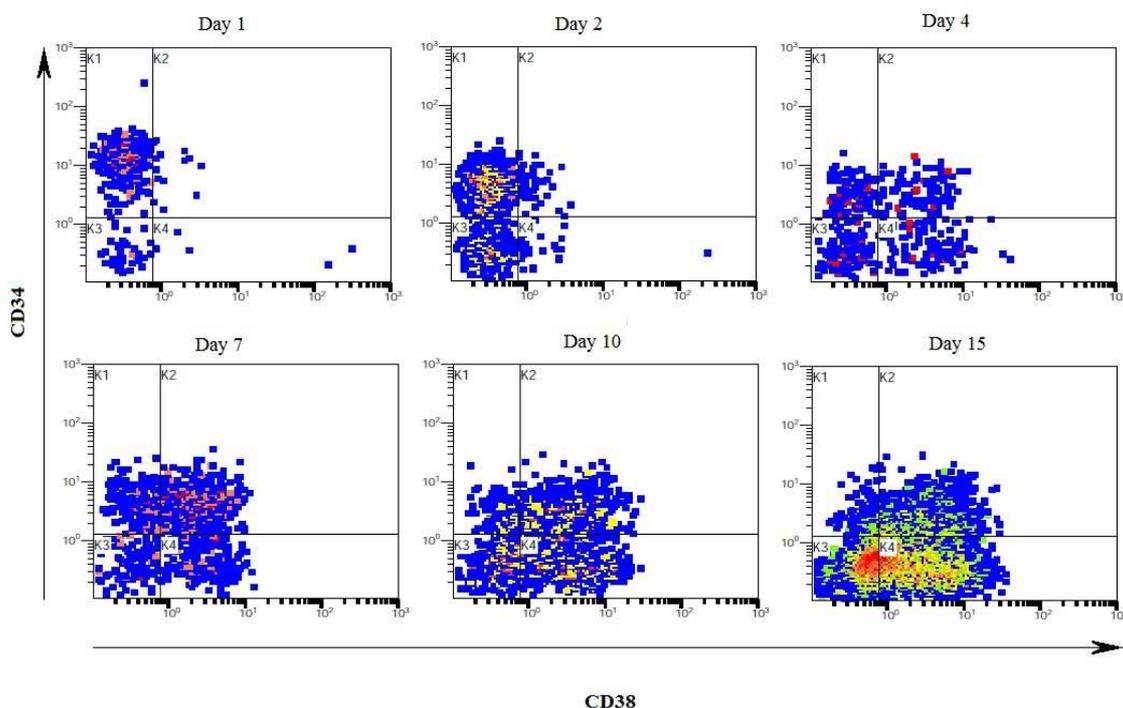


Figure 28: The most typical (density) plots from 15 day culture – we can see how the marker expression (CD34/CD38) is changing through time (day 1, 2, 4, 7, 10, 15)

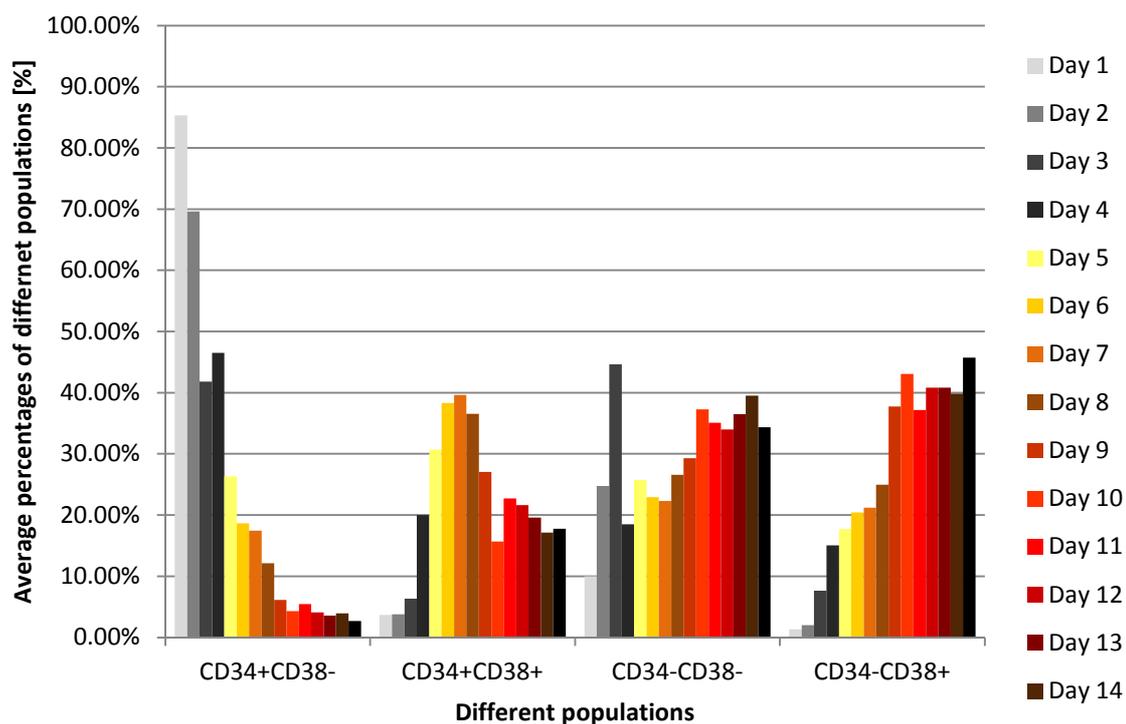


Figure 29: The average percentages of different populations (CD34⁺CD38⁻, CD34⁺CD38⁺, CD34⁻CD38⁺, CD34⁻CD38⁻) during 15 days TPO culture

In Figure 29 we can see, how the average proportion of different populations are changing through 15 day TPO culture. The CD34⁺CD38⁻ population is dropping by time, the CD34⁺CD38⁺ has its peak at days 6-8. After day four, the CD34⁻CD38^{+/-} populations are rising in percentage and after this time, they start to decrease.

Figure 30 clearly shows that the ratio of divided and non-divided cells is growing with every day.

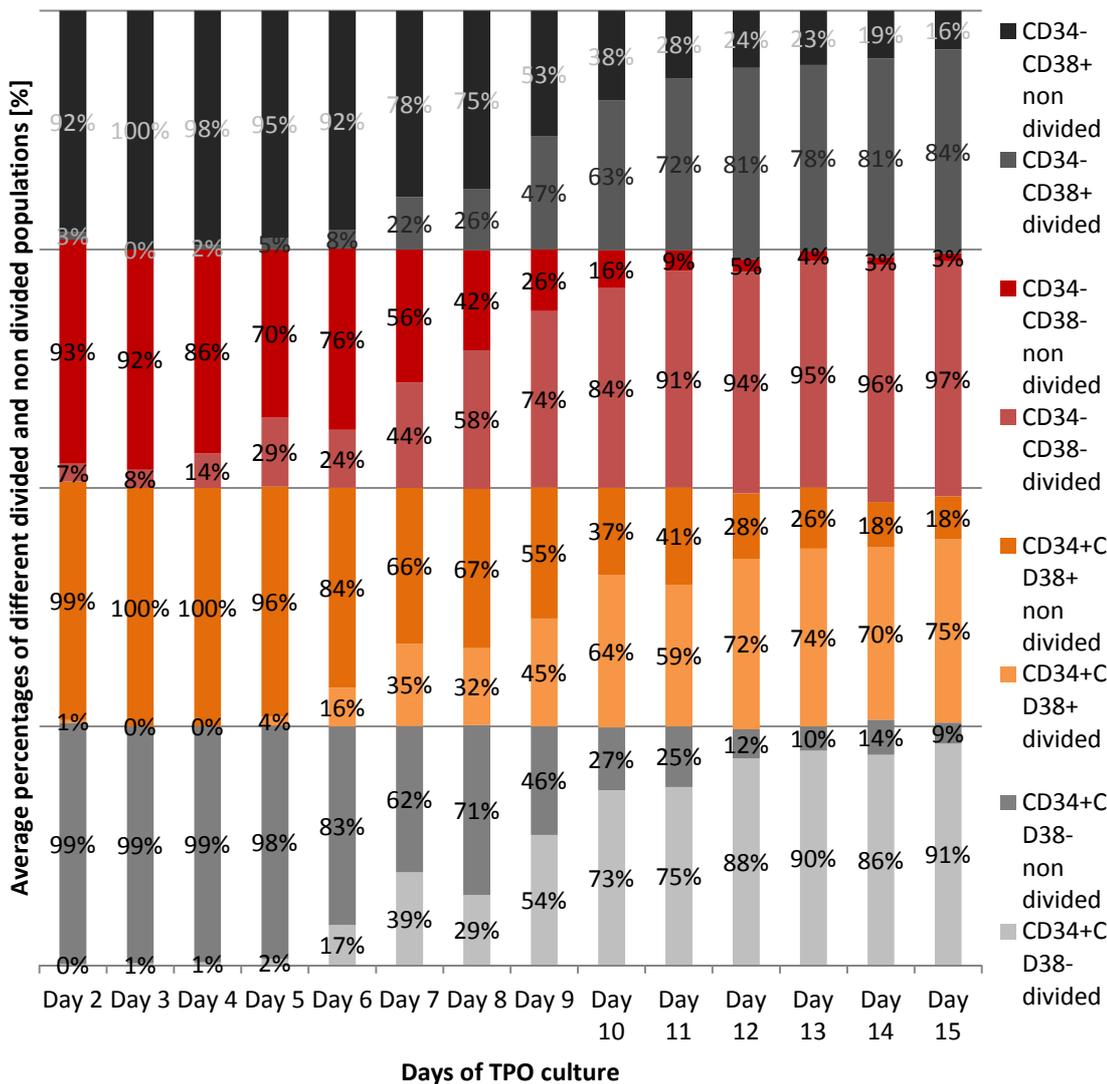


Figure 30: The average populations of different divided and non-divided ($CD34^+CD38^+$, $CD34^+CD38^-$, $CD34^-CD38^+$, $CD34^-CD38^-$) populations in 15 days TPO culture

4.5 CD9/CD61 MARKER COMBINATION

As we can see in Figure 32, the CD61 and CD9 marker expression is changing through time as well. After 15 day culture we saw a typical shape: a linear population and a population underneath it (see Figure 26). After day 5 or 6 of TPO culture, we can observe the linear population, which is double positive ($CD61^+CD9^+$). After day 8 or 9, the second population (under the linear population) appears. The $CD61^-CD9^-$ population does not disappear at the end of 15 day culture.

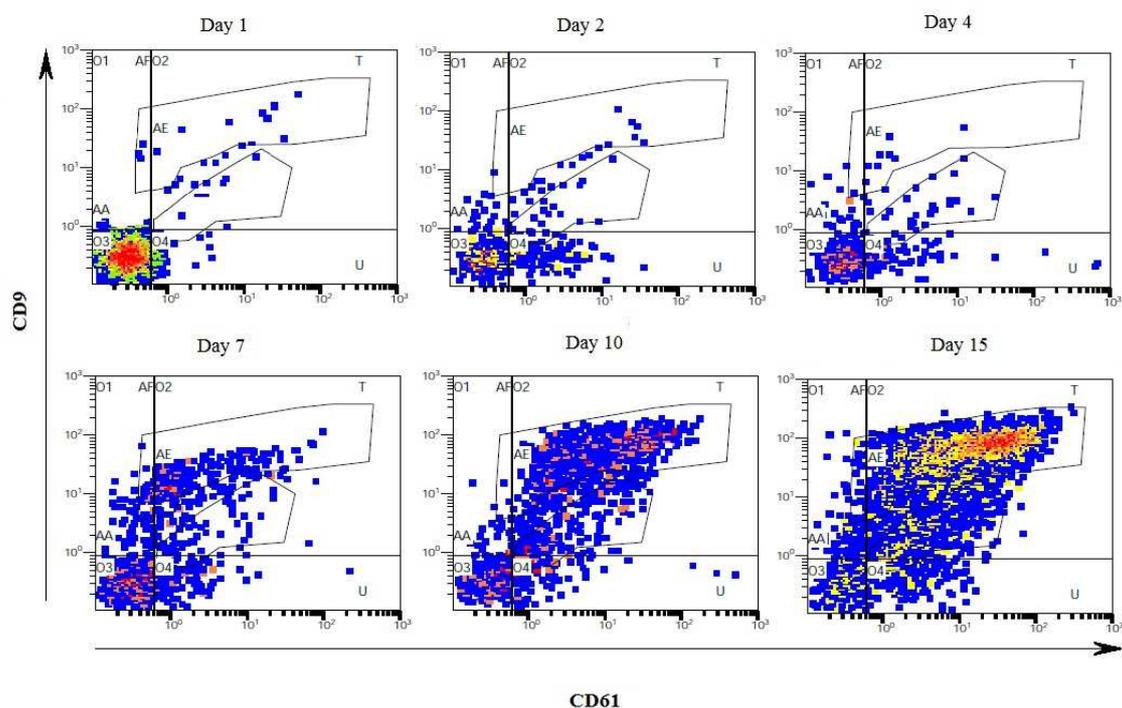


Figure 31: The most typical (density) plots from 15 day culture – we can see how the marker expression (CD61/CD9) is changing through time (day 1, 2, 4, 7, 10, 15)

4.6 EXPANSION OF CD34⁺CD38⁻ AND CD34⁺CD38⁺ POPULATIONS

We were interested in the differences between primitive HSCs (CD34⁺CD38⁻) and more mature HSCs (CD34⁺CD38⁺); therefore, we sorted the isolated HSCs to these two fractions. After 10 days of TPO culture we analyzed the results. We had some trouble with having enough cells to put in culture (in the thawing and labelling procedure we have lost many cells) that is why we have results just from two cords.

4.6.1 Expansion rate

From the positive (CD34⁺CD38⁺) fraction we observed in average a $1,9 \pm 0,1$ expansion rate, and from the negative (CD34⁺CD38⁻) fraction a $4,7 \pm 1,2$ expansion rate after 10 days of TPO culture (see Figure 32).

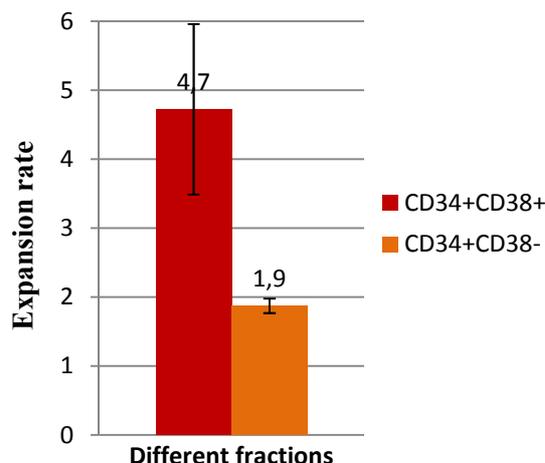


Figure 32: The average expansion rates and their standard deviations of CD34⁺CD38⁺ and CD34⁺CD38⁻ fractions with standard deviation after 10 days of TPO culture

4.6.2 Analysis of the CD34⁺CD38⁻ and CD34⁺CD38⁺ fractions

4.6.2.1 The CD34/CD61 marker combination

The CD34⁺CD38⁻ and CD34⁺CD38⁺ fractions are indeed differentiating differently. As we can see in Figure 33, the populations express same markers, but their proportions are different. The CD34⁺CD38⁻ fraction contains after 10 days of TPO culture in average 10,7% ± 4,4% of CD34⁺CD61⁻ cells, 5,1% ± 0,3% of CD34⁻CD61⁻ population, 72,9% ± 10,7% of CD34⁻CD61⁺ population and 11,4% ± 6,5% of CD34⁺CD61⁺ population. Moreover, in the CD34⁺CD38⁺ fraction we observed in average 9,1% ± 9,7 of CD34⁺CD61⁻ population, 51,9% ± 21,4% of CD34⁻CD61⁻ population, 32,3% ± 4,2% of CD34⁻CD61⁺ population and 6,8% ± 7,5% of CD34⁺CD61⁺ population.

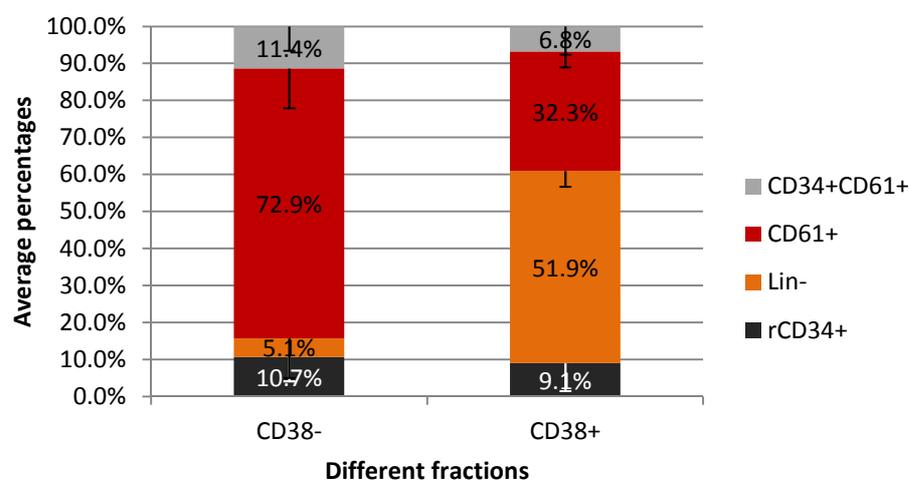


Figure 33: The average percentages and their standard deviations of different populations (rCD34⁺, Lin⁻, CD61⁺, CD34⁺CD61⁺) in the CD38⁻ and CD38⁺ fractions

4.6.2.2 The CD34/CD9 marker combination

As we can see in Figure 34, there are differences between average percentages in populations of CD34/CD9 marker combination in the CD34⁺CD38⁻ and CD34⁺CD38⁺ fractions. The CD34⁺CD38⁻ fraction after 10 days of TPO culture contains in average 9,5% ± 6,1% of CD34⁺CD9⁻ population, 8,4% ± 2,0% of CD34⁻CD9⁻ population, 70,1% ± 13,6% of CD34⁻CD9⁺ population and 11,9% ± 5,5% of CD34⁺CD9⁺ population. Moreover, in the CD34⁺CD38⁺ fraction we observed in average 8,1% ± 9,3% of CD34⁺CD9⁻ population, 22,3% ± 6,5% of CD34⁻CD9⁻ population, 62,1% ± 23,4% CD34⁻CD9⁺ population and 7,6% ± 7,6% of CD34⁺CD9⁺ population.

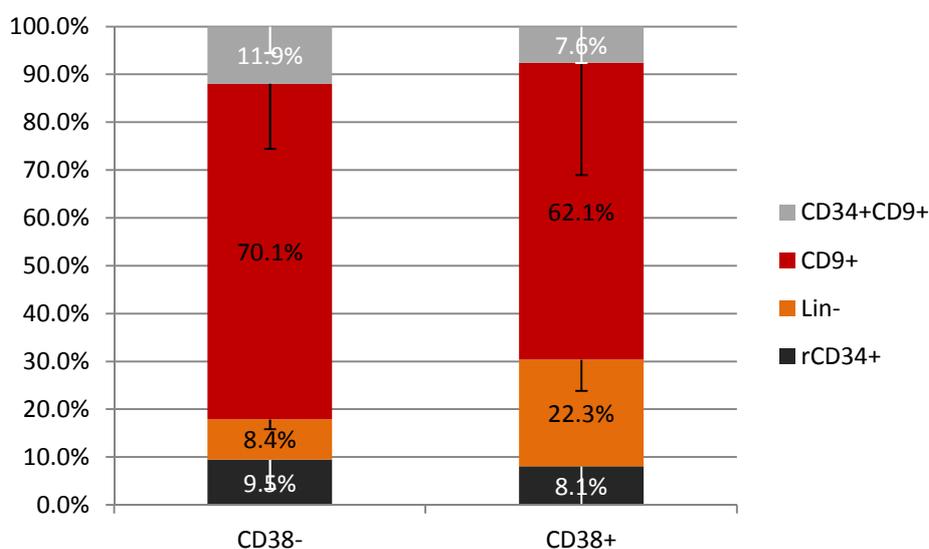


Figure 34: The average percentages and their standard deviations of different populations (rCD34⁺, Lin⁻, CD9⁺, CD34⁺CD9⁺) in the CD38⁻ and CD38⁺ fractions

4.6.2.3 The CD34/CD38 marker combination

In the CD34/CD38 marker combination populations there are differences as well (see Figure 35). The CD34⁺CD38⁻ fraction after 10 days of TPO culture contains in average 3,4% ± 0,2% of CD34⁺CD38⁻ population, 29,3% ± 16,5% of CD34⁺CD38⁺, 14,2% ± 4,9% of CD34⁻CD38⁻ and 53,2% ± 11,6% of CD34⁻CD38⁺. Moreover, in the CD34⁺CD38⁺ fraction we observed in average 0,5% ± 0,8% of CD34⁺CD38⁻ population, 12,9% ± 11,9% of CD34⁺CD38⁺, 14,8% ± 10,4% of CD34⁻CD38⁻ and 71, ± 2,0% of CD34⁻CD38⁺.

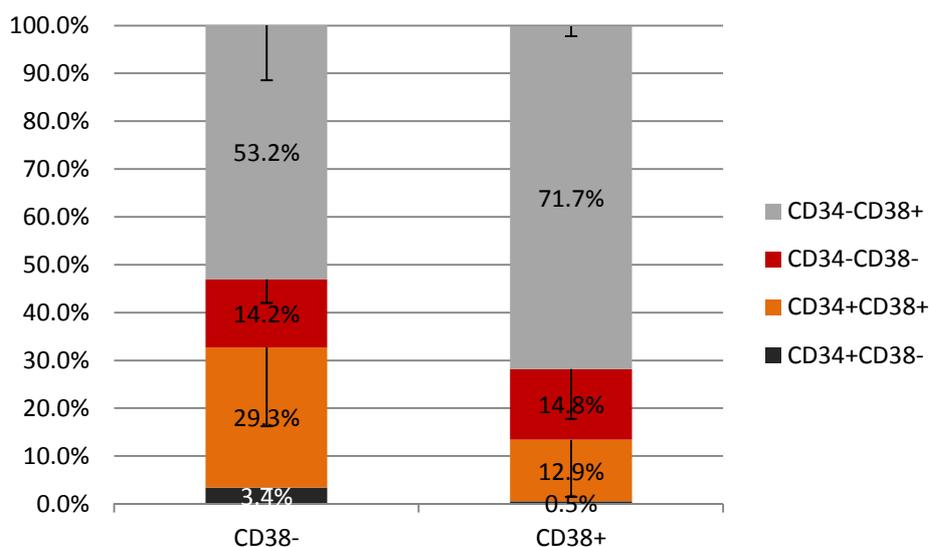


Figure 35: The average percentages and their standard deviations of different populations (CD34⁺CD38⁺, CD34⁺CD38⁻, CD34⁻CD38⁺, CD34⁻CD38⁻) in the CD38⁻ and CD38⁺ fractions

4.7 LONG TERM STEM CELL CAPACITY PRESERVATIONS – CAFC ASSAY

4.7.1 CAFC colonies after 4 and 5 weeks of CAFC culture

After 4 and 5 weeks of CAFC assay, we scored the 96 well plates. Because we didn't have enough cells to make dilutions, we just put one concentration to grow. That is why the results are given in percentages. One well out of ten cultured wells is equal to 10%.

Table 11: Scored CAFC wells after 4 and 5 weeks of culture

| Cord number | Week 4 | | Week 5 | |
|-------------|-------------------|-------------------|-------------------|-------------------|
| | CD34 ⁺ | CD34 ⁻ | CD34 ⁺ | CD34 ⁻ |
| UCB 11-196 | 100% | 90% | 100% | 50% |
| UCB 11-054 | 100% | 0% | 100% | 0% |
| UCB 11-165 | 100% | 40% | 100% | 10% |
| UCB 11-232 | 100% | 70% | 100% | 10% |

It seems that our cells indeed preserved their long term engrafting capacity, since after week 5, the CD34⁺ wells all contained cobble stone cells, in the CD34⁻, there still are some cells (see Table 11). There appeared positive CD34⁻ cells as well, but those cells are smaller in size and in number. It is also clearly seen on Figure 36.

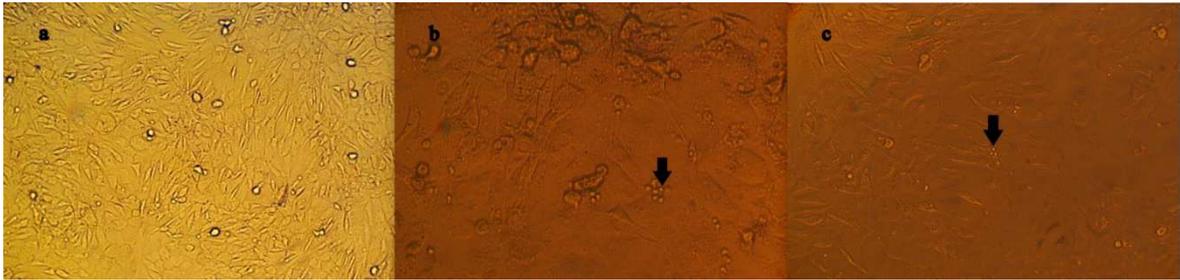


Figure 36: Pictures of CAFC cultures: a – just feeder cells, the negative control (200× zoom), b – LT-SCs in the CD34⁺ population (200× zoom), c – cobble stone cells in the CD34⁻ fraction (200× zoom) (Kristina Marton, 2012)

5 DISCUSSION AND CONCLUSIONS

Ex vivo expansion of CD34⁺ cells isolated from UCB with TPO is one of strategies to reach better SC and progenitor engraftment in the BM and therefore shorter delay in platelet production. To date, studies around this topic are promising (Schipper et al., 1998; Schipper et al., 2003; Hensbergen et al., 2006; Mattia et al., 2008; Schipper et al., 2012), but before further studies are carried out, it is important to predict the responsibility of the UCB isolated CD34⁺ cells on TPO. In this study, we evaluated the ability of those cells to expand in vitro in the presence of TPO into the megakaryocyte lineage, and explore their capacity to preserve long-term repopulating capacity.

5.1 DISCUSSION

5.1.1 Expansion rate

After 10 days of TPO culture we observed a 13, 9 ± 7, 5 fold mean expansion, as a response of CD34⁺ cells on TPO. After analyzing the expansion rates with a non-paired two sided t-test, we can conclude that our sample's data does not oppose to Schipper's data (assuming unequal standard deviations, $\alpha=0,01$). They are reporting a 12, 5 ± 5, 9 fold mean expansion.

5.1.2 CD34/CD61 marker combination

Before culturing, we observed a very small double positive (CD34⁺CD61⁺) population (0, 6% ± 0, 7%), smaller than reported by Schipper et al. from 2003 (6% ± 6%, but they were observing CD41 instead of CD61, which are expressed simultaneously). As a result of 10 days TPO culture of CD34⁺, we observed differentiation. Four populations were emerging: 1) a pro-megakaryocytic population expressing CD34⁻CD61⁺ (CD61⁺ population), in average 69,2% ± 13% of the cells; 2) the CD34⁻CD61⁻ (Lin⁻) population, in average 18,1% ± 12,1% of the cells; 3) the remaining CD34⁺ cells (CD34⁺CD61⁻, rCD34⁺), in average 6,5% ± 4,9% of all cells; and 4) the double positive population CD34⁺CD61⁺, in average 6,3 ± 7,1% of cells (shown in Figure 16). We obtained with a non-paired two sided t-test (assuming unequal standard deviations, $\alpha=0,05$) that the average percentages for the rCD34⁺, Lin⁻ and CD61⁺ population doesn't oppose to Hensbergen's data, however, we can claim that the CD34⁺CD61⁺ population is bigger. Therefore, we partially confirmed our second hypothesis. The bigger double positive population is probably due to the differences in UCB samples, as it appeared just in two of nine UCB samples.

The results from day 15 suggest that the differentiation is going further towards the megakaryocyte lineage, as the CD61⁺ population is bigger (90,1% ± 4,4%) and the other

populations smaller ($4,6\% \pm 2,2\%$ of Lin^- , $1,0\% \pm 0,5\%$ of rCD34^+ and $4,3\% \pm 4,2\%$ of $\text{CD34}^+\text{CD61}^+$) (shown in Figure 16).

The plots (shown in Figure 18) from 15 day-to-day culture suggest that the differentiation goes from the isolated CD34^+ cells ($\text{CD34}^+\text{CD61}^-$), follows with loosing the CD34^+ marker at day 2 towards Lin^- ($\text{CD34}^-\text{CD61}^-$) and after day 5-7 gaining the CD61^+ marker starts. After 12-13th day of culture two populations in the CD61^+ population are visible (see Figure 18). The same conclusions are suggested by the average percentages of different population in the 15 day culture with TPO (Figure 19), where the rCD34^+ population is dropping day-to-day, the Lin^- population peaks in day 3-6, and the CD61^+ population is growing every day. This phenomenon, which is just the opposite form BM-derived CD34^+ cells, where the differentiation follows with gaining the CD61^+ and then with loosing the CD34^+ , was also observed by Schipper and colleagues (Schipper et al., 2003). The small double population peaking at days 6 to 9 (Figure 19) suggests that some cells of the UCB choose to differentiate the other way around. This means that both of the scenarios can take place, but rarely. The CD61^+ population does not provide platelet production (Schipper et al., 2012); therefore, it represents more mature cells, the pro-megakaryocytes and their progenitors. Starting from the differentiation course model suggested by Schipper et al., the CD61^+ cells are more mature than the Lin^- (Schipper et al., 2003). Schipper and colleagues reported about a high megakaryocyte ($\text{CD34}^-\text{CD41}^+$, the CD41 and CD61 are expressed simultaneously) suspension after 14 days of TPO culture, which was 85% of all cells, our data does not oppose to this (in average $86,9\% \pm 4,9\%$ of CD61^+ on day 14).

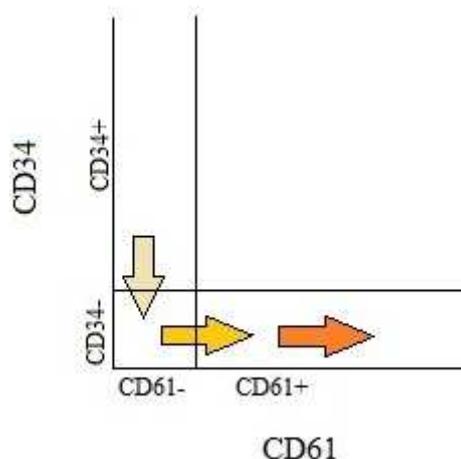


Figure 37: The $\text{CD34}/\text{CD61}$ differentiation starts from CD34^+ cells, which lose their CD34^+ marker and gain CD61^+ : we can see two CD61^+ populations

Schipper et al. report that the rCD34^+ and the Lin^- populations are responsible for long-term engraftment. They state that the composition of the expanded product is more important than the number of it: more rCD34^+ and Lin^- cells are in a product, better it is. Therefore, when looking at our data from 15 day-to-day cultures, the optimal time for

maximizing the ratio of rCD34⁺ and Lin⁻ populations in a sample should be cultures that last for 3-5 or 6 days, depending on expansion.

With the PKH26 label, we had insight in the cell divisions as well. From those results we could make some conclusions, how the differentiation was happening (Figure 20). In general, the percentages of the undivided cells are dropping through time. The percentages of the divided cells are rising – logically.

As Schipper et al. reported the HSCs and progenitors, which enable long-term engraftment, are located in the rCD34⁺ and Lin⁻ populations after 10 days of TPO culture. Therefore, we can conclude that after culture, the rCD34⁺ cell population contains stem cells. The same can be concluded for the Lin⁻ population, but here we can find SCs and progenitors, which did not express CD34⁺ marker. The majority of the rCD34⁺ after 10 days of TPO culture should be divided cells (asymmetric cell division), in average, there were 91,1% ± 5,6% of the CD34⁺ cell population after 10 days of TPO culture divided as shown in Figure 17. The undivided cells represent those CD34⁺ cells, which did not respond with expansion on TPO, 9, 2% ± 6, 3%. With the existence of undividing populations we confirmed our first hypothesis.

We expected the Lin⁻ population to be expanded after 10 days of TPO culture. Our data shows that in average just 75, 8% ± 13, 5% of the Lin⁻ population was divided and in average 26, 87% ± 10, 53% of them was not (Figure 17). This can be due to impurities or constituting that cells can lose their markers without expanding.

The CD61⁺ cell population after 10 days of TPO culture represents pro-megakaryocytes or their progenitors. We expect that cells gain their markers with divisions (94, 0% ± 3, 8% divided), but we still saw some undivided cells, in average 6, 0% ± 4, 2% (Figure 17). This implies that the differentiation can take place without cell division. Because of some reason, some cells just lose their marker and gain some other, without divisions. For a long time it was believed that differentiation without proliferation cannot take place. However, it cleared out that the processes of proliferation and differentiation are regulated independently (Brown et al. 2003; Andäng et al., 2008). Maybe something similar was going on in our cultures as well. Differentiation without proliferation was observed in embryonic SCs and neural crest SCs, peritoneal B cells, T cells, skeletal muscle cells, the cells of PC12 cell line (rat neuronal cells), and myeloid cells, but not for megakaryocytes (O'Neill and Stockdale, 1972; Rudkin et al., 1989; Carson et al., 1999; Cox et al., 2006; Andäng et al., 2008). In addition, we prepared a model of how differentiation could go on in a 15 day TPO culture, shown in Figure 38. In more details, the outcome after culturing the cells with TPO can be as follows: 1) the cells remain undivided, they don't change their markers; 2) some of the undivided cells can contain CD61 right in the beginning after the isolation; 3) the isolated cells can divide asymmetrically, one daughter cell can lose the

CD34 marker or rarely, the CD34 marker is kept and the CD61 is gained, and the other daughter cell remains CD34⁺; 4) the differentiation can go on without cell division as well, the cells can lose their CD34 marker and gain CD61, without cell division; 5) the divisions can be symmetric as well, from one CD34⁺ cell, two Lin⁻ daughter cells can occur which can differentiate and proliferate to CD61⁺ cells; 6) the differentiation can sometimes go towards the double positive population as well, first, the CD61 marker is gained and after it the CD34 wanes.

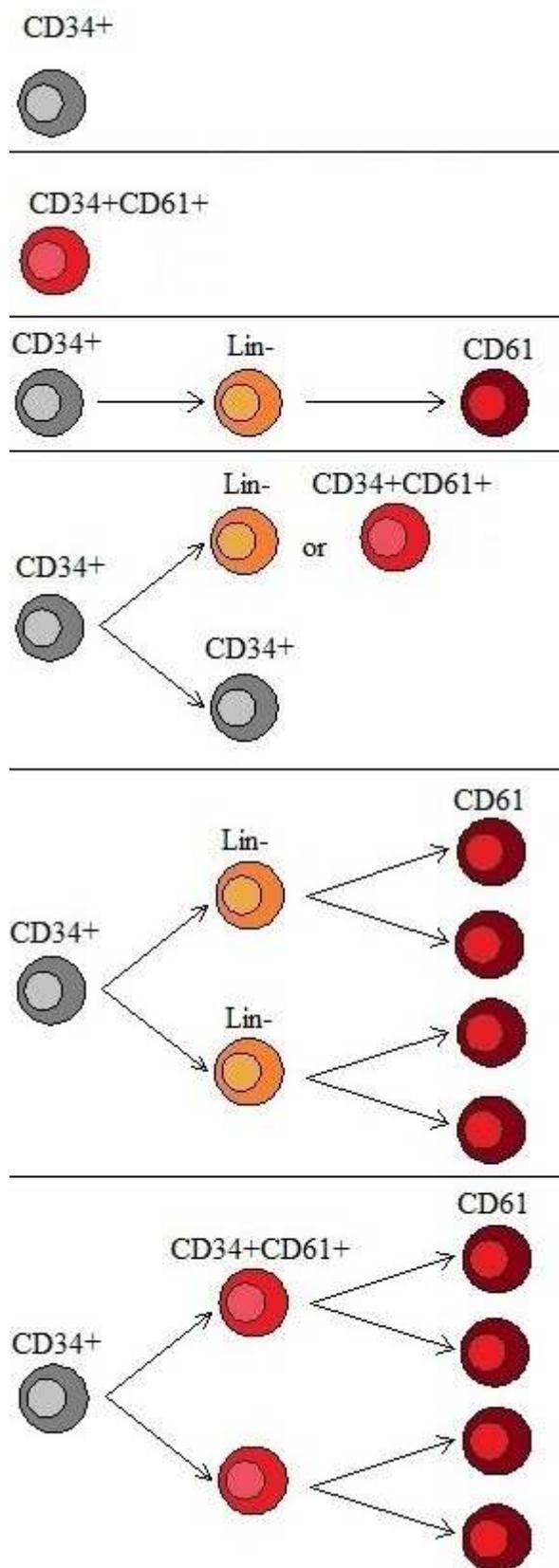


Figure 38: Possible outcomes of differentiation of UCB HSCs while TPO culture

5.1.3 CD34/CD9 marker combination

The CD9 and CD61 are expressing similarly, the CD9 is expressed a little bit earlier (Le Naour et al., 1997). Therefore, it is not a surprise that the results are very similar as well. Before culturing, we observed a double positive population here as well (CD34⁺CD9⁺, 0, 4% ± 0, 5%). After analyzing the correlation between CD34/CD61 and CD34/CD9 marker combinations, we can claim that the average percentages for the different CD34/CD9 populations doesn't oppose from the average proportions of CD34/CD61 populations (non-paired two sided t-test, $p < 0,1$). Due to 10 days of TPO culture with CD34⁺ cells, we observed four populations as well: 1) a pro-megakaryocytic population expressing CD34⁻CD9⁺ (CD9⁺ population), in average 67,6% ± 6,1% of the cells; 2) the CD34⁻CD9⁻ (Lin⁻) population, in average 19,5% ± 8,4% of the cells; 3) the remaining CD34⁺ cells (CD34⁺CD9⁻, rCD34⁺), in average 5,1% ± 2,7% of all cells; and 4) the double positive population CD34⁺CD9⁺, in average 7,9 ± 7,2% of cells (shown in Figure 21). Even the double positive population was similar.

The differentiation in CD34/CD9 combination after 15 days was progressing more compared to 10 day culture as well as in CD34/CD61 marker combination. In addition, the differentiation course is very much the same. The differentiation starts with losing the CD34 marker is on the 2nd day as well, the CD9 marker gaining is intensively starting on day 5 too, but the CD9⁺ population gains two visible populations right after 7th day, five days sooner (Figure 23). Interestingly, if we have a better look on Figure 24, the Lin⁻ is peaking later, on days 5 to 9. In brief, the differentiation pattern is similar to CD34/CD61; the differences are just in the days of changes. With these results, we confirmed the data from Le Naour.

Moreover, the average of divided and undivided cells in different populations was not different then in CD34/CD61 marker combination (non-paired two-sided t-test, $p < 0, 1$). The undivided cells in all population were present as well. Some of them represent the CD34⁺ cells, which did not respond to TPO, and obviously, there was a cell population which responded to TPO with differentiation towards megakaryocytes without cell division, too.

5.1.4 CD34/CD38 marker combination

The CD38 marker can be a tool to distinguish between primitive SCs and more mature ones. Usually the very early HSCs from UCB express CD34 marker but not CD38 (CD34⁺CD38⁻) and the more mature HSCs express them both (CD34⁺CD38⁺) (Ishikawa et al., 2003). Belvedere and colleagues reported that usually in the CD34⁺ UCB cells, there are 96,08% ± 3,18% of CD34⁺CD38⁺ cells, the rest is CD34⁺CD38⁻ of course (Belvedere et al., 1999). However, our results represent a complete opposite story, since our data

shows an 85, 3% \pm 3, 2% of CD34⁺CD38⁻ population. Maybe it is due to some differences in UCB samples and as a result of a too small sample size without representative samples.

The CD34⁺ cells from UCB responded on TPO after ten days of culture with differentiation. Four populations emerged: 1) the CD34⁺CD38⁻ population, usually containing remaining primitive HSCs, 6,0% \pm 4,1%, the smallest population, probably while they are having many asymmetric cell division (one daughter stem cell, the other a more mature cell); 2) the CD34⁻CD38⁻ population, usually the most mature cells (34,5% \pm 16,5%; 3) the CD34⁻CD38⁺ population, 39,3% \pm 16,1%; and 4) the CD34⁺CD38⁺ population, usually containing more mature HSCs, 20,3 \pm 14,9% (Figure 26). It is interesting that the proportion of the CD34⁻CD38⁺ population was not statistically different ($p < 0, 1$) from the CD34⁻CD38⁻ population. If we compare the proportions from the CD34⁻CD61^{+/-} with the CD34⁻CD38^{+/-} population we can state that there are no differences in proportions ($p < 0, 1$). However, the CD34⁺CD38^{+/-} population is bigger than the CD34⁺CD61^{+/-} ($p < 0, 1$). This is probably due to the fact that in the database of CD34/CD61 samples were bigger than from CD34/CD38; in the CD34/CD61 were some additional UCB samples. The CD38 marker is believed to be expressed on immune cells, including CD4⁺, CD8⁺, B-cell and natural killer cells (Malavasi et al., 1994), but probably here they weren't, since the growth factor TPO led the differentiation towards platelets.

The results from day 15 suggest further differentiation, as the CD34⁺CD38^{+/-} populations are smaller than at day 10 and the CD34⁻CD38^{+/-} population bigger. The both of CD34⁻CD38⁻ and CD34⁻CD38⁺ populations had not different proportions ($p < 0, 05$). After 15 days of TPO culture we would expect that the latter will be bigger as it was more differentiated (Figure 26). Again, the CD34⁺CD38^{+/-} population compared to CD34⁺CD61^{+/-} was bigger and here the CD34⁻CD38^{+/-} population was smaller than the CD34⁻CD61^{+/-}. As mentioned before, this was probably due to a small sample size, because we did not compare same samples (the samples were not representative).

The differentiation pathway of CD34/CD38 is normally expected to be as follows: CD34⁺CD38⁻ \rightarrow CD34⁺CD38⁺ \rightarrow CD34⁻CD38⁺ \rightarrow CD34⁻CD38⁻. This suggested our data as well; if we look at Figure 28, the CD34⁺CD38⁻ population was decreasing through time and on days 4-5 all four population emerged. After the 11th day, the CD34⁺CD38^{+/-} populations started to disappear and from day 8 on the CD34⁻CD38⁻ population started to strengthen. For better visualizing of the differentiation course see Figure 39. In addition, in Figure 29 we can see that the CD34⁺CD38⁻ population was dropping through time, due to the asymmetric cell division their number stays same, however the others number increases, so compared to the whole sample, their percentage is dropping. At days 6-8 the double positive population (CD34⁺CD38⁺) peaks, after this time, it starts to decrease, which is an expected phenomenon, since the TPO induces differentiation, the number of mature cells is rising. After day four, the CD34⁻CD38^{+/-} populations were rising in

percentage, as a result of differentiation: the cells are dividing, gaining megakaryocyte markers. The odd point, the third day in the CD34⁻CD38⁻ we cannot explain.

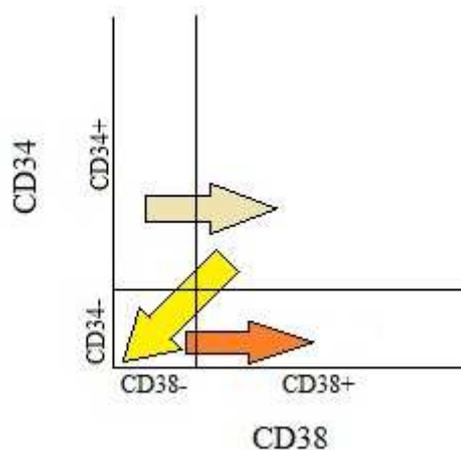


Figure 39: The differentiation model of CD34/CD38 marker combination

As previously told, the CD34⁺CD38⁻ population is the population that is thought to contain multi-potent and primitive progenitor cells, with long-term repopulating capacity. They are usually highly divided, however in our case just 73, 5% ± 26, 2% of this population was divided after 10 days of TPO culture (Figure 27).

The CD34⁺CD38⁺ cell population represents the more mature stem cells, which does not show long-term repopulating capacity. When their source is UCB, they divide many times (Hao et al., 1995). This wasn't the case in our study, since the average percentage of the CD34⁺CD38⁺ divided cells was 64,1% ± 27,4% (Figure 27).

The CD34⁻CD38⁻ and CD34⁻CD38⁺ cell populations were mature cells in different maturation stages. This is clearly shown in our data for CD34⁻CD38⁻, but not for CD34⁻CD38⁺, as the cells are in the latter just 63, 1% ± 22, 9% divided (Figure 27).

However, there were many cells which lost their CD34 or CD38 markers or gained the CD38 without expansion, so again: cells can lose and gain their markers without cell division.

In Figure 30, we can clearly observe that the percentage of the undivided cells is dropping through time. The percentages of the divided cells were rising – logically. The percentages are similar to data from other marker combinations.

With the PKH26 label, we had insight in the cell divisions as well. From those results we could make some conclusions, how the differentiation was going on (Figure 40). In more details, the response of the CD34⁺ isolated cells from UCB can be as follows: 1) some cells

don't respond to TPO; 2) the most primitive cells ($CD34^+CD38^-$) are probably dividing asymmetrically, one of the daughter cells gains a CD38 marker and the other remains as it's mother cell; 3) we isolate the $CD34^+CD38^+$ cells as well (with $CD34^+$ isolation) and some of them do not respond to TPO; 4) the $CD34^+CD38^+$ cells can divide symmetrically with or without differentiation, which starts with losing the CD34 marker, and after the CD38 as well; 5) the $CD34^+CD38^+$ cells can divide asymmetrically as well, one daughter cell loses its CD34 marker and the other remains as it's mother cell; 6) we observed differentiation without cell proliferation as well. We saw that there is a $CD34^+CD38^-$ population, which did not expand, cells, which did not respond to TPO. There were $CD34^+CD38^-$ expanded cells as well. From this, we can conclude that the $CD34^+CD38^-$ population is not homogeneous. The interesting observation was the undivided $CD34^+CD38^+$. Obviously, there are beside the divided $CD34^+CD38^+$ cells also cells, which are not responding to TPO. The undivided $CD34^+CD38^-$ was a surprise. Obviously, beside the CD34, CD61, CD9, the CD38 marker can be lost without division as well. As we expected, the $CD34^-CD38^-$ divided population was observed as well. The $CD34^-CD38^+$ divided cells were not a surprise, since the CD34 is usually vanishing with divisions, but sometimes not, as we can conclude from $CD34^-CD38^+$ undivided cells.

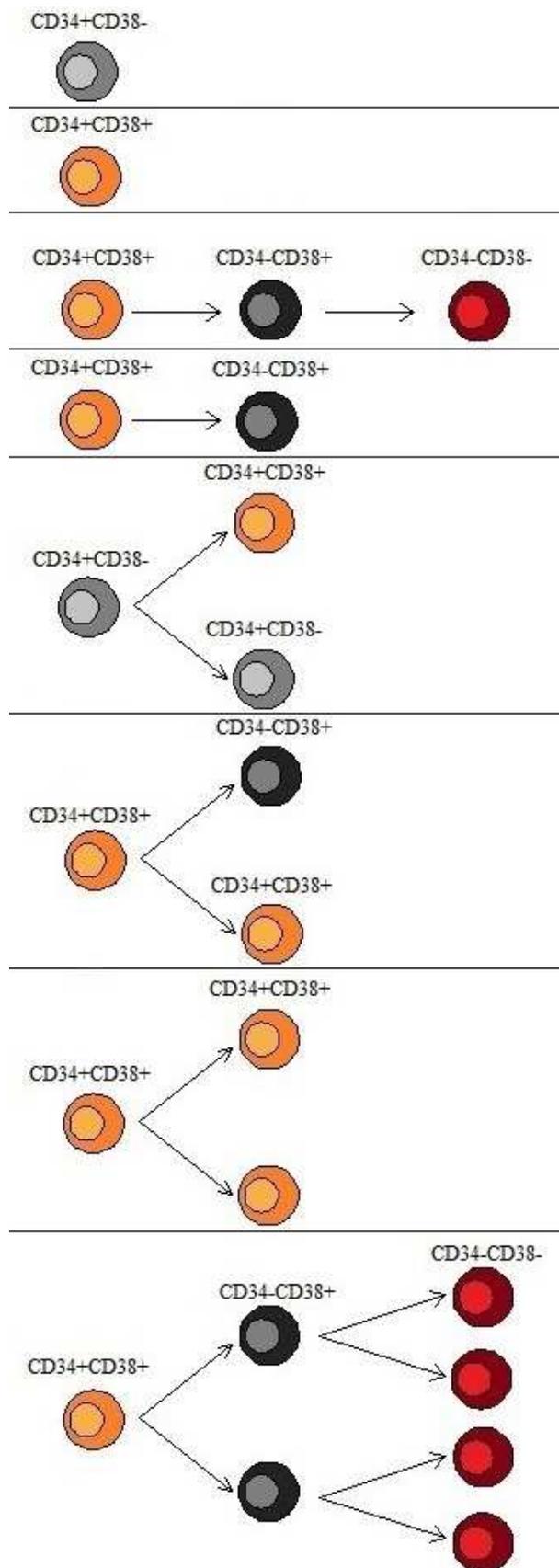


Figure 40: Possible outcome of differentiation of UCB HSCs while TPO culture

5.1.5 CD61/CD9 marker combination

The CD61 and the CD9 markers are expressed almost simultaneously; the CD9 appears earlier (Le Naour et al., 1997). This is suggested as well by our data, since the usually plot after 10 days of culture are of a specific plot pattern, as follows in figure 31. There is always a linear population on the diagonal between CD61 and CD9, as they are expressed simultaneously. The population above the linear one is the consequence of CD9 appearing earlier.

5.1.6 Expansion of CD34⁺CD38⁻ and CD34⁺CD38⁺ populations

The CD34⁺CD38⁻ population is thought to be very early HSC population, which is more reactive on factors, such as TPO for example. In this chapter, we discuss the differences between the two fractions after separate 10 day of TPO culturing.

5.1.6.1 Expansion rate

With the results, we confirmed our third hypothesis, there is indeed a difference in the expansion between the positive and negative fraction. As we expected, the negative fractions (CD34⁺CD38⁻, CD38⁻) divided more times as the positive fraction (CD34⁺CD38⁺, CD38⁺) (CD34⁺CD38⁻ fraction $4,7 \pm 1,2$ fold, the CD34⁺CD38⁺ fraction $1,9 \pm 0,1$ fold, Figure 33). The positive population is dividing as well, because there are heterogenic, but not that much as the negative fraction. However, Schipper et al. reported about a 50 fold expansion of CD34⁺CD38⁻ fraction after 14 days of culture. Even if we would leave the cultures for four more days, we probably could not reach the same expansion.

Anyway, if we compare the expansion rate to not separated fractions, the expansion calculated by us, is smaller, since Hensbergen and colleagues reported a $7,3 \pm 1,2$ fold expansion (range 3,8 to 12,8). The lower average is probably due to the small sample number.

5.1.6.2 CD34/CD61 marker combination

The negative and the positive fractions are indeed differentiating differently, they respond to TPO with a different rate of differentiation. In Figure 33 we can see that four emerging populations are the same, but their proportions are different. The CD38⁻ fraction is differentiated more (contained more CD61⁺ cells); statistically it is not different from the CD34/CD61 or CD34/CD9 results ($p < 0, 1$). Consequently we confirmed our forth hypothesis. The proportions of populations in the CD38⁻ fraction, however, were different. It looks that the positive fraction differentiated slower, since the rCD34⁺'s and the double

positive's population rate were not different, but the CD61⁺ population in the positive fraction was smaller and the Lin⁻ bigger. This was suggested by Hao et al. as well (Hao et al., 1995). Therefore it looks that the negative fraction contributes to overall differentiation mostly, the differentiation from the positive fraction just fill it. However, it is of course mostly dependent on the expansion rate.

5.1.6.3 The CD34/CD9 marker combination

Le Naour et al. stated that the CD9 is expressing earlier during megakaryocyte differentiation than CD61. This is clearly seen in Figure 34 compared to 33, since the results from the positive fractions contain more CD9⁺ population than in the negative fraction. For further investigation, it would be interesting to follow the differentiation of those different fractions every day. In addition, the data from the negative fraction did not differ from the CD34/CD61 marker combination in the negative fraction ($p < 0, 1$). Moreover, the differentiation in the positive fraction compared to the negative, seems to be slower as well (Figure 34).

5.1.6.4 The CD34/CD38 marker combination

The remaining CD34⁺CD38⁻ population in the negative fraction is probably due to the asymmetric division. When compared the negative fraction to the 10th day results from 15 day culture (CD34/CD38) we can only state for the CD34⁺CD38⁻ population that has statistically same result, with $p < 0, 1$. The others appear in different ratio. In average, all populations are more differentiated. This is completely logical, as we expected. However, when the negative fraction compared to the positive, the CD34⁻CD38⁻ average percentage is statistically same, the CD34⁺CD38⁻ is logically higher, the CD34⁻CD38⁺ is lower. Therefore, we can conclude that the differentiation (lost of CD34 and CD38) is slower than in CD38⁺ (Figure 35). However, literature states the opposite (Hao et al., 1995). This was probably due to the small sample size.

5.1.7 Long term stem cell capacity preservations – CAFC assay

Usullay after HSC transplantation we would like to have a long lasting engraftment. However, sometimes after *ex vivo* expansion the cells loose their capacity of long-term engrafting that is why it is important to predict it. One of the possibilities is the CAFC assay. It seems that our cells indeed perserved their long term engrafting capacity, since after week 5 all CD34⁺ wells contained cobble stone cells (shown in Table 11). Hereby we confirmed our fourth hypothesis. However, in the CD34⁻, there still are some cobble stone cells, wchich were smaller in size and in number (see Figure 36). The positive CD34⁻ scores appeared probably because of not 100% pure sorting, or they are the positive cells from Lin⁻ population, Schipper et al. reported about.

5.2 CONCLUSIONS

The majority of CD34⁺ cells respond to TPO with expansion and differentiation towards megakaryocyte lineage. In the CD34/CD61 marker combination, four populations emerge: rCD34⁺, Lin⁻, CD61⁺ and CD34⁺CD61⁺, where the differentiation mostly goes as follows: CD34⁺ → Lin⁻ → CD61⁺, rarely, the cells choose an other way: CD34⁺ → CD34⁺CD61⁺ → CD61⁺, which is typical for MB-derived CD34⁺ cells. The CD34⁺ UCB cells after 10 days of TPO culture divide and differentiate on different ways. Furthermore, we observed differentiation without proliferation, which was not known for CD34⁺ UCB expanded cells towards megakaryocyte lineage until now. We can make similar conclusions for CD34/CD9 marker combination.

In the CD34/CD38 marker combination, four populations emerged and differentiated as follows: CD34⁺CD38⁻ → CD34⁺CD38⁺ → CD34⁻CD38⁺ → CD34⁻CD38⁻. Again, we observed differentiation without proliferation, the cell division and differentiation combinations are different.

The CD34⁺CD38⁻ fraction of UCB, the fraction, where the primitive HSCs are believed to be, expand more than the CD34⁺CD38⁺ fraction, where the more mature HSCs are believed to be. In addition, the differentiation in the CD34⁺CD38⁻ is more progressed as in the CD34⁺CD38⁺ fraction.

The remaining CD34⁺ cells after 10 days of TPO culture are keeping their long-term repopulation activity.

We can conclude that this kind of optimizing strategy can be a promising one, since the cells are keeping their long-term repopulating activity (there are still LT-HSCs), and are expanded as well. Schipper and colleagues stated that the best engraftment is due to rCD34⁺ and Lin⁻ cells, which was observed in 3-6 day TPO culture, so the choice should be in that interval of days. Of course, further investigation is necessary.

6 SUMMARY (POVZETEK)

6.1 SUMMARY

UCB is an important HSC source. Its bigger disadvantage is that in an average unit (around 100 ml) of UCB it does not contain enough cells to treat an adult patient, which often results in platelet production delay after engraftment. Therefore, some strategies to optimize engraftment were developed; one of them is TPO expansion of CD34⁺ cells. In order to carry out further investigation with this approach, we explored the response of CD34⁺ cell on TPO.

The aim of our study was to determine how the CD34⁺ isolated cells from UCB respond to TPO. In more details, we were interested in how much can they expand and differentiate under the influence of TPO, and if they are keeping their long-term repopulation capacity, for which the CD34⁺CD38⁻ population is believed to be responsible. That is why we were interested in the differences between expansion and differentiation capacity of the CD34⁺CD38⁻ and CD34⁺CD38⁺ fractions.

The CD34⁺ were isolated from UCB with the method MACS (Mini-Macs, Milteny Biotec, GmbH, Bergisch-Gladbach, Germany), following the manufacturer's instructions.

In order to determine the response of the CD34⁺ isolated cells from UCB to TPO (KIRIN; Brewery Ltd, Japan), we coloured the CD34⁺ cells with PKH26 fluorescent dye (Sigma, Rotterdam, following the manufacturer's instructions), which binds equally to the cell membrane and therefore it is a good marker of cell divisions. The coloured cells were cultured 10 (to know the general response after culture to TPO) or 15 days (to follow all the markers every day) under standard culture conditions with addition of 5 ng/ml TPO. The properties of the expanded cells were analyzed with a flow cytometer (CYTOMICS FC 500, Beckman Coulter). We determined the markers using monoclonal antibodies: anti-CD9-FITC (megakaryocytes), anti-CD34-PC5 (SCs), anti-CD38-FITC (primitive/more mature SCs), anti-CD61-PC7 (megakaryocytes) (Beckman Coulter, Netherlands, following the manufacturer's instructions); and the intensity of the PKH26 dye in different appearing populations with the CXP, 2.0 software.

The CD34⁺CD38⁻ population is responsible for the long-term repopulating capacity, but we were interested in differences between CD34⁺CD38⁻ and CD34⁺CD38⁺ fraction. We determined it with separating the CD34⁺ cell into these two fractions with a cell sorter (FACS Aria, Becton, Dickinson and Company). We coloured each of the fractions with the PKH26 fluorescent dye and cultured them separately under standard culture conditions with addition of 50 ng/ml TPO. The cultured cells were analyzed with a flow cytometer on markers listed above.

The long term repopulating capacity was determined with the CAFC assay.

The majority of CD34⁺ cells respond to TPO with expansion and differentiation towards megakaryocyte lineage. In the CD34/CD61 marker combination, four populations emerge: rCD34⁺, Lin⁻, CD61⁺ and CD34⁺CD61⁺. In addition, the differentiation mostly goes as follows: CD34⁺ → Lin⁻ → CD61⁺, rarely, the cells choose an other way: CD34⁺ → CD34⁺CD61⁺ → CD61⁺, which is typical for MB-derived CD34⁺ cells. The CD34⁺ UCB cells after 10 days of TPO culture divide and differentiate on different ways. Furthermore, unexpectedly we saw differentiation without proliferation, which was not clear for CD34⁺ UCB expanded cells towards megakaryocyte lineage until now. The results from CD34/CD9 marker combination suggest the same conclusion from CD34/CD61 marker combination with slight differences, since the CD9 and CD61 are expressed simultaneously, the CD9 marker appears earlier.

In the CD34/CD38 marker combination, because of CD34⁺ cell's response to TPO, four populations emerged and differentiated as follows: CD34⁺CD38⁻ → CD34⁺CD38⁺ → CD34⁻CD38⁺ → CD34⁻CD38⁻. Again, we observed differentiation without proliferation, the cell division and differentiation combinations are different.

The CD34⁺CD38⁻ fraction of UCB, the fraction, where the primitive HSCs are believed to be, expand more than the CD34⁺CD38⁺ fraction, where the more mature HSCs are believed to be. In addition, the differentiation in the CD34⁺CD38⁻ is more progressed as in the CD34⁺CD38⁺ fraction.

The remaining CD34⁺ cells after 10 days of TPO culture are keeping their long-term repopulation activity.

We can conclude that this kind of optimizing strategy can be a promising one, since the cells are keeping their long-term repopulating activity (there are still LT-HSCs), and are expanded as well. Schipper and colleagues stated that the best engraftment is due to rCD34⁺ and Lin⁻ cells, which was observed in 3-6 day TPO culture, so the choice should be in that interval of days. Of course, further investigation is necessary.

6.2 POVZETEK

Število presaditev krvotvornih matični celic (KMC) se čez čas povečuje zaradi njihove pomembne vloge pri zdravljenju levkemije, raka, anemij in ostalih bolezni (Laughlin et al., 2004, cit. po Broxmeyer, 2010). Klasičen vir KMC je kostni mozeg ali mobilizirana periferna kri. Kasneje se je začela tudi uporaba KMC iz popkovnične krvi. Izolirane KMC iz kostnega mozga imajo kar nekaj slabosti kot so: visok zahtev po ujemanju človeškega levkocitnega antigena (HLA), tveganje za darovalca, tveganje prenašanja bolezni z

darovalca na prejemika in zapozneta razpoložljivost. Prav zaradi teh lastnosti predstavljajo KMC, izolirane iz popkovnične krvi, prednost, saj so razpoložljivi za 25-36 dni prej kot KMC iz kostnega mozga, ujemanje HLA je potrebno na štirih lokusih izmed šestih, frekvenca KMC v vzorcu je večja (Rocha et al., 2010).

Poleg vseh prednosti, ki jih KMC iz popkovnične krvi predstavljajo, imajo tudi nekaj slabosti, ki so vse povezane z nizkim številom KMC v povprečni enoti popkovnične krvi (okoli 100ml) v primerjavi s povprečno 500 ml enoto krvnega mozga ali imobilizirane periferne krvi. Te slabosti so zapoznelo vraščanje nevtrofilcev, limfocitov T, najbolj pa trombocitov (Schipper et al., 2003). Da povzamem; v eni enoti popkovnične krvi je premalo KMC za zdravljenje odraslega pacienta, kar v večini primerov privede do potrebnih transfuzij trombocitov. Prav zato poteka razvoj novih pristopov za izboljšanje vraščanja izoliranih celic, kot je uporaba specifičnih zaviralnih in aktivirajočih peptidov v kostnem mozgu darovalca (Campbell et al., 2007, cited by Rocha et al., 2010), injekcija KMC iz popkovnične krvi direktno v kostni mozeg pacientovih dolgih kosti (Frassoni et al., 2008, cited by Rocha et al., 2010), transplantacija dveh enot popkovnične krvi (Barker et al., 2005, cited by Rocha et al., 2010), koinfuzija s haploidentičnimi od T limfocitov osiromašenimi vzorci (Fernandez et al., 2003, Bautista et al., 2009), koinfuzija z multipotentnimi mezenhimalnimi matičnimi celicami (Macmillan et al. 2009) ali nemieloalativno kondicioniranje pacientov (Del Toro et al., 2004). Rezultati teh pristopov so še vedno v postopku ocenjevanja.

Ex vivo ekspanzija KMC iz popkovnične krvi v megakariocitno celično linijo, torej v prekursorje trombocitov s trombopoietinom (TPO), je ena izmed možnosti za izboljšanje vraščanja transplantatov, bolj natančno trombocitov. Največja skrb z *ex vivo* namnoženimi celicami je riziko izčrpanosti matičnih celic, kar lahko privede do izgube dolgotrajne repopulacijske sposobnosti (Boiron et al., 2006). Rezultati van Hensbergena in sodelavcev so pokazali hitrejša vgradnja trombocitov po transplantaciji *ex vivo* namnoženih KMC s TPO v NOD/SCID mišjem modelu (van Hensbergen et al., 2006). Zadnje študije kažejo, da populacije, pridobljene z *ex vivo* pomnoževanjem CD34⁺ celic s TPO, prispevajo k boljšemu vraščanju trombocitov in dolgotrajni repopulaciji KMC v kostni mozeg. Ti populaciji sta rCD34⁺, ki sta ohranile sioj marker po gojenju in Lin⁻, kjer celice ne izražajo nobenega linijsko značilnega markerja po namnoževanju. Populacija CD61⁺ (to so celice, ki izražajo marker, značilen za megakariocitne celice) ne vpliva na izboljšavo vrščanja, je pa najbolj številna po gojenju s TPO (Schipper et al., 2012). Dolgotrajna repopulacijska sposobnost je lastnost, ki jo pripisujejo populaciji CD34⁺CD38⁻, ki naj bi vsebovala primitivne in multipotentne progenitorje (Sutherland et al., 1989; Hao et al., 1995; Ishikawa et al., 2003). Na kratko, Schipper in sod. menijo, da celice, ki so bile delno diferencirane v smeri megakariocitov, izboljšajo vraščanje MC in omogočajo boljše preživetje trombocitov.

Namen te diplomske naloge je bilo ugotoviti, kako točno se odzivajo izolirane KMC iz popkovnične krvi na gojenje s TPO, v kolikšni meri se pomnožujejo in diferencirajo zaradi TPO ter v kakšni meri ohranjajo svojo dolgotrajno repopulacijsko sposobnost, za kar bi naj bila odgovorna populacija $CD34^+CD38^-$. Zato nas je zanimala tudi razlika med stopnjo diferenciacije in ekspanzije populacij $CD34^+CD38^-$ in $CD34^+CD38^+$. Postavili smo štiri hipoteze:

H_1^0 : Po deset dnevem gojenju (oziroma po petnajstih dnevih gojenja) izoliranih $CD34^+$ celic iz popkovnične krvi s TPO se bosta pojavili dve populaciji: a) populacija, ki se bo odzvala na TPO, b) populacija, ki se ne bo. Odzivna populacija bo rezultat delečih se primitivnih KMC in ostalih progenitorjev na TPO.

H_2^0 : Odzivna populacija se bo odzvala z delitvijo in diferenciacijo v tri populacije: 1) v populacijo, ki bo izražala megakariocitne površinske označevalce ($CD34^-CD61^+$; $CD61^+$); 2) v populacijo, ki ne bo izražala nobenih površinskih označevalcev ($CD34^-CD61^-$; Lin^-); in 3) v populacijo, ki bo še zmeraj izražala površinski označevalec CD34 ($CD34^+CD61^-$; $rCD34^+$).

H_3^0 : Med bolj primitivnimi ($CD34^+CD38^-$) in bolj zreliimi ($CD34^+CD38^+$) KMC bo obstajala razlika v ekspanziji in diferenciaciji. Bolj primitivne se bodo hitreje delile in diferencirale.

H_4^0 : $CD34^+$ celice bodo ohranile dolgotrajno repopulacijsko sposobnost po 10 dneh gojenja s TPO, kar bomo preverili v CAFC kulturi.

KMC smo iz popkovnične krvi izolirali z metodo MACS (Mini-Macs, Milteny Biotec, GmbH, Bergisch-Gladbach, Nemčija) po navodilih proizvajalca.

Da bi ugotovili, kako se odzivajo izolirane KMC iz popkovnične krvi na TPO (KIRIN Brewery Ltd, Japonska), smo KMC obravali s fluorescentnim barvilom PKH26 (Sigma, Rotterdam, po navodilih proizvajalca), ki se enakomerno veže na membrano in je zato tudi dober indikator celičnih delitev, in jih gojili 10 (da bi na splošno določili odzivnost celic na TPO) oziroma 15 dni (da smo spremljali vse površinske označevalce iz dneva v dan) pod standardnimi pogoji gojenja s 50 ng/ml TPO. Ekspandirane celice smo analizirali s pretočnim citometrom (CYTOMICS FC 500, Beckman Coulter) in določili površinske označevalce z monoklonskimi protitelesi. anti-CD9-FITC (megakariociti), anti-CD34-PC5 (matične celice), anti-CD38-FITC (primitivne/bolj zrele matične celice), anti-CD61-PC7 (megakariociti) (Beckman Coulter, Nizozemska, po navodilih proizvajalca) ter KH26 v različnih nastalih populacijah s pomočjo programa CXP, 2.0.

Za dolgotrajno repopulacijsko sposobnost so odgovorne dolgo živeče matične celice (CD34⁺CD38⁻), nas pa je zanimala razlika med dolgoživečimi in kratkoživečimi matičnim celicami. To smo določili tako, da smo izolirane CD34⁺ celice s celičnim sorterjem (FACS Aria, Becton, Dickinson and Company) razdelili na CD34⁺CD38⁻ in CD34⁺CD38⁺ populaciji. Vsako posebej smo obarvali s PKH26 in gojili pod standardnimi pogoji s 50 ng/ml TPO. Gojene celice smo analizirali s pretočnim citometrom z že prej naštetimi markerji in barvili.

Ohranitev dolgorajne repopulacijske sposobnosti tudi po desetih dneh gojenja s TPO smo preverili s CAFC testom. Vzorce izoliranih CD34⁺ celic smo najprej pobarvali s PKH26 in jih gojili 10 dni pod standardnimi pogoji z dodatkom 50 ng/ml TPO. Po gojenju smo celice ločili s celičnim sorterjem na populacijo, ki je še vedno izražala CD34 in ostalo. En dan pred ločitvijo preiskovanih celic je bilo potrebno nanesti feeder layer celice, ki so se pritrdile na dno luknjice. Dolgo živeče matične celice imajo sposobnost repopulacije kostnega mozga, ki je posledica sposobnosti migracije. Prav zaradi te lastnosti so dolgoživeče matične celice migrirale pod feeder layer, ostale pa prosto plavale v gojišču. Z osveževanjem gojišča vsak teden smo odstranili plavajoče celice in tako so nam ostale samo dolgo živeče matične celice, ki smo jih prepoznali po značilni obliki pod invertnim mikroskopom.

Ugotovili smo, da se izolirane CD34⁺ celice iz popkovnične krvi odzovejo na gojenje s trombopoietinom z ekspanzijo.

Drugi odziv gojenih celic je diferenciacija. Začeli smo gojiti CD34⁺ celice ter jih po deset oziroma petnajst dnevem gojenju pregledali z različnimi kombinacijami markerjev: CD34/CD61, CD34/CD9, CD34/CD38 ter CD9/CD61. Pri markerski kombinaciji CD34/CD61, smo po 10. dnevu gojenja opazili veliko populacijo CD61⁺ celic (CD34⁻CD61⁺), manj je bilo celic, ki niso izražale nobenega markerja (Lin⁻), nekaj je bilo takih, ki so ostale CD34⁺, brez, da bi pridobile CD61 (rCD34⁺), nekaj je bilo tudi takih, ki so hkrati pridobile še megakariocitni marker CD61 (CD34⁺CD61⁺). Po 15ih dneh gojenja je diferenciacija napredovala še bolj, celic z megakariocitnim markerjem CD61 je bilo procentualno še več, ostalih populacij pa logično manj. Celic, ki so se delile, je bilo čez čas vedno več, nedeležih se celic pa logično manj, kar smo spremljali z barvilom PKH26. Z vsakodnevnim spremljanjem grafa intenzitete signalov CD34-PC5 od CD61-PC7 smo spremljali diferenciacijo. Opazili smo, da se je CD34 marker začel zgubljeni drugi dan, od petega do sedmega dne se je začel izražati marker CD61, ki je po 12. dnevu prisoten celo v dveh populacijah. Površinski označevalec CD9 se pojavi v diferenciaciji malenkost prej, zato se rezultati z markersko kombinacijo CD34/CD9 statistično ne razlikujejo od rezultatov kombinacije CD34/CD61. Markerska kombinacija CD34/CD38 nam je dala informacije o stopnji zrelosti CD34^{+/-} celic. Prvi dan smo imeli ogromno CD34⁺CD38⁻ celic, katerih število je po 10 ali 15 dnevem gojenju precej padlo. Procent populacij s

CD34⁺ je čez čas padal, procent populacije s CD34⁻ pa rasel. Dvojno pozitivna populacija (CD34⁺CD38⁺) je dosegla svoj vrh v dneh 6-8. Po četrtem dnevu je procent CD34⁻CD38^{+/-} rasel. Čez čas je bilo vedno več deležih se celic, kar smo spremljali z barvilom PKH26. Markerska kombinacija CD9/CD61 pri celicah, gojenih s trombopoietinom, ima posebno obliko populacij na grafu intenzitete signalov CD9-FITC od CD61-PC7. Prvi dan vidimo, da so vse celice CD9⁻CD61⁻, po 5-6 dneh gojenja s TPO pa opazimo linearno populacijo, ki je dvojno pozitivna (CD9⁺CD61⁺). Po 8-9. dnevu se pojavi malo nižje še ena dvojno pozitivna populacija.

Zanimala nas je tudi razlika med ekspanzijo in diferencijsko sposobnostjo dolgo živečih matičnih celic (CD34⁺CD38⁻, primitivnih KMC) in kratko živečih matičnih celic (CD34⁺CD38⁺). Primitivna populacija (CD34⁺CD38⁻) izoliranih CD34⁺ celic se deli večkrat kot bolj zrela populacija (CD34⁺CD38⁺) izoliranih CD34⁺ celic iz popkovnične krvi. Kot pri analizah prej, smo tudi tu preverili razlike še v markerskih kombinacijah: CD34/CD61, CD34/CD9, CD9/CD61. Pri markerski kombinaciji CD34/CD61 smo ugotovili, da pod vplivom TPO obe frakciji razvijeta že prej omenjene populacije (CD61⁺, Lin⁻, rCD34⁺, CD34⁺CD61⁺), vendar diferenciacija napreduje pri CD34⁺CD38⁻ frakciji bolj, procentualno je več CD61⁺ celic. Markerska kombinacija CD34/CD9 kaže na podobno zgodbo, vendar je tu razlika v diferenciaciji med obema frakcijama manjša. Pri markerski kombinaciji CD34/CD38 so se po deset dnevnom gojenju v frakciji CD34⁺CD38⁻ pojavile vse populacije (CD34⁺CD38⁻, CD34⁺CD38⁺, CD34⁻CD38⁺, CD34⁻CD38⁻), pri frakciji CD34⁺CD38⁺ pa je CD34⁺CD38⁻ manjkala.

Po petih tednih CAFC testa smo videli, da populacija, ki je ohranila svoj CD34 marker po deset dnevnom gojenju s TPO (rCD34⁺), vsebuje dolgo živeče matične celice. Take celice smo opazili tudi pri ostanku, vendar ne v vsakem vzorcu.

CD34⁺ izolirane celice iz popkovnične krvi se na gojenje s trombopoietinom odzivajo s ekspanzijo, ki je primerljiva z raziskavami Schipperja in van Hesnbergena (Schipper et al., 2012; van Hensbergen, 2006).

Prav tako se CD34⁺ celice iz popkovnične krvi odzovejo na gojenje s TPO z diferenciacijo, ki smo jo spremljali skozi različne kombinacije površinskih označevalcev: CD34/CD61; CD34/CD9, CD34/CD38 in CD9/CD61. Pri markerski kombinaciji CD34/CD61 nastanejo po 10 oziroma 15 dnevnom gojenju štiri populacije, s čimer smo delno potrdili svojo hipotezo številka 2: 1) celice, ki ohranijo svoj CD34 marker (rCD34⁺, CD34⁺CD61⁻); 2) take, ki izgubijo oba markerja (Lin⁻); 3) take, ki pridobijo CD61 marker, brez da bi izgubili CD34 (CD34⁺CD61⁺) in 4) celice, ki so pridobile CD61 in izgubile CD34 (CD34⁻CD61⁺). Najbolj primitivna populacija med njimi je CD34⁺CD61⁻, če smo zelo površinski, bi lahko rekli, da je to populacija matičnih celic, ki se diferencira tako, da se CD34 izgublja in pripelje do Lin⁻ populacije (CD34⁻CD61⁻). Ta ne izraža nobenega

linijskega markerja, se pa diferencira dalje do celic, ki izražajo megakariocitne markerje CD61⁺ (CD34⁻CD61⁺). Včasih diferenciacija poteka tako, da celice najprej pridobijo CD61 marker, komaj nato izgubijo CD34, kar je vzrok za nastanek dvojno pozitivne populacije (CD34⁺CD61⁺). Izolirane CD34⁺ celice iz popkovnične krvi se lahko odzovejo na TPO na različne načine. Po deset dnevnem gojenju imamo lahko v gojišču naslednje celice: 1) izolirane celice CD34⁺CD61⁻, ki se ne odzovejo na TPO (s tem smo potrdili hipotezo številka 1); 2) neodzivajoče se CD34⁺ celice, ki so bile že ob izolaciji CD61⁺ (CD34⁺CD61⁺); 3) celice CD34⁺CD61⁻, ki se lahko delijo asimetrično: ena hčerinska celica ostane CD34⁺, druga pa ali izgubi svoj CD34⁺ in tako postane Lin⁻ ali pa brez izgubljanja CD34 pridobi CD61 in tako postane dvojno pozitivna (CD34⁺CD61⁺); 4) diferenciacija se lahko zgodi tudi brez celične delitve: celica izgubi svoj CD34 marker in pridobi CD61 brez celične delitve; 5) celična delitev je lahko tudi simetrična: po celični delitvi CD34⁺CD61⁻ nastaneta dve enaki hčerinski celici, ki lahko izgubita svoj CD34 in tako postaneta Lin⁻ in se lahko diferencirata dalje; 6) diferenciacija pa v redkih primerih lahko poteka tudi tako, da celica začne najprej izražati CD61 in nastane dvojno pozitivna celica (CD34⁺CD61⁺), šele nato izgubi CD34. Schipper in sodelavci so mnenja, da je sestava produkta za transplantacijo bolj pomembna kot njihovo število: več ko je rCD34⁺ in Lin⁻ celic, boljši je transplantat. V tem smislu rezultati vsakodnevnega analiziranja kažejo, da bi bilo najbolj optimalno gojiti celice 3-5 ali 6 dni, odvisno od ekspanzije, seveda. V času se vse več celic deli, vendar so med njimi še vedno takšne, ki se niso delile in ohranile svoj CD34 marker ali tudi takšne, ki so pridobile CD61. Rezultati raziskav kažejo, da se proliferacija in diferenciacija regulirata posebej (Brown in sod., 2003; Andäng in sod., 2008), zato ni nujno, da se ta dva dogodka zgodita v točno določenem zaporedju. Diferenciacijo brez celične delitve so opazili že pri embrionalnih matičnih celicah, limfocitih B in T, skeletnih celicah, vendar ne pri megakariocitih.

Diferenciacijo smo spremljali tudi z markersko kombinacijo CD34/CD9. CD9 je prav tako megakariocitni površinski označevalac, ki se začne izražati malenkost prej kot CD61, vendar se izraža precej podobno, tudi rezultati so zelo podobni, zato so tudi zaključki precej podobni.

Poleg kombinacij CD34/CD61 in CD34/CD9 smo diferenciacijo spremljali tudi s kombinacijo površinskih označevalcev CD34/CD38, s katerimi smo lahko določili stopnjo zrelosti matičnih celic. Belvedere in sodelavci poročajo, da je v populaciji izoliranih CD34⁺ celic iz popkovnične krvi večina CD34⁺CD38⁺ celic. Kakorkoli, naši rezultati kažejo na obratno zgodbo, kar je verjetno zaradi razlik v vzorcih ali pa zaradi majhnega števila vzorcev, ki niso bili reprezentativni. Kot odziv celic na 10 oziroma 15 dnevno gojenje s TPO smo opazili štiri populacije: 1) majhna CD34⁺CD38⁻ populacija celic, zaradi veliko asimetričnih delitev, to je populacija, ki ponavadi vsebuje primitivne KMC; 2) CD34⁺CD38⁺ populacija, ki po navadi predstavlja malo bolj zrele KMC; 3) CD34⁻CD38⁺ populacija, ki je malenkost bolj zrela; ter 4) CD34⁻CD38⁻ populacija, ki je najbolj zrela

izmed vseh nastalih populacij. Diferenciacija poteka od najbolj primitivnih celic, ki izražajo CD34⁺CD38⁻, te najprej pridobijo CD38 (CD34⁺CD38⁺), izgubijo CD34 (CD34⁻CD38⁺), nato pa še CD38 (CD34⁻CD38⁻). Ob primerjanju populacije CD34⁺CD38^{+/-} z CD34⁺CD61^{+/-} smo ugotovili, da se velikosti statistično ne razlikujejo, kar pa ne moremo reči za CD34⁻CD38^{+/-} in CD34⁻CD61^{+/-}. Tako kot pri kombinaciji CD34/CD61, smo pri CD34/CD38 postavili model diferenciacije in proliferacije. Obstajajo različni odzivi na TPO, ki rezultirajo v naslednje tipe celic v gojišču po deset dnevnom gojenju s TPO: 1) CD34⁺CD38⁻ in CD34⁺CD38⁺ celice, ki se ne odzovejo nanj; 2) CD34⁺CD38⁻ celice, ki so prisotne v CD34⁺ populaciji, ki se delijo asimetrično, ena hčerinska celice začne izražati CD38 (CD34⁺CD38⁺), druga ostane enaka materinski celici; 3) delitev lahko poteka tudi simetrično, iz CD34⁺CD38⁺ nastaneta dve hčerinski celici, ki izražata enake markerje kot materinska; 4) CD34⁺CD38⁺ celice se diferencirajo dalje v CD34⁻CD38⁺, te pa v CD34⁻CD38⁻; 5) tudi pri tej kombinaciji smo opazili diferenciacijo brez proliferacije; 6) CD34⁺CD38⁺ celice se lahko delijo tudi asimetrično.

Razlika med bolj in manj zreli KMC (CD34⁺CD38⁺, CD34⁺CD38⁻) je v tem, da imajo manj zrele KMC večjo sposobnost ekspanzije in diferenciacije (s tem smo potrdili hipotezo številka 3). Sicer sta obe populaciji razvili enake populacije, če gledamo kombinacijo markerjev CD34/CD61, vendar je drugačno njihovo razmerje, bolj primitivne KMC (CD34⁺CD38⁻) so po desetih dneh gojenja bile bolj diferencirane, več je bilo celic, ki so izražale megakariocitne markerje. Kot kaže, je CD34⁺CD38⁻ populacija v večji meri odgovorna za diferenciacijo. Povsem podobni so rezultati za kombinacijo markerjev CD34/CD9. Ko smo na istih celicah pregledovali kombinacijo CD34/CD38, smo ugotovili, da v populaciji CD34⁺CD38⁺ ni bilo CD34⁺CD38⁻, kar je popolnoma logično, saj smo populaciji predhodno ločili in je slednja populacija bolj primitivna, v katero se CD34⁺CD38⁻ ne more več pretvoriti. Populacija CD34⁺CD38⁻ se je diferencirala hitreje kot CD34⁺CD38⁺ populacija.

V CAFC testu smo dokazali, da ima populacija, ki po deset dnevnom gojenju še izraža CD34⁺, dolgoročno repopulacijsko sposobnost. S tem smo dokazali, da deset dnevno gojenje s TPO KMC ne izčrpa, ampak le-te ohranijo svojo dolgoročno repopulacijsko sposobnost in s tem potrdili hipotezo številka 4.

7 LITERATURE

- Ades E.W., Zwerner R.K., Acton R.T., Balch C.M. 1980. Isolation and partial characterization of the human homologue of Thy-1. *The Journal of Experimental Medicine*, 151, 2: 400-406
- Ali H., Bahbahani H. 2010. Umbilical cord blood stem cells – potential therapeutic tool for neural injuries and disorders. *Acta Neurobiologiae Experimentalis*, 70: 316-324
- Andäng M., Hjerling-Leffler J., Moliner A., Lundgren T.K., Castelo-Branco G., Nanou E., Pozas E., Bryja V., Halliez S., Nishimaru H., Wilbertz J., Arenas E., Koltzenburg M., Charnay P., Manira A.E., Ibañez C.F., Ernfors P. 2008. Histone H2AX-dependent GABA_A receptor regulation of stem cell proliferation. *Nature*, 451: 460-464
- Arai F., Hiara A., Ohmura M., Sato H., Matsuoka S., Takubo K., Ito K., Koh G.Y., Suda T. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell*, 118: 149-161
- Archimbaud E., Ottmann O., Liu J.A., Lechner K., Dombret H., Sanz M.A., Herrmann F., Gruss H., Feneaux P., Ganser A., Heil G., Kanz L., Brugger W., Sims T., Olsen K., Hoelzer D. 1996. A randomized, double blind, placebo controlled study using PEG-rHuMGDF as an adjunct to chemotherapy for adults with de-novo acute myeloid leukemia: early results. *Blood*, 88: 447
- Baum C.M., Weissman I.L., Tsukamoto A.S., Buckle A.M., Peault B. 1992. Isolation of a candidate human hematopoietic stem-cell population. *Proceedings of the National Academy of Science of the United States of America*, 89: 2804-2808
- Bautista G., Cabrera J.R., Regidor C., Forés R., García-Marco J.A., Ojeda E., Sanjuán I., Ruiz E., Krsnik I., Navarro B., Gil S., Magro E., de Laiglesia A., Gonzalo-Daganzo R., Martín-DOnaire T., Rico M., Millán I., Fernández M.N. 2009. Cord blood transplants supported by co-infusion of mobilized hematopoietic stem cells from a third-party donor. *Bone Marrow Transplantation*, 43: 365-373
- Becker A.J., McCulloch E.A., Till J.E. 1963. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*. 197: 452-454
- Beckmann J., Scheitza S., Wernet P., Fischer J.C., Gielbel B. 2007 Asymmetric cell division within the human haematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating progenitor. *Blood*, 109: 5494-5501
- Beerman I., Bhattacharya D., Zandi S., Sigvardsson M., Weissman I.L., Bryder D., Rossi D.J. 2010. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proceedings of the National Academy of Science of the United States of America*, 107, 12: 5465-5470
- Belvedere O., Feruglio C., Malangone W., Bonora M.L., Donini A., Diritea L., Tonutti E., Rinaldi C., Pittino M., Baccarani M., Frate G.D., Biffoni F., Sala P., Hilbert

- D.M., Degrassi A. 1999. Phenotypic characterization of immunomagnetically purified umbilical cord blood CD34⁺ cells. *Blood Cells, Molecules and Diseases*, 25, 9: 140-145
- Betschinger J., Knoblich J.A. 2004. Dare to be different: asymmetric cell division in *Drosophila*, *C. elegans* and vertebrates. *Current Biology*, 14: 674-685
- Bhatia M., Wang J.C., Kapp U., Bonnet D., Dick J.E. 1997. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *The Proceedings of the National Academy of Sciences Online (USA)*, 94: 5320-5325
- Bissels U., Wild S., Tomiuk S., Hafner M., Scheel H., Mihalovic A., Choi Y.H., Tuschl T., Bosio A. 2011. Combined characterization of microRNA and mRNA profiles delineates early differentiation pathways of CD133⁺ and CD34⁺ hematopoietic stem and progenitor cells. *Stem Cells*, 29: 847-857
- Bjornson C.B., Rietze R.L., Reynolds B.A., Magli M.C., Vecovi A.I. 1999. Turning brain into blood: a haematopoietic fate adopted by adult neural cells *in vivo*. *Science*, 283: 534-537
- Boiron J.M., Dazey B., Cailliot C., Launay B., Attal M., Mazurier F., McNiece I.K., Ivanovic Z., Caraux J., Marit G., Reiffers J. 2006. Large-scale expansion and transplantation of CD34⁺ hematopoietic cells: *In vitro* and *in vivo* conformation of neutropenia abrogation related to the expansion process without impairment of the long term engraftment capacity. *Transfusion*, 46: 1934-1942
- Bone marrow donors worldwide. 2012. <http://www.bmdw.org/> (28 Apr. 2012)
- Boxall S.A., Cook G.P., Pearce D., Bonnet D., El-SHERbiny D., Blundell Y.M., Howe M.P., Leek S.J., Markham J.P., Wynter A.F. 2009. Haematopoietic repopulating activity in human cord blood CD133⁺ quiescent cells. *Bone Marrow Transplantation*, 43: 627-635
- Bradley M.B., Satwani P., Baldinger L., Morris E., van de Ven C., Del T.G., Garvin J., George D., Bhatia M., Roman E., Baxter-Lowe L.A., Schwartz J., Qualter E., Hawks R., Wolownik K., Foley S., Militano O., Leclere J., Cheung Y.K., Cairo M.S. 2007. Reduced intensity allogeneic umbilical cord blood transplantation in children and adolescent recipients with malignant and non-malignant diseases. *Bone Marrow Transplant*, 40, 7: 621-631
- Breems D.A., Blokland E.A., Neben S., Ploemacher R.E. 1994. Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay. *Leukemia*, 8: 1095-1104
- Brown G., Hughes P.J., Michell R.H. 2003. Cell differentiation and proliferation – simultaneous but independent?. *Experimental Cell Research*, 291: 282-288
- Broxmeyer H.E. 2009. Cord blood transplantation: a mini review celebrating the 20th anniversary of the first cord blood transplant. *Hematologist*, 6: 7
- Broxmeyer H.E. 2010. Umbilical Cord Transplantation: Epilogue. *Seminars in Hematology*, 47, 1: 97-103

- Broxmeyer H.E., Cooper S., Hass D.M., Hathaway J.K., Stehman F.B., Hangoc G. 2009. Experimental basis of cord blood transplantation. *Bone Marrow Transplantation*, 44: 627-633
- Broxmeyer H.E., Gluckman E., Auerbach A.D., Douglas G.W., Friedman H., Cooper S., Hangoc G., Kurtzberg J., Bard J., Boyse E.A. 1990. Human umbilical cord blood: A clinically useful source of transplantable hematopoietic stem/progenitor cells. *The International Journal of Cell Cloning*, 8: 76-91
- Broxmeyer H.E., Lee M.R., Hangoc G., Cooper S., Prasain N., Kim Y.J., Mallett C., Ye Z., Witting S., Cornetta K., Cheng L., Yoder M.C. 2011. Haematopoietic stem/progenitor cells, generation of induced pluripotent stem cells, and isolation of endothelial progenitors from 21-23,5 year cryopreserved cord blood. *Blood*, 117: 47734777
- Bruno S. Gunetti M., Gammaintoni L., 2004. Fast but durable megakaryocyte repopulation and platelet production in NOD/CSID mice transplanted with *ex-vivo* expanded human cord blood CD34+ cells. *Stem Cells*, 22: 135-143
- Cairo M.S., Wagner J.E. 1997. Placental and/or umbilical cord blood: an alternative source of hematopoietic stem cells for transplantation. *Blood*, 90, 12: 4665-4678
- Can A. 2008. A concise review on the classification and nomenclature of stem cells. *Turkish Journal of Hematology*, 25: 57-59
- Carson M.J., Sutcliffe J.G., Campbell I.L. 1999. Microglia stimulate naïve T-cell differentiation without stimulating T-cell proliferation. *Journal of Neuroscience Research*, 55 ,1: 127-134
- CD34 molecule (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/gene/947> (29 Apr. 2012)
- CD38 molecule (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/gene/952> (29.4.2012)
- CD9 molecule (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/gene/928> (29 Apr. 2012)
- Challen G.A., Boles N.C., Chambers S.M., Goodell M.A. 2010. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell Stem Cell*, 6, 3: 265-278
- Chang Y., Bluteau D., Debili N., Vainchenker W. 2007. From hematopoietic stem cells to platelets. *Journal of Thrombosis and Haemostasis*, 5, 1: 318-327
- Christersen J.L., Weissman I.L. 2001. Flk-2 is a marker in haematopoietic recovery in haematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98: 14541-14546
- Christopherson K.W., Hangoc G., Mantel C.R., Broxmeyer H.E. 2004. Modulation of haematopoietic stem cell homing and engraftment by CD26. *Science*, 305: 1000-1003
- Cosman D. 1993. The hematopoietin receptor superfamily. *Cytokine*, 5: 95-106

- Cox K.O., Daenke S., Samcewicz B. 2006. Peritoneal B cells differentiate without proliferation into autoantibody secretors under the influence of factors released by other cells. *Scandinavian Journal of Immunology*, 20, 6: 527-532
- Cramer E.M., Norol F., Guichard J., Breton-Gorius J., Vainchenker W., Massé J.M., Debili N. 1997. Ultrastructure of platelet formation by human megakaryocytes cultured with the Mpl ligand. *Blood*, 89: 2336-2346
- Daley G.Q., Goodell M.A., Snyder E.Y. 2003. Realistic prospects for stem cell therapeutics. *American Society of Hematology Education Program Book*, 1: 398-418
- Del Toro G., Satwani P., Harrison L., Chaeung Y.K., Brigid B.M., George D., Yamashiro D.J., Garvin J., Skerrett D., Bessmertny O., Wolownik K., Wischhover C., van der Ven C., Cairo M.S. 2004. A pilot study of reduced intensity conditioning and allogeneic stem cell transplantation from unrelated cord blood and matched family donors in children and adolescent recipients. *Bone Marrow Transplantation*, 33, 6: 613-622
- Delaney C., Heimfeld S., Brashem-Stein., 2010. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nature Medicine*, 16: 232-236
- Delaney C., Ratajczak M.Z., Laughlin M.J. 2010. Strategies to enhance umbilical cord blood stem cell engraftment in adult patients. *Expert Review of Hematology*, 3, 3: 273-283
- Delaney C., Varnum-Finney B., Aoyama K., Brashem-stein C., Bernstein I.D. 2005. Dose-dependent effects of the Notch ligand Delta1 on *ex vivo* differentiation and *in vivo* marrow repopulation ability of cord blood cells. *Blood*, 106, 8: 2693-2699
- Drake A.C., Khoury M., Leskov I., Iliopoulou B.P., Fragoso M., Lodish H., Chen J. 2011. Human CD34⁺ CD133⁺ hematopoietic stem cells cultured with growth factors including angptl5 efficiently engraft adult NOD-SCID Il2r γ ^{-/-} (NSG) mice. *PLoS ONE*, 6, 4: e18382
- Duchez P., Chevalerey J., Vlaski M., Dazey B., Bijou F., Lafarge X., Milpied N., Boiron J.M., Ivanovic Z. 2011. Thrombopoietin to replace megakaryocyte-derived growth factor: impact on stem and progenitor cells during *ex vivo* expansion of CD34⁺ cells mobilized in peripheral blood. *Transfusion*, 51: 313-318
- Dykstra B., Olthof S., Schreuder J., Ritsema M., de Haan G. 2011. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *Journal of Experimental Medicine*, 208, 13: 2691-2703
- Fernandez M.N., Regidor C., Cabrera R., Carcía-Marco J.A., Forés R., Sanjuán I., Gayoso J., Gil S., Ruíz E., Little A.M., McWhinnie A., Madrigal A. 2003. Unrelated umbilical cord blood transplants in adults: early recovery of neutrophils by supportive co-transplantation of a low number of highly purified peripheral blood CD34⁺ cells from a HLA-haploidentical donor. *Experimental Hematology*, 31: 535-544

- Fibbe W., Heemskerk D., Laterveer L., Pruijt J. Foster D., Kaushansky K., Willemze R. 1995. Accelerated reconstitution of platelets and erythrocytes after syngenic transplantation of bone marrow cells derived from thrombopoietin pre-treated donor mice. *Blood*, 86: 3308-3313.
- FLT3 fms-related tyrosine kinase 3 (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/gene/2322> (29 Apr. 2012)
- Fox N., Priestley G.V., Papayannopoulou T., Kaushansky K. 2002 Thrombopoietin expands haematopoietic stem cells after transplantation. *Journal of Clinical Investigation*, 110: 389-394
- Gluckman E. 2009. Ten years of cord blood transplantation: from bench to bedside. *British Journal of Haematology*, 147: 192-199
- Gluckman E., Broxmeyer H.E., Auerbach A.D., Friedman H., Douglas G.W., Devergie A., Esperou H., Thierry D., Socie G., Lehn P., Cooper S., English D., Kurtzberg J., Bard J., Edward A.B. 1989. Haematopoietic reconstitution in a patient with Fanconi anemia by means of umbilical-cord blood from an HLA-identical sibling. *The New England Journal of Medicine*, 321: 1174-1178
- Golde D.W. 1991. The stem cell. *Scientific American*, 265: 86-93
- Hall P.A., Watt F.M. 1989. Stem cells: the generation and maintenance of cellular diversity. *Development*, 106: 619-633
- Hao Q.L., Shah A.J., Thiemann F.T., Smogorzewska E.M., Crooks G.M. 1995. A functional comparison of CD34⁺CD38⁻ cells in cord blood and bone marrow. *Blood*, 86, 10: 3745-3753
- Hao Q.L., Smogorzewska E.M., Barsky L.W., Crooks G.M. 1998. In vitro identification of single CD34⁺CD38⁻ cells with both lymphoid and myeloid potential. *Blood*, 91: 4145-4151
- Hao Q.L., Thiemann F.T., Petersen D., Smogorzewka E.M., Crooks G.M. 1996. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood*, 88: 3306-3313
- Hoffmeister C.C., Zhang J., Knight K.L., Le P., Stiff P.J. 2007. *Ex vivo* expansion of umbilical cord blood stem cells for transplantation: Growing knowledge from the haematopoietic niche. *Bone Marrow Transplant*, 39: 11-23
- Holyoake T.L., Nicolini F.E., Eaves C.J. 1999. Functional differences between transplantable human haematopoietic stem cells from fetal liver, cord blood and adult marrow. *Experimental Haematology*, 27, 9: 1418-1427
- Horan P.K., Slezak S.E. 1989. Stable cell membrane labeling. *Nature*, 340: 167-168
- Hosokawa K., Arai F., Yoshihara H., Nakamura Y., Gomei Y., Iwasaki H., 2007. Function of oxidative stress in the regulation of hematopoietic stem cell-niche interaction. *Biochemical and Biophysical Research Communications*, 363, 3: 578-583
- Howell W.H. 1890. Observation upon the occurrence, structure, and function of the giant cells of the marrow. *Journal of Morphology*, 4: 117-130

- Huang S., Terstappen L.W. 1994. Lymphoid and myeloid differentiation of single human CD34⁺, HLA-DR⁺, CD38⁻ hematopoietic stem cells. *Blood*, 83: 1515-1526
- Ishikawa F., Livingston A.G., Minamiguchi, H., Wingard, J. R., Ogawa, M. 2003. Human cord blood long-term engrafting cells are CD34⁺CD38⁻. *Leukemia*, 17: 960-964
- ITGA2B integrin, alpha 2b (platelet glycoprotein IIb or IIb/IIIa complex, antigen CD41) (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=3674> (29 Apr. 2012)
- ITGB3 integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=3690> (29 Apr. 2012)
- Ivanović Z. 2010. Hematopoietic stem cells in research and clinical applications: The CD34 issue. *World Journal of Stem Cells*, 2, 2: 18-23
- Junt T., Schulze H., Chen Z., Massberg S., George T., Krueger A., Wagner D., Graf T., Italiano J.J., Shivdasani R., von Andrian U. 2007. Dynamic visualization of thrombopoiesis within bone marrow. *Science*, 317: 1767-1770
- Kang Y.J., Yang S.J., Park G., Cho B., Min C.K., Kim T.Y., Lee J.S., Oh I.H. 2007. A novel function of interleukin-10 promoting self-renewal of hematopoietic stem cell. *Stem Cell*, 25: 1814-1822
- Kato T., Matsumoto A., Ogami K., Tahara T., Morita H., Miyazaki H. 1998. Native thrombopoietin: structure and function. *Stem Cells*, 16 (suppl 2): 11-19
- Kaushansky K. 2008. Historical review: megakaryopoiesis and thrombopoiesis. *Blood*, 111: 981-986
- KIT v- kit Hardy-Zuckerman 4 feline sarcoma oncogene homolog (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/gene/3815> (29 Apr. 2012)
- Kojika S., Griffin J.D., 2001. Notch receptors and haematopoiesis. *Experimental Hematology*, 29, 9: 1041-1052
- Kurtzberg J., Laughlin M., Graham M.L., Smith C., Olson J.F., Halperin E.C., Ciocci G., Carrier C., Stevens C.E., Rubinstein P. 1996. Placental blood as a source of haematopoietic stem cells for transplantation into unrelated recipients. *New England Journal of Medicine*, 335: 157-166
- Larochelle A., Vormoor J., Hanenberg H., Wang J.C., Bhatia M., Lapidot T., Moritz T., Murdoch B., Xiao X.L., Kato I., Williams D.A., Dick J.E. 1996. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy. *Nature Medicine*, 2: 1329-1337
- Laughlin M.J., Eapen M., Rubinstein P., Wagner J.E., Zhang M. J., Champlin R.E., Stevens C., Barker J.N., Gale R.P., Lazarus H.M., Marks D.I., van Rood J.J., Scaradavou A., Horowitz M.H. 2004. Outcomes after transplantation of cord blood

- or bone marrow from unrelated donors in adults with leukemia. *The New England Journal of Medicine*, 351: 2265-2275
- Le Naour F., Francastel C., Prenant M., Lantz O., Boucheix C., Rubinstein E. 1997. Upregulation of CD9 expression during TPA treatment of K562 cells. *Leukemia*, 11, 8: 1290-1297
- Li L., Xie T. 2005. Stem cell niche: structure and function. *The Annual Review of Cell and Developmental Biology*, 21: 605-631
- Locatelli F., Rocha V., Reed W., Bernaudin F., Ertem M., Grafakos S., Brichard B., Li X., Nagler A., Giorgiani G., Haut P.R., Brochstein J.A., Nugent D.J., Blatt J., Woodard P., Kurtzberg J., Rubin C.M., Miniero R., Lutz P., Raja T., Roberts I., Will A.M., Yaniv I., Vermylen C., Tannoia N., Garnier F., Ionescu I., Walters M.C., Lubin B.H., Gluckman E. 2003. Realted umbilical cord blood transplantation in patients with thalassemia and sickle cell disease. *Blood*, 101: 2137-2143
- Lok S., Kaushansky K., Holly R., Kuijper J.L., Lofton-Day C.E., Oort P.J., Grant F.J., Heipel M.D., Burkhead S.K., Kramer J.M., Bell L.A., Sprecher C.A., Blumberg H., Buddle M.M., Osborn S.G., Evans S.J., Sheppard P.O., Presnell S.R., O'hara P.J., Hagen F.S., Roth G.J., Foster D.C. 1994. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production *in vivo*. *Nature*, 369: 565-568
- Long M.W., Heffner C.H., Williams J.L., Peters C., Prochownik E.V. 1990. Regulation of megakaryocyte phenotype in human erythroleukemia cells. *Journal of Clinical Investigations*, 85: 1072-1084
- Macmillan M.L., Blazar B.R., DeFor T.E., Wagner J.E. 2009. Transplantation of *ex-vivo* culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of phase I-II clinical trial. *Bone Marrow Transplantation*, 43: 447-454
- Majeti R., Park C.X., Weissman I.L. 2007. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell*, 1, 6: 635-645
- Malavasi F., Funaro A., Roggero S., Horestein A., Calosso L., Metha K. 1994. Human CD38: glycoprotein in search of a function. *Immunology Today*, 15, 3: 95-97
- Mathe G., Jammet H., Pendic B., Schwarzenberg L., Duplan J.F., Maupin B., Latarjet R., Larrieu M.J., Kalic D., Djukic Z. 1959. Transfusions and grafts of homologous bone marrow in humans after accidental high dosage irradiation. *Revue Francaise D'etudes Cliniques et Biologiques*, 4: 226-238
- Matsumoto K., Yasui K., Yamashita N. 2000. In vitro proliferation potential of AC133 positive cells in peripheral blood. *Stem Cells*, 18: 196-230
- Mattia G., Milazzo L., Vulcano F., Pascuccio M., Macioce G., Hassan J.H., Giampaolo A. 2008. Long-term platelet production assessed in NOD/SCID mice injected with cord blood CD34+ cells, thrombopoietin-amplified in clinical grade serum-free culture. *Experimental Hematology*, 36: 244-252

- McDonald T.P. 1992. Thrombopoietin. Its Biology, Clinical Aspects, and Possibilities. *The American Journal of Pediatric Hematology/Oncology*, 14: 8-21
- McGukin C.P., Basford C., Hanger K., Habibollah S., Forraz N. 2007. Cord blood revelations: the importance of being a first born girl, big, on time and to a young mother! *Early Human Development*, 83, 12: 733-741
- McKenna D.H., Brunstein C.G. 2011. Umbilical cord blood: current status and future directions. *Vox Sanguinis*, 100: 150-162
- McKenzie J.L., Gan I.O., Doedens M., Dick J.E. 2005. Human short-term repopulating stem cell are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of CD122+ cells. *Blood*, 106, 4: 1259-1261
- McNiece I., Briddell R. 2001. *Ex vivo* expansion of haematopoietic progenitor cells and mature cells. *Experimental Hematology*, 29: 3-11
- Metcalf D., MacDonald H.R., Odartchenko N., Sordat B. 1975. Growth of mouse megakaryocyte colonies in vitro. *The Proceedings of the National Academy of Sciences Online*, 72: 1744-1748
- Methia N., Louache F., Vainchenker W., Wendling F. 1993. Oligodeoxynucleotides antisense to the proto-oncogene *c-Mpl* specifically inhibit in vitro megakaryocytopoiesis. *Blood*, 82: 1395-1401
- Mezey E., Chandross K.J., Harta G., Maki R.A., McKercher S.R. 2000. Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science*, 290: 1779-1782
- Michel G., Rocha V., Chevret S., Arcese W., Chan K.W., Filipovich A., Takahashi T.A., Vowels M., Ortega J., Bordigoni P., Shaw P.J., Yaniv I., Machado A., Pimentel P., Fagioli F., Verdeguer A., Jouet J.P., Diez B., Ferreira E., Pasquini R., Rosenthal J., Sievers E., Messina C., Iori A.P., Garnier F., Ionescu I., Locatelli F., Gluckman E. 2003. Unrelated cord blood transplantation for childhood acute myeloid leukemia: a Eurocord Group analysis. *Blood*, 102: 4290-4297
- Miller J.S., McCullar V., Punzel M., Lemischaka I.R., Moore K.A. 1999. Single adult human CD34⁺/Lin⁻/CD38⁻ progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, ad myeloid cells. *Blood*, 93: 96-106
- Molineux G., Hartly C., McElroy P., McCrea C., McNiece K. 1996. Megakaryocyte growth and development factor accelerates platelet recovery in peripheral blood progenitor cell transplant recipients. *Blood*, 88: 366-376
- Morita Y., Ema H., Nakauchi H. 2010. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *Jurnal of Experimental Medicine*, 207, 6: 1173-1182
- Morrison S.J., Kimble J. 2006. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature*, 44: 1068-1074
- Morrison S.J., Shah N.M. 1997. Regulatory mechanisms in stem cell biology. *Cell*, 88: 287-298

- MPL myeloproliferative leukemia virus oncogene (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/gene/4352> (29 Apr. 2012)
- Nabhan S., Rocha V., Labopin M., Arcese W., Sirvent A., Ionescu I., Bizzetto R., Boudjedir K., Chaves W., Herr A.L., Gluckman E., Sanz G. 2008. Influence of myeloablative conditioning regimens on outcomes after single unrelated cord blood transplantation for adults with leukemia: an analysis on behalf of eurocord EBMT-netcord-blood. *Blood*, 112: abstract number 155
- Notta F., Doulatov S., Laurenti E., Poeppl A., Jurisica I., Dick J.E. 2011. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science*, 333, 6039: 218-221
- O'Neill M.C., Stockdale F.E. 1972. Differentiation without cell division in cultured skeletal muscle. *Developmental Biology*, 29, 4: 410-418
- Oh I.H., Humphries R.K. 2012. Concise review: Multidimensional regulation of the hematopoietic stem cell state. *Stem Cell*, 30: 82-88
- Peled T., Landau E., Mandel J., Glukhman E., Goudsmid N.R., Nagler A., Fibach E. 2004. Linear polyamine copper chelator tetraethylenepentamine augments long-term *ex vivo* expansion of cord blood-derived CD34+ cells and increase their engraftment potential in NOD/SCID mice. *Experimental Hematology*, 32: 547-555
- Peled T., Landau E., Prus E., Treves A.J., Nagler A., Fibach E. 2002. Cellular copper content modulates differentiation and self-renewal in cultures of cord blood-derived CD34+ cells. *British Journal of Haematology*, 116: 655-661
- Petzer A.L., Hogge D.E., Landsdorp P.M., Reid D.S., Eaves C.J., 1996. Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) in vitro and their expansion in defined medium. *The Proceedings of the National Academy of Sciences Online (USA)*, 93: 1470-1474
- Potten C.S., and Loeffler M. 1990. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* 110: 1001-1020
- PTPRC protein tyrosine phosphatase, receptor type, C (*Homo sapiens*). Gene. NCBI. <http://www.ncbi.nlm.nih.gov/gene/5788> (29 Apr. 2012)
- Ramalho-Santos M., Willenbring H. 2007 On the origin of the term "stem cells". *Cell Stem Cell*, 1: 35-38
- Raslova H., Roy L. Vourc'h C., Le Couedic J.P., Brison O., Metivier D., Feunteun J., Kriemer G., Debili N., Vainchenker W. 2003. Megakaryocyte polyploidization is associated with a functional gene amplification. *Blood*, 101: 541-544
- Reca R., Mastellos D., Majka M., Marquez L., Ratajczak J., Franchini S., Glodek A., Honczarenko M., Spruce L.A., Janowska-Wieczorek A., Lambris J.D., Ratajczak M.Z. 2003. Functional receptor for C3a anaphylatoxin is expressed by normal haematopoietic stem/progenitor cells, and C3a enhances their homing-related responses to SDF-1. *Blood*, 101, 10: 3784-3793
- Reems J.A. 2011. A journey to produce platelets in vitro. *Transfusion*, 51: 169S-176S (supplement)

- Rocha V., Broxmeyer H.E. 2010. New approaches for Improving Engraftment after Cord Blood Transplantation. *Biology of Blood and Marrow Transplant*, 16: 126-132
- Rocha V., Labopin M., Sanz G., Arcese W., Schwerdtfeger R., Bosi A., Jacobsen N., Ruutu T., de Lima M., Finke J., Frassoni F., Gluckman E. 2004. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *New England Journal of Medicine*, 351: 2276-2285
- Rogers I., Casper R.F. 2004. Umbilical cord blood stem cells. *Best Practice & Research Clinical Obstetrics and Gynaecology*, 18, 6: 893-908
- Rubinstein P., Carrier C., Scaradavou M., Kurtzber J., Adamson J., Migliaccio A.R., Berkowitz R.L., Cabbad M., Ludy D., Taylor P.E., Rosenfield R.E., Stevens C.E. 1998. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *New England Journal of Medicine*, 339, 22: 1565-1577
- Rubinstein P., Dobrila L., Rosenfield R.E., Adamson J.W., Migliaccio G., Migliaccio A.R., Taylor P.E., Stevens C.E. 1995. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *The Proceedings of the National Academy of Sciences Online (USA)*, 92: 10119-10122
- Rudkin B.B., Lazarovici P., Levi B.Z., Abe Y., Fujita K., Guroff G. 1989. Cell cycle-specific action of nerve growth factor in PC12 cells: differentiation without proliferation. *The EMBO Journal*, 8, 11: 3319-3325
- Salles I.I., Thijs T., Brunaud C., De Meyer S.F., Thys J., Vanhoorelbeke K., Deckmyn H. 2009. Human platelets produced in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice upon transplantation of human cord blood CD34⁺ cells are functionally active in an *ex vivo* flow model of thrombosis. *Blood*, 114, 24: 5044-5051
- Schipper L.F., Brand A., Fibbe W.E., Hensbergen Y. 2012 Functional characterization of TPO-expanded CD34⁺ cord blood cells identifies CD34⁻CD61⁻ cells as platelet-producing cells early after transplantation in NOD/SCID mice and rCD34⁺ cells as CAFC colony-forming cells. *Stem Cells*, 30: 988-996
- Schipper L.F., Brand A., Reiners N., Melief C.J.J., Willemze R., Fibbe, W.E. 1998. Effects of thrombopoietin on the proliferation and differentiation of primitive and mature haematopoietic progenitor cells in cord blood. *British Journal of Haematology*, 101: 425-435
- Schipper L.F., Brand A., Reiners N., Melief C.J.J., Willemze R., Fibbe W.E. 2003. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. *Experimental Hematology*, 31: 324-330
- Schöler H. R. 2007. The potential of stem cells: an inventory. V: Human biotechnology as social challenge. Knoepffler N., Schipanski D., Sorgner S.L. (eds.). Hampshire, Ashgate Publishing: 27-56
- Seita J., Weissman I.L. 2010. Haematopoietic stem cell: self-renewal versus differentiation. *Wiley interdisciplinary reviews. Systems Biology and Medicine*, 6: 640-53

- Siddiqui N.F.A., Shabrani N.C., Kale V.P., Limaye L.S. 2011. Enhanced generation of megakaryocytes from umbilical cord blood-derived CD34⁺ cells expanded in the presence of two nutraceuticals, docosahexanoic acid and arachidonic acid, as supplements to the cytokine-containing medium. *Cytotherapy*, 13: 114-128
- Silvestri F., Banavali S., Baccarani M., Preisler H.D. 1992. The CD34 hematopoietic progenitor cell associated antigen: biology and clinical applications. *Haematologica*, 77: 265-273
- Smith R.J., Sweetenham J.W. 1995. A mononuclear cell dose of 3×10^8 /kg predicts early multilineage recovery in patients with malignant lymphoma treated with carmustine, etoposide, Ara-C and mephalan (BEAM) and peripheral blood progenitor cell transplantation. *Experimental Hematology*, 23: 1581-1588
- Souyri M., Vigon I., Penciolelli J.F., Heard J.M., Tambourin P., Wendling F. 1990. A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes haematopoietic progenitors. *Cell*, 63: 1137-1147
- Sprangrude G.J., Heimfeld S., Weissman I.L. 1988. Purification and characterisation of mouse haematopoietic stem cells. *Science*, 241: 58-62
- Sujata L., Chaudhuri S. 2008. Stem cell niche, the microenvironment and immunological crosstalk. *Cellular and Molecular Immunology*, 5, 2: 107-112
- Sullivan M.J. 2008. Banking on cord blood stem cells. *Nature Reviews Cancer*, 8, 7: 555-563
- Summers Y.J., Heyworth C.M., de Wynter E.A., Hart C.A., Chang J., Testa N.G. 2004. AC133⁺ G0 cells from cord blood show a high incidence of long-term culture-initiating cells and a capacity for more than 100 million-fold amplification of colony-forming cells *in vitro*. *Stem Cells*, 22: 704-715
- Sutherland H.J., Eaves C.J., Eaves A.C., Dragowska W., Landsdorp P. 1989. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis *in vitro*. *Blood*, 74: 1563-1570
- Szilvassy S.J., Humphries R.K., Lansdorp P.M., Eaves A.C., Eaves C.J. 1990. Quantitative assay for totipotent reconstituting haematopoietic stem cells by a competitive repopulation strategy. *Proceedings of the National Academy of Science*, 87: 8736-8740
- Takahashi S., Iseki T., Ooi J., Tomonari A., Takasugi K., Shimohakamada Y., Yamada T., Uchimarui K., Tojo A., Shirafuji N., Kodo H., Tani K., Takahashi T., Yamaguchi T., Asano S. 2004. Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. *Blood*, 104, 12: 3813-3820
- Tanavde V.M., Malehorn M.T., Lumkul R., Gao Z., Wingard J., Garrett E.S., Civin C.I. 2002. Human stem-progenitor cells from neonatal cord blood have greater haematopoietic expansion capacity than those from mobilized adult blood. *Experimental Hematology*, 30, 7: 816-823

- Tarasova A., Haylock D., Winkler D. 2011. Principal signaling complexes in haematopoiesis: Structural aspects and mimetic discovery. *Cytokine and Growth Factor Reviews*, 231-253
- Till J.E., McCulloch E.A. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Research*, 14: 213-222
- Till J.E., McCulloch E.A. 1980. Hemopoietic stem cell differentiation. *Biochimica et Biophysica Acta*, 605: 431-459
- Till J.E., McCulloch E.A., Siminovitch L. 1964. A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. *Proceedings of the National Academy of Sciences (USA)*, 51, 1: 29-36
- Uchida N., Tsukamoto A., He D., Frieria A.M., Scollay R., Weissman I.L. 1998. High doses of purified stem cells cause early haematopoietic recovery in syngeneic and allogeneic hosts. *The Journal of Clinical Investigations*, 101: 961-966
- van Hensbergen Y., Schipper L.F., Brand A., Slot M.C., Welling M., Nauta A.J., Fibbe W.E. 2006. *Ex vivo* culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. *Experimental Hematology*, 34: 943-950
- Vandhan-Raj S., Patel S., Broxmeyer H., Buesoramos C., Reddy S.P., Papaddopolous N., Burgess A., Johnson T., Yang T., Paton V., Hellman S., Benjamin R.S. 1996. Phase I-II investigation of recombinant human thrombopoietin (rhTPO) in patients with sarcoma receiving high dose chemotherapy with adriamycin and ifosfamid. *Blood*, 88: 448
- Varnum-Finney B., Purtion L.E., Yu M., Brashem-Stein C., Flowers D., Staats S., Moore K.A., Le Roux I., Mann R., Gray G., Artavanis-Tsakonas S., Brenstein I.D. 1998. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood*, 91: 4084-4091.
- Verneris M.R., Brusntein C.G., Barker J., MacMillan M.L., DeFor T., McKenna D.H., Burke M.J., Blazar B.R., Miller J.S., McGlave P.B., Weisdorf D.J., Wagner J.E. 2009. Relapse risk after umbilical cord blood transplantation: enhanced graft versus leukemia effect in recipients of two units. *Blood*, 114: 4293-4299
- Vigon I., Moron J.P., Cocault L., Mitjavila M.T., Tambourin P., Gisselbrecht S., Souyri M. 1992. Molecular cloning and characterization of *MLP*, the human homolog of the *v-mlp* oncogene: identification of a member of the haematopoietic growth factor receptor superfamily. *The Proceedings of the National Academy of Science Online (USA)*, 89: 5640-5644
- Watt F.M., Hogan B.L.M. 2000. Out of eden: Stem cells and their niches. *Science*, 287: 1427-1430
- Weigman A., Corbeil D., Hellwig A., Huttenr W.B. 1997. Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. *The Proceedings of the National Academy of Science Online (USA)*, 94: 12425-12430

- Weissman I.L., Anderson D.J., Gage F. 2001. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annual Review of Cell and Developmental Biology*, 17: 387-403
- Weksberg D.C., Chambers S.M., Boles N.C., Goodell M.A. 2008. CD150⁻ side population cells represent a functionally distinct population of long-term hematopoietic stem cells. *Blood*, 111, 4: 2444-2451
- Wendling F., Varlet P., Charon M., Tambourin P. 1986. A retrovirus complex inducing an acute myeloproliferative leukemia disorder in mice. *Virology*, 149: 242-246
- Wickrema A., Crispino J.D. 2007. Erythroid and megakaryocytic transformation. *Oncogene*, 26: 6803-6815
- Williams N., Jackson H. 1978. Regulation of the proliferation of murine megakaryocyte progenitor cells by cell cycle. *Blood*, 52: 163-170
- Wobus A.M., Boheler K.R. 2005. Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiological Reviews*, 85: 635-678
- Yang L., Bryder D., Adolfsson J., Nygren J., Månsson R., Sigvardsson M., Jacobsen S.E.W. 2005. Identification of Lin⁻Sca1⁺kit⁺CD34⁺Flt3⁻ short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood*, 105, 7: 2717-2723
- Yin A.H., Miraglia S., Zanjani E.D., Almeida-Porada G., Ogawa M., Leary A.G., Olweus J., Kearney J., Buck D.W. 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*, 90: 5002-5012

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