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Generation of autologous hematopoietic stem cells from induced pluripotent stem cells

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Abstract

Leukemia is mainly treated with chemotherapy, irradiation and hematopoietic stem cell transplantations (HSCTs). Unfortunately, leukemia can still be fatal, due to relapses and therapy-related mortality. This is partly due to the complications that come along with HSCTs. Allogeneic HSCTs can result in rejection and graft-versus-host disease (GVHD). Moreover, autologous HSCTs can result in relapses, since the collected cells can be contaminated with leukemic cells. Therefore, other forms of HSCTs should be developed to improve leukemia treatment. This could be provoked when induced pluripotent stem cells (iPSCs) could be used as a new source for hematopoietic stem cells (HSCs). In this bachelor thesis, an overview of current findings and challenges in this field has been provided. Studies have investigated multiple ways to generate HSCs from iPSCs. One way to attempt this is through genetic manipulation. Ectopic expression of the HOXB4 gene in iPSCs resulted in generation of HSCs and engraftment in mouse models. Another method is relying on the microenvironment of the iPSCs. Co-injection of iPSCs with stromal cells and hematopoietic cytokines in teratoma-bearing mice is shown to result in HSCs generation in both peripheral blood and bone marrow. A microRNA-based approach was also investigated as a technique to obtain iPSC-derived HSCs. Numerous miRNAs showed stimulation of self-renewal and suppression of differentiation in HSCs. These different techniques for generating iPSCs-derived HSCs hold great potential but still contain some limitations. When these limitations can be overcome, iPSCs can be used as a new source for autologous HSCs and hold great therapeutic potential in leukemia treatment.

Context

Introduction

Blood is one of the biggest tissues in the human body. An average adult human has around 5 L of blood in his/her body. Blood has multiple important functions, for example the transport of nutrients and oxygen to other tissues and organs in the body, but also transport of immune cells to infection sites[1,2]. Blood adapts to the body's needs: during exercise, more blood is pumped through the body to provide more oxygen to the muscles. Because of its variated functions, our blood is very precious[2]. Blood cells derive from hematopoietic stem cells (HSCs) in the bone marrow[3]. In leukemia, these cells remain undifferentiated and continuously proliferate, where they would normally develop into different types of blood cells[4].

Leukemia is mostly treated with chemotherapy and irradiation, followed by hematopoietic stem cell transplantation (HSCT)[5]. HSCT is performed in order to restore the damage caused by the irradiation and chemotherapy. Different forms of HSCT can be performed: autologous or allogeneic. For autologous HSCT, stem cells from the patient's own bone marrow are obtained before executing chemotherapy and irradiation and are transplanted after the treatment. For allogeneic transplantations, HSCs from other people are used[6].

Unfortunately, leukemia can still be fatal due to relapses and therapy-related mortality[7]. This is related to the fact that HSCTs do not always appear to be effective. Autologous HSCT does not cure leukemia. Autologous HSCTs show low toxicity, since the cells are not rejected by the patient's immune system. However, one major issue with autologous HSCTs after treating leukemia is that the collected cells are likely to be contaminated with residual leukemic cells. Reinfusing these cells in the patient will result in reoccurrence of the cancer. Even after purging the collected bone marrow from leukemic cells with different types of techniques (chemotherapy, monoclonal antibodies), autologous transplantations are shown to still lead to relapses[5].

Allogenic HSCTs result in graft-versus-host disease (GVHD) and rejection and are therefore more toxic than autologous HSCTs. On the other hand, allogeneic HSCTs can also cause an anti-leukemic effect, the graft-versus-leukemia effect (GVL)[5,8]. This is caused by genetic disparity between donor and recipient. Donor-derived T cells recognize alloantigens on the recipient's tumour cells, resulting in an immune response against the tumour cells. GVL does not occur in autologous transplantations[9]. Although allogeneic HSCTs, paired with a combination of GVHD and GVL, are shown to result in lower incidence of recurrent leukemia, it is not yet clear if this benefits the patient, as GVHD results in an increased mortality[5]. Studies are examining strategies for separating GVHD from GVL to improve the outcome of allogenic HSCTs[9].

Another limitation is the restricted availability of suitable donors for allogenic transplantations. This weights down the application of this approach in clinical therapies, on account of needing a big amount of transplantable HSCs to acquire a successful treatment[10].

Since both autologous and allogenic HSCTs show serious complications, an alternative should be developed to make this crucial part of leukemia treatment more effective and less toxic. In the last couple of years, a lot of research has focussed on using induced pluripotent stem cells (iPSCs) as a source for HSCTs[10]. The field of iPSCs has enormously grown[11]. iPSCs derived from reprogramming somatic cells broaden the horizons in generating patient-specific stem cells. If these cells could be used for generation of autologous HSCs, there might be a way to treat hematologic diseases without restriction from immune incompatibility, relapses and ethical concerns. However, the difficulty of obtaining iPSC-derived HSCs that have a strong capacity to sustain engraftment should not be underestimated[10]. Therefore, it could be questioned if generation of autologous HSCs from iPSCs can be realized. Since a lot of work is put into improving hematopoietic differentiation from iPSCs, an overview of the current findings and challenges is provided in this bachelor thesis.

1. The hematopoietic system

To indicate how important HSCTs are in treating leukemia and to illustrate how hematopoietic stem cells are generated under natural conditions in the human body, some general information about the hematopoietic system will be covered in this first chapter.

1.1. Development of the hematopoietic system

The development of the hematopoietic system starts in the embryo. In mammals, this development is a gradually process in which cells progressively lose their plasticity and gain characteristics specific for certain blood lineages. First, the pluripotent inner cell mass divides into the primitive endoderm and epiblast. The epiblast develops into the primitive streak, which will differentiate into three primary germ layers: ectoderm, mesoderm and endoderm. Hematopoietic cells originate from the mesoderm layer. In the yolk sac, these mesoderm cells will form blood islands, which support the primitive hematopoiesis of the embryo. Mesoderm cells in the aorta-gonad-mesonephros (AGM) region will differentiate into hemogenic endothelium. Through endothelial-to-hematopoietic transition, these cells will generate HSCs, which are the precursors for the definitive hematopoiesis. The HSCs will enter the circulation and eventually migrate to the bone marrow. Here they function as progenitor cells for hematopoiesis throughout adult life. An overview of this process is illustrated in figure 1[10].

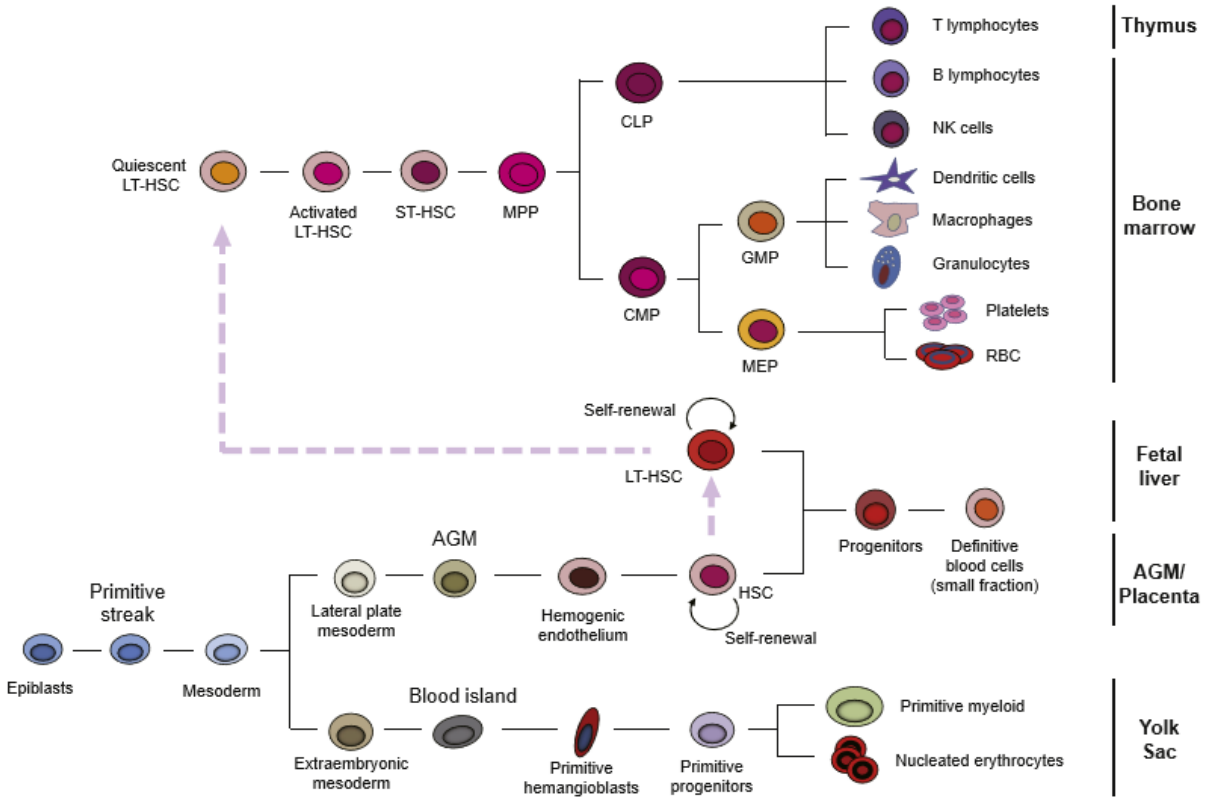

Figure 1. Overview of the development of the hematopoietic system. *ST-HSC, short-term HSCs; MPP, multipotent progenitors; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; MEP, megakaryocyte/erythrocyte progenitors; GMP, granulocyte/macrophage progenitors; RBC, red blood cells.*[10]

When located in the bone marrow, these long-term HSCs (LT-HSCs) develop into multipotent progenitors (MPPs). These MPPs differentiate into various lineage-restricted cells that make up the entire hematopoietic system. These cells are able to repopulate the entire blood system after transplantation. This is an essential feature of stem cell therapies that are being used to treat hematologic diseases[12].

1.2. Hematopoietic stem cells (HSCs)

It has been shown that HSCs play an important role during the development of the hematopoietic system in embryos[10], but how big is their part in the adult hematopoietic system? Stem cells are characterised by their capability to self-renew and differentiate. To illustrate how active these HSCs are: the turnover of hematopoietic cells in a man weighing 70 kg is around 1 trillion cells per day. This indicates that renewal rate in the hematopoietic system is high[13]. Differentiated cells are incapable of self-renewal[14]. Therefore, this remarkable renewal process is driven by the undifferentiated HSCs in the bone marrow. However, in steady state, only a few HSCs supply the production of the hematopoietic cells. Most of the HSCs are dormant in the cell cycle. This gives rise to the next question, why only certain HSCs differentiate, while others remain dormant[13]. Multiple studies provoked different models for the faith of HSCs[14,15,16].

Till et al. (1964) developed the 'stochastic' model, stating that the decision of an individual stem cell to self-renew or to differentiate is a stochastic, random process. This was shown by experiments in which cell suspensions of mouse bone marrow, spleen or fetal liver are injected into heavily irradiated recipient mice. Newly formed colonies in the spleen were analysed and the colony-forming cells (stem cells) were studied. These experiment showed that colony-forming stem cells are heterogenous distributed amongst the new colonies. An explanation for this could be that HSCs act following the birth-and-death process: an entity, for example a single cell, may either give rise to offspring like itself ("birth") or be expelled in some way ("death") and these two events occur randomly. During the development of the colonies, a small fraction of colony-forming cells and a large number of differentiated cells are being formed. Many of the daughter cells of the colony-forming cell lose their capacity to form colonies, as they are differentiated cells. The loss of this colony-forming ability can be considered as a 'death' process. Production of a small amount of daughter cells that maintain this colony-forming ability and therefore maintain self-renewal, can be considered as a 'birth' process. This confirms that not all HSCs are equally active in producing hematopoietic cells, but it also suggests that individual HSCs in a population are not strictly regulated. This is in contrast with the orderly behaviour that normal hematopoietic tissues show. It could therefore be possible that the whole population is regulated and not individual cells[14].

Another model is the hemopoietic inductive microenvironment (HIM) model proposed by Trentin (1971). This states that the development of HSCs into particular monopotent progenitors is determined by a specific microenvironment that surrounds the individual stem cells and that this decision is highly directed instead of random. Experiments showed that bone marrow stroma and spleen stroma can direct differentiation of stem cells[16]. This could also explain the orderly behaviour of the whole population in contrast to the stochastic behaviour of individual cells: neighbouring HSCs show similar decisions in self-renewal and differentiation as a result of encountering the same extrinsic signals. However, other studies showed contrary results that suggest that a structurally intact microenvironment is not obligatory to guide stem cells into differentiation[17]. It could therefore be proposed that the microenvironment has a more supportive role[15].

For both models, there is evidence that supports, but also evidence that contradicts the authenticity of each model. Therefore it remains unknown which model is most truthful. Perhaps, the most viable assumption would be that the decision of HSCs to go into differentiation or not is a combination of directed and stochastic factors[15].

1.3. HSCs in leukemia

Having an idea how of HSCs work under normal conditions, a closer look can be taken into the behaviour of leukemic HSCs.

One huge question in cancer research is whether every cell within a neoplasm has tumour-initiating capacities or whether this is limited to only a rare subset of cells, so called cancer stem cells (CSCs)[18]. Evidence for the existence of CSCs in leukemia came from studies that used primary leukemic tumour tissue and showed that only a small part of cancer cells has extensive proliferative capacities. This states that there is heterogeneity within leukemic tumours[19–21]. Because only a

minority of leukemic blasts can produce colonies, similar to normal hematopoiesis, an hierarchic organisation is maintained in which the CSCs continuously refill the bulk population of leukemic blasts[18].

There are, again, different models that explain the development of heterogeneity in leukemic tumours. The stochastic model states that the decision between differentiation and renewal is random and that every tumour cell has a low but equal probability to behave as a stem cell, where the stem cell model states that there are distinct cell classes within a tumour with different capacities to self-renew and only a small group of cells can initiate tumour growth as a CSC, or leukemic stem cell (LSC)[22,23]. However, evidence from acute myeloid leukemic (AML) cells supports the stem cell model[24,25].

LSCs and HSCs share many properties, but also show some differences. LSCs lack a couple of surface markers that are expressed on normal HSCs (such as Thy-1 and c-kit)[26,27]. Also, some LSCs classes have a much higher self-renewal capacity than HSCs. This suggests that the normal balance in the decision between renewal and differentiation is not present in LSCs. This may be due to mutations in genes involved in hematopoietic development[28,29]. These genetic changes result in increased proliferation. Remarkably, the effects of these genetic changes are principally noticeable in the downstream progenitors, since LSCs show a more dormant status[30–32]. Therefore it remains difficult to treat these cells, since most therapies rely on proliferating cells as their target.

1.4. Hematopoietic stem cell transplantations (HSCTs) in leukemia

In order to cure leukemia, LSCs should be eliminated and normal hematopoiesis should be restored. Unfortunately, achieving this with a low toxicity and high effectiveness remains quite difficult. Treatment of leukemia mostly consist out of chemotherapy and radiation to clear out all highly proliferating cells. This method is not LSC specific, but also affects normal HSCs and other kinds of stem and progenitor cells. Normal hematopoiesis is damaged as a consequence of the therapy. This increases the mortality, instead of decreasing it[5]. Besides, LSCs are not really that proliferative[30–32]. This makes current treatment of leukemia highly toxic and less effective and specific.

As an additional therapy, HSCTs are performed after chemotherapy and/or radiation in order to restore normal hematopoiesis. This part is crucial. Chemotherapy and radiation also cause death of normal HSCs, resulting in decreased hematopoiesis[5]. Fewer new hematopoietic cells can be produced, having immense consequence for the physiology of the human body.

As mentioned earlier, many limitations lay around these transplantations. Autologous HSCTs may result in relapses and do not cure leukemia, where allogenic HSCTs show high toxicity and the amount of donors is limited. Therefore, ongoing investigations look into other options to rebuild the hematopoietic system via HSCT, which brings us to the next topic: induced pluripotent stem cells[5,8,10].

2. Induced pluripotent stem cells (iPSCs)

Using induced pluripotent stem cells (iPSCs) as a new source for HSCs could be very interesting, since this technique helps to avoid multiple obstructions. To understand the unique potential of iPSCs, it is explained how the development of these cells has arisen. Another remarkable matter in this subject is that the use of iPSCs to produce certain cell types is no longer only based on speculations, but is also examined in advanced studies that are almost ready for clinical trials.

2.1. The invention of iPSCs

Embryonic stem (ES) cells are able to proliferate while maintaining pluripotency and can differentiate into cell types of all three germ layers. These ES cells could therefore be used to treat diseases such as Parkinson's disease and diabetes, as these malignancies result from disfunction of certain cell types. This disfunction can be restored by using pluripotent stem cells to generate normal functioning cells[33]. This sounds very promising. Sadly, some complications limit the use of these cells in clinical applications. ES cells are collected from human embryos, giving rise to ethical difficulties. In addition, implantation of ES cells results in tissue rejection[34]. Another reason why the clinical use of ES cells is limited, is because of the ability of undifferentiated ES cells to form teratomas in vivo, which is very dangerous and unwanted[35,36].

A way to avoid these obstructions was found when researchers discovered techniques to develop pluripotent cells from the patient's own cells. Shinya Yamanaka and his lab were the first to accomplish this in 2006. In the study of Takahashi and Yamanaka, reprogramming of somatic cells (differentiated adult cells) was established by the introduction of several transcription factors: Oct3/4, Klf4, Sox2 and c-Myc[34]. Oct3/4 and Sox2 play a role in the maintenance of pluripotency in ES cells[37,38]. Klf4 and c-Myc are oncogenes. c-Myc has multiple downstream targets that stimulate proliferation and transformation[39]. Klf4 has both tumour-suppressive and oncogenic properties, but initiation of Klf4 has one major effect: Klf4 represses p53, which in turn has been shown to repress the transcription factor Nanog during ES cell differentiation[40,41]. In this way Klf4 indirectly stimulates Nanog and other ES cell-specific genes[34]. Nanog also functions in the maintenance of pluripotency[42], but is not essential for generating iPSCs[34].

Figure 2 gives an illustration on how the introduction of the four transcription factors results in reprogramming of somatic cells. c-Myc facilitates an open, active chromatin structure through epigenetic changes. As a result, Oct4 and Sox2 have access to their target loci. The oncogenic function of c-Myc results in a cancer-like transformation of the somatic cells, giving the cells proliferative potential. p53 tumour suppressor proteins promote senescence, apoptosis and cell cycle inhibition and have an inhibitory effect on iPSCs generation on that account. c-Myc increases p53 levels, where Klf4 can block this apoptotic effect by suppressing p53 expression. This is not the only aspect where c-Myc and Klf4 work contrary: Klf4 activates p21, where c-Myc inhibits p21. p21 has an anti-proliferative effect. It seems that a balance between the expression of Klf4 and c-Myc is necessary to obtain reprogramming. Oct3/4 change the cell fate from tumour cells to ES-like cells. Sox2 drives pluripotency[43].

Expression of these four 'Yamanaka factors' can be introduced by using viral expression systems. Through this system, genes coding for these transcription factors can be integrated in the cell genome and will be expressed. Initially, this was done in mouse embryonic fibroblasts. These transcription factors contribute to the maintenance of pluripotency and thereby facilitate resetting of somatic cell cultures to generate iPSCs[34]. Later on, this was also established with adult human fibroblasts[44].

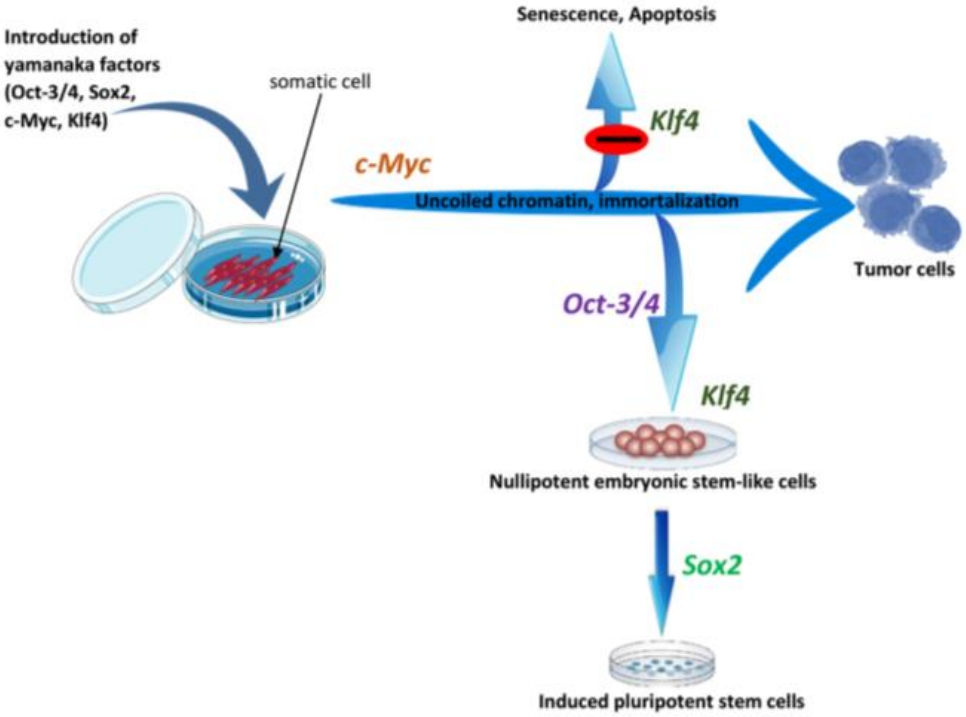

Figure 2. An diagrammatic illustration showing the roles of 'Yamanaka' factors in the induction of iPSCs. *A balance between the expression of c-Myc and Klf4 results in initial reprogramming of somatic cells and stimulates proliferation. Oct3/4 transforms the cell faith from tumour cells to ES-like cells. Sox2 helps to drive pluripotency. Altogether, these four factors can cause somatic cells to become iPSCs* [43].

2.2. Successful experiments using iPSCs

When it became clear that somatic cells can be reset to iPSCs, which could give rise to almost every other cell type, multiple biomedical delegations recognized the potential of these cells. For example, in Japan it is already accomplished to generate cardiomyocytes from iPSCs. These cells were even used for clinical application[45,46]. Human iPSCs have also been used to successfully generate retinal pigment epithelium cells[47]. These studies indicate that iPSCs can indeed be used to generate all kinds of cell types.

The therapeutic potential of iPSCs is also acknowledged in research into large-scale construction of blood products with expectancy of clinical application. In this field, it is researched if in vitro production of red blood cells is possible in order to develop a new interesting alternative to classic blood transfusions. When production of red blood cells could be accomplished through iPSCs, an adequate supply would be developed with specific production of blood cells with particular phenotype and infection and immunization risks might possibly be reduced. This would be of great importance, since the requirement of red blood cells lays around 80-90 million units in the world every year[48].

The most logic manner to produce red blood cells in vitro would be to use stem cells that are already programmed to give rise to blood cells: HSCs. Research into the feasibility of this approach is already at industrial level[49]. However, this approach is restricted to the constrained supply of HSCs. Identification of an unlimited source of stem cells would therefore be a huge breakthrough[50]. Since iPSCs can be obtained by reprogramming somatic cells, this could be a potential source for unlimited blood production.

Multiple studies have already shown that it is possible to obtain complete maturation of the erythroid blood line in vitro starting from iPSCs. These studies show differences in methods that they used, but both achieve production of mature functioning erythrocytes[50].

The study of Lapillonne et al. (2010) used iPSC lines that were obtained from human fetal and adult fibroblasts. The iPSCs were driven to differentiate into erythroid cell types by using cytokines and human plasma. Requirements for this protocol contain obligatory passage through a hematopoietic progenitor stage, co-culturing on a cellular stroma and the use of proteins of animal origin. When these criteria are followed, successful complete maturation of erythroid blood cells is obtained, starting from iPSCs. This was the first study achieving this complete maturation[50]. Generating iPSCs from fibroblasts is estimated to give a low frequency[34]. This may be due to rare tissue stem/progenitor stem cells that coexist in the used fibroblast cultures, since it has been described that stem cells have been isolated from the skin[51–53].

Hansen and colleagues from the Sanquin Research Lab designed a method to create three major myeloid blood cell types from iPSCs. In this study they showed that differentiation of iPSCs into hematopoietic lineage cells can be achieved with 3D- or 2D-methods[54]. Clustering of iPSCs into an embryoid body (EB) mimics in some aspects the complex gathering during embryogenesis[55]. By controlling the EB size or by using an extracellular matrix and/or growth factors, differentiation towards certain cell types can be directed[56]. However, an EB is a 3D-structure and hinders isolation of cells for further use. Therefore, differentiation of iPSCs could be performed in 2D monolayers on feeder-free matrix coated dishes. In this way Hansen et al. (2018) proposed a novel, feeder-free, single iPSC-derived monolayer differentiation system to culture 3 major myeloid blood cell types, which can mature into erythroid, megakaryocytic and myeloid cells[54].

First they performed single cell-seeding with RevitaCell™ Supplement[54], which is optimized for use with pluripotent stem cells. RevitaCell™ improves cell viability and growth when performing single cell-passage[57]. This results in colonies equal in size and number within iPSC lines. Unwanted variability in culture outcome are thereby avoided. Next, they harvested cells from the colony culture. They followed the standardized hematopoietic induction scheme illustrated in figure 3. Basic hematopoietic growth factors (IL-6, IL-3, hSCF, TPO) and lineage specific growth factors (EPO, IL-1β, FLT-3) were administrated after mesoderm differentiation. This resulted in differentiation to erythroid cells, megakaryocyte cells and myeloid cells[54].

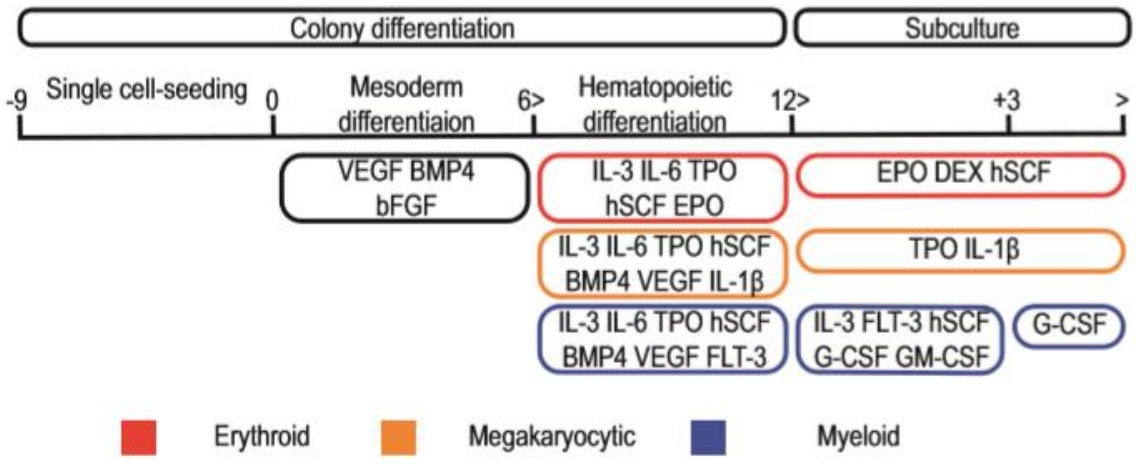

Figure 3. Standardized hematopoietic induction scheme used in the study of Hansen et al. *Shown is single cell-seeded iPSC colony differentiation towards hematopoietic cell types. Maturation of cells to erythroid- (red), megakaryocyte- (yellow) and myeloid cells (blue) are individually shown*[54].

As already mentioned in the study of Lapillonne and colleagues[50], to acquire fully maturated erythrocytes from iPSCs, cells were obliged to passage through a hematopoietic progenitor stage. HSCs and progenitor cells are closely related, but differ in potency and differentiation[58]. In order to produce differentiated hematopoietic cells, the process is directed towards these progenitors and passes by the HSC-stage. It can therefore be speculated that when this process could be terminated earlier, HSCs can be obtained. The next and last chapter will get to this point.

3. iPSCs and HSCs

It has already been mentioned why in vitro generation of autologous HSCs from iPSCs shows a great potential: it could provide new therapeutic approaches for replacing present HSCTs, since no rejection or GVHD will occur and there will be no contaminating leukemic cells in the graft . A lot of work has been put in research into this topic in the last couple of years. In previous chapters, general information about HSCs and iPSCs has been given and it has been explained how these cells arise. Combining all this information, the question remains: are their studies showing that generation of HSCs from iPSCs is possible? This last chapter will provide current findings in research into this field and challenges will be addressed.

3.1. Methods to generate iPSC-derived HSCs

Where generation of mature erythrocytes from iPSCs is already accomplished, generation of HSCs from iPSCs remains more difficult. Ex vivo culturing of HSCs without loss of their stem cell characteristics is challenging[59]. One reason for this difficulty is that the microenvironment has an huge impact on the behaviour of HSCs. Reproduction of the microenvironment, necessary for development of hematopoietic lineage cells, is quite difficult since not all the cell types in the HSC niche are known[60]. However, some genes that are normally expressed in HSCs have already been indicated to be responsible for maintaining stemness. Both factors, microenvironment (extrinsic) and genetics (intrinsic), are used as targets in research into generating iPSC-derived HSCs.

3.1.1.Genetic manipulation

One approach to generate HSCs is through genetic manipulations. It is shown that ectopic expression of transcription factors that are normally expressed in HSCs maintain stemness and expansion[61–63]. That is why it has been tried to make HSCs from mouse ES cells by ectopic expression of HOXB4. The HOXB4 gene is shown to result in an expansion of HSCs in mouse models[64]. HOXB4 overexpression also promotes differentiation of ES cells to HSCs[65]. Therefore, it would be interesting to study the effect of ectopic expression of HOXB4 in iPSCs and if this also results in HSCs generation. However, ectopic expression of HOXB4 in mouse ES cells did not result in normal hematopoiesis, since lymphoid and erythroid differentiation were inhibited. It was also shown that the amount of HOXB4 expression influences the lineage distribution. High expression levels of HOXB4 result in reduced capability of erythroid differentiation[59]. This study used ES cells to generate HSCs.

Other studies also examined the effect of ectopic expression of HOXB4 in iPSCs. In the study of Izawa et al. (2013) it was seen that ectopic expression of HOXB4 in murine iPSCs resulted in HSCs generation and that these cells are able to repopulate for over two months in recipient mice[65].

3.1.2.Microenvironment

Regarding the problem concerning the unknown microenvironment of HSCs, Suzuki and colleagues (2013) tried to obtain HSCs from mouse and human iPSCs by using teratomas. Teratomas were considered as differentiation sites of hematopoietic lineage cells, since they are tumours containing differentiated tissues of all three germ layers. Within these tumours, erythrocytes, megakaryocytes and blood vessels are formed. Therefore, they hypothesized that such a microenvironment might give rise to iPSC-derived HSCs[60].

Exploring this within immunodeficient mice, they first used mouse iPSCs with a high hematopoietic potential ($Lnk^{-/-}$ iPSCs). These cells were obtained by reprogramming of tail tip fibroblasts of $Lnk^{-/-}$ mice with Oct3/4, Sox2 and Klf4. These iPSCs were injected into teratoma-bearing mice. Although injection of iPSCs alone did not generate iPSC-derived HSCs, co-culturing and co-injection with stromal cells and particular hematopoietic cytokines enhanced the induction efficiency. Hematopoietic cells originating from injected iPSCs were found in both peripheral blood and bone marrow. These hematopoietic cells consisted out of multipotent progenitors, hematopoietic stem progenitor cells and long-term HSCs. This suggests that generation of iPSC-

derived HSCs was succeeded through teratoma formation and enhanced through supplementation with hematopoietic cytokines and stroma cells. In addition, this study also showed that these HSCs are capable of homing to the host bone marrow. Secondary transplantation of bone marrow cells from primary recipients resulted into self-renewal of these HSCs. The experiment was also performed with wildtype mouse iPSCs and similar results were observed[60].

Furthermore, human iPSCs were injected into non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice in a similar way: co-cultured and co-injected with hematopoietic cytokines and stroma cells. This resulted in engraftment of human iPSCs-derived hematopoietic cells (both HSCs and differentiated blood cells) in the peripheral blood and bone marrow. This study showed that injected iPSCs differentiate into HSCs in teratomas and migrate into the bone marrow. The latter is very interesting, since no intra-bone marrow delivery method is required, in contrast to other studies[60].

3.1.3.miRNAs

Other studies have tried to achieve generation of all kinds of cell types from iPSCs via the use of micro RNAs (miRNA/miR). For example, it has already been accomplished to obtain endothelial cells from iPSCs by overexpressing miR-21[66]. These small non-coding RNA molecules bind to messenger RNA (mRNA), resulting in decreased protein expression. miRNAs play important roles in cell differentiation, proliferation and apoptosis[67]. miRNAs have also been reported to affect reprogramming of somatic cells by stimulating the expression of pluripotent reprogramming factors (Yamanaka factors)[68–70]. There are numerous studies investigating all different sorts of miRNAs in relation to HSC development[67].

A couple of these findings are summarized in figure 4. Ectopic expression of miR-125a in human MPPs resulted in an increase in self-renewal. When these cells were transplanted in mice, they showed high engraftment[71]. miR-125b targets LIN-28A. Therefore, high expression of miR-125b results in a very aggressive form of myeloid leukemia in mice[72]. miR-155 targets multiple transcription factors genes (ETS1, MEIS1, CEBPB (encoding for C/EBPβ), CREB1, JUN, SPI1, AGTR1, AGTR2, FOS). ETS1 and MEIS are involved in megakaryocyte (MK) proliferation and differentiation. Through this pathway, miR-155 inhibits MK development. The other genes targeted by miR-155 are involved in the regulation of HSC differentiation into myeloid progenitor cells (MPCs) and lymphoid progenitor cells (LPCs). miR-155 functions as a negative regulator for normal myelopoiesis and erythropoiesis[73]. Both miR-181 and miR-223 are shown to have crucial functions in myeloid and lymphoid lineage development[74–76]. miR-221 and miR-222 modulate the expression of the Kit receptor, resulting in down-modulation of the Kit protein. This results in down regulation of erythropoiesis[77,78].

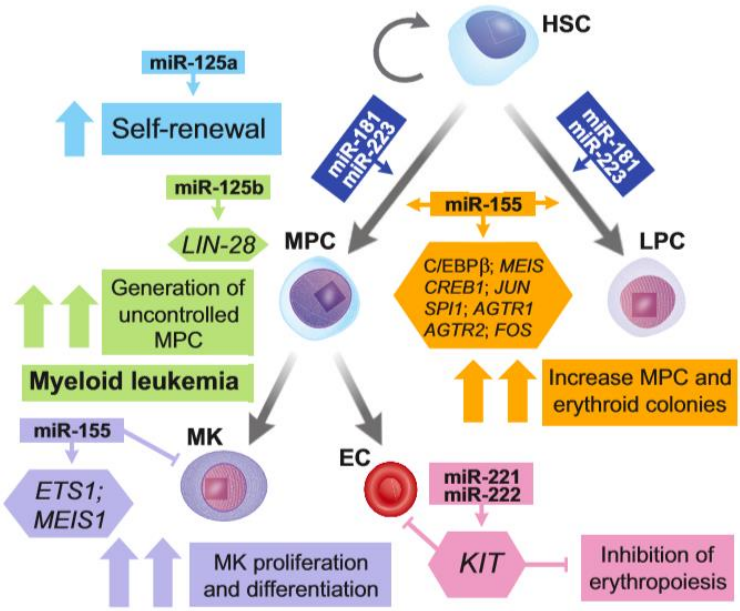

Figure 4. Schematic summary of findings from multiple studies investigating the role of miRNAs in HSC self-renewal and differentiation. *Expression of miR-125a stimulates self-renewal in human MPPs (bright blue). Overexpression of miR-125b results in uncontrolled proliferation of myeloid progenitor cells (MPCs), resulting in myeloid leukemia (green). mi-155 has multiple genetic targets, leading to MK inhibition via affecting ETS1 and MEIS1 (greyish) and increases the formation of MPCs and erythroid colonies (orange). miR-181 and miR-223 regulate MPC and LPC differentiation of HSCs (dark blue), where miR-221 and miR-222 inhibit erythropoiesis by targeting the KIT protein (pink)[67].*

All miRNAs mentioned in the above part are somehow involved in maintaining stemness and differentiation in HSCs, some stimulating maintaining stemness, other stimulating differentiation. Sadly, there are little findings about this method in iPSCs. It could therefore be interesting to express these miRNAs in iPSCs, to see what kind of effect this would have. Since these miRNAs are related to HSC self-renewal and differentiation, it could be hypothesized that they could be used to stimulate iPSC-derived HSC generation.

Since there are multiple mechanisms playing a role in HSC maintenance, multiple techniques to convert iPSCs into HSCs are examined. In this thesis, techniques have been discussed that concern both intrinsic and extrinsic mechanism. The ectopic expression of genes in iPSCs relies on genetic manipulation, where iPSCs could also be guided to differentiation into HSCs with microRNAs. The use of certain microenvironmental factors to generate HSCs from iPSCs relies on extrinsic effects on the iPSCs. However, these technique still show some limitations. One of the biggest challenges remains to obtain engraftable HSCs from iPSCs.

Discussion & Conclusion

The need for another approach for HSCT is very big. Current types of HSCT in leukemia treatment result in high levels of rejection and GVHD and often result in relapses due to contamination of the graft with leukemic cells. In order to invent another type of HSCT, a lot of effort is put in examining different methods to obtain iPSC-derived HSCs. If iPSCs could be used as a source for autologous HSCs, the above-mentioned constrictions will not occur since cells from the patient-self can be used and contamination of the transplant with leukemic cells can be excluded. Nevertheless, to move this field from speculations to clinical applications, standardized protocols should be set to convert iPSCs into HSCs. In order to achieve this, numerous studies are trying to address factors that are able to cause this conversion.

In this bachelor thesis, the focus was put on three sorts of methods for iPSC-derived HSC generation. These types rely on genetic changes, changes in the microenvironment of iPSCs and micro-RNA based approaches. All three types show great potential in generation of HSCs, but unfortunately also show some limitations.

Regarding the genetic manipulation of iPSCs in order to obtain HSCs, ectopic expression of HOXB4 in murine iPSCs resulted in HSC generation and therefore hold great potential. However, one huge hazard is that the vectors that are being used can also integrate into the genome and potentially introduce mutations at the insertion site. This might lead to tumorigenesis[79]. Fortunately, this constraint has already been improved. Strategies such as mRNA transfection, miRNAs, non-integrating vector technologies and recombinant membrane-crossing proteins have been developed in order to perform genetic manipulation. These strategies are more clinical proof and are less likely to cause tumorigenesis[80].

Besides, studies have shown that is remains quite difficult to generate HSC through induction of certain genes. This is often seen in studies trying to manipulate cells through genetic modification, since most biological processes are under the control of multiple genes. Moreover, induction of genes in mice can result in promising shows findings, but does not generate the same yield when they are examined in human cells, or the other way around[59,81].

The study showing that injection of iPSCs in teratoma-bearing animals induces HSC development from iPSCs, gives insight in the importance of extracellular factors in this process[60]. Yet the idea of introducing teratomas into humans does not seem very viable. Although this study has shown that transplantation of these cells did not result in leukemia or other forms of cancer[60], a contrary feeling is commencing when treatment of a certain type of cancer would consist out of injecting cells from another tumour type. Nevertheless, if the construction of teratomas would be better understood, this information could be used to generate an environment in which iPSCs can be stimulated to develop into HSCs.

Concerning the studies exploring the use of miRNAs, similar to the genetic manipulations, it should be taken into account that such changes can also give an opposite effect and cause tumorigenesis. This was also shown in the study of Chaudhuri et al (2012). They showed that high expression of miR-125b results in leukemia in mice[72]. This indicates that the expression range of these microRNAs should be regulated very carefully. In addition, the role of miRNAs in regulation of cell differentiation and proliferation should be explored in greater depth, to avoid unwanted side effects.

In addition to the constrictions in converting iPSCs into HSCs, there are also some limitations in the initial generation of iPSCs. Generation of iPSCs is shown to have a low efficiency[34]. Explanations for this finding could be the presence of a small amount of tissue stem cells, and that only these cells seem to be sensitive for the reprogramming factors, or that only a narrow expression range of the reprogramming factors results in successful reprogramming of somatic cells[34].

Other limitations in iPSC production concern the key aspects of this process, for example reprogramming factors, method of cell delivery, target tissue, culture conditions and the biological assay to validate the resulting cell pluripotency potential. All these processes are very time consuming and expensive[82].

However, the low frequency of iPSC generation might not be that problematic. Generation of only one iPSC might be enough to generate eternal somatic cells. This is ensured by the capacity of the iPSC to self-renew.

Another, maybe even bigger, limitation is that the origin of the iPSC might influence the efficiency of the differentiation process. In the study of Hansen et al., hematopoietic cells were used as origin for iPSCs[54], but Lapillonne et al. used fibroblast as origin for the iPSCs[50]. Both studies generated iPSC in order to create HSCs, but differed in the type of cells that they used as origin for their iPSCs. It has been shown that iPSCs conserve the epigenetic memory of their ancestor. This may influence their differentiation capacity. When iPSCs are generated from ancestors that are not closely related to the desired cell type, it might be very difficult to obtain iPSC-derived HSCs with high efficiency[83].

Ongoing research by PhD student Daniëlle Luinenburg in Gerald de Haan's lab is investigating the role of miR-125a in HSCs. In this study, immunodeficient mouse models are transplanted with human cord blood cells with ectopic expression of miR-125a. Overexpression of miR-125a has already been shown to increase the proliferative ability of HSCs and to induce a more stem cell-like phenotype in MPPs[71]. The studies of Wojtowicz et al. (2019) showed that miR-125a increased number, size, longevity and migration of the clones, which resulted in a symmetric distribution of clones throughout the peripheral blood[84]. Luinenberg also observed increased proliferation and a more stem cell-like character in the cord blood cells, confirming that miR-125a stimulates HSC generation. The next thing to do, is to examine if ectopic expression of miR-125a in iPSC-derived hematopoietic progenitors also induces a more stem cell-like phenotype and stimulates the proliferation of these cells. When this is the case and the mechanism behind miR-125a is more understood, miR-125a might be used to generate HSCs from iPSCs.

Although there are still a lot of limitations in generating HSCs from iPSCs, this approach does show a lot potential. Multiple studies addressed ways to generate iPSC-derived HSCs, showing that it is possible to use iPSCs as a new source for HSCs. The biggest advantage of this approach is that there does not occur immunization against the iPSC-derived HSCs, since they are originating from the patient itself. However, there is still a long way to go. Studies concerning this topic are still centred in mouse models, which does not always show high efficiency. If we would like to bring this approach to clinical applications, we first should overcome the hurdle of translating these findings in mice to humans, next to solving the remaining complications. It would therefore be of great importance to address more research into generation of HSCs from iPSCs, since this holds great potential in leukemia treatment.

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