Weill Cornell Medical College | NewYork-Presbyterian Hospital Weill Cornell Medical Center | Fachhochschule Salzburg University of Applied Sciences

2nd Bachelor Thesis

GENERATION OF INDUCED PLURIPOTENT STEM CELLS FOR THE TREATMENT OF HEMOGLOBINOPATHIES

AS OBTAINMENT OF THE ACADEMIC DEGREE

„BACHELOR OF SCIENCE IN HEALTH STUDIES“

Author: Manuela Passrugger

1010434017

Submitted in June 2013

1st Supervisor: Stefano Rivella, PhD

2nd Supervisor: Gertie Janneke Oostingh, PhD

Biomedical Science

Fachhochschule Salzburg GmbH

Bischofshofen, June 5th, 2013

Eidesstattliche Erklärung

Ich versichere an Eides statt, dass ich die vorliegende Bachelorarbeit ohne fremde Hilfe und ohne Benutzung anderer als der angegebenen Quellen und Hilfsmittel angefertigt und alle aus ungedruckten Quellen, gedruckter Literatur oder aus dem Internet im Wortlaut oder im wesentlichen Inhalt übernommenen Formulierungen und Konzepte gemäß den Richtlinien wissenschaftlicher Arbeiten zitiert, bzw. mit genauer Quellenangabe kenntlich gemacht habe.

Diese Arbeit wurde in gleicher oder ähnlicher Form weder im In- noch im Ausland in irgendeiner Form als Prüfungsarbeit vorgelegt und stimmt mit der durch die BegutachterInnen beurteilten Arbeit überein.

....

Ort, Datum Unterschrift

Index

1. Zusammenfassung/Abstract

Zusammenfassung

Hemoglobinopathien werden von einem Fehler in der genetischen Information hervorgerufen. Das Globin-Gen ist so mutiert, dass es entweder zu einer falschen Struktur, oder zu einer mangelhaften Produktion von Globin-Ketten kommt. Die am häufigsten vorkommenden genetisch bedingten Hemoglobinopathien sind die Sichelzellanämie und die Thalassämie. Weltweit sind 7% der Bevölkerung Träger einer dieser Krankheiten.
Die β-Thalassämie-PatientInnen sind auf eine Langzeitbehandlung angewiesen (Bluttransfusionen oder Knochenmarkstransplantationen). Die Krankheit wird trotz vorhandener Therapie immer noch mit hoher Morbidität und Mortalität verbunden. Der Großteil der β-Thalassämien wird durch eine Punktmutation hervorgerufen und daher eignen sie sich hervorragend für eine Gentherapie.

Stammzellen besitzen die Eigenschaft, sich in alle Zellen differenzieren zu können. Diese Eigenschaft wird als Pluripotenz bezeichnet. Da die Verwendung von Stammzellen sehr umstritten und aus ethischen Gründen erschwert durchführbar ist, wurden Experimente durchgeführt, mit dem Ziel, somatische Zellen, in Stammzellen zurück zu verwandeln. Dafür werden spezielle Faktoren in, aus einem Retrovirus hergestellten Vektor, verpackt und in das Genom transportiert. Die dabei entstehenden Zellen nennen sich induzierte pluripotente Stammzellen (iPSCs). Weites haben homologe Rekombinationsexperimente gezeigt, dass es möglich ist, das krankhaft mutierte β-globin Gen in iPSCs, mit einem Gesunden zu ersetzen. Diese iPSCs werden aus β-Thalassemie-PatientInnen kreiert.

Mit diesem Hintergrundwissen ist die Idee, periphere Blutzellen von einem/einer β-Thalassemie-Patienten/Patientin zu gewinnen und umzuprogrammieren, um anschließend gentherapeutisch behandeln zu können, entstanden. Das Ziel dieser Bachelorarbeit ist es, die iPSCs aus peripheren Blut eines β-Thalassämie-Patienten herzustellen.
Die Blutprobe wurde von einem β-Thalassämie-Patienten, der sich in Behandlung im New York Presbyterian Krankenhaus befindet, zur Verfügung gestellt und die Zellen wurden mit einer integrierenden Methode und einer nicht integrierenden Methode induziert.
Die Pluripotenz wurde mit mehreren Tests nachgewiesen und die Ergebnisse können als positiv interpretiert werden.

Schlüsselwörter: gene therapy, hemoglobinopathies, induced pluripotent cells

Abstract

Hemoglobinopathies originate from a malfunction in the globin gene that results in structural defects or decreased production of globin chains. The most common inherited hemoglobin disorders are sickle cell disease (SCD) and the thalassemias. Worldwide there are approximately 7% carriers for such disorders.
Beta-Thalassemia patients are depend on a long-term treatment (blood transfusion and bone marrow (BM) transplantation) and are still associated with morbidity and mortality. Most of the β-Thalassemias are caused by point mutations; this fact makes them a great target for genetically based therapies.

Due to their pluripotent nature, embryonic stem cells (ESc) hold great potential for a variety of diseases. However, due to their controversial sourcing, efforts have been made to create induced pluripotent stem cells (iPSCs) from somatic cells. IPSCs are mature cells reprogrammed to an embryonic-like state by using defined factors.
The idea is to create these cells, correct the point mutation and treat the patient.
In particular, combining gene transfer with iPSC technology means independence from finding a compatible BM donor and the use of patients' own cells can prevent graft versus host disease (GVHD).

The aim of the study is to create iPS cells from a β-Thalassemia patient and to verify their pluripotency. The generation of iPSCs in this study requires only 30ml peripheral blood sample from a β-Thalassemia patient. If successful, this will be sufficient to reprogram hematopoietic cells to iPSCs,

The blood sample was provided from a β-Thalassemia patient and iPSCs were induced with lentiviral as well as episomal vectors expressing the four iPSC-creating genes.
The embryonic-like state is evaluated with various assays, such as RT-PCR and FACS for ES-specific markers, the functional teratoma assay and AP-staining. A Karyotype is determined and morphology is observed microscopically.

The results confirm the ESc-like state, which means that iPS cells were created successfully.

Key words: gene therapy, hemoglobinopathies, induced pluripotent cells

2. Introduction

Hypothesis:
IPSCs can be created from a ß-Thalassemia patients' peripheral blood cell sample.

Stem cells were always of great interest for many scientists driven to understand better their main characteristics: their ability to self-renew or multiply while maintaining the potential to develop into other types of cells. However there has always been an ethical difficulty regarding the use of human embryos. This was circumvented when Takahashi et al. in 2006, established a method to reprogram patient own cells into embryonic stem cell – like cells (ES-like), also called induced pluripotent stem cells (iPSCs). Shortly thereafter, scientists worldwide started to derive iPSC from several human and mouse tissue to modify and improve the method.
The ability of iPSCs to divide for long periods and retain their ability to make all cell types within the organism provides a broad scientific potential in terms of studying diseases and also the effects of pharmaceuticals. In addition, the patient's own tissue or cells can be used for reprogramming to adult tissues. This would also solve the problem of graft versus host disease (GVHD) and the limitation of finding compatible donors.
The aim of this Thesis is to create and verify the pluripotency of the iPSCs induced from a β-Thalassemia patient's peripheral blood sample. The first chapter talks about the background of Hemoglobinopathies, as that is the main focus of the research lab where the author performed the internship. In addition, the source of cells utilized to generate the iPSCs was from blood of β-Thalassemia patients. This chapter will also describe how the patients are presently treated in the clinic and how the use of iPSCs might modify the way they are treated in the future. The aim of the Thesis is mentioned in the second chapter along with the overall review of the project.
The study procedure is introduced in the third chapter, when the steps from gaining the blood samples until the final verification of the created iPSC are explained. How these steps could be accomplished and what was needed for it is all written down in the fourth chapter "Materials and Methods".
In the fifth chapter, the results of verification are presented with the support of figures. Afterwards, the results are discussed in the penultimate chapter when the author is also reviewing the study structure and possible problems.
The last chapter, chapter 7, is discussing about potential application of iPSc.
The table of figures and all the references can be found in the end of the Thesis.

2.1. Hemoglobinopathies with focus on beta-Thalassemia

In a healthy human being, the normal Hemoglobin (Hb) is responsible for nourishing the tissues with oxygen. It is also responsible for providing the color to the red blood cells (erythrocytes). Hb binds oxygen, which is then transported in circulation by the erythorcytes (Jamison et al., 2006. Chapter 34).

During the human development different forms of haemoglobin can be identified:

- An unborn has embryonic Hb which binds oxygen with very high affinity. There are different types built in the first 8 weeks:
  Gower 1 (ζ2ε2), **Gower 2** (α2ε2)
  Hb **Portland I** (ζ2γ2), Hb **Portland II** (ζ2β2) (Steinberg et al., 2009. page 119)
- HbF = fetal hemoglobin, which is expressed until a few months after a child was born. It is built in spleen and liver. **HbF** contains two alpha- and two gamma-chains (**$\alpha_2\gamma_2$**)
  (Jamison et al., 2006. Chapter 34)
- HbA = adult hemoglobin, which is built in the bone marrow (BM) and consists of four chains. This structure represented in figure 1 is also known as an adult tetramer. There are two forms of the adult tetramer:
  HbA_1 is made out of two alpha- and two beta-chains (**$\alpha_2\beta_2$**)
  HbA_2 is made out of two alpha- and two delta-chains (**$\alpha_2\delta_2$**)

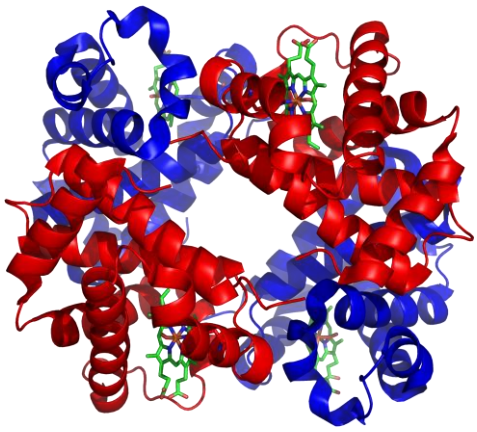

Figure 1: Tetramer. Wikipedia, 03/2013

Each of the different globin chains is controlled by a specific genetic locus in the human genome. Two genes exist for α- and γ-chains and one for each of the other globin encoding chains. The structure and the region of the genes are already well known and have been determined in several studies (Jamison et al., 2006. Chapter 34).

Dong et al., 2013 summarized that Hemoglobinopathies originate from a malfunction in the globin gene that results in structural defects or decreased production of globin chains. The most common inherited hemoglobin disorders are sickle cell disease (SCD) and the thalassemias. Worldwide there are approximately 7% carriers for such disorders.
The main difference between these two disorders is that SCD originates from a single mutation leading to a **structural defect** in the β-globin chain, while the thalassemias from mutations leading to **limited or absent synthesis** of the hemoglobin molecule. Furthermore thalassemias are classified based on the defect leading to the globin chain's reduced synthesis. For instance, α-Thalassemia and β-Thalassemia are characterized, respectively, by mutations in the α-globin and the β-globin genes (Breda et al., 2009).

A-Thalassemia can be silent when only 1 or 2 globin genes are mutated (silent thalassemia carriers), while when 3 genes out of four are mutated this can result in Hemoglobin H disease (moderate to severe anemia and hepatosplenomegaly). Deletion of all four globin genes leads to α-Thalassemia major. The latter is the most severe form and is associated with death in utero. Most of the α-Thalassemias are caused by gene deletions (Breda et al., 2009).

In β-Thalassemia, this disorder can be classified based on the severity of the mutation (where β+ and β0 indicates, respectively some or no β-globin synthesis) and combination of these two mutant alleles: β0/0, +/0 or +/+. In particular these three groups associated respectively with no, very low or low to moderate β-globin synthesis.
From the clinical standpoint, β-Thalassemia can be classified as β-Thalassemia major or Cooley's anemia, which is the most severe form and β-Thalassemia intermedia. In β-Thalassemia major patients are dependent on red blood cell transfusion for survival a lifelong. A greater number of β-globin chains and red cell synthesis characterize β-Thalassemia intermedia. Only infrequent or no transfusions are required in this case. Most of the β-Thalassemias are caused by point mutations.

2.1.1. Current Treatment

The patient's anemia is treated with red blood cell transfusion. Transfusions if provided frequently lead to iron overload and transfusion-associated infections and required iron chelation. These options have improved the quality of life of patients, but they still depend on a long-term treatment and are still associated with morbidity and mortality (Dong et al.,

2013). A transplantation of allogenic bone marrow (BM) is the definitive cure for Hemoglobinopathies. In 1982 the first successful treatment was reported by Case et al. (Breda et al., 2009). One of the largest cohorts of patients treated with allogenic bone marrow transplant was treated by Dr. Lucarelli and co-workers in Italy (Breda et al., 2009). Although it can lead to Thalassemia free survival, this approach is of limited suitability because a matching donor is needed and also graft versus host disease (GVHD) may occur (Dong et al., 2013).

2.1.2. Treatment aim

Because of the flaws of the afore mentioned methods, treatments need to improve to provide a better quality of life for these patients.
Considering genetically based therapies, these might offer a variety of benefits, such as:

- Use of patients' own hematopoietic stem cells (HSCs) can prevent GVHD (figure 2)
- Independence from finding a compatible BM donor
- Definitive cure
- In addition, this might provide a way to cure monogenic disorders
  (Breda et al., 2009)

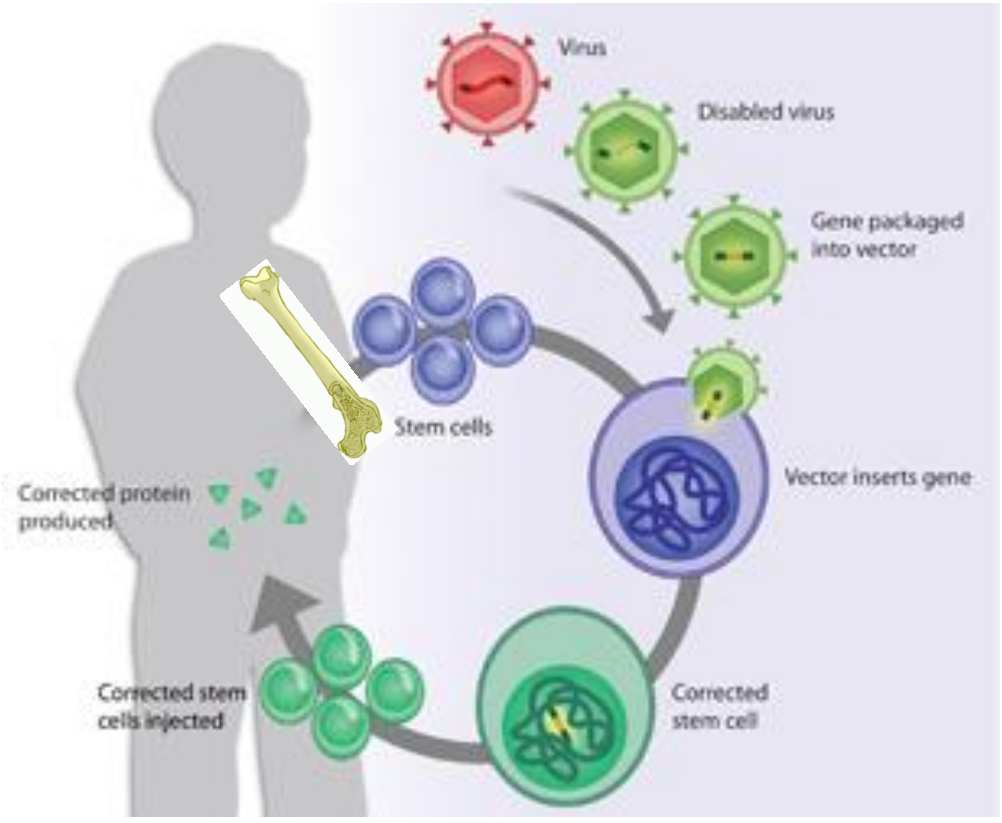

Figure 2: Hemoglobinopathies and gene therapy. Wikipedia, 04/2013

In particular, combining gene transfer with iPSC technology might reduce the risks for the patient presently associated with the gene transfer in BM cells. This approach also requires a minimal amount of tissue from the patient.
Notably, as will be shown later, generation of iPSCs requires only 30ml peripheral blood sample from a β-Thalassemia patient. If successful, this will be sufficient to reprogram hematopoietic cells to iPSCs, correcting them and then transplanting them back into the patient.

2.2. Induction of pluripotent Stem Cells (iPSCs)

Figure 3 shows, in a schematic representation of the most relevant part progresses that have been made over several years related to the manipulation of stem cells (Graf, T., 2011).

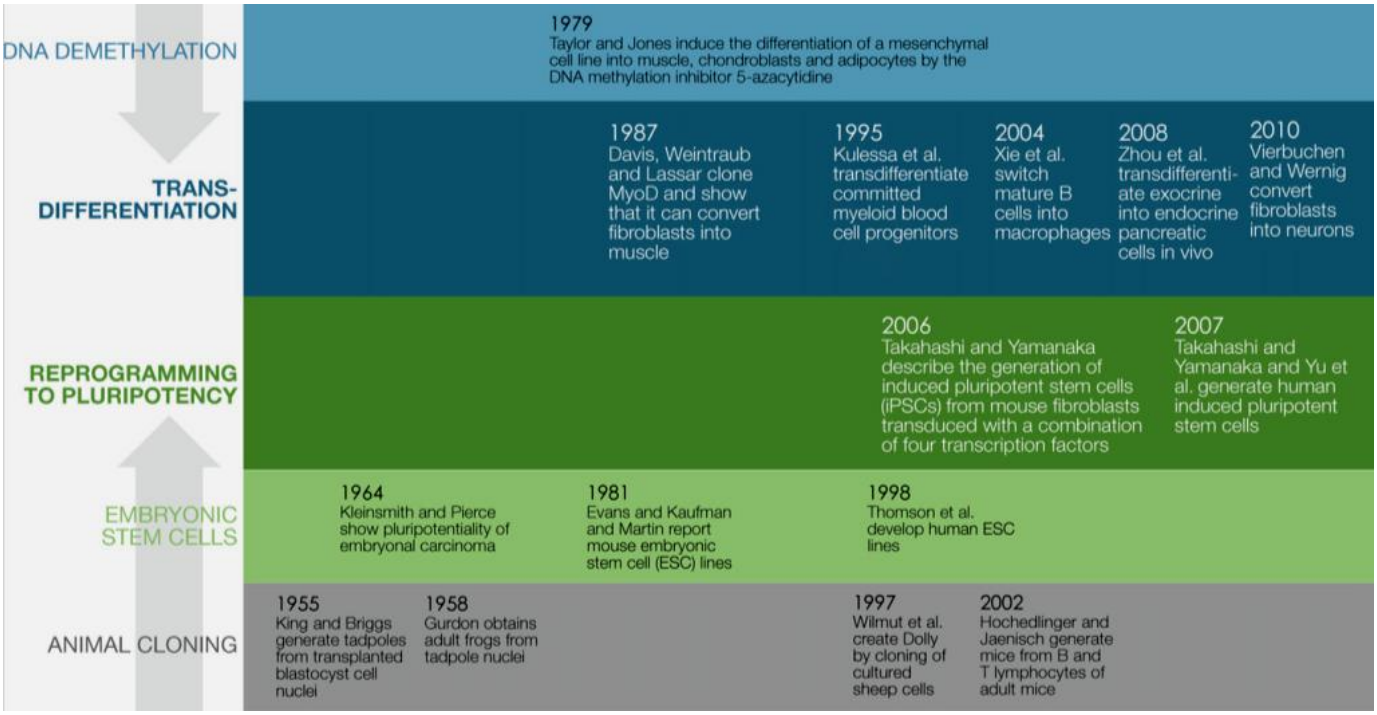

Figure 3: Research Enabling the Discoveries of Transdifferentiation and Reprogramming to Pluripotency. Graf, T., 2011

As shown in the figure already in 1955 genomic experiments were successfully conducted, showing that tadpoles could be generated from transplanted blastocyst cell nuclei. A few years later an adult frog was raised from tadpole nuclei. The animal cloning had its peak in 1997 when cultured sheep cells were utilized to clone the sheep Dolly.
Currently, animal cloning advances represent a great success and reflect the improved knowledge about genetics. This knowledge enabled scientists to differentiate a mesenchymal cell line into muscle, chondroblastic and adipocyte cells (Graf, T., 2011).

Fibroblasts could be transdifferentiated into muscle cells (Davis et al., 1987) and mature B cells into macrophages (Xie et al., 2004). The key advances in embryonic stem cell research together with the transdifferentiation achievements lead to the great development of iPSCs (Graf. T., 2011).

In 2006 the Nobel Prize winning paper with the title “Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors” was published by Takahashi, K. with colleague Yamanaka, S. and since then pluripotent cells were induced in loads of studies for many different reasons.

Pluripotency is the capability of forming cells derived from all three germ layers:

- Ectoderm,
- Mesoderm
- and Endoderm (Takahashi et al., 2006).

This state can be reached by different approaches, until the year of 2006 two methods had been established and are still in use:

1. Nuclear contents can be transferred into Oocytes or
2. a cell can be fused with embryonic stem (ES) cells (Fig. 4)

Also shown in Figure 4 is the direct reprogramming as gained by Takahashi et al..

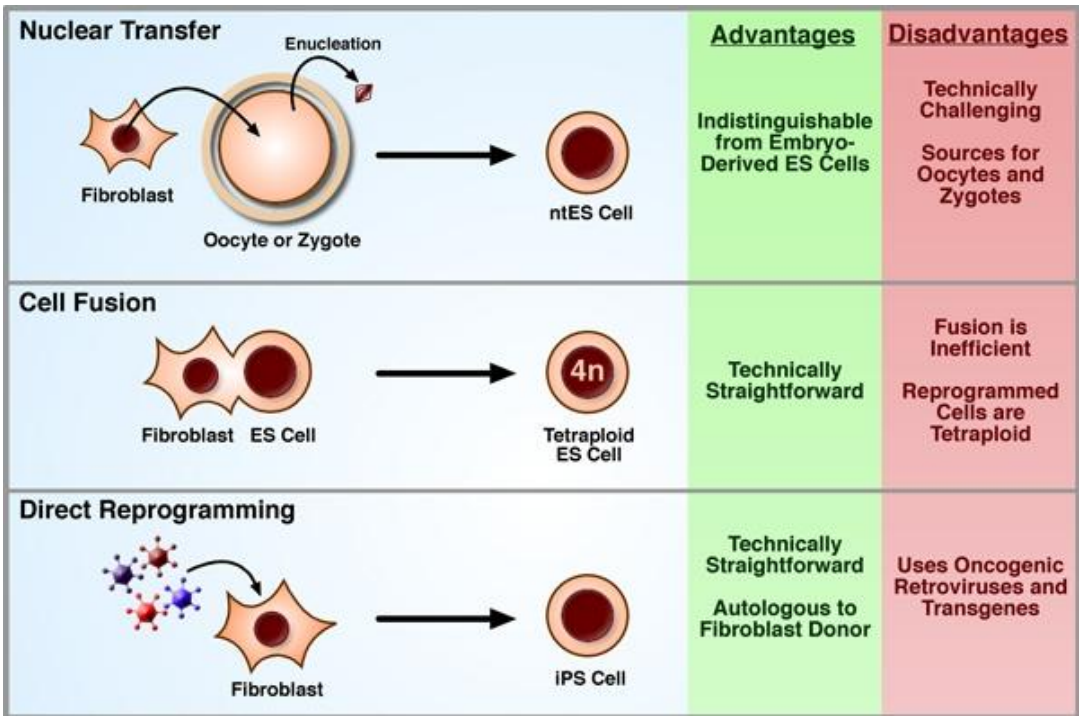

Figure 4: Inducing pluripotency. StemBook, 04/2013

2.3. What has been done until today?

Little was known about reprogramming factors in 2006. The idea originated because of ethical difficulties accompanied by the use of human embryos, as well as the problem of GVHD. The knowledge of the two above mentioned methods led to the hypothesis that unfertilized eggs (Oocytes) and ES cells contain factors that can confer pluripotency. Thus, by using these factors somatic cells can be reprogrammed to an embryonic-like state.

Discovery of reprogramming factors:

Yamanaka et al. chose 24 genes, some according to previous literature but most of them were new identified, as candidates for factors that induce pluripotency in somatic cells.
They made different combinations of the genes and tested the potential of inducing pluripotency by establishing an assay system. The best combinations were transduced retrovirally into mouse embryonic fibroblasts (MEFs) and cultured. By observing the morphology and performing further analysis and experiments (e.g. RT-PCR and DNA Microarray analysis of ES marker gene expression, injection of iPS cells into nude mice and histological analysis of the growing teratoma) the four essential factors **Oct3/4**, **Klf4**, **Sox2** and **c-Myc** were indentified.

The following year a paper about iPS cells from human dermal fibroblasts was published by the same group (Takahashi et al., 2007). An additional aim of this study was to optimize the transduction method. Retroviral vectors were used in both studies 2006 and 2007.

Using retroviral transduction:

In 2007 the sickle haemoglobin allele was corrected in iPSc created from a sickle cell anemia mouse model. The mouse model was rescued by re-transplanting the corrected iPSc. However, flaws concerning retrovirus for reprogramming and risks of tumor formation were still barriers that needed to be conquered before iPS cells could be considered for human therapy (Hanna et al., 2007).

Series of studies followed in that mouse and human cells are reprogrammed in vitro into pluripotent stem-cell like cells through retroviral transduction:

- In 2008 Kim et al. reprogrammed human neural stem cells by using only 2 factors trying to reduce the risk of tumorgenesis.

- The same year Yu et al. worked with oct4, sox2, nanog and lin28 as reprogramming factors, excluding c-myc because of potential cause for differentiation and death.

As a result they both got proof of pluripotency in several analyses and a similarity in morphology to human ES cells. This shows that there is more than one way to successfully create iPS cells. However, to proceed to use the cells in clinical trials, additional work is required to avoid vectors that integrate into the genome, potentially introducing mutations at the insertion site.

Establishing episomal transduction:

4 years later in 2011 Okita et al., a group that Takahashi and Yamanaka (above mentioned in 2006 and 2007) were part of, published a paper about the generation of integration-free iPS cells. They introduced a reprogramming by episomal plasmid vectors. Starting with seven combinations of episomal vectors one combination, namely Y4, showed the best results. In Y4 the 5 transcription factors are delivered in 3 separated plasmid vectors (1: OCT3/4; 2: SOX2 and KLF4; 3: L-MYC and LIN28). This combination resulted in significantly more iPSc clones than did any of the others.

By the insertion of p53 suppressor (master regulator of cancer) and using L-Myc instead of c-Myc a decrease in risk of tumor formation and an enhanced efficiency could be reached. Although this is a safer approach to induce pluripotency, the efficiency of iPS cell derivation is significantly reduced, and the reprogrammed cell types are limited (Okita et al., 2011).

More iPSC work:

Creating iPSC provides a great potential in many, still unexplored, ways. Soon scientists thought about the broad field what to use them for.

The therapeutic potential of induced pluripotent stem (iPS) cells for neural cell replacement strategies was explored in 2008 by Wernig et al. In their study they show, that iPS cells can be efficiently differentiated into neural precursor cells, giving rise to neuronal and glial cell types in culture. Upon transplantation into the fetal mouse brain, the cells migrate into various brain regions and differentiate into glia and neurons.

But still, the problems associated with using retroviruses and oncogenes for reprogramming need to be resolved before iPS cells can be considered for human therapy (Wernig et al., 2008)

Relevant for the purpose of this study Dong et al. mentioned that several laboratories have shown lentiviral vectors, carrying the human β or γ-globin gene and its fundamental regulatory elements, were able to cure and rescue animal models for β-thalassemia intermedia and

major. Much progress has been made in the treatment in vitro of both human SCD and β-thalassemic cells with lentiviral vectors.

With the extensive research on human immunodeficiency virus-1, it has been realized that lentivirus, engineered by removing any pathogenic elements, can become efficient gene transfer vectors (Breda et al., 2011).

Considering the HIV vector and also the episomal method as the safest and most efficient vectors to deliver the transcription factors, both of them are used in this study to induce pluri-potent stem cells.

3. Aim of the thesis

The aim of the thesis is to create iPS cells from a β-Thalassemia patient. To understand the subject better, background information about the disease and iPS cell development is provided in the first chapter.

The author is visiting the Rivella Lab at Weill Cornell Medical College and is part of accomplishing the first milestone (blue circle) in the 5 years Phd project (Fig. 5) of Phd student Alisa Dong.

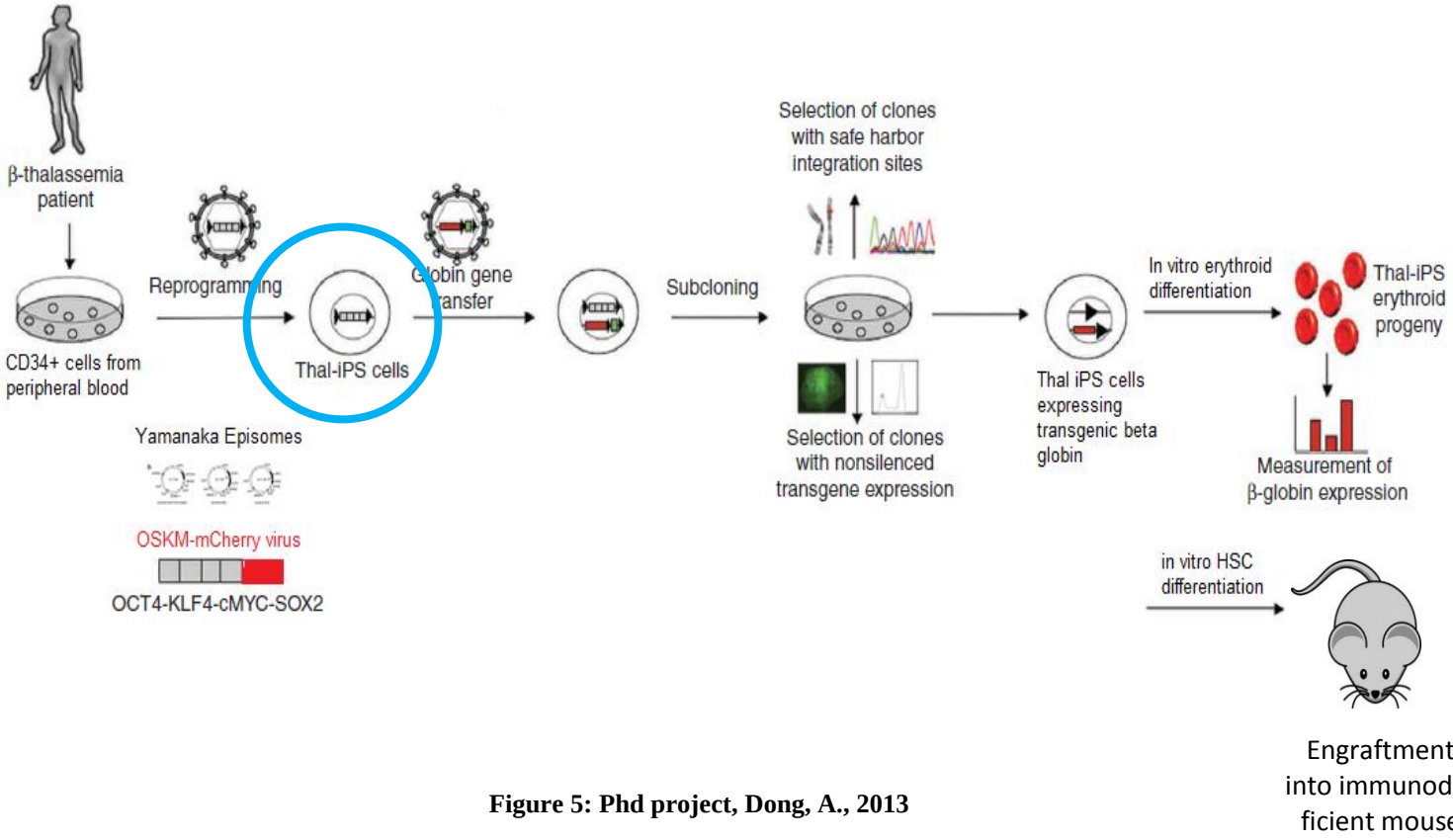

Figure 5: Phd project, Dong, A., 2013

4. Study structure (Procedure)

In this chapter the author explains the steps to get and verify iPS cells. The subchapters are in a chronological order and a red thread is running through the procedures for a better understanding.

4.1. Obtaining and preparing blood samples

The blood sample is provided from a β-Thalassemia patient, who is in treatment at the New York Presbyterian Hospital. For the retroviral infection first the cells need to be separated. The CD34+ cells are selected from the mononuclear cells (Methods are explained in Chapter 4: Materials and Methods). The rejected CD34+ cells can now be infected, meaning the 4 factors of pluripotency are delivered either into the genome or integration-free.
Because of random integration, the genomic change takes place on different locations,. About two million cells are infected with the virus and eight clones (virus A, B, C, D, E, F, G, H;) can be detected using the integrating method and two (epi A, B;) for the non-integrating episomal method.

4.2. Adding 4 factors of pluripotency

Genes of induction:

- Oct-3/4 plays a crucial role in maintaining pluripotency. Its' presence gives rise to the pluripotency and the differentiation potential of embryonic stem cells. It is exclusively expressed in pre-implantation embryos and primordial germ cells.
- The Sox family is known to regulate early developmental programs. Similar to Oct-3/4 Sox 2 is associated with maintaining pluripotency outstanding with the highest efficiency of the Sox family.
- The Myc family of genes are proto-oncogenes (normal gene that can become an oncogene due to mutation) implicated in cancer. C-myc has been discovered as one of the key factors for inducing pluripotent stem cells. C-myc shows similar properties to genes that are involved in metabolism, cell growth proliferation and apoptosis.
- The role of Klf4 is less clear, but is suggested that it could act as a tumor suppressor gene in iPS cells and may also act via repression of p53 which leads to activation of Nanog and other ESC-associated genes. (Tam et al., 2008)

- Nanog was considered to be a key factor for iPS cells until Takahashi et al. 2006 proofed that it is dispensable. Nonetheless it is a gene expressed is ES cells and detected expression can be used to proof the ES-like state after induction.

The relevance of c-Myc and Klf4 targets appears to be more speculative, nevertheless cells have been successfully reprogrammed by Takahashi and Yamanaka 2006 and no other combination of transcription factors with efficiency that high has been found yet.

4.2.1. Integrating Virus

Viral vectors have gained their popularity in basic research and gene therapy applications because of their high rates of gene transfer that are far superior to those achieved with non-viral methods. Numerous types of virus-derived gene transfer systems are available these days. These genetic vehicles are based either on DNA viruses or on RNA viruses (Sommer et al., 2009).
In this study the HIV virus is modified and used as single lentiviral vector, a genetic vehicle to deliver the pluripotency factors. Lentivirus-based vectors can efficiently infect non-dividing and slowly dividing cells. This vector derives from a re-engineered pHAGE, third-generation lentiviral vector, modified to express a ‘‘stem cell cassette’’ (STEMCCA) composed of the four transcription factors**, Oct4, Klf4, Sox2, and cMyc.**

As shown in figure 6, the engineered stem cell cassette consists of a single multicistronic mRNA transcribed under the control of a doxycycline-inducible TetO-miniCMV promoter. The mRNA contains an IRES element separating two fusion cistrons.
The two cistrons consist of Oct4 and Sox2 coding sequences fused to Klf4 and cMyc, respectively, through the use of intervening sequences encoding self-cleaving 2A peptides (F2A and E2A) (Sommer et al., 2009).

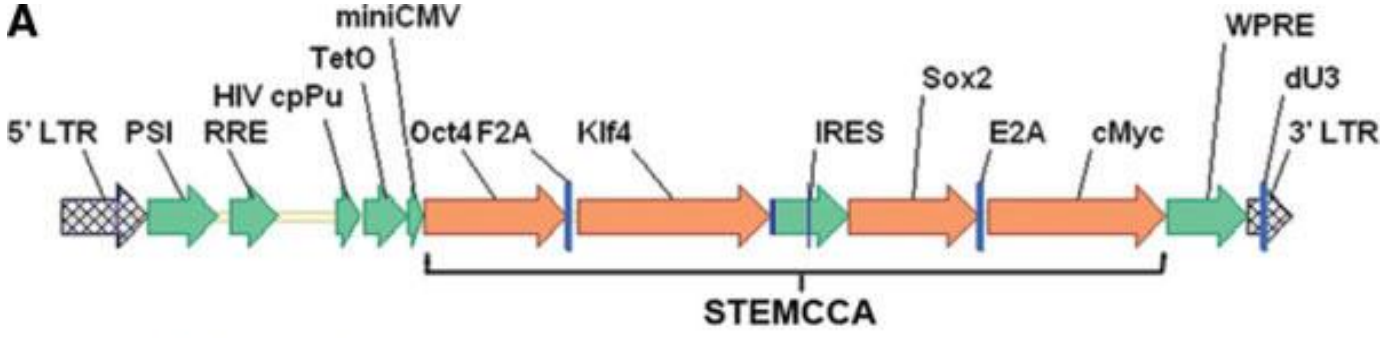

Figure 6: Schematic representation of pHAGE-STEMCCA. Sommer et al., 2009

4.2.2. Non-integrating Virus

The corresponding vectors either integrate into the host genome (previous subchapter) or express their genetic information episomally.
Episomal transmitted transcriptions means that the TF are delivered in Plasmids and the Genome can be reprogrammed without integration. The advantage of using the episomal method is that they are considered to be safer (Okita et al., 2011).
For this study the method established in 2011 by Okita et al. has been used, i.e. 3 Plasmids with 5 TF. In the Okita paper also called the Y4 combination. CAG, CAG are different promoters and the WPRE, woodchuck hepatitis post-transcriptional is used as regulatory element.

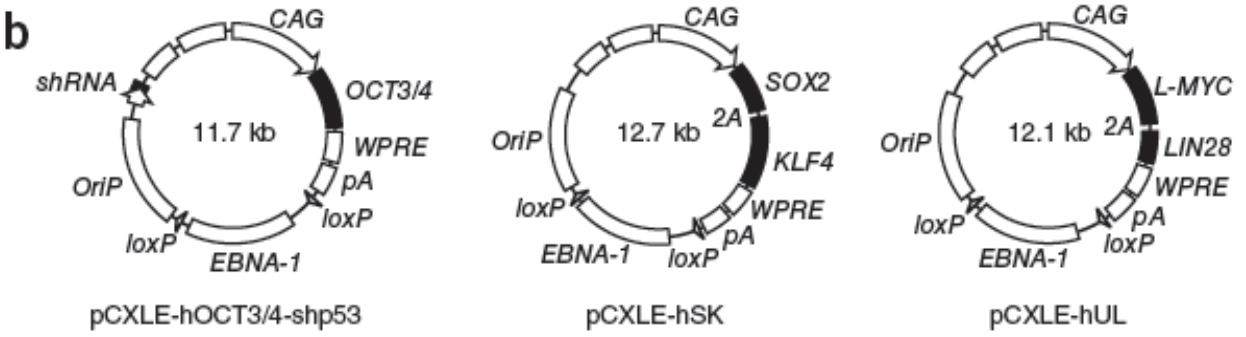

Figure 7: Combinations of episomal vectors used in this study. Okita et al., 2011

4.3. Growing cells on feeders (MEF) vs. Growing cells on Matrixgel

Human ES and iPS cells require a specific culture system for successful expansion. Originally, human ES cells were cultured on a feeder layer of mouse embryonic fibroblasts (MEFs). MEFs are cells obtained from a mouse embryo (see chapter 4: Materials and Methods). MEFs cells are required to support the growth of undifferentiated mouse or human ES cells, iPS cells. They also support self-renewal either by providing necessary factors or by removing inhibitory factors.
While MEFs provide a robust surface for long-term culture of hES cells, there are a number of concerns associated with the use of a cell feeder layer. Feeder cells may secrete undefined components into the media and cause batch-to-batch variability in feeder layers.

Despite this, the continued use of animal-derived components will hinder the development of clinical applications due to the presence of immunogenic material and the risk of xenogenic contamination (Stemcell, 2012).

Therefore, BD Matrigel™ matrix has been established and can provide culture conditions that support the proliferation and culture of human ES and iPS cells without the need for feeders. Recently, BD Matrigel matrix has been used for culturing undifferentiated human iPS cells in a feeder-free environment. This has been accomplished using BD Matrigel matrix in conjunction with MEF-conditioned medium, culture additives or defined media such as mTeSR®1. STEMCELL Technologies has developed mTeSR1 and TeSR™2 as standardized media for feeder-independent maintenance of human pluripotent stem cells (hPSCs) (Stemcell, 2012).
Still, it occurs that very sensitive clones have to be thawed onto feeders and can later be transferred onto Matrigel plates.

4.4. Verification of Plurypotency

This chapter explains different validating methods carried out in this study to ensure that iPS cells are pluripotent.

4.4.1. Morphology

About 8-9 days from the reprogramming, when cells are plated on MEFs, some colonies start to appear. They look like cobblestone deposits at the very beginning. When colonies are fully formed (about three weeks after having seeded them onto MEFs) their edges should be easy to distinguish; clear an easy to see by eye under the microscope.

IPS cells are meant to look alike ESCs, characteristics:

- Round shape
- Large nucleolus (high nuclear-to-cytoplasm ratio)
- Scant cytoplasm
- Compact multicellular colony and multilayer in the center of colony (Stemcell, 2012)

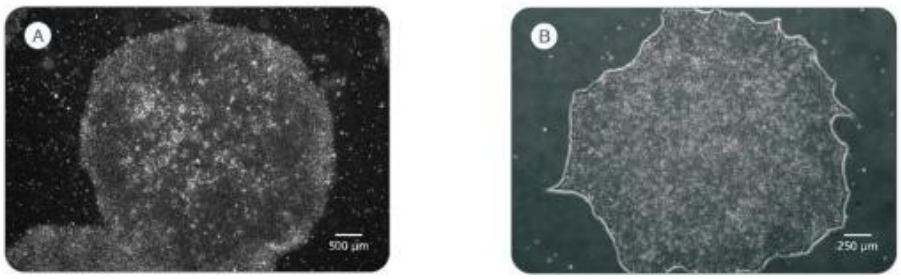

Figure 8: (A) A human ES colony and (B) a human iPS colony. Stemcell, 2012.

Above listed bullet points and a picture like figure 6 are an evidence of healthy and undifferentiated iPS and ES cell colonies. It is important to observe the morphology of the created iPS cells thoughtfully during all the passages. Differentiation can be noticed by an expert eye and removed immediately to keep healthy colonies.

4.4.2. AP staining

The undifferentiated state of embryonic stem (ES) and induced pluripotent stem (iPS) cells can be characterized by a high level of alkaline phospatase (AP) expression. Along with surface markers it indicates undifferentiated cells with the potential to self-renewal. AP is a hydrolyse enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions. When fixed ES or iPS cells are stained using the AP Staining Kit, undifferentiated cells appear red or purple, whereas differentiated cells appear colourless.
The AP Staining Kit is functionally tested on human and mouse ES cells to ensure product quality (Stegment, 2012).

4.4.3. Pluripotency factor expression

A human embryonic stem cell is defined by the expression of several transcription factors and cell surface proteins. Expression of pluripotency factors of ES cells has been studied well and to verify that iPS cells are in an ESc-like state different factors are expected to be expressed. In order to assess these pluripotency factors we are using PCR and FACS analysis to manifest the expressed factors. It is crucial to characterize the identity and potency status of pluripotent stem cells by measuring molecular markers during cultivation.

4.4.3.1. PCR

Commercial available primers can be used to find and bind the right gene parts. Those parts are amplified and can be detected by base pair length when PCR sample is loaded on an Agarose-Gel and Electrophoresis is applied. Primers corresponding to the OCT 3/4, KLF 4, SOX 2 and C-myc genes are used.

4.4.3.2. FACS

Pluripotent stem cells can be identified by the expression of specific cell surface antigens such as the glycolipids SSEA-3 and SSEA-4, and the keratan sulfate antigens TRA-1-60 and TRA-1-81. Antigenes are found by antibody-antigene reaction and fluorescent detection (Stemcell Technologies, 2012).
In this study SSEA-4, TRA-1-60, TRA-1-81 and OCT 3/4, the latter is a core marker, are used.

4.4.4. Karyotype

A Karyotype describes the number of chromosomes, and what they look like under a light microscope. It's a laboratory test used to study an individual's chromosome make-up. Chromosomes are separated from cells, stained, and arranged in order from largest to smallest so that their number and structure can be studied. That can help identify genetic problems as the cause of a disorder or disease. Therefore structural changes in chromosomes are observed and the number of chromosomes is counted.
The basic number of chromosomes in the somatic cells of an individual or a species is called the somatic number and is designated 2n. Thus, in humans 2n = 46. In the germ-line (the sex cells) the chromosome number is n (humans: n = 23) (Wikipedia, 2013).

4.4.5. Histological/Teratoma

Teratomas are solid, defined tumors, often derived from a germ-line. Teratomas are composed of the highly organized differentiated cells and tissues containing representatives of the three developmental germ layers. The three germ layers can also be generated artificially by transplanting pluripotent stem cells into immunodeficient mice

As part of this methodology, the generated iPSCs are usually transplanted intramuscular or subcutaneous in an immune deficient mouse. After a period of at least three weeks, the mature teratomas are excised out of the animal. Histological and Immunohistochemical analysis are applied to verify the presence of cells derived from the three germ layers.

The internship of the author didn't provide enough time to apply the histological verification by Teratoma, but still, it will be conducted in the following months.

5. Materials and Methods

Seperation of peripheral blood mononuclear cells (PBMCs)

For the seperation of the PBMCs the sep-mate system by Stem Cell Technologies (Catalog #15460) was used. 30ml blood was collected in heparinized tubes and mixed (in 50 ml conical tube) with 1.5 volumes PBS (45ml of PBS for 30ml of blood, volume can be adjust proportionally if starting with a different amount of blood). All blood-PBS mix was added on top of 15ml of ficoll as suggested by manufacturer protocol.

Manufacturer protocol was followed up to the separation by centrifugation of the PBMCs. After which the upper phase was directly poured into a fresh tube to which was filled up with PBS and centrifuged at 300xg for 5 minutes (max acceleration and brake on). The supernatant was discarded and the cell pellet disrupted by scraping the tube against a ribbed surface (like the surface of the TC hood by the air flow). To eliminate all the platelets the tube was refilled with PBS and centrifuged another time at 300xg for 5 minutes plus at 200xg for 5minutes. The finally disrupted PBMCs pellet was resuspended in 600µl of cold (sterile) 1 % BSA[PBS and transfered in a clean 15ml conical tube.

Positive Selection of CD34+ cells from PBMCs

100µl of anti-human CD34+ beads (Miltenyi, #130-046-702) was added to the previous resuspended PBMCs and incubated for 15 minutes in a cold room on ice. The pre-chilled (for at least 3h) Midimacs separator and Multistand (Miltenyi #130-042-302 and #130-042-303) were transferred under the TC hood and the LS colums (2 columns/specimen) (Milteniy #130-042-401) were equilibrated with 5ml of ice cold and filtered beading buffer (BB: 0.5% BSA, 2mM EDTA, PBS; can be stored in fridge for max 10 days). The flow-through was discarded and the columns were incubated for 15min in a capped 50ml tube in a cold room on ice. After incubation BB was added to the cell-bead mix by filling the tube up and after 5min centrifugation (300xg at 4°C) the supernatant was aspirated and the tube was refilled and spun again. Finally the cells were resuspended in 1ml of cold BB.

The Midimacs separator and Multistand were next used to place the pre-chilled column onto for loading the cell suspension, first column 0.5ml cells with 1.5ml of BB wash; second column 2.5ml and 2.5ml BB wash (as many times as nessecary to use up the suspension). Each column was washed twice with 3ml of cold BB and after removing the column from the stand it is important to place it as quickly as possible on top of a clean 15ml conical tube and load 5ml of BB. The cells were flushed by inserting the plunger into the column and the column was centrifuged 5min at RT at 300xg. To resuspend the pellet 3ml Stem-span (Stem

cell technologies #09650), 30μl of CC-100 (Stemcell technologies #02690), 3μl Erythropoietin (2U/final concentration, available for purchase through Amgen, Epogen, Epoietin alpha, recombinant 10x 2000units/ml single use vials, NDC 55513-126-10), 3μl 10-3 M dexamethasone (10-6 M final concentration, can be purchased through Sigma) and 1% Pen-Strep.

Cells were propagated at a concentration lower than 0.5million/ml in the media specified above, density was checked 3 days after the initial isolation and media was changed (change in media in general twice a week).

By the day ten we did analysis and cells were C-KIT+, CD71+, GPA- and Band3- by flow. We let calls expand for at least 14 days before starting any differentiation.

Retroviral Infection

Cells were counted, cetrifuged at 300xg and resuspended in 1ml of expansion media supplement with 1x polybrene in eppendorf tube. 3 MOI and up to 4x of concentrated virus (minimum multiplicity of infection, MOI, 1x108) was added and incubated in cell incubator for 6 hours. Cell-virus mix was pipetted up and down in TC hood every hour. After the 6 hour incubation cells were spun down at 300xg for 5 minutes at RT and resuspended in expansion media.

Freezing/Thawing CD34+

Freezing: Cell pellet was collected by centrifuging at 300xg for 5 minutes and resuspended quickly in ice-cold freezing medium: Iscove Modified Dulbecco's Medium (IMDM, Corning Cellgro #10-016-CV) 10% dimethylsulphoxid, 50% Fetal Bovine Serum (FBS). After storing them in -80C for 1 day, they were moved to liquid nitrogen.

Thawing: CD34+ cells were thawed by rapidly incubating them at 37C and then adding 10x volume of IMDM 5% FBS dropwise. Before centrifuging at 945rpm for 10min and resuspending in an appropriate volume of expansion medium, cells recovered 5-10min.

Obtaining mouse embryonic fibroblasts (MEFs) from the mouse:

Pregnant females were ordered from Charles River (CF1 code 023). Typically females of this strain are shipped at day 12, feeders can be made up to day 14. Most of the times they have 5 to 12 pups. The embryos were taken out on a dish, the head was separated first and the hematopoietic organs (the red parts) were taken out next. To 5 embryos Trypsin was added and mixed by pipetting up and down with a 2ml pipette in a 10cm dish. Trypsin was inactivated with MEF medium (DMEM, 10% FBS BFU-E tested) and cells were plated in 10ml of

MEF medium. When confluent cells were split 1 to 5 (passage #2, Trypsin for detaching cells) and frozen (freezing medium: hESC media, 105DMSO in KO serum Gibso #774119).

Cell Culture

IPS cells were growing on feeder layers (MEFs) and/or on plates coated with matixgel (BD Biosciences, hESC-qualified Matrix, 5 ml *LDEV-Free #354277).
Cells were maintained on 10cm plates in a hypoxia incubator and 8ml Medium (Stem Cell Technologies, mTsR #5870 for matrixgel and MEF Medium for feeder) had to be changed every day.

Passaging iPSCs

Plate was observed under the microscope to mark any parts that are differentiating. When media was aspirated and marked parts were sucked off. After washing with base media DMEM F12 (Hyclone, #SH30023.02) Dispase (Stem Cell Technologies, #7923) was added and incubated until the edges of the iPS started to come up (2-5min). When passaging from feeders, they detached completely and were aspirated out when washed twice with base media to dilute Dispase out. Original media mTsR (Stem Cell Technologies, #5870) was added and cells were scraped off with cell lifter (Genemat CAT No. T-2443-4) and collected in a 15ml tube.

Freezing/Thawing iPSCs

Freezing: The 15ml tube was centrifuged and the pellet resuspended in 250µl of Solution 1 for one freezing tube. 250µl of Solution 2 was added dropwise while agitating tube, capped and inverted twice. The tubes were frozen in a rate-controlled freezing container (-1°C/min) for 24 hours and then moved into liquid nitrogen.
Solution 1: KOSR (Invirtogen, #10828-028) + 10µM ROCK Inhibitor (Stemgent, # 04-0012)
Solution 2: Solution 1 + 20% DMSO (Sigma, # 154938)
Thawing: The tube was rapidly thawed in 37°C water bath, emptied into a 15 ml conical tube and then 7ml of mTsR were added dropwise. 15ml tube was spun down (300xg for 5min), supernatant aspirated and pellet resuspended in 0.5ml of thawing media.
Thawing media: hESC Media + 20ng/ml basic fibroblast growth factor (bFGF) + Stemgent Cloning & Recovery Supplement (01-0014-500)

PCR for marker genes

RNA was purified using the PURELINK RNA MINI KIT (Life technologies, #12183018A). Cells were collected and spun down 5minutes at 300xg. The pellet was resuspended in 600

µl lysis buffer (for 1 ml lysis buffer + 10µl 2-Betamercaptoethanol) and then manufacturer's instructions was followed. The quantity of the purified RNA was measured with the NanoVue plus Spectrophotometer (GE Healthcare Life Science) and the quality by running a 1.5% gel with 1µg RNA loaded.

For retrotranscription 1µg of total RNA was used and for a treatment for a final volume of 10µl 1µl DNAse, 1µl Buffer and 1µl RNAse out were added. The thermocycler ran 20'at 37°C, 10' at 70°C and stayed at 4°C in the 1st step. Step 2: 10µl volume from previous step plus 1µl dNTPs, 1µl hexomers ran for 5' at 65°C and stayed at 4°C. Step 3: 12µl from previous step plus 4µl MgCl2, 2µl DTT, 1µl Buffer, 1µl SSII ran for 10' at 25°C, 50' at 50°C, 5' at 85°C and steyed at 4°C. In the last step, step 4, 1µl of RNAse was added and the thermocycler ran for 20' at 37°C. Sample was either frozen at 20°C or PCR was performed immediately. For the PCR anneling temperature and elongation time were individually calculated according to the primer length, G=C contents and bp amount of the product. The cycles were as follows: 1. 1' at 95"C; 2. 45'' at 94°C; 3. 45'' at 60°C or 62°C; 4. 30'' or 15'' at 72°C; 5. go to step 2 and rep 30x cycles; 6. 5' at 72°C and 7. stay at 4°C.

FACS for marker genes

For FACS cells were detached by adding Trypsin, which was later inactivated with 2%FBS (diluted with 1xPBS). The collected cells were counted, appropriate amount (200.000/analysis) aliquoted out and spun down at 850rpm for 10min. The pellet was the resuspended in 39µl of 2% FBS PBS, accordingly to the number of stainings required, and 1µl of Fc/39 µl of cell solution was added and left on ice for 10min.

In the next step, when working with Antibodies (Ab) it is important to work with the lights off. 40µl were pipetted out and together with the Ab added to a 1.5ml tube, 0.5µl SSEA-4, 3µl TRA-1-60, 5µl TRA-181 or 3µl OCT 3/4 with an incubation time of 15min in the dark.

For the 3 surface markers, sample were washed with 1ml of 2% FBS BPS, spun down and resuspended in 5% FBS 0.1% NaN3 0.1% PFA.

For OCT 3/4, which is a core marker, cells needed to be permeabilizated. For permeabilization the commercially available FIX&PERM CELL PERMEABILIZATION REAGENTS were ordered and manufacturer's instructions were followed (Invitrogen, 2007)

AP Staining

For AP Staining the Stegment Alkaline Phosphatase Staining Kit was used. The following procedure describes staining one well of a 12-well plate for AP dedection.

First the culture medium is aspirated and the cells are washed twice with 2 ml of PBST. Then 1ml of Fix Solution is added and incubated at room temperature for 1 to 2 minutes. Aspirating

and washing with PBST is repeated and cells are finally stained with 1ml freshly prepared AP Staining Solution.
For the 10 to 20 minutes incubation it is important to wrap the plate with foil or to keep them in a dark container, as stain is very sensible when exposed to light.
The reaction is stopped by aspirating the AP Staining Solution and washing the wells twice with 1ml of 1xPBS. To maintain the plate, cells must be covered with 1xPBS, to prevent drying and plate can be stored at 4°C (Stegment 2012).

PBST ist prepared by adding 5µl of Tween 20 to 10ml of 1x PBS for a final concentration of 0.05%.
The **AP staining solution** is a 1:1 ratio of AP Staining Solution A and AP Staining Solution B. For optimal results, the AP Substrate Solution should be used within 5 minutes after preparation (Stegment 2012).

Karyotype

The karyotyping has been performed at the Memorial Sloan-Kettering Cancer Center in New York City.

6. Results

IPS cells were successfully created and verified by the following analysis.

- Morphology
  In figure 9 iPSC colonies are shown in different shapes observed during culturing. Also pictures from colonies the way they should not be are included to transfer a better picture of the whole process of culturing IPS.

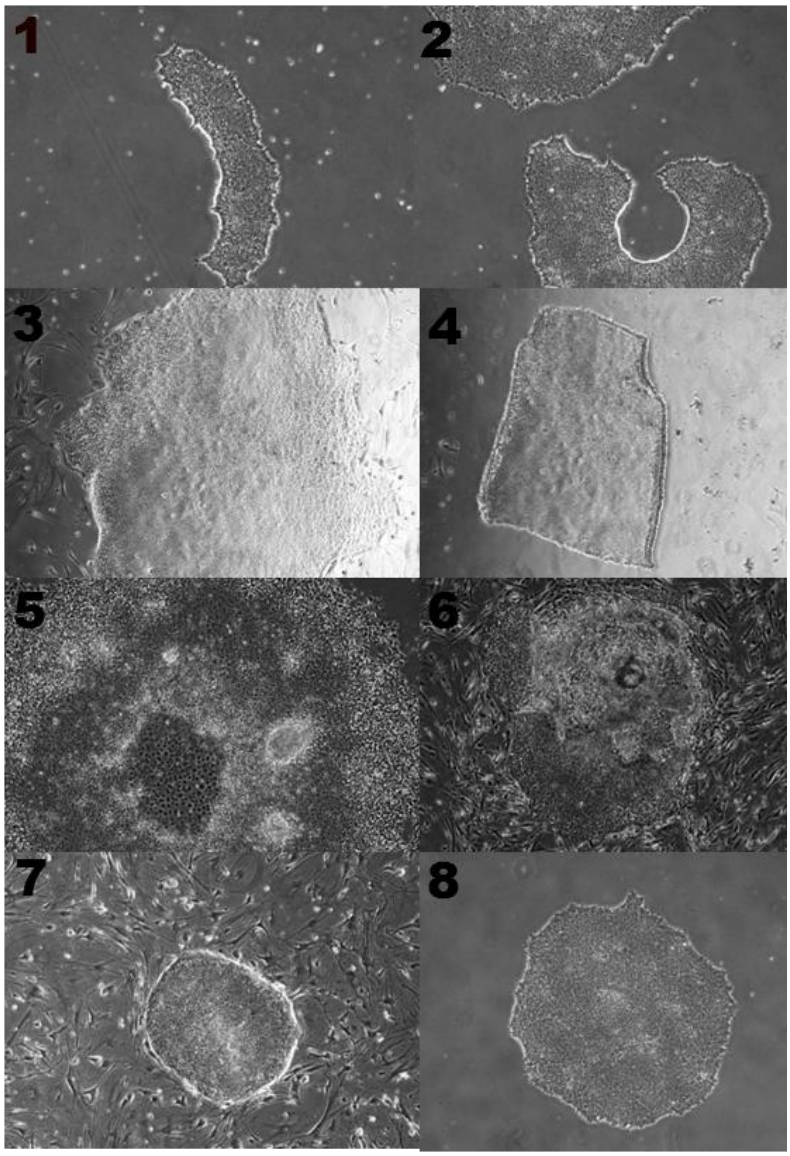

Figure 9: Oblong colony (1), c-shaped colony (2), colony with differentiating parts (3), colony after microsurgery (with pipette under microscope) (4), differentiating colony, non-ES like (5,6), ES-like colony on feeder cells (7), ES-like colony on BD Matrigel (8);

- AP staining

  Induced pluripotent stem cells, maintained on BD Matrigel coated plates in an undifferentiated stage, as indicated by the high AP activity (fig. 10)

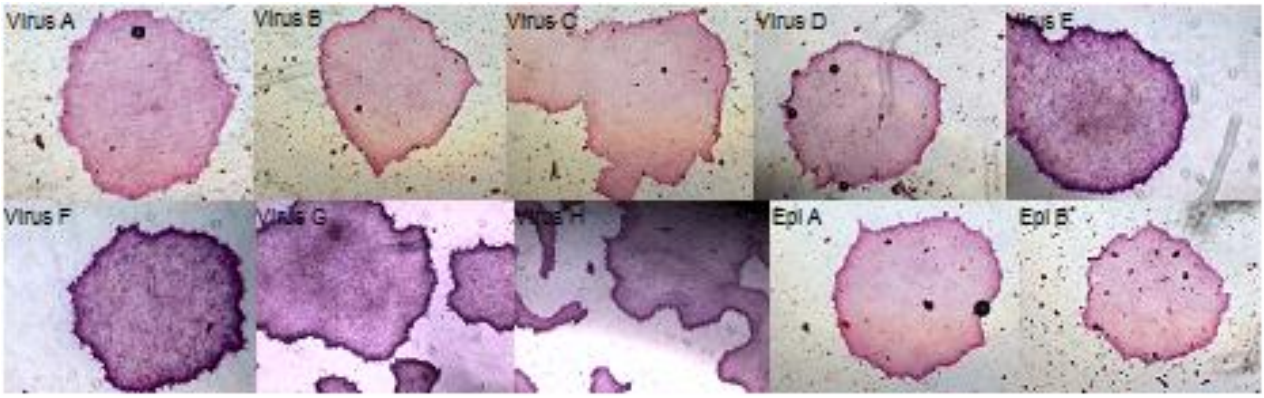

Figure 10: AP stained iPSCs on martigel-coated plates

- PCR

  Figure 11 shows the 4 factors of pluripotency with which the cells were induced and later expressed as in ES cells. The pictures represent the PCR product, detected on an Aragose-Gel.

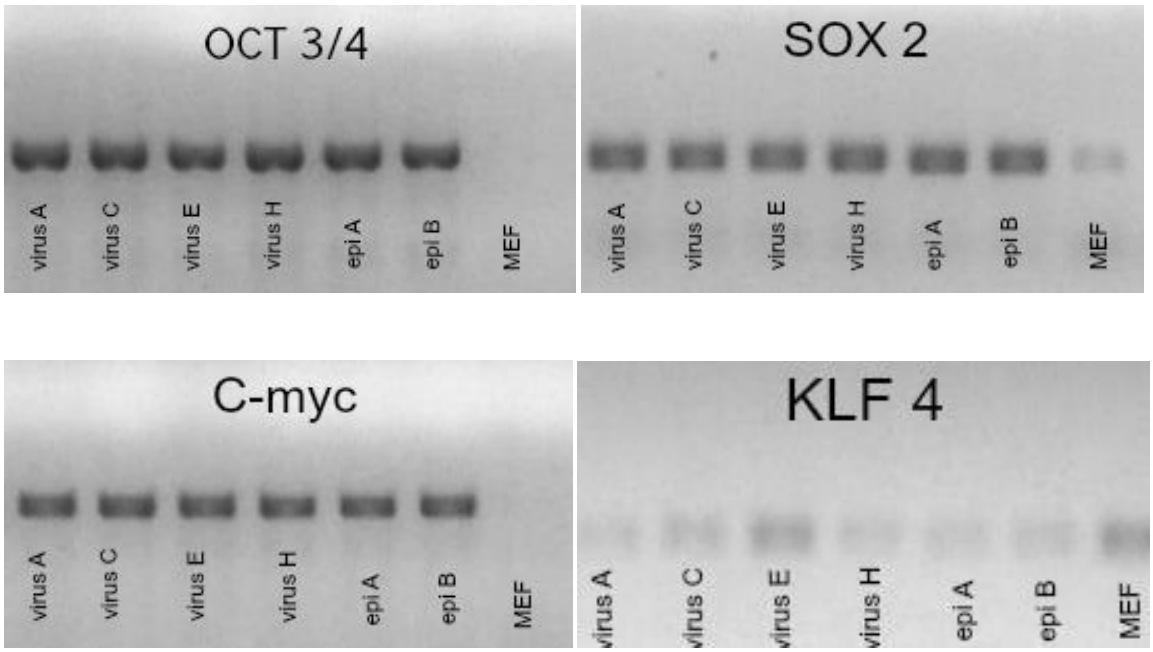

Figure 11: PCR Gel

- FACS

  Characterization of iPSCs by flow cytometry (fig. 12) shows Epi A and it's positive surface marker expression of SSEA-4, TRA-1-80, TRA-1-81 and positive core marker expression of OCT 3/4 representing the ES-like state.

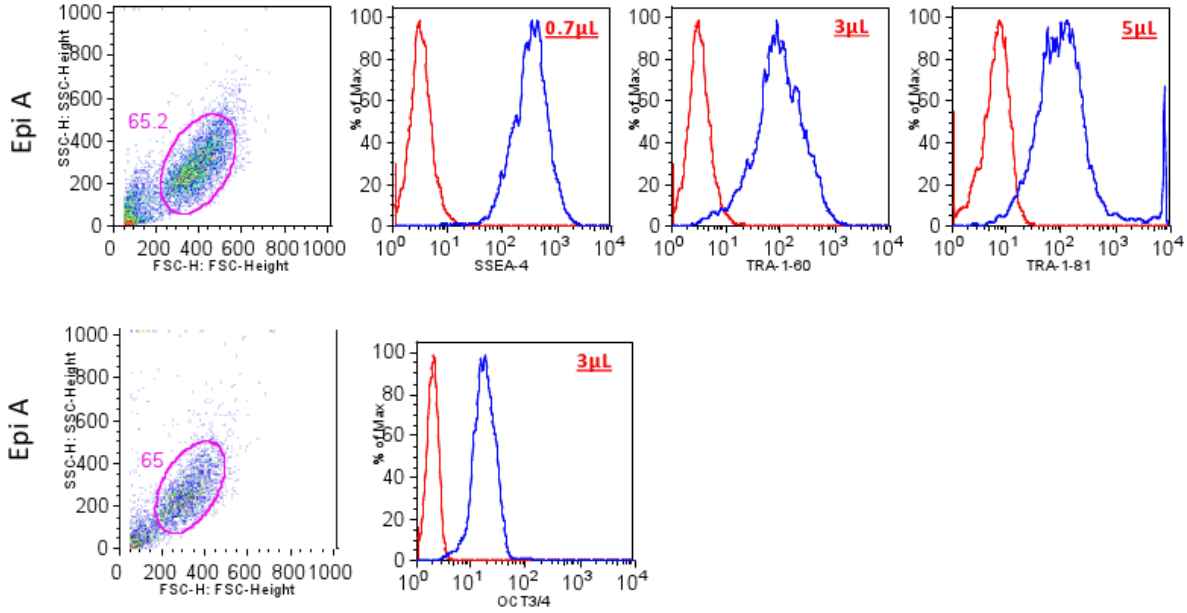

Figure 12: FACS epiA

- Karyotype

  Chromosome analysis was performed on a minimum of 15 DAPI-banded metaphases. All metaphases were fully karyotyped (

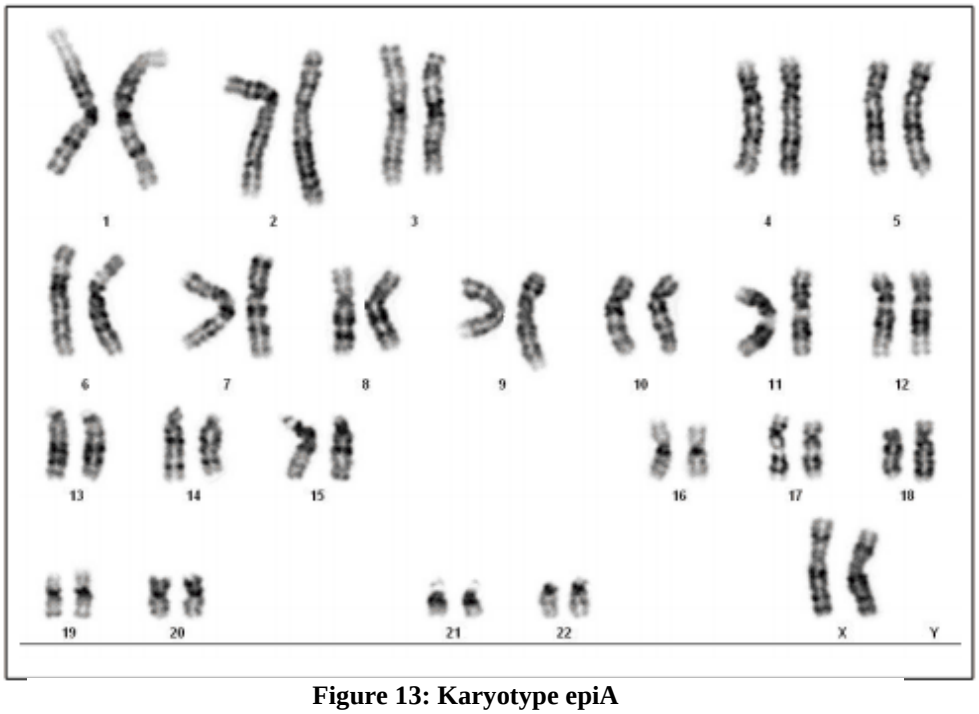

Figure 13: Karyotype epiA

7. Discussion

The results of analyses carried out to verify the pluripoteny and thus the ES-like state of the iPSCs can be interpreted as positive. IPSCs have been created successfully.

The desired morphology could be reached but required a lot of microsurgery and aspirating out the differentiating colonies. The c-shaped colonies (fig. 9) originated from temporary problems coating the plates with Matrigel. Matrigel was insufficient dissolved and formed chunks on the plates which later came off and left an empty hole behind. The question why the colonies sometimes grow in different shapes (oblong colony) remains unsolved but is not relevant to the morphology and the quality of the iPSCs.

For the AP staining clear results could be reached at the very first try for all clones.
PCR shows clear positive results for the presence of OCT 3/4, C-myc and SOX 2. There is a visible band in the MEFs for SOX 2 and the result of KLF 4 in questionable. However, the only statements that can be made are that there is no contamination in MEFs and that the analysis needs to be repeated with **human** non-ES-like cells as a negative control. Also expanding the test with a primer for the Nanog-gene is recommended to reach more significant results.

Additional molecularbiological detection by FACS shows positive surface marker expression of SSEA-4, TRA-1-80, TRA-1-81 and positive core marker expression of OCT 3/4 representing the ES-like state.

The preliminary karyotypic analysis of 15 metaphases in each of the four iPS samples submitted did not reveal gross/major numerical or structural abnormalities.

8. Conclusion (Future aims)

Overall the author has to say, that the three month internship in the Rivella lab went very well and satisfying in order to the obtained results. IPSCs are very sensitive cells and challenging to work with in cell culture. Referring to the morphology (fig. 9) a better picture can be transferred what someone has to go through to get flawless clones, which can be used to conduct analyses. On account of this the gained data is most appreciated.

During culturing the cells, techniques for freezing and thawing the iPSCs have been optimized and trained. A stock of frozen iPSCs could be established to conduct further analysis, for example the injection of the cells into mice for the Teratoma, which is the next step that will be taken soon.

The cells are further used to work with, in the Phd project as shown in figure 5, which means they will be infected with a healthy ß-globin gene. The long-term goal of nuclear transfer or alternative reprogramming approaches is to create patient-specific donor cells. These can be used for transplantation therapy, avoiding immunorejection, a major complication in current transplantation medicine.
Homologous recombination experiments have already demonstrated that it is possible to correct the mutated ß-globin gene in iPSCs derived from thalassemia-patients (Puthenveetil et al., 2004). However, the efficiency of correction is still very low, and the iPSCs show very little expression of the corrected gene.
Furthermore, generating corrected iPSCs using the current technologies entails several drawbacks, including the potential disruption of oncogenes and tumour suppressors. Unfortunately, this would mean that SCD and ß-thalassemia could be cured, but that this might lead to the development of cancer or other diseases. Now it is very important to find a procedure to generate corrected iPSCs that do not express enhanced levels of oncogenes or tumour suppressors. Therefore, there is still a great amount of research to be done.

In general induced pluripotent stem cells (iPSC), as well as human embryonic stem cells (hESC), are of great interest for regenerative medicine and drug discovery due to their potential to differentiate into a wide range of specialized cells.

Figures

References

Breda, L., Gambari, R., Rivella, S. (2009). Gene Therapy in Thalassemia and Hemoblobinopathies. Mediterr J Hematol Infect Dis. 2009 Nov 13;1(1):e2009008; http://www.ncbi.nlm.nih.gov/pubmed/21415990

Breda, L., Casu, C., Gardenghi, S., Bianchi, N., Cartengi, L., Narla, M., Yazdanbakhsh, K. (2012). Therapeutic Hemoglobin Levels after Gene Transfer in β-Thalassemia and Sickle Cells Disease Patients. PLoS One. 2012;7(3):e32345; http://www.ncbi.nlm.nih.gov/pubmed/22479321

Dong, A., Rivella, S., Breda, L. (2013). Gene therapy for hemoglobinopathies: progress and challenges. Transl Res. 2013 Apr;161(4):293-306; http://www.ncbi.nlm.nih.gov/pubmed/23337292

Graf, T. (2011). Historical Origins of Transdifferentiation and Reprogramming. Cell Stem Cell. 2011 Dec 2;9(6):504-16; http://www.cell.com/cell-stem-cell/retrieve/pii/S1934590911005406

Hanna, J., Wernig, M., Markoulaki, S., Sun, .CW., Meissner, A., Cassady, J.P., Beard, C., Brambrink, T., Wu, L.C., Townes, T.M., Jaenisch, R.(2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science. 2007 Dec 21;318(5858):1920-3. Epub; http://www.ncbi.nlm.nih.gov/pubmed/18063756

Jamison, D.T., Breman, J.G., Measham, A. R., Alleyne, G., Claeson, M., Evans, D.B., Jha P., Mills, A., and Musgrove, P. (2006). Disease Control Prioritiers in Developing Countries, 2nd edition. Washington (DC): World Bank; http://www.ncbi.nlm.nih.gov/books/NBK11728/

Kim, J.B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Araúzo-Bravo, M.J., Ruau, D., Han, D.W., Zenke, M., Schöler, H.R. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature. 2008 Jul 31;454(7204):646-50. Epub; http://www.ncbi.nlm.nih.gov/pubmed/18594515

Kit, T. R. (2008). Inducing pluripotency. In **StemBook**. Cambridge (MA): Harvard Stem Cell Institute; http://www.ncbi.nlm.nih.gov/books/NBK27044/

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., Shibata, T., Kunisada, T., Takahashi, M., Takahashi, J., Saji, H., Yamanaka, S. (2011). A more efficient method to generate integration-free human iPS cells. Nat Methods. 2011

Puthenveetil, G., Scholes, J., Carbonell. D., Qureshi, N., Xia, P., Zeng, L., Li, S., Yu, Y., Hiti, A.L., Yee, J.K., Malik, P. (2004). Successful correction of the human beta-thalassemia major phenotype using a lentiviral vector. Blood. 2004 Dec 1;104(12):3445-53. Epub 2004 Aug 3; http://www.ncbi.nlm.nih.gov/pubmed/21460823

Sommer, C.A., Stadtfeld, M., Murphy, G.J., Hochedlinger, K., Kotton, D.N., Mostoslavsky, G. (2009) Induced pluripotent stem cell generation using a single lentiviral stem cell cassette.Stem Cells. 2009 Mar;27(3):543-9; http://www.ncbi.nlm.nih.gov/pubmed/19096035

Stegment. (2012). Product Specification Sheet, v2.0. Cat.num. 00-0009. San Diego, CA 92121; www.stegment.com

Steinberg, M.H., Forget, B.G., Higgs, D.R., Weatherall, D.J. (2009). Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management. Second Edition. Cambridge University Press 2009

Stemcell Technologies. (2012). Maintenance of Human Pluripotent Stem Cells in mTeSR™1 and TeSR™2. Technical Manual, Vers. 3.0.0, Document #29106; http://www.stemcell.com/~/media/Technical%20Resources/B/C/A/2/B/29106MAN_3_0_0.pdf

Tam, W.l., Lim, B. (2008). Genome-wide transcription factor localization and function in stem cells. In **StemBook**. Cambridge (MA): Harvard Stem Cell Institute;

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007 Nov 30;131(5):861-72.; http://www.ncbi.nlm.nih.gov/pubmed/18035408

Wernig, M., Zhao, J.P., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., Broccoli, V., Constantine-Paton, M., Isacson, O., Jaenisch, R. (2008). Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci U S A 2008;105:5856–5861; http://www.pnas.org/content/105/15/5856.long

Acknowledgements

First of all I want to thank PhD student Alisa Dong for letting me be part of this step of her PhD project. For trusting me in my work and judgements, for answering all my questions about science and beyond, being the most inspiring conversational partner in the last three months and infecting me with her passionate about science. I wish her all the best and a lot of success with her future studies plus vigorousness as well as imperturbation for both, work and life. It's been a pleasure meeting her and hopefully we'll see us again someday in the future.

Next I want to thank associate Professor Laura Breda and Professor Stefano Rivella. Laura, for teaching me, spreading wisdom and high spirits and making tough days passing by easily. Stefano for accepting my request for the internship, the beginning of a memorable and horizon broadening experience, for being supportive and having an open ear/door at all time.
Many thanks to all the great people in the Lab, creating a wonderful environment and making the Lab a place I loved to go to every day.

I also want to thank Dr. Oostingh, head of degree program Biomedical Science at the University of Applied Science in Salzburg, Austria, for being open and flexible, understanding and on top of that, being supportive for her more adventurous students and making it possible having my internship abroad.
The financial support in terms of the scholarship provided by the Austrian Marshall Plan foundation is most appreciated and without the accommodation provided by the most generous Mr. and Mrs Melville Straus, an internship in NYC would never have been possible.

Last but not least many thanks to my family and friends in Austria, I know time difference has been a little bit of a burden and I'm officially sorry for arousing one or another in the middle of the night.