Generation of induced pluripotent stem cells from patients' somatic cells using two different approaches

by

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Abstract

Stem cells are the source of all tissues in the human body – therefore the study of their properties is essential for understanding humans and their diseases.

The two main types of stem cells that exist are embryonic stem cells and adult stem cells. The difference lies in their potency and their potential to self-renew. Embryonic stem cells have the potential to differentiate into any cell type, meaning they are pluripotent, and have the capability of self-renewal. In contrast adult stem cells can only differentiate into some specialized cell types of the tissue or organ in which they are found. Since they cannot give rise to cell types other than that of their originating tissue their clinical use would be more limited. As embryonic stem cells can be exclusively found in the inner cell mass of a blastocyst, using them for research has raised many ethical concerns and in many countries research has been slowed or banned by regulatory restrictions.

The possibilities and interests of using stem cells in the field of disease treatment increased dramatically when Shinya Yamanaka produced induced pluripotent stem (iPS) cells from mouse fibroblast cells in 2006, and in 2007, from human cells by overexpressing four transcription factors. It is since then that research laboratories all over the world started working intensively with so-called induced pluripotent stem cells – adult somatic cells being reprogrammed to pluripotent state. This ability to experimentally create cells showing the same properties as embryonic stem cells could offer an unlimited source of cells for therapeutic purposes. Moreover, they provide an invaluable tool for disease modeling and drug screening. This gives hope for patients with degenerative diseases, such as Alzheimer's, Parkinson's or Huntington's disease.

The aim of this thesis was the successful generation of disease-specific iPS cell lines from T-cells, mesenchymal stem cells and fibroblasts using the Sendai virus method as well as microRNA enhanced mRNA technology to do so. After successful derivation of iPS cell lines, all cells were cultivated in feeder conditions and subsequent characterization was done to prove embryonic stem cell-like characteristics. Also, Sendai virus mediated reprogramming of mesenchymal stem cells was done using two different reprogramming kits, CytoTune^{TM}-iPS and

CytoTune™-iPS 2.0 Sendai Reprogramming Kit, to compare the resulting iPS cells.

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List of abbreviations

α-hCD3	anti-human CD3
AP	Alkaline Phosphatase
βFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CiPSCs	chemically induced pluripotent stem cells
CIU	cell infectious units
CPT	cell preparation tube
CRISPR	clustered regularly interspaced short palindromic repeat
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium, Nutrient mixture F12
DMSO	dimethyl sulfoxid
DPBS	Dulbecco's phosphate-buffered saline
EB	embryoid body
EpiSC	epiblast stem cell
(h)ES or (h)ESC	(human) embryonic stem (cell)
FBS	fetal bovine serum.
GFP	green fluorescent protein
HN	Hemagglutinin-neuraminidase
ICC	immunocytochemistry
ICM	inner cell mass
IgG	Immunoglobulin G
iPS(C)	induced pluripotent stem (cell)
Klf4	Kruppel-like factor 4
KOSR	Knockout Serum Replacement
LIF	leukemia inhibitor factor
MEF	Murine embryonic fibroblasts
MEM-NEAA	Minimum Essential Medium-Non-Essential Amino Acids
miRNA	microRNA
MOI	multiplicity of infection
mRNA	Messenger RNA

(h)MSC	(human) mesenchymal stem cell
NT-hESC	Nuclear transfer-human embryonic stem cells
NuFF cells	Newborn Human Foreskin Fibroblast cells
Oct4	Octamer-binding transcription factor 4
Pen/Strep	Penicillin/Streptomycin
PBMC	peripheral blood mononuclear cell
PFA	Paraformaldehyde
RISC	RNA induced silencing complex
ROCK inhibitor	Rho-associated protein kinase inhibitor
RQ	relative quantification
RT-PCR	Reverse Transcriptase PCR
(RT)-qPCR	(real time) quantitative-PCR
SeV	Sendai virus
SCNT	somatic cell nuclear transfer
SRT	short tandem region
TALEN	transcription activator-like effector nuclease

1. Introduction

In 2012, Dr. John B. Gurdon and Dr. Shinya Yamanaka were awarded the Nobel Prize in Physiology or Medicine for their groundbreaking discovery that mature, somatic cells could be dedifferentiated back into the pluripotent state. These findings led to a complete change in our knowledge of cellular differentiation. Mammalian development is unidirectional: embryonic stem cells develop progressively into more specific cells due to epigenetic changes. This was first modeled by Conrad Hal Waddington in 1957, who illustrated the cellular differentiation as an epigenetic landscape. He compared pluripotent stem cells with marbles on top of a mountain – they roll down in valleys to reach their destinations as differentiated cells. On their way down, they have the potential to turn into any type of differentiated cells. Waddington's model nicely illustrates that mammalian development is a unidirectional process, since marbles cannot move back towards the top of the hill, meaning the cells normally would not dedifferentiate (Waddington, 1957). This is illustrated in figure 1 below.

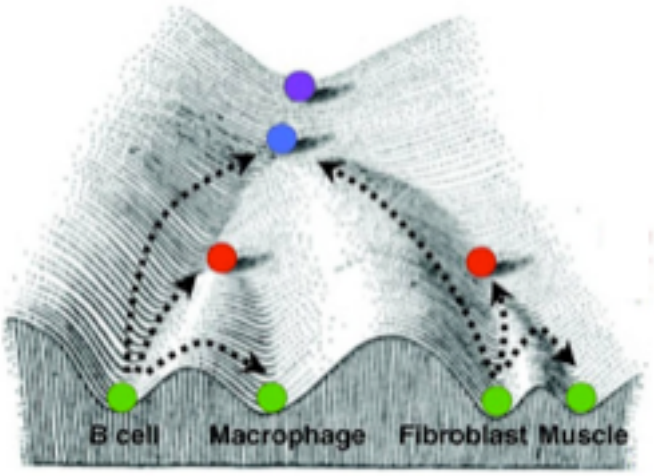

Figure 1: The model for illustrating the epigenetic landscape of cells at different stages of development

This illustration, originally from Waddington, here adapted from Hochedlinger *et al*., shows the idea that during mammalian development, cells and their differentiation potential behave like marbles.
The different colors of the "marbles" stand for their different differentiation states: purple means totipotent, blue means pluripotent, red means multipotent and green means unipotent. The arrows show the procedure of reprogramming– meaning to dedifferentiate mature cells back into an earlier potency state (modified after: Hochedlinger *et al.*, 2009)

In the 1950's, the common understanding was that mature cells permanently remain in their differentiated state. In 1962 John B. Gurdon's outstanding experiments first showed that it was possible to revert adult somatic cells into pluripotent stem cells via nuclear transfer in an enucleated frog oocyte. His discovery was the starting point for a completely new research area: somatic cell reprogramming. In 2006 and 2007, Shinya Yamanaka and his team reprogrammed adult mouse and human cells by simply introducing a small set of transcription factors thereby creating so-called induced pluripotent stem (iPS) cells.
The discoveries made by John Gurdon and Shinya Yamanaka are the two most fundamental findings in the field of stem cell research, offering exciting new opportunities.

This thesis should provide an overview of the development from the past years and the current findings in the field of stem cell research and somatic cellular reprogramming. Also the generation of patient-specific iPS cell lines using the Sendai virus and miRNA enhanced mRNA reprogramming methods is included.

1.1. Embryogenesis and the origin of stem cells

A huge number of the bodies cells are differentiated, meaning they have developed into a specific cell type with properties and functions unique for that cell, and they are unable to give rise to any other type of cell in the human body. Skin, muscle, blood, bone and the nervous system are all made up of populations of differentiated cells. However, a less differentiated type of cells exists, known as stem cells. As the name already indicates, stem cells build the foundation of tissues and organs in the human body, they are the "stem" from which specific cells can arise. Their name refers to their function: they are precursor cells from which mature cells in our body develop, which later on carry out specific functions in different tissues and organs of the body.

Development of the human body starts with two gametes, an egg and a sperm cell, both being haploid. With the fertilization of the egg with the sperm cell a zygote is formed, which will immediately start to experience cleavage. This cleavage does not lead to an increase in size, but via mitosis many cell divisions cause the zygote to turn into a solid ball of cells, which keeps splitting. This mass of cell is referred to as the morula. As the cells continue to divide and to reorganize, the embryo becomes a hollow ball, known as blastocyst. This blastocyst consists of an inner cell mass, ICM, also known as embryoblast, later forming the embryo, and the trophoblast, the outer layer of cells, which later becomes the placenta (Gilbert, 2010, Nüsslein-Volhard, 2006).

Next, the embryo undergoes gastrulation. During this stage the single-layered blastocyst reorganizes into three different germ layers: ectoderm, mesoderm and endoderm. Each of these layers will later give rise to all adult tissues and organs. The endoderm will develop into the digestive system, the liver, the pancreas and the lungs. The mesoderm gives rise to somites, which will form muscle, the heart, the spleen and the bone marrow. The nervous system, epidermis and mammary glands derive from the ectoderm layer (Gilbert, 2010, Thomson *et al.*, 1998).

Figure 2 below shows the development of a mammal from fertilization till gastrulation, as well as the consequent changes in potency of stem cells occurring.

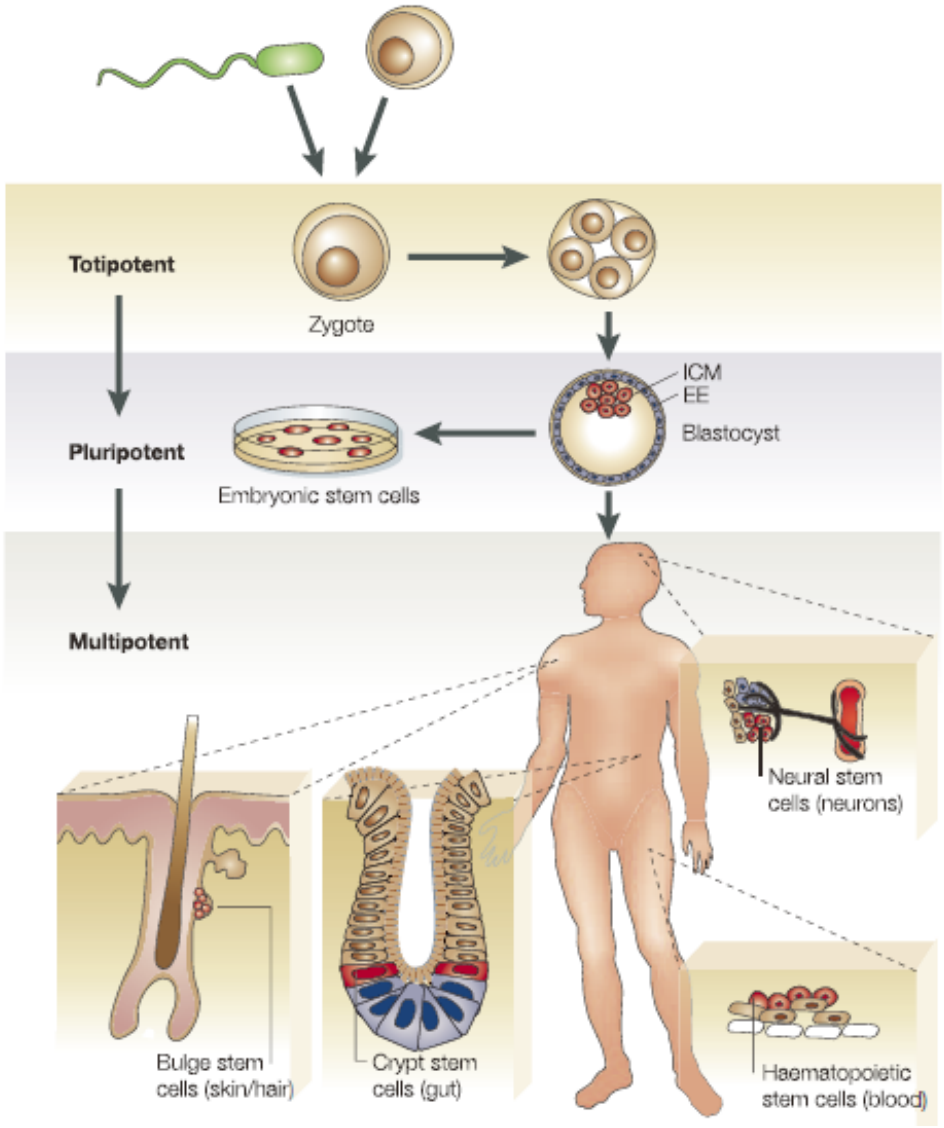

Figure 2: Embryogenesis and the differentiation of human tissue

Illustration showing how after fusion of the two gametes a fertilized egg, the zygote, forms and develops in mammals. During development of the embryo, the cells get more lineage restricted, generating tissue-specific multipotent stem cells. The cells develop into all three germ layers, the ectoderm, mesoderm and endoderm, leading to the formation of organs and tissues in the human body (not shown). Examples for such tissue-specific stem cells include epidermal stem cell, forming skin and hair, haematopoietic stem cells, giving rise to all types of blood cells, neural stem cells, and gastrointestinal stem cells (Eckfeldt *et al.*, 2005)

1.1.1. Properties of stem cells

Stem cells can be distinguished from other somatic cell types by two distinctive properties: Their capability of self-renewal and their potency. (Zhang *et al.*, 2008).

1.1.1.1. Self-renewal

The property of self-renewal describes the ability of a cell to undergo several cycles of mitotic cell division while still remaining in an undifferentiated state – that means cell division results in at least one daughter cell with the same developmental potential as the mother cell. With on-going division this process leads to the formation of a stem cell pool. This capability to create more stem cells differs between the type and age of a stem cell: Embryonic stem cells (ESC) as well as induced pluripotent stem cells (iPSCs) are known to have an unlimited capacity for self-renewal under certain conditions. Adult multipotent stem cells have a more restrictive self-renewal capacity.

The presence of a sufficient number of stem cells in different types of tissues and organs of the human body is important to ensure the maintenance and repair of lost or damaged cells and tissue. Adult stem cell self-renewal is regulated via several cell-extrinsic signals coming from the stem cell niche. The niche is the microenvironment interacting with stem cells to regulate their function in tissues (He *et al.*, 2009).

1.1.1.2. Potency

Potency of a cell describes its potential to differentiate into any specialized cell type of the body. Cells that are able to give rise to every cell in the embryo, and also to the trophoblastic cells of the placenta are said to be totipotent (Gilbert, 2010). Totipotency gets lost with on-going embryogenesis, and therefore embryonic cells within the first couple of cell divisions after fertilization are the only cells that are totipotent (zygote to morula).

Cells with the ability to differentiate into any other cell type except for the placenta are pluripotent. This includes embryonic stem cells isolated from the inner cell mass of the blastocyst (Thomson *et al.*, 1998), and induced pluripotent stem cells. (Vazin *et al.*, 2010).

As embryogenesis continues, several signaling molecules trigger the development of more specialized cells from ESCs, known as adult stem cells. These cells are multipotent, meaning they are lineage-restricted cells of either the blood, muscle, brain, bone or other tissues (Gonzalez *et al.*, 2012). These multipotent cells are also known as progenitor cells. For example, blood stem cells can differentiate into several types of blood cells, but cannot differentiate into muscle cells, bone cells, or brain cells – they are restricted to hematopoietic cells (Seaberg *et al.*, 2003). At this stage of development, the cells have lost their ability of unlimited self-renewal, and can only divide few times before differentiating (Gilbert, 2010).

Figure 3 below shows how the differentiation potential of a cell changes over development of a specialized cell using the development of a neuron as an example.

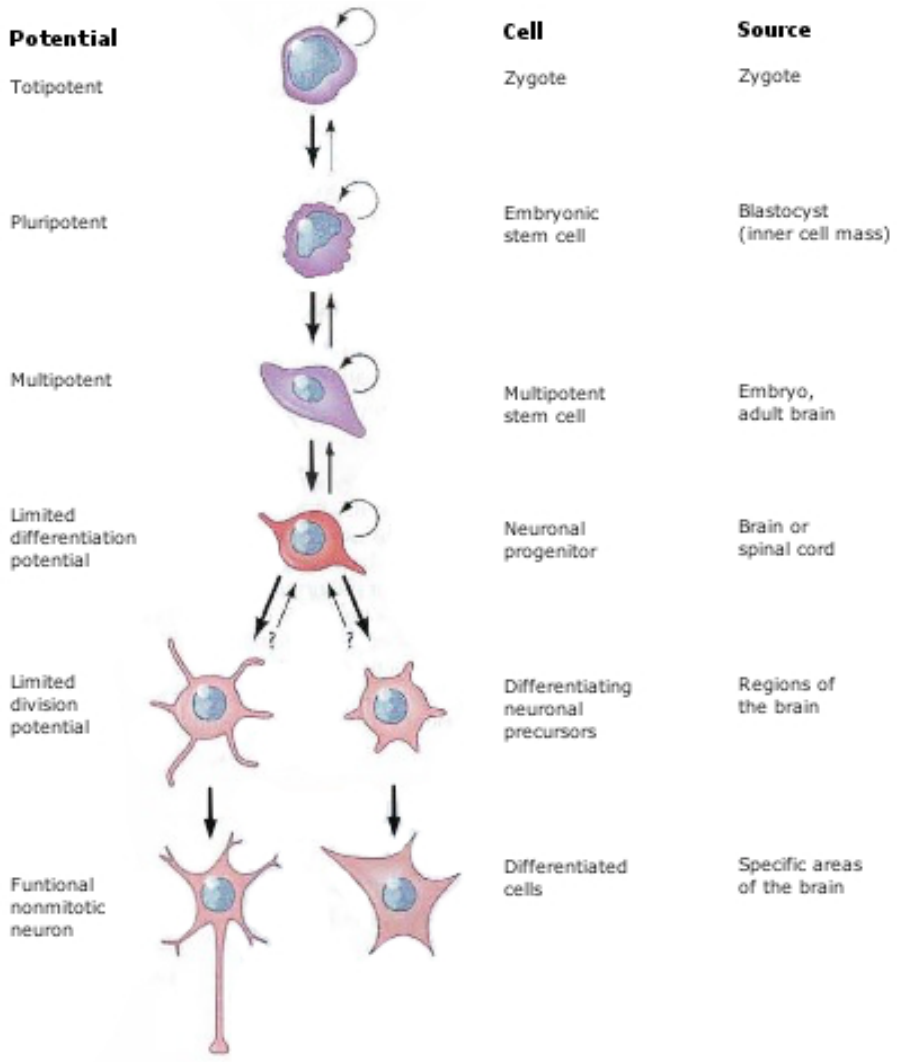

Figure 3: Hierarchy of stem cells with their change in potency over development

Maturational steps of stem cells, here shown with the example of a neuronal cell. Also the change in differentiation potential over development is shown. (modified after: Gilbert, 2010)

1.2. Types of Stem Cells

In the next paragraphs, we will cover the two major types of stem cells in more detail: Embryonic stem cells and adult stem cells. They differ in terms of potency and their ability to self-renew (Gilbert, 2010).

1.2.1. Adult Stem Cells

Adult stem cells can be found throughout the body in various organs and tissues. They can be isolated from the bone marrow, the stroma, fat, muscle and nervous tissue (Gilbert, 2010, Lanza *et al.*, 2006). They are limited to differentiate into specialized cell types of their organ or tissue of origin. Hematopoietic stem cells for example can generate precursors to every type of blood cells, but they cannot differentiate into other cell types. The primary role of adult stem cells is to generate differentiated progenitor cells, which are important in replacing and repairing tissues of that particular organ, thereby maintaining homeostasis. Adult stem cells normally remain in a quiescent, non-dividing state until they are activated via several processes. These processes depend on a mix of genetic and environmental factors, operating in the cells environment, at its surface membrane and within its nucleus, and they lead to asymmetric cell division of the adult stem cell (Burdon *et al.*, 1999, Li *et al.*, 2011).

Since adult stem cells cannot give rise to cell types other than that of their originating tissue their clinical use is more limited than that of embryonic stem cells.

1.2.2. Human embryonic stem cells

Embryonic stem cells are cells with the potential to give rise to any cell type, which has been demonstrated in vitro and in vivo. (Wobus *et al.*, 2005).

Compared to adult stem cells, their potential to self-renew has been shown to be unlimited. ES cells are normally found in the inner cell mass of a mammalian blastocyst, or they can be derived from fetal germ cells (Gilbert, 2010).

In 1981 the first ES cells were isolated and cultured from mouse blastocysts (Evans *et al.*, 1981, Martin, 1981), and in 1998 *Thomson et al*. first managed to isolate embryonic stem cells from human inner cell mass and under appropriate conditions cultured ES cells in a laboratory. Over time, the methods used to isolate and culture human ES cells developed further, and researchers were able to gain more information about somatic differentiation in vitro (Reubinoff *et al.*, 2000, Thomson *et al.*, 1998).

Due to their limitless ability to self-renew while remaining in an undifferentiated, pluripotent state, ESCs are an ideal source of cells for research and transplantation therapy, and to study cell differentiation and development of cells (Keller, 2005, Lok, 2012, Sykova *et al.*, 2013, Walsh *et al.*, 2012).
However, the use of human embryonic stem cells has been limited due to ethical concerns. During the process of hESC isolation from the inner cell mass, the human embryo is destroyed, raising ethical issues. Therefore in many countries research has been slowed or banned by regulatory restrictions (Sommer *et al.*, 2010).

1.3. Experimentally-induced pluripotency

The study and understanding of stem cells plays an important role for understanding different diseases, their underlying causes and potential cures.
Due to the aforementioned ethical concerns, and the limited use of hESC to create patient- and disease-specific tissues (Miyazaki *et al.*, 2012, Walsh *et al.*, 2012), the stem cell community put a lot of effort into finding other sources of human pluripotent stem cells.

In the past few years different approaches have been described to reprogram differentiated cells into a pluripotent state. Some of these approaches are depicted in figure 4.

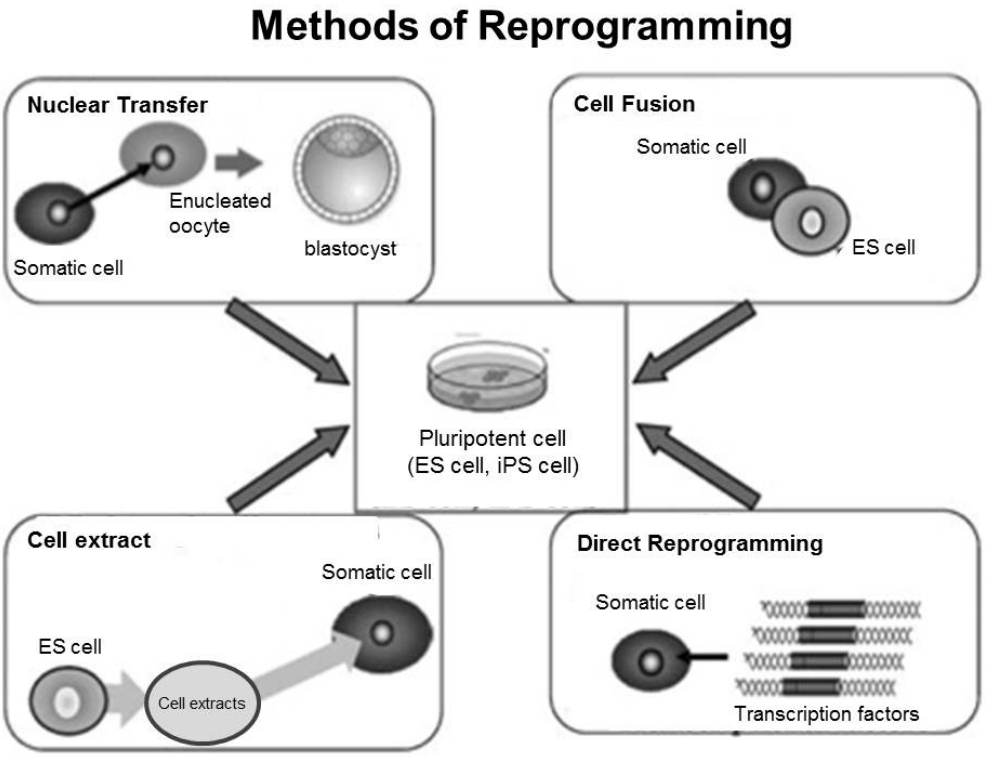

Figure 4: Four main strategies used for reprogramming somatic cells back into the pluripotent state.

First, nuclear transfer describes the transfer of a somatic nucleus into an enucleated oocyte. This can, when inserted into a surrogate mother, lead to the development of a clone or, when explanted in culture, give rise to ES cells. Second, fusion of a somatic cell with an ES cell leads to the formation of a tetraploid hybrid showing all characteristics of ES cells. Third, reprogramming via insertion of cell extracts from pluripotent stem cells also leads to an epigenetic change of the somatic cell, bringing it back into pluripotent state. Last, direct reprogramming methods, which include integrating, non-integrating and DNA-free methods, being the most prominent reprogramming approaches so far (modified after: Miyazaki *et al.*, 2012).

The first successful nuclear reprogramming was described in 1962, when *Gurdon et al.* successfully transferred the blastula nuclei of early-stage frog embryos into enucleated eggs, and thereby created tadpoles (Gurdon, 1962). The most prominent example for reverting a committed cell fate was the development of Dolly the sheep in 1997. It was the first cloned mammal from an adult cell

(Hochedlinger *et al.*, 2006). The general procedure for somatic cell nuclear transfer (SCNT) consists of the enucleation of an egg - thereby all the genetic material gets eliminated – followed by the transfer of the nucleus of an adult somatic cell into the enucleated egg. (Miyazaki *et al.*, 2012). This procedure could allow the generation of genetically compatible cells, tissues and organs of patients for transplantation therapy, and it could also provide new tools for drug testing and drug development, but it comes with several drawbacks (Nichols *et al.*, 1998, Takahashi *et al.*, 2006). The creation of patient-matched nuclear transfer human embryonic stem cells (NT-hESC) has not been possible until 2013, due to several challenges: lack of donated oocytes, technical challenges such as early embryonic arrest of the SCNT embryos, (Niwa *et al.*, 1998) and low efficiency (Avilion *et al.*, 2003, Hochedlinger *et al.*, 2006, Stadtfeld *et al.*, 2010a).

Another reprogramming method is the cellular fusion of a somatic cell with an embryonic stem cell. During cellular fusion the embryonic stem cell can reprogram a somatic cell by changing its properties (Miyazaki *et al.*, 2012). The somatic cell epigenome gets overwritten with properties of ES cells, which happens after two days through the reactivation of ES cells essential genes: Oct4 and Nanog (Li *et al.*, 2005, Takahashi *et al.*, 2003). Those genes are responsible for maintaining the ES cells in an undifferentiated state (Miyazaki *et al.*, 2012). This approach proved that ES cells have all the factors necessary to induce pluripotency.
However, there are several downsides to this approach, including technical hurdles, a low fusion rate, and the possibility of immune rejection due to the tetraploid nature of the hybrid cells (Hochedlinger *et al.*, 2006, Mitsui *et al.*, 2003, Miyazaki *et al.*, 2012).

Reprogramming using cell extracts is another method. Here cell extracts, consisting of factors from pluripotent stem cells are introduced into somatic cells, leading to the reprogramming of the somatic cell nucleus (Miyazaki *et al.*, 2012). This reprogramming method has been tested with mouse and human somatic cells together with mouse ES cell extract. While in 2005 and 2008 two groups were able to show the expression of pluripotency genes, Neri *et. al* could not confirm this

(Cartwright *et al.*, 2005, Neri *et al.*, 2007, Vogt *et al.*, 2012). Additionally it has been shown that the reprogrammed cell could not differentiate into all three germ layers *in vivo* (Miyazaki *et al.*, 2012).

The simplest and most promising reprogramming method is the direct reprogramming method by overexpressing the four “Yamanaka” transcription factors: Oct4, Sox2, Klf4 and cMyc. Different approaches have been used to introduce these factors, including integrative, excisable, non-integrative and DNA-free methods. All of these approaches will be further discussed in chapter 1.5.

1.4. Induced pluripotent stem cells

When Yamanaka first generated induced pluripotent stem cells from mouse somatic cells in 2006 (Takahashi *et al.*, 2006) and from human fibroblasts in 2007 (Takahashi *et al.*, 2007) by introducing a few defined transcription factors, one of the most powerful and exciting discoveries in the field of somatic reprogramming was made. Induced pluripotent stem cells are adult cells like skin or blood cells, which have been genetically reprogrammed to show pluripotent properties of embryonic stem cells. The resulting iPS cells have been shown to be morphologically and characteristically very similar to ES cells. Therefore this technique provides human pluripotent stem cells without the need for a human embryo, and with the potential to create autologous patient-specific cells. These cells have great potential for many medical applications like drug discovery and regenerative medicine.

Over the past years, many different genes have been identified to be specifically expressed in ES cells and either contributing to the maintenance of pluripotency or to the unlimited proliferation of these cells, including Oct3/4, Sox2, Nanog, Stat3, Klf4, c-Myc, LIN28 and more (Avilion *et al.*, 2003, Cartwright *et al.*, 2005, Chambers *et al.*, 2003, Li *et al.*, 2005, Maruyama *et al.*, 2005, Mitsui *et al.*, 2003, Nichols *et al.*, 1998, Niwa *et al.*, 1998, Niwa *et al.*, 2000, Vogt *et al.*, 2012).

Takahashi and Yamanaka selected a set of 24 genes associated with pluripotency and tested whether their overexpression into somatic cells could induce their reprogramming. Although the efficiency was very low, they were able to obtain a few colonies with the properties of ES cells. They were then able to narrow it down to a combination of four genes: Oct3/4, Sox2, Klf4 and c-Myc, commonly referred to as the "Yamanaka factors." Using retroviral transduction to express these four factors, Takahashi *et al*. were the first to create pluripotent stem cells directly from mouse embryonic and adult fibroblasts. Even though these cells were similar to embryonic stem cells in terms of morphology, proliferation and teratoma formation, once transplanted into a blastocyst, the iPS cells were unable to give rise to adult chimeric mice (Takahashi *et al.*, 2006). However, in 2007 four different research groups reported the successful generation of iPS cells competent for adult and germline chimeras by using a more stringent selection marker, Nanog (Maherali *et al.*, 2007, Meissner *et al.*, 2007, Okita *et al.*, 2007, Wernig *et al.*, 2007).

This major discovery enabled an amazing way to change the fate of a cell by forcing epigenetic changes. It is a powerful technology for creating autologous patient-specific cells, thereby eliminating the risk of rejection by the immune system after cell transplantation.

1.4.1. Cellular reprogramming factors

Reprogramming via nuclear transfer was the first indicator that epigenetic modifications occurring during development could be reversed (Hochedlinger *et al.*, 2002). With the successful reprogramming of somatic cells by fusion with an embryonic stem cell, it was proven that certain factors present in ES cells could mediate the reprogramming of differentiated cells into an embryonic state. With the discovery of the four Yamanaka factors, Oct3/4, Sox2, Klf4 and c-Myc in 2006 there was a great breakthrough in the history of cellular reprogramming (Takahashi *et al.*, 2006). Overexpression of these factors in adult cells has been shown to effectively generate pluripotent stem cells from human (Takahashi *et al.*, 2007), monkey (Liu *et al.*, 2008), pig (Ezashi *et al.*, 2009) and rat somatic cells (Liao et al., 2009; Rajarajan *et al.*, 2012).

Since the initial discovery in 2006, successful reprogramming of murine and human somatic cells has been described using different combinations of transcription factors (Kim *et al.*, 2009, Li *et al.*, 2010, Park *et al.*, 2008b, Takahashi *et al.*, 2007, Wernig *et al.*, 2008a, Yu *et al.*, 2007). Using chromatin immunoprecipitation it has been shown that several factors important for reprogramming somatic cells bind DNA in clusters, with their binding sites often overlapping (Chen *et al.*, 2008, Kim *et al.*, 2008).

1.4.1.1. Oct4

Oct4, short for Octamer-binding transcription factor 4, also known as Oct3, Oct3/4 or POU5f1 is one of the transcription factors known to regulate and maintain the pluripotency of human embryonic stem cells in vivo, and was shown to function by the formation of a heterodimer with Sox2 in ES cells (Avilion *et al.*, 2003, Takeda *et al.*, 1992). Also, Oct4 is critically involved in the self-renewal of stem cells (Lewitzky *et al.*, 2007).

This member of the POU protein family is expressed in the ICM of the blastocyst during mammalian development. Since embryonic stem cells are derived from the ICM, Oct4 is detected in mouse and human embryonic stem cells, but also in primordial germ cells (Okamoto *et al.*, 1990, Rosner *et al.*, 1990, Schöler *et al.*, 1990). Oct4 is specifically expressed in cells important for the generation of germline lineage, and it has been shown that its expression is down-regulated during differentiation (Pesce *et al.*, 1998, Rosner *et al.*, 1990), all indicating its importance for maintaining pluripotency in ES cells (Schöler *et al.*, 1990).

Although embryos lacking Oct4 were able to develop until the blastocyst stage, their inner cell mass did not contain pluripotent cells (Nichols *et al.*, 1998). In 2003, POU5f1 was detected in human germ cell tumors and other tumors containing cells with pluripotent potential (Looijenga *et al.*, 2003). Recently it has been shown that Oct4 is able to regulate pluripotency of embryonic cells by inactivating the tumor suppressor gene p53 (Zhang *et al.*, 2013b).

Howsoever, the expression of Oct-4 must be closely regulated, since it determines the fate of ES cells: Overexpression of Oct4 leads to differentiation to mesoderm

and endoderm, while depletion of Oct4 leads to trophoectoderm differentiation (Niwa *et al.*, 2000).

1.4.1.2. Klf4

Kruppel-like factor 4 (Klf4) is a transcription factor belonging to the Krueppel-type zinc-finger family. It is known to be essential for ES cell self-renewal and maintenance of pluripotency (Lewitzky *et al.*, 2007). In 1988, two research groups identified a factor, which was able to enhance proliferation of mouse ES cells in vitro and limit their differentiation: leukemia inhibitor factor (LIF) (Smith *et al.*, 1988, Williams *et al.*, 1988). Binding of LIF to a membrane receptor complex leads to the activation of intrinsic Jak-Stat3 cascade and to the subsequent phosphorylation of Stat3. This phosphorylation activates the transcription of several target genes, including Klf4. Up-regulation of Klf4 leads to an increased capacity to self-renew, and additionally to sustained expression of Oct4, maintaining pluripotency in ES cells (Li *et al.*, 2005). Additionally, Klf4 indirectly regulates the expression of Nanog, by repressing p53, thereby preventing cell differentiation (Rowland *et al.*, 2005, Zhang *et al.*, 2010). Recently, it has been shown that Klf4 is a component in the regulation of Tert via β-catenin signaling. Tert is a telomerase subunit, controlling telomere length. Tert-deficient mice have shown a decrease in lifespan and a concurring loss of tissue renewal. This indicates that the ability of stem cells to regenerate and repair tissue is related to telomerase activity and therefore telomere length (Blasco, 2007, Hoffmeyer *et al.*, 2012).

1.4.1.3. Sox2

Sox2, also known as SRY (sex determining region Y)-box2 is the only known Sox-protein important for embryonic development and maintenance of ES cell pluripotency (Avilion *et al.*, 2003, Kiefer, 2007, Lewitzky *et al.*, 2007). Several studies showed that expression of genes important for development and pluripotency of ES cells are regulated by a combination of Sox2 and Oct4 (Chew *et al.*, 2005, Nishimoto *et al.*, 1999, Yuan *et al.*, 1995). The same is true for Sox2 and Nanog (Boyer *et al.*, 2005). Also silencing of either Sox2 or Oct4 would promote differentiation of ES cells, suggesting that both are essential for keeping

ES cells in a pluripotent state (Chew *et al.*, 2005). Furthermore the differentiation potential of epiblast stem cells (EpiSCs) has been shown to be lower compared to ES cells. This may be explained by the lower level of Sox2 in EpiSCs, indicating the importance of Sox2 to maintain the pluripotent state of the cells (Han *et al.*, 2013).

1.4.1.4. c-Myc

The transcription factor c-Myc, or Myc, is one of the three proto-oncogenic Myc family members, the others being N-Myc and L-Myc. It contains two dimerization motifs: the helix-loop-helix and the leucine zipper (Davis *et al.*, 1993). Through these motifs c-Myc specifically dimerizes with Myc-associated factor-X (Max), forming a complex that enables DNA binding and thereby activating transcription and modulating DNA activities (Amati *et al.*, 1993, Shafa *et al.*, 2010).

C-Myc is known to play a major role in cell growth, differentiation and proliferation (Lewitzky *et al.*, 2007). It has been shown to inhibit differentiation, but it also plays an important role in the self-renewal of stem cells (Satoh *et al.*, 2004, Varlakhanova *et al.*, 2010, Waikel *et al.*, 2001).

Like Klf4, c-Myc is also a downstream target of activated STAT3 (Kidder *et al.*, 2008). Its ability to block differentiation is also regulated via the Wnt signalling cascades (Marson *et al.*, 2008). The contribution of the Wnt signaling to self-renewal and pluripotency has already been shown for murine and human ES cells (Cai *et al.*, 2007, Ogawa *et al.*, 2006, Reya *et al.*, 2005, Sato *et al.*, 2004, Singla *et al.*, 2006).

One reason why retrovirally reprogrammed iPS cells are not suitable for clinical use is their risk for tumorigenic potential due to cMyc expression. In 2007, Okita et al. showed that they could obtain chimeric mice by injecting iPS lines into mouse blastocysts. However, about 20% of offspring mice obtained from germline competent chimeric mice developed teratomas after reactivation of retroviral expression of c-Myc. This indicates that c-Myc is directly related to the iPS cells' tumorigenic potential (Okita *et al.*, 2007). By regulating expression of genes involved in cell growth and cell adhesion, c-Myc often promotes tumor formation and thereby induces tumorigenesis (Gartel *et al.*, 2003). For this reason, this

proto-oncogene is often replaced by other transcription factors or simply omitted in reprogramming method (Judson *et al.*, 2009, Nakagawa *et al.*, 2008, Wernig *et al.*, 2008a).

1.4.1.5. L-Myc

L-Myc, another proto-oncogenic protein from the Myc family, is a transcription factor with a basic helix-loop-helix domain (Ikegaki *et al.*, 1989). Like c-Myc, L-Myc regulates transcription through dimerization with Max and subsequent binding to DNA (Blackwood *et al.*, 1991, Fitzgerald *et al.*, 1999). L-Myc was found to be overexpressed in a number of small cell lung carcinoma cell lines (Ikegaki *et al.*, 1989). This might suggest similar risks in terms of tumor development in iPS as detected for c-Myc. However, L-Myc was shown to be more efficient than c-Myc for iPSC generation, but less tumorigenic in chimeric mice (Nakagawa *et al.*, 2010).

Nowadays, L-Myc is often used to replace c-Myc for reprogramming somatic cells using episomal vectors. In fact, reprogramming of human fibroblasts and CD34 cells was successfully done using episomal vectors with different integrated transcription factors including L-Myc (Mack *et al.*, 2011, Okita *et al.*, 2011).

1.4.1.6. LIN28

LIN28 is a microRNA (miRNA) inhibitor being critically involved in growth and early human development (Wilbert *et al.*, 2012). MicroRNAs are very important for successful human development, and an abnormal miRNA expression is found in embryonic stem cells, embryonal carcinoma cells and primary tumors. LIN28 is one of the proteins that binds and blocks a hairpin-like structure on the pri-let-7 miRNA (Viswanathan *et al.*, 2008, Wilbert *et al.*, 2012).

Yu *et al.* were the first to use LIN28 in combination with other transcription factors (Oct4, Sox2 and Nanog) for reprogramming human somatic cells back into the pluripotent state. (Yu *et al.*, 2007). LIN28 is highly expressed in ES cells, and its downregulation during ES cell differentiation indicates its importance for maintaining pluripotency (Richards *et al.*, 2004).

1.5. Direct reprogramming methods

Since Yamanaka's initial discovery of direct reprogramming of somatic cells by introducing the four transcription factors (OKSM) via a retroviral system many new and improved technologies were developed. All of these methods are summarized in figure 5.

Figure 5: Timeline showing the development of new reprogramming methods since 2006

Illustration shows all different methods for reprogramming either mouse somatic cells (M) or human somatic cell (H) into iPS cells since the first successful approach in 2006. It also includes the corresponding first author or principal investigator of the first relevant paper published. (modified after: https://www.stemgent.com/knowledge/cellular_reprogramming)

The major changes were made in the delivery methods of the transcription factors into the cell.
Generally, these reprogramming strategies can be classified in four categories: Integrative, excisable, non-integrative, and DNA-free reprogramming (Robinton *et al.*, 2012).

The original delivery approaches used retrovirus or lentivirus which are integrated into the host's genome. These integrative methods come with several drawbacks including reproducibility, which could affect the quality of the reprogrammed iPS cells (Takahashi *et al.*, 2007, Takahashi *et al.*, 2006). The iPS cell lines generated with these methods have a higher risk of being tumorigenic due to random integration of viral constructs into the host DNA, which could cause deregulation of nearby genes, and the possible residual expression of the c-Myc transgene in iPS lines or their derivatives (Okita *et al.*, 2007, Varas *et al.*, 2009). This makes integrative methods not applicable for clinical use.

Several strategies to excise the provirus after the generation of the iPS lines have been described. Using a loxP/Cre Recombinase approach, several groups have shown that it was possible to eliminate the transgenes following reprogramming. However, this appoach is unefficient and labour intensive since it requires the selection of iPS lines with low copy number and the screening of many colonies to confirm the excision of the transgenes.

Recently, new non-integrative delivery systems have been reported. First, the Sendai virus, a negative sense RNA virus, was shown to efficiently reprogram fibroblast and blood cells (Fusaki *et al.*, 2009). Another method, using episomal vectors, proved to be suitable for the derivation of safer iPS lines by allowing prolonged expression of the transgenes but also permitting the elimination of the plasmids by dilution in iPS lines. Although they are extrachromosomal elements, a certain risk remains for the vectors to integrate into host's genome (Harui *et al.*, 1999). A third method for non-integrative reprogramming consists of the

transfection of modified mRNA. This method allowed the efficient generation of transgene-free iPS lines.

Additional DNA-free strategies based on protein transduction or chemical treatment were also described.

The iPS core facility at Harvard University is currently providing three different non-integrative reprogramming methods: Sendai viral reprogramming, episomal reprogramming vector, and microRNA enhanced messenger RNA (mRNA) reprogramming system.

1.5.1. Sendai virus reprogramming

The Sendai virus, also known as Hemagglutinating Virus of Japan, is an enveloped single stranded non-integrative negative-sensed RNA virus, belonging to the Paramyxoviridae family (Lamb *et al.*, 2001). It was first isolated in 1953 in Sendai, Japan (Kuroya *et al.*, 1953a, Kuroya *et al.*, 1953b). Its replication happens independently of cell division in the cytoplasm of a cell. It does not go through a DNA phase therefore integration into the host's genome is not occurring, making it safer than integrative-viruses. On its surface, the virus carries Hemagglutinin-neuraminidase (HN) proteins, which attach to sialic acid receptors on the host cell's surface (Fusaki *et al.*, 2009). Sialic acid is expressed in many different cells of mammals or other animal species, hence it has a very high potential for infecting cells (Markwell, 1991). The general replication life-cycle of Sendai virus compared to integrative viruses is illustrated in figure 6.

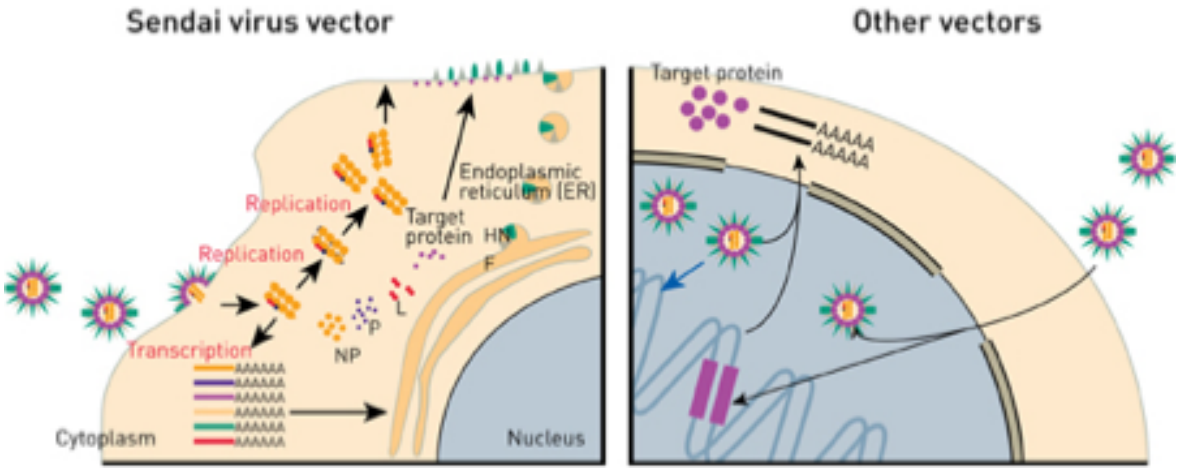

Figure 6: Differences in lifecycles of Sendai virus vector and integrating vectors

Infection of cells via the Sendai virus happens in the cytoplasm. No integration of viral particles into the host cells genome occurs. For other, integrating vectors, viral integration into the genome is necessary for the cell to become infected.
(http://www.lifetechnologies.com/us/en/home/communities-social/blog/blogs/what-are-induced-pluripotent-stem-cells-ipscs-and-how-is-Sendai-virus-used-to-generate-ipscs.html)

With the first successful reprogramming of somatic cells using the Sendai virus, Fusaki et al. could eliminate several of the drawbacks coming along with other viral systems. Compared to virus-integrative systems, the Sendai virus eliminates the major problem of random viral integration into the host's chromosomes, which might cause alterations in essential gene functions (Fusaki *et al.*, 2009). Moreover, the efficiency of somatic cell reprogramming with the Sendai virus is increased compared to retroviral/lentiviral methods. (Robinton *et al.*, 2012). Due to its simplicity of use, as well as its safety and efficiency, Sendai viral reprogramming is one of the most commonly used reprogramming methods.

1.5.2. Episomal vector reprogramming

Another non-integrative method for the derivation of iPS cells uses episomal vectors. These Epstein-Barr virus-based vectors can be introduced into the somatic cells by a single transfection. In the cells, they behave as an extra-chromosomal element. They can replicate and therefore allow prolonged expression of the Yamanka factors. The three episomal vectors first described by Yamanaka's group are depicted in figure 7. These vectors include Oct4, Klf4, Sox2, LIN28 and L-Myc, as well as a p53 knockout mRNA cassette. Removal of

the tumor suppressor p53 during reprogramming significantly increases efficiency (Marion *et al.*, 2009).

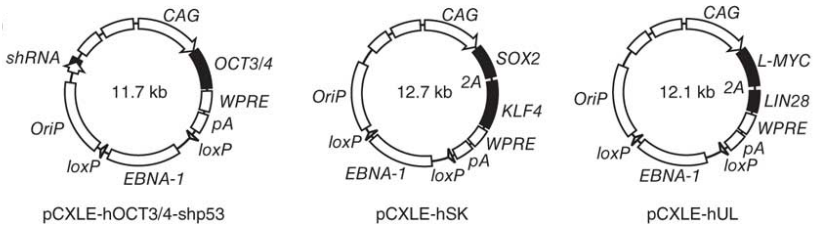

Figure 7: Construction of episomal expression vectors as used at the iPS core facility

In 2009, Thomson and colleagues generated induced pluripotent stem cells from fibroblasts with the use of episomal vectors for the first time (Yu *et al.*, 2009). Since then episomal vector reprogramming was successfully done for blood cells and fibroblasts, and its procedure is relatively simple as only one transfection is necessary (Zhang *et al.*, 2013a). Nevertheless, compared to Sendai-viral reprogramming, the efficiency is low, with approximately 0.0005%, and although the process is non-integrative, there is still a possibility that vector pieces integrate the genome (Sommer *et al.*, 2010).

1.5.3. MicroRNA enhanced mRNA reprogramming

The use of induced pluripotent stem cells for therapeutic and clinical applications is limited due to several problems most methods for iPSC derivation bring along. This does not only include the risk of viral integration into the cells' genome, and thereby alteration of gene expression and the subsequent risk of mutagenesis, but also the relatively low efficiency (Kim *et al.*, 2009, Okita *et al.*, 2008, Stadtfeld *et al.*, 2008, Yu *et al.*, 2009, Zhou *et al.*, 2009a).

With the successful reprogramming via modified mRNA transfection in 2011, it was possible to generate iPS lines devoid of transgenes with high efficiency (up to 3%) (Warren *et al.*, 2010).

The modified mRNA used for the derivation of iPS cells encode the four Yamanaka factors: Oct4, Sox2, Klf4, c-Myc as well as Lin28. Upon transfection, the proteins are rapidly translated but since the mRNAs are not stable, the

expression of these proteins is transient (~24 hours) (Stadtfeld *et al.*, 2010a). Therefore this protocol requires daily transfection for 12 to 15 consecutive days.
In mammalian cells exogenous single-stranded RNA trigger antiviral immune responses through interferon- and NF-KB pathways (Angel *et al.*, 2010, Diebold *et al.*, 2004, Hornung *et al.*, 2006, Kawai *et al.*, 2007, Uematsu *et al.*, 2007). To attenuate these innate antiviral responses, the mRNAs were synthetically modified and B18R, an interferon suppressor, was added to the cells prior transfection.

MicroRNAs are a type of small RNA, consisting of approximately 18-24 nucleotides in humans. Most miRNAs come from RNAs being transcribed in the nucleus. Here the RNAs are getting folded and processed by Dosha, to form a double stranded precursor miRNA (pre-miRNA), which gets exported in the cytoplasm. The double stranded pre-miRNAs bind to Dicer, an endonuclease protein that cuts the RNA into short segments, thereby generating mature miRNAs (Bernstein *et al.*, 2001). The short double stranded RNA then assembles with proteins called Argonaute. One strand of the RNA is selected and remains bound to Argonaute. The combination of the RNA and Argonaute along with other proteins is called the RNA induced silencing complex, or RISC (Hammond *et al.*, 2001). MiRNAs guide RISC to mRNAs, where usually only part of a miRNA pairs with the target mRNA. This imprecise matching allows miRNA to target hundreds of endogenous mRNAs. The targeting by a miRNA can lead to mRNA degradation or inhibition of translation (Filipowicz, 2005, He *et al.*, 2004, Miyazaki *et al.*, 2012, Rana, 2007, Wilson *et al.*, 2013, Zamore *et al.*, 2000).
In 2011, the first successful reprogramming using microRNAs (miRNAs) was reported (Miyoshi *et al.*, 2011). MicroRNAs have already been known to play an important role in pluripotent stem cell maintenance by silencing the translation of selected mRNAs. Also, it has been shown that some types of miRNAs are specifically expressed in ES cells, while showing a decreased expression during differentiation (Laurent *et al.*, 2008, Stadler *et al.*, 2010, Yang *et al.*, 2011).

With a great efficiency (1-4.4%) (Robinton *et al.*, 2012) and safety, mRNA reprogramming seems to be a very convenient approach to generate iPS lines for

disease modeling, regenerative medicine and basic research (Warren *et al.*, 2010). However, as mentioned above, the miRNA/mRNA transfection needs to be repeated every day for several days, which is associated with a high workload and high costs (Robinton *et al.*, 2012). Moreover, the cytotoxicity associated with the mRNA transfection varies from sample to sample, thus the reliability of this reprogramming technology needs to be improved.

Nevertheless, combination of mRNA and miRNA reprogramming, which is offered in a kit from Stemgent, has been shown to result in a high number of fully reprogrammed iPS cells, using a safe approach.

1.6. Induced pluripotent stem cells in tissue culture

After successful reprogramming of somatic cells to iPS cells, individual colonies are picked for further expansion. It is important to maintain these cells in a pluripotent undifferentiating state using adequate culture conditions. Following expansion, the newly-derived iPS lines must be characterized to ensure their pluripotent stem cells.

The most important factor to be considered before iPSCs can be applied clinically is the production of iPS cells in an efficient, standardized and reproducible manner. This is influenced by many factors, beginning with the type of somatic cell, the cocktail of reprogramming factors and their stoichiometry, the methods used to deliver these factors, culture conditions (like oxygen levels), the choice of medium and the use of reprogramming enhancers like Vitamin C or histone deacetylase inhibitor (Carey *et al.*, 2011, Gonzalez *et al.*, 2011, Maherali *et al.*, 2008, Stadtfeld *et al.*, 2012, Yoshida *et al.*, 2009).

1.6.1. Cultivation and maintenance of iPS cells

The expansion of hiPS cells demand optimal handling and culturing condition, including medium and supplements. (Akutsu *et al.*, 2006, Maherali *et al.*, 2008)

Generally, human embryonic stem cells and iPS cells are cultured on irradiated mouse embryonic fibroblast (MEFs) feeder layer or medium containing fibroblast-derived factors to assure pluripotency and a steady proliferation rate (Richards *et al.*, 2006). However, more defined feeder-free culture conditions (some of them

xeno-free) have been developed in the past few years. (Richards *et al.*, 2006). The main factor maintaining human pluripotent stem cell undifferentiated is bFGF. It is added to the cells daily at a concentration of 10ng/ml.

Induced pluripotent stem cells require new medium and nutrition factors every day. Maintaining iPS cells in an undifferentiated state includes checking the cultures daily for normal medium colour, absence of any visible contamination, colony size and density as well as overall quality of the cells and differentiation. It is not unusual to have some differentiated iPS colonies in the plate, but if the culture contains more than approximately 5% differentiating colonies these cells should be cleaned up by manually scraping away the differentiated area. Differentiated cells can be distinguished from iPS cells by their morphology. Differentiated human iPS cells are usually larger and more spread out cells, while healthy iPS cells are smaller, tightly packed and colonies should have clean and defined edges.. An example for a differentiated and a healthy iPS cell and their difference in morphology can be seen in figure 8.

To maintain healthy cultures of cells, passaging at optimal time is of great importance since merging colonies might increase differentiation rate. Passaging is normally done every four to seven days, depending on the confluency of the cells on a plate.

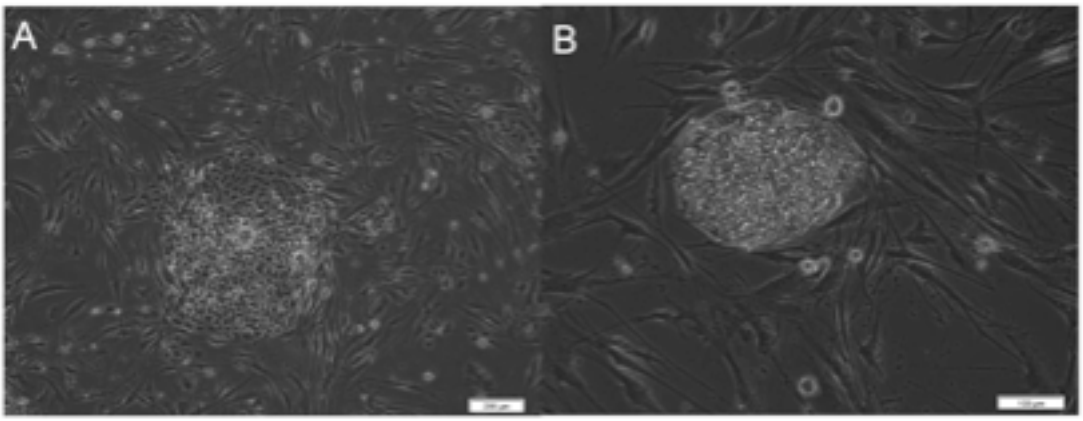

Figure 8: Morphological differences between a differentiated iPS cell and a healthy iPS cell

Microscopic illustration of *(A)* differentiated iPS cell. Cells are larger, loosen, and no defined edges can be seen. Scalebar: 200 µm an*d (B)* Healthy iPS cell. Cells are small, tightly packed, and grow on a monolayer. Clean and defined edges visible. Scalebar: 100 µm.

Both cells show iPS cells derived from T-cells with the use of Sendai virus. Cells are cultured on MEF.

Induced pluripotent stem cells are usually cultured at 37 degrees Celsius with 5% CO2, on feeder conditions to maintain pluripotency and enhance self-renewal (Kent, 2009). In 2009, it was shown that reprogramming efficiency was increased under hypoxic conditions (5% oxygen) (Yoshida *et al.*, 2009).
Although in most laboratories iPS cultivation and maintenance is done in tissue culture, Shafa et al. showed in 2012 the successful and efficient maintenance of induced pluripotent stem cells in stirred suspension bioreactors for four years (Shafa *et al.*, 2012). This might offer a new possibility to develop consistent and more efficient method.

1.7. Challenges and current findings in stem cell research

With the successful reprogramming of somatic cells into pluripotent state, the major issues regarding human embryonic stem cell research, including ethical concerns, could be eliminated. Induced pluripotent stem cells offer an amazing potential for disease modeling, drug discovery and cell therapy. Even though the generation of iPS cells allows development of patient specific cells, thereby eliminating the risk of immune rejection after transplantation, its potential use in industrial and clinical applications comes with its own hurdles, some being shared with ES cells, others being unique. Therefore there is still a need to further understand and optimize reprogramming processes.

One of the biggest challenges for iPSCs clinical application is to produce iPS cells in an efficient, safe, standardized and reproducible manner.
Although the reprogramming methods have definitely improved efficiency and safety aspects, reprogramming processes still only result in about 1% fully reprogrammed iPSCs. Additionally, studies in mice reported that murine iPS cells often come along with epigenetic abnormalities, while still keeping a transient epigenetic memory of their donor cells. Therefore additional study of molecular and functional properties of human iPSCs is critical (Kim *et al.*, 2010, Polo *et al.*, 2010, Stadtfeld *et al.*, 2010b).

Also, some of the reprogramming methods like miRNA enhanced mRNA reprogramming come along with a heavy workload due to daily transfection of the cells, making processing of many samples more difficult.

Even though there is no consensus yet on which reprogramming method is the most suitable one, some new promising research reports might offer new insights and possibilities.

One of these new findings is the generation of human iPS cells with the use of a synthetic self-replicative RNA. This positive single stranded RNA could successfully derive human iPS cells from newborn or adult human fibroblasts by expressing four reprogramming factors: Oct4, Klf4, Sox2, c-MYC or GLIS1. Additionally, to assure retention of the RNA replicon and iPSC generation, the self-replicative RNA included the immune-suppressor B18R. The effect of self-replication after single transfection of the donor cells leads to consistent expression of all reprogramming factor genes at high threshold levels, and would therefore eliminate the repetitive transfection when using miRNA enhanced mRNA reprogramming, saving time and costs. However, side-by-side studies would be necessary to compare efficiency with other reprogramming methods like Sendai virus (Yoshioka *et al.*, 2013).

Also, in 2013 Hou et al. were the first to report the successful reprogramming of mouse somatic cells by small-molecule compounds. The combination of seven small-molecule compounds and chemical induction of the somatic cells leads to the derivation of so-called chemically induced pluripotent stem cells (CiPSCs). This method might offer a very simple tool for somatic cellular reprogramming, but so far derivation of human CiPSCs has not been reported (Hou *et al.*, 2013).

The most promising finding in terms of efficiency was shown in 2013 by Rais *et al*.. Upon depletion of a single factor called Mbd3, reprogramming efficiency increased up to 100%, while efficiency with which somatic cells convert to iPS cells was so far ranging from 0.01-5% (Brumbaugh *et al.*, 2013, Rais *et al.*, 2013). Their findings conclude that in a normal reprogramming process Mbd3 directly interacts with the reprogramming factors Oct4, Klf4, Sox2 and c-Myc, and thereby suppresses their activation. However, it still needs to be tested whether Mbd3

limits reprogramming with the use of alternative transcription factors or chemicals (Rais *et al.*, 2013).

1.8. Potential uses of stem cells

With the generation of human iPS cells, it is not only possible to create patient specific cells, but also to study disease and perform drug screen for many different types of diseases.

The idea of using embryonic stem derived cells for cell-replacement in patients with genetic and has been reported before (Doss *et al.*, 2004). However, since these embryonic stem cells appear “foreign” to the patient’s body, such therapy will be followed with immune rejection fighting against the ESCs. Therefore being able to generate autologous stem cells has been a long-desired goal in the field of regenerative medicine. An overview of possible applications for patient-specific iPS cells is summarized in figure 9.

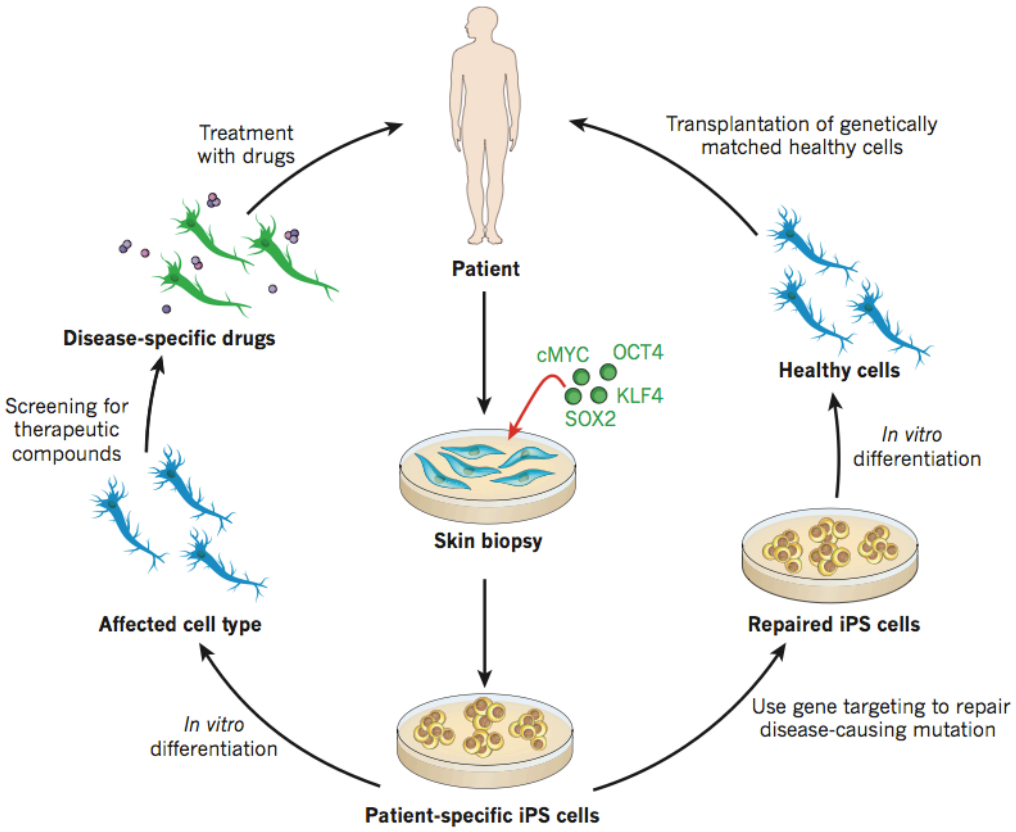

Figure 9: Possible applications of human iPS cells

Patient-specific iPS cells can potentially be used to model and treat human diseases. The derived iPS cells can be used for two different approaches:
If the mutation causing the disease is unknown (left pathway), *in vitro* differentiation of the autologous iPS cells can be done to create the affected cell type. This allows modeling of the patient's disease to understand the molecular mechanism triggering the disease. Additionally, the cells can be screened for potential drugs, which would help in the development of new therapeutic compounds.
If the cause of the mutation is known (right pathway), genome editing could be done to repair the DNA sequence. The healthy iPS cells could then be differentiated *in vitro* to develop into healthy autologous cells, which could potentially be transplanted into the patient. (Robinton *et al.*, 2012)

Patient-specific iPS cells can help in identifying disease-associated cellular phenotypes and studying and understanding the molecular mechanisms underlying these phenotypes. In vitro differentiation of autologous iPS cells to various specialized cell fates is an important tool in disease modeling, and hence also for drug screening and drug discovery. For some diseases it might be possible to determine the effects of candidate drugs and new compounds on disease-specific iPS cells, which will be an immense help when developing new disease therapies. Moreover, performing cardiac, neural and liver toxicity tests will

be useful to analyze cellular toxic responses to drugs, important for the development and validation of therapeutic compounds (Bellin *et al.*, 2012).
Patient-specific induced pluripotent stem cells have been successfully produced from a wide range of diseases. These include Parkinson disease, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophy, Huntingtons disease, Timothy syndrome, long QT syndrome, juvenile onset type I diabetes mellitus, trisomy 21 (Park *et al.*, 2008a), schizophrenia (Robicsek *et al.*, 2013), spinal muscular atrophy, Rett syndrome, amyotrophic lateral sclerosis, familial hypercholesterolemia, LEOPARD syndrome (Onder *et al.*, 2012) and multiple sclerosis (Song *et al.*, 2012). For some of these disease models, relevant phenotypes were observed (Brennand *et al.*, 2011, Israel *et al.*, 2012, Koch *et al.*, 2011, Marchetto *et al.*, 2010, Rashid *et al.*, 2010).
Although the potential usage of human iPS cells for replacement therapy in patients with degenerative disorders sounds promising, there are still several hurdles that need to be overcome. These obstacles highly depend on the reprogramming method used, since the resulting iPS cells clearly vary in safety and efficiency. Therefore further work is required to assure that using cells generated from patient-specific iPS cells is safe for therapy. Additionally, several research groups focused on organ regeneration with the help of stem cells (Kobayashi *et al.*, 2011). However, this will be an even greater challenge, and will require much more time and effort to develop.

Another possible application of patient or disease specific induced pluripotent stem cells is to use gene targeting to repair the disease-causing mutation, and to differentiate the repaired iPS cells *in vitro* into healthy cells for transplantation. This genetic manipulation can be done through homologous recombination either using zinc-finger nucleases (ZFNs) (Zhou *et al.*, 2009a), transcription activator-like effector nucleases (TALENs) (Ding *et al.*, 2013a, Miller *et al.*, 2011) or clustered regularly interspaced short palindromic repeats (CRISPRs) (Ding *et al.*, 2013b). These methods allow genome editing by either integrating targeted mutations into human ES cells to study diseases, or to correct mutations (Soldner *et al.*, 2011). However, genome editing is only applicable if the cause of the mutation is known.

2. Objective

The main idea for using iPS in disease treatment is to create induced pluripotent stem cells directly from patients suffering a certain disease. These autologous iPS cells can then be genetically corrected in vitro and transplanted back into a damaged tissue or organ, to allow differentiation into the cell that is needed at this site. The creation of iPS cells from patients' somatic cells is already being achieved, and the iPS core facility of the Harvard Stem Cell Institute is currently using three different reprogramming methods to do so: Sendai viral reprogramming, episomal reprogramming vector, and microRNA/mRNA technology.

Nevertheless, induced pluripotent stem cell research is still in its infancy and struggles with several technological hurdles. Although the reprogramming methods used at the iPS core facility are non-invasive, they still involve risks. Since they rely on genes and delivery vectors there would be a risk of a patient developing cancer after such therapy. Therefore more efforts in basic research are required and different methods need to be developed before being able to routinely use iPS cells for disease treatment. Until then reprogramming will be done using the three methods mentioned above, as they've been shown to be the most effective non-invasive methods for reprogramming fibroblasts and blood cells so far.

The interest of the research in this thesis will be to reprogram disease-specific cells with different techniques and the subsequent cultivation and characterization of the produced iPS cells in a standardized and reproducible manner. Additionally, a new kit for more efficient reprogramming using the Sendai virus is tested and compared to the current kit on the market.

3. Materials and Methods

All described procedures in this section were conducted aseptically unless otherwise stated.

3.1. Standard Operating Procedures for the culture of human iPS cells in feeder-dependent cell culture system

All experiments were carried out on feeder-dependent cell culture systems using irradiated CF1 mouse embryonic fibroblasts (GlobalStem, 2M: Cat# GSC-6201G, 4M: Cat# GSC-6001G), or MEFs, as feeder cells. After successful reprogramming of somatic cells into iPS cells the correct caring, including passaging and feeding, for hiPS cells is essential to maintain their hESC-like properties.

3.1.1. Media preparation

For successful cultivation, expansion and characterization of human induced pluripotent stem cells, the use of different culture media was necessary.

3.1.1.1. 10% FBS/DMEM Media

10% FBS/DMEM medium, also called fibroblast or MEF medium, was used for culturing MEFs. All the necessary reagents for preparation of MEF medium are listed in table 1 below. All the components were sterile filtered through a filter bottle (VWR, Cat# 28199-778) consisting of a polyethersulfone membrane with a 0.22 µm pore size. If not used medium was stored at 4°C and aliquots were prepared and pre-warmed in a 37°C water bath before each usage.

Table 1: Fibroblast Medium

Table lists all reagents needed for the preparation of ~500 mL fibroblast medium. Also final concentrations of the reagents, product information including supplier and catalog number of each reagent used are stated. DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum; Pen/Strep: Penicillin/Streptomycin.

Components	Volume	Final concentration	Product information
DMEM	450 mL	/	Life Technologies, Cat# 11995-073
FBS	50 mL	10%	VWR, Cat# 97068-100
L-Glutamine	5 mL	/	Life Technologies, Cat# 25030-081
Pen/Strep	5 mL	/	Life Technologies, Cat# 15140-122

3.1.1.2. Standard human embryonic stem cell medium

Human iPSCs have specific requirements for growth conditions in order to maintain their unique qualities. Therefore they are cultured in standard human embryonic stem cell medium containing essential supplements for growth. All the necessary reagents for preparation of hESC medium are listed in table 2. All the components, except for the basic fibroblast growth factor βFGF, were sterile filtered through a filter bottle consisting of a polyethersulfone membrane with 0.22 µm in pore size. If not used medium was stored at 4°C and aliquots were prepared and pre-warmed in a 37°C water bath before each usage. βFGF was always added directly prior use in a concentration of 10 ng/mL.

Table 2: Components for the human embryonic stem cell medium used for culturing cells

Table lists all reagents needed for the preparation of ~500 mL hESC medium. Also final concentrations of the reagents, product information including supplier and catalog number of each reagent used are stated. DMEM/F12: Dulbecco's Modified Eagle Medium, Nutrient mixture F12; KOSR: Knockout Serum Replacement; MEM-NEAA: Minimum Essential Medium-Non-Essential Amino Acids.

Components	Volume	Final concentration	Product information
DMEM/F12	400 mL	/	Life Technologies, Cat# 11330-057
KOSR	100 mL	20%	Life Technologies, Cat# 10828-028
L-Glutamine	5 mL	/	Life Technologies, Cat# 25030-081
Pen/Strep	5 mL	/	Life Technologies, Cat# 15140-122
MEM-NEAA	5 mL	/	Life Technologies, Cat# 11140-050
2-Mercaptoethanol	500 µL	0.1 mM	Life Technologies, Cat# 21985-023
βFGF	500 µL	10 ng/mL	Life Technologies, Cat# PHG0261

3.1.2. Culturing MEF feeder cells

Plates seeded with MEFs always have to be prepared one day prior to use to assure attachment of MEFs to the plate. In this section preparation of 6-well plates containing feeder cells is explained. Amounts of reagents used vary with different types of plates or dishes and their difference in diameters. With 2M irradiated MEFs two 6-well plates can be prepared.

For this the wells were coated with 1 mL 0.1% gelatin (Millipore, Cat# ES-006-B) for 20 minutes at room temperature. Then 9 mL 10% FBS/DMEM medium were pre-warmed in a 37°C water bath. 2 million MEFs were taken out of the liquid nitrogen storage tank and were thawed by immersing the bottom of the cryovial in a 37°C water bath until only small ice crystal remained in the vial. From the pre-

warmed 10% FBS/DMEM 1 mL was taken and added dropwise to the cryovial containing the MEFs. Cells were then transferred dropwise into the conical tube containing the rest of the 10% FBS/DMEM and centrifuged for 5 minutes at 200 x g. In the meantime the gelatin from the pre-coated wells was aspirated and 1 mL pre-warmed 10%FBS/DMEM medium was added into each well. The supernatant was aspirated and the cell pellet was resuspended in 12 mL pre-warmed 10% FBS/DMEM and 1 mL was plated onto each well of the prepared 6-well plates. The plates were gently rocked to assure equal distribution of the MEFs in the wells. The plates were then incubated overnight under standard cell culture conditions (37°C, 5% CO_2).

3.1.3. Passaging of feeder cells

Passaging of cells was necessary in case of high confluence (~80%) of cells or after seven to ten days on the same MEF plates. The splitting was normally done in ratios of 1:1, 1:2 or 1:3, depending on the density of the cells. Before splitting differentiated colonies were removed manually by pipet scraping in a sterile biosafety cabinet equipped with a dissection microscope. Passaging of iPS cells grown on MEFs combines enzymatic methods with mechanical force. Medium was aspirated and cells were washed with Dulbecco's phosphate-buffered saline, DPBS. Then DPBS was aspirated and 1 mL of Collagenase IV (StemCell Technologies, Cat# 07923) per well of a 6-well plate was added and incubated at 37°C for 10 minutes. The enzyme was aspirated and the cells were washed once with DPBS. 1 mL of hESC medium was added per well of a 6-well plate and cells were detached from the plate by scraping the entire well with a cell lifter (VWR, Cat# 29442-200). The solution was transferred into a conical tube, and the well was washed with additional hESC medium to collect all the cells. Everything was collected in one conical tube and centrifuged at 200 x g for 2 minutes. The supernatant was aspirated and the pellet was resuspended in 1 mL hES medium with βFGF per well of a 6-well plate intended to plate, depending on the splitting ratio chosen. Pellet was pipetted up and down to get small cell fragments. However, too intensive pipetting was avoided in order to prevent cell death. New plates seeded with MEFs (see section 3.1.2.) were washed with DPBS and 1 mL

of hESC medium with βFGF was added to it. From the resuspended cell-pellet 1 mL was plated into each well of a 6 well plate containing MEFs. The cells were then cultured under standard cell culture conditions.

3.1.4. Freezing of cells on feeder plates

Cells were regularly frozen to establish a backup of the created iPSCs. Before freezing the first stock of cells they was tested with the MycoAlert® Assay Control Set (Lonza, Cat# LT07-518) to assess the negativity of mycoplasma contamination. This was conducted with 24-hour old medium from the cell culture according to manufacturer's protocol.

For freezing, the cells were prepared, collected and pelleted in a conical tube in the same manner as described in section 3.1.3. "Passaging of feeder cells". The medium from the pellet was aspirated and 500 µL of hESC medium without βFGF was added for every vial intended to freeze. Then 500 µL of 2X freezing medium, consisting of 8 mL FBS and 2 mL dimethyl sulfoxid or DMSO (Sigma, Cat# D-2650), for each vial intended to freeze was added and the pellet was gently resuspended by pipetting up and down two times. 1 mL of the solution was transferred into one cryovials (VWR, Cat# 82050-180) and placed inside a Mr. Frosty (VWR, Cat# 55710-200), an isopropanol-containing freezing container. The freezing container was then stored at -80°C for 24 to 48 hours and afterwards the vials were transferred into a liquid nitrogen storage tank.

3.2. Sendai virus mediated reprogramming of T-cells

This section describes the reprogramming of T-cells with the use of the CytoTune™-iPS Reprogramming Kit (Life Technologies, Cat# A13780-01) to efficiently derive integration-free iPS cells. Figure 10 below should give a rough outline for the CytoTune™-iPS reprogramming experiment according to the protocol established at the Harvard Stem Cell Institute, iPS Core Facility, Cambridge, MA.

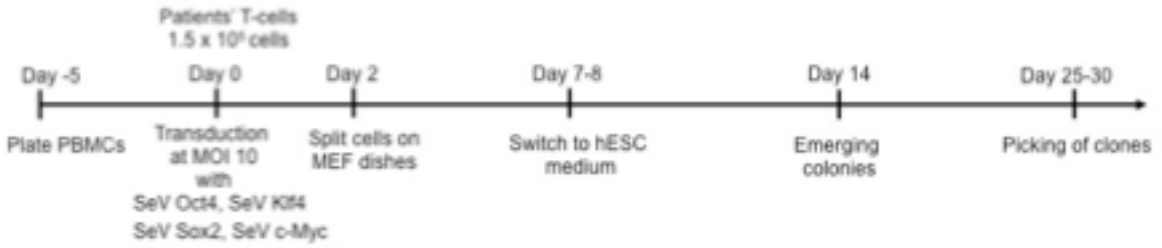

Figure 10: Experimental timeline for the Sendai virus mediated reprogramming of Peripheral Blood Mononuclear Cells (PBMCs)

This timeline shows the experimental layout of the reprogramming of T-cells using the CytoTune™-iPS Reprogramming Kit. The timeline is based on the manufacturer's protocol and some adaptions made by the iPS Core Facility, Cambridge, MA. SeV: Sendai Virus

3.2.1. Material preparation

3.2.1.1. Pre-coating of wells for plating isolated PBMCs

Per sample to reprogram 1 well of a 12-well plate (VWR, Cat# 82050-930) was pre-coated with 10 µg/mL anti-human CD3, α-hCD3, (eBioscience, Cat# 16-0037-81) in DPBS (Life Technologies, Cat# 14190-250), for one hour at 37°C. Then the plate was blocked with 2% bovine serum albumin, BSA, (Sigma Aldrich, Cat# A7159-50mL) in PBS for 30 minutes at 37°C. Before plating of isolated PBMCs, the well was rinsed twice with DPBS.

3.2.1.2. X-Vivo Complete Media

X-Vivo Complete Media for T-cell expansion has to be prepared fresh for each experiment. For preparation of 20 mL of X-Vivo Complete Media the components listed in table 3 were mixed together. If not used medium was stored at 4°C and aliquots were prepared and pre-warmed in a 37°C water bath before each usage.

Table 3: Components of the X-Vivo Complete Media used for T-cell expansion and transduction

The table includes all the reagents needed for the expansion of T-cells from PBMCs and for the transduction with the Sendai virus. The volumes stated are used to make 20 mL of X-Vivo Complete Media. Also, product information including supplier and catalog number of each reagent used are listed.

Components	Volume	Product information
X-Vivo 10 with Gentamicin & Phenol Red	18.8 mL	Lonza, Cat# 04-380Q
Human Serum, Type AB, Heat Inactivated	1 mL	Valley Biomedical, Cat# HP1022HI
Penicillin/Streptomycin	200 µL	Life Technologies, Cat# 15140-122
Anti-Human CD3	8 µL	eBioscience, Cat# 16-0037-81
Anti-Human CD28	8 µL	eBioscience, Cat# 16-0289-85
Interleukin-2	20 µL	BD, Cat# 356043

3.2.2. Isolation of PBMCs from fresh blood samples

Fresh blood samples from patients suffering from Multiple Sclerosis were used as a starting material for the generation of iPSCs. Four milliliters of whole blood were transferred into a BD Vacutainer® Cell Preparation Tube (CPT) with Sodium Citrate (BD, Cat# 362760). Samples were centrifuged for 30 minutes at 1650 x g for the separation of mononuclear cells from whole blood. After centrifugation red blood cells should be at the bottom, and above the polyester gel should be a white layer of PBMCs and plasma. The PBMCs were resuspended into the plasma by inversion of the CPT. The contents above the polyester gel were transferred into a 15 mL conical tube containing 10 mL DPBS. The mixture was centrifuged for 15 minutes at 300 x g. Supernatant was aspirated, leaving a pellet. The pellet was resuspended in 10 mL of DPBS, and a cell count was done to evaluate the number of cells. The resuspended PBMCs were centrifuged for 10 minutes at 200 x g, and supernatant was aspirated without disturbing the cell pellet. $1*10^7$ cells

were plated in the pre-coated well of a 12-well plate in 1 mL X-Vivo complete media.

3.2.3. Expansion of T-cells from PBMCs

Cells were cultured for approximately 5 days, to allow activated T-cells to reach 80-90% confluence. Activated T-cells do not attach to the well surface, therefore when media turned yellow, cell clumps were dissociated by pipetting and 1 mL X-Vivo complete media was added. After 1-2 days, media turned yellow again, and cell clumps were dissociated and then spun down for 5 minutes at 200 x g. Cells were then split 1:2 using the original well and a new α-hCD3 coated well in 1 mL X-Vivo complete media per well.

3.2.4. Transduction of T-cells with the sendai virus

At day 0, the cell clumps were dissociated again and spun down for 5 minutes at 200 x g. Cells were counted with Trypan blue (Cellgro, Cat# 25-900-CL) and $1.5*10^5$ live activated T-cells were plated in 1 well of a 12 well plate in X-Vivo complete media. These cells were transduced using Sendai viruses provided by the CytoTune™-iPS Reprogamming Kit. The reprogamming kit contains 4 Sendai virus-based reprogramming vectors: CytoTune™ Sendai hOct3/4, CytoTune™ Sendai hSox2, CytoTune™ Sendai hKlf4 and CytoTune™ Sendai hc-Myc. To ensure transduction of high number of cells, a multiplicity of infection (MOI) of 10 and a total volume (Sendai virus and cell suspension) of 700 µL were used.

Table 4: Given titers for each virus of the CytoTune™-iPS Sendai Reprogramming Kit

These titers were provided by Life Technologies and they are associated to the viruses in the CytoTune™-iPS Sendai Reprogramming Kit with the Lot number 1234222A. Titers were needed to calculate the volume of virus suspension needed for transduction. CIU: cell infectious unit.

Component	Titer (CIU/mL)
CytoTune™ Sendai hOct3/4	$4.5*10^7$
CytoTune™ Sendai hSox2	$7.6*10^7$
CytoTune™ Sendai hKlf4	$6.4*10^7$
CytoTune™ Sendai hc-Myc	$8.6*10^7$

The volume of virus suspension needed for transduction was calculated with the following formula:

$$Volume\ of\ virus\ suspension\ (\mu L) = \frac{MOI\ \left(\frac{CIU}{cell}\right) * number\ of\ cells}{titer\ of\ virus\ \left(\frac{CIU}{mL}\right) *\ 10^{-3}\ (\frac{\mu L}{mL})}$$

For each well transduced, a 15 mL conical tube containing the correct amount of pre-warmed X-Vivo Complete media was prepared (700µL – volume of virus needed). Then one set of CytoTune™ Sendai tubes was removed from -80°C storage, and each tube at a time was thawed for 10 seconds in a 37°C water bath. The tubes were then placed at room temperature to allow complete thawing. Each tube was briefly centrifuged and stored on ice. The calculated amount of each of the four Sendai viruses was added to the conical tube containing the X-Vivo Complete media. To ensure that the solution is well mixed, mixture was mixed by gently pipetting up and down. The X-Vivo Complete media on the prepared T-cells was aspirated and 700 µL of the Sendai virus-medium mixture were added to it. The cells were then incubated under standard cell culture conditions overnight. All tools in contact with the viral solution were treated with bleach (VWR, Cat# 37001-060) and discarded.

On the following day (D1), the cells were spun down for 5 minutes at 200 x g to remove the Sendai virus. The cells were then gently re-plated in the same well of the 12-well plate in 1 mL of 10% FBS/DMEM medium. Cells were cultured an additional 24 hours under standard cell culture conditions. For each sample transduced one 10-cm petri dish (VWR, Cat# 82050-916) pre-coated with 0.1% gelatin was pre-seeded with $2*10^6$ MEFs (see section 3.1.2).

Two days after transduction (D2), T-cells were harvested and plated on the prepared MEF culture dishes. Therefore the cells were collected in a 15 mL conical tube and spun down for 5 minutes at 200 x g. In the meanwhile the medium from the cultured MEFs was removed, and the feeder-cells were washed with 7 mL DPBS. The supernatant was aspirated and the T-cell pellet was gently

resuspended in 10 mL 10% FBS/DMEM medium without dissociating cell clumps. The DPBS on the seeded MEF 10-cm dish was removed and the resuspended T-cells were re-plated on the 10 cm dish with the MEFs in 10% FBS/DMEM medium.

On day five after transduction (D5) the 10% FBS/DMEM medium was changed to recover suspended cells. For this cells were spun down again and then re-plated in fresh medium.

Seven days post-transduction the medium was changed to standard human embryonic stem cell media with basic fibroblast growth factor at a concentration of 10 ng/mL. Media was changed for seven days every other day, then every day until colonies appeared that were ready to be picked. 10-cm dishes were observed every day under a microscope to check for the emergence of cell clumps indicative of transformed cells. Media change was done with the spin-down methods until live cells were no longer visible in the supernatant.

3.2.5. Picking and cultivation of newly derived iPSC colonies

Usually three to four weeks after cells were transduced, large and compact colonies should have formed. After about ten days post-transduction the first morphological changes of the cells were observed, and picking was first done on day 18. One day prior to picking, MEF were seeded on gelatin-coated 6-well plates and cultured over night at normal culture conditions. The colonies to be picked were examined under a microscope and marked on the bottom of the culture dish. Picking was done in a sterile biosafety cabinet equipped with a dissection microscope. Colonies were manually picked by the use of glass picking tools. These picking tools were made by pulling Pasteur pipettes (VWR, Cat# 14673-043) to a closed, angled end over the flame of a Bunsen burner. The colony to be picked was detached from the surrounding fibroblasts by circling the area to be picked with the prepared picking tool. The detached colony was then cut into small pieces in a grid-like pattern and with the use of a pipettor with a sterile 200 µL filter tip passaged onto the prepared and with DPBS pre-washed 6-well MEF feeder plate. The cells were cultivated in hESC medium with βFGF and 10 µM Rho-

associated protein kinase (ROCK) inhibitor Y27632 (EMD Chemicals Inc, Cat# 688001-500UG). ROCK inhibitor was only added to the media on the day of picking to increase cell viability and attachment. Cells were incubated under normal conditions. Cells were checked on the following day as some attached cells might already be visible at this time point. However, to allow full attachment of the colonies to the culture plate, cells were not fed on the day after picking. Medium change was done two days after picking with fresh human ESC medium containing βFGF. After that medium was changed every day.

3.3. Reprogramming of MSCs using the Sendai virus

This section describes the reprogramming of mesenchymal stem cells (MSCs) with the use of the CytoTune™-iPS Reprogramming Kit (Life Technologies, Cat# A13780-01) as well as the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Life Technologies, Cat# A16517) to efficiently derive integration-free iPS cells. The newly released CytoTune™-iPS 2.0 Sendai Reprogramming Kit should increase reprogramming efficiency, lower cytotoxicity and eliminate the Sendai virus faster. This was tested for the first time at the iPS Core facility with this experiment on the basis of two samples. Figure 11 below should give a rough outline for the CytoTune™-iPS reprogramming experiment according to the protocol established at the Harvard Stem Cell Institute, iPS Core Facility, Cambridge, MA.

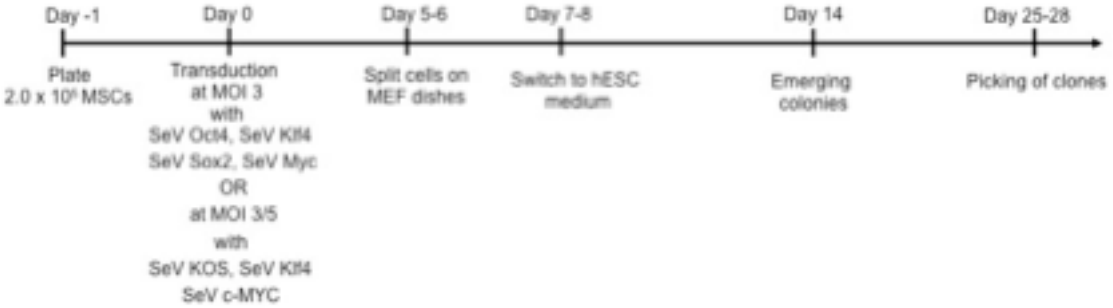

Figure 11: Experimental timeline for the Sendai virus mediated reprogramming of MSCs

This timeline shows the experimental layout of the reprogramming of MSCs using both, the CytoTune™-iPS Reprogramming Kit and the CytoTune™-iPS 2.0 Sendai Reprogramming Kit. For the CytoTune™-iPS Reprogramming Kit the four Yamanaka factors are expressed by the introduced Sendai viruses at an MOI of 3. For the CytoTune™-iPS 2.0 Sendai Reprogramming Kit only three Sendai virus-based reprogramming vectors are used: SeV KOS and SeV c-Myc both with an MOI of 5, and SeV Klf4 with an MOI of 3.

3.3.1. Media preparation: Mesenchymal Stem Cell Growth Medium

Human mesenchymal stem cells (hMSCs) require a specific medium, the mesenchymal stem cell growth medium, containing growth supplement promoting the growth and cell viability of MSCs. All the necessary reagents for preparation of MSC growth medium are listed in table 5. If not used medium was stored at 4°C and aliquots were prepared and pre-warmed in a 37°C water bath before each usage. Medium can be used up to four weeks after production. All components necessary for production of the medium can be either purchased single or in the MSCGM Bullet Kit (Lonza, Cat# PT-3001).

Table 5: Components of the MSCGM™ Mesenchymal Stem Cell Growth Medium used for MSC expansion and transduction

The table lists all the reagents needed for the expansion of MSCs and for the transduction with the Sendai virus. The volumes stated are used to make 500 mL of MSC growth medium. Also, product information including supplier and catalog number of each reagent used are listed. GA-1000: Aqueous solution of Gentamicin Sulfate and Amphotericin-B, used as a bacterial growth inhibitor;

Components	Volume	Product information
MSCBM: hMSC Basal Medium	440 mL	Lonza, Cat# PT-3238
L-glutamine	10 mL	Lonza, Cat# PT-4105
GA-1000	500 µL	Lonza, Cat# PT-4105
Growth supplement	50 mL	Lonza, Cat# PT-4105

3.3.2. Thawing and culturing of MSCs

Two frozen MSC samples were received from the investigator in cryovials on dry ice. Until usage, the cells were stored in the -80°C liquid nitrogen storage tank. The cells received had a cell number of 450000 cells per vial, and therefore one vial was plated in a T75 flask (VWR, Cat# 82050-856). For this one 15 mL conical tube containing 9 mL pre-warmed MSC medium was prepared. The cryovial containing MSCs was taken out of the liquid nitrogen tank and was thawed by immersing the bottom of the tube in a 37°C water bath until only small ice crystal remained in the vial. From the pre-warmed MSC growth medium 1 mL was taken and added dropwise to the cryovial containing the MEFs. Cells were then transferred dropwise into the conical tube containing the rest of the medium and

centrifuged for 5 minutes at 200 x g. In the meantime 10 mL of pre-warmed MSC growth medium was added to the T75 flask. The supernatant was aspirated and the cell pellet was resuspended in 5 mL pre-warmed MSC growth medium and the solution was added to the T75 flask The flask was gently rocked to assure equal distribution of the MSCs and the flask was then incubated overnight under standard cell culture conditions (37°C, 5% CO_2).

One day before transduction (D-1), cells were collected in a conical tube via trypsinization (Invitrogen, Cat# 25300-062) for 3-4 minutes at 37°C. The cells were spun down for 5 minutes at 200 x g and the pellet was resuspended in pre-warmed MSC medium. Cells were counted with trypan blue and 2 x 10^5 cells were seeded in a well of a 6-well plate coated with 0.1% gelatin. Cells were cultured overnight in MSC growth medium.

3.3.3. Transduction of cells with the CytoTune-iPS reprogramming kit

At the day of transduction (D0), cells were checked again to confirm good quality in terms of morphology. The MSCs were transduced using Sendai viruses provided by the CytoTune™-iPS Reprogamming Kit. The reprogramming kit contains 4 Sendai virus-based reprogramming vectors: CytoTune™ Sendai hOct3/4, CytoTune™ Sendai hSox2, CytoTune™ Sendai hKlf4 and CytoTune™ Sendai hc-Myc. To ensure transduction of high number of cells, a multiplicity of infection of 3 and a total volume (Sendai virus and cell suspension) of 1 mL were used.

Transduction of the cells was performed as described in section 3.2.4, with the only difference that cells were cultured in MSC growth medium.

On the following day (D1), the cells were refreshed by removing the MSC growth medium and adding new 2 mL MSC growth medium per well. Cells were cultured an additional 24 hours under standard cell culture conditions. This was repeated on day 3 and day 4 after transduction.

For each sample transduced one 10-cm petri dish pre-coated with 0.1% gelatin was pre-seeded with $1*10^6$ MEFs (see section 3.1.2).
Five days after transduction (D5), MSCs were harvested and plated on the prepared MEF culture dishes. Therefore the cells were detached from the well by adding 1 mL trypsin and by incubating the plate for 4 min at 37°C. Detachment of the cells was checked under the microscope. To neutralize the action of trypsin and stop detachment 1 mL of MSC growth medium was added to the wells. The entire volume of neutralized trypsin was transferred into a 15 mL conical tube. The well was then rinsed with an additional 1-2 mL MSC growth medium and combined in the 15 mL conical tubes with the cells. Solution was spun down for 5 minutes at 200 x g. In the meanwhile the medium form the cultured MEFs was removed, and the feeder-cells were washed with 7 mL D-PBS. The D-PBS was removed and 5 mL of pre-warmed MSC growth medium were added on the 10-cm petri dish. The supernatant was aspirated and the pellet with the transduced MSCs was gently resuspended in an additional 5 mL MSC growth medium. The cells were counted with Trypan blue and 250000 resuspended MSCs were re-plated on the 10 cm dish with the MEFs. Cells were cultured under standard cell culture conditions overnight.

On the next day the medium was changed to standard human embryonic stem cell media with basic fibroblast growth factor. Media was changed for seven days every other day, then every day until colonies appeared. 10-cm dishes were observed every day under a microscope to check for the emergence of cell clumps indicative of transformed cells.

Picking of the newly-derived iPSC colonies was performed as described in section 3.2.5. Cultivation of the picked iPSC colonies was done as needed for maintaining the quality and properties of the cells.

3.3.4. Transduction of cells with the CytoTune™-iPS 2.0 Sendai Reprogramming Kit

At the day of transduction (D0), cells were checked again to confirm good quality in terms of morphology. The MSCs were transduced using Sendai viruses provided by the CytoTune™-iPS 2.0 Sendai Reprogamming Kit. The reprogramming kit contains 3 Sendai virus-based reprogramming vectors: CytoTune™ Sendai hKOS, CytoTune™ Sendai hc-Myc and CytoTune™ Sendai hKlf4. To ensure transduction of high number of cells, a multiplicity of infection of 3 and 5 respectively and a total volume (Sendai virus and cell suspension) of 1 mL were used.

Table 6: Given titers and MOI for each virus of the CytoTune™-iPS 2.0 Sendai Reprogramming Kit

These titers were provided by Life Technologies and they are associated to the viruses in the CytoTune™-iPS 2.0 Sendai Reprogramming Kit with different lot numbers stated below. Also, multiplicity of infection is listed. Titers and MOI were needed to calculate the volume of virus suspension needed for transduction. CIU: cell infectious unit.

Virus	Titer (CIU/mL)	Lot number	Multiplicity of infection
CytoTune™ Sendai hKOS	$1.0*10^8$	TS12(PM-KOS)013-01d	5
CytoTune™ Sendai hc-Myc	$1.0*10^8$	TS15(HNL-cMYC)013-04	5
CytoTune™ Sendai hKlf4	$1.2*10^8$	TS(Hs-KLF4)013-01	3

To calculate the volume of virus suspension needed for the transduction, the formula mentioned in section 3.2.4 was used.

Transduction was performed the same manner as described in section 3.2.4, using the Sendai viruses listed in table 6. Cells were then cultured in MSC medium over night.

After transduction, cells were handled in the exact same manner as described in section 3.3.3.

3.4. Reprogramming of a somatic cell by mRNA/miRNA transfection

This section describes the reprogramming of fibroblasts from three patients suffering from multiple system atrophy, as well as the reprogramming of fibroblasts from two patients with an unknown disease using the miRNA-enhanced mRNA reprogramming method developed from Stemgent to efficiently derive integration-free iPS cells. Figure 12 gives a rough outline for the experiment according to the protocol provided by Stemgent.

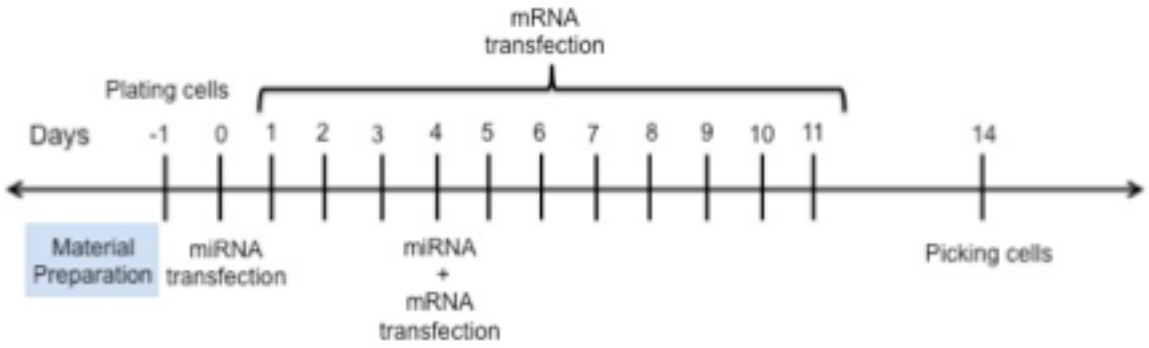

Figure 12: Experimental timeline for the miRNA-enhanced mRNA reprogramming

This provides an overview of the experimental layout for reprogramming fibroblasts according to the microRNA-enhanced mRNA protocol from Stemgent.

The following protocol describes the material preparation for the reprogramming of 5 fibroblast samples plated in 5 wells of a 6-well plate.

3.4.1. Material preparation

The reprogramming of fibroblasts with miRNA-enhanced mRNA transfection required quite some preparation. For the successful conduction of the experiment three different kits were required, listed in table 7.

Table 7: Required materials for successful generation of iPSCs from fibroblasts through miRNA-enhanced mRNA reprogramming

The table lists all the reagents needed for the transfection of fibroblasts with miRNA and mRNA. Product information of each kit including supplier and catalog number used are listed.

Product description	Concentration	Product information
Stemgent mRNA Reprogramming Kit		Stemgent, Cat# 00-0071
mRNA Reprogramming Factors Set	/	
B18R Recombinant Protein	0.5 mg/mL	
Pluriton™ Supplement	2500X	
Pluriton™ Medium	/	
Stemgent microRNA Booster Kit		Stemgent, Cat# 00-0073
microRNA Reprogramming Cocktail	/	
B18R Recombinant Protein	0.5 mg/mL	
Stemgent Stemfect RNA™ Transfection Kit		Stemgent, Cat# 00-0069
Stemfect™ RNA Transfection Reagent	/	
Stemfect™ Buffer	/	

3.4.1.1. Preparation of NuFF conditioned Pluriton™ medium

The use of conditioned medium is extremely important to the overall health of cells undergoing reprogramming. Stemgent Reprogramming-Qualified Newborn Human Foreskin Fibroblast (NuFF) cells (Stemgent, Cat# GSC-3006G) contribute immensely to the successful growth and proliferation of the derived iPSCs. For the miRNA-enhanced mRNA reprogramming NuFF cells were used as feeder cells to produce conditioned Pluriton™ medium.

As a starter the 500 mL bottle of Pluriton™ medium was thawed for two days at 4°C. Then 5 mL of penicillin/streptomycin were added to it and it was stored at 4°C. For the generation of NuFF-conditioned Pluriton Medium, 4 million irradiated NuFF cells were thawed as described in section 3.3.2, and plated in 35 mL FBS/DMEM onto a T150 flask (VWR, Cat# 29186-106). The cells were incubated over night under standard cell culture conditions to allow attachment. On the following day the 10% FBS/DMEM medium was aspirated and the cells were washed with 10 mL DPBS. DPBS was aspirated and then 35 mL Pluriton Medium containing βFGF to give a final concentration of 4 ng/mL were added to the NuFF cells. To generate NuFF conditioned Pluriton medium, the medium was incubated for 24 hours to become "conditioned." Therefore after a 24-hour period of incubation the NuFF conditioned PluritonTM medium was collected in a 50 mL conical tube (VWR, Cat# 21008-940) and stored at -20°C. 35 mL of fresh pre-warmed Pluriton medium with βFGF was added to the T150 flask and incubated for another 24 hours. This process was repeated until the whole Pluriton™ medium was conditioned. Afterwards the NuFF cells were discarded. The NuFF-conditioned Pluriton™ medium was then filtered through a filter bottle with a 0.22 µM pore size. Aliquots of 25 mL were created and re-frozen at -20°C until use. Before usage the medium was thawed at 4°C.

3.4.1.2. Preparation of reagents for miRNA enhanced mRNA reprogramming system

As in this experiment the transfection of five different samples was conducted, reagents needed for the reprogramming were always prepared as for six samples.

3.4.1.2.1. Pluriton™ Supplement

200 µL of the Pluriton™ Supplement were delivered in one vial. As it was needed everyday for transfection, 40 single-use aliquots containing 5 µL each were prepared in 1.5 mL microcentrifuge tubes (VWR, Cat# 89000-028). For every day of transfection one of these aliquots was thawed on ice and 4.8 µL were added to 12 mL of NuFF-conditioned Pluriton medium. Aliquots were stored at -80°C until use.

3.4.1.2.2. B18R Recombinant Protein

For the B18R Recombinant Protein, included in the Stemgent mRNA Reprogramming Kit and the Stemgent microRNA Booster Kit, 21 single-use aliquots containing 7.5 µL each were prepared. One vial was thawed on ice for everyday transfection was done and 7.2 µL B18R protein were added to 12 mL of NuFF-conditioned Pluriton^{TM} medium. Aliquots were stored at -80°C until use.

3.4.1.2.3. mRNA Reprogramming Cocktail

The mRNA Reprogramming Cocktail was produced from the following mRNA factors included in the mRNA reprogramming factors set: Oct4, Sox2, Klf4, c-Myc, Lin28 and nGFP with a molar stoichiometry of 3:1:1:1:1:1 respectively. Therefore the factors were added together and 20 single-aliquots with 50 µL mRNA reprogramming cocktail each were prepared. Table 8 below lists the volumes of reprogramming factors mixed to produce the mRNA reprogramming cocktail. Aliquots were stored at -80°C until use.

Table 8: Components and volumes necessary for preparation of mRNA reprogramming cocktail

mRNA transfection factor	volume [µL]
Oct4 mRNA	400
Sox2 mRNA	123.8
Klf4 mRNA	162
c-Myc mRNA	153.5
Lin28 mRNA	85.7
nGFP mRNA	115.0
mRNA reprogramming cocktail	1040

3.4.1.2.4. microRNA Cocktail

The miRNA Cocktail was delivered already prepared containing mature microRNAs in RNase-free water, and was included in the Stemgent microRNA Booster Kit. Therefore the miRNA cocktail was thawed on ice and four single-use

aliquots containing 17.5 µL each were prepared. The aliquots were stored at -80°C until use.

3.4.1.3. Coating of plates with matrigel (feeder-free system)

One cryovial containing 250 µL of matrigel (BD Bioscience, Cat# 354277) was thawed on ice for one to two hours. The matrigel was then pipetted into 25 mL of cold DMEM F12. The mixture was used to coat 25 wells of a 6-well plate, meaning 1 mL was added per one well of a 6-well plate. The solution was equally spread and the plates were left at room temperature for one hour. If not used immediately, plates were wrapped in parafilm (VWR, Cat# 52858-000) and stored at 4°C in the fridge.

3.4.2. Plating target cells

One hour before plating the cells, the matrigel-coated 6-well plates were placed at room temperature to allow it to equilibrate. The target cells were plated into matrigel-coated 6-well plates a day before the first transfection (D-1). For this the matrigel was aspirated and $5*10^4$ cells per well were plated in NuFF-conditioned Pluriton™ medium. The cells were then incubated at low oxygen conditions (5% O_2) at 37°C overnight. As reprogramming was also done under low oxygen conditions NuFF-conditioned Pluriton™ medium was equilibrated overnight. Therefore 12.5 mL of NuFF-conditioned Pluriton™ medium was added into a 10 cm petri dish and placed in the incubator under low oxygen conditions overnight. Fresh 12.5 mL medium were added everyday after transfection to a petri dish to equilibrate the medium needed for the following day.

3.4.3. Transfection of fibroblasts with miRNA

30 minutes before each transfection one single-use aliquot of the B18R recombinant protein and the Pluriton™ supplement were placed on ice to allow thawing. Also, the Stemfect™ Transfection Reagent and the Stemfect Buffer, included in the Stemgent Stemfect RNA™ Transfection Kit, were placed on room temperature for 30 minutes to equilibrate. Before each transfection medium of the cells was changed. Therefore 12 mL of the equilibrated NuFF-conditioned Pluriton™ medium were transferred into a 15 mL conical tube. 4.8 µL or the

Pluriton™ supplement and 7.2 µL B18R were added to it. Then the medium from the target cells was aspirated and 2 mL of the NuFF-conditioned Pluriton™ medium with B18R and supplement were added to each well of a 6 well plate.

For the miRNA transfection (D0), the 17.5 µL single-aliquot containing the miRNA Cocktail was thawed on ice. To this 107.5 µL of equilibrated Stemfect Buffer were added and gently mixed via pipetting.
In a second sterile 1.5 mL microcentrifuge tube, henceforth referred to as tube 2, 105 µL of Stemfect Buffer and 20 µL of Stemfect RNA Transfection Reagent were added. The solution was pipetted gently to thoroughly mix the reagents. Then the contents of tube 2, 125 µL, were transferred into the microcentrifuge tube containing the miRNA Cocktail and the Stemfect Buffer. The solution was gently pipetted 3 to 5 times to mix. The resulting microRNA transfection complex was then incubated at room temperature for 15 minutes to allow the microRNA to properly complex with the transfection reagent. Then the plate containing the samples to be transfected was brought into a 45 degree angle position, and 50 µL of the miRNA transfection complex was added dropwise to the medium of each sample. Afterwards the plates were gently rocked from side to side and front to back to assure distribution of the transfection complex. The cells were then incubated at 37°C and 5 % oxygen level for 24 hours. Cells were transfected at the same time each day.

3.4.4. Transfection of fibroblasts with mRNA

Transfection of the cells with the mRNA transfection complex was done on days 1 to 3 and days 5 to 11. Before each transfection, reagents were placed either on ice or at room temperature to thaw or equilibrate respectively and medium was changed as described in section 3.4.3
For the mRNA transfection, the 50 µL mRNA Cocktail was thawed on ice. To this 75 µL of equilibrated Stemfect Buffer was added and gently mixed via pipetting.
In a second sterile 1.5 mL microcentrifuge tube, henceforth referred to as tube 2, 105 µL of Stemfect Buffer and 20 µL of Stemfect RNA Transfection Reagent were added. The solution was pipetted gently to thoroughly mix the reagents. Then the

contents of tube 2, 125 µL, were transferred into the microcentrifuge tube containing the mRNA Cocktail and the Stemfect Buffer. The solution was gently pipetted 3 to 5 times to mix. The resulting mRNA transfection complex was then incubated at room temperature for 15 minutes. Then the plate containing the samples to be transfected was brought into a 45 degree angle position, and 50 µL of the mRNA transfection complex was added dropwise to the medium of each sample. Afterwards the plates were gently rocked from side to side and front to back to assure distribution of the transfection complex. The cells were then incubated at 37°C and 5 % oxygen level for 24 hours. Cells were transfected at the same time each day.

3.4.5. Transfection of fibroblasts with miRNA and mRNA

On day 4 after plating the cells, co-transfection was done with both, miRNA and mRNA. For this miRNA transfection complex and mRNA transfection complex were prepared and added to the samples as described in step 3.4.3 and 3.4.4. Plates were then incubated for 24 hours at 37°C and 5 % oxygen level.

3.4.6. Picking and passaging iPSC colonies

Picking was performed approximately two weeks after the first transfection in the same way as described in section 3.2.5. Cells were picked on a 6-well plate pre-seeded with MEF and cultivation of the picked iPSC colonies was done as needed for maintaining the quality and properties of the cells.

3.5. Characterization of reprogrammed iPS cell lines

Characterization of the derived iPS cells was necessary to confirm the human embryonic-like properties of the cells. All the listed characterization processes were used in at least one of the three performed projects presented in this thesis.

3.5.1. Alkaline Phosphatase staining

The cells were tested for alkaline phosphatase (AP) expression with the Alkaline Phosphatase Detection Kit (Millipore, Cat. No. SCR004). Around five to seven days before AP testing each cell line was plated on one well of a 12-well plate (VWR, Cat# 82050-930) pre-coated with MEFs. As soon as the cells reached a

medium cell density and nice colonies were visible, the cells were processes at non-sterile conditions and medium was aspirated. The cells were then fixed in 4% Paraformaldehyde, PFA, for 1 – 1 1/2 minutes maximum, then one well was rinsed with 500 µL of 1X Rinse buffer, consisting of 50 mL DPBS and 25 µL Tween 20 (Sigma Aldrich, Cat# P1379-100mL). A mix of Fast Red Violet with Naphtol AS-BI phosphate solution (included in the AP Detection Kit) and Ultra-Pure™ DNase-RNase-Free Distilled Water (Life Technologies, Cat# 10977) was prepared in a 2:1:1 ratio. 500 µL of the mixture was added to each well and the plate was incubated in the dark at room temperature for 15 minutes. The staining solution was then aspirated and the wells rinsed with 500 µL 1 X Rinse Buffer. Cells were covered with DPBS to prevent drying out. All the samples were examined under a microscope, where iPSC colonies appeared pink, while differentiated colonies appeared colorless. Pictures were taken and stored to document the AP expression.

Alkaline Phosphatase images were captured with an Olympus CK40 Culture microscope equipped with an Optronics MicroFIRE Digital Microscope Camera and analyzed with Picture Frame™ 2.3 Software.

3.5.2. Immunocytochemistry Assay for Pluripotency Markers

Around five to seven days before immunocytochemistry (ICC) each cell line to be analyzed was plated on six wells of a 48-well plate (VWR, Cat# 82051-004) pre-coated matrigel and seeded with MEFs. As soon as the cells reached a medium cell density and nice colonies were visible, the cells were moved to non-sterile conditions and immunocytochemistry assay was started. For this medium was aspirated and the cells were washed 3 times with DPBS (500 µL/well). The cells were then fixed in 4% PFA for 20 min at room temperature, and washed three times with DPBS/0.05% Tween 20. A permeabilization was performed by adding 500 µL DPBS/ 0.1% Triton X-100 (Sigma Aldrich, Cat# X100-100 mL) to each well and incubating it for 15 min at room temperature. The cells were washed three times with DPBS/0.05% Tween 20. To block non-specific binding sites, 500 µL of 4% Donkey Serum (Jackson Immuno Research Laboratories, Cat# 017-000-121)

in DPBS was added to each well and the plate was incubated at 4°C overnight. On the next day, each well was washed three times with DPBS/0.05% Tween 20. Then 100 µL of each primary antibody diluted in 4% Donkey Serum/DPBS was added to each well of a cell line. The types of primary and secondary antibodies used with respective dilutions are stated in table 9. The plate was then incubated for one hour at room temperature. Afterwards, cells were washed three times with 500 µL DPBS/0.05% Tween 20 per well to remove unbound primary antibodies. 100 µL of each secondary antibody diluted in DPBS was added to the corresponding wells. The plate was incubated for another hour at room temperature in the dark to avoid. photobleaching. After that cells were washed three times with 500 µL DPBS/ 0.05% Tween 20 and 100 µL of DAPI (Life Technologies, CAT# D3571) solution (1µl of DAPI in 10 ml DPBS) was added to each well to stain the nuclei. The staining solution was left on the well and gene expression of iPSCs was analyzed under a fluorescence microscope. Pictures were taken and stored to document the gene expression of the iPSCs.

Immunocytochemistry images were captured with an Olympus IX71 inverted microscope equipped with a Olympus TH4-100 camera and analyzed with the Olympus cellSens Dimension software.

Table 9: Primary antibodies and corresponding secondary antibodies used for immunocytochemistry

Table lists all the primary antibodies and their matching secondary antibodies used for immunocytochemistry. Primary antibodies dilutions are given and prepared in 4% Donkey Serum/DPBS. All secondary antibodies are diluted 1:500 in D-PBS. Also product information including supplier and catalog number of each antibody used are listed. IgG: Immunoglobulin G

Primary Antibody	Product information	Dilution of primary antibody	Secondary antibody	Product information
Oct 4	Abcam, Cat# ab19857	1:100	Alexa Flour ® 488 donkey anti-rabbit IgG	Invitrogen, Cat# A21206
Nanog	Abcam, Cat# ab21624	1:50	Alexa Flour ® 488 donkey anti-rabbit IgG	Invitrogen, Cat# A21206
SSEA 3	Millipore, Cat# MAB4303	1:200	Alexa Flour ® 594 goat anti-rat IgM	Invitrogen, Cat# A21213
SSEA 4	Millipore, Cat# MAB4304	1:200	Alexa Flour ® 488 goat anti-mouse IgG	Invitrogen, Cat# A21121
TRA-1-60	Millipore, Cat# MAB4360	1:200	Alexa Flour ® 555 goat anti-mouse IgM	Invitrogen, Cat# A21426

3.5.3. Pluripotency Marker analysis by quantitative PCR

To analyze the pluripotency of the derived iPSCs, real time quantitative PCR (RT-qPCR) was conducted. Therefore primers specific for the pluripotency markers Dmntb3, hTERT, Nanog, Oct4, Rex1 and Sox2 were used. Their primer sequences and annealing temperature used are listed in table 10. All primers were received from Invitrogen, however, no catalogue number is listed, as each primer used was produced according to customers' specifications. For analysis of the pluripotency, iPSCs were collected, RNA extraction was performed and from this RNA complementary DNA (cDNA) was synthesized.

3.5.3.1. RNA Extraction

RNA extraction was performed with the RNeasy® Mini Kit (Qiagen, Cat# 75106) and the RNase-free DNase Kit (Qiagen, Cat# 79254). All the reagents needed for successful RNA extraction were included in these kits except for 70 % ethanol (Sigma-Aldrich, Cat. No. E7023, diluted with Ultra- Pure^{TM} DNase-RNase-Free distilled water to reach a concentration of 70%). For RNA extraction one to two wells of a 6 well plate containing iPS cells were collected with collagen and spun down for 5 minutes at 200 x g. The supernatant was aspirated and the pellet was resuspended in 350 µL of Buffer RLT. Cells were vortexed for 1 minute and 350 µL of 70% ethanol was added to the mixture. To mix everything equally, the solution was pipetted up and down and then transferred into a RNeasy spin column. After centrifuging the column for 15 seconds at 600 x g, the flow-through was discarded and 350 µL of Buffer RW1 were added to the RNeasy spin column. The column was centrifuged for another 15 seconds at 600 x g. In the meantime 10 µL DNase and 70 µL Buffer RDD were mixed in a 1.5 mL microcentrifuge tube. After the centrifugation, the flow-through was discarded and the prepared DNase mix was added to the RNeasy column and incubated for 15 minutes at room temperature. Then 350 µL of Buffer RW1 were added to the RNeasy spin column and centrifuged for 15 seconds at 600 x g. Flow-through was removed again and 500 µL Buffer RPE were added to the RNeasy spin column. After centrifugation for 15 seconds at 600 x g flow-through was discarded and an additional 500 µL Buffer RPE were added to the RNeasy spin column. The column was centrifuged for two minutes at 600 x g. The flow-through together with the collection tube was discarded and the RNeasy spin column was placed in a new 2 mL collection tube and centrifuged for another minute at 800 x g. After that the RNeasy spin column was placed in a 1.5 mL collection tube and 30 µL RNase-free water were added onto the spin column membrane. After centrifuging the column for one minute at 10.000 rpm, the RNeasy spin column was discarded and the 1,5 mL collection tube containing the extracted RNA was kept. Then RNA concentration was measured with a spectrophotometer (NanoVue plus spectrophotometer, GE Healthcare Life Sciences) and the sample was stored at -80°C.

3.5.3.2. cDNA synthesis

cDNA synthesis was done with the qScript™ cDNA SuperMix (Quanta Biosciences, Cat# 95048-025). For this 1 µg RNA and 4 µL qScript cDNA SuperMix (5x) were combined in a 0.2 mL micro- tube (VWR, Cat# 20170-004). The mixture was filled up with Ultra-Pure™ DNase-RNase-Free distilled water to a final volume of 20 µL. Afterwards, reaction tubes were briefly centrifuged to collect all components. Then the samples were placed in PCR machine and incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes for 85°C and a final holding step at 4°C. After completion of cDNA synthesis the contents were transferred in a 1.5 mL microcentrifuge tube, labeled and stored at -20°C until used.

3.5.3.3. Analysis of Pluripotency Markers using qPCR

For the quantitative PCR, triplicates of 10 µL reactions were run in a MicroAmp® Optical 384-Well Reaction Plate with Barcode (Life Technologies, Cat# 4309849) with Fast SYBR® Green reagent (Life Technologies, CAT# 4385612). Therefore the following components needed were placed on ice to thaw: Fast SYBR® Green, Actin, Oct4, Nanog, Sox2, Rex1, hTERT and Dnmt3b primer reverse and primer forward, and the prepared cDNA. When analyzing the pluripotency of iPSCs with the six primers specific for the pluripotency markers and actin primer as a control in triplicates, 21 wells per sample are needed. Therefore after calculating the number of wells needed, for each well 5 µL SYBR® Green, 1 µL of a mix of primer reverse and forward with a concentration of 5 µM, and 2 µL Ultra-Pure™ DNase-RNase-Free were mixed in a 1.5 mL microcentrifuge tube. 8 µL of this mixture were transferred into each well of the 384-well plate needed. Then 2 µL of cDNA diluted 1:10 were loaded to each well. The plate was covered with an adhesive film for PCR plates (VWR, Cat# 60941-078) and centrifuged at 300 x g for 5 minutes. Quantitative PCR was then done with the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Life Technologies) at the following cycles: 1 initial hold stage of 20 seconds at 95°C followed by 40 cycles consisting of 1 second at 95 °C and 20 seconds at 60°C each. The qPCR was finished with 15 seconds at 95°C, followed by 1 minute at 60°C and final 15 seconds at 95°C. Results were analyzed with the ViiA™ 7 software.

Table 10: Forward and reverse PCR primers for pluripotency markers

All the primers needed for detection of pluripotency-specific gene products by qPCR are listed with their respective primer sequences. Also for the PCR program annealing temperature varies for each primer and is therefore included in the table. Actin was used as a control for gene expression

Primer	Sequence		Annealing temperature
Actin	Forward	GGACTTCGAGCAAGAGATGG	60°C
	Reverse	AGCACTGTGTTGGCGTACAG	
Oct4	Forward	GTGGAGGAAGCTGACAACAA	56°C
	Reverse	CAGGTTTTCTTTCCCTAGCT	
Nanog	Forward	TCCAACATCCTGAACCTCAG	58°C
	Reverse	GACTGGATGTTCTGGGTCTG	
Sox2	Forward	TTGTCGGAGACGGAGAAGCG	58°C
	Reverse	TGACCACCGAACCCATGGAG	
Rex1	Forward	TGGACACGTCTGTGCTCTTC	60°C
	Reverse	GTCTTGGCGTCTTCTCGAAC	
hTERT	Forward	TGTGCACCAACATCTACAAG	57°C
	Reverse	GCGTTCTTGGCTTTCAGGAT	
Dnmt3b	Forward	ATAAGTCGAAGGTGCGTCGT	56°C
	Reverse	GGCAACATCTGAAGCCATTT	

3.5.4. Differentiation Marker analysis by Embryoid body formation

To analyze the differentiation potential of the derived iPSCs into all three germ layers, quantitative PCR was conducted. Therefore two primers specific for differentiation markers of each germ layer were used. These primers include AFP, Brachyury, GATA2, Map2, Pax6 and Sox17. Their primer sequences and annealing temperatures used are listed in table 11. All primers were received from Invitrogen, however, no catalogue number is listed, as each primer used was produced according to customers' specifications. For analysis of the differentiation potential, in-vitro differentiation of iPSCs was performed by creating embryoid bodies, EBs. The EBs were then collected, RNA extracted, cDNA synthesized and analyzed with qPCR.

3.5.4.1. EB formation in-vitro

To determine the differentiation ability of human iPSCs, cells were collected with collagenase and centrifuged for 5 minutes at 200 x g. The pellet was resuspended in hESC medium without βFGF without destroying too many cell chunks. The cells collected from 1 well of a 6 well plate were plated onto one well of a 6-well ultra low attachment plate (Corning, Cat# 3471). Here cells were cultured for one week under normal culture conditions, and fed every other day with hESC medium without FGF. As cells do not attach on these plates feeding was done by gathering the EBs in the middle of a well through circular rocking of the plate. Then the medium was removed by pipetting at the edges of the wells, and fresh medium was added. After 7 days the EBs were seeded on a gelatin-coated well of a 6-well plate in 10% FBS/DMEM medium for another week. The medium was changed every other day. EBs were collected via trypsinization, total RNA was extracted and cDNA synthesize was done as described in sections 3.5.3.1 and 3.5.3.2.

3.5.4.2. Analysis of Differentiation Markers using qPCR

For the quantitative PCR, triplicates of 10 µL reactions were run in a 384-well plate with Fast SYBR® Green reagent. This was done as described in section 3.5.3.3 with the following primers: Actin, AFP, Brachyury, GATA2, Sox17, Map2 and Pax6 primer reverse and primer forward

Table 11: Forward and reverse PCR primers for differentiation markers

All the primers needed for detection gene products specific for differentiation into all three germ layers by qPCR are listed with their primer sequences. Also, for the PCR program, annealing temperature varies for each primer and is hence included in the table. Product information including supplier and catalogue number are stated too. Actin was used as a control to prove gene expression and successful working of qPCR itself

Germ layer	Primer	Sequence		Annealing temperature
	Actin	Forward	GGACTTCGAGCAAGAGATGG	60°C
		Reverse	AGCACTGTGTTGGCGTACAG	
Mesoderm	Brachyury	Forward	AATTGGTCCAGCCTTGGAAT	62°C
		Reverse	CGTTGCTCACAGACCACA	
	GATA2	Forward	GCAACCCCTACTATGCCAACC	58°C
		Reverse	CAGTGGCGTCTTGGAGAAG	
Ectoderm	Pax6	Forward	TCTAATCGAAGGGCCAAATG	57°C
		Reverse	TGTGAGGGCTGTGTCTGTTC	
	Map2	Forward	CAGGTGGCGGACGTGTGAAAATTGAGAGTG	58°C
		Reverse	CACGCTGGATCTGCCTGGGGACTGTG	
Endoderm	Sox17	Forward	CTCTGCCTCCTCCACGAA	60°C
		Reverse	CAGAATCCAGACCTGCACAA	
	AFP	Forward	AGCTTGGTGGTGGATGAAAC	58°C
		Reverse	CCCTCTTCAGCAAAGCAGAC	

3.5.5. Testing for Sendai Virus elimination by RT-PCR

The assurance that the Sendai virus used for reprogramming was completely eliminated from the iPSCs was absolutely essential to proceed with Karyotyping and teratoma formation experiments. Therefore the loss of the Sendai virus vector in the iPS cells was detected with a Reverse Transcriptase PCR (RT-PCR) using specific SeV primers and a subsequent analysis of the result with agarose gel electrophoresis was performed.

For the RT-PCR cells were collected and processed to extract RNA and synthesize cDNA as described in chapters 3.5.3.1 and 3.5.3.2. All the reagents needed were placed on ice to thaw. Then per sample to be checked for SeV elimination one PCR tube was needed. Into each tube 23 µL Platinum Blue PCR Mix (Invitrogen, Cat# 12580-023), 0.5 µL primer forward, 0.5 µL primer reverse and 1 µL of the prepared cDNA were added. The samples were then processed using the C1000™ Thermal Cycler (BioRad). Also, to prove that cDNA synthesis was successful each sample to be tested was also prepared with Actin primers.

3.5.5.1. Agarose Gel Electrophoresis

For the agarose gel electrophoresis a 1.5% agarose gel was used. Therefore depending on the size of the gel the correct amount of TAE 1X, diluted from a 50X stock (Invitrogen, Cat# 24710030) and agarose powder (Fisher Scientific, Cat# BP1356-500) were mixed together and heated up in the microwave for about two minutes. The solution was cooled down for about five to ten minutes and then 1 µL ethidium bromide dye (Fisher Scientific, Cat. No. 1302-10) was added under a chemical fume hood. Then the gel was poured into the assembled gel chamber and a comb was inserted. After the gel was polymerized it was placed in the electrophoresis gel apparatus with TAE 1X buffer (diluted from UltraPure™ DNA Typing Grade® 50X TAE Buffer, Invitrogen, Cat# 24710-030).

Then, 10 µL of 1 kb Plus DNA ladder (Life Technologies, Cat# 10787-026) as well as 10 µL of the samples were loaded onto the gel. The DNA ladder was diluted in 10X Blue Juice™ Gel Loading Buffer (Life Technologies, Cat# 10977) and Ultra-Pure™ DNase-RNase-Free distilled water. The gel was run at approximately 80 volts for about 30 to 40 minutes and then analyzed under UV light. For documentation pictures were taken and stored.

3.5.6. Karyotyping

To examine the genetic stability of derived iPS cells, cells were sent to be karyotyped to Cell Line Genetics (Madison, Wisconsin, USA). Therefore one well of cell line intended for karyotyping was collected and plated on a T25 flask pre-coated with 0.1% gelatin and seeded with a layer of MEF. The flask was incubated

at 37°C and 5% CO2 for about five to seven days until cells reached a confluence of approximately 70%. On the day of shipping the T25 flask was completely filled up with hESC medium with βFGF and sealed with parafilm in order to be sent to the testing facility.

3.5.7. DNA fingerprinting

DNA fingerprinting was performed by Cell Line Genetics with the same cells sent for karyotyping. Also 1 well of patients' somatic cells was collected before reprogramming and DNA was extracted from these cells and sent to cell line genetics together with the cells for karyotyping. DNA extraction was done according to the protocol provided from Qiagen using the QIAamp® DNA Mini kit (Qiagen, Cat# 51304).

3.5.8. Differentiation evaluation by teratoma formation in vivo

Teratoma formation is the most accurate test available in vivo for testing the differentiation potential of iPS cells. Therefore iPS cells from one 10-cm petri dish were collected with collagenase in a 15 mL conical tube. Cell suspension was centrifuged at 200 x g for 5 minutes and washed with DPBS/0.5% BSA. Centrifugation was repeated and afterwards supernatant was aspirated until about 50 µL remained on top of the cell pellet. The pellet was gently resuspended in those 50 µL DPBS/BSA to keep the cells mostly pelleted and in large cell clumps. The cells were then delivered to the Harvard Genome Modification Facility, Cambridge, MA, USA. Here the processed cells were surgically implanted into the kidneys capsules of three immunodeficient mice per cell lines to be tested. After 8-9 weeks after teratoma formation teratomas were sent to the HSCRB Histology Core, Cambridge, MA, USA, for haematoxylin and eosin staining.

4. Results

Regenerative medicine is a broad field of science that focuses on how, when, where and why diseased or damaged tissues in the human body heal. It studies how certain diseases develop and aims to find potential therapies for such disorders. Cellular replacement, a promising field of regenerative medicine, has always been an interesting aspect for treating debilitating diseases involving neurodegeneration. With the discovery of the generation of patient-specific induced pluripotent stem cells, the potential use of cellular replacement therapies have been improved drastically. However, before being able to use iPS cells in a clinical setting several technological hurdles need to be overcome to produce iPS cells in an efficient, safe standardized and reproducible manner. (Blelloch, 2008)

Due to the tremendous potential of stem cells, the aim of this thesis was to produce induced pluripotent stem cells from patients' somatic cells using different methods. As the iPS core facility works upon request of external investigators, reprogramming and characterization was done as commissioned in their contract. This thesis includes three different projects, using different somatic cell types as a starting material: T-cells, fibroblasts and mesenchymal stem cells. They were cultured and iPS cells were derived via Sendai virus mediated reprogramming or miRNA-enhanced mRNA reprogramming. Each project required diverse types of characterization assays. Additionally, one project was used two compare two different Sendai virus reprogramming kits, which was done for research purposes for the HSCI iPS core facility and was not part of any contract with an external investigator. The typical workflow for the generation and subsequent characterization of iPS cells is depicted in figure 13.

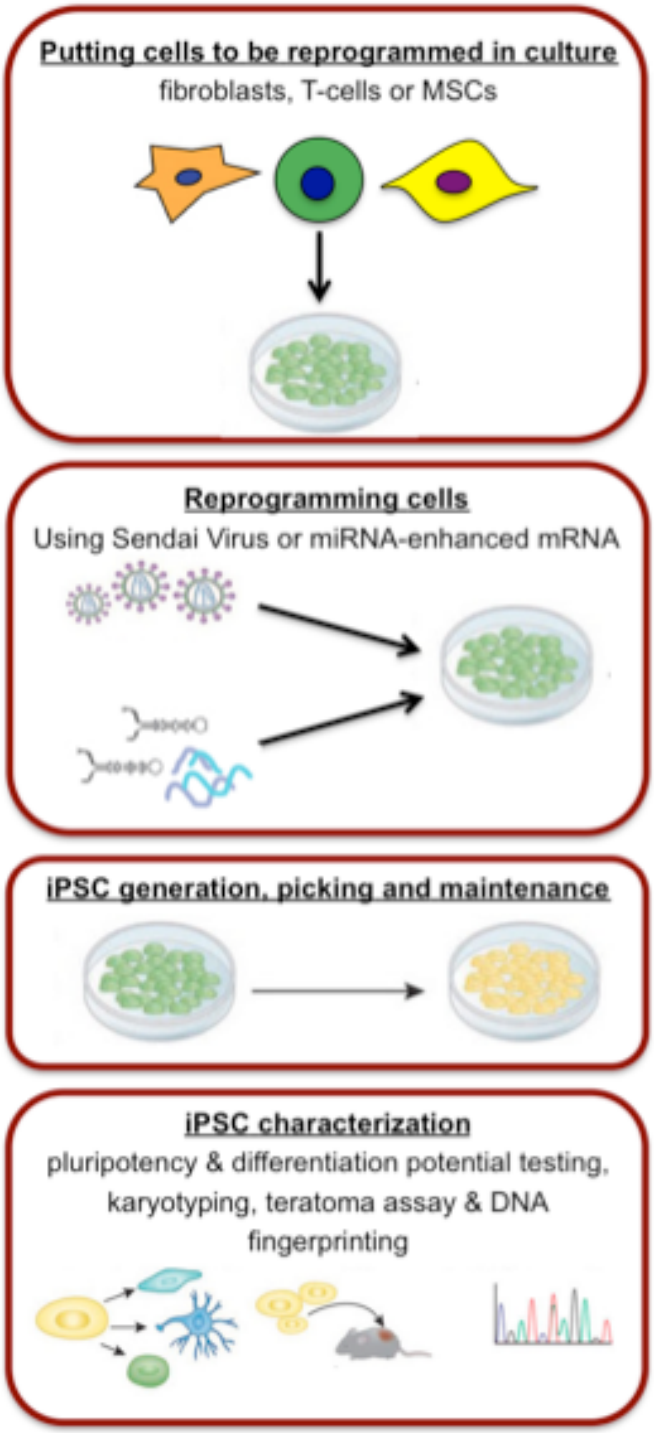

Figure 13: Generation and characterization of iPS cells

The flow chart shows a typical work flow for the generation of induced pluripotent stem cells, their cultivation and different characterization methods. (Parts of figure: http://www.nature.com/nm/journal/v17/n12/full/nm.2504.html?WT.ec_id=NM-201112)

4.1. Project 1: Sendai virus mediated reprogramming of T-cells

In this project fresh blood samples of three different patients suffering from Multiple Sclerosis were processed. The samples, GXT-1, LEP-1 and JSD-1 were received from the Brigham and Women's Hospital, Boston, MA, USA. From the obtained samples peripheral blood mononuclear cells were collected and T-cells were isolated. These T-cells were reprogrammed with the Sendai virus having the four Yamanaka factors introduced to derive iPS cells. Therefore 150,000 cells were transduced using the CytoTune^{TM} iPS Reprogramming Kit with a multiplicity of infection of 10. The cells were requested to be cultivated on feeder conditions.

After transduction of the samples, cells were daily checked for morphological changes. These became visible by means of colony formation, which was first observed about 7 days after transduction. The first iPS cell clone was picked on day 18 post transduction. Figure 14 below shows the morphological changes of one colony of cells over a time course of almost three weeks for all three samples, GXT-1, LEP-1 and JSD-1.

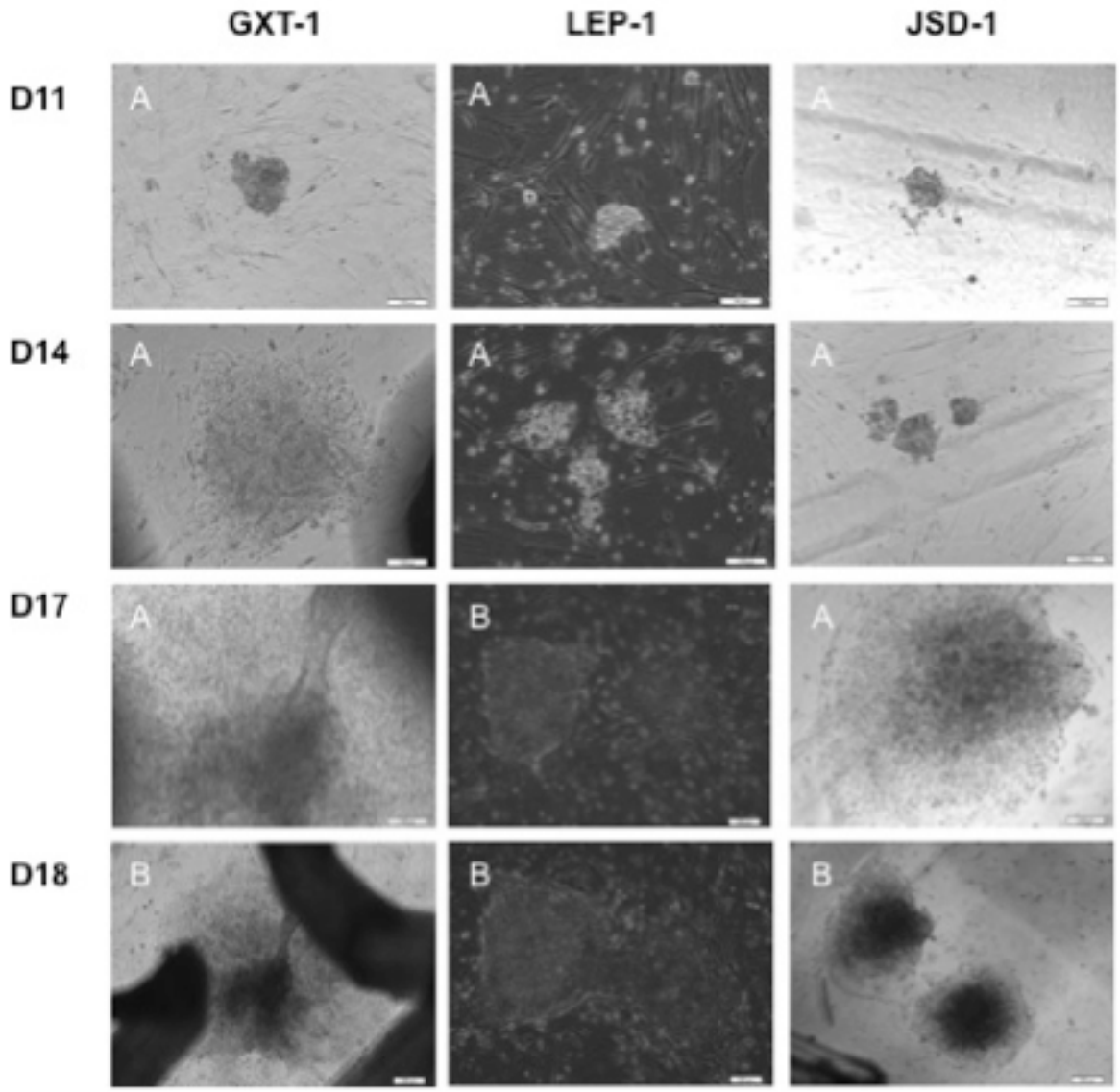

Figure 14: Development of iPS colonies over a timecourse of 18 days for GXT-1, LEP-1 and JSD-1

Figure shows the formation and morphological changes of one colony for each sample, GXT-1, LEP-1 and JSD-1. Development is shown in a timecourse of 11 days after reprogramming.until 18 days after reprogramming, where picking was done for the first colonies. Pictures were taken at (A) 10x magnification and (B) 4x magnification. Scale bar: (A) 100 µm, (B) 200 µm.

For each sample five colonies were picked and cultured on 6-well plates. For the sample LEP-1 for example the picked colonies were named LEP1-A, -B, -C, -D, and –E respectively and one of these cell lines was used for characterization, while the rest was expanded and frozen. The following three cell lines were kept in culture: GXT1-C, LEP1-A and JSD1-C, and analyzed with the hereinafter listed

characterization assays: ICC and AP staining, EB formation to check for differentiation potential using qPCR, qPCR with pluripotency genes, karyotyping, DNA fingerprinting and Teratoma formation.

4.2. Characterization of cells:

As mentioned earlier, fully reprogrammed iPS cells should have similar characteristics as hES cells. During the reprogramming process of somatic cells many colonies emerge, but only few of them actually are fully reprogrammed. Thus, it is important to select the right colonies for picking. This can be done based on morphology: round and tightly packed colonies with defined edges or by live staining using an antibody against pluripotent markers such as TRA-1-60 or SSEA4. To confirm that the picked clones are fully reprogrammed iPS cells and to check the genomic integrity, standard characterization tests are performed: 1. Verification of the expression of pluripotent markers, 2. In vitro and/or in vivo differentiation to check their potential to develop into cells of the three germ layers, 3.karyotype and 4. DNA fingerprinting (Maherali *et al.*, 2008).

4.3. Alkaline Phosphatase Staining

AP staining is a simple, sensitive and rapid tool for staining undifferentiated iPS cells. Therefore alkaline phosphatase stains were done to assess the pluripotent state of all three newly-derived iPS cell lines and are depicted in figure 15.

The figure clearly shows alkaline phosphatase activity of undifferentiated cells resulting in a pink coloring of the cells.

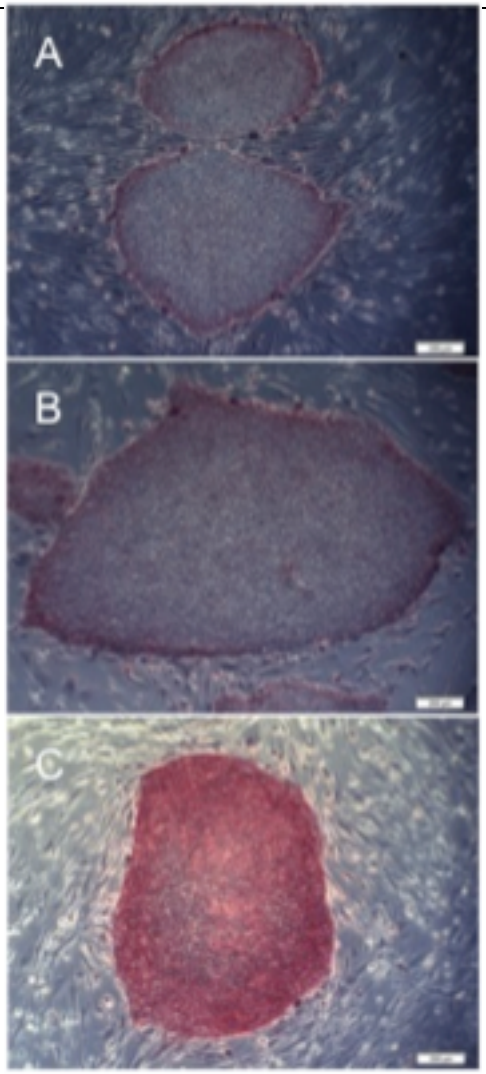

Figure 15: Alkaline Phosphatase staining of the derived iPS cell lines

Microscopic pictures of AP stained iPS cell lines (A) GXT1-C, (B) LEP1-A and (C) JSD1-C are shown. Scale bar: 200 µm.

4.4. Immunocytochemistry assay

Immunocytochemistry assay for pluripotent antigens specific to human iPS cells was performed for all three cell lines derived to assess the pluripotency potential of the cells. The two transcription factors Nanog and Oct4, and the three cell surface markers SSEA-3, SSEA-4 and Tra-1-60 were examined. The obtained results for these markers with the three cell lines GXT1-C, LEP1-A and JSD1-C are presented in figures 16, 17 and 18 respectively.

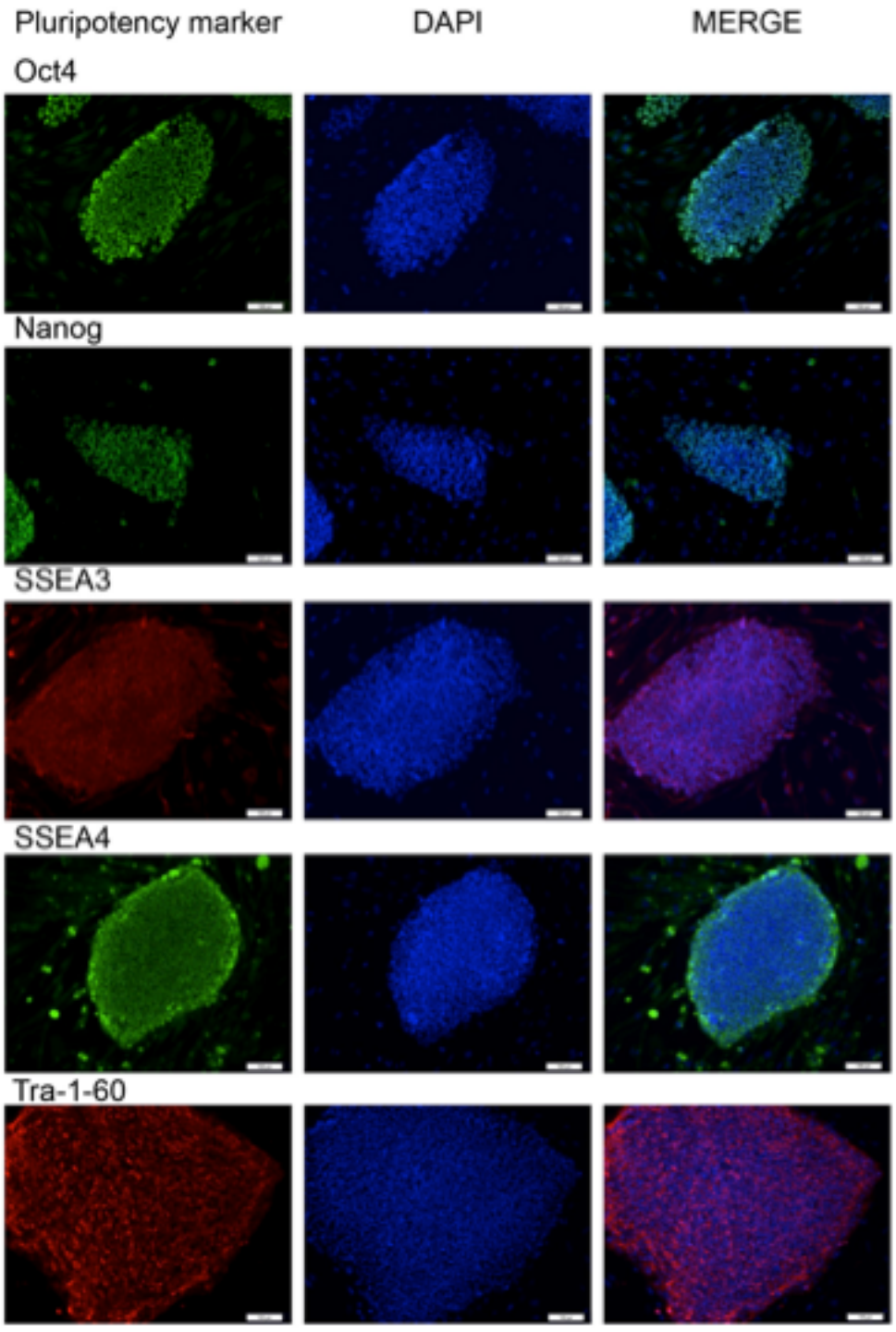

Figure 16: Immunocytochemistry showing expression of pluripotency markers for iPS cell line GXT1-C

Immunocytochemistry analyses of derived iPSCs for cell line GXT1-C at passage 5 indicates expression of Oct4, Nanog, SSEA3, SSEA4 and Tra-1-60. Not only primary antibody staining, but also DAPI nuclear counterstain and an overlay image of both at 10x magnification is shown. Scale bar: 100 µm

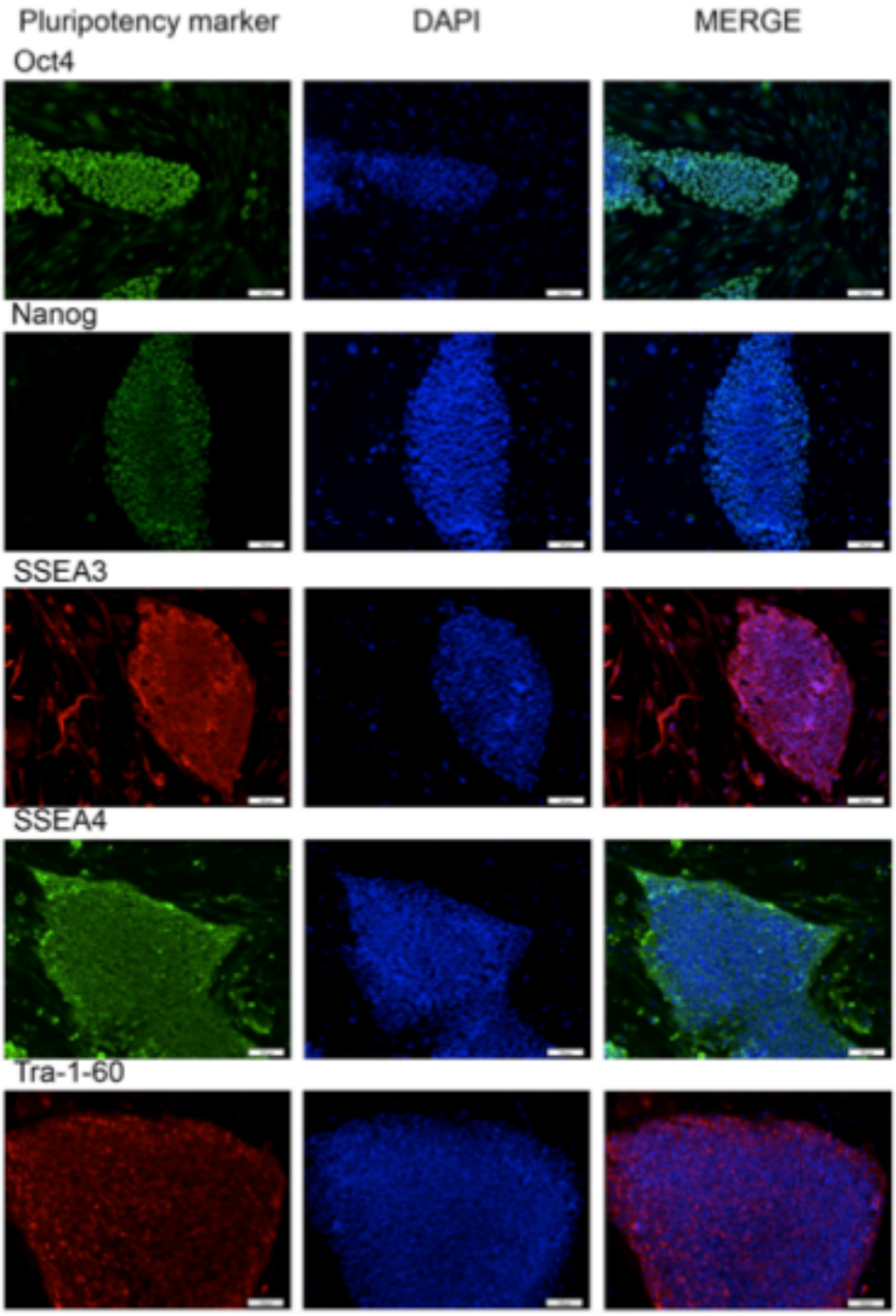

Figure 17: Immunocytochemistry showing expression of pluripotency markers for iPS cell line LEP1-A

Immunocytochemistry analyses of derived iPSCs for cell line LEP1-A at passage 6 indicates expression of Oct4, Nanog, SSEA3, SSEA4 and Tra-1-60. Not only primary antibody staining, but also DAPI nuclear counterstain and an overlay image of both at 10x magnification is shown. Scale bar: 100 µm

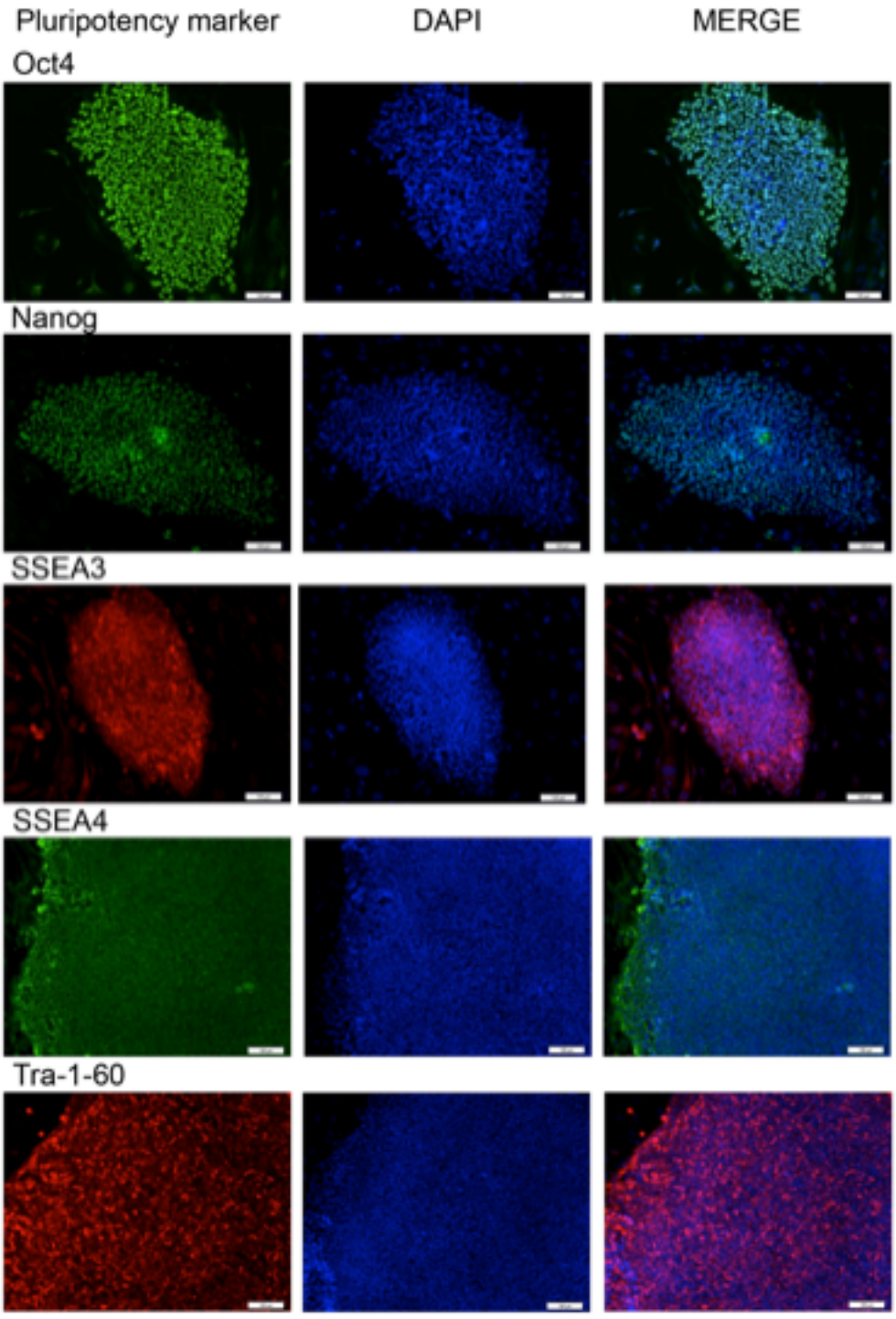

Figure 18: Immunocytochemistry showing expression of pluripotency markers for iPS cell line JSD1-C

Immunocytochemistry analyses of derived iPSCs for cell line JSD1-C at passage 5 indicates expression of Oct4, Nanog, SSEA3, SSEA4 and Tra-1-60. Not only primary antibody staining, but also DAPI nuclear counterstain and an overlay image of both at 10x magnification is shown. Scale bar: 100 µm

All three iPS cell lines tested were strongly positive for a number of molecular markers of undifferentiated pluripotent stem cells including pluripotency nucleus and cell surface markers. The expressed fluorescence of the tested markers clearly indicates embryonic stem cell-like properties, confirming the result of the AP staining.

4.4.1.1. In vitro pluripotency assay by qPCR

For verifying the pluripotency of the iPS cells in vitro, expression of key pluripotency-related genes Oct4, Nanog, Sox2, hTERT, Rex1 and Dnmt3b are assessed. The expression of these genes was checked using q-PCR run with fast SYBR® Green reagent. The resulting gene expression is depicted in figure 19 below.

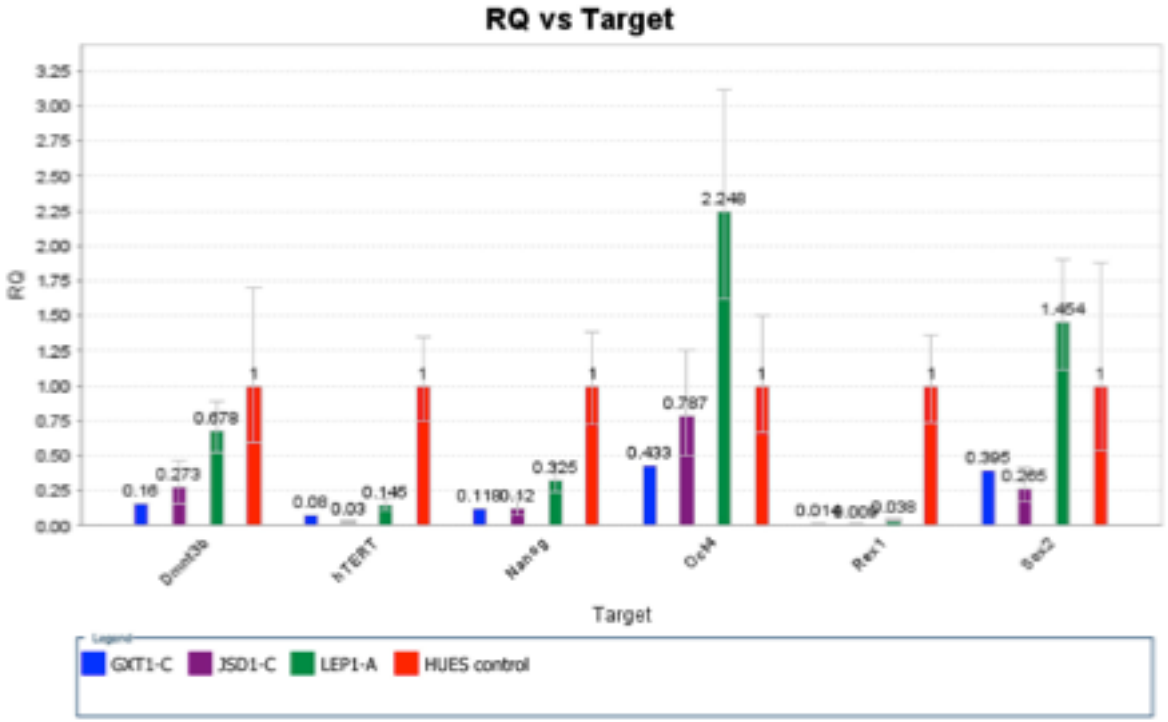

Figure 19: Expression of pluripotency markers of GXT1-C, LEP1-A and JSD1-C using qPCR

Results of in-vitro pluripotency assay of derived iPSCs for cell lines GXT1-C (blue), LEP1-A (green) and JSD1-C (purple) indicate expression of Oct4, Nanog, Dmnt3b, hTERT, Rex1 and Sox2. Changes in gene expression of the produced iPS cells relative to the ES reference sample are shown. Data was normalized to the Actin expression of a control composed of cDNA from three different ES cell lines (red). On the x-axis, the tested pluripotency genes can be seen. Relative quantification (RQ) of the samples is indicated on y-axis.

4.4.1.2. In vitro differentiation assay: Embryoid body formation

To check for the differentiation potential of iPS cells in vitro, iPS cells were differentiatied into embryoid bodies. Therefore cells were first seeded onto low-attachment plates with normal hESC lacking bFGF. 3D aggregates started to form, and after one week, these aggregates are transferred onto a gelatin-coated plate for another week. After two weeks of differentiation, EBs get collected and from its extracted RNA, cDNA is prepared. The expression of the genes specific for all three germ layers was assessed using quantitative real time PCR. To check the differential potential into all three germ layers, two expression markers for each germ layer are used: Brachyury and GATA2 for mesoderm, AFP and Sox17 for endoderm and PAX6 and Map2 for ectoderm differentiation (Otsuki *et al.*, 2013). The resulting gene expression is shown in figure 20.

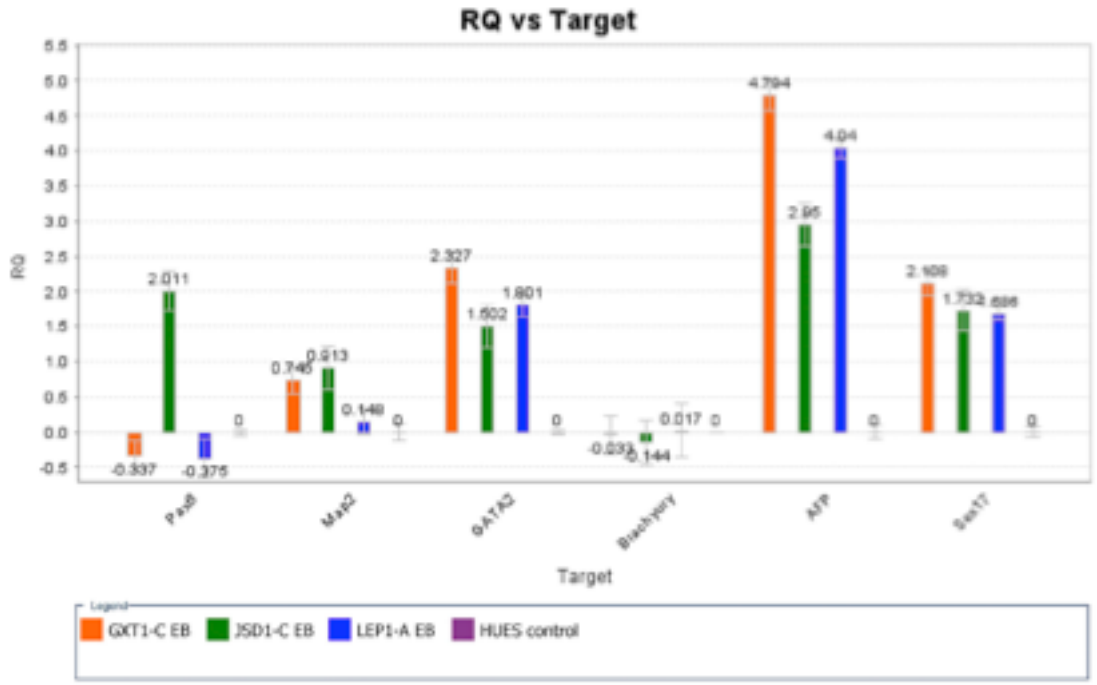

Figure 20: Expression of differentiation markers of GXT1-C, LEP1-A and JSD1-C using qPCR

In vitro differentiation assay using EB formation of derived iPSCs for cell lines GXT1-C (orange), LEP1-A (blue) and JSD1-C (green) indicate expression of all molecular markers. Changes in gene expression in the produced iPS cells relative to the ES reference sample are shown. Data was normalized to the Actin expression of a control composed of cDNA from three different ES cell lines (purple). On the x-axis the tested pluripotency genes can be seen. Relative quantification (RQ) of the samples is indicated on y-axis.

The graph above indicates that derived iPS cells are able to differentiate into all three germ layers.

4.5. Sendai virus elimination testing

The elimination of the Sendai virus is crucial for several experiments to proceed, therefore the loss of Sendai virus vector in cells was performed using a RT-PCR with SeV-specific primers.

Figure 21 below shows the elimination of the Sendai virus over several passages for each cell line on a 1.5% agarose gel. For GXT1-C, LEP1-A and JSD1-C virus was completely eliminated with passage 9, 10 and 7 respectively. Also, β-Actin expression was tested for each cell line to prove that cDNA synthesis was successful.

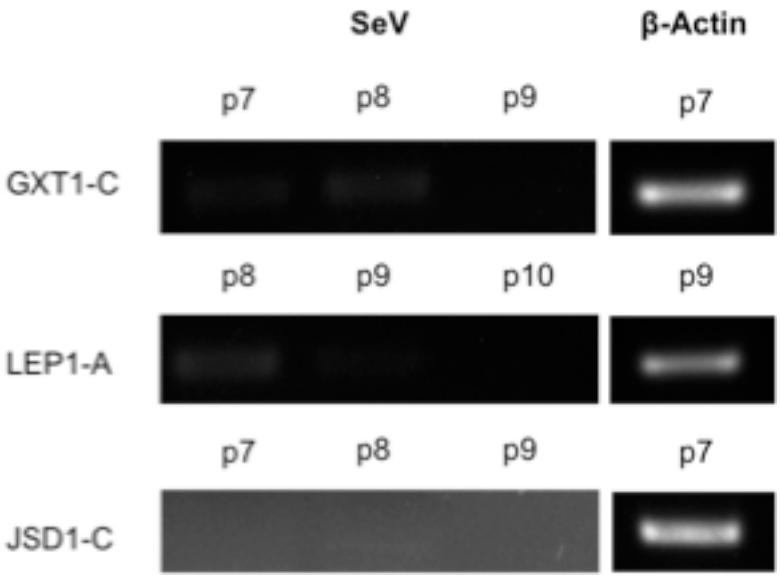

Figure 21: Testing of iPS cell lines GXT1-C, LEP1-A and JSD1-C for Sendai virus gene expression

Gene expression profiles for Sendai virus was performed over three passages on all three cell lines kept for characterization. β-Actin was used as housekeeping gene to prove that the cDNA synthesis was successful. Here β-Actin is only shown for one passage of each cell line tested.

4.5.1.1. DNA Fingerprinting

DNA fingerprinting was performed by Cell Line Genetics with the same cells sent for karyotyping. To confirm patient-specific origin, iPS cells were compared to DNA extracted from somatic cells of the same patient prior to reprogramming. Results were transmitted via a report, stating that each cell line used for characterization matched the patients somatic cells.

4.5.1.2. Karyotype analysis

Karyotype analysis was done by Cell Line Genetics to assess chromosomal stability of the produced iPS cell line. Figure 22 depicts the G-band karyotyping analysis for the cell lines GXT1-C, LEP1-A and JSD1-C. While GXT1-C and JSD1-C led to a normal female karyotype, LEP1-A resulted in an abnormal human

female karyotype with a duplication on the long-arm of chromosome 12 from band q24.1 to band q24.31.

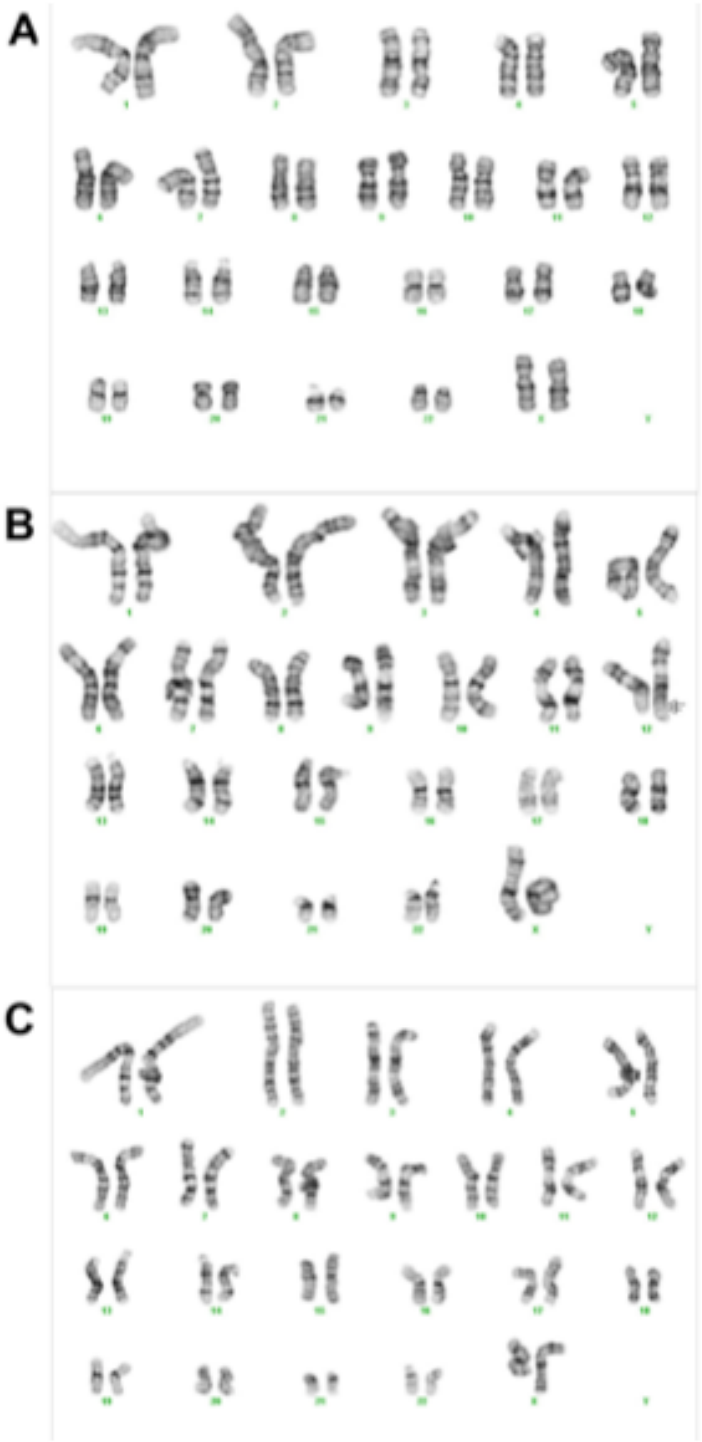

Figure 22: G-band karyotyping analysis of the iPS cell lines GXT1-C, LEP1-A and JSD1-C

Karyotype results for all (A) GXT1-C, (B) LEP1-A and (C) JSD1-C are shown. GXT1-C, tested at passage 13, and JSD1-C, tested at passage 10, resulted in a normal female karyotype. LEP1-A however, tested at passage 14, resulted in an abnormal human female karyotype with a duplication on the long-arm of chromosome 12 from band q24.1 to band q24.31.

4.6. Project 2: Reprogramming of mesenchymal stem cells using the CytoTune™ –iPS 1.0 & 2.0 Sendai Reprogramming Kit

Aim of this project was to derive functional iPS cells from two different mesenchymal stem cell samples: One sample originating from the bone marrow (BMAC), and one sample originating from the umbilical cord (UCD144). In both cases, MSCs were obtained from healthy volunteers and received from the Experimental and Clinical Cell Therapy Institute, Spinal Cord & Tissue Regeneration Center Salzburg, Austria. Samples were obtained in frozen condition and reprogrammed with the Sendai virus.

The kit used for Sendai virus mediated reprogramming at the iPS Core Facility so far was the CytoTune™ 1.0-iPS Sendai Reprogramming Kit by Life Technologies. However, Life Technologies offers a new version of this kit on the market, CytoTune™-iPS 2.0 Sendai Reprogramming Kit, which promises to be less cytotoxic, more efficient and faster in terms of virus elimination. Therefore this project was also used to compare CytoTune 1 with CytoTune 2. Hence, both samples were reprogrammed once with the CytoTune 1 and once with the Sendai Virus CytoTune 2.0 kit. This part of the project was only for research purposes of the HSCI iPS Core Facility in Cambridge, MS, USA and not part of the contract with the external investigator.

For reprogramming 200,000 cells were transduced with the CytoTune™ 1.0 iPS reprogramming kit containing four Sendai virus-based reprogramming vectors, each competent of expressing Oct4, Sox2, Klf4 or c-Myc with a multiplicity of infection of 3 and with the CytoTune™-iPS 2.0 Sendai reprogramming kit using SeV KOS, SeV Klf4 and SeV c-Myc at a MOI of 3 and 5.

After transduction of the samples, cells were daily checked for morphological changes. These became visible about eight days after transduction for CytoTune 1.0 and about three days after transduction for samples treated with CytoTune 2.0. The first iPS cell clone was picked ten days after reprogramming.

For each sample five to ten colonies were picked and cultured on 6-well plates. Cell lines picked were labeled according to the originating sample, the Sendai virus kit used for reprogramming and a unique identification letter for each individual cell line picked, i.e. for the sample UCD144 transduced with the CytoTune™-iPS 2.0 Sendai Reprogramming Kit, cell lines were labeled UCD144 CT2-A, -B, -C and so on.

Five cell lines for each sample and reprogramming method used were kept in culture until passage 4, and at every passage cells from one well of a 6-well plate were collected and processed for testing of Sendai Virus elimination using RT-PCR. After passage 4, only one cell line of each sample was kept in culture for characterization, while the rest was frozen. The following two cell lines were kept for characterization: UCD144 CT2-C and BMAC CT2-B. They were analyzed with karyotyping assay and for teratoma formation.

4.6.1. Comparison of CytoTune 1.0 and CytoTune 2.0

As mentioned earlier, with the development of CytoTune™-iPS 2.0 Sendai Reprogramming Kit, Life Technologies claimed that Sendai virus mediated reprogramming is supposed to be less cytotoxic, more efficient and at the same time elimination of the virus occurs faster. To test this, both samples, UCD144 and BMAC were reprogrammed using CytoTune™-iPS 1.0 Sendai reprogramming kit and CytoTune™-iPS 2.0 Sendai reprogramming kit. The results were directly compared to confirm the improvements of the reprogramming method. Reprogramming with the CytoTune™-iPS 2.0 Sendai reprogramming kit worked well for both samples, however, the transduction of BMAC using CytoTune™-iPS 1.0 Sendai reprogramming kit did not lead to the formation of colonies. Therefore upon picking, project was continued with UCD144 CT1, UCD144 CT2 and BMAC CT2.

4.6.1.1. Cytotoxicity

Cytotoxicity is a common occurring drawback of Sendai virus mediated reprogramming, influencing its efficiency. It occurs especially during the first 24 to

48 hours after transduction of the cells, and can affect over 50% of the cells. Dying of cells is normally an indication of high uptake of the virus. Prevention or reduction of cytotoxicity would result in a higher number of colonies emerging, and therefore in an increase in efficiency.

Figure 23 shows the difference in density for both samples treated with CytoTune 1 and CytoTune 2, clearly indicating that CytoTune^{TM}-iPS 2.0 Sendai reprogramming kit is less toxic.

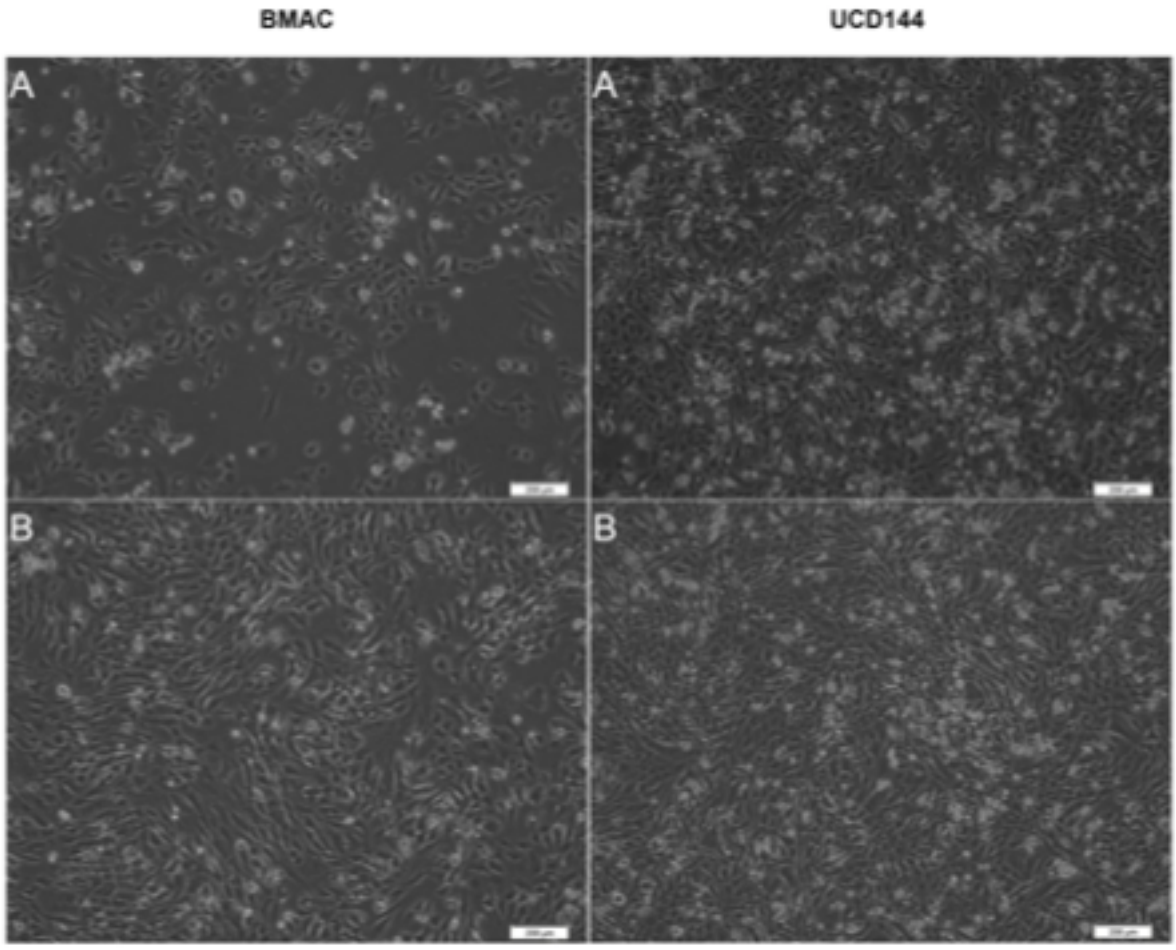

Figure 23: Difference in density for BMAC and UCD144 after reprogramming with CytoTune 1 and CytoTune 2

Difference in the cytotoxicity between (A) CytoTune 1 and (B) CytoTune 2 three days post transduction. For all four samples reprogrammed, same amount of cells was plated. However, a clear difference in density and the number of dead swimming cells can be seen when comparing CytoTune 1 with CytoTune 2 for both samples. Scalebar: 200 µm

4.6.1.2. Efficiency:

To determine the efficiency of each reprogramming process, the number of colonies per line was counted and efficiency was calculated. Therefore colonies on the 10 cm petri dish were counted, and efficiency was calculated with the initial amount of cells transduced, here being 250,000. The resulting efficiency is shown in table 12.

Table 12: Efficiency for transduced samples BMAC and UCD144 with CytoTune 1 & CytoTune 2

Number of colonies counted in 10 cm dishes for the two different samples with both Sendai virus reprogramming kits and the resulting efficiency are listed. Efficiency was calculated using 250,000 cells as starting cell number for transduction.

Sample	Sendai Virus Reprogramming Kit used	Number of colonies in 10 cm dish	Efficiency [%]
BMAC	CytoTune 1	~85	0.034
	CytoTune 2	~2400	0.96
UCD144	CytoTune 1	8	0.0032
	CytoTune 2	12	0.0048

As mentioned earlier, we were not able to successfully reprogram BMAC using the CytoTune^{TM}-iPS 1.0 Sendai reprogramming kit. We encountered problems with our feeder cells for this sample, which is why all cells visible (~85) at the beginning started to die several days post transduction.

However, BMAC CytoTune 2 showed an extremely high number of colonies, suggesting that CytoTune 2 leads to an increase in efficiency.

For UCD144, CytoTune 1 showed a low, but expected number of colonies. After reprogramming, when the cells were split onto 10 cm dishes pre-seeded with MEF, the cell pellet of UCD144 CytoTune 2 was accidentally almost completely aspirated. Therefore the calculated number of colonies is much lower than it would have been, and the calculated efficiency cannot be taken into consideration when comparing the sample.

4.6.1.3. SeV elimination testing

To compare at which passage the Sendai virus was eliminated, using both kits, 1 well of cells was collected for each cell-line of all three samples. From this RNA was extracted and cDNA was synthesized. In a further step RT-PCR was performed with the Sendai virus-specific primers to detect the loss of the Sendai virus in the cells. Elimination of the SeV was visualized on a 1.5% agarose gel and is depicted in figure 24.

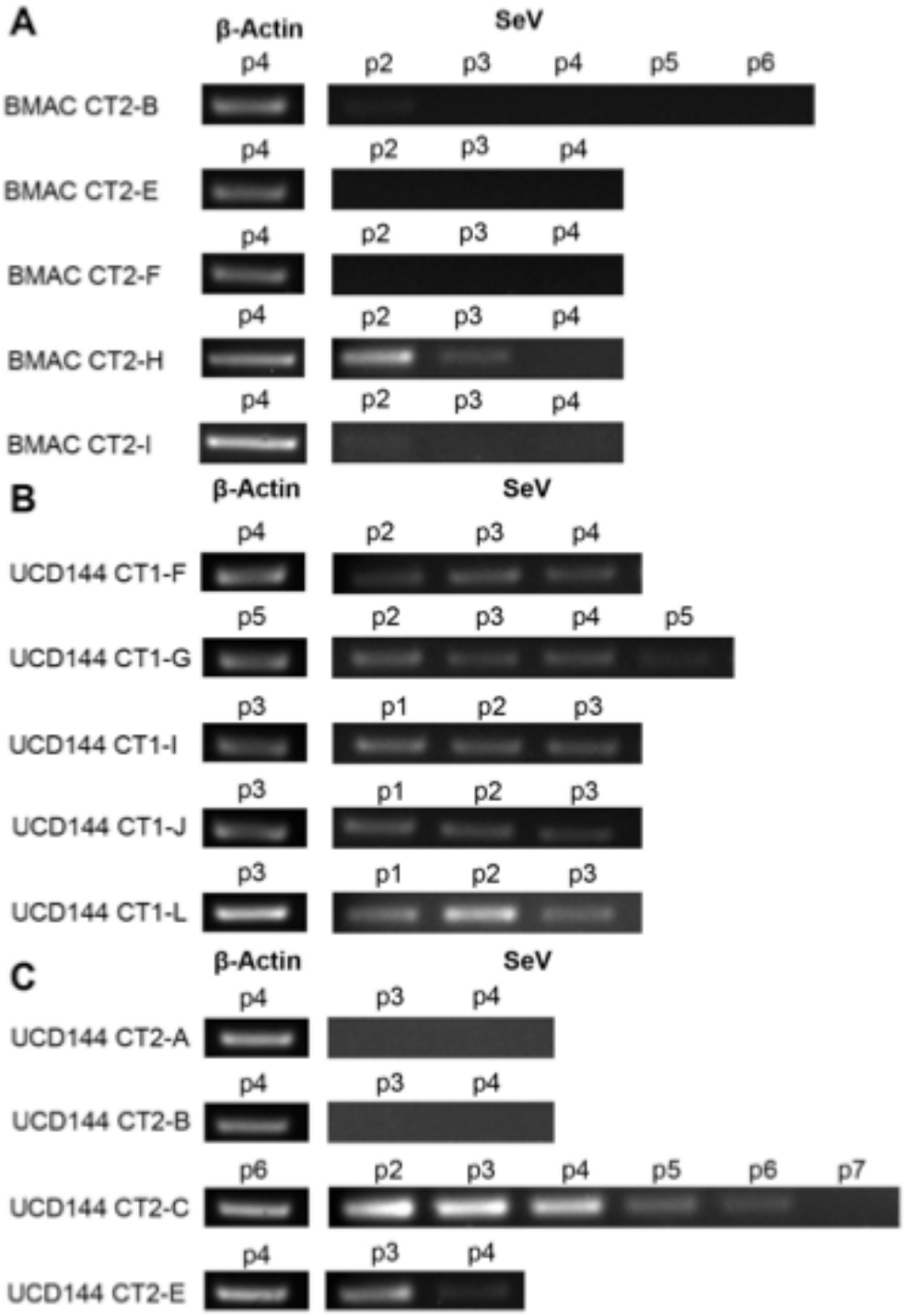

Figure 24: Testing of iPS samples BMAC CT-2, UCD144 CT-1 and UCD144 CT-2 for Sendai virus gene expression

Gene expression profiles for Sendai virus was performed over several passages on all cell lines in culture for all three samples (A) BMAC CT-2, (B) UCD144 CT-1 and (C) UCD144 CT-2. β-Actin was used as a housekeeping gene to prove cDNA synthesis was successful. Here β-Actin is only shown for one passage of each cell line tested

The results indicate that for most cell lines derived with the CytoTune 2.0 reprogramming kit, Sendai virus was eliminated around passage 3 to passage 4. For all cell lines produced with the CytoTune 1.0 reprogramming kit however, Sendai virus is still clearly present at passages 4 and 5. This suggests that using the CytoTune 2.0 kit for Sendai virus mediated reprogramming results in an earlier loss of the Sendai virus backbone.

4.7. Project 3: Reprogramming of fibroblasts using miRNA/mRNA transfection

iPS cells were derived from fibroblasts obtained from one patient with Parkinson's Disease (ND-19) one patient with Frontotemporal Dementia (ND-6) and three patients with an unknown disease (Hff001, Hff002 and Hff004) using the miRNA-enhanced mRNA reprogramming method. Therefore 50,000 cells were seeded in one well of a 6-well plate and cultured in NuFF-conditioned Pluriton^{TM} medium under low oxygen conditions at 37°C. Cells were transfected with microRNA and/or an mRNA cocktail consisting of Oct4, Sox2, Klf4, c-Myc, Lin28 and nGFP mRNA for 12 days.

Changes in morphology were first observed about three days after the first transfection, and based on morphological changes, first colonies were picked 12 days post transfection.

The mRNA cocktail used for transfection included nGFP mRNA, encoding a green fluorescent protein (GFP), which specifically localizes to the nucleus of cells. Therefore nGFP expression can be seen as a positive control for mRNA transfection, and was checked daily. Figure 25, 26 and 27 below show the nGFP expression of the transfected fibroblasts compared to the bright field image showing morphological changes over a time course of 7 to 11 days for three of the five samples, ND-19, Hff002 and Hff004 respectively.

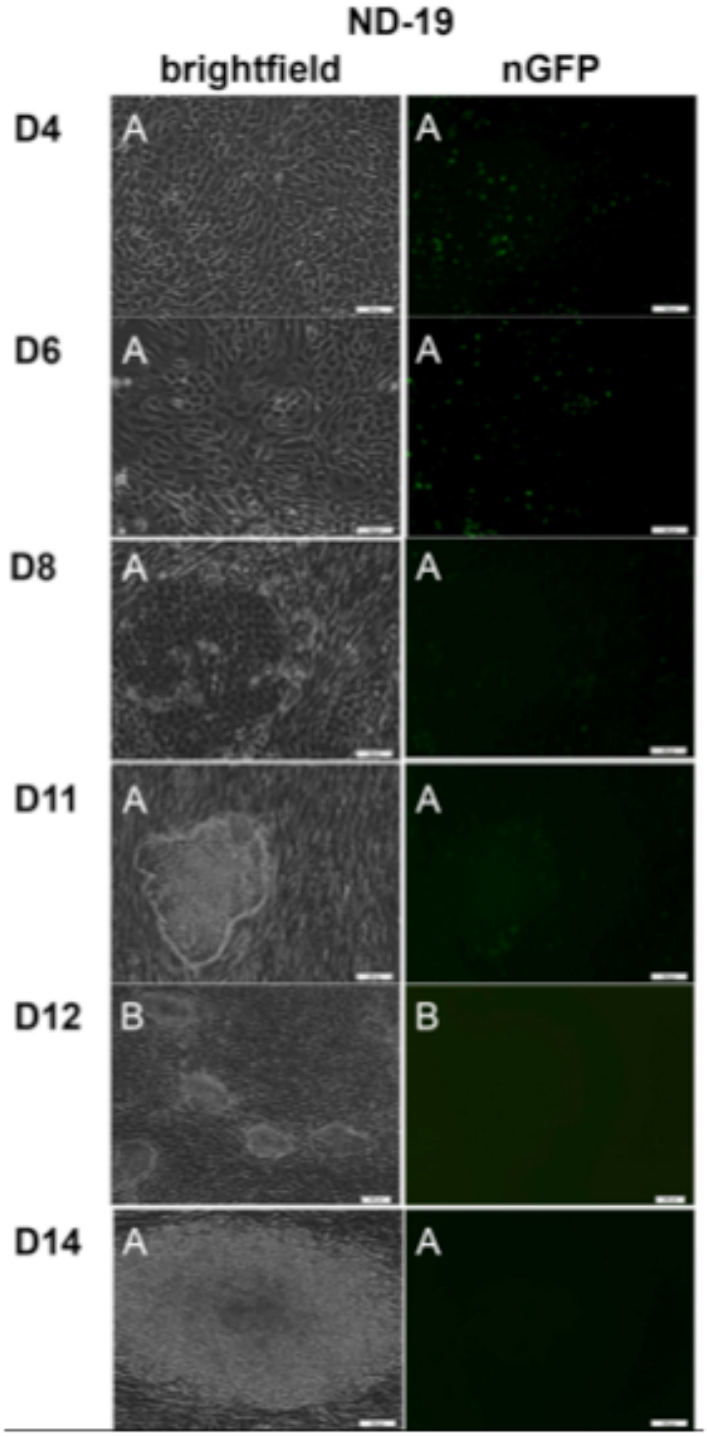

Figure 25: nGFP expression of ND-19 fibroblasts over a time course of 11 days

Morphological changes for ND-19 miRNA enhanced mRNA transfected fibroblasts and the nGFP expression of the cells four days to 14 days after transfection are shown. Scalebar: (A) 100 µm; (B) 200 µm.

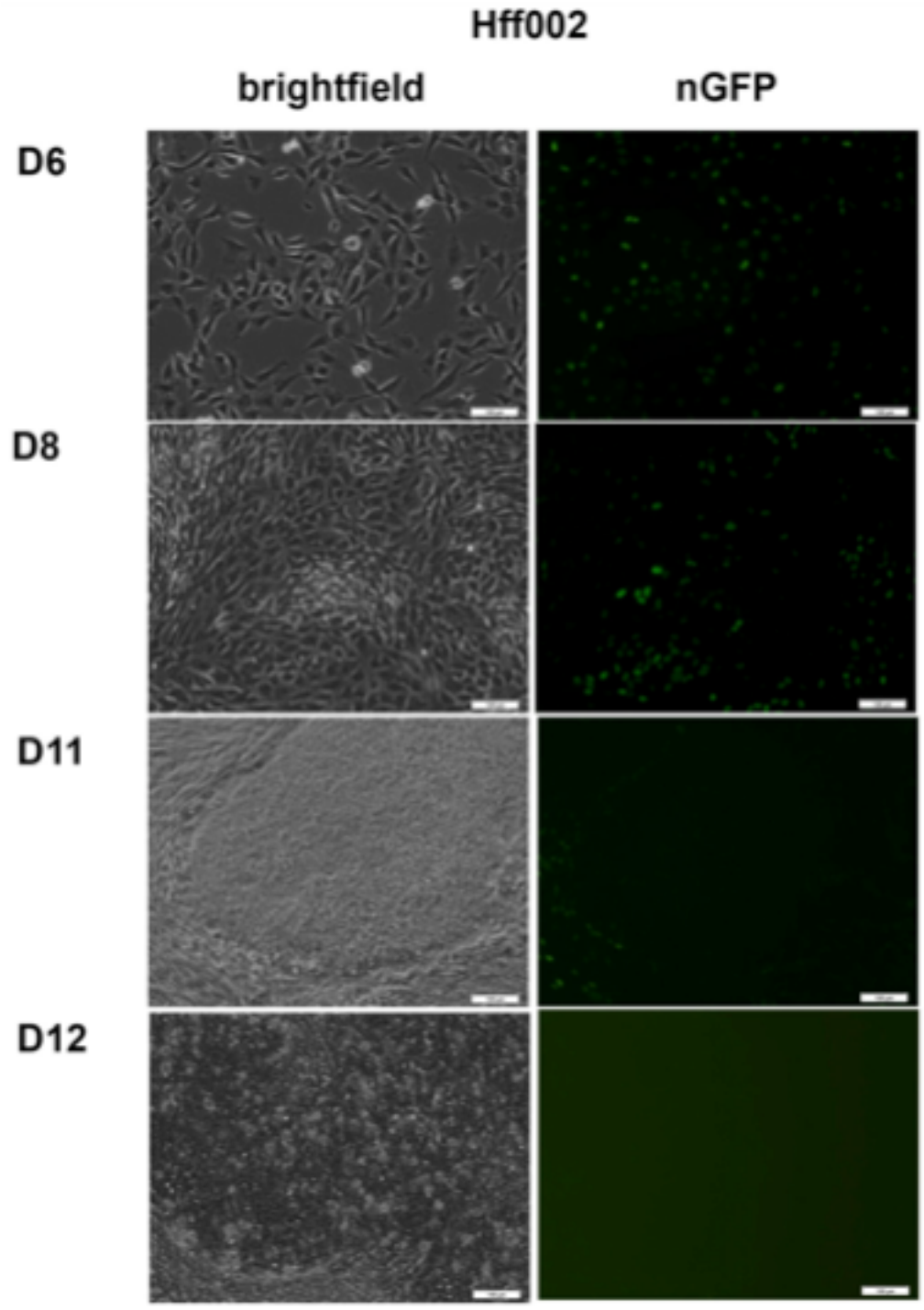

Figure 26: nGFP expression of Hff002 fibroblasts over a time course of 7 days

Morphological changes for Hff002 miRNA enhanced mRNA transfected fibroblasts and the nGFP expression of the cells six days to twelve days after transfection are shown. Scalebar: 100 µm.

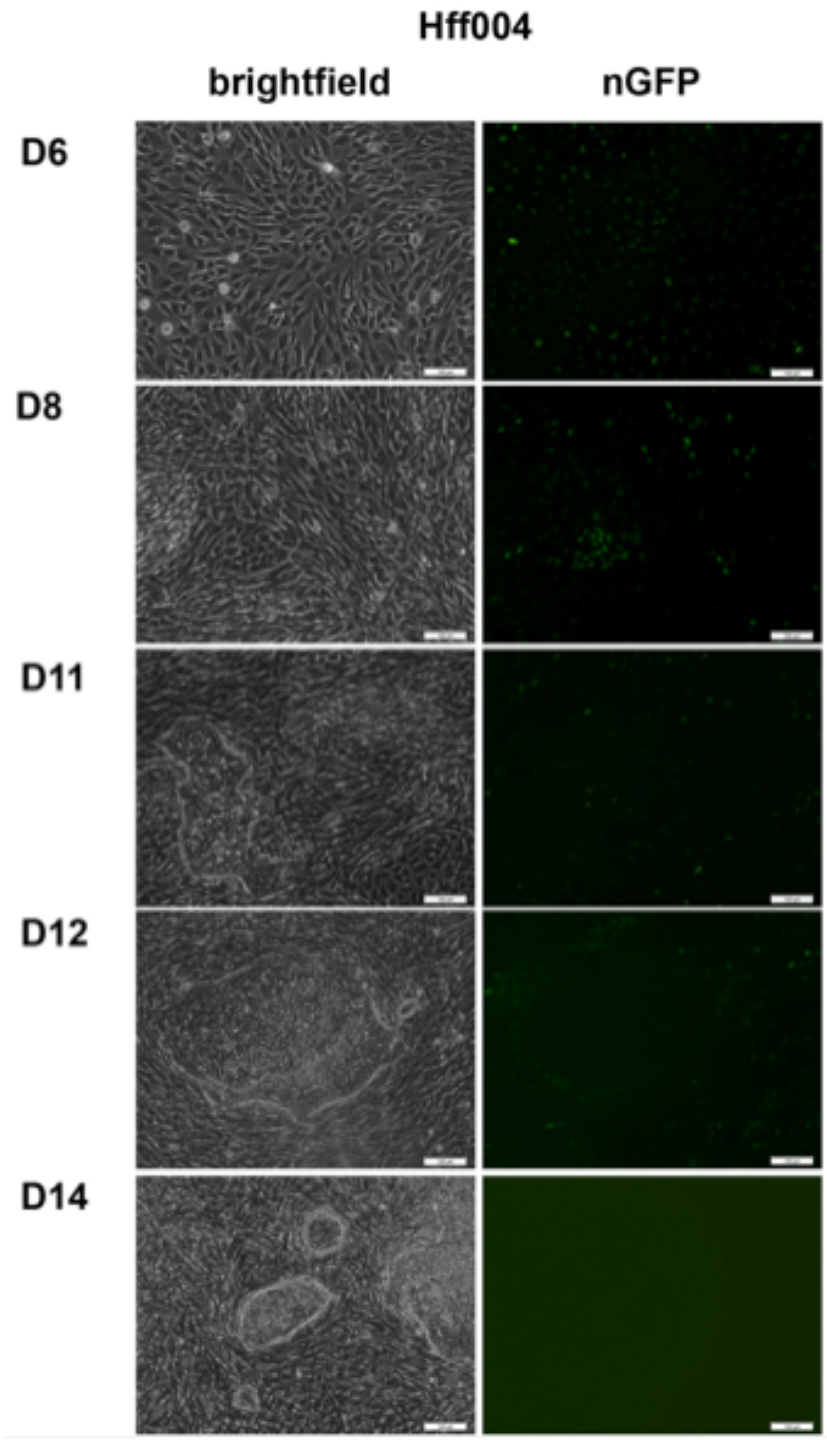

Figure 27: nGFP expression of Hff004 fibroblasts over a time course of 9 days

Morphological changes for Hff004 miRNA enhanced mRNA transfected fibroblasts and the nGFP expression of the cells six days to 14 days after transfection are shown. Scalebar: 100 µm.

The derivation of fully reprogrammed iPS cell colonies was successful for all five samples. Two to six cell lines were picked for each sample and cultured in feeder condition. For the iPS cell lines derived from ND-6 and ND-19, live cultures were sent to the investigators, and no characterization was performed. For the three samples Hff001, Hff002 and Hff004, two cell lines per sample were characterized using ICC and AP staining assays. To assess the gene expression of the key pluripotency-related genes Oct4, Nanog, Sox2, hTERT, Rex1 and Dnmt3b two cell lines per sample derived were checked by a RT-PCR. Also, to evaluate the pluripotency and differentiation potential of the produced iPSCs, EBs were produced and the expression of two endoderm, two ectoderm and two mesoderm marker genes was checked by RT-PCR

All these characterization assays were successfully performed, indicating the ES-like characteristics of the produced iPS cells. However, due to a restricted outline, these results are not included in this thesis.

5. Discussion and implications

In 1998 James Thomson was the first to develop a technique to isolate and grow human embryonic stem cells, offering very unique potential for the field of developmental biology, drug discovery and transplantation medicine (Thomson *et al.*, 1998), The unlimited potential of embryonic stem cells to self-renew, and their pluripotent character, have always made them very promising to study, understand and ultimately treat chronic and degenerative diseases. However, their origin has raised moral and political issues, banning them for clinical use. Therefore the successful direct reprogramming of adult stem cells into iPS cells revolutionized the fields of stem cell biology and regenerative medicine.

The work presented in this thesis describes the successful reprogramming of mesenchymal stem cells, fibroblasts and T-cells back into the pluripotent state, as well as their expansion and characterization. It covers two widely-used direct non-integrative reprogramming methods, the Sendai virus method and miRNA-enhanced mRNA reprogramming. Additionally, a newly-available kit for Sendai virus mediated reprogramming was tested and compared to the kit used for reprogramming so far at the iPS Core Facility.

5.1. Project 1: Sendai virus mediated reprogramming of T-cells

The first part of this project was the derivation of induced pluripotent stem cells from isolated T-cells from three different Multiple Sclerosis patients. Here, according to morphology, nice iPSC colonies appeared, suggesting that reprogramming T-cells with the use of the Sendai virus carrying the four “Yamanaka factors” was successful. Picking and subsequent cultivation and maintenance of the produced iPS cell lines was performed as expected. For all three samples, GXT-1, LEP-1 and JSD-1, five cell lines were successfully established. To confirm the ES-like characteristics of the iPS cells, one cell line for each sample was characterized using several characterization assays.

First the pluripotency state of the cells was checked using alkaline phosphatase staining. Alkaline phosphatase is a hydrolase enzyme expressed in most cell

types. However, for pluripotent stem cells including ESCs, iPSCs and embryonic germ cells, a characteristic elevation of alkaline phosphatase levels on the cell membrane is found. Therefore after staining of the fixed cells, undifferentiated cells turn red or purple, while differentiated cells stay colourless. (O'connor et al., 2008). Alkaline phosphatase is responsible for dephosphorylating many types of molecules, such as nucleotides and proteins under alkaline conditions. One reaction triggered by AP is the hydrolysis of p-nitrophenylphosphate into phosphate and p-nitrophenol. The resulting amount of p-nitrophenol produced is equivalent to the amount of AP present in the cell. Figure 15 clearly indicates that cells for all three samples tested turned pink after staining, suggesting alkaline phosphatase activity, which is typical for undifferentiated cells.

The second characterization assay performed was immunocytochemistry to check the expression of specific proteins in cells. Therefore antigen-specific antibodies were used to assess the pluripotency potential of cells (Nethercott *et al.*, 2011). With evolving research in the field of stem cells, a broad range of cell surface markers for undifferentiated ESCs and iPSCs have been reported. The most critical markers for human ES cells include the surface marker proteins SSEA-3, SSEA-4, TRA-1-60 and TRA1-81 (Zhao *et al.*, 2012). For the characterization of the derived iPS cells at the iPS Core Facility, the expression of five key pluripotency marker proteins, surface and nuclear, was used to assess the pluripotency potential of iPS cells: Nanog, Oct4, SSEA-3, SSEA-4 and TRA-1-60.
Nanog is a transcription factor that is highly expressed in murine and human ES cells, as well as in embryonal carcinoma cells. The expression of Nanog has been reported to be essential for maintenance of pluripotency (Chambers *et al.*, 2003, Hatano *et al.*, 2005, Mitsui *et al.*, 2003).
Primary antibodies specific for the antigens produced in pluripotent stem cells are added. Then secondary antibodies with an attached fluorochrome recognize and bind the primary antibodies (Nethercott *et al.*, 2011).
Following this procedure, the individual cells were visualized within a colony, thereby allowing an overall assessment of expression of the particular pluripotency marker. The strength of staining is proportional to the expression of the marker,

hence verifying the pluripotency potential of stem cells (Fenderson *et al.*, 2006, Pera *et al.*, 2003).
Figures 16, 17 and 18 show that cells for all three samples derived were strongly positive for all tested molecular markers of undifferentiated pluripotent human stem cells, including Oct4, SSEA-3, SSEA-4, TRA-1-60 and Nanog.

To further verify the pluripotency of the iPS cells in vitro, expression of key pluripotency-related genes Oct4, Nanog, Sox2, hTERT, Rex1 and Dnmt3b using qPCR. Real time q-PCR was performed with the fast SYBR Green reagent, which binds to each new copy of double-stranded DNA. The result is an increase in fluorescence intensity proportional to the amount of PCR product produced, and a quantification of the initial number of gene copies of the targeted gene. The resulting gene expression analysis is depicted in figure 19 and indicates an expression of all pluripotency markers for all three samples. Even though some markers are only slightly expressed for some samples (Rex1 and hTERT), the overall outcome suggests that all three samples were able to express the tested pluripotency-related genes. Especially Oct4, Nanog and Sox2 play a major role in the pluripotency state of ES cells, and those three are nicely expressed by all three cell lines tested. As a further control, all three samples were again checked for expression of Oct4, Nanog and Sox2 using RT-PCR (not shown), and all samples resulted in a clear band on the gel.

Additionally, the differentiation potential of iPS cells into lineages of mesoderm, endoderm and ectoderm was assessed by the use of specific markers expressed in these three germ layers after in vitro differentiation for two weeks into embryoid bodies.
Embryoid bodies are three-dimensional aggregates of pluripotent stem cells. Upon seeding of pluripotent stem cells into low-attachment plates with normal hESC medium lacking bFGF, 3D aggregates start to form. After one week these aggregates are transferred to gelatin-coated 6-well plates. This adherent surface leads to further differentiation of the cell into all three germ layers. To check the differential potential of the produced EBs into all three germ layers, two expression

markers specific for each germ layer are used: Brachyury and GATA2 for mesoderm, AFP and Sox17 for endoderm and PAX6 and Map2 for ectoderm differentiation (Otsuki *et al.*, 2013). Figure 20 shows the resulting gene expression upon qPCR analysis. The two endodermal expression markers Sox17 and AFP clearly show that all three derived iPS cell lines are capable of differentiating into endodermal cells. For mesodermal differentiation all three samples clearly expressed GATA2, while Brachyury was only expressed to a very small extent. For this experiment, however, several samples, some of which were not part of this study, were tested with the same genes, and Brachyury was not expressed for any of the samples. This suggests that there was a problem with the primers upon sample preparation, and not with the samples per se. Since expression of GATA2 was very high for all three samples, it can be stated that cells have the potential to differentiate into mesoderm cells. Looking at the expression of the ectoderm markers PAX6 and Map2, all three samples expressed them to a certain extent, indicating that they are capable of differentiating into ectodermal cells.

At least one expression marker unique for each germ layer was expressed by each cell line. Therefore it can be concluded that cells are capable of differentiating into all three germ layers *in vitro*. Part of the project was also to investigate the potential of the derived iPS cell lines to differentiate into ectodermal, mesodermal, and endodermal cell types in vivo through teratoma formation after injection of processed cells under the kidney capsules of immunocompromised mice. However, teratoma formation is a process requiring several weeks, hence these results were not yet gathered upon completing this report and are therefore not included. Before the cells were sent to Harvard Genome Modification Facility for teratoma formation, cells were tested for Sendai virus elimination first. The elimination of the Sendai virus is essential before starting teratoma formation, since it could infect the mice in the facility. Since CytoTune is based on a replication-incompetent version of the Sendai virus, it gradually gets lost as the cells proliferate. As shown in figure 21, the Sendai virus was completely eliminated at passages 7, 9 and 10 for cell line JSD1-C, GXT1-C and LEP1-A.

To assure the generation of stem cell lines free from cross-contamination and to confirm the patient-specific origin as well as genomic integrity, DNA fingerprinting and karyotype analysis are performed.

DNA fingerprinting, also known as DNA profiling, was first described in 1984 by Alec Jeffreys (Jeffreys *et al.*, 1985b, Jeffreys *et al.*, 1985a). It is a method that allows identification of individuals by analyzing the sizes of DNA fragments, so-^ 433ASWDF1QA<called short tandem repeats (STRs), which are unique for each individual of the same species.

DNA fingerprinting is an important tool in stem cell research to guarantee the genetic identity of the stem cell line. This is done by cutting out single short tandem repeats using restriction enzymes, and amplifying them via PCR. With the use of gel electrophoresis, these DNA fragments are analyzed according to their sizes, giving detailed information about their genetic makeup.

Over the years, several cases have been reported that showed stem cell contamination. This might for example occur for stem cells grown on feeder-plates due to a cross-contamination of the stem cells with MEF. However, since normally irradiated MEF are used, the risk of cross-contamination is low. The most common issues might occur due to cross-contamination of two iPSC lines mixed together or when two iPSC lines are swapped. To avoid the usage of such contaminated stem cells in research and to verify patient-specific origin of each cell line derived, DNA fingerprinting is an important tool used (Salguero, 2008).

Results obtained for the derived cell lines matched the results of the patients' somatic cells, therefore patient-specific origin can be confirmed.

Karyotype analysis is done in order to assess the chromosomal stability of the produced iPS cell line. Karyotype generally is the visual appearance and number of chromosomes present in the nucleus of a eukaryotic cell. The basic number of chromosomes in somatic cells of humans is 46, and in germ cells 23.

Overall, about 10% of newly-derived iPSC lines have abnormal karyotypes. This could be gained through the reprogramming process or already present in the somatic cell of origin. Moreover, pluripotent stem cells can acquire genetic

changes throughout passaging. Therefore it is important to check the karyotype after large expansion.

Abnormal karyotypes of cultured human embryonic stem cells have been reported before. Human ES lines have recurrent gains of specific chromosomes and regions of chromosomes, like 17q and 12. It has been shown that stem cells from later passage are more likely to develop karyotype changes than cells from earlier passage. Any changes of chromosomal stability in the cell would exclude this produced cell line from cell-based therapy, since it has been shown that in vivo atypical karyotypes are often associated with tumorigenesis (Buzzard *et al.*, 2004, Draper *et al.*, 2004).

Karyotype analysis revealed that two of the three cell lines tested, GXT1-C and JSD1-C, had a normal female complement of 46 chromosomes, see figure 22. However, LEP1-A resulted in an abnormal human female karyotype with a duplication on the long-arm of chromosome 12 from band q24.1 to band q24.31 indicated in figure 22.B by small arrows.

Since this karyotypic abnormality has never been experienced by the iPS Core Facility, it is suspected that the duplication is maybe not a result of the passaging and culturing the iPS cell line itself. The patient from whom the derived iPS cell line, LEP1-A, originates was an elderly woman, suffering from a certain blood disease which might be the reason for an abnormal karoytype. Even though there is no proven evidence that this disease correlates with these karyotypic abnormalities, it might still originate from the patient herself. Therefore, to prove or disprove this theory, a different derived cell line from the same sample, LEP1-C, was placed back in culture and will be tested for genomic integrity in a few weeks.

Mutations of chromosome 12 reported so far are normally always connected to Nanog, which has a cytogenetic location of chromosome 12 on band p13.31. In our case however, the abnormality was found on chromosome 12 from band q24.1 to q24.31, therefore independent from the genomic location of Nanog.

5.2. Project 2: Reprogramming of mesenchymal stem cells using the CytoTune™ –iPS 1.0 & 2.0 Sendai Reprogramming Kit

For this study, iPS cells were successfully derived from mesenchymal stem cells using the CytoTune™ 1.0-iPS Sendai Reprogramming Kit as well as the CytoTune™-iPS 2.0 Sendai Reprogramming Kit. Cells from two different healthy volunteers were used as starting samples, one originating from the bone marrow (BMAC) and one originating from the umbilical cord (UCD144). For both samples and both reprogramming methods 250,000 cells were reprogrammed and cultured under the normal cell conditions. The new version of the Sendai virus reprogramming kit, CytoTune 2.0, consists of three Sendai virus-based reprogramming vectors, hKOS, hc-Myc and hKlf4, and transduction was performed using an MOI of 5, 5 and 3 respectively.

Life Technologies claims that with the use of the new kit, reprogramming is less cytotoxic, more efficient and elimination of the Sendai virus occurs faster. Therefore all samples were compared for these three factors.

Figure 23 depicts the difference in cell density for both samples after reprogramming with both kits. These pictures clearly indicate that for the samples reprogrammed with CytoTune 1.0, cells are less dense and additionally more dead cells can be seen, suggesting that reprogramming with CytoTune 2.0 results in higher survival rate of the cells, which might directly indicate a better efficiency.

For the cells reprogrammed with the CytoTune 2.0 kit, first colonies emerged only three days after transduction in a much higher number than usual. For the samples reprogrammed with the CytoTune 1.0 kit, few colonies were first visible around 8 days after transduction. To determine the efficiency of the reprogramming, the total number of colonies appearing on the 10 cm dish was determined, and efficiency was calculated with the use of the original cell number transduced (250,000). After transferring the 10 cm dishes onto MEF with hESC medium, we encountered a problem with the quality of the feeder cells, which is why for one sample, BMAC CytoTune 1, even though ~85 colonies were counted,

they started to die several days after transduction. For BMAC reprogrammed with CytoTune 2, an approximate number of 2400 colonies was counted, leading to an efficiency about 28 times as high as for CytoTune 1.0 reprogrammed samples.
For the second patient sample, UCD144, CytoTune 1 reprogramming resulted in only eight colonies. This extremely low number might either be also due to difficulties encountered with the feeder cells, or simply the low efficiency resulting from reprogramming with Sendai CytoTune 1.0. Several days after reprogramming, cells always get transferred onto the MEF-coated 10 cm dishes. Here, while transferring, a large amount of the UCD144 CytoTune 2.0 cell pellet was accidentally aspirated. Therefore it is no surprise that the number of colonies emerged after transferring was, with 12 colonies only, very low, and the calculated efficiency could therefore not be taken into consideration when comparing samples.

Testing for Sendai virus elimination was done for four to five cell lines per sample using RT-PCR. The results were displayed on a 1.5% agarose gel. The intensity of the bands corresponds to the gene expression itself, meaning that brighter, more intense bands indicate a higher gene expression. β-Actin was also tested for each sample at each passage to confirm that cDNA synthesis was successful. For BMAC CT2 (indicating that cells were reprogrammed with the CytoTune 2.0 kit) five cell lines were tested starting at passage 2 up to passage six. For three out of five cell lines, Sendai virus was already completely eliminated at passage 2, and the other two cell lines showed elimination of the virus at passage 4. All five cell lines tested for UCD144 CT1 showed that Sendai virus was still integrated at passages 3 to 5. For UCD144 CT2 only four cell lines were tested, two of which show a clear elimination of the virus at passage three already, while the other two showed that the virus was still integrated at passage 4 and passage 6. Looking at all these results it can be said that out of nine cell lines derived with the CytoTune 2.0 kit, one cell line still expressed the Sendai virus at passage 6. From experience at the iPS Core Facility with the CytoTune 1.0 kit however, it is normally the case that only 1 out of 8 samples does not have the Sendai virus integrated anymore at passage 6. Therefore it can be suggested that with the use

of the CytoTune 2.0 kit, Sendai virus gets eliminated faster. This might be of great use, especially in terms of time needed for finishing projects and characterization. Normally for testing differentiation potential in vivo via teratoma formation, it has to be assured that the Sendai virus is completely gone. With the use of the CytoTune 1.0 kit, this usually happens around passages 7 to 12 or even later, meaning keeping cells for weeks in culture to wait for the Sendai virus to be eliminated.

In summary, these results suggest that using CytoTune^{TM}-iPS 2.0 Sendai Reprogramming Kit, the derived iPS cells were less cytotoxic, showed a higher efficiency and eliminated the Sendai virus faster, confirming the statement of Life Technologies.

5.3. Project 3: Reprogramming of fibroblasts using miRNA/mRNA transfection

The reprogramming of fibroblasts using miRNA-enhanced mRNA was performed for five different patient samples. Transfection of the cells with either miRNA, mRNA or a combination of both, was successfully done for twelve days. The first morphological changes were observed about three days after the first transfection of the cells. After medium change, cells were checked daily. As mRNA cocktail contains nGFP, cells were checked daily for the expression of green fluorescent protein. For three out of the five transfected cell samples pictures were taken, once with bright field microscopy, and once with fluorescence microscopy to detect the GFP expression of the cells. The timeline for all three samples shown in figures 25, 26 and 27, clearly shows that after some days of transfections, cells nicely start to express GFP, indicating that transfection was successful.

Upon successful transfection of all five samples, two to six cell lines per sample could be picked. For three of the five samples, two cell lines per sample were characterized using ICC and AP staining. Also, their expression of key-pluripotency genes was tested using RT-PCR and their in-vitro differentiation potential was assessed by the production of EBs and subsequent analysis of several differentiation genes using RT-PCR. All these characterization assays

were successful, hence it can be stated that miRNA-enhanced mRNA transfection worked well.

6. Conclusion

Looking at the data presented in this thesis it can be stated that the generation of induced pluripotent stem cells from T-cells, MSCs and fibroblasts worked as expected. All the characterization assays performed indicate the ES-like characteristics of the derived cells, thereby confirming their pluripotent character.

The iPS cell technology holds an unprecedented potential to model and treat human diseases. Not only can it be used for drug screening, but also with the use of genome editing, healthy iPS cells might offer a tremendous possibility for regenerative medicine.

Also the clinical potential disease-specific iPS cells hold is enormous. There are several major barriers that need to be overcome before iPS cells can be used as a standard treatment in clinics. One of the biggest issues is still the relatively low efficiency rate for most techniques, less than 0.1%. Also, the complexity of most reprogramming methods is still not fully understood. However, the results comparing two kits of Sendai virus mediated reprogramming show that with ongoing research, new and more efficient techniques of deriving iPS cells are being explored.

With the world's first clinical trial using iPS cells to create retinal cells for replacement of damaged parts of the eye, which started in July 2013, foundation has been laid. This raises hope that iPS cells might be used in clinical applications someday in the future.
However, a lot more research is needed to improve safety of iPS cells for human applications.

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