# Generation of disease-specific human induced pluripotent stem cell lines by Sendai virus mediated reprogramming using mouse embryonic feeder cells and a feeder free system

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## STATUARY DECLARATION

I declare in a lieu of an oath that I have written this master thesis myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This master thesis has not been submitted elsewhere for examination purposes.

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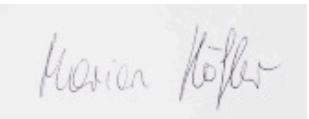

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# ABBREVIATIONS

| | |
|---|---|
| ALS | Amyotrophic Lateral Sclerosis |
| AP | Alkaline Phosphatase |
| bFGF | Basic Fibroblast Growth Factor |
| BP | Base Pair |
| BMD | Becker Muscular Dystrophy |
| BMP | Bone Morphogenic Protein |
| cDNA | Complementary Deoxyribonucleic acid |
| CB-derived | Cord Blood derived |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMEM/F12 | Dulbecco's Modified Eagle Medium, Nutrient mixture F12 |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Scid |
| DNMT3B | DNA (cytosine-5-)-Methyltransferase 3 Beta |
| DNTP | Deoxynucleotide Triphosphate |
| DPBS | Dulbecco's Phosphate Buffered Saline |
| DS | Down Syndrome (Trisomy 21) |
| EB | Embryoid Bodies |
| EDTA | Ethylenediaminetetraacetic Acid |
| ESC | Embryonic Stem Cells |
| FBS | Fetal Bovine Serum |
| FLK1 | Fetal Liver Kinase 1 |
| FRV | Fast Red Violet |
| GATA | Transcription Factor Family binding to the DNA sequence GATA |
| GF | Growth Factor |
| GFP | Green Fluorescence Protein |
| HD | Huntington's Disease |
| HDAC | Histone Deacetylase |
| hES | Human Embryonic Stem Cell Medium |
| HESC | Human Embryonic Stem Cells |
| HECs | human embryonal carcinoma cells |
| HF | Human Fibroblasts |
| HSCI | Harvard Stem Cell Institute |
| hTERT | Human Telomerase Reverse Transcriptase |
| ICC | Immunocytochemistry |
| ICM | Inner Cell Mass |
| iPSCs | Induces Pluripotent Stem Cells |
| k/o | Knock out |

| | |
|---|---|
| KLF4 | Krueppel-like Factor |
| KOSR | Knockout Serum Replacement |
| LIN-28 | Human Protein encoded by the gene LIN28 |
| MEF | Mouse Embryonic Fibroblasts |
| MEF-CM | MEF conditioned medium |
| miRNAs | Micro RNAs |
| MOI | Multiplicity of Infection |
| MYC | Oncogenic Transcription Factor |
| NANOG | Transcription Factor |
| NCAM | Neural Cell Adhesion Molecule |
| OCT-3/4 | Octamer-Binding Transcription Factor 3/4 |
| OSKM | “Yamanaka Factors”: A Combination of Transcription Factors Oct4, Sox2, Klf4, Myc |
| OSKML | Combination of Transcription Factors Oct4, Sox2, Klf4, c-Myc and Lin28 |
| P21 | Cyclin-dependent Kinase Inhibitor 1 |
| PAX6 | Paired Box and Homeobox Protein 6 |
| PBS | Phosphate Buffered Saline |
| PBMC | Peripheral Mononuclear Blood Cell |
| PCR | Polymerase Chain Reaction |
| piPS | protein induced pluripotent stem cells |
| P/S | Penicillin/Streptomycin |
| PSC | Pluripotent Stem Cells |
| qPCR | real time PCR |
| REX1 | Zinc-finger protein 42 homolog |
| RiPS | RNA produced iPS cells |
| RNA | Ribonucleic Acid |
| ROCK | Rho-associated Protein Kinase |
| RPM | Rotations Per Minute |
| RT | Room Temperature OR Reverse Transcriptase |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| RV | Retrovirus |
| SOX-2 | SRY (sex-determining region Y)-box 2 |
| SSEA | Stage-Specific Embryonic Antigen |
| SMA | Spinal Muscular Artrophy |
| SV | Sendai Virus |
| TAE | Tris Base, Acetic Acid, EDTA |
| TF | Transcription Factor |
| VE-CAD | Vascular-Endothelial Cadherin |
| VPA | Valproic Acid |
| µg | Microgram |
| µL | Microliter |
| µm | Micrometer |

# ABSTRACT

The generation of induced pluripotent stem cells (iPSCs) from human somatic cells has revolutionized the field of stem cell research. Stem cell therapy has become a promising hope for regenerative medicine and the possible cure of degenerative diseases.
In 1998, researchers first developed a technique to isolate and grow human embryonic stem cells in cell culture, which was a breakthrough for stem cell research. As these cells, as well as induced pluripotent stem cells, are able to differentiate into any cell type of the human body, they offer the potential for the development of medical treatments for a wide range of conditions. But, there is still controversy and ethical issues, as the origin of human embryonic stem cells are human embryos.
The breakthrough for human induced pluripotent stem cells was in 2007, where researchers reprogrammed somatic cells into a pluripotent, embryonic stem cell-like state by the overexpression of certain transcription factors.
The potential of iPSCs is enormous, but there is still relatively little known about the molecular and functional equivalence of iPSCs to ESCs. Many obstacles remain before medical and pharmaceutical applications for iPSCs can be fully applied.

This thesis describes the generation of disease-specific induced pluripotent stem cells from human fibroblasts and erythroblasts. Different reprogramming methods, as well as iPSC culture conditions on feeder cells and in feeder free conditions are described. The generated induced pluripotent stem cell lines are fully characterized by specific assays to show the pluripotency of the cells.

# 1 INTRODUCTION

The discovery of the generation of induced pluripotent stem cells (iPSCs) from somatic cells demonstrated that adult mammalian cells can be reprogrammed to a pluripotent state by the enforced expression of a few embryonic transcription factors. It was a major breakthrough and provides researchers with a unique platform to dissect the mechanisms of cellular reprogramming.

In 2006, a major breakthrough occurred when Shinya Yamanaka reported the successful reprogramming of adult, fully differentiated mouse fibroblasts into pluripotent embryonic stem cell-like cells: induced pluripotent stem cells (Takahashi and Yamanaka, 2006). One year later, Takahashi and Yamanaka were successful in the transformation of human fibroblasts into iPSCs using the same cocktail of transcription factors, named the “Yamanaka Factors”: Oct4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007). Yamanaka received the Nobel Price for this discovery in 2012.

Soon after this breakthrough for regenerative medicine, several other groups reported the generation of induced pluripotent stem cells (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007).

Induced pluripotent stem cells are not identical to human embryonic stem cells (hESCs), but they are similar in proliferation, morphology and their expression of pluripotent cell marker genes. Furthermore, iPSCs have a similar transcription profile and both have the ability to form teratomas. Recently, differences have been reported about the epigenetic modification and global methylation patterns. However, hESCs are regarded as the “gold standard” in regenerative medicine, but the ethical issues of the origin of these cells, namely human embryos, makes the application of hESCs controversial. iPSCs can be easily derived from a patient’s skin biopsy, and even more simply from blood cells, which is a rather novel technique. Furthermore, iPSCs can be directly derived from the patient. This fact would overcome the second main disadvantage of hESCs of their potential graft-versus-host reaction where the immune cells in the tissue recognize

the recipient as “foreign” and the transplanted immune cells then attack the host’s body cells.

The reprogramming protocol, initiated by Yamanaka and Takahashi, is based on a retroviral vector, carrying four transcription factors. As the retrovirus integrates into the genome of its host, it makes the derived iPSCs unusable for clinical applications. In the last years, and even months, scientists were able to expand the field of induced pluripotent stem cells. Researchers all over the world already made a step further into the derivation of safe and for clinical applications acceptable iPSCs (Fusaki et al., 2009).

This thesis shows an overview of the field of iPSCs and the generation of disease-specific induced pluripotent stem cells by different techniques, such as by the Sendai virus, first employed by Fusaki et al. in 2009, new feeder-free culture systems, and novel techniques, like the microRNA-enhanced mRNA transfection or the reprogramming of erythroblasts to iPSCs.

## 1.1 Human Embryonic Stem Cells

Embryonic stem cells are pluripotent cells and originate from the inner cell mass (ICM) or epiblast. In 1981, two groups successfully cultured these cells from mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). The ability of ES cells to contribute to all cell lineages has been demonstrated in vitro and in vivo (Wobus and Boheler, 2005). This means, that ES cells have the ability to differentiate into any derivative of the three embryonic germ layers: the endoderm, mesoderm and ectoderm germ layer (Figure 1). But ES cells cannot differentiate into extraembryonic tissue and lack the ability to form an embryo itself.

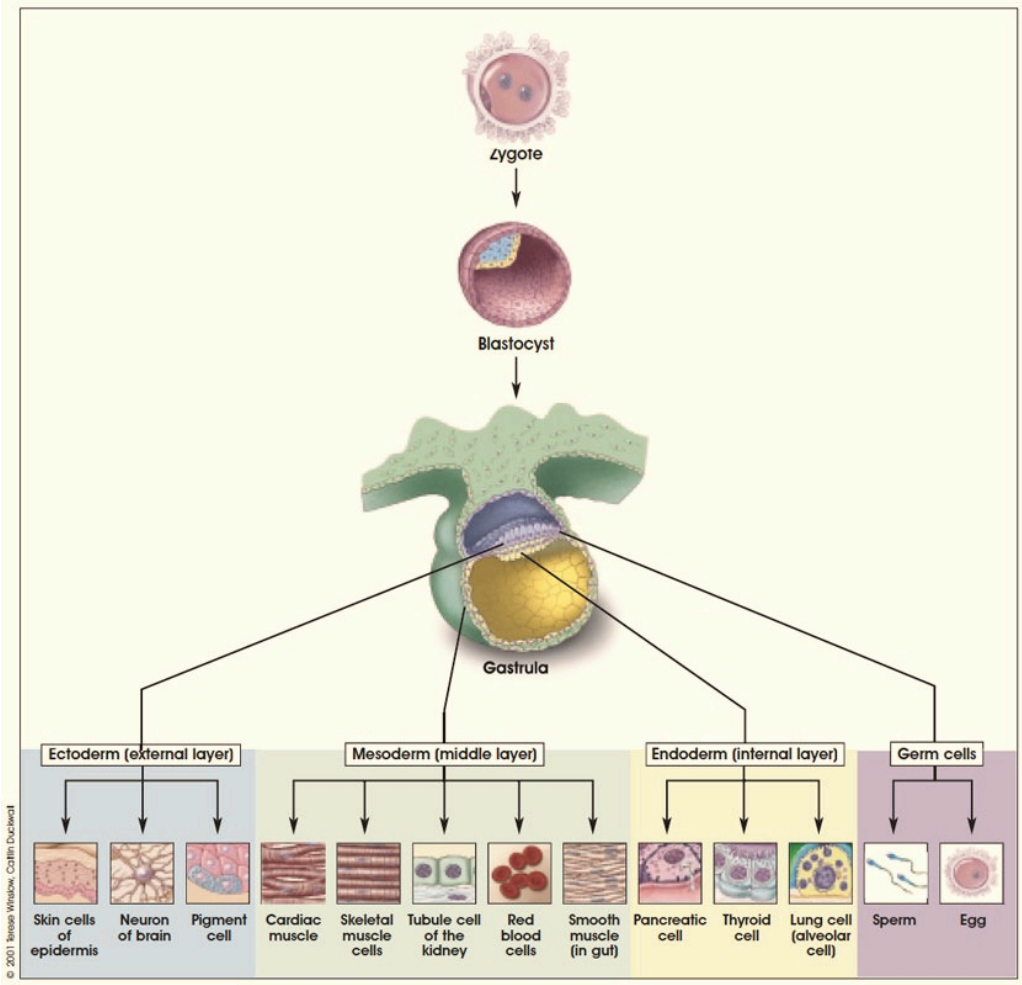


**Figure 1. Differentiation of human tissue.**

The three germ layers in human development: ectoderm (external layer), mesoderm (middle layer) and endoderm (internal layer). During early embryogenesis in humans and in almost all animal species, three germ layers are formed and developed later into specific parts and cells of the human body (Winslow, 2001)

Human ES cells (Thomson, 1998) share together with induced pluripotent stem cells (Takahashi et al., 2007) the pluripotency, but both have different origins and also different characteristics and functionalities.

Under specific culture conditions, hES cells proliferate indefinitely without senescence. Furthermore, they are able to differentiate into almost all tissue-specific cell lineages, what makes them attractive candidates for cell replacement therapy and open exciting new opportunities to model human embryonic development in vitro (Keller, 2005). ES cell models open, besides the developmental biology and cell-based therapy,

applications in the areas of drug discovery and development (Gorba and Allsopp, 2003). ES cells can be derived and maintained in an undifferentiated state and could be expanded for an infinite time range (Thomson, 1998). Summarizing all the advantages for regenerative medicine, ES cells are describes as the “gold standard” for biomedical research. But there is still the controversial fact, that these cells are derived from human embryos.

## 1.2 Induced Pluripotent Stem Cells

### 1.2.1 Somatic Cell Reprogramming

Somatic cell reprogramming is defined as the transformation of somatic cells into an induced pluripotent state by the overexpression of defined factors known to be involved in determining pluripotency in cells (Lewitzky and Yamanaka, 2007).

In the 1950s, the first cellular reprogramming was reported by Briggs et al. and King et al. when they demonstrated the developmental potential of single nuclei isolated from late-stage frog embryos by the transplantation into enucleated oocytes (Briggs and King, 1952; King and Briggs, 1955). In 1997, the first mammal was cloned: the sheep “Dolly” (Wilmut et al., 1997), followed by several other mammals and the reprogramming of terminally differentiated cells (Eggan et al., 2004; Hochedlinger and Jaenisch, 2002). These successful experiments, although many cloned animals showed abnormalities with regard to phenotypes and gene expression, indicating errors in the reprogramming process, were the proof of the principle that fully differentiated cells remain genetically totipotent. In 1964, the fusion of somatic cells with pluripotent embryonal carcinoma cells was another breakthrough (Miller and Ruddle, 1976) and in 1981 the first embryonic stem cells were isolated (Evans and Kaufman, 1981; Martin, 1981)

### 1.2.2 Yamanaka Factors

One strategy of reprogramming somatic cells into induced pluripotent stem cells is the fusion of somatic cells with embryonic stem cells (Miller and Ruddle, 1976). This method indicates that embryonic stem cells have factors that induce pluripotency (Yamanaka, 2007). Yamanaka reported the testing of 24 different candidate factors and this analysis led to the demonstration that retrovirus-mediated introduction of four transcription factors into mouse embryonic or adult fibroblasts results in the generation of iPSCs: Oct-3/4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007).
The more stringent selection marker Nanog has demonstrated a significant improvement of these four factors (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). The expression of Oct4, Sox2 and Nanog blocks the differentiation and promotes self-renewal (Biswas and Hutchins, 2007; Ivanova et al., 2006). Also other combinations of factors were used to generate iPSCs (Yu et al., 2007). But the Yamanaka-Factors (often used without c-Myc), remain as the standard method for reprogramming somatic cells into iPSCs.

#### 1.2.2.1 Oct4

Oct4 is the abbreviation for Octamer-binding transcription factor-4 (also known as Pou5f1 (POU class 5 homeobox 1) or Oct-3/4) and is a member of the POU protein family. All Oct family transcription factors contain the POU domain. Oct4 was identified as protein of the Oct family, which is specifically expressed in embryonic cells, in early embryos and germ cells (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990) and is essential for the maintaining of a pluripotent state in ESCs as well as the formation of the pluripotent inner cell mass. The Oct4 expression is associated with tumors and with an undifferentiated phenotype. Hence, the knockdown of Oct4 promotes differentiation and tests in mouse embryos that are Oct4-deficient fail to form the inner cell mass and die (Niwa et al., 2000; Yamanaka, 2007). Oct4 demonstrates an essential role in human embryonic stem cell self-renewal and for that reason also in induced pluripotent stem cells.

#### 1.2.2.2 Sox2

The protein Sox2 was identified as a Sox (SRY-related HMG box) protein in embryonic cells (Yuan et al., 1995). It is also known as Sox-2 or SRY (sex determining region Y)-box 2 protein and is as transcription factor essential in embryonic stem cells for pluripotency, the undifferentiated state and for maintaining self-renewal. The HMG (high mobility group) domain is a DNA binding domain, which binds DNA with little or no sequence specificity. Sox2 is, like Oct4, also a marker for the pluripotent lineage of the early embryo. This protein is expressed in the inner cell mass, the epiblast and in germ cells. Unlike the transcription factor Oct4, it is also expressed by multipotent cells of the extraembryonic ectoderm. Sox2 is highly involved in the embryonic development and governing cell fate decisions (Avilion et al., 2003; Brazel et al., 2005; Pevny and Placzek, 2005; Pevny et al., 1998). Avilion et al. reported that Sox2 null embryos die due to a failure of epiblast development (Avilion et al., 2003) and the deletion of Sox2 in embryonic stem cells results in differentiation (Masui et al., 2007).

Furthermore, Sox2 forms a heterodimer with Oct4 and regulates the transcription of specific downstream targets (Kamachi et al., 2000; Wilson and Koopman, 2002). In addition, it has been reported that these two proteins coregulare themselves (Chew et al., 2005; Tomioka et al., 2002), as well as Nanog (Kuroda et al., 2005; Rodda et al., 2005). Oct4, Sox2 and Nanog are essential regulators in early embryonic development and in the maintenance of plurpotency.

#### 1.2.2.3 Klf4

Klf4 is a zinc-finger protein and belongs to the Krüppel-like factors (Schuh et al., 1986). It is expressed in differentiated cells of the skin and the gastrointestinal tract and in fibroblasts of MEF and NIH3T3 cells (Shields et al., 1996). Klf4 is also expressed in undifferentiated mouse ES cells and can function furthermore as tumor suppressor and

as oncogene. Segre et al. and Katz et al. reported, that Klf4 null embryos develop normally, but an impaired differentiation in the skin and colon and death in the first 15 hours after birth is shown in newborn Klf4 null mice (Katz et al., 2002; Segre et al., 1999). Klf4 is associated with oncogenesis and tumor suppression and plays an essential role in the shift between differentiation and proliferation.

#### 1.2.2.4 c-Myc

c-Myc is the fourth important factor in the reprogramming of somatic cells to iPSCs. Controversially, it is known as a proto-oncogene, what means that it is a normal gene that can become an oncogene due to mutations or increased expression and was found for the first time in human cancers (Dalla-Favera et al., 1982).
Myc is located on chromosome eight in the human genome and binds after dimerization with its partner protein Max to DNA enhancer box sequences (E-boxes). It also recruits histone acetyltransferases, which means that Myc also regulates global chromatin structures by the regulation of histone acetylation besides its function as classical transcription factor.
c-Myc null mice embryos die after 10 days of gestation and it causes defects in vasculogenesis and primitive erythropoiesis (Baudino et al., 2002). Despite, earlier-stage embryos and embryonic stem cells show normal proliferation as well as self-renewal without c-Myc. Hence, it might be the case that other Myc family members compensate the function of c-Myc (Cartwright et al., 2005).

The Harvard Stem Cell Institute iPS core facility uses c-Myc as fourth transcription factor for the reprogramming of somatic cells to iPSCs despite the fact that c-Myc can cause cancer. The reprogramming without c-Myc is possible but would take up to three weeks longer than with OSKM and the efficiency is much lower.

### 1.2.3 Cell Types for Reprogramming

Several factors have to be considered when choosing the optimal cell type for a given application. Many studies reported that this choice plays an important role in the efficiency and in the kinetics of the whole reprogramming process (Gonzalez et al., 2011; Maherali and Hochedlinger, 2008).
It is very important to consider the ease at which the reprogramming factors can be introduced. Also if the cells are available and the ease of derivation of the given cell type have to be considered. Furthermore, the age and source are critical points. Older cells that have undergone several passages in culture may have genetic lesions, which decrease the therapeutic potential of the resulting iPSCs.
The most common cell type used for reprogramming are still fibroblasts. But obtaining fibroblasts normally requires a skin punch biopsy followed by expansion of the cells in culture. Recently, a number of groups reported the reprogramming of human peripheral blood cells into iPSCs. In contrast to fibroblasts, the collection of blood cells can be performed by minimally invasive procedures, the discomfort and the stress for the patient greatly reduced.

This assay shows the Sendai virus mediated reprogramming of the most common cell type for reprogramming: fibroblasts. As wells as the reprogramming of peripheral mononuclear blood cells (PBMCs) expanded to erythroblasts.

#### 1.2.3.1 Fibroblasts

The first cells used for reprogramming were mouse and human fibroblasts and have been reprogrammed for the first time by nuclear transfer in mouse (Wakayama et al., 1998) and cell fusion in mouse and human (Tada et al., 2001). The derivation of fibroblasts is technically simple and the cells can be cultured in ESC culture conditions. Their ability to expand, endure for multiple passages in culture, and receptiveness to efficient infection by viruses expressing a combination of transcription factors for

reprogramming make them to the predominate source material for the development of the process to generate induced pluripotent stem cells.
A number of mouse cell types besides fibroblasts were successfully reprogrammed in the last years: stomach and liver cells (Aoi et al., 2008), pancreatic β cells (Stadtfeld et al., 2008), neural progenitor cells (Eminli et al., 2008; Kim et al., 2008), as well as human keratinocytes (Aasen et al., 2008).

#### 1.2.3.2 Peripheral Blood Cells

For clinical applications, it is desired to find alternative cell types for the reprogramming to iPSCs by the overexpression of several transcription factors. In contrast to fibroblasts, blood is a cells source that can be easily obtained from most patients. The procedure to collect blood is minimally invasive and the stress for the patient is maximally reduced.
In 2010 Hanna et al. reported the reprogramming of lymphocytes (Hanna et al., 2010) and in 2009 another group described the successful reprogramming of human CD34+ hematopoietic stem cells (Loh et al., 2010). But these cells were isolated from peripheral blood by previous mobilization of G-CSF. This procedure is frequently associated with side effects, such as headache, bone pain, fatigue and nausea.
Haase et al. described the derivation of iPSCs from human cord blood (CB)-derived endothelial celsl by means of lentiviral overexpression of defined factors (Haase et al., 2009).
Researchers then set the target on the reprogramming of human peripheral blood cells and in 2010, Staerk et al. and Loh et al. reported the successful reprogramming of T Cells from peripheral blood (Loh et al., 2010; Staerk et al., 2010). Peripheral blood would circumvent all the issues from the reprogramming of CD34+ cells. Furthermore, peripheral blood is the most accessible tissue and permits access to numerous frozen samples already stored at blood banks. Mononuclear blood cells are isolated by Ficoll-Hypaque density gradient centrifugation (Vissers et al., 1988) and the samples are then expanded to T-Cells with defined medium and growth factors. The hurdles, reported by these two groups, are the relatively low efficiency of reprogramming blood cells and that

blood cells are difficult to infect. To increase the efficiency, a higher multiplicity of infection (MOI) was used.
The ability to reprogram peripheral blood cells will greatly facilitate the development of efficient and safe ways of generating patient-specific pluripotent stem cells.

In 2012, two groups reported protocols for the reprogramming of erythroblasts, expanded from PBMCs, to iPSCs (Sommer, 2012; Yang, 2012). The Harvard Stem Cell Institute adapted these protocols and the successful reprogramming of erythroblasts is described in this thesis.

### 1.2.4 Reprogramming Methods

There are various reprogramming strategies reported and studied for reprogramming somatic cells to induced pluripotent stem cells. The choice of the right reprogramming strategy is one of the main criteria for a successful derivation of iPSCs. It depends on the given cell type and the further use of the iPSCs to be generated.
In general, there are main groups of reprogramming methods. The methods are basically grouped based on the occurring integration of the transgenes into the host genome or not.

Table 1 shows an overview of reprogramming methods currently used. The methods are divided into three main groups: integrative, non-integrative and DNA-free methods.
The probably most known reprogramming method is the integrative retroviral reprogramming. It was the first approach for reprogramming somatic cells by the delivery of defined factors. The factors are constitutively employed into active retroviral vectors, which integrate into the genome of the host cell. Yamanaka and colleagues reported the very first approach for retroviral reprogramming in 2006 for mouse fibroblasts and in 2007 for human fibroblasts (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).
Other integrative methods are based on the lentivirus and excisable vector systems. The problem with these integrative reprogramming strategies is the fact that the virus is

randomly integrated into the genome. This could result in an alteration of the gene expression of oncogenes or tumor suppressor genes. That is the reason why iPSCs derived from these methods, are excluded for clinical applications.

In 2009, Fusaki et al. reported the first derivation of iPSCs by Sendai virus mediated reprogramming of somatic cells. The Sendai virus is a non-integrative virus and is the most frequent method used for somatic cell reprogramming. Other non-integrative methods, such as adenoviral vectors or plasmids for induction of iPSCs were developed, but the risk of integration still remains as far as DNA-type vectors are used (Harui et al., 1999).

The focus of this thesis is set on the relatively young Sendai virus mediated reprogramming and the novel microRNA enhanced mRNA transfection.

**Table 1. Overview - Reprogramming Methods.**

A summery of currently used methods for the reprogramming of somatic cells to induced pluripotent stem cells is shown. The strategies are divided into three main groups based on the integration of the vector into the genome of the host.

| | **Reprogramming Strategy** |
|---|---|
| Integrative | Retroviral Vector |
| | Lentiviral Vector |
| | Excisable Vector Systems |
| | - Piggy Bac Transposons |
| | - Lentiviral System using Flox sites |
| Non-integrative | Sendai virus-based vectors |
| | Episomal vector |
| | Adenoviral vector |
| DNA-free Methods | mRNA Transfection |
| | microRNA-enhanced mRNA Transfection |
| | Protein-mediated Reprogramming |

#### 1.2.4.1 Sendai virus

The integration of foreign genes into the host genome is a technical hurdle for the clinical applications of the derived iPSCs. In 2009, Fusaki et al. reported for the first time

the Sendai virus mediated reprogramming, an efficient solution for generating safe iPSCs (Fusaki et al., 2009). The Sendai virus is an RNA virus and carries no risk of altering the host genome. Sendai virus vectors replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells. They do not go through a DNA phase nor integrate into the host genome. Because the Sendai virus infects cells by attaching itself to the sialic acid receptor present on the surface of many different cells, it can infect a wide range of cell types.

Additional advantages are the moderate toxicity of the Sendai virus (Li et al., 2000) and the exogenous gene expression capabilities are well controllable (Tokusumi et al., 2002). These facts make the Sendai virus to a preferable method to reprogram safe iPSCs and have been used for studying various diseases (Ferrari et al., 2007).

With this method, Fusaki and colleagues were able to enter a new stage by the development of a method for epigenetic reprogramming without genetic modification. The only drawback shown by the Sendai virus, is that it can take up to 20 passages to eliminate viral transgenes entirely. However, compared to the integrative methods available, has the Sendai virus significant advantages for its safety, efficiency and convenience.

#### 1.2.4.2 microRNA-enhanced mRNA Transfection

To generate induced pluripotent stem cells for therapeutic applications such as disease modeling, drug discovery and regenerative medicine, the development of efficient DNA-free reprogramming techniques are required (Angel and Yanik, 2010). Also, the low efficiency of viral reprogramming is still a drawback (Li and Rana, 2012; Warren et al., 2010). The success of DNA-free strategies to reprogram fibroblasts into iPSCs has provided an opportunity to potentially overcome the obstacles carried by iPSCs reprogrammed by viral methods.((Lai et al., 2011).

One technique that avoids using DNA is called protein-mediated reprogramming. It utilizes the four Yamanaka factors in the form of proteins fused with peptides. In 2009, Zhou et al. reported the first successful generation of piPS cells: protein-induced

pluripotent stem cells (Kim et al., 2009; Zhou and Freed, 2009). Purified recombinant reprogramming factor proteins expressed in *E.Coli* inclusion bodies were fused to polyarginine peptide tags to allow the passage of the proteins through the membrane when added to the culture medium. This very complicated technique had a low induction efficiency, which must be overcome for clinical applications (Miyazaki et al., 2012).

In 2010, Warren et al. described another approach for somatic cell reprogramming that avoids the use of DNA, viral vectors, or plasmids (Warren et al., 2010). They developed a strategy for reprogramming based on the administration of synthetic modified mRNA with conversions and kinetics superior to DNA-based methods. By using Lin28 in addition to the four Ymanaka factors (OSKML), Warren and colleagues were able to achieve efficiencies of 2-4 % in the derivation of RNA-produced iPS cells (RiPS). Colonies appeared in half of the time needed for the viral reprogramming of somatic cells. Despite these advantages of mRNA transfection, there are disadvantages such as the daily transfection for more than two weeks and the oncogenic effects of the high dosage of reprogramming factors (Yakubov et al., 2010).

The roles of microRNAs in the control of pluripotent stem cells were clearly established by the discovery that embryonic stem cells lacking mature microRNAs show defects in proliferation and differentiation (Kanellopoulou et al., 2005; Murchison et al., 2005). Recent studies show that microRNAs play an important role in the gene networks controlled by the pluripotency factors Sox2, Oct4 and Nanog (Barroso-delJesus et al., 2008; Marson et al., 2008). Judson et al. first described the effects of microRNA expression to promote somatic cell reprogramming to iPSCs (Judson et al., 2009). Other strategies, likethe reprogramming of somatic cells by microRNA clusters in the absence of exogenous reprogramming transcription factors, were tested (Anokye-Danso et al., 2011; Miyoshi et al., 2011). Anokye-Danso's work involved using lentiviral integration to express microRNA clusters and Miyoshi et al. were able to deliver synthetic microRNAs to reprogram somatic cells with low efficiency.

In 2012, the scientists at Stemgent combinedthese two approaches that used mRNA and microRNA into a successful method for somatic cell reprogramming: microRNA-enhanced mRNA transfection. This system includes microRNA and five transcription factors (OSKML). In less than two weeks, patient fibroblasts are reprogrammed to iPSCs at a high efficiency on a feeder-free cell culture system. Also, culturing iPSCs in a hypoxic environment (5 % O2) has been shown to influence the cellular reprogramming by increasing the efficiency to up to four times (Yoshida et al., 2009). The successful execution of this novel method is shown in this thesis.

### 1.2.5 Cultivation of iPSCs

Culture and derivation conditions are essential parameters when working with iPSCs. Medium compositions and the decision if culturing the cells in a feeder culture system or without feeder cells has the ability to regulate and influence the entire reprogramming process and its efficiency, as well as the further expansion and characterization of the cells.

Human iPSCs and mouse iPSCs need to be cultured under the same culture conditions used for ESC maintenance (Akutsu et al., 2006; Cowan et al., 2004; Lerou et al., 2008; Maherali and Hochedlinger, 2008). It is essential to support growth, self-renewal and especially the maintenance of the pluripotent state of iPSCs with the right choice of the culture conditions. (Levenstein et al., 2006; Xu et al., 2005). Reports show the addition of valproic acid (VPA), which is a histone deacetylase inhibitor, increase the reprogramming efficiency (Huangfu et al., 2008). Other groups report the better survival of the cells by the addition of Rho-assiciated kinase (ROCK) (Li et al., 2009; Park et al., 2008; Watanabe et al., 2007).

Many typical components for culturing iPSCs are still from animal sources, such as fetal bovin serum (FBS), bovine serum albumin (BSA), irradiated mouse embryonic feeder cells (MEFs) or animal-derived enzymes like trypsin. These products, derived from animals, make iPSCs unsafe and unsuitable for clinical applications (Rao and Zandstra,

2005). Many stem cell scientists pay now great attention to feeder- and xeno-free derivation and maintenance of hiPSCs to make them suitable for potential clinical applications and to fulfill mandatory good manufacturing practice standards (GMP) (Unger et al., 2008).

In the last years and even months, new protocols for feeder-free induced pluripotent stem cell derivation were created, new medium was established. That is the reason why one focus of this thesis is the comparison of iPSC derivation and maintenance on a traditional feeder-cell culture system and on new feeder-free culture systems.

#### 1.2.5.1 Feeder-free Culture System

Previous studies show the growth of iPSCs on Matrigel coated plates in MEF-conditioned medium (MEF-CM) (Xu et al., 2001). But the derivation of iPSCs still took place on a MEF layer as the reprogrammed cells were transferred from the feeder-cell layer to Matrigel coated plates, which is includes again cells from animals.
In 2011, Chen et al. reported a step further into an iPSC culture system completely without animal derived products. They developed a xeno-free and feeder-free medium for the growth and expansion of human pluripotent stem cells: E8 medium (Chen et al., 2011). This medium was then adapted and extensively tested by Life Technologies as Essential 8$^{TM}$ Medium (Prototype). One year later, Beers et al. reported the application of this medium for iPSC growth and expansion on vitronectin coated cell culture plates. In addition to the application of this medium with just eight components, they demonstrated the passaging of iPSCs without enzymes. They show a protocol for using EDTA for passaging iPSCs, which achieves maximum cell survival without enzyme neutralization or centrifugation and in a short time of 6-7 minutes (Beers et al., 2012).

This assay shows the cultivation of iPSCs and also the reprogramming on vitronectin with E8 medium. As the E8 medium contains substances, which block the reprogramming of fibroblasts into iPSCs, a second medium for feeder free reprogramming is demonstrated: N2B27. In 2006, this medium, that is ususally a

medium for the expansion of neuronal cells, was used from Liu et al. for the undifferentiated growth of hESCs (Liu et al., 2006). The Harvard Stem Cell Institute iPS core facility adapted the N2B27 medium for a reprogramming trial of somatic cells by retroviral transduction.

### 1.2.6 Characterization of iPSCs

To ensure the identity of fully reprogrammed induced pluripotent stem cell lines, a detailed characterization is required. To determine chromosomal abnormalities, gene expression, differentiation capabilities and expression of antigens, several commonly accepted assays have to be performed in order to ensure the generation of fully reprogrammed iPSCs (Daley et al., 2009; Ellis et al., 2009).
The Harvard Stem Cell Institute iPS Core facility determined an array of methods, which are used for this thesis, to ensure pluripotent iPS cells have been generated.

iPSCs must be morphologically similar to ESCs and must present identical features to ES cells as for example the round shape of the cells. The cells have to form sharp-edged, flat, tightly-packed colonies similar to that of ESCs and demonstrate unlimited self-renewal. iPSCs need to express cell surface antigenic markers expressed on ESCs such as SSEA3, SSEA4 and Tra-1-60. They have to show gene expression profiles of key pluripotency genes as Oct4, Nanog, Dnmt3b and others. On a functional level, iPSCs must have the ability do differentiate into all three germ layers (endoderm, mesoderm, ectoderm), which is assessed by in-vitro differentiation into embryoid bodies and in-vivo differentiation into teratomas in immunodeficient mice.

#### 1.2.6.1 G-Band Karyotyping

Genetic abnormalities in the derived iPSCs have to be excluded by G-band karyotyping analysis to detect potential chromosomal abnormalities.
iPSCs are telomerase positive and display extensive capabilities for self-renewal. Because of this extensive replication, stem cells have the potential for mutations that

may subsequently alter cellular functions (Henson et al., 2005). Another reason for potential chromosomal abnormalities in iPSCs is the prolonged culturing of the cells, recently reported by two groups (Gore et al., 2011; Lister et al., 2011). This long-term culturing can cause point mutations or chromosomal translocations in the genome of iPSCs. Additionally, Lerou et al. reported in 2007 that ES cells have a tendency to harvest genetic abnormalities in general (Lerou et al., 2008).

#### 1.2.6.2 Alkaline Phosphatase Staining

It is necessary to proof the undifferentiated state of iPSCs and ESCs. An easy method to characterize the undifferentiated state of pluripotent stem cells is the alkaline phosphatase staining (AP). Pluripotent stem cells have an increased level of AP on their cell membrane. AP is a stem cell membrane marker and elevated expression of this enzyme is associated with undifferentiated pluripotent stem cell. All primate pluripotent stem cells express alkaline phosphatase activity and the loss of AP expression is an early indicator for differentiation (Palmqvist et al., 2005).
AP catalyzes the removal of 5' phosphate groups from DNA, RNA, proteins and alkaloids. The process of removing the phosphate group is called dephosphorylation and the enyme alkaline phosphatase is most effective in an alkaline environment and exists in all body tissues. The hydrolysis of p-nitrophenylphosphate into phosphate and p-nitrophenol is catalyzed by the enzyme. The amount of p-nitrophenol produced is relative to the amount of the AP present in the reaction. It can be used for quantitative and qualitative analysis of AP.

There are different methods of detecting alkaline phosphatase. The method used in this thesis is based on an enzymatic reaction followed by fast red violet dye.

#### 1.2.6.3 Immunocytochemistry

Immunocytochemistry (ICC) is a technique that assesses the presence of proteins or antigens in cells by the use of specific antibodies (Marchenko and Flanagan, 2007). The

antibodies target specific peptides or antigens in the cells via these specific antibodies. There are different methods for detection, the common one is immunofluorescence and also used in this thesis. With ICC one can evaluate whether or not cells express the specific antigen and if yes, the determination of which sub-cellular compartments are expressing the antigen is possible.

In this thesis a so-called “indirect” method, which is more common in the iPS research, is used for ICC of pluripotent and differentiation markers. In an indirect method, the primary antibody binds to the antigen, which is then amplified by the use of a secondary fluorescent antibody, which binds to the primary one. Additionally, a nuclear counterstaining with DAPI is done.
The “direct” method uses a primary fluorescent antibody that binds to the target protein or antigen and can be visualized after one incubation period with UV light.

#### *1.2.6.3.1. Pluripotent Stem Cell Markers*

Induced pluripotent stem cells need to express the proteins associated with pluripotency. Specifically, the two transcription factors Oct4 and Nanog and the three cell surface markers SSEA3, SSEA4 and Tra-1-60 are targeted. These markers are known to be expressed in hESCs and for that reason they should also be expressed in iPSCs. The secondary fluorescent antibody are detected by immunofluorescence.

#### *1.2.6.3.2. Differentiation Stem Cell Markers*

Upon differentiation, induced pluripotent stem cells can form cells of all three germ layers. To ensure that derived iPSCs are fully reprogrammed, the expression of differentiation markers are checked on Embryoid Bodies (EBs). Three differentiation markers, one for each germ layer, are tested in this thesis: β-Tubulin for the ectoderm germ layer, SMA for the mesoderm germ layer and SOX17 for the endoderm germ layer were examined.

#### 1.2.6.4 Analysis for the Expression of Pluripotent Genes

Another important assay to prove that the derived iPS cells are pluripotent and to determine the molecular criteria for pluripotency is the characterization of a gene expression profile of iPSCs for pluripotency markers. Six important key pluripotency related genes are assessed by gene expression: Oct4, Nanog, Sox2, Dnmb3b, hTERT and Rex1.
It is absolutely necessary to test the gene expression of iPSCs as the use of incomplete reprogrammed cells can result in the inability of full differentiation. Furthermore, the risk of the formation of immature teratoma tumors increases. That is the reason why iPSCs that can potentially be used for therapeutic purposes have to be tested in detail for their pluripotency (Li et al., 2008).

Polymerase chain reactions (PCR) with specific primer sequences are set up to exponentially amplify specific genes. The gene expression is determined by the extraction of RNA from iPS cell colonies. cDNA is synthesized and the expression profile of pluripotency-associated genes is analyzed by PCR with specific cycles with defined temperatures. The amplified cDNA can be then qualitatively analyzed by agarose gel electrophoresis.

#### 1.2.6.5 In-vitro Differentiation and Analysis of differentiation Markers

The full differentiation potential of reprogrammed iPS cells has to be tested on a functional level. In-vitro differentiation of iPS cells to Embryoid Bodies (EBs) and the analysis for differentiation markers of all three germ layers is performed. This assay describes the testing for two markers for each germ layer: the mesoderm markers FLK and GATA2, the endoderm markers AFP and GATA4, and the ectoderm markers PAX6 and N-CAM.
EBs are formed by plating iPSCs onto low-attachment cell culture plates without anti-differentiation factors to initiate EB formation. The EBs are then further cultured on a gelatin layer to promote the differentiation into cells of all germ layers.

EBs are three-dimensional aggregates of pluripotent stem cells and formed by the binding of E-cadherin, which is expressed in a high value on undifferentiated iPSCs or ESCs. Pluripotent stem cells initiate spontaneous differentiation towards the three germ layers by aggregating to form EBs when they are cultured without anti-differentiation factors. EBs summarize many aspects of cell differentiation during early embryogenesis (Itskovitz-Eldor et al., 2000; Kurosawa, 2007; Sheridan et al., 2012).

#### 1.2.6.6 In-vivo differentiation by Teratoma Formation

Teratoma formation is a landmark test for pluripotency and is considered as the “gold standard” for demonstrating differentiation potential of pluripotent hESCs and iPSCs (Lensch et al., 2007). It is the most accurate test for pluripotency in induced pluripotent stem cells, as teratoma tumors consist of multiple lineages containing tissue derived from the three germ layers (endoderm, mesoderm, ectoderm). This characteristic is unique, as other tumors typically form tissue derived from only one cell type (Gutierrez-Aranda et al., 2010; Meng et al., 2010).
iPSCs are injected or transplanted into immunodeficient mice and form spontaneously teratomas after approximately eight weeks. The tumors are explanted and are histological and immunohistochemical stained for tissue derived from all three germ layers.

In this thesis, iPSCs are surgically implanted into the sub-renal capsule, a fibrous layer surrounding the kidney, of immunodeficient mice.

The characteristic of teratoma formation that makes iPSCs a powerful tool in the future of medicine, creates on the other hand major clinical hurdles. The hurdle of the fine line that both separates and connects pluripotency and tumorigenicity is still not taken. Human embryonal stem cells, as well as induced pluripotent stem cells share many characteristics with embryonal carcinoma cells (hECs). On the other hand, teratoma

formation form hESCs or iPSCs has not yet been reported due to the predominantly pre-clinical stage where stem cell research currently is located. (Schwartz et al., 2012). However, in 2009, a report of a multifocal brain and spinal cord tumor in a male patient was reported after the transplantation of fetal neural stem cells for treatment of ataxia-telangiectasia in 2001 (Amariglio et al., 2009). There is still the need to assess the safety of such therapies to reach the full potential of hESCs and iPSCs in medicine and it is absolutely necessary to remove undifferentiated cells from the transplant to remove the risk of teratoma formation.

#### 1.2.6.7 Analysis for the Expression Level of Pluripotent and Differentiation Markers by real time PCR

Additionally to the gene expression profiles of key pluripotency and key differentiation genes by PCR, the expression level of pluripotent and differentiation markers can be tested by real time PCR (qPCR). Compared to the gene expression profile with PCR, the real time PCR brings quantitative information about the gene expression level, fold change and other additional information.
This assay shows the preparation and the analysis of real time PCR kits from Qiagen for iPSC pluripotency validation and embryoid body differentiation. The used kits consists of 96-well plates. The gene expression level of 19 different pluripotency or differentiation related genes of four cell lines can be analyzed on one plate at a time.

### 1.2.7 Applications

The iPS cell technology has the potential to overcome two important hurdles associated with human embryonic stem cells: immune rejection after transplantation and ethical concerns regarding the use of human embryos (Yamanaka, 2012). But the clinical application of iPS cells faces many obstacles. Some of them are shared with ES cells, some are new. Teratoma formation is one of the shared obstacles (Li et al., 2008). Teratomas can be formed out of a very small number of differentiated cells, so an important goal is to leave differentiated hESCs or iPSCs behind. There is also still little known whether or not nuclear reprogramming is complete for each iPS cell clone. Another issue is the presence of transgenes in the iPS cells, as many of the derived iPSCs are still reprogrammed by the retro- or lentivirus (Daley, 2012; Lister et al., 2011).

One short-term goal for iPSCs is to use this technology for drug or toxicology screens in vitro and for creating disease models in culture. These two concepts of future applications for iPSCs are shown in Figure 2 using Spinal Muscular Atrophy (SMA) as example (Stadtfeld and Hochedlinger, 2010).

The generation of in vitro disease models using iPSCs will be useful not only for drug screening, it will also be useful elucidating mechanisms of disease pathogens. Also the problem associated with studying degenerative diseases that reasearchers are usually confined to cell material from patients whose disease has already progressed to advanced stages at the time of diagnosis could be overcome with iPSC technology (Amabile and Meissner, 2009).

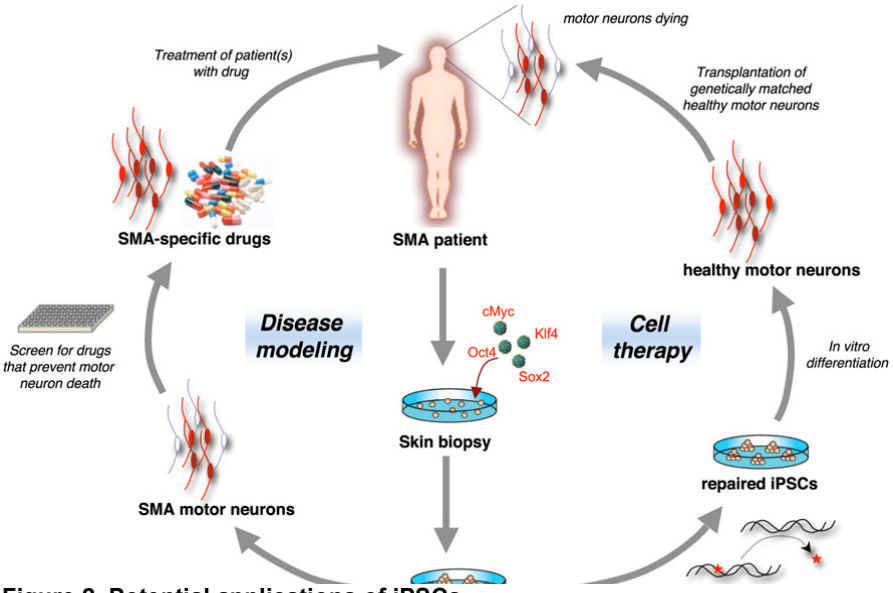


**Figure 2. Potential applications of iPSCs**

Shown are potential applications of iPSCs for disease modeling and cell therapy using Spinal Muscular Atrophy (SMA) as example. Potential therapies for SMA patients in the future could be established by deriving iPSCs from SMA patients, to differentiate them into motor neurons in vitro and to generate a culture model of the disease. With iPSCs it is also possible to screen for new drugs. Another possibility would be the reparation of a known disease-specific mutation in iPSCs by gene

The iPS cell technology is still in its infancy, but the potential of this technology is enormous. It will be essential to improve the methodologies for iPS cell generation and to precisely evaluate each clone and subclone of iPSCs for their safety and efficacy.

# 2 MATERIALS AND METHODS

## 2.1 Somatic Cell reprogramming on feeder using a Sendai Virus method

This section describes the generation of disease specific induced Pluripotent Stem Cells by the reprogramming of human fibroblasts using a Sendai Viral vector. The protocol that was used was described by Fusaki and colleagues in 2009 (Fusaki et al., 2009), and adapted by the Harvard Stem Cell Institute (HSCI) iPS Core Facility, Cambridge, MA, USA. The Sendai virus kit is commercialized by Life Technologies Corporation. A schematic timeline of the reprogramming process of diseased fibroblasts with Sendai virus is shown in Figure 3.

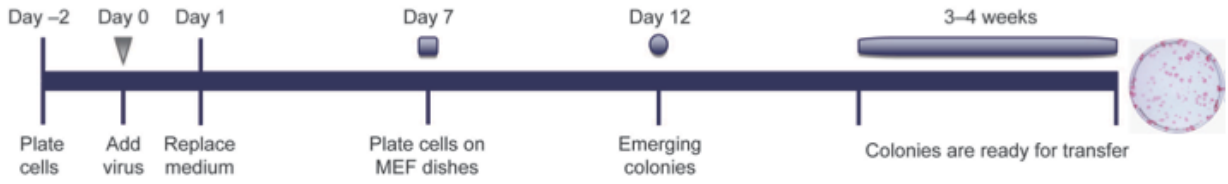


**Figure 3. Schematic timeline of the reprogramming process using Sendai virus.**

Timeline of cellular reprogramming using Sendai viruses involving the following major steps: seeding of donor fibroblasts, viral transduction, selection of reprogrammed cells and subsequent cultivation.

Primary fibroblasts from three patients with Type 1 diabetes, were received for reprogramming from an external investigator as part of a contract with the HSCI iPS core facility in Cambridge, MA, USA.

### 2.1.1 Transduction of fibroblast lines with the Sendai Virus

Frozen fibroblasts were obtained from the investigator and received in cryotubes on dry ice about one week before transduction. For thawing the cells, two 15 mL conical tubes

(VWR, Cat. No. 21008-936) were prepared. One tube contained 9 mL pre-warmed DMEM 10 % FBS (450 mL DMEM (Life Technologies, Cat. No. 11995-073), 50 mL FBS (VWR, Cat. No. 45000-734), 5 mL P/S (Life Technologies, Cat. No. 15140-122) and 5 mL L-Glutamine (Life Technologies, Cat. No. 25030-081) filtered with a 0.22 µm filter bottle (VWR, 28199-778) and one was empty. The frozen cells were partially thawed at 37 °C in the waterbath until only a small piece of ice remained. The pre-warmed medium was added dropwise to the cryotube 1 mL at a time. The liquid containing the cells was then transferred into the second empty tube. This was repeated until all 9 mL of the media were used. The cells were spun down at 1,200 rpm for 2 min. Depending on the size of the cell pellet, cells were either plated into one well of a 6-well cell culture multiwell plate (VWR, Cat. No. 82050-842) or into a T25 cell culture flask (VWR, Cat. No. 82051-074) by aspirating the media and resuspending the cells in 2 mL or 7 mL respectively of DMEM 10 % FBS. The fibroblasts were incubated in standard cell culture conditions (37 °C, humidified, 5 % $CO_2$/Air).

All human fibroblast samples were tested for mycoplasma prior to transduction. In order to do this test, the cells were cultured in DMEM 10 % FBS without antibiotics (P/S) overnight. The next day, the medium was tested for mycoplasma using the MycoAlert® Mycoplasma Detection Kit (Lonza, Cat. No. LT07-418).

The fibroblasts were trypsinized (Life Technologies, Cat. No. 25300-062) by washing the cells once with DPBS (Life Technologies, Cat. No. 14190-250), adding 1 mL of Trypsin and incubating for 3-4 minutes at 37 °C. The cells were looked under the microscope to determine if they had detached. When the cells were fully detached, 1 mL of DMEM 10 % FBS was added to neutralize the Trypsin. Next, the cells were collected into a 15 mL conical tube and centrifuged at 1,200 rpm for 5 minutes. After centrifugation, the media was aspirated and the cell pellet was resuspended in pre-warmed DMEM 10 % FBS. The number of cell was determined by an automated cell counter (Bio Rad Laboratories, CA, USA). For each sample of fibroblasts, 250,000 cells were plated into 1 well of a 6-well plate. The cells were incubated in standard cell culture conditions overnight.

On the day of transduction (D0) the media of the fibroblasts was changed to pre-warmed DMEM 10 % FBS without P/S at least 4 hours prior to transduction.
The Sendai virus used for reprogramming was the CytoTune™-iPS Sendai Reprogramming Kit, which contains the four viruses that encode the four transcription factors: Oct4, Sox2, Klf4 and c-Myc (Life Technologies, Cat. No. A1378001). For each well to be transduced, a 15 mL conical tube containing 1 mL of DMEM 10 % FBS without P/S was prepared and pre-warmed at 37 °C. One CytoTune™ Kit was taken out of the -80°C storage. The tubes were thawed one at a time by immersing the bottom of the tube in the 37 °C water bath for 10 seconds, spinning it down quickly and thawing it at room temperature before placing it on ice. For the transduction of fibroblasts with the Sendai virus a multiplicity of infection (MOI) of 3 was used. According to the different titers of each virus, the necessary volume was calculated to add $7.5 \times 10^5$ CIU (colony forming units) to the 1 mL of DMEM medium. Subsequently, the medium was aspirated from the well of fibroblasts and 1 mL of the viral vector solution was added. The cells were incubated at standard cell culture conditions overnight. All tools that came in contact with the viral solution were immediately treated with vesphene (VWR, Cat. No. 21899-870) and discarded.

The following day (24 hours after transduction on D0) the wells were washed twice with DPBS and the media was replaced with fresh DMEM 10 % FBS with antibiotics. The medium was then changed every other day.

Six days after transduction (D6), two 10-cm cell culture dishes (VWR, Cat. No. 82050-916) were coated with 0.1 % gelatin (Millipore, Cat. No. ES-006-B) at room temperature for 20 minutes for each sample. After coating, 1 million irradiated mouse embryonic feeder cells (MEFs) (GlobalStem, Cat. No. GSC-6201G) was plated onto each plate. The MEFs were thawed, spun down and plated the same way as the frozen fibroblasts were plated.

On day 7 (D7), the transduced fibroblasts were harvested and replated onto the 10-cm cell culture dishes plated on D6. The cells were washed once with DPBS, trypsinized for 5 minutes, and scraped with a cell scraper to detach any remaining cell (VWR, Cat. No. 29442-200), collected into a 15 mL conical tube and spun down at 1,200 rpm for 5 minutes. In the meantime the medium of the prepared cell culture dishes with MEFs was replaced to 5 mL of DMEM 10 % FBS each. The cells were resuspended in 10 mL of DMEM 10 % FBS and 5 mL were added to each of the 10-cm dishes to reach a total volume of 10 mL per dish.
24 hours later, the cells were washed with DPBS, and the medium was changed to hESC medium (400 mL DMEM/F-12 (Life Technologies, Cat. No. A1377801), 100 mL KnockOut$^{TM}$ Serum Replacement (Life Technologies, Cat. No. 10828-028), 5 mL P/S, 5 mL L-Glutamine, 5 mL MEM Non-Essential Amino Acids Solution (Life Technologies, Cat. No. 11140-050) and 500 µL 2-Mercaptoethanol (Life Technologies, Cat. No. 21985023) filtered with a 0.22 µm filter bottle). Before adding the medium to the cells βFGF (Life Technologies, Cat. No. PHG0261) was added at a concentration of 10 ng/mL. Additionally one 10-cm plate per cell line was treated with VPA (Calbiochem, Cat. No. 67630) at a concentration of 1 µM for one week. The medium was then changed every other day for one week, followed by daily feedings until colony formation was observed (3 to 4 weeks after transduction, D25-30).

### 2.1.2 Picking and Cultivation of newly derived iPSC colonies

Starting at 10 to 15 days after transduction, visible changes in morphology were observed. Starting on D29, fully reprogrammed iPSC colonies were manually transferred to a feeder layer of irradiated MEFs in one well of a 6-well plate prepared one day before as described in 1.2. Selection of fully reprogrammed cells was based on morphology only. For one cell line a live-cell ICC imaging was carried out as described in 2.1.2.1.

Colonies were transferred by means of glass Pasteur pipettes shaped into “picking tools” (VWR, Cat. No. 14673-043) using a dissection microscope located in a biological

safety cabinet. Each colony was treated as one distinct cell line and therefore was picked one at a time by cutting the colony into pieces and transferring it to one well of a 6-well plate. Each colony was cultivated in hESC medium with βFGF with a one-time addition of 10 µM ROCK inhibitor (500X, Y-27632, EMD Chemicals Inc, Cat. No. 688001-500UG) in order to prevent cell death and increase attachment. The colony was labelled as passage 0 and cultured in standard conditions. The day after picking, the colony was not fed to ensure complete attachment of the colony pieces to the MEF layer.

#### 2.1.2.1 Live-cell ICC imaging of human pluripotent stem cells

For one plate in this reprogramming trial, a live-cell ICC imaging was performed because the plate was overgrown and visual identification of fully reprogrammed colonies by morphology was not possible.

For this live staining, the antibody Alexa Fluor ® 488 Mouse IgG1 Tra-1-60 (Biolegend, Cat. No. 303015) was briefly spun down for 30 seconds at room temperature and diluted at 1:50 in DMEM/F12 phenol redfree based hESC medium (400 mL DMEM/F12 phenol red free medium (Life Technologies, Cat. No. 11039-047), 100 mL KOSR, 5 mL P/S, 5 mL L-Glutamine, 5 mL NEAA and 500 µL 2-Mercaptoethanol) (for one 10 cm plate 100 µL Antibody and 5 mL hESC growth medium without phenol red were used). The diluted antibody was spun down at 1,200 rpm for 5 min. The cells were washed with DMEM/F12 phenol redfree medium, the diluted antibody was added, and the cells were incubated at 37 °C for 2 hours. After incubation, the cells were washed 3 times with DMEM/F12 phenol redfree medium and 5 mL of DMEM/F12 phenol redfree hESC medium were added. Images were taken under an inverted epifluorescence microscope (10X objective) to identify fully reprogrammed iPSC colonies. These colonies were picked with a Picking tool and transferred using a dissection microscope located in a biological safety cabinet to 1 well of a 6-well cell culture plate. The plate was previously coated with 0.1 % gelatin and an additional MEF layer. After picking the iPSC colonies, the remaining cells in the 10-cm plate were washed 3 times with DMEM/F12 phenol

redfree medium and 10 mL of regular hESC medium were added. The culture was continued in standard cell culture conditions.

## 2.2 Somatic Cell reprogramming on a feeder free system

This section describes the generation of disease specific induced Pluripotent Stem Cells by the reprogramming of human fibroblasts on two different feeder free systems.

### 2.2.1 Somatic cell reprogramming using a Sendai virus on Vitronectin in combination with the Prototype Essential 8™ Medium.

For the reprogramming of human fibroblasts to induced Pluripotent Stem Cells, a Vitronectin layer and the Prototype Essential 8™ Medium were used.

Fibroblasts, derived from a patient with Type 1 diabetes, were received from an external investigator. The reprogramming on this feeder-free system was only for research purposes of the HSCI iPS core facility in Cambridge, MA, USA and not part of a contract with the external investigator.

#### 2.2.1.1 Transduction of fibroblast lines with Sendai Virus

The method used for the transduction of the fibroblasts with the Sendai viruses is described in chapter 2.1.1, until D6 of the reprogramming process.

Seven days after transduction (D7), one 10-cm cell culture dish was coated with a Vitronectin layer (Truncated Recombinant Human (VTN-N) (prototype) Protein, Life Technologies, Cat. No. A14701SA). The 10-cm cell culture dish was coated with 60 µL of Vitronectin at a concentration of 0.5 mg/mL (60 µL of vitronectin diluted in 6 mL DPBS). The plate was incubated for 1 hour at room temperature in a biological safety cabinet.

First, the transduced fibroblasts were harvested: The cells were washed once with DPBS, and 1 mL of 0.5 mM Ultra Pure™ EDTA (Life Technologies, Cat. No. 15575) diluted in DPBS, was added to the well. The cells were incubated for 5 minutes at 37°C, until visible holes were formed in the layer of fibroblasts. The EDTA was aspirated, 1 mL of DMEM 10 % FBS was added, and the cells were detached by pipetting the medium up and down. The fibroblasts were collected in a 15 mL conical tube and spun down at 1,200 rpm for 5 minutes. After centrifuging the cells, the medium was aspirated and the cells were resuspended in 10 mL Essential 8™ Medium (Life Technologies, Cat. No. A14666SA, including DMEM/F-12 (HAM) 1:1, Cat. No. A14625DJ and Essential 8™ Supplement (50X), Cat. No. A14626SA). For the preparation of the E8 Medium, the Essential 8™ Supplement was thawed at 4 °C overnight and slowly added to the DMEM/F-12 (HAM). 5mL of P/S were added to the medium. Prior to use, the necessary amount of E8 medium was aliquot and pre-warmed at room temperature.
The vitronectin was aspirated and the 10 mL cell solution was added to the cell culture dish. The cells were fed every other day for one week followed by daily feedings until colony formation was observed (3-4 weeks after transduction, D25-D30).

The picking of newly derived iPSC colonies was performed as described in part 1.3. The only differences were the Vitronectin layer, the E8 medium and the splitting of the colonies with 0.5 mM EDTA. The general techniques for the cultivation of the iPSC colonies are as described in chapter 2.1.2.

### 2.2.2 Somatic Cell reprogramming on Vitronectin in combination with the N2B27 Medium using Retro Virus

For the reprogramming of human fibroblasts to induced Pluripotent Stem Cells, a Vitronectin layer and N2B27 medium were used. From D15 after transduction, the N2B27 medium was changed to the Prototype Essential 8™ Medium described in part 2.2.1.1.

Fibroblasts (MIN31621) derived from a patient with Dystonia, were received for reprogramming from an external investigator. The reprogramming on this feeder free system was only for research purposes of the HSCI iPS core facility in Cambridge, MA, USA and was not part of a contract with the external investigator.

#### 2.2.2.1 Transduction of fibroblast lines with retrovirus

Retroviral transduction was the method used for this fibroblast line. This method is now rarely used because the retrovirus is an integrative virus and stays in the genome. The methods for the transduction of fibroblasts with the retrovirus are not described in this paper.

As with the Sendai virus, the transduced fibroblasts are replated on a mouse embryonic feeder layer or on a Vitronectin layer. The focus of this chapter is D4 after transduction, when the cells are replated. Two wells of a 6-well cell culture plate, with 100,000 fibroblast cells each, were transduced for this experiment.

Four days after the transduction, two 10-cm cell culture dishes were coated with a Vitronectin layer. Each dish was coated with 60 µL of Vitronectin, at a concentration of 0.5 mg/mL (60 µL of vitronectin diluted in 6 mL DPBS). The plate was incubated for 1 hour at room temperature in a biological safety cabinet.

The transduced fibroblasts were harvested and replated onto 10-cm cell culture dishes, 1 well on 1 dish. The cells were collected using the protocol described in part 2.2.1.1. After centrifuging the cells, the medium was aspirated, and the cells were resuspended in 20 mL N2B27 Medium (470 mL DMEM/F-12, 5 mL N-2 Supplement (100X) (Life Technologies, Cat. No. 17502-048), 10 mL B-27® Supplement (50X) (Life Technologies, Cat. No. 17504-044), 5 mL MEM Non-Essential Amino Acids Solution, 5 mL L-Glutamine, 5 mL P/S and 500 µL 2-Mercaptoethanol, filtered with a 0.22 µm filter bottle).

Prior to use, the necessary amount of N2B27 medium was aliquoted and pre-warmed at 37 °C in the waterbath.

The Vitronectin was aspirated and 10 mL of the cell solution were added to each cell culture dish.

24 hours later, the medium was changed to fresh N2B27 medium containing 0.1 µL βFGF per 1 mL medium (10 ng/mL). Additionally, VPA was added to one 10-cm plate at a concentration of 1 µM medium for one week. The medium was changed every other day for 1 week. 15 days after plating the cells on a Vitronectin layer, the N2B27 medium was changed to Essential 8$^{TM}$ Medium and the cells were fed every other day until colony formation was observed (15-21 days after transduction).

The picking of newly-derived iPSC colonies was performed as described in part 1.3. The only differences were in the Vitronectin layer, the E8 medium and the splitting of the colonies with 0.5 mM EDTA. The medium N2B27 was just used for the reprogramming process. The general techniques for the cultivation of the iPSC colonies are as described in chapter 2.1.2.

## 2.3 Characterization of Reprogrammed iPS Cell Lines

To ensure the identity of fully reprogrammed induced pluripotent stem cell lines, a detailed characterization is required. To check chromosomal abnormalities, gene expression, differentiation capabilities and expression of antigens, several commonly accepted assays were carried out and results were analyzed.

### 2.3.1 Karyotyping

Each characterized iPS cell line was karyotyped in order to detect potential chromosomal abnormalities.
One well of a 6-well cell culture plate of the cells was split into one T25 cell culture flask coated with 0.1 % gelatin and a layer of irradiated mouse embryonic feeder cells. The medium (hESC with βFGF) was changed every day and cells were cultured in standard cell culture conditions until approximately 70 % confluency was reached after 5-7 days. The cells were then sent in the T25 cell culture flask to an external laboratory for

karyotyping analysis (Cell Line Genetics, 510 Charmany Drive, Suite 254, Madison, WI 53719). The results were usually received within 1 to 2 weeks.

### 2.3.2 Alkaline Phosphatase

As the undifferentiated state of iPS cells are characterized by a high level of alkaline phosphatase (AP) expression, an alkaline phosphatase assay was done to distinguish between undifferentiated and differentiated iPS cell colonies.
The assay was performed using an Alkaline Phosphatase Detection Kit (Millipore, Cat. No. SCR004) according to the manufacturer's protocol.

Around 5-7 days prior to doing the AP assay, approximately 5 colonies were manually picked with a Picking tool and transferred to 1 well of a 12-well cell culture plate (VWR, Cat. No. 82050-930) (the wells were coated with gelatin 0.1 % and a MEF layer, approximately 80,000 cells per well, the day before, as described). On the day the colonies were picked, the well of the 12-well plate was washed with DPBS and the medium was changed to hESC with βFGF. Cells were fed every day except the day after picking with hESC containing βFGF and cultured at standard cell culture conditions.

Approximately 5-7 days after picking the colonies the Alkaline Phosphatase Assay was performed.
The medium was aspirated and the cells were fixed with 500 µL 4 % Paraformaldehyde (PFA) in DPBS for 1-2 minutes under a chemical fume hood. It was important to not overfix the cells because this would result in the inactivation of AP. The fixative was aspirated and the cells were rinsed once with 500 µL 1 X rinse buffer (50 mL DPBS + 25 µL TWEEN®20 (Sigma-Aldrich, Cat. No. P1379)). Subsequently, the reagents for AP staining were prepared according to the manufacturer's protocol. To avoid the cells drying out, the well was covered with 500 µL 1 X Rinse Buffer during the preparation of the staining solution.

- Staining Solution
    - 2 parts Fast Red Violet Solution (delivered as part of AP detection kit - Millipore)
    - 1 part Naphtol AS/BI phosphate Solution (delivered as part of AP detection kit - Millipore)
    - 1 part Ultra-Pure™DNase-RNase-Free Distilled Water (Life Technologies, Cat. No. 10977)

The rinse buffer was aspirated and enough staining solution was added to cover the well (250 µL). The plate was incubated for 15 minutes at room temperature in the dark. After the incubation, the staining solution was aspirated, the well was rinsed once with 1 X rinse buffer, and the cells were covered with 500 µL DPBS to prevent drying. Each cell line was examined under a microscope for positive alkaline phosphatase expression and pictures were taken at 4X magnification for documentation.

### 2.3.3 Immunocytochemistry – Pluripotency Markers

Induced pluripotent stem cells express proteins that are associated with pluripotency. The expression was shown by immunocytochemistry and immunofluorescence. The two transcription factors, Nanog and Oct4, and the three cell surface markers, SSEA-3, SSEA-4 and Tra-1-60 were examined.

Five to seven days prior to performing the Immunocytochemistry Assay (ICC), around 3 colonies (VWR, Cat. No. 82050-004) were manually picked and transferred to 1 well of a 48-well cell culture plate. Six wells per cell line were prepared for analysis (the wells were coated with gelatin 0.1 % and a MEF layer, approximately 30,000 cells per well, the day before, as described). On the day the colonies were picked, the wells of the 48-well plate were rinsed with DPBS and the medium was changed to hESC with βFGF. The cells were fed every day except the day after picking with hESC containing βFGF and cultured at standard cell culture conditions.

Approximately 5-7 days after picking the colonies, the Immunocytochemistry Assay was performed.

First, the medium was aspirated and the cells were washed three times with 500 µL DPBS. The cells were fixed with 150 µL 4 % PFA in DPBS for 20 minutes under a chemical fume hood. After fixing the cells, the wells were washed with 500 µL PBS/0.05 % Tween®20 (50 mL DPBS + 25 µL TWEEN®20) three times and permeabilized with 500 µL PBS/0.1 % Triton® X-100 (10 mL DPBS + 10 µL Triton® X-100 (Sigma-Aldrich, Cat. No. X100)) for 15 minutes at room temperature. After permeabilization, the wells were rinsed three times with 500 µL PBS/0.05 % Tween®20 and non-specific binding sites were blocked by adding 500 µL 4 % Donkey Serum/PBS (15 mL DPBS + 600 µL Donkey Serum (Jackson Labs, Cat. No. 017-000-121)) to each well. The plate was wrapped in Parafilm (VWR, 52858-000) and stored overnight at 4 °C.

On the following day, the cells were washed three times with 500 µL DPBS and 100 µL of primary antibodies (Table 2), diluted in 4 % Donkey Serum, were added. One of the 6 wells of each cell line was treated with 4 % Donkey Serum without antibody, serving as negative control. After the addition of the diluted antibodies, the plates were incubated for 1 hour at room temperature.

**Table 2. Primary antibodies and dilution factors.**

The primary antibodies used for the immunocytochemistry assay were iluted in 4 % donkey serum.

| Primary Antibodies | Dilution |
|---|---|
| Oct4 (AbCam, Cat.No. ab19857) | 1:100 |
| Nanog (AbCam, Cat.No. ab21624) | 1:50 |
| SSEA-3 (Millipore, Cat.No. MAB4303) | 1:200 |
| SSEA-4 (Millipore, Cat.No. MAB4304) | 1:200 |
| Tra-1-60 (Millipore, Cat.No. MAB4360) | 1:200 |

After the incubation of the primary antibodies, the cells were washed three times with PBS/0.05 % Tween®20. 100 µL of the following secondary antibodies, diluted 1:500 in DPBS and listed in table 3, were added to one well for each cell line. The negative control was treated with DPBS without antibodies. The plates were wrapped in tinfoil and incubated for 1 hour at room temperature.

**Table 3. Primary antibodies and complementary secondary antibodies with excitation and emission spectra (in mm).**

All Secondary Antibodies were diluted 1:500 in PBS and purchased from Life Technologies.

| Primary Antibody | Secondary Antibody | Excitation [nm] | Emission [nm] |
|---|---|---|---|
| Oct4 | Alexa Flour ® 488 donkey anti-rabbit IgG (Cat. No. A21206) | 495 | 519 |
| Nanog | Alexa Fluor ® 488 donkey anti-rabbit IgG (Cat. No. A21206) | 495 | 519 |
| SSEA-3 | Alexa Fluor ® 594 goat anti-rat IgM (Cat. No. 21213) | 590 | 617 |
| SSEA-4 | Alexa Fluor ® 488 goat anti-mouse IgG (Cat. No. A21121) | 495 | 519 |
| Tra-1-60 | Alexa Fluor ® 555 goat anti-mouse IgM (Cat. No. A21426) | 555 | 565 |

The secondary antibodies were aspirated and the cells were washed three times with PBS/0.05 % Tween®20. Afterwards, the cells were counterstained by the addition of 100 µL of DAPI solution to each well for 20-30 seconds.

Each cell line was examined under a fluorescence microscope for the expression of the pluripotency markers tested and pictures were taken at 10X magnification for documentation.

### 2.3.4 Immunocytochemistry – Differentiation Markers

Upon differentiation, induced pluripotent stem cells should form cells of all three germ layers. To ensure that our newly derived lines are fully reprogrammed, we check the expression of three different markers: β-Tubulin for the ectoderm germ layer, SMA for the mesoderm germ layer and SOX17 for the endoderm germ layer were examined. The expression was shown by immunocytochemistry and immunofluorescence.

Two weeks prior to performing the ICC assay, embryoid bodies were formed in vitro.
In order to form EBs, 3 wells of a 6-well plate of iPS cell colonies were collected.
The cells were washed once with DPBS, and 1 mL of collagenase (Stem Cell Technologies, Cat. No. 07909) was added to each well. The plate was incubated at 37 °C for 10 minutes. The enzyme was aspirated and 1 mL pre-warmed hESC without βFGF was added. Using a cell scraper, the cells were detached from the plate and cells from all three wells were transferred into a 15 mL conical tube. After the cells were centrifuged at 1,200 rpm for 5 minutes, the medium was aspirated and the cells were gently resuspended in 6 mL hESC without βFGF, keeping the clumps as large as possible. The cells were then transferred to 3 wells of a 6-well ultra-low attachment plate (Fisher Scientific, Cat. No. 3471). The cells were cultured in standard cell culture conditions and fed every three days with hESC medium without βFGF. After one week, the cells were replated on three 0.1 % gelatin coated wells in DMEM 10 % FBS (without MEF layer) by transferring the cell clumps with a pipette (PT1000). For one week, the medium was changed every three days with DMEM 10 % FBS and the EBs were cultured at standard cell culture conditions.

After feeding the EBs for one week with DMEM 10 % FBS, the ICC assay was done. First, the medium was aspirated and the cells were washed three times with 1 mL DPBS. The cells were fixed with 500 µL 4 % PFA in DPBS for 20 minutes under a chemical fume hood. After fixing the cells, the wells were washed with 1 mL PBS/0.05 % Tween®20 three times and permeabilized with 1 mL PBS/0.1 % Triton® X-100 for 15 minutes at room temperature. After permeabilization, the wells were rinsed three times with 1 mL PBS/0.05 % Tween®20 and non-specific binding sites were blocked by

adding 1 mL 4 % Donkey Serum/PBS to each well. The plate was wrapped in parafilm and stored overnight at 4 °C.

On the following day, the cells were washed three times with 1 mL DPBS and 500 µL of primary antibodies (Table 4), diluted in 4 % Donkey Serum, were added. After the addition of the diluted antibodies, the plate was incubated for 1 hour at room temperature.

**Table 4. Primary and complementary secondary antibodies used for immunocytochemistry of EBs.**

Listed are the germ layers with their corresponding primary and secondary antibodies. The primary antibodies were diluted 1:200 in 4 % donkey serum. The secondary antibodies were diluted 1:500 in DPBS.

| Germ Layer | Primary Antibodies | Secondary Antibodies |
|---|---|---|
| Ectoderm | β-Tubulin (Neuronal Class III β-Tubulin Clone Tuj1, Covance Research Products, Cat.No. MMS-435P) | Alexa Fluor ® 488 Goat Anti-Mouse IgG2a (Life Technologies, Cat. No. A21131) |
| Mesoderm | SMA (Monoclonal Anti-Actin, α-Smooth Muscle antibody produced in mouse, Sigma-Aldrich, Cat.No.A5228) | Alexa Fluor ® 488 Goat Anti-Mouse IgG2a (Life Technologies, Cat. No. A21131) |
| Endoderm | SOX17 (Human SOX17 Affinity Purified Polyclonal Ab, R&D Systems, Cat.No. AF1924) | Alexa Fluor ® 594 Chicken Anti-Goat IgG |

After the incubation of the primary antibodies, the cells were washed three times with 1 mL PBS/0.05 % Tween®20. 500 µL of the secondary antibodies, diluted 1:500 in DPBS and listed in table XY, were added. The plate was wrapped in tinfoil and incubated for 1 hour at room temperature.

The secondary antibodies were aspirated and the cells were washed three times with 1 mL PBS/0.05 % Tween®20. Afterwards, the cells were counterstained by the addition of 500 µL of DAPI solution to each well for 20-30 seconds.
Each cell line was examined under a fluorescence microscope for the expression of the differentiation markers tested and pictures were taken at 10X magnification for documentation.

### 2.3.5 Testing for Pluripotency Markers

In order to prove that the reprogrammed iPS cells were pluripotent, a polymerase chain reaction (PCR) was performed using primers specific for pluripotent markers. The PCR products were run in an Agarose Gel via Electrophoresis. To perform PCR analysis, RNA was extracted from the reprogrammed cells and cDNA (complementary DNA) was synthesized.

#### 2.3.5.1 RNA Extraction

For the extraction of RNA, the Qiagen RNeasy Mini Kit (Qiagen, Cat. No. 74106) and the RNase-free DNase Kit (Qiagen, Cat. No. 79254) were used. Everything needed for the RNA Extraction was provided in the kits except 70 % Ethanol.

For the RNA Extraction, the cells from two wells of a 6-well cell culture plate were used per cell line.

First, the cells were trypsinized for 5 minutes, collected in a 15 mL conical tube and spun down at 1,200 rpm for 5 minutes. The supernatant was aspirated and 350 µL of Buffer RLT was added to the cells. The cells were then vortexed for 1 minute. After vortexing, 350 µL of 70 % Ethanol (Sigma-Aldrich, Cat. No. E7023, diluted with Ultra-Pure™DNase-RNase-Free Distilled water to reach a concentration of 70 %) were added, cells were pipetted up and down to mix, and 700 µL of the cell solution were transferred to an RNeasy spin column. Afterwards, the RNeasy spin column was centrifuged for 15 seconds at 10,000 rpm, and the flow through was discarded. 350 µL of Buffer RW1 were added to the column followed by a centrifugation step for 15 seconds at 10,000 rpm. During the centrifugation, a mix of 10 µL DNase and 70 µL Buffer RDD (both provided in the RNase-free DNase Kit) was prepared. After discarding the flow-through, the DNase mix was added to the RNeasy column and incubated for 15 minutes at room temperature.

Then, the flow-through was discarded, 350 µL of Buffer RW1 were added to the RNeasy spin column and the column was centrifuged at 10,000 rpm for 15 seconds. Afterwards, the flow-through was discarded and 500 µL Buffer RPE were added to the column,

followed by centrifugation at 10,000 rpm for 15 seconds. Again, 500 µL of Buffer RPE were added and the column was centrifuged at 10,000 rpm for 2 minutes. The flow-through was then discarded along with the 2 mL collection tube. The column was placed into a new 2 mL collection tube and centrifuged at 12,000 rpm for 1 minute. Afterwards, the RNeasy spin column was placed into a new 1.5 mL collection tube, 30 µL RNase-free water were added directly to the RNeasy spin column membrane and centrifuged at 10,000 rpm for 1 minute. This time, the RNeasy spin column was discarded and the collection tube containing the extracted RNA was kept. The RNA concentration was measured by means of a spectrophotometer (NanoVue, GE Healthcare, Buckinghamshire, GB), recorded and the tubes were stored at -80 °C to be used for cDNA Synthesis or further analysis.

#### 2.3.5.2 cDNA Synthesis

Following RNA extraction, complementary DNA was made by using the SuperScript® III Reverse Transcriptase Kit (Life Technologies, Cat. No. 18080-044).

The following reagents were needed for cDNA Synthesis: DTT 0.1 M, 5X First Strand Buffer, Reverse Transcriptase (RT) (provided by the SuperScript® III Reverse Transcriptase Kit), dNTP Mix 10 mM (Life Technologies, Cat. No. 18427-088), Random Hexamers 50 ng/µL (Applied Biosystems, Cat. No. N8080217), and RNase Out™ Recombinant RNase Inibitor (Life Technologies, Cat. No. 10777019). The enzyme Reverse Transcriptase was kept at -20 °C in a laboratory cooler until its use.

First, two PCR tubes and caps (VWR, Cat. No. 82050-976 respectively -416) were labelled with “+” and “-“ for each cell line to be analyzed. One µg of the previously extracted RNA was added to each of the two PCR tubes and Ultra-Pure™DNase-RNase-Free distilled water was added to reach a volume of 11 µL. Subsequently, 1 µL dNTP and 1 µL Random Hexamers were added to each tube followed by an heat-inactivation step at 65 °C for 5 minutes in the PCR machine (C1000 Thermal Cycler, Bio-Rad Laboratories, NJ, USA). The tubes were quickly incubated on ice for 1 minute. Afterwards, the following reagents were added to the tube labelled with “+” and to the negative control labelled with “-”:

| | | | |
|---|---|---|---|
| Tube "+" : | 4 µL 5 X Buffer | Tube "-" : | 4 µL 5 X Buffer |
| | 1 µL DTT | | 1 µL DTT |
| | 1 µL RNase Out | | 1 µL RNase Out |
| | 1 µL RT | | 1 µL UltraPure Water |

The tubes were spun down for 10 seconds and placed into the PCR machine. The program used for generating cDNA included a 5 min holding step at 25 °C, a 45 min holding step at 50 °C, a 15 min holding step at 70 °C, and a final holding step at 4 °C. The synthesized cDNA was then transferred to 1.5 mL microcentrifuge tubes (VWR, Cat. No. 14231-062), labelled and stored at -20 °C for further analysis.

#### 2.3.5.3 Testing of Pluripotent Markers via PCR

To analyze the pluripotency of the reprogrammed iPS cells, PCR reactions with the primer sequences listed in table 5 were performed. The cDNA Synthesis in 2.3.5.2 was used.

**Table 5. Forward and reward primer sequences for detection of pluripotency-specific gene products by PCR.**

PCR programs were set up with an initial denaturation step at 94 °C for 5 minutes followed by a repeated number of cycles (listed below, temperature for the annealing step and cycle number varies), an extended elongation period at 72 °C and a final holding step at 4 °C. Except Sox2 and Oct 4, which target the endogenous expression of the genes, primers listed in table 5 target exogenous gene products. All primers were purchased from Life Technologies.

| Primer | Sequence | | Annealing Step [° C] | Cycle Number |
|---|---|---|---|---|
| | Forward | Reverse | | |
| Actin | GGACTTCGAGCAAGAGATGG | AGCACTGTGTTGGCGTACAG | 60 | 25 |
| Nanog | TCCAACATCCTGAACCTCAG | GACTGGATGTTCTGGGTCTG | 58 | 33 |
| Rex1 | TGGACACGTCTGTGCTCTTC | GTCTTGGCGTCTTCTCGAAC | 58 | 33 |
| Sox2 | TTGTCGGAGACGGAGAAGCG | TGACCACCGAACCCATGGAG | 58 | 33 |
| Oct4 | GTGGAGGAAGCTGACAACAA | CAGGTTTTCTTTCCCTAGCT | 56 | 30 |
| Dnmt3B | ATAAGTCGAAGGTGCGTCGT | GGCAACATCTGAAGCCATTT | 56 | 30 |
| hTERT | TGTGCACCAACATCTACAAG | GCGTTCTTGGCTTTCAGGAT | 56 | 30 |

In order to prove that the cDNA synthesis was successful, a PCR reaction with Actin primers was performed, followed by an agarose gel electrophoresis described in

2.3.5.4. After cDNA synthesis was proved to be successful, and pictures of the gel were taken for documentation, PCR reactions with pluripotency markers were performed.

As described in 2.3.5.2, 2 PCR tubes per sample were labelled, one with "+" and one, acting as negative control, with "-". 23 µL of Platinum® Blue PCR SuperMix (Life Technologies, Cat. No. 12580-023), 0.5 µL of forward primer, 0.5 µL of reverse primer and 1 µL of cDNA were added to each tube. According to the labelling of "+" and "-" when carrying out the cDNA synthesis, cDNA "+" was added to the PCR tube labelled with "+" and the one with "-" to the PCR tube labelled with "-". The tubes were spun down for 10 seconds and placed into the PCR machine. The program with the corresponding annealing step found in table XY was selected and run. After the amplification, samples were stored at 4 °C or analyzed by means of agarose gel electrophoresis.

2.3.5.4 Agarose Gel Electrophoresis

Qualitative Agarose Gel Electrophoresis was carried out to verify the presence of the pluripotency markers amplified in 1.4.4.3.

To prepare a 1.5 % agarose gel with 28 wells, 1.5 g Agarose powder (Fisher Scientific, Cat. No. BP1356-500) was dissolved in 100 mL 1X Tris-acetate-EDTA (TAE) buffer, diluted from a 50X stock (Life Technologies, Cat. No. 24710030). The solution was heated for approximately 2 minutes in the microwave and cooled for 5-10 minutes under a chemical fume hood. After cooling, 2 µL of 1 % Ethidium Bromide solution (Fisher Scientific, Cat. No. 1302-10) were added, the solution was slowly transferred into a prepared gel carrier, and combs were inserted. After a cooling time of approximately 20 minutes, the gel carrier was placed into an electrophoresis gel apparatus, and 1X TAE Buffer was added. The first well was loaded with 10 µL of a 1 Kb Plus DNA Ladder (Life Technologies, Cat. No. 10787-026), diluted in 10XBlueJuiceTM Gel Loading Buffer (Life Technologies, Cat. No. 10977) and Ultra-Pure$^{TM}$DNase-RNase-Free Distilled Water in a ratio of 1:1:8 to serve as a size marker control. For each sample, 10 µL of the PCR reaction were loaded. The voltage was set at approximately 80 volts. After 20 minutes, the gel was analyzed under UV light and pictures were taken for documentation.

### 2.3.6 Differentiation Marker analysis by Embryoid Body Formation

The full differentiation potential of reprogrammed iPS cells was tested by embryoid body formation and PCR analysis of markers of different germ layers.

#### 2.3.6.1 Embryoid Body Formation in-vitro

In order to form Embryoid Bodies, two wells of a 6-well plate of iPS cell colonies per line were collected.

Cells were washed once with PBS, and 1 mL collagenase was added to each well. The plates were incubated at 37 °C for 10 minutes. The enzyme was aspirated and 1 mL pre-warmed hESC without βFGF was added. Using a cell scraper, the cells were detached from the plate and cells from both wells were transferred into a 15 mL conical tube. After the cells were centrifuged at 1,200 rpm for 5 minutes, the medium was aspirated and the cells were gently resuspended in 4 mL hESC without βFGF, keeping the clumps as large as possible. The cells were then transferred to 2 wells of a 6-well ultra low attachment plate. The cells were cultured in standard cell culture conditions and fed every three days with hESC medium without βFGF. After one week, the cells were replated on two 0.1 % gelatin coated wells in DMEM 10 % FBS by transferring the cell clumps with a pipette. For one week, the medium was changed every three days with DMEM 10 % FBS and the Embryoid Bodies were cultured at standard cell culture conditions.

After one week, the EBs were trypsinized for 5 minutes at 37 °C and centrifuged. The cell pellet was stored either at -80 °C or used immediately for further analysis.

#### 2.3.6.2 Analysis of Differentiation Markers using PCR

PCRs were performed to test for the expression of six differentiation markers representing the three germ layers (endoderm, ectoderm, and mesoderm).

The processes of RNA extraction, cDNA Synthesis, PCR, and Agarose gel electrophoresis needed for the analysis of differentiation markers were performed as described in chapters 2.3.5. The primer sequences are listed in Table 6.

**Table 6. Forward and reward primer sequences for detection of differentiation-specific gene products by PCR.**

PCR programs were set up with an initial denaturation step at 94 °C for 5 minutes followed by a repeated number of cycles (listed below, temperature for the annealing step and cycle number varies), an extended elongation period at 72 °C and a final holding step at 4 °C. For characterization 2 primers each germ layer were used. All primers were purchased from Life Technologies.

| Lineage | Primer | Sequence | | Annealing Step [° C] | Cycle Number |
|---|---|---|---|---|---|
| | | Forward | Reverse | | |
| | **Actin** | GGACTTCGAGCAAGAGATGG | AGCACTGTGTTGGCGTACAG | 60 | 25 |
| **Endoderm** | **AFP** | AGCTTGGTGGTGGATGAAAC | CCCTCTTCAGCAAAGCAGAC | 58 | 34 |
| | **Gata4** | CTAGACCGTGGGTTTTGCAT | TGGGTTAAGTGCCCCTGTAG | 61 | 32 |
| **Mesoderm** | **Flk1** | AGTGATCGGAAATGACACTGGA | GCACAAAGTGACACGTTGAGAT | 61 | 32 |
| | **GATA2** | GCAACCCCTACTATGCCACC | CAGTGGCGTCTTGGAGAAG | 58 | 34 |
| | **VECAD** | CAGCCCAAAGTGTGTGAGAA | TGTGATGTTGGCCGTGTTAT | 58 | 33 |
| | **PECAM** | CCCAGCCCAGGATTTCTTAT | ACCGCAGGATCATTTGAGTT | 58 | 33 |
| **Ectoderm** | **β-Tubulin** | CAGATGTTCGATGCCAAGAA | TGCTGTTCTTGCTCTGGATG | 58 | 33 |
| | **NCAM** | ATGGAAACTCTATTAAAGTGAACCTG | TAGACCTCATACTCAGCATTCCAGT | 68 | 33 |
| | **Pax6** | TCTAATCGAAGGGCCAAATG | TGTGAGGGCTGTGTCTGTTC | 57 | 35 |

### 2.3.7 Differentiation evaluation by teratoma formation in vivo

Teratoma formation is the most accurate test for pluripotency in induced pluripotent stem cells, as teratoma tumors consist of multiple lineages containing tissue derived from the three germ layers (endoderm, mesoderm, ectoderm).

The iPS cell colonies of one 10-cm cell culture dish and 3 wells of a 6-well plate were collected with collagenase and spun down. The medium was aspirated until a small amount of medium remained on top of the cell pellet. The small amount of medium was gently pipetted up and down to keep the clumps as large as possible and to avoid cell death.

The cells were then sent to the Harvard Genome Modification Facility, MA, USA. The iPS cells were surgically implanted into the sub-renal capsule, a fibrous layer surrounding the kidney, of 3 immunodeficient mice per cell line. The teratomas were received in 4 % PFA around 9 weeks after implantation and sent to the HSCRB Histology Core, Cambridge, MA, USA, for a histological and immunohistochemical staining.

### 2.3.8 Real Time PCR – Expression Level of Pluripotent and Differentiation Markers

In order to quantify the expression of the pluripotency markers and the 6 differentiation markers, Real Time PCR (qPCR) was performed using two Real Time PCR Kits from Qiagen.

- Pluripotency Markers:
  - iPSC pluripotency validation (Qiagen, Cat. No. 337221 IPHS-100)
- Differentiation Markers:
  - Embryoid Body Differentiation (Qiagen, Cat. No. 337221 IPHS-101)

The layout of both plates included in the Real Time PCR kits is shown in figure 4. Three rows represent 1 sample to be analyzed.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | 9 | 17 | 1 | 9 | 17 | 1 | 9 | 17 | 1 | 9 | 17 |
| B | 2 | 10 | 18 | 2 | 10 | 18 | 2 | 10 | 18 | 2 | 10 | 18 |
| C | 3 | 11 | 19 | 3 | 11 | 19 | 3 | 11 | 19 | 3 | 11 | 19 |
| D | 4 | 12 | 20 | 4 | 12 | 20 | 4 | 12 | 20 | 4 | 12 | 20 |
| E | 5 | 13 | 21 | 5 | 13 | 21 | 5 | 13 | 21 | 5 | 13 | 21 |
| F | 6 | 14 | 22 | 6 | 14 | 22 | 6 | 14 | 22 | 6 | 14 | 22 |
| G | 7 | 15 | 23 | 7 | 15 | 23 | 7 | 15 | 23 | 7 | 15 | 23 |
| H | 8 | 16 | 24 | 8 | 16 | 24 | 8 | 16 | 24 | 8 | 16 | 24 |

**Figure 4. Plate Layout of Real Time PCR Kits iPSC pluripotency validation and Embryoid Body Differentiation (Qiagen).**

Each 3 rows are representing 1 sample to be analyzed

All reagents needed to perform the qPCR analysis were thawed on ice. First, an experimental cocktail was prepared for each sample:

| | |
|---|---|
| Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix<br>(Agilent, Cat. No. 600882) | 350 µL |
| Reference Dye (0.3 µL per well → total)<br>contained in SYBR® Green, used as 1:500 dilution in UltraPure Water | 28.8 µL |
| cDNA<br>used as 1:5 dilution of synthesized cDNA in 1.4.4 and 1.4.5 | 28 µL |
| $H_2O$ | 322 µL |
| Total | 728.8 µL |

Afterwards, 25 µL of the experimental cocktail were added to its corresponding well (24 wells per sample per plate). It is important to avoid bubbles, which could modify the results. The plate was covered with an adhesive film for PCR plates (VWR, Cat. No. 60941-078) and centrifuged at 1,200 rpm for 5 minutes. The plate was placed in the qPCR machine (Agilent Technologies, Stratagene Mx3005P) and the following PCR program was set up: 1 initial denaturation cycle of 10 minutes at 95 °C followed by 40 cycles consisting of 15 seconds at 95 °C and 1 minute at 60 °C each.

## 2.4 Cell reprogramming of Erythroblasts using the Sendai Virus

This section describes the generation of disease specific induced Pluripotent Stem Cells from human erythroblasts using a Sendai Viral vector. The protocols that were used were described by Yang et al and Sommer et al (Sommer, 2012; Yang, 2012)and adapted by the Harvard Stem Cell Institute (HSCI) iPS Core Facility, Cambridge, MA, USA.

The blood of two randomly selected donors was received from the Blood Donor Center, Massachusetts General Hospital (MGH), Gray 1 Lobby, 55 Fruit Street, Boston, MA.

The expansion of the PBMCs to erythroblasts and the reprogramming using the Sendai virus was for research purposes of the HSCI iPS core facility in Cambridge, MA, USA only, and not part of a contract with an external investigator.

The received blood samples were processed and the PBMCs were frozen by the person responsible for blood reprogramming in the HSCI iPS Core facility, Cambridge, MA, USA.

### 2.4.1 Expansion of Erythroblasts from Peripheral Blood Mononuclear Cells (PMBCs)

To expand erythroblasts from frozen PBMCs, one cryovial containing 1 million cells was thawed per cell line. For thawing the cells, two 15 mL conical tubes were prepared. One tube contained 9 mL pre-warmed basal medium (Table 7), and one was empty. 50 mL of basal medium were prepared in advance and were stored at 4 °C. The frozen cells were partially thawed at 37 °C in the waterbath until only a small piece of ice remained. The pre-warmed medium was added dropwise to the cryotube, 1 mL at a time. The liquid containing the cells was then transferred into the second empty tube. This was repeated until all 9 mL of the medium were used. The cells were spun down at 1,200 rpm for 5 minutes. The cells were then resuspended in 2 mL expansion medium (Table

7) and each cell line was plated into 1 well of a 12-well cell culture plate. The growth factors for the expansion medium were added fresh daily to the basal medium.

**Table 7. Reagents for Basal Medium and Expansion Medium used to expand and transduce Erythroblasts with the Sendai virus.**

Listed are the reagents which were needed for the expansion and transduction of erythroblasts with the Sendai Virus. The final concentration, the volume of the reagent needed to make 1 mL of medium, as well as the supplier and the catalog numbers are listed in table 7.

| Reagent | Final Concentration | Volume | Supplier | Cat. No. |
|---|---|---|---|---|
| **Basal Medium** | | | | |
| **QBSF-60 (serum free medium)** | | 1 mL | VWR | 101641-762 |
| **Primocin** | 100 µg/mL | 2 µL | Invivogen | ant-pm-1 |
| **L-Ascorbic Acid per (AA)** | 50 µg/mL | 10 µL | Sigma-Aldrich | A4544-25G |
| **Expansion Medium** | | | | |
| **Basal Medium** | | 1 mL | | |
| **Recombinant human SCF** | 50 ng/mL | 1 µL | R&D Systems | 255-SC-010/CF |
| **Recombinant human IL-3** | 10 ng/mL | 0.1 µL | R&D Systems | 203-IL-010/CF |
| **Recombinant human EPO** | 2 U/mL | 10 µL | Life Technologies | PHC9634 |
| **Recombinant human IGF-1** | 40 ng/mL | 10 µL | Life Technologies | PHG0078 |
| **Dexamethasone** | 1 µM | 10 µL | Sigma-Aldrich | D8893-1MG |

The cells were fed every other day with pre-warmed expansion medium for 9 days. Some of the cells were in suspension and some of the cells were attached to the well. When feeding the cells, the medium with the cells was transferred to a 15 mL conical tube and spun down at 1,200 rpm for 5 minutes. After transferring the cells to the conical tube, 1 mL of expansion medium was added to the well to prevent the attached cells from drying out. After the centrifugation, the medium was aspirated and the cells were resuspended in 1 mL expansion medium. The solution with the cells was then added to the well with the 1 mL of medium.

### 2.4.2 Transduction of Erythroblast lines with the Sendai Virus

On the tenth day after thawing the PBMCs (D0) and expanding the erythroblasts with expansion medium, which contains growth factors for erythroblasts, the cells were collected in a 15 mL conical tube. To detach the cells, the medium was pipetted up and down several times. The well was washed 2 times to collect adherent cells. Then, the cells were counted by an automated cell counter. For each sample of erythroblasts, 150,000 cells were collected in a 15 mL conical tube. The erythroblasts were spun down at 1,200 rpm for 5 minutes and resuspended in 1 mL of expansion medium. The cells were kept in the conical tube during the transduction.

The Sendai virus that was used for reprogramming was the CytoTune$^{TM}$-iPS Sendai Reprogramming Kit, which contains the four viruses that encode the four transcription factors: Oct4, Sox2, Klf4 and c-Myc. The tubes were thawed one at a time by immersing the bottom of the tube in the 37 °C water bath for 10 seconds, spinning it down quickly and thawing it at room temperature before placing it on ice. For the transduction of the erythroblasts with the Sendai virus, a multiplicity of infection of 10 was used. According to the different titers of each virus, the necessary volume was calculated to add 1.5 x $10^6$ CIU (colony forming units) to the 1 mL of cells in expansion medium. The suspensions of the 2 transduced lines were then transferred to 1 well of a 12-well plate and a spinoculation was performed. The plates were centrifuged at 2,250 rpm for 90 minutes. The cells were incubated at standard cell culture conditions overnight. All tools that came in contact with the viral solution were immediately treated with vesphene and discarded.

The following day (24 hours after transduction on D0) the Sendai virus was washed off. The cells were collected in a 15 mL conical tube, spun down at 1,200 rpm for 10 minutes and resuspended in 2 mL pre-warmed expansion medium.

The day after (D2), 6 wells of a 6-well cell culture plate were plated with MEFs for each cell line.

The next day (D3) the erythroblasts were collected into one 15 mL conical tube. To detach the cells, the medium was pipetted up and down several times. The well was washed twice to collect adherent cells. To compare two different cell culture conditions, the cell solution was gently mixed and half of the solution was pipetted into an empty 15 mL conical tube. Both tubes were spun down at 1,200 rpm for 5 minutes. The cells in tube number 1 were then resuspended in 6 mL hES medium, which contained βFGF, ascorbic acid and growth factors according to table 7. The cells in tube number 2 were resuspended in 6 mL DMEM 10% FBS medium, which contained βFGF, ascorbic acid, and growth factors according to table 7. The cells were replated onto three wells of MEFs for each culture condition. The plate was then centrifuged at 500 rpm for 30 minutes. The cells were incubated in standard cell culture conditions overnight.

On D5, the medium was changed to fresh hES or DMEM 10 % FBS depending on the sample, with βFGF and ascorbic acid (without growth factors). The medium with the cells was transferred to a 15 mL conical tube and spun down at 1,200 rpm for 5 minutes. After transferring the cells to the conical tube, 1 mL of fresh medium was added to each well to prevent the attached cells from drying out. After the centrifugation, the medium was aspirated and the cells were resuspended in 1 mL of fresh medium. The solution with the cells was then added to the well with the 1 mL of medium.

Every other day (D7 and D9), the cells were fed with hES or DMEM 10 % FBS depending on the sample, with βFGF and ascorbic acid (without growth factors). Since the cells were already attached to the MEFs, the medium was replaced by aspiration as usual.

Starting on day 11 after transduction (D11), the cells on both conditions were fed with fresh hES with βFGF (without ascorbic acid) every other day.

Starting at 19 days after transduction, the first fully reprogrammed iPSC colonies were manually transferred to a feeder layer of irradiated MEFs in one well of a 6-well plate

prepared one day before as described in 2.1.1. Selection of fully reprogrammed cells was based on morphology only.

The picking and cultivation of the newly derived iPSC colonies was performed as described in chapter 2.1.2.

The number of colonies per line was counted and the efficiency was calculated.

## 2.5 Somatic Cell reprogramming using a microRNA enhanced mRNA Reprogramming System

This section describes the generation of disease specific induced Pluripotent Stem Cells from human fibroblasts using a microRNA enhanced mRNA reprogramming system from Stemgent. The protocol that was used was also received from Stemgent and adapted by the Harvard Stem Cell Institute iPS Core facility, Cambridge, MA, USA. This reprogramming procedure was performed for Stemgent in order to test their new product.

Fibroblasts derived from 3 patients with an unknown disease, were received from an investigator. Two cell lines were derived from living patients and one cell line was derived at the autopsy of a deceased patient. Two cell lines were reprogrammed by the microRNA enhanced mRNA transfection only. One of these three cell lines was reprogrammed by the microRNA enhanced mRNA transfection and was compared to transfection with mRNA only. Fibroblasts from a BJ cell line that had previously been successfully reprogrammed, was used as a positive control.
The following protocol describes the materials needed for the reprogramming of fibroblasts of 5 wells in a 6-well cell culture plate. Three wells were reprogrammed by microRNA enhanced mRNA transfection, two wells were reprogrammed by mRNA transfection only.

A summary of the transfection schedule is shown in table 8.

Before starting the transfection, the necessary reagents, the microRNA, and the mRNA were aliquoted and stored according to the instruction manual. Human fibroblast conditioned Pluriton™ Medium was prepared using Nuff cells (Human Fibroblasts Irradiated, Donor 11 = NuFF cells, Global Stem, Cat. No. GSC-3001G).

**Table 8. Sample transfection schedule for the microRNA enhanced mRNA reprogramming system.**

A daily schedule for the tasks that have to be performed daily during the microRNA enhanced mRNA transfection of fibroblasts is shown. Listed are the days, the day numbers and the tasks.

| Number of the Day | Task |
|---|---|
| **Day 0** | Plate Target Cells |
| **Day 1** | Transfect microRNA only |
| **Day 2** | Transfect mRNA |
| **Day 3** | Transfect mRNA |
| **Day 4** | Transfect mRNA |
| **Day 5** | Transfect microRNA + mRNA |
| **Day 6** | Transfect mRNA |
| **Day 7** | Transfect mRNA |
| **Day 8** | Transfect mRNA |
| **Day 9** | Transfect mRNA |
| **Day 10** | Transfect mRNA |
| **Day 11** | Transfect mRNA |
| **Day 12** | Transfect mRNA |
| **Picking - Starting on Day 15** | |

### 2.5.1 Preparation of Reagents for the microRNA enhanced mRNA Transfection of Fibroblasts

*Pluriton Supplement*

The Pluriton™ Supplement (200 µL) was delivered with the Pluriton™ Medium (Stemgent, Cat. No. 00-0070), thawed on ice and aliquoted in 40 x 5 µL single-use vials. The vials were stored at -80 °C. One vial was thawed each day of the reprogramming

schedule to supplement the NuFF conditioned Pluriton™ medium. 4 µL Pluriton Supplement were used to supplement 10 mL medium.

*B18R Recombinant Protein*

The B18R Recombinant Protein (80 µL) (Stemgent, Cat. No. 03-0017), was thawed on ice and aliquoted in 13 x 6 µL single-use vials. The vials were stored at -80 °C. One vial was thawed each day of the reprogramming schedule to supplement the NuFF conditioned Pluriton™ medium. 6 µL B18R protein were used to supplement 10 mL medium.

*mRNA Reprogramming Cocktail*

A master mRNA cocktail was prepared and aliquoted in single-use volumes to reprogram 5 wells of a 6-well cell culture plate. All mRNA factors were thawed on ice and combined according to the volumes in table 9. The final mixture was and aliquoted into 20 x 50 µL mRNA cocktail.

The mRNA cocktail, as prepared below, has a molar stoichiometry of 3:1:1:1:1:1 for Oct4, Sox2, Klf4, c-Myc, Lin28 and nGFP mRNAs, respectively.

**Table 9. mRNA reprogramming factors combined to a mRNA reprogramming cocktail.**

Listed are the mRNA reprogramming factors needed for the transfection of fibroblasts and the volumes to reach a molar stoichiometry of 3:1:1:1:1:1 for Oct4, Sox2, Klf4, c-Myc, Lin28 and nGFP mRNAs, respectively.

| mRNA reprogramming factor | Volume [µL] |
|---|---|
| **Oct4 mRNA** | 385.1 |
| **Sox2 mRNA** | 119.2 |
| **Klf4 mRNA** | 155.9 |
| **c-Myc mRNA** | 147.7 |
| **Lin28 mRNA** | 82.5 |
| **nGFP** | 110.6 |
| **mRNA cocktail mix** | 1000.0 |

One vial was thawed each day of the reprogramming schedule, except on the day with microRNA transfection only.

*microRNA Reprogramming Cocktail*

The microRNA reprogramming cocktail was thawed on ice and aliquoted in single-use volumes to reprogram 3 wells of a 6-well cell culture plate. The cocktail was aliquoted into 6 vials containing 11.5 µL microRNA each.

One vial was thawed on D1 and D5 of the reprogramming schedule for the microRNA transfection.

### 2.5.2 Preparation of NuFF conditioned Pluriton™ Medium

Eight days before the reprogramming (day -8), one vial of 4 million NuFF irradiated cells was thawed in DMEM 10 % FBS medium as described in 1.1.1. The cells were plated into two T75 cell culture flasks (VWR, Cat. No. 82050-856) and cultured in standard cell culture conditions overnight. After allowing the NuFF cells to attach overnight, the cells were washed with 10 mL DPBS and 20 mL Pluriton™ medium with βFGF (final concentration of 4 ng/mL) were added. After 24 hours of incubation, the NuFF conditioned Pluriton™ medium was collected into a 50 mL conical tube and stored at -20 °C. 20 mL of fresh pre-warmed Pluriton medium were added per T75 cell culture flask and incubated overnight. This process was repeated for six days. After the collection of the medium on the day before reprogramming, the NuFF cells were discarded.

### 2.5.3 microRNA enhanced mRNA Transfection - Schedule

A summary of the schedule for the microRNA enhanced mRNA transfection starting on D0 is shown in 2.5.

#### 2.5.3.1 Day 0 – Plating Target Cells

On the day before the first day of the transfection (D0), the target cells were plated into a 6-well cell culture plate.

To ensure that the microRNA and the mRNA are only received by the target cells, the cells were plated on a matrigel layer (BD Biosciences, Cat. no. 35277) and not on feeder cells. An aliquot of matrigel for 5 wells of a 6-well cell culture plate was thawed on ice and diluted with DMEM/F12 medium (1:100). Each well of a 6-well plate was coated with 1 mL matrigel and incubated for 1 hour at room temperature. Then, the matrigel was aspirated and 50,000 fibroblast cells were plated per well (according to 1.1.1). The cells were incubated in standard cell culture conditions overnight.

#### 2.5.3.2 Day 1 – microRNA Transfection

The protein B18R, which is essential to modulate the cells' innate immune response by blocking the cells' interferon response, was supplemented in the cell culture medium to a final concentration of 200 ng/mL during each mRNA and microRNA transfection. It improves the health of the culture throughout the reprogramming experiment. For that reason, the cells were pretreated with the B18R protein to pre-suppress the cells' interferon response.

Two hours before the transfection, the medium of the target cells was changed to pre-warmed NuFF-conditioned medium with B18R and Pluriton™ Supplement. From this point on, the cells were cultured in a low $O_2$ (5 % $O_2$) incubator because the reprogramming efficiency was proven to be higher with decreased oxygen levels.

Every day, Pluriton™ medium was prepared for the next day to let the medium equilibrate to attain the desired oxygen tension. 10 mL medium were added to a 10 mm dish and stored in the low oxygen incubator overnight prior to use. The Pluriton™ Supplement and the B18R protein were added to the medium after equilibration, just prior to exchanging the transfection medium, in order to avoid degradation of these components.

The transfection was performed at the same time all 12 days. 30 minutes before the transfection was planned, the Stemfect buffer and the Stemfect RNA reagent (Stemfect™ RNA Transfection Kit, Stemgent, Cat. No. 00-0069) were pre-warmed at room temperature. To thaw one 11.5 µL aliquot of the microRNA cocktail, it was put on ice 30 minutes prior to the transfection.

*microRNA Transfection*

The microRNA complex was prepared for 3 wells of a 6-well cell culture plate. One empty, sterile 1.5 mL microcentrifuge tube was prepared and marked with “tube 2”. The tube with the thawed microRNA was marked as “tube 1”.

Tube 1, with the microRNA cocktail, was removed from the ice and 64 µL Stemfect buffer were added and gently mixed. The tube was kept at room temperature. 75 µL Stemfect buffer were then added to tube 2, followed by the addition of 12 µL Stemfect reagent. The complex was pipetted up and down to mix, as the transfection reagent seemed to adhere to the pipette tip. The content from tube 2 was carefully transferred to tube 1 and gently mixed. To allow the complex formation, the mix was incubated at room temperature for 15 minutes. After the incubation, the content of the tube was gently mixed again. 50 µL of the mixture were added to each of the 3 wells by distributing the complex dropwise on the surface of the NuFF conditioned medium across the well. Immediately after the addition of the complex to 1 well, the plate was rocked 2-3 times in both the x and y direction. After the transfection of all 3 wells, the cells were incubated overnight in the hypoxic incubator. The remaining microRNA transfection complex was discarded.

#### 2.5.3.3 Days 2-4 – mRNA Transfection

To give the cells time to recover from the toxic transfection cocktail, the medium was changed 4 hours before the next transfection every day. The 10 mL NuFF conditioned Pluriton™ medium, which were prepared the day before and stored overnight in the low oxygen incubator, were transferred to a 15 mL conical tube. The Pluriton Supplement and the B18R protein were added to the medium. The medium with the transfection complex was aspirated from the cells and replaced with the fresh medium. New NuFF

conditioned medium was added to the 10 mm dish and stored in the hypoxic incubator overnight

nGFP was part of the mRNA cocktail. 4-5 hours after the transfection, the nGFP expression appears uniform throughout the transfected cells. The nGFP expression of the 3 wells that were transfected on D1, was checked under the microscope after changing the medium. Starting on day 3, the nGFP expression of all wells was checked daily after changing the medium four hours prior to the transfection. Of note, early morphology changes began as early as day 3. Pictures were taken for documentation.

*mRNA Transfection*

One 50 µL aliquot of the mRNA cocktail was thawed on ice 30 minutes prior to transfection. As described in 1.5.3.2, the Stemfect buffer and the Stemfect reagent were pre-warmed at room temperature 30 minutes before the transfection.

The mRNA complex was prepared for 5 wells. One empty, sterile 1.5 mL microcentrifuge tube was prepared and marked with “tube 2”. The tube with the thawed mRNA was marked as “tube 1”.

Tube 1, with the mRNA cocktail, was removed from the ice and 75 µL Stemfect buffer were added and gently mixed. The tube was kept at room temperature. 125 µL Stemfect buffer were then added to tube 2, followed by the addition of 20 µL Stemfect reagent. The complex was pipetted up and down to mix, as the transfection reagent seemed to adhere to the pipette tip. The contents of tube 2 were carefully transferred to tube 1 and gently mixed. To allow the complex to form, the mix was incubated for 15 minutes at room temperature. After the incubation, the contents of the tube were gently mixed again. 50 µL of the mixture were added to each of the 5 wells by distributing the complex dropwise on the surface of the NuFF-conditioned medium. Immediately after the addition of the complex to one well, the plate was rocked a couple of times in both the x and y direction. After the transfection of all three wells, the cells were incubated overnight in the hypoxic incubator.

#### 2.5.3.4 Days 5 – microRNA and mRNA Transfection

As on all other days of the transfection before, the medium was changed 4 hours prior to the transfection, the changes in morphology of the cells and the nGFP expression were checked under the microscope.

The target cells were sequentially transfected with both the microRNA and mRNA transfection complexes. The complexes were made in parallel, as described in 2.5.3.2 and 2.5.3.3, applied, and distributed within 5 minutes of each other.
The cells were incubated in the low oxygen incubator overnight.

#### 2.5.3.5 Days 6-12 – mRNA Transfection

The mRNA transfection on day 6 to day 12 were performed as on day 2 and as described in 2.5.3.3.

After the transfection on day 12, the cells were incubated at low oxygen overnight.
The next day, the medium was changed to fresh NuFF conditioned medium with Pluriton$^{TM}$ Supplement (without B18R).

On the third day after the series of transfections (D15), the cells were washed once with DPBS and hES medium was added to the wells. From now on, the cells were fed every day with pre-warmed hES medium and cultured in the low oxygen incubator. The changes in morphology were checked every day.

The first fully reprogrammed iPSC colonies were picked on D15 as described in 2.1.2.

# 3 RESULTS

The Somatic Cell reprogramming on mouse embryonic feeder cell layer using a Sendai virus and the Characterization of Reprogrammed iPS Cell Lines were performed as part of two separate projects. These projects were part of the contract between external investigators and the Harvard Stem Cell Institute iPS Core Facility, Cambridge, MA, USA. The projects on feeder free systems and the reprogramming of Erythroblasts with the Sendai Virus were for research purposes of the HSCI iPS core facility in Cambridge, MA, USA and were not part of a contract with an external investigator.

## 3.1 Generation of Human Induced Pluripotent Stem Cells from fibroblasts with the Sendai virus on Mouse Embryonic Feeder Layer

Human fibroblasts, derived from patients with Type 1 diabetes, were transduced with the four Yamanaka factors: Oct4, Sox2, Klf4 and c-Myc. The cells were transduced at a density of 250,000 cells per well of a 6-well cell culture plate, using a CytoTune$^{TM}$ iPS Reprogramming Kit at a multiplicity of infection of 3.

Approximately one week after the transduction, the first morphological changes were observed. The first fully reprogrammed iPS cell colonies were picked on D29, based on morphology.

Figure 5 and 6 show the time courses of the morphological changes of the lines Control3, D262 and 721P, observed after the transduction of the fibroblasts with the Sendai virus.

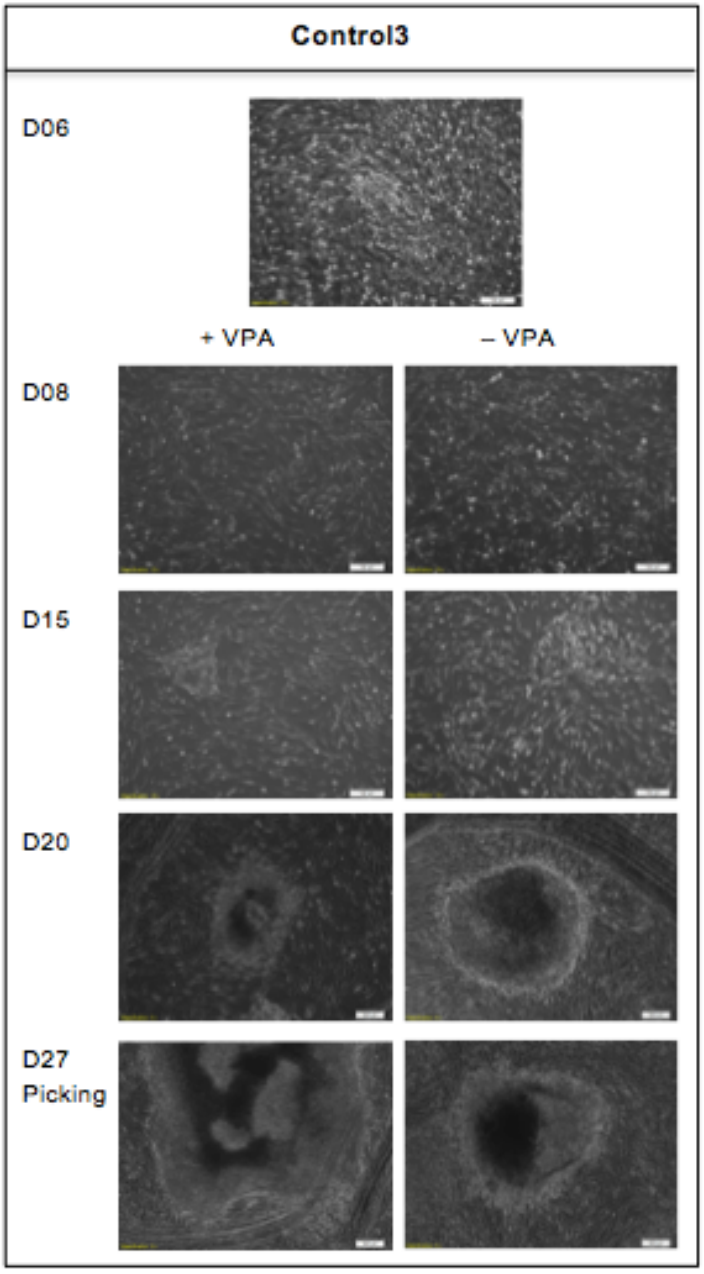


**Figure 5. A time course of morphological changes observed after the transduction of fibroblasts with the Sendai virus of line Control3.**

The first visible changes were observed approximately one week after transduction of 250,000 fibroblast cells with the Sendai virus. The images show morphological changes from D6 after transduction to D29 when colonies were picked.

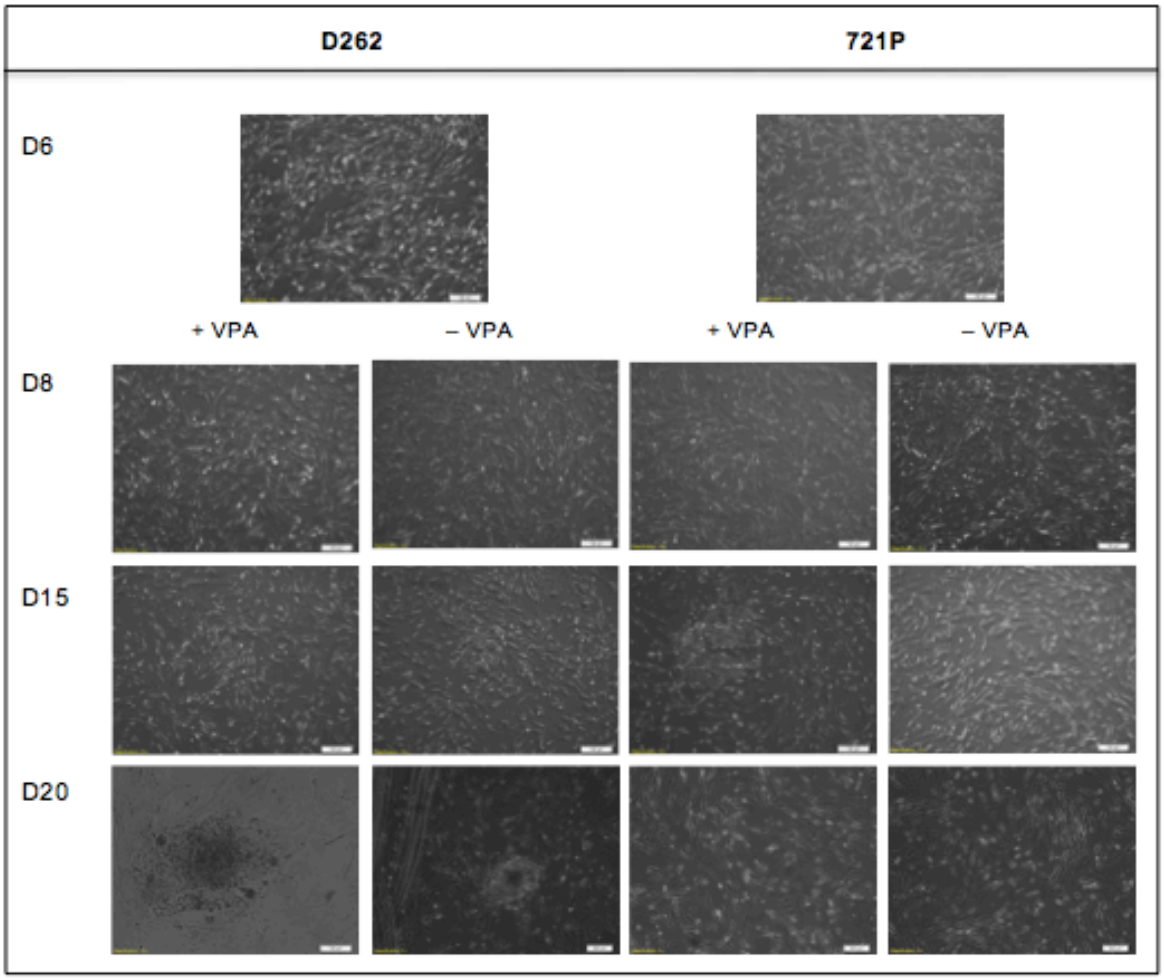


**Figure 6. A time course of morphological changes observed after the transduction of fibroblasts with the Sendai virus of lines D262 and 721P.**

The first visible changes were observed approximately one week after the transduction of 250,000 fibroblast cells with the Sendai virus. The images show morphological changes from D6 to D20 after transduction.

Fully reprogrammed human induced pluripotent stem cell colonies were derived from the Control3 and D262 lines. In sample 721P morphological changes were observed but no iPSC colonies could be derived.

For sample Control3, 29 reprogrammed iPSC colonies were observed. 10 hESC-like clones were picked from the original plates and labelled as individual cell lines from A to

J. For sample D262, 2 reprogrammed iPSC colonies were observed. The colonies were picked and labelled as A and B. The lines were then expanded in 6-well cell culture plates. As the characterization of these lines was not part of the contract between the external investigator and the HSCI iPS core facility, frozen vials and live cell cultures were given to the external investigator.

### 3.1.1 Live-cell Immunocytochemistry imaging of human induced pluripotent stem cells

For one plate of the sample Control3, a Live-cell ICC imaging was performed because the plate was overgrown and it was difficult to identify the fully reprogrammed iPSC colonies by morphology.
Live-cell ICC imaging was performed with the antibody Alexa Fluor ® Mouse IgG1 Tra-1-60.

Figure 7 shows Live-cell ICC pictures of 2 iPSC colonies taken with brightfield and fluorescence microscopy.

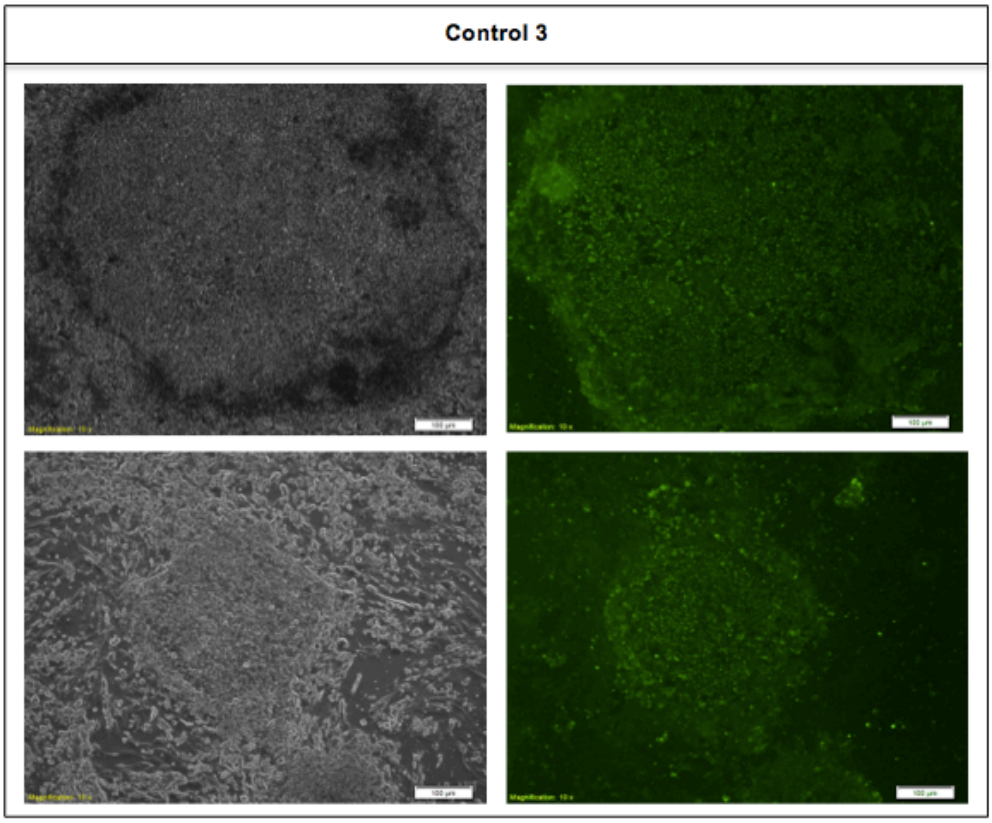


**Figure 7. Live-cell imaging of human induced pluripotent stem cells.**

The live-cell imaging was performed using the antibody Alexa Fluor ® 488 Mouse IgG1 Tra-1-60. Images on the left side show fully reprogrammed iPSC colonies of sample Control3 on D29 after transduction of 250,000 fibroblast cells with the Sendai Virus with the brightfield. Pictures on the right side show the colonies under a fluorescence microscope

## 3.2 Somatic Cell Reprogramming on Essential 8™ Medium with Sendai Virus

For research purposes of the HSCI iPS core facility in Cambridge, MA, USA only, the sample Control3 that was reprogrammed on MEF cells in chapter 2.1 was also reprogrammed on a feeder free system. The reprogramming was performed as on the feeder cell layer until D7 after transduction. On the day of replating the cells onto a 10-cm cell culture dish, the 125,000 transduced fibroblasts were replated onto a Vitronectin layer and Essential 8™ Medium.

One fully reprogrammed induced pluripotent stem cell colony was derived and was picked on a Vitronectin coated well of a 6-well cell culture plate in E8 medium. The Line was expanded and frozen for further analysis.

Figure 8 shows the fully reprogrammed iPSC colony on the day of picking (D30 after transduction of fibroblasts with the Sendai virus).

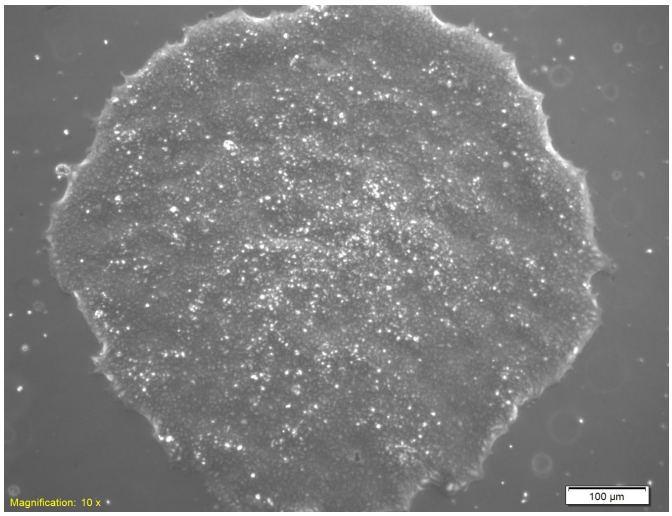


**Figure 8. Fully reprogrammed iPSC colony on Vitronectin and Essential 8TM Medium after transduction of 125,000 fibroblasts with the Sendai virus.**

Shown is a fully reprogrammed iPSC colony derived from 125,000 fibroblast cells of sample Control3 on a Vitronectin layer and E8 medium transduced with the Sendai virus

## 3.3 Somatic Cell reprogramming on Vitronectin using Retrovirus and N2B27 medium

For research purposes of the HSCI iPS core facility in Cambridge, MA, USA only, a sample from a patient with an unknown disease, received from an external investigator, was reprogrammed using a retrovirus system on a feeder-free system in parallel to feeder system. The reprogramming was performed as on the feeder cell layer until D4 after transduction. 100,000 transduced fibroblasts were replated onto a vitronectin layer and N2B27 medium in a 10 cm dish.

Figure 9 shows the time course of the morphological changes of the transduced fibroblasts that were observed after the transduction with the retrovirus. No reprogrammed induced pluripotent stem cell colonies were derived using this protocol. The experiment was stopped 24 days after the transduction of the fibroblasts with the retrovirus.

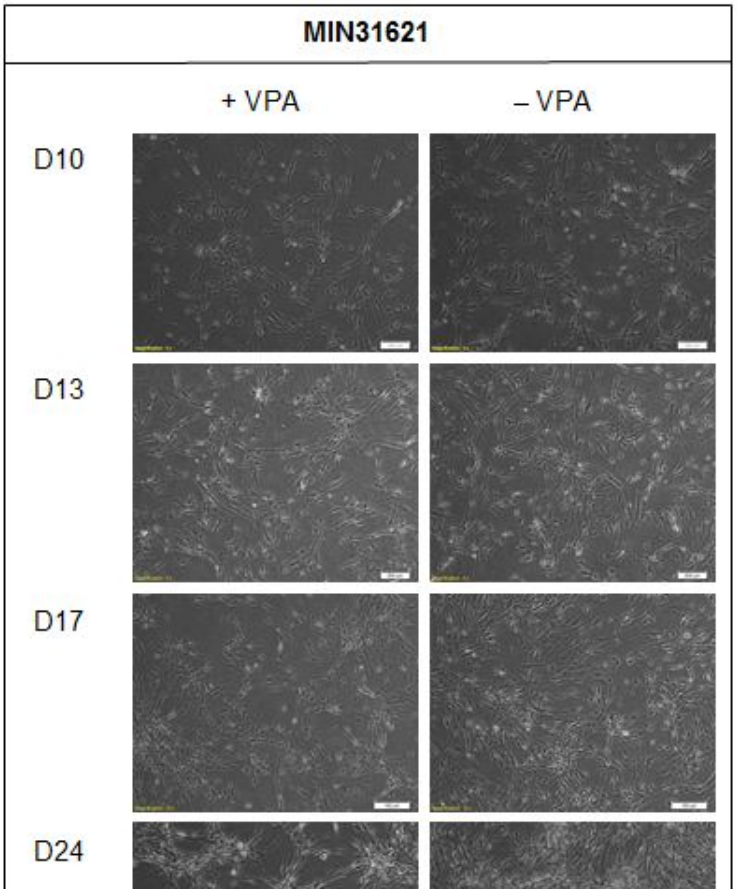


**Figure 9. A time course of morphological changes observed after the transduction of the fibroblast line MIN31621 with the retrovirus for the reprogramming on N2B27 medium with VPA and without VPA**

The first visible changes were observed approximately one week after the transduction of 100,000 fibroblast cells with the retrovirus. The images show morphological changes from D10 to D24 after transduction. The images on the left side show visible changes with the addition of VPA for 1 week, the ones on the right side without the addition of VPA

.

## 3.4 Characterization of the Induced Pluripotent Stem Cell Lines derived with the Sendai virus method

To verify the identity of the reprogrammed induced pluripotent stem cell lines, a detailed characterization and quality-control assays are required.

### 3.4.1 Karyotyping

In order to look for mutations or an abnormal number of chromosomes, each characterized iPS cell line was analyzed by G-band karyotyping.

Figure 10 shows the G-band karyotyping analysis for the cell lines MIN30857-C and MIN30857-D. Both lines had a normal, male karyotype.

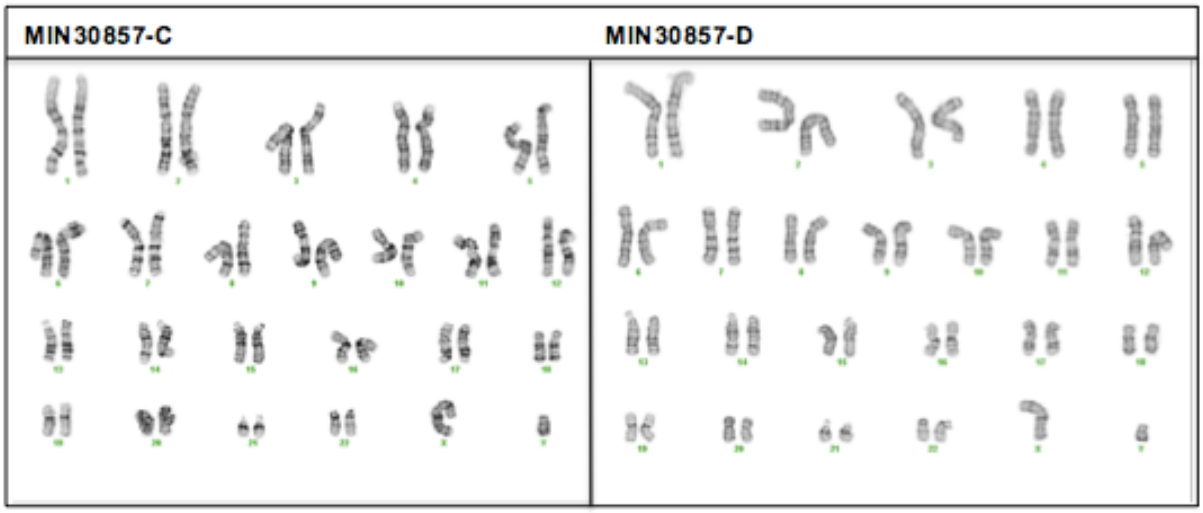


**Figure 10. G-band karyotyping analysis of the iPS cell lines MIN30857-C and MIN30857-D.**

All cell lines that were tested had a normal, male karyotype. The analysis was performed at passage number 4. The karyotype was analyzed by Cell Line Genetics, Madison WI, USA

### 3.4.2 Alkaline Phosphatase Staining

A high level of alkaline phosphatase expression is a characteristic of induced pluripotent stem cells. In order to show that the iPS cells were undifferentiated, an Alkaline Phosphatase Staining was performed using an Alkaline Phosphatase Detection Kit.

In figure 11, undifferentiated iPSC colonies of the lines MIN30857-C and -D, which exhibit increased levels of AP expression (appear in red), are shown.

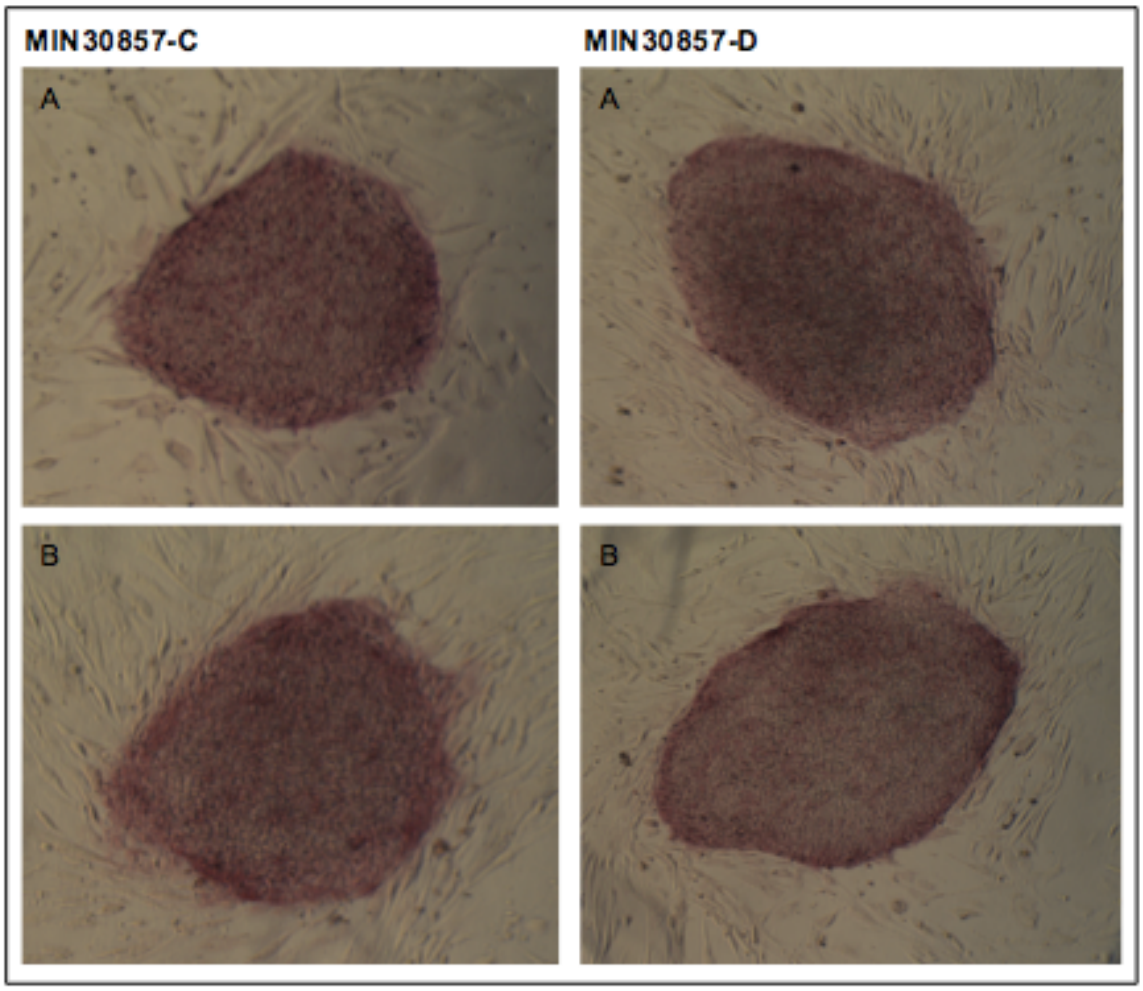


**Figure 11. Alkaline Phosphatase staining of the iPS cell lines MIN30857-C and MIN30857-D.**

That cell lines MIN30857-C and –D are pluripotent and undifferentiated, an alkaline phosphatase staining, using the Alkaline Phosphatase Staining Kit, was performed. 2 stained colonies per cell line are shown

.

### 3.4.3 Analysis by Immunocytochemistry – Pluripotency Markers

To show that the reprogrammed iPS lines are pluripotent, immunocytochemistry and immunofluorescence was performed to verify the expression of the stem cell markers associated with pluripotency. Two transcription factors, Nanog and Oct4, and three cell surface markers, SSEA-3, SSEA-4 and Tra-1-60 were examined. In figure 12 and 13, the primary antibodies, the DAPI nuclear counterstain and the merged images for the cell lines MIN30857-C and –D are shown.

All induced pluripotent stem cell lines that were tested expressed pluripotent markers. A positive expression of the transcription factors and the cell surface markers was displayed.

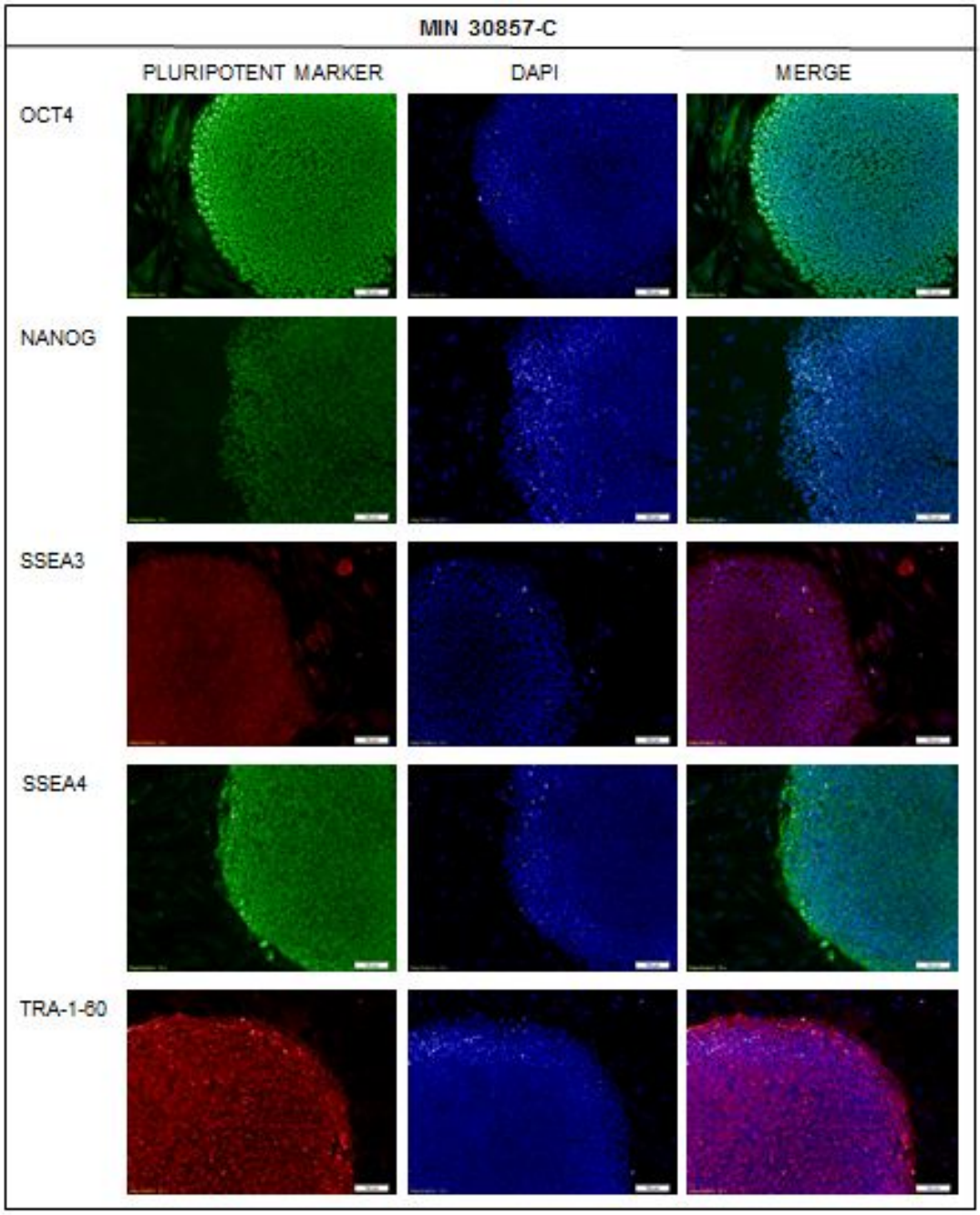


**Figure 12. Immunocytochemistry for pluripotent markers – Cell line MIN30857-C**

The primary antibodies, the DAPI nuclear counterstain and the merged images for the cell line MIN30857-C are shown. Two transcription factors, Nanog and Oct4, and three cell surface markers, SSEA-3, SSEA-4 and Tra-1-60 were examined.

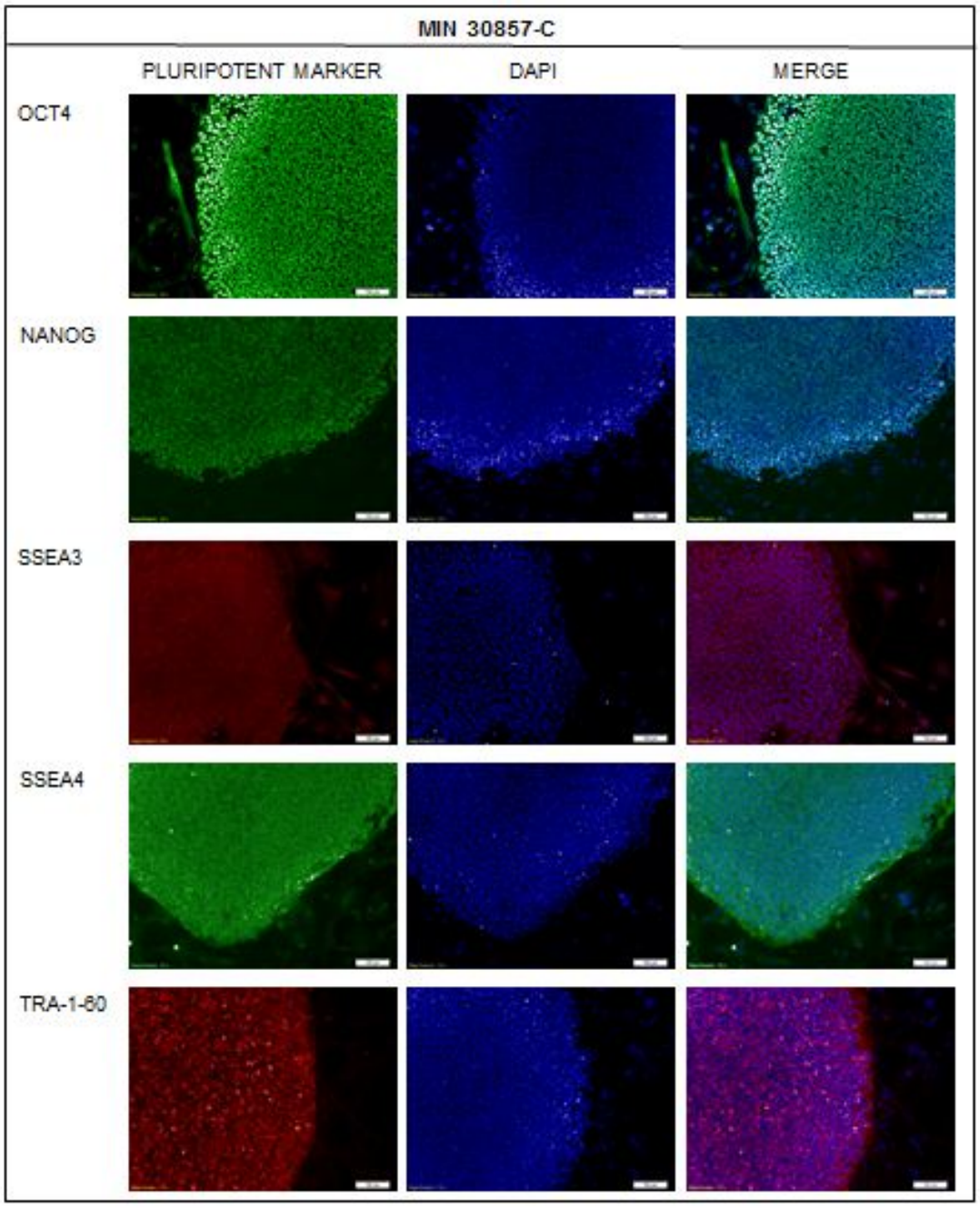


**Figure 13. Immunocytochemistry for pluripotent markers – Cell line MIN30857-D.**

The primary antibodies, the DAPI nuclear counterstain and the merged images for the cell line MIN30857-D are shown. Two transcription factors, Nanog and Oct4, and three cell surface markers, SSEA-3, SSEA-4 and Tra-1-60 were examined.

### 3.4.4 Analysis by Immunocytochemistry – Differentiation Markers

To verify that the iPSCs can form all three germ layers, three markers: β-Tubulin for the ectoderm germ layer, SMA for the mesoderm germ layer and SOX17 for the endoderm germ layer were examined by ICC immunofluorescence. In figure 14, the primary antibodies, the DAPI nuclear counterstain and the merged images for the cell line MIN30857-D are shown.

The iPSC line that was tested expressed the markers for the three germ layers.

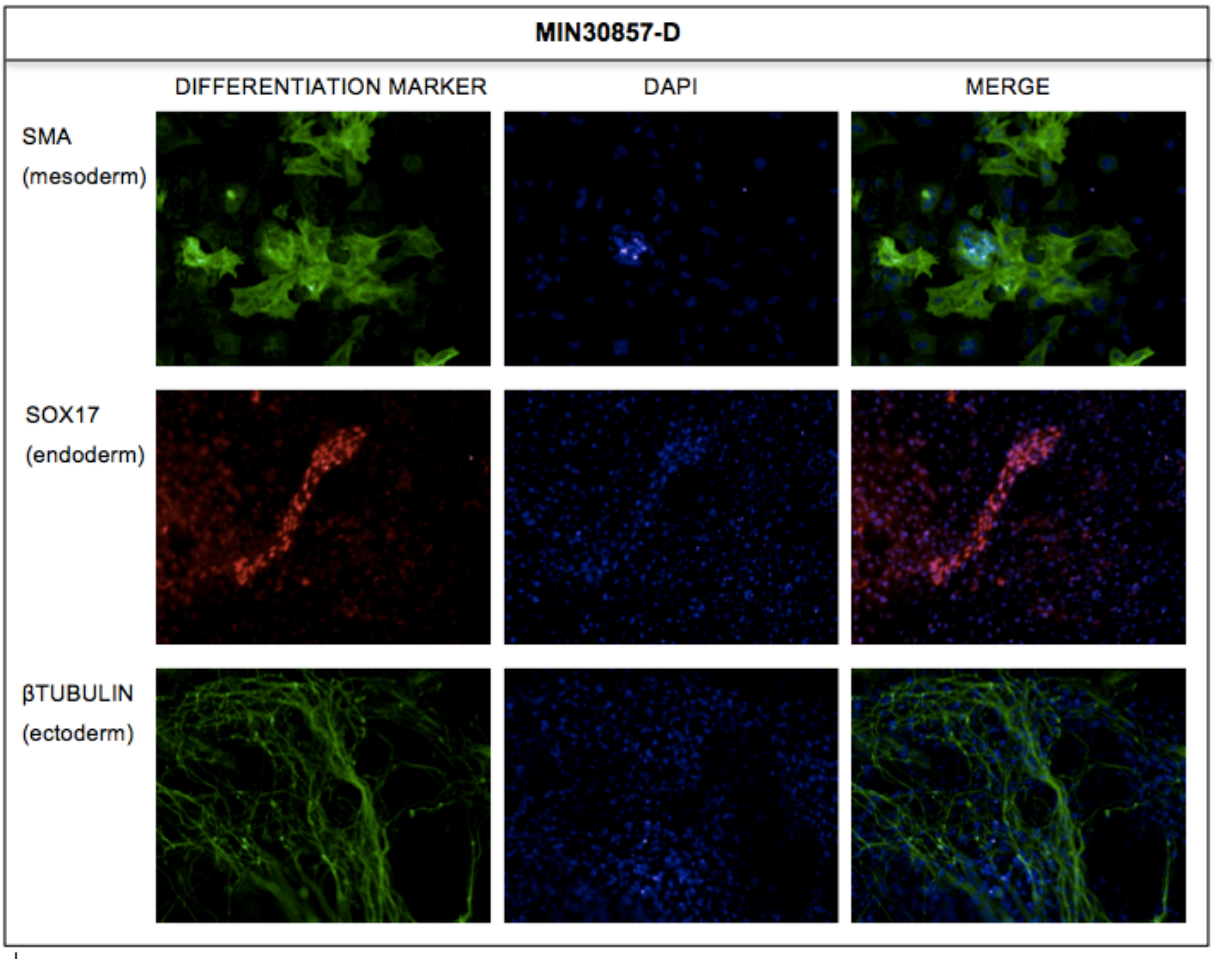


**Figure 14. Immunocytochemistry for differentiation markers on EBs – Cell line MIN30857-D.**

The primary antibodies, the DAPI nuclear counterstain and the merged images for the cell line MIN30857-D are shown. Three markers: β-Tubulin for the ectoderm germ layer, SMA for the mesoderm germ layer and SOX17 for the endoderm germ layer were examined.

### 3.4.5 Analysis of the Expression of Pluripotency Genes

Another method, to show that the reprogrammed iPS cells are pluripotent is a PCR using specific primer sequences for pluripotent markers. The PCR products were run on an agarose gel via electrophoresis.

RNA was extracted from iPS cell colonies. cDNA was synthesized and the expression profile of pluripotency-associated genes was analyzed via PCR.

Figure 15 shows the bands of the pluripotency markers run on the agarose gel via electrophoresis. The parts with no bands represent the negative controls. β-Actin was used as a housekeeping gene to prove that the cDNA synthesis was successful.

The results confirm the pluripotency of the derived iPS cell lines on a molecular level. All iPS cell lines that were analyzed show distinct pluripotency based on the expression of key pluripotent genes.

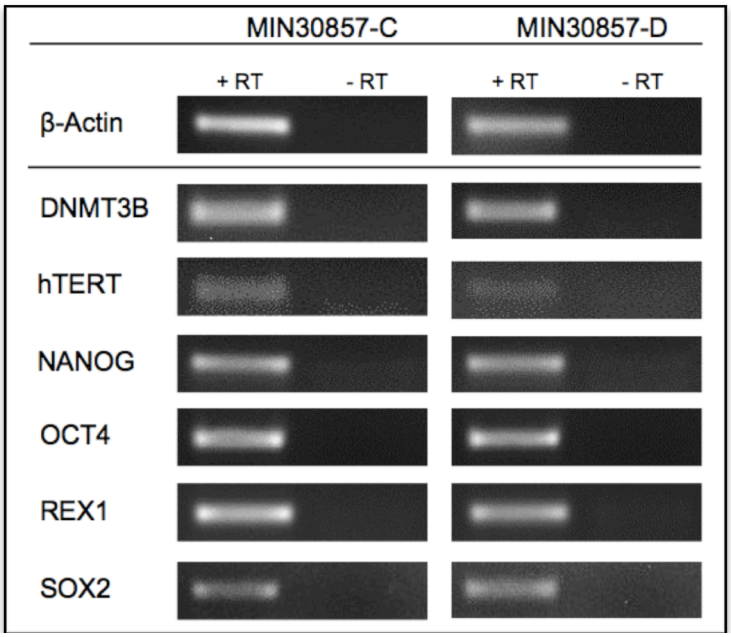


**Figure 15. Pluripotent gene expression profiles of iPS cell lines MIN30857-C and –D.**

Gene expression profiles of the key pluripotent genes for iPS cell were performed for DNMT3B, hTERT, NANOG, OCT4, REX1, SOX2. β-Actin was used as housekeeping gene to prove that the cDNA synthesis was successful.

### 3.4.6 Analysis of the Differentiation Potential

In order to test the full differentiation potential of reprogrammed iPS cells, the gene expression of markers from the three germ layers was analyzed in differentiated iPS cells.

In-vitro differentiation of iPS cells by EBs formation, and the analysis for differentiation markers of all three germ layers was performed. The mesoderm markers FLK and GATA2, the endoderm markers AFP and GATA4, and the ectoderm markers PAX6 and N-CAM were tested. β-Actin was used as a housekeeping gene to prove that the cDNA synthesis was successful.

The results confirm that the iPS lines tested could differentiate into the 3 germ layers. The differentiation capacity is shown by the expression of differentiation genes (Figure 16).

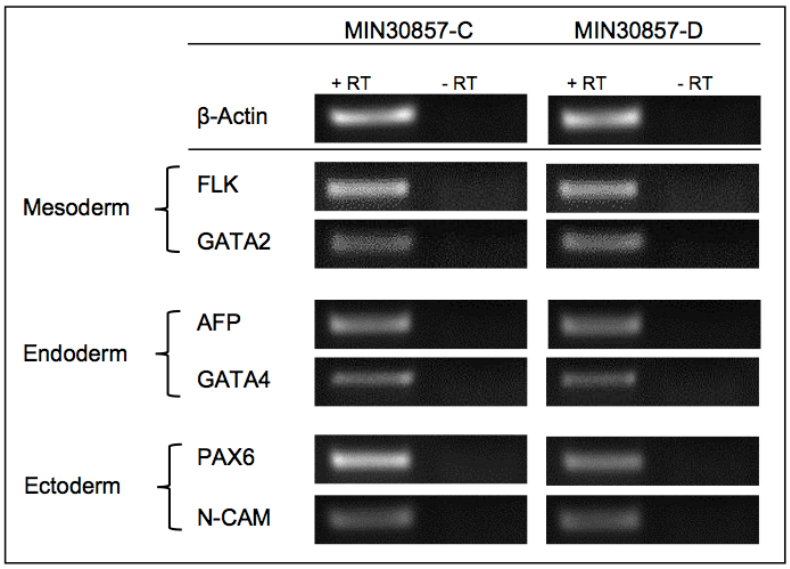


**Figure 16. Differentiation gene expression profile of iPS cell lines MIN30857-C and –D.**

Gene expression profiles of the key differentiation-associated genes for iPS cell were performed for the mesoderm markers FLK and GATA2, the endoderm markers AFP and GATA4 and the ectoderm markers PAX6 and N-CAM. β-Actin was used as a housekeeping gene to prove that the cDNA synthesis was successful.

### 3.4.7 Differentiation Evaluation by Teratoma Formation

Teratoma formation is the most accurate test for pluripotency in induced pluripotent stem cells, as teratoma tumors consist of multiple lineages containing tissue derived from the three germ layers (endoderm, mesoderm, ectoderm).
Reprogrammed iPSC colonies were sent to the Harvard Genome Modification Facility, MA, USA. The iPS cells were surgically implanted into the sub-renal capsule of three immunodeficient mice per cell line. The teratomas were received around nine weeks after implantation and sent to the HSCRB Histology Core, Cambridge, MA, USA, for a histological and immunohistochemical staining.

The teratoma formation of both lines was successful. Cells of all three germ layers were found in the teratomas.

Images of the teratoma slices of the cell lines MIN30857-C and D which show different origins of cell types that correspond to the three germ layers: endoderm, mesoderm and ectoderm, are shown in figure 17 and 18.

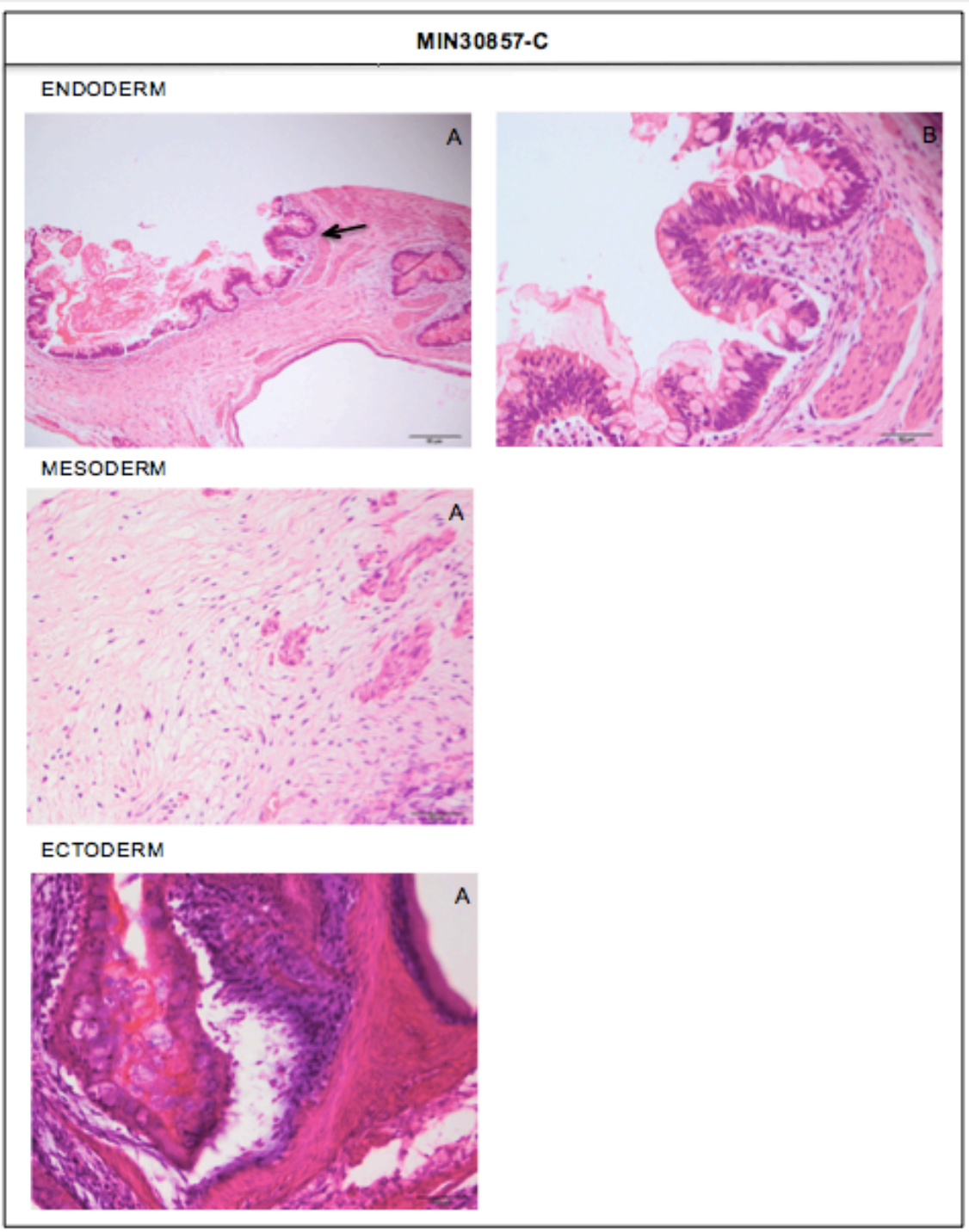


**Figure 17. Teratoma slices of the cell line MIN30857-C that correspond to the three germ layers: endoderm, mesoderm and ectoderm.**

Images of slices of the teratomas of MIN30857-C which show different origins of cell types that correspond to the three germ layers. The images in the left column (A) were taken at 10X magnification. The images in the right column (B) were taken at 40X magnification

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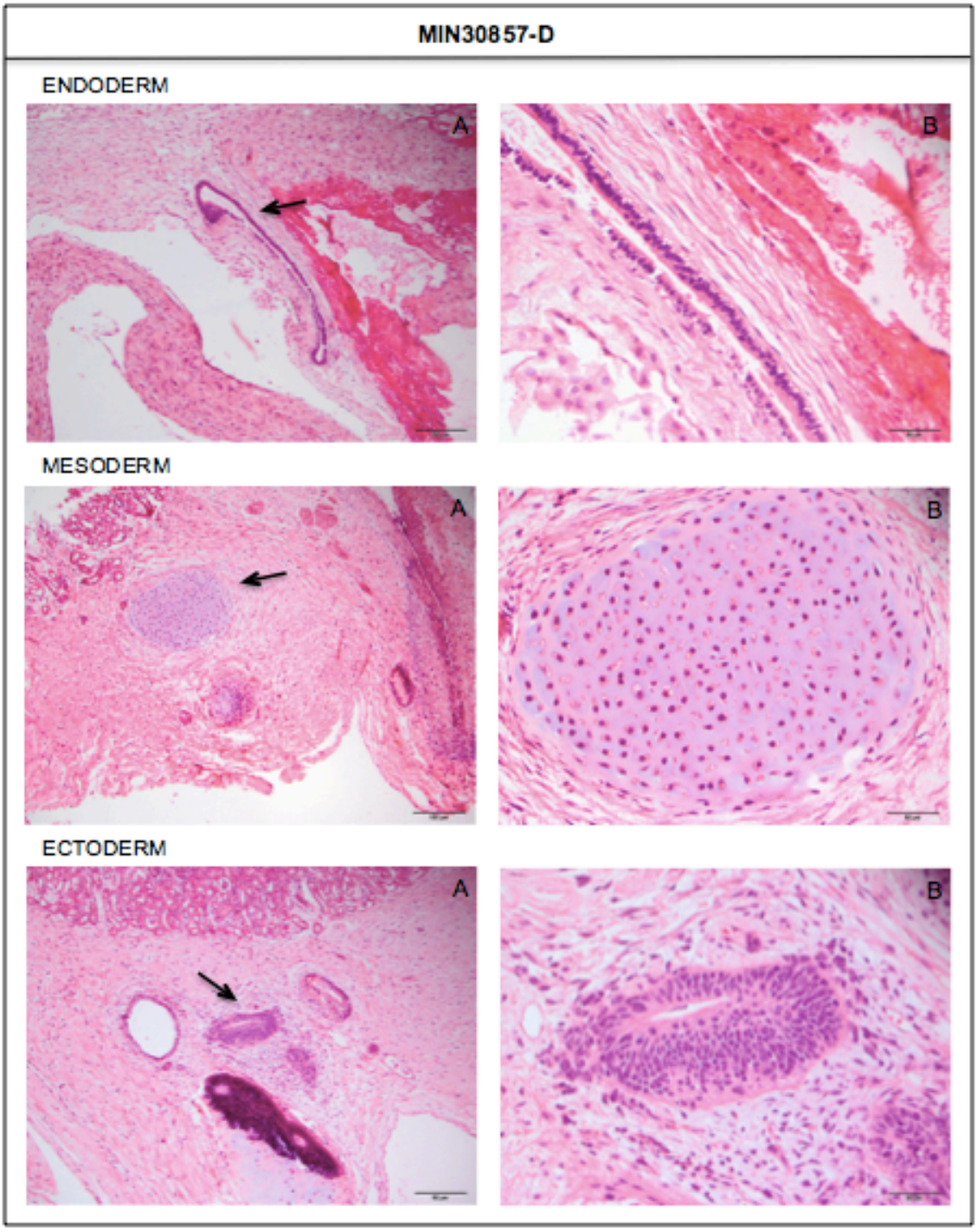


**Figure 18. Teratoma slices of the cell line MIN30857-D that correspond to the three germ layers: endoderm, mesoderm and ectoderm.**

Images of slices of the teratomas of MIN30857-C which show different origins of cell types that correspond to the three germ layers. The images in the left column (A) were taken at 10X magnification. The images in the right column (B) were taken at 40X magnification.

### 3.4.8 Analysis of the Expression Level of Pluripotent and Differentiation Markers using real time PCR

#### 3.4.8.1 iPSC pluripotency validation

To test the quantitative expression of pluripotent-associated genes, a real time PCR using real-time PCR Kits from Qiagen was performed.

19 specific pluripotency-related genes were tested with the qPCR Kit. The graph in figure 19 shows the relative expression of these 19 genes compared to NAT1, which functions as a housekeeping gene. The bars in pink and yellow demonstrate the relative gene expression of the induced pluripotent stem cell lines MIN30857-C and –D. The bars in purple show the expression of these genes in fibroblasts and the green bars shown the expression in human embryonic stem cells for comparison. On the x-axis, the genes that were tested can be seen. A logarithmic fold change for logarithmic fold changes to NAT1 can be seen on the y-axis.

The gene expression of the gene XIST is shown in the negative area because it is only expressed in female lines and the two iPS cell lines that were analyzed, as well as the fibroblast line, were derived from a male donor, while the hESC line was a female line These results confirm the pluripotency of the derived iPS cell lines MIN30857-C and –D as all bars are in the positive area of the graph.

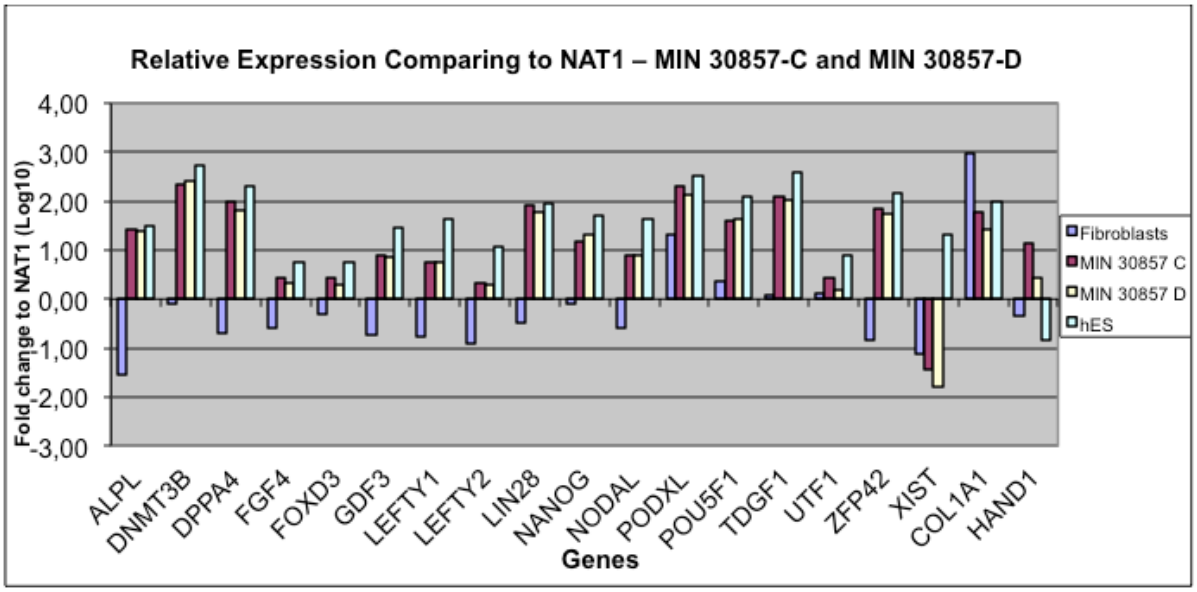


**Figure 19. Relative gene expression of pluripotency-related genes compared to NAT1 of MIN30857-C and -D.**

Shown is the relative expression of 19 genes compared to NAT1, which functions as a housekeeping gene. The bars in pink and yellow demonstrate the relative gene expression of the induced pluripotent stem cell lines MIN30857-C and –D. The bars in purple show the expression of these genes in fibroblasts and the green bars show expression in human embryonic stem cells as comparison. On the x-axis, the genes that were tested can be seen. A logarithmic fold change for logarithmic fold changes to NAT1 can be seen on the y-axis.

### 3.4.8.2 Embryoid Body Differentiation

To test the quantitative expression level of differentiation-associated markers, a real time PCR using real -time PCR Kits from Qiagen was performed.

19 genes were tested with the qPCR Kit. 16 genes are specific differentiation-related markers. The 3 last markers in the graph are pluripotency genes and serve as negative control. The graph, shown in figure 20, shows the fold change compared to a control sample. The bars in pink and yellow demonstrate the relative fold change of the embryoid bodies formed from the induced pluripotent stem cell lines MIN30857-C and –D. The bar in green shows the fold changes embryoid bodies from a fully characterized iPS line (FB7) and serves as positive control. A good negative control would be undifferentiated hESC or iPS lines. On the x-axis, the genes that were tested can be seen. Logarithmic fold change to NAT1 can be seen on the y-axis. A logarithmic fold change for logarithmic fold changes to NAT1 can be seen on the y-axis.

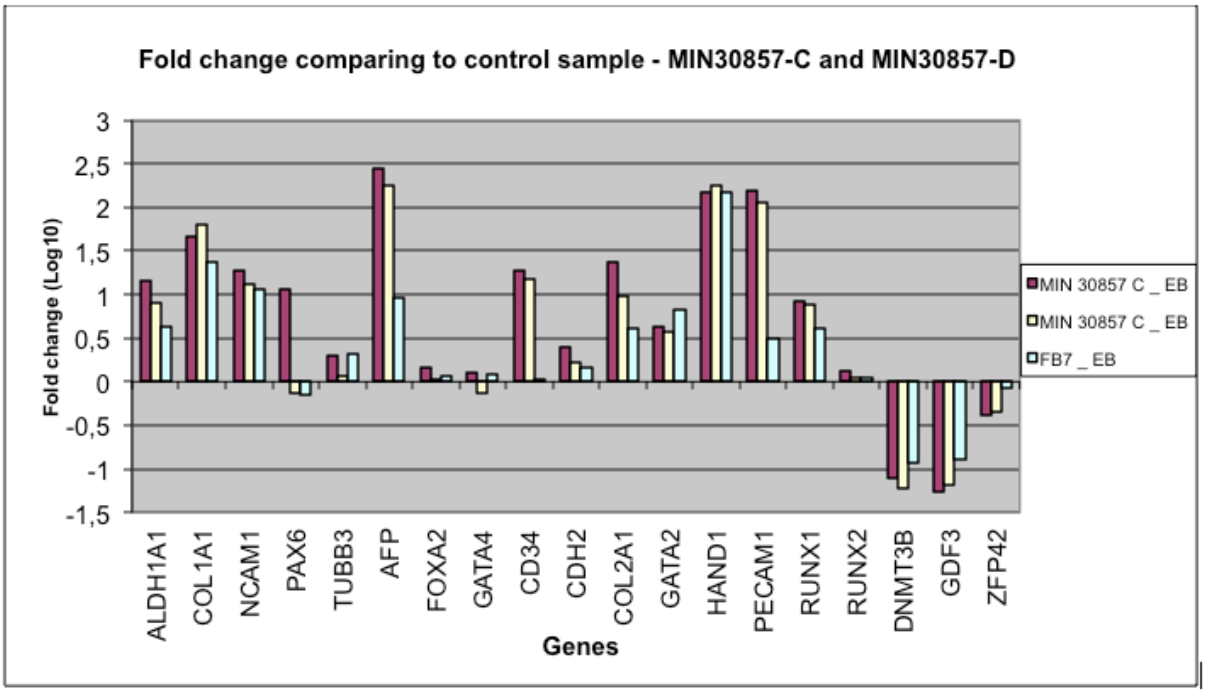


**Figure 20. Fold change of differentiation-related genes compared to a control sample of MIN30857-C and -D.**

Shown are 19 genes tested with the qPCR Kit. 16 genes are specific differentiation-related, 3 markers serve as negative control and are pluripotency markers (last 3 genes). The graph, shown in figure XY, shows the fold change compared to a control sample. The bars in pink and yellow demonstrate the relative fold change of the embryoid bodies formed from the induced pluripotent stem cell lines MIN30857-C and –D. The bars in green show fold change in Fibroblasts and serve as negative control. On the x-axis, the genes that were tested can be seen. A logarithmic fold change for logarithmic fold changes to NAT1 can be seen on the y-axis.

These results confirm the differentiation potential of the newly derived iPS cell lines MIN30857-C and –D.

## 3.5 Generation of Human Induced Pluripotent Stem Cells from Erythroblasts with the Sendai virus

Human erythroblasts, derived from healthy donor, were transduced with the Sendai virus carrying the four Yamanaka factors: Oct4, Sox2, Klf4 and c-Myc. The cells were transduced at a density of 150,000 cells per well of a 12-well cell culture plate, using a CytoTune$^{TM}$ iPS Reprogramming Kit at a multiplicity of infection of 10.

Three days after the transduction, the cells were plated on MEFs and cultured either on hES medium or on DMEM 10 % FBS medium. The first morphological changes were observed approximately one week after the transduction. The first fully reprogrammed iPS cell colonies were picked on D19 after the transduction, based on morphology.

Figure 21 and 22 show the time courses of the morphological changes of the lines ED3 and ED4, observed after the transduction of the erythroblasts with the Sendai virus.

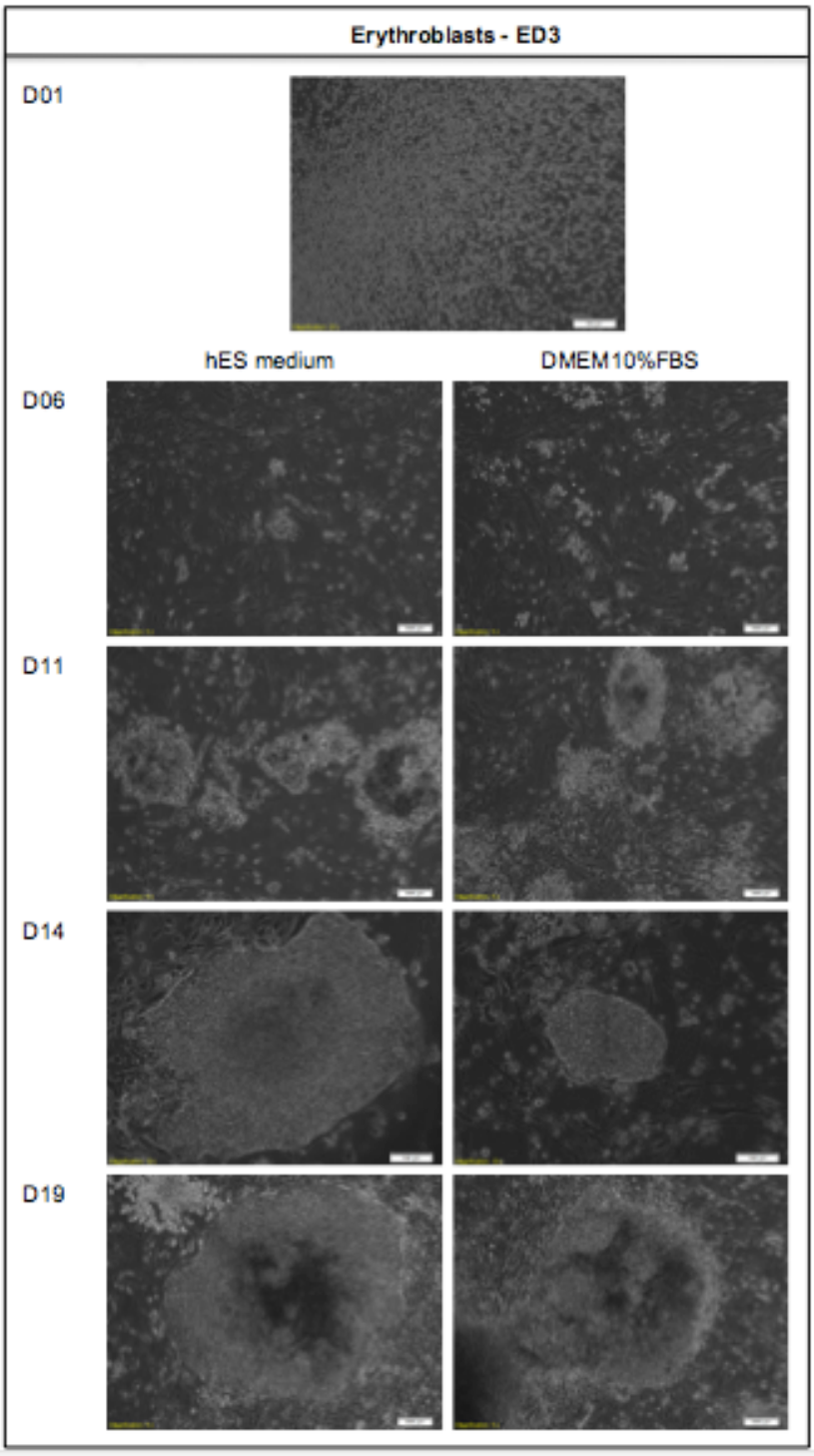


**Figure 21. A time course of morphological changes observed after the transduction of the erythroblast line ED3 with the Sendai virus on hES and DMEM 10 % FBS medium.**

The first visible changes were observed approximately one week after the transduction of 150,000 erythroblast cells with the Sendai virus. The images show morphological changes from D1 to D19 after transduction

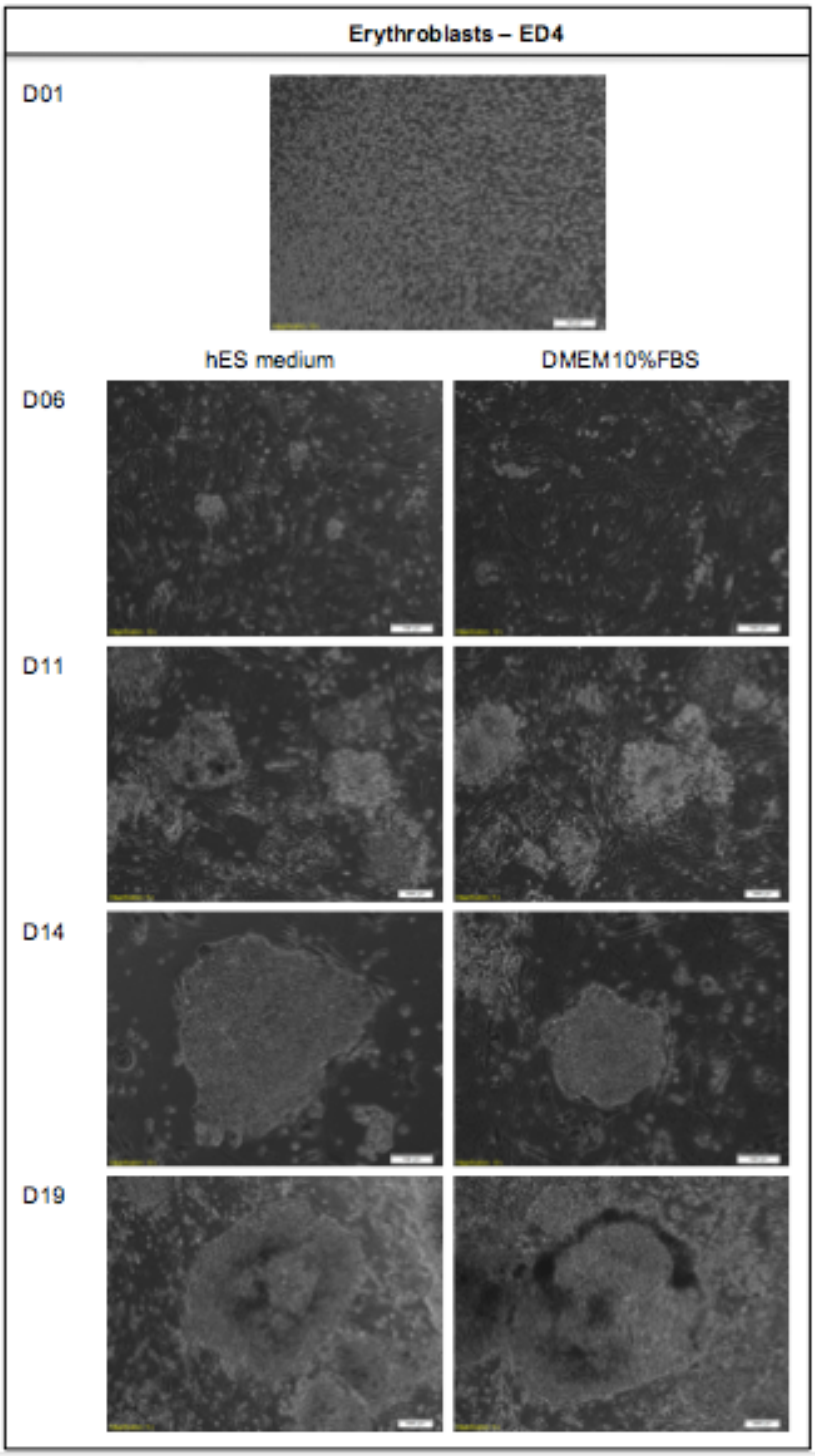


**Figure 22. A time course of morphological changes observed after the transduction of the erythroblast line ED4 with the Sendai virus on hES and DMEM 10 % FBS medium.**

The first visible changes were observed approximately one week after the transduction of 150,000 erythroblast cells with the Sendai virus. The images show morphological changes from D1 to D19 after transduction.

The colonies in one well of each culture condition (hES medium and DMEM 10 % FBS medium) were counted. (The efficiency is calculated using 150,000 transduced cells and the total number of iPSC colonies. (shown in Table 10).

**Table 10. The efficiency of 100,000 transduced erythroblast cells of the lines ED3 and ED4 transduced with the Sendai virus.**

The number of colonies counted and the culture mediums are listed. The efficiency was calculated using 150,000 transduced cells and the total number of iPSC colonies.

| **Erythroblast Line** | **ED3** | | **ED4** | |
|---|---|---|---|---|
| **Medium** | **hES** | **DMEM10%FBS** | **hES** | **DMEM10%FBS** |
| **Number of derived iPSC colonies in 3 wells of a 6 well cell-culture plate** | 213 | 129 | 156 | 126 |
| **Total** | 342 | | 282 | |
| **Efficiency** | **0.228 %** | | **0.180 %** | |

## 3.6 microRNA enhanced mRNA Transfection

Fibroblasts derived from three patients with an unknown disease, were received for reprogramming from an investigator. Two cell lines were derived from living patients, one cell line was derived at the autopsy of a deceased patient. Two cell lines were reprogrammed by the microRNA enhanced mRNA transfection. The line MSA1 (autopsy) was reprogrammed by the microRNA enhanced mRNA transfection and was compared to transfection with mRNA only. Fibroblasts from a BJ cell line that had previously been successfully reprogrammed, was used as a positive control.

Changes in morphology were observed starting on day 3 of the experiment. The first fully reprogrammed iPS colonies were picked on day 15, based on morphology.

Figure 23 and 24 show the time courses of the morphological changes of the lines MSA1, MSA5, C2B and BJ (positive control) observed after the transfection. MSA1 was reprogrammed by microRNA enhanced mRNA transfection and was compared to

transfection with mRNA only. MSA5 and C2B were reprogrammed by microRNA enhanced mRNA transfection. The BJ line served as positive control as a it has already been successfully reprogrammed by mRNA transfection and was reprogrammed with mRNA only.

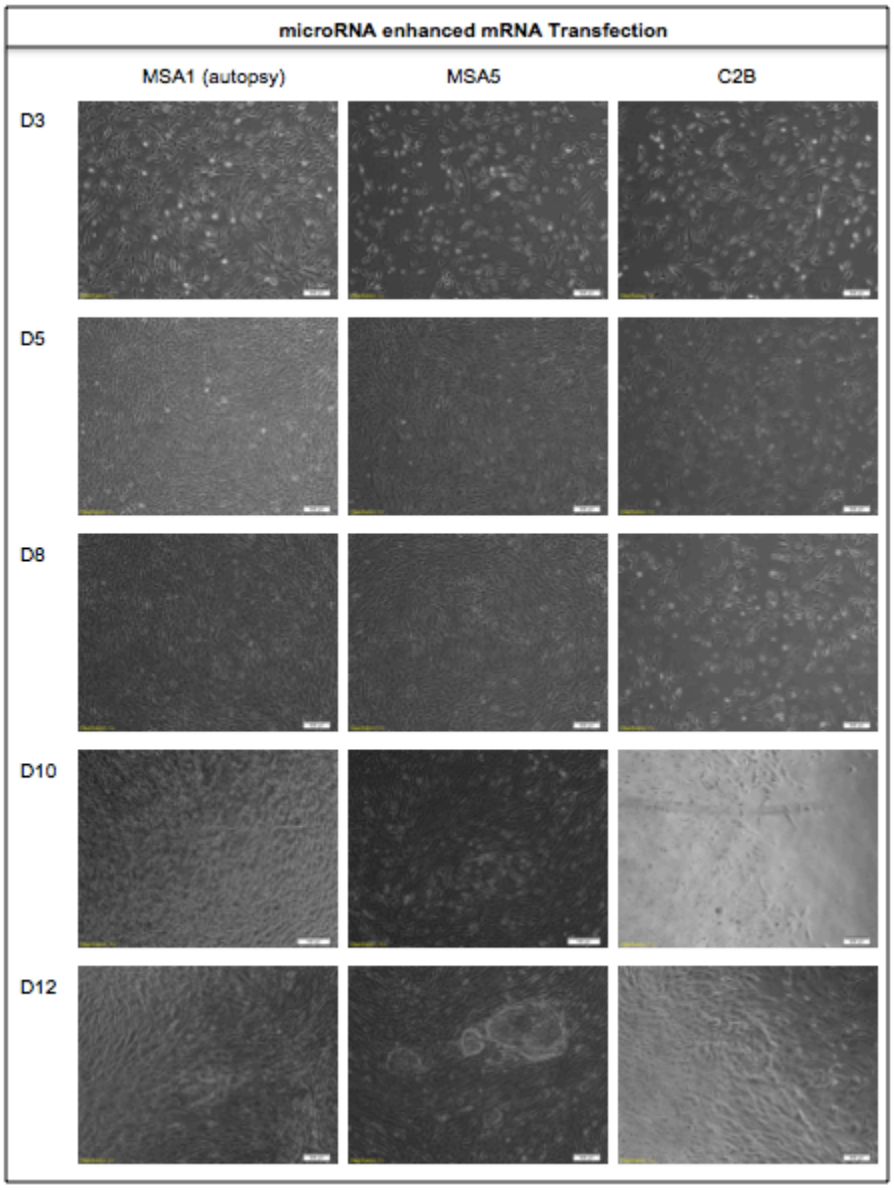


**Figure 23. A time course of morphological changes observed at the microRNA enhanced mRNA transfection of fibroblasts of the lines MSA1, MSA5 and C2B.**

Changes in morphology were observed starting on day 3 of the experiment. 50,000 fibroblasts were transfected. Morphological changes from D3 to D12 are shown. The first colonies were picked on day 15 of the experiment. Images are shown in 4X magnification.

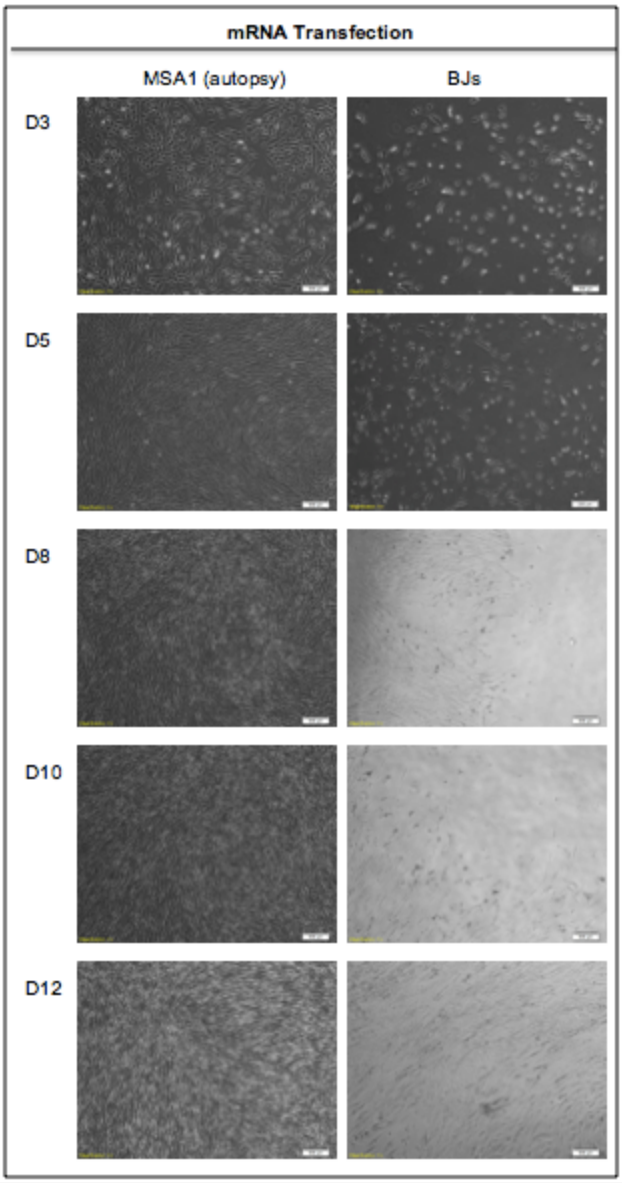


**Figure 24. A time course of morphological changes observed at mRNA transfection of fibroblasts of the lines MSA1 and BJs.**

Changes in morphology were observed starting on day 3 of the experiment of the transfection of 50,000 fibroblasts. Morphological changes from D3 to D12 are shown. No colonies were picked or expanded for these lines. Images are shown in 4X magnification.

Fully reprogrammed induced pluripotent stem cell colonies were successfully derived from all four lines. iPSC colonies were expanded from the lines MSA1, MSA5 and C2B. The BJ line just served as positive control and has been already reprogrammed.
For the line MSA1, which was reprogrammed with mRNA only and with microRNA and mRNA, colonies were derived from the microRNA enhanced mRNA transfection but not from the mRNA transfection, confirming that this new system is more robust.

The nGFP expression of the cells was checked every morning after changing the medium to fresh NuFF conditioned Pluriton$^{TM}$ medium. The time course of the nGFP expression of the transfected cells is shown in figure 25, 26 and 27. Figure 25 shows the nGFP expression of the fibroblast line MSA1, which was reprogrammed under both conditions. Figure 26 shows the nGFP expression of the 2 fibroblast lines MSA5 and C2B, which were reprogrammed by microRNA-enhanced mRNA transfection. In figure 27, the nGFP expression is shown for the BJ line that was reprogrammed by mRNA transfection and served as positive control.

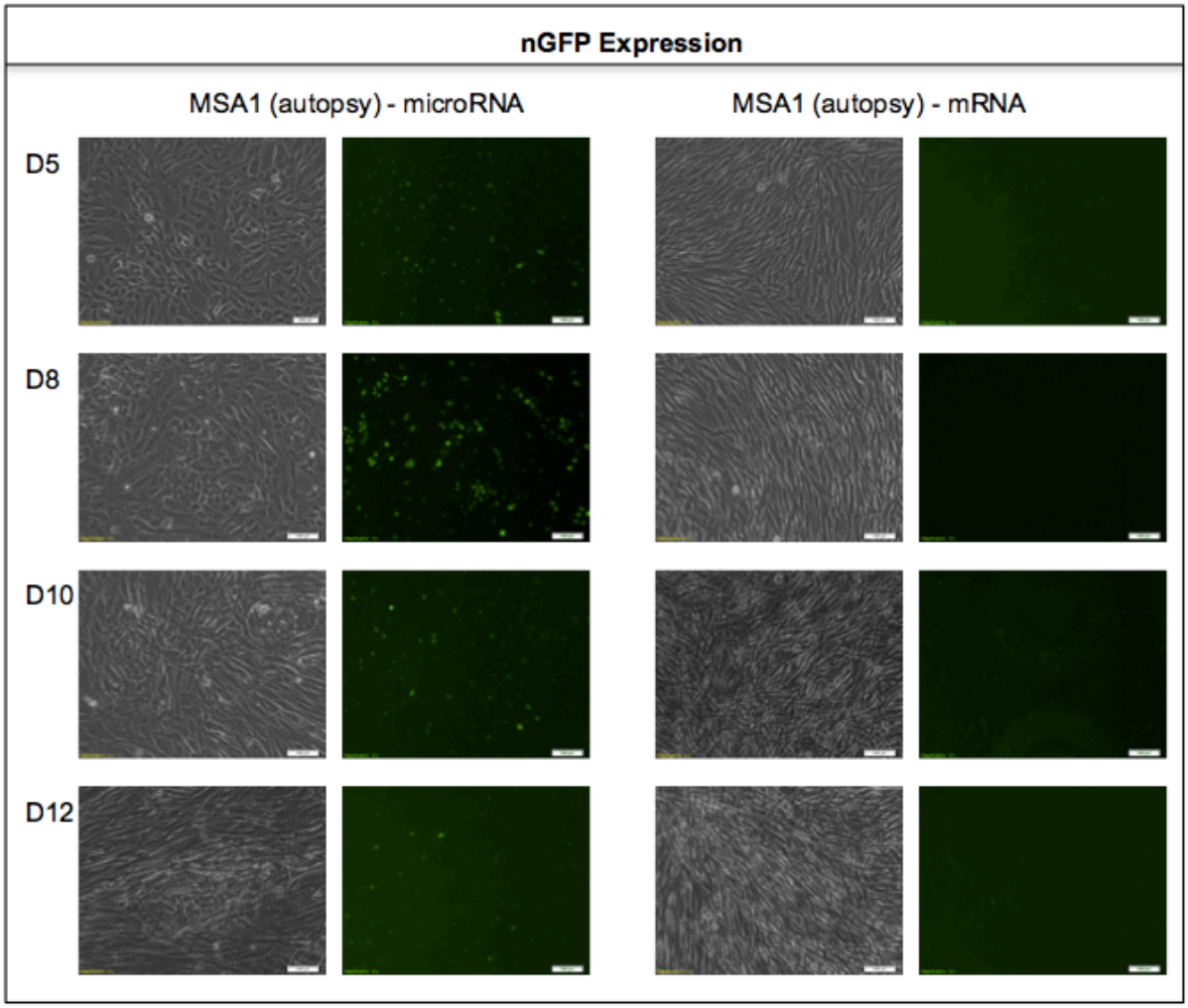


**Figure 25. A time course of the nGFP expression observed at microRNA enhanced mRNA transfection and mRNA transfection of fibroblasts of the line MSA1.**

Morphological changes and the nGFP expression of the line MSA1 from D5 to D12 are shown. This line was reprogrammed by microRNA enhanced mRNA transfection and was compared to transfection with mRNA only. Images are shown in 10X magnification.

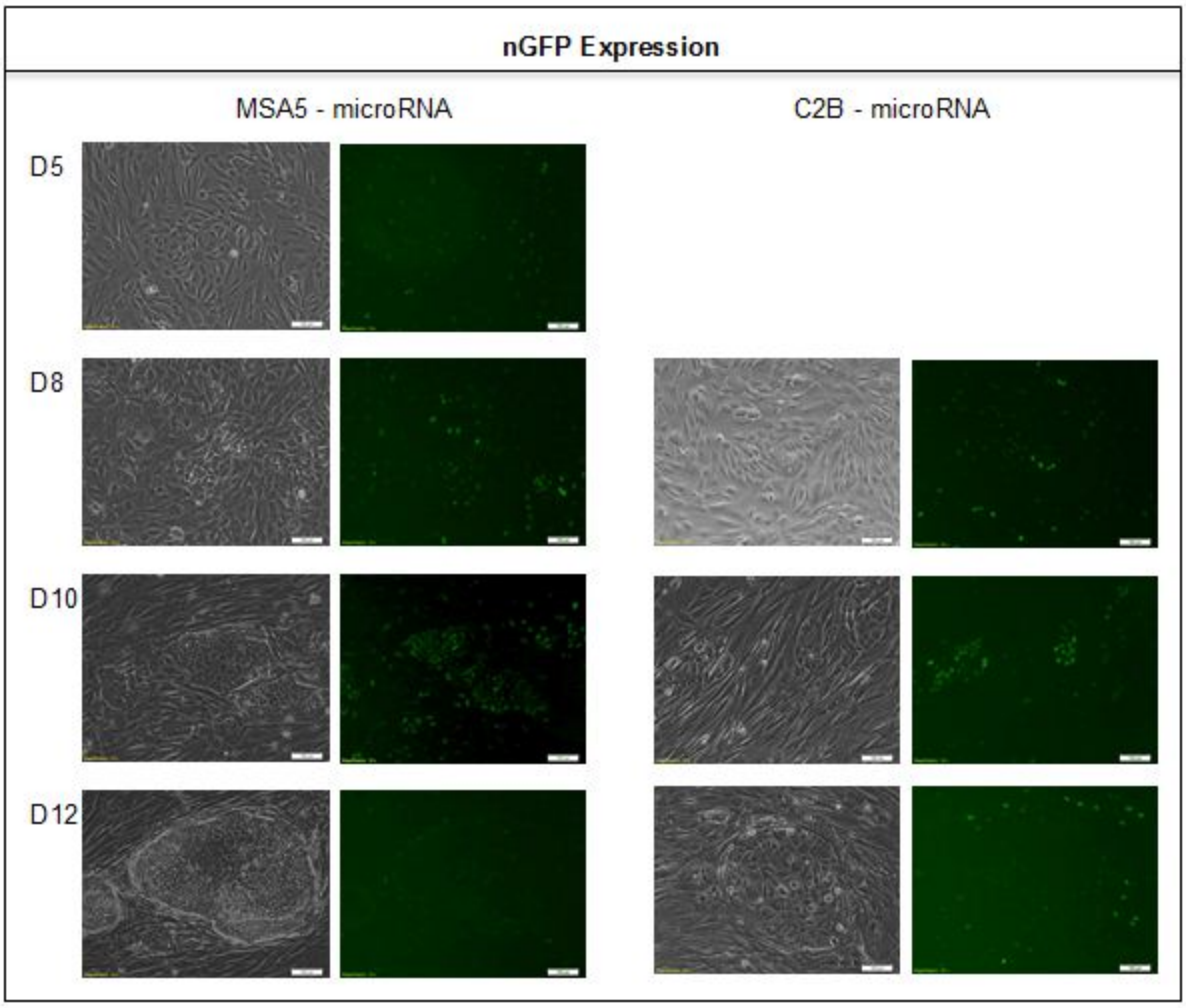


**Figure 26. A time course of the nGFP expression observed at microRNA enhanced mRNA transfection of fibroblasts of the lines MSA5 and C2B.**

Morphological changes and the nGFP expression of the lines MSA5 and C2B from D5 to D12 are shown. These lines were reprogrammed by microRNA enhanced mRNA transfection. Images are shown in 10X magnification.

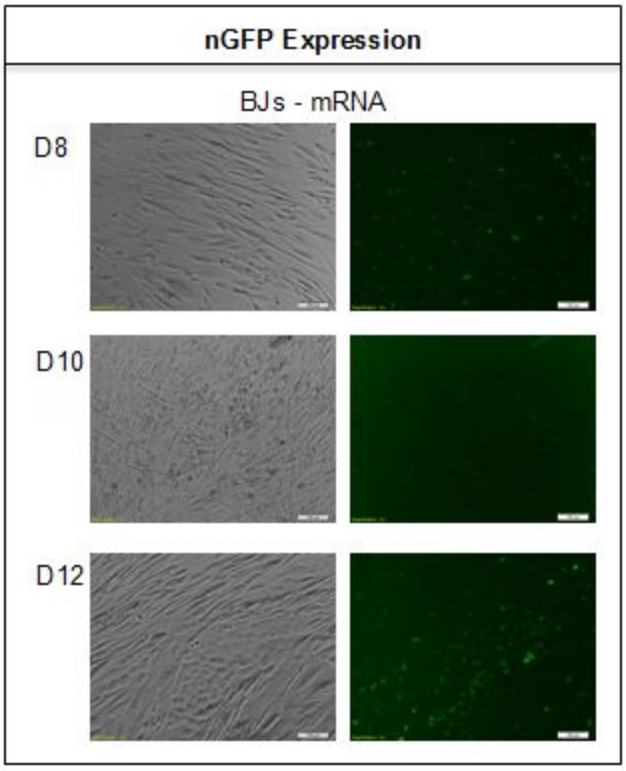


**Figure 27. A time course of the nGFP expression observed at mRNA transfection of fibroblasts of a BJ cell line.**

Morphological changes and the nGFP expression of a BJ cell line from D5 to D12 are shown. This line was reprogrammed by mRNA transfection and served as positive control. Images are shown in 10X magnification.

18 induced pluripotent stem cell lines were successfully derived from three patient samples.

# 4 DISCUSSION

The generation of human disease-specific induced pluripotent stem cells from fibroblasts was successful. The pluripotency of all iPSC lines was assessed and confirmed.

Human fibroblasts were reprogrammed by the transduction of fibroblasts with the Sendai virus carrying four transcription factors: the “Yamanaka Factors” Oct4, Sox2, Klf4 and c-Myc. These transcription factors are overexpressed in the cells. The cells were cultured on mouse embryonic feeder cells. The pluripotency of the cells was successfully shown by the completion of specific assays.

The first assay was G-band karyotyping, which could detect an abnormal number of chromosomes or mutations in the cells. A normal, male karyotype was shown for the two characterized iPSC lines. In order to show the overexpression of alkaline phospotase (a characteristic necessary to show that a line is pluripotent), an alkaline phosphatase staining was successfully performed. To confirm the pluripotency of the reprogrammed cells, an immunocytochemistry and immunofluorescence assay for the expression of markers related to pluripotency was done. Two transcription factors (Nanog and Oct4) and three cell surface markers (SSEA-3, SSEA-4 and Tra-1-60) were examined. All iPSC lines showed a clear expression of all of the markers tested. A slight difference in the expression level could be seen. A reason for that could be the different concentrations of the antibody stock solutions or variations in the performance of the assays. But because of the positive overexpression of alkaline phosphatase in combination with the successful immunocytochemistry assay, a pluripotent character of the lines MIN30857-C and D can be already presumed.

Another assay used to determine pluripotency was a PCR to test the expression of pluripotent markers. Six key pluripotency genes were examined: Nanog, Oct4, Sox2, Dnmt3b, hTERT and Rex1. RNA was extracted from the cells and a polymerase chain reaction was done to generate cDNA. The expression of key pluripotency genes was

observed. Differences in the intensity of the bands could be seen. This variation could have been caused by pipetting errors during the set up of the PCR, general variations in PCR, or the agarose gel electrophoresis. Another reason could be the actual variation in the expression of the genes in the cells. Because of that possibility, an expression profile of six different pluripotency genes was performed.

In order to test the full differentiation potential of reprogrammed iPS cells, the gene expression in differentiated iPS cells was analyzed. The iPS cells were differentiated in-vitro into embryoid bodies and the expression of differentiation markers was analyzed by PCR. The mesoderm markers FLK and GATA2, the endoderm markers AFP and GATA4, and the ectoderm markers PAX6 and N-CAM were tested. All genes were expressed in the EBs of both iPSC lines. Variations in the intensity of the bands could be seen. Reasons for that were mentioned above in the discussion of PCR for pluripotency markers. For both PCR analyses, the variations in the intensity of the bands can be neglected, as the purpose of these assays was not the quantitative expression of the genes but the general expression, which was shown for all genes in both assays.

The next assay, also done on in-vitro differentiated iPS cell EBs, was an immunocytochemistry assay for differentiation markers. To confirm the differentiation potential of the reprogrammed cells, an immunocytochemistry and immunofluorescence assay for the expression of markers related to the cells of the three germ layers was done. The marker βTubulin was used for cells of the ectoderm germ layer, the marker SMA was used for cells of the mesoderm germ layer and the marker SOX17 was used for cells of the endoderm germ layer. All iPSC lines showed a clear expression of all of the markers tested. A slight difference in the expression level could be seen. Reasons for that are mentioned above in the discussion of the immunocytochemistry for pluripotency markers.

Another assay was the analysis of the expression level of pluripotent differentiation markers by real time PCR. Real time PCR kits from Qiagen were used. Both assays confirmed the pluripotency and the differentiation potential of the derived iPSCs.

As the final and most accurate characterization assay, teratoma formation was performed to evaluate the differentiation potential of the iPS lines. Teratoma tumors consist of multiple lineages containing tissue derived from the three germ layers (endoderm, mesoderm, ectoderm). Reprogrammed iPSC colonies were sent to the Harvard Genome Modification Facility, MA, USA. The iPS cells were surgically implanted into the sub-renal capsule of three immunodeficient mice per cell line. The teratomas were then analyzed by the HSCRB Histology Core by a histological and immunohistochemical staining. The teratoma formation for both lines was successful and cells of all three germ layers were found in the tumors.

Because of the number and accuracy of all assays which were performed to characterize the generated induced pluripotent stem cells, it can be said that the generated iPS cell lines MIN30857-C and D are fully pluripotent on a molecular and functional level.

For comparison, fibroblasts of a different patient were reprogrammed to iPSCs using two different transduction methods (the Sendai virus and the retrovirus) and were cultured on a feeder free system.
Fibroblasts of one cell line, which were reprogrammed using the Sendai virus, were replated on a vitronectin layer and fed with Essential 8$^{TM}$ Medium on day 7 of the reprogramming process. One fully reprogrammed iPS cell colony was successfully derived, which is a low efficiency. This colony was expanded in Essential 8$^{TM}$ Medium on a vitronectin layer, but it could not be expanded for characterization and freezing, as the cells did not survive. After detailed research, it was found that one component in the Essential 8$^{TM}$ Medium blocks the formation of iPS colonies. According to the literature, this medium is not usually used during the reprogramming. It is only used for general culturing of iPSCs.
After the failure of this reprogramming experiment, fibroblasts of another patient were reprogrammed with the retrovirus and cultured on a different feeder-free medium during the reprogramming process. On day 4 of the reprogramming process, the transduced fibroblasts were replated on a vitronectin layer and cultured in N2B27 medium. The

experiment was stopped 24 days after the transduction of the fibroblasts because no colonies could be seen. As the same transduced fibroblasts were reprogrammed and cultured on mouse embryonic feeder cells, and fully reprogrammed iPSC colonies were derived, the reason for the failure of this experiment was not the reprogramming method. One reason could be that the only protocol that exists for the reprogramming of fibroblasts in N2B27 medium was performed with an episomal vector and not with a virus.
In conclusion, these feeder-free reprogramming systems are less-expensive and do not contain animal. These two systems need to be improved before researchers and laboratories can switch to the reprogramming and culturing of iPSCs on these feeder-free systems.

As another technique to derive induced pluripotent stem cells from fibroblasts, the microRNA enhanced mRNA transfection, a novel technique from Stemgent, was tested. The HSCI iPS core facility received the transfection kit and the fibroblasts for the test from Stemgent.
Four fibroblast lines of different patients were reprogrammed by a 12 day reprogramming schedule of microRNA-enhanced mRNA transfection. Five lines per fibroblast sample were expanded and a frozen stock was made. This reprogramming method is faster than the transduction of fibroblasts with the Sendai virus. In addition, a faster growth rate of the iPS cells and a lower differentiation potential on the MEF feeder layer could be seen.
Because this experiment was successful, Stemgent will sell the microRNA-enhanced mRNA transfection kit. This system is non-integrative and non-viral, which is advantageous to the researcher and a more preferable option to the viral methods.

Besides the reprogramming of human fibroblasts to iPSCs, PBMCs were expanded to erythroblasts and the erythroblasts were transduced with the Sendai virus. The reprogramming process was performed in two conditions, hES medium and DMEM 10 % FBS. In both conditions, fully reprogrammed iPSC colonies were derived, expanded and frozen.

Overall, the reprogramming of fibroblasts with different reprogramming methods and the reprogramming of erythroblasts with the Sendai virus were successful. Also, two iPSC lines, derived by Sendai viral transduction, were characterized in detail. The pluripotency of both lines could be verified.

# 5 CONCLUSION

Induced pluripotent stem cells were generated from fibroblasts by the Sendai virus and the microRNA-enhanced mRNA transfection. Two reprogrammed iPSC lines were successfully characterized. Erythroblasts from two patients were successfully reprogrammed by the Sendai virus.

The generation of induced pluripotent stem cells (iPSCs) from human somatic cells has become a promising hope for regenerative medicine and the possible cure of degenerative diseases.
For a long period of time, the only methods to reprogram somatic cells into iPSCs were based on a virus containing the four Yamanaka factors. The first virus used in 2007 was the retrovirus, a virus which integrates into the genome. In 2009, improvements were made with the non-integrative Sendai virus. Improvements are still needed to make the cells safe for therapy. In 2012, Stemgent developed the microRNA-enhanced mRNA transfection kit, a non-viral and non-integrative reprogramming method for fibroblasts.
There is still need for improvement. The efficiency of viral transduction is still quite low and the costs for the reprogramming process and for the characterization are extremely high.

More research will be necessary to improve the safety of iPSCs and to assess all of the molecular and functional properties of these cells. Researchers all over the world know about the potential of these cells, whose use is restricted for now. Some preliminary clinical trials have been initiated in this field. At the present time, iPSCs offer an alternative to embryonic stem cells in their disease-modeling abilities. They eliminate the moral and ethical objections of embryonic stem cells. A better understanding of cellular reprogramming is needed and it will probably take decades to use iPSCs for gene therapy and other applications.

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