

# **GENERATION OF DISEASE-SPECIFIC HUMAN INDUCED PLURIPOTENT STEM CELL (iPSC) LINES BY USING TWO DIFFERENT REPROGRAMMING APPROACHES: SENDAI VIRUS AND RETROVIRUS**

**MASTER THESIS**

Submitted at the  
IMC University of Applied Sciences Krems

**Master Degree Programme**  
*Medical and Pharmaceutical Biotechnology*

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Area of emphasis/focus/special field: Stem Cell Biology, Regenerative Biology

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Submitted on: 29.01.2012

**This Master Thesis was performed at Harvard University and The Harvard Stem Cell Institute, in the HSCI iPS Core Facility in Cambridge, Massachusetts, USA, under the supervision of Dr. Laurence Daheron.**

## 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.*

Date: 29.01.2012

A handwritten signature in black ink, appearing to read 'Elisa Wolf', written over a horizontal line.

Signature

## **ACKNOWLEDGEMENTS**

First of all, I would like to express my utmost gratitude to Dr. Laurence Daheron, Head of the iPS Core Facility. Laurence has not only given me the opportunity to conduct my research fellowship internship under her supervision in the iPS Core Facility of the Harvard Stem Cell Institute in Cambridge (USA). She also willingly shared her vast knowledge about stem cell biology with me and provided support to me whenever I asked for it. In addition, Laurence spent time proofreading my thesis and shared valuable comments with me.

I would also like to thank Dr. Chad Cowan, principal investigator at the Harvard Stem Cell Institute and the iPS Core Facility, for giving me the chance to come to Cambridge and work in an incredibly enriching environment such as the Harvard University community.

Special thanks are dedicated to my supervisor in Austria, Prof.(FH) Priv.Doiz. Dr. Andreas Eger, for his support during the entire course of my fellowship.

Besides that, I am most grateful to the entire team of the iPS Core, for their strong and enthusiastic support during my time there and their willingness to help me out with whatever troubles or questions I had.

Apart from working fulltime on a daily basis, I somehow managed to have a social life besides, which I mostly shared with my lovely roomies Purvi, Bianca, and Shangwen. These girls made my past couple of months to unforgettable ones, especially when it comes to sharing cultural experiences and values. Not only am I addicted to Taiwanese hot pot and Indian cuisine now, I also gained three extraordinary, lovely and hopefully lifelong friends.

In addition, all my gratitude goes to my family in Austria, who I know will never stop supporting me in whatever I decide to do in my life. I appreciate it a lot!

I must also not forget to mention my dear Marcus, who was once more willing to let me leave the country in order to develop myself as well as my future career.

Last but not least, special thanks go to the Austrian Marshall Plan Foundation in Vienna, which provided generous financial support to me in order to conduct my research fellowship in the United States.

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## ABBREVIATIONS

ADA-SCID	Adenosine Deaminase Deficiency-Related Severe Combined Immunodeficiency
AFP	Alpha Fetoprotein
ALS	Amyotrophic Lateral Sclerosis
AP	Alkaline Phosphatase
bFGF	Basic Fibroblast Growth Factor
BP	Base Pair
BMD	Becker Muscular Dystrophy
BMP	Bone Morphogenic Protein
cDNA	Complimentary DANN
DAPI	4',6-diamidino-2-phenylindole
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium, Nutrient mixture F12
DMSO	Dimethyl Sulfoxide
DNA	Dioxyribonucleic Acid
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 Beta
DNTP	Deoxynucleotide Triphosphate
DOX	Doxycycline
DPBS	Dulbecco's Phosphate Buffered Saline
DS	Down Syndrome; Trisomy 21
E2F	Family of Transcription Factors
EB	Embryoid Bodies
EDTA	Ethylenediaminetetraacetic acid
EGC	Embryonic Germ Cells
ESC	Embryonic Stem Cells
ESCC	ESC-specific cell-cycle regulating miRNAs
FBS	Fetal Bovine Serum
FLK1	Fetal Liver Kinase 1
FMDV	Foot-and-Mouth Disease Virus
FRV	Fast Red Violet
GAG	Group-specific antigen
GATA	Transcription Factor Family
GD	Gaucher Disease
GF	Growth Factor
GFP	Green Fluorescence Protein
HD	Huntington's Disease

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HDAC	Histone Deacetylase
HESC	Human Embryonic Stem Cells
HF	Human Fibroblasts
HFF	Human Foreskin Fibroblasts
HLA	Human Leukocyte Antigen
HSCI	Harvard Stem Cell Institute
hTERT	Human Telomerase Reverse Transcriptase
ICC	Immunocytochemistry
ICM	Inner Cell Mass
iPSCs	Induced Pluripotent Stem Cells
IRES	Internal Ribosomal Entry Site
IVF	In-vitro Fertilization
JDM	Juvenile-Onset Type 1 Diabete Mellitus
k/o	Knockout
KLF4	Krueppel-like Factor 4
KOSR	Knockout Serum Replacement
LIN-28	Human Protein
LTR	Long Terminal Repeat
MEF	Mouse Embryonic Fibroblasts
MEK	Mitogen-activated protein kinase kinase
MIG	MSCV-IRES-GFP
miRNAs	Micro RNAs
MMLV	Moloney Murine Leukemia Virus
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
ORI	Origin of Replication
OSK	Combination of Transcription Factors Oct4, Sox2, Klf4
OSKM	Combination of Transcription Factors Oct4, Sox2, Klf4, Myc; also called „Yamanaka Factors“
OSNL	Combination of Transcription Factors Oct4, Sox2, Nanog, Lin28
P21	Cyclin-dependent Kinase Inhibitor 1
P53	Tumor Protein 53
PAX6	Paired Box Protein 6
PB	PiggyBac
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PD	Parkinson's Disease
PGC	Primordial Germ Cells
P/S	Penicillin/Streptomycin
PSC	Pluripotent Stem Cells
RB	Retinoblastoma
REX1	Zinc-finger protein 42 homolog
RNA	Ribonucleic Acid
ROCK	Rho-associated Protein Kinase
RPE	Retinal Pigment Epithelial Cells
RPM	Rotations Per Minute
RT	Room Temperature/Reverse Transcriptase
RT-PCR	Real-time PCR OR Reverse Transcription
SBDS	Shwachman-Bodian-Diamond Syndrome
SMA	Spinal Muscular Atrophy
SOX-2	SRY (sex-determining region Y)-box 2
SSEA	Stage-Specific Embryonic Antigen
SV40LT	SV40 Large T Antigen
TAE	Tris Base, Acetic Acid, EDTA
TAT	Transactivator of Transcription
TF	Transcription Factor
TRA	Human Embryonic Carcinoma Marker
UTF1	Undifferentiated Embryonic Cell Transcription Factor 1
UV	Ultraviolet
VE-CAD	Vascular-Endothelial Cadherin
VPA	Valproic Acid
VSV	Vesicular Stomatitis Virus
µg	Microgram
µL	Microliter
µm	Micrometer

## **ABSTRACT**

Over the past decades, stem cell research has revolutionized the field of regenerative medicine. The discovery of human embryonic stem cells in 1998 struck the beginning of a new era, because these cells possess the ability to morph into virtually any cell type of the human body. Novel proposals of treating devastating human diseases including diabetes, Parkinson's, Alzheimer's, and spinal cord injuries arose amongst the scientific community. Despite the many promises embryonic stem cells hold for regenerative purposes, the field has been the focus of controversy and fierce debates due to the source of these cells: human embryos.

New hopes for stem cell research were raised in 2007 when a process was discovered to reprogram human somatic cells into a pluripotent, embryonic stem cell-like state, terming the cells "induced pluripotent stem cells" or "iPSCs". Although they resemble human embryonic stem cells very closely, slight but important differences have been discovered in recent years, positioning this new type of stem cell somewhere in between embryonic and adult stem cells.

Despite research on cellular reprogramming still being in its infancy, iPSCs are already being employed as useful tools for modeling human diseases as well as improving drug development. Their autologous character, anticipated to eliminate any rejection reactions upon transplantations, makes them highly valuable for purposes aiming at personalized medicine. However, due to the mostly viral character for generating iPSCs from human body cells, using them for cell replacement therapies in humans remains yet a wish for the future.

This thesis describes the generation of disease-specific induced pluripotent stem cells from human fibroblasts derived from patients with a history of bipolar disease and constitutional mosaic trisomy 8. Cellular reprogramming was achieved by employing Sendai virus and retrovirus, respectively. The generated iPS cell lines were subsequently fully characterized by specific assays to assess their pluripotent character. The successful generation of patient-specific and disease-specific iPS cells enables their further use in patient-specific therapy approaches as well as individualized drug screenings.

## ZUSAMMENFASSUNG

In den letzten Jahrzehnten wurde das große Potential der Stammzellforschung für den Bereich der regenerativen Medizin erkannt. Die Entdeckung von humanen embryonalen Stammzellen 1998 zeichnete den Beginn einer neuen Ära, da diese Zellen das Potential haben, sich in jede beliebige Zelle des menschlichen Körpers zu verwandeln. Neuartige Visionen, wie man verheerende menschliche Krankheiten wie Diabetes Mellitus, Morbus Parkinson, Morbus Alzheimer oder Rückenmarksverletzungen behandeln könnte, tauchten unter den Wissenschaftlern auf. Obgleich der vielen Versprechen für die regenerative Medizin war die Thematik der embryonalen Stammzellforschung auch immer schon Fokus zahlreicher heftiger Debatten und Kontroversen, nicht zuletzt aufgrund der Tatsache, dass die Gewinnung dieser Zellen aus menschlichen Embryos erfolgt.

Neue Hoffnungen in der Stammzellforschung entstanden 2007, als ein Weg entdeckt wurde, menschliche somatische Zellen in pluripotente, embryonale stammzellähnliche Zellen, sogenannte "induzierte pluripotente Stammzellen" oder "iPSCs" zu reprogrammieren. Obwohl diese Zellen große Ähnlichkeit zu embryonalen Stammzellen aufweisen, wurden in den letzten Jahren strukturell kleine, jedoch wesentliche Unterschiede entdeckt, die diesen neuen Typ von Stammzellen sehr wahrscheinlich zwischen embryonalen und adulten Stammzellen positionieren.

Obwohl die Forschung mit iPSCs noch in den Kinderschuhen steckt, werden diese Stammzellen bereits als hilfreiche Werkzeuge für das Modellieren von menschlichen Krankheiten und zur Entwicklung von Medikamenten verwendet. Ihr autogenetischer Charakter, der zum Beispiel Immunreaktionen bei Transplantationen verhindern soll, verleiht diesen Stammzellen enormes Potential für Anwendungen im Bereich der personalisierten Medizin. Da iPS Zellen aber meistens mit Hilfe von Viren hergestellt werden, bleibt der Gedanke, diese Zellen für Zellersatz-Therapien in Patienten zu verwenden bis dato noch ein Wunsch für die Zukunft.

In dieser Arbeit wird die Herstellung von krankheitsspezifischen induzierten pluripotenten Stammzellen von menschlichen Fibroblasten beschrieben, die von ausgewählten Patienten isoliert wurden, welche unter bipolaren Störungen oder

Trisomie 8 litten. Zelluläres Reprogrammieren wurde durch die Anwendung einer Methode mit Sendaiviren und Retroviren erzielt. Die geschaffenen iPS Zellen wurden im Folgenden mit spezifischen Tests charakterisiert um ihre pluripotenten Eigenschaften zu bestimmen. Durch die erfolgreiche Herstellung von krankheits- sowie patientenspezifischen iPS Zellen wurde deren weitere Nutzung in Bezug auf patientenspezifische Therapieansätze und individualisierte Medikamentenentwicklung- und Selektion ermöglicht.

## 1 INTRODUCTION

A major breakthrough occurred in 2006 when Shinya Yamanaka, a Japanese scientist claimed to have transformed somatic cells into pluripotent stem cells (Takahashi et al., 2006). More precisely, he reported the generation of pluripotent embryonic stem cell-like cells from fully differentiated mouse fibroblasts. This was achieved by the enforced expression of four transcription factors, which are known to play important roles in maintenance of a pluripotent state.

In 2007, the first human induced pluripotent stem cell (iPSC) lines were derived from fibroblasts using either the same cocktail of factors (Takahashi et al., 2007) or a different one: Oct4, Sox2, Nanog, Lin28 (Yu et al., 2007). Soon after the first human iPSC lines were generated, multiple scientists all over the world reported to have reproduced the miraculous induced stem cells (Okita et al., 2007; Wernig et al., 2007; Maherali et al., 2007). The hallmark of these cells is their very close resemblance, if not identical appearance, to human embryonic stem cells (hESCs), set as the gold standard in regenerative medicine. iPSCs are similar to hESCs in morphology, proliferation, expression of pluripotent cell marker genes, transcription profiles, and teratoma formation. Despite the many characteristics that these two stem cell types share, differences have been reported mostly concerning epigenetic modifications and global demethylation patterns.

One great advantage that iPSC cells hold is the fact that they can simply be derived from a patient's skin biopsy. In comparison, hESCs are derived from the inner cell mass (ICM) of embryonic blastocysts, requiring the donation of extramural/aborted or low-quality in-vitro fertilization (IVF) embryos and therefore creating ethical hurdles. The second main advantage over embryonic stem cells is the ability to generate autologous cells. Human ESCs have raised serious concerns in transplantations due to potential immune reactions in the recipients. As iPSC cells can be derived from the patient himself, eventually they may be used for cell or tissue therapy in the very same patient.

The initial reprogramming protocol comprised the use of four retroviruses, each carrying a transgene encoding one target transcription factor. As the retrovirus is transduced into the cell, the transgenes are randomly integrated into the host's genome at high copy number and subsequently their proteins are produced. This method provides adequate reprogramming efficiencies of about 0.01% but has



major drawbacks such as its integrative character, shown to result in genetic aberrations in many iPS cells (derived by this approach). As a consequence, new techniques were established to deliver the transcription factors into the host cell such as lentiviruses, transposons, Sendai virus, plasmids, proteins, or mRNA. Major attention was paid to increase the overall transduction efficiency as well as advance the techniques to allow the derivation of integration-free iPSCs.

At the same time, the initial cell type of origin, namely fibroblasts, has been replaced. So far, mouse iPSCs have been generated from bone marrow cells (Takahashi et al., 2006), pancreatic beta cells (Stadtfeld et al., 2008a), hepatocytes and gastric epithelial cells (Aoi et al., 2008), B lymphocytes (Hanna et al., 2008), neural stem cells (Kim et al., 2008; Silva et al., 2008a). In comparison, so far human iPSCs have been established from skin fibroblasts (Takahashi et al., 2007), keratinocytes (Aasen et al., 2008), blood progenitor cells (Loh et al., 2010), cord blood stem and endothelial cells (Eminli et al., 2009; Haase et al., 2009), adipose-derived stem cells (Sugii et al., 2010) neural stem cells (Kim et al., 2009b), hepatocytes (Liu et al., 2010), and amniotic cells (Li et al., 2009).

The field of induced pluripotent stem cell research has tremendously expanded since its discovery five years ago, and numerous researchers in the whole world are now dedicated to find simpler and more efficient ways to transform somatic body cells back into a pluripotent state. As of now, two distinct applications of iPSCs should be considered realistic in the near future. One relates to the in vitro usage of iPS cells for the development of disease models as well as drug and toxicology screenings. Various stem cell lines from patients with diseases such as Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), Diabetes Mellitus Type I and many other have been established, some of which carry the diseased phenotype. These disease-specific cell lines provide a perfect opportunity for deciphering the molecular mechanisms of these diseases in vitro and study their characteristics. Drug and toxicology screenings can be performed, which would drastically speed up the finding of new molecular therapeutic compounds.

The use of iPSCs in regenerative medicine is the other potential application in the future. Differentiated, autologous iPS cells could be used for replacing lost or damaged tissue or even parts of or whole organs. As the cells used for therapy are derived from the patient himself, immune reactions can most certainly be ruled out. Nevertheless, safety remains the biggest hurdle to be overcome as single, undifferentiated cells or transgene integrations, resulting from the generation

process of the cells, have the potential to create teratomas and tumors, respectively, when implanted into humans.

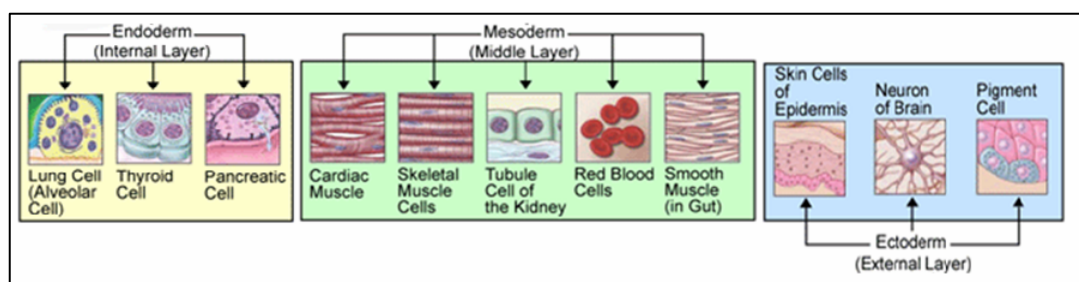
In summary, great advancements have been achieved in the field of iPSC research in the past few years. Nevertheless, numerous factors concerning the generation and potential applications of human iPS cells remain to be elucidated.

Here a compact overview of the relatively young history of iPSCs and the generation of disease-specific iPS cell lines by applying two different approaches is provided.

### **1.1. Human Embryonic Stem Cells**

Pluripotent embryonic stem cells derived from mouse blastocysts were the first instruments of their kind to be used for studying (early) embryonic development and exploring functions of genes (Evans et al., 1981; Martin, 1981). With the help of transgenic animal models, experiments allowing insights into mammalian biology were and still are carried out. For ethical reasons, such experiments are for the majority restricted to animal models.

Pluripotency refers to a cell's ability to differentiate into any derivative of the three embryonic germ layers, namely endoderm, mesoderm, and ectoderm but not into extraembryonic tissue and thus lack the ability to form an embryo itself (Smith, 2000) (Figure 1). Human embryonic stem cells (Thomson et al., 1998) together with embryonal carcinoma (EC) cells (Solter et al., 1970; Stevens, 1964), embryonic germ (EG) cells (Shamblott et al., 1998) and induced pluripotent stem (iPS) cells (Takahashi et al., 2007) share a pluripotent existence but have different origins and therefore different characteristics and functionalities.



**Fig. 1 The three germ layers in human development: endoderm, mesoderm, and ectoderm and their differentiation potentials.**

Germ layers form during early embryogenesis in humans and other vertebrates and develop later on into specific parts and lineage-specific cells of the human body (Winslow et al., 2009).

In vivo, pluripotent stem cells exist only transitionally in the early embryo. However, upon extraction at the early embryonic stage, embryonic stem cells can be derived and maintained in an undifferentiated state and theoretically they can be expanded for an infinite amount of time (Thomson et al., 1998). Their broad differentiation potential and easy expansion capabilities in vitro make them the “gold standard” for biomedical research and regenerative medicine. Gaining a deeper understanding of genetic and environmental factors governing pluripotency in a cell would allow important and novel insights into the basics of human development.

Therapeutic applications have so far been vastly hindered due to unsolved ethical issues and allogenicity of human embryonic stem cells. Nevertheless, individual clinical trials in the US and Great Britain using human iPSC- or hESC-derived differentiated cells have been started in the last few years.

### 1.1.1. Hallmarks of ES Cells

In contrast to multipotent adult stem cells (SCs), mouse and human embryonic stem cells are derived from the inner cell mass (ICM) of the blastocyst (Evans et al., 1981; Martin, 1981). These cells share two important characteristics that make them unique: self-renewal, that is the ability to expand in culture for a long time while maintaining an undifferentiated state and pluripotency, which is the ability to develop into cell types of all three germ layers (Thomson et al., 1998; Reubinoff et al., 2000).

The pluripotent potential of mouse ESCs is typically assessed by their ability to a) differentiate in vitro into lineages of all three germ layers, b) form teratomas upon injection into immune-deficient mice, c) contribute to chimera formation, d)

germline transmission, and e) ability to create tetraploid complementation. For human ESCs, the pluripotency potential is assessed by various assays determining the expression of pluripotency markers and genes. The differentiation potential is assessed by embryoid body (EB) formation to determine the formation of lineages of all three germ layers. Due to the fact that neither chimera formation nor germline transmission or tetraploid complementation, all known as the most rigorous tests for pluripotentiality in mice, cannot be demonstrated in humans for ethical reasons, *in vivo* assays consist of teratoma formation upon injection of undifferentiated cells into immune-deficient mice (Jaenisch et al., 2008; Daley et al., 2007).

Under defined culture conditions, hESCs can be constantly maintained in culture for more than 300 population doublings, while maintaining a normal karyotype (Odorico et al., 2001; Shambloott et al., 2001). Extrinsic factors such as leukemia inhibitory factor (LIF) and bone morphogenic protein 4 (BMP4) for murine ES cells (Williams et al., 1988) and basic fibroblast growth factor (bFGF/FGF-2) as well as transforming growth factor- $\beta$  (TGF- $\beta$ )/activin/nodal signalling for human ES cells (Rao et al., 2005; Levenstein et al., 2006; Xiao et al., 2006; Xu et al., 2005) play a detrimental/essential role in maintaining a pluripotent and undifferentiated state. Mouse as well as human ESCs are routinely cultured using irradiated murine embryonic fibroblast feeder layers (Thomson et al., 1998), secreting all essential factors for maintaining pluripotentiality. In the absence of a fibroblast-supporting layer, stem cells require a gelatinous protein matrix and addition of the above-mentioned soluble factors (Xu et al., 2001). This feeder- and xenofree environment would be favored not only because it is accompanied with more efficient and consistent culture maintenance but also to facilitate future cell-based therapies employing hESCs or hiPSCs, for which any animal-derived components used for maintaining the cells in culture need to be eliminated entirely (Ludwig et al., 2006).

### **1.1.2. Applications and Drawbacks**

Versatile ways of applying human pluripotent stem cells in research and medicine are theoretically possible. A few approaches have actually been realized for research purposes and even fewer for medical and clinical applications.

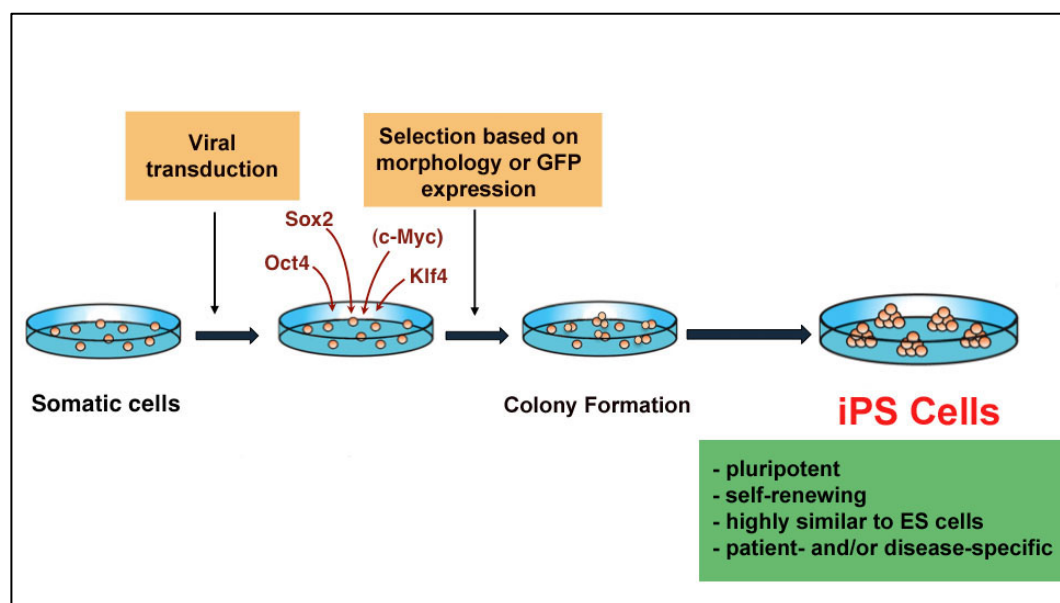
Understanding the genetic and environmental factors controlling pluripotency would shed incredible light on the basics of development not to mention the possibility for defining sources for therapeutically useable cells and tissues. Additionally, the pathways controlling pluripotency in normal cells may become

perturbed in certain abnormal biological conditions including germ cell-related tumors of the testes or ovary or other diseases affecting the human genome. –

## **1.2. Induced Pluripotent Stem Cells**

### **1.2.1. Somatic Cell Reprogramming**

Somatic cell reprogramming is defined as the conversion of somatic cells into an induced pluripotent state by ectopic expression of specific factors (Figure 2). Important events in the past decades were pivotal for the discovery of induced pluripotency. Cellular reprogramming was first described in the 1950s, when Briggs and King demonstrated the developmental potential of single nuclei isolated from late-stage frog embryos by transplanting them into previously enucleated oocytes (Briggs et al., 1952; King et al., 1955), a method later termed somatic cell nuclear transfer (SCNT) or “cloning”. The main conclusion from these and following experiments was that differentiated cells retain the same genetic information as early pluripotent/embryonic cells. This meant that reversible epigenetic changes rather than irreversible genetic changes take place during cellular differentiation. The cloning of the first mammal, the sheep Dolly (Wilmut et al., 1997), and later on also other mammals and the reprogramming of terminally differentiated cells (Hochedlinger et al., 2002a; Eggan et al., 2004; Li et al., 2004) was the proof of principle that fully differentiated cells remain genetically totipotent. However, many cloned animals present abnormalities in regards to phenotypes and gene expression, concluding errors in the reprogramming process (Wakayama et al., 1999; Hochedlinger et al., 2002b).



**Fig. 2 Steps involved in reprogramming of somatic cells by defined factors.**

Reprogramming of somatic cells by defined factors is accomplished by transducing donor cells with specific transcription factors known to be important for a pluripotent state, in this case Oct4, Sox2, Klf4, and c-Myc (often omitted). The viral transduction step is followed by the selection for reprogrammed cells, either by GFP expression (retrovirus) or morphology (Sendai virus) in this experiment. Subsequently, colony formation can be observed and after several weeks fully reprogrammed iPS cells can be expanded and characterized.

Despite the initial success of somatic cell nuclear transfer, this process was technically challenging and not ideal for extensive biochemical and genetic studies. A great invention in the field arose when immortal pluripotent cell lines were isolated from teratocarcinomas, which are tumors formed by germ cells. The pluripotent embryonal carcinoma cells (ECCs) derived from these tumors (Kleinsmith et al., 1964; Stevens, 1964) were capable of expanding in culture while maintaining pluripotency (Finch et al., 1967; Kahan et al., 1970). When ECCs were fused with somatic cells (Miller et al., 1976), the resulting hybrid cells acquired developmental and biochemical properties of ECCs, suggesting the presence of certain/powerful factors within ECCs capable of transforming somatic cells.

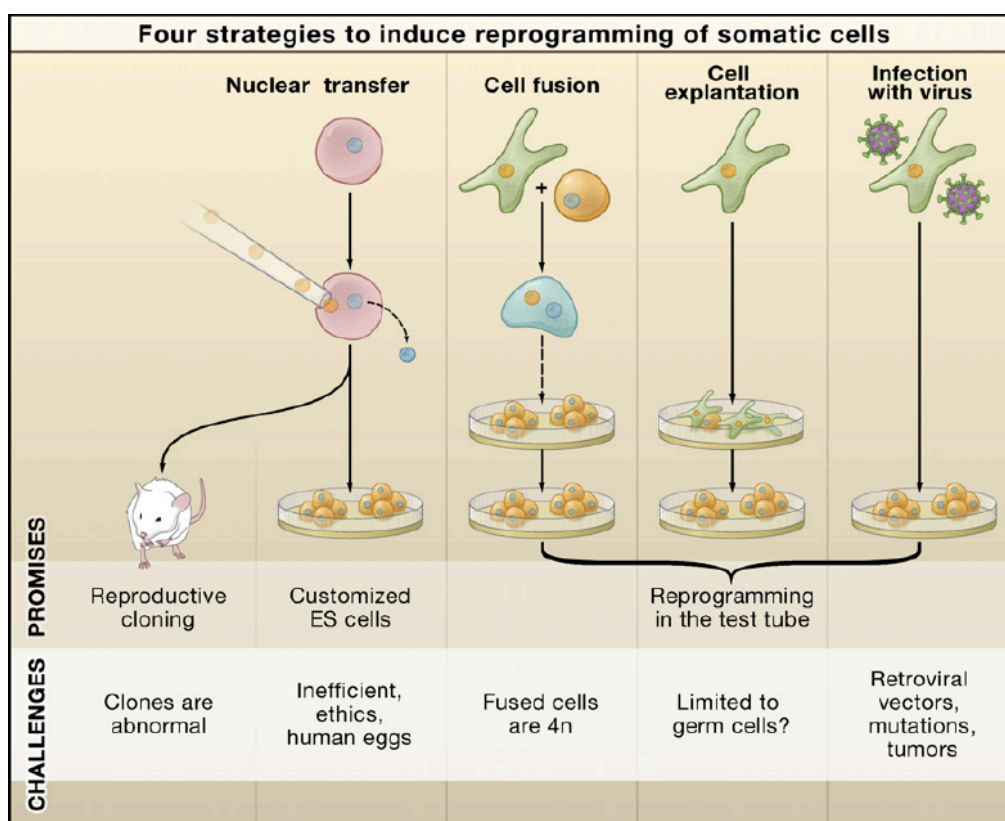
In 1981 first attempts were made to directly isolate pluripotent cells from early-stage mouse embryos and the first embryonic stem cells (ESCs) were isolated. ESCs in contrast to ECCs contribute fully to all three germ layers and possess normal karyotypes (Evans et al., 1981; Martin, 1981). Besides ESCs and ECCs, pluripotent cell lines have also been derived from post-implantation embryos termed epiblast-derived stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007), from primordial germ cells (PGCs) termed embryonic germ cells (EGCs)

(Matsui et al., 1992; Resnick et al., 1992), and from explanted mouse testicular cells termed multipotent germline stem cells (mGSCs) (Kanatsu-Shinohara et al., 2004; Guan et al., 2006). One common feature shared by all these cell types is the expression of Oct4, a protein required for early development and pluripotency (Takeda et al., 1992). Despite this similarity, ESCs and ECCs are the only cell types capable of passing the most stringent assay in terms of developmental potential, which is tetraploid blastocyst complementation.

### **1.2.2. Reprogramming By Defined Factors**

The far most powerful event in the field of somatic reprogramming occurred when the generation of murine and soon afterwards human iPSCs was reported in 2006 (Takahashi et al., 2006; Takahashi et al., 2007). Terminally differentiated somatic cells were converted to a pluripotent-like state with the help of specific transcription factors. These findings present a major breakthrough in the field of somatic cell reprogramming as this offers a way for the generation of autologous, patient-specific cells.

Previously established methods for somatic cell reprogramming such as nuclear transplantation and fusion of somatic cells all possess major hurdles (Figure 2). Nuclear transplantation (NT), also termed somatic cell nuclear transfer, demonstrated that epigenetic changes occurring in normal development can be reversed by exposing a somatic nucleus to the environment of an enucleated egg (Briggs et al., 1952). Despite its power to study developmental potential of cells, SCNT is technically challenging and inefficient (Hochedlinger et al., 2002a; Eggan et al., 2004).



**Fig. 3 Strategies for reprogramming of somatic cells.**

Four main strategies for reprogramming of somatic cells into an ESC-like state. Nuclear transfer describes the injection of a somatic nucleus into an enucleated oocyte, which can give rise to a clone upon transfer into a pseudopregant mother, or to genetically matched embryonic stem (ES) cells upon explanation in culture (somatic cell nuclear transfer (SCNT)). Fusion of somatic cells with ES cells results in the formation of hybrid cells that represent all features of pluripotent ES cells. Explantation of somatic cells in culture allows to select for immortal cell lines that may be multipotent or pluripotent. Transduction of somatic cells with defined factors can initiate reprogramming into a pluripotent, ESC-like state. Each method has various advantages for certain applications but also limitations (Jaenisch et al., 2008).

Fusion of somatic cells with embryonic stem cells is the second method of choice in the production of pluripotent cells (Miller et al., 1976). Hybrid cells can be obtained by the fusion of somatic cells with embryonic carcinoma cells (Solter, 2006), embryonic germ cells, or ES cells (Zwaka et al., 2005; Cowan et al., 2005; Yu et al., 2006). They were shown to have various features like morphology, gene and antigen expression patterns, and differentiation ability in common with their parental embryonic cells, suggesting a dominant pluripotent phenotype (Jaenisch et al., 2008; Cowan et al., 2005). Nevertheless, shortcomings of this approach are the very low efficiency and the tetraploid nature of the hybrids, eliminating them as potential cells for customized cell therapy.



Reprogramming by defined factors overcomes most of the drawbacks of already established methods for generating pluripotent stem cells. The conversion of somatic cells into a pluripotent-like state by specific transcription factors has been shown to require a resetting of the epigenetic state of somatic cells (Kim et al., 2010; Papp et al., 2011). Initially performed with mouse fibroblasts and comprising a set of 24 pluripotency-associated candidate genes, the core set of transcription factors necessary to reprogram human somatic cells into an embryonic stem cell-like state was initially reduced to only four (Takahashi et al., 2007). Shortly after the presentation of the four transcription factors, soon to be known as “Yamanaka Factors”, another research team defined a set of different factors also capable of reprogramming somatic cells, namely Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007). More recently, iPSCs were derived with the help of different combinations of factors and reducing them to a few or even just one single transcription factor (Kim et al., 2009b; Nakagawa et al., 2008; Wernig et al., 2008b; Yu et al., 2007; Ichida et al., 2009).

Overexpression of specific transcription factors (Oct4, Sox2, Klf4, and c-Myc) has been shown to convert somatic cells into pluripotent-like stem cells, exhibiting very similar characteristics to pluripotent human embryonic stem cells. Through ectopic expression of the four factors, a series of poorly defined events are initiated in the cells leading to the reactivation of endogenous, previously silenced pluripotency genes encoding for Oct4, Sox2, and Nanog. Furthermore, a somewhat autoregulatory loop/mechanism is turned on inside the cells, vastly caused by the strong DNA-binding properties of the transcription factors used. With the help of this somehow pluripotency-governing regulatory circuitry formed (Jaenisch et al., 2008), a pluripotent state is maintained in the reprogrammed cells even after successful shut down of the transgenes (Stadtfield et al., 2008b). Silencing of viral transgenes seems to depend greatly on two events: activation of DNA (Lei et al., 1996) and histone methyltransferases (Matsui et al., 2010).

### **1.2.3. Yamanaka Factors**

Pluripotency and self-renewal in embryonic cells is regulated by a distinct set of transcription factors including Oct4, Sox2, and Nanog. The expression of these genes blocks the differentiation into specialized cells and promotes self-renewal (Thomson et al., 1998; Darr et al., 2006; Biswas et al., 2007; Ivanova et al., 2006).

Initially performed with mouse fibroblasts and comprising a set of 24 candidate genes, the core set of transcription factors necessary to reprogram human somatic

cells into an embryonic-like stem cell state comprises Oct3/4, Klf4, Sox2, c-Myc (Takahashi et al., 2007). Despite the fact that other combinations of factors were also shown to generate iPSCs (Yu et al., 2007), the initially proposed four or three factors (no c-Myc) remain the standard method for producing iPSCs.

#### 1.2.3.1. Oct4

Oct4 (Octamer-binding transcription factor-4) or also called Pou5f1 (POU class 5 homeobox 1) or Oct-3/4 is a transcription factor and a member of the POU protein family, containing a highly conserved POU domain and a homeodomain. Oct4 is expressed throughout early mammalian development and is essential for the formation of the pluripotent ICM as well as for maintaining a pluripotent state in ESCs. The protein is expressed in unfertilized oocytes, pluripotent stem cells including ESCs and EGCs, as well as multipotent germ cells (Nichols et al., 1998; Scholer et al., 1989; Okamoto et al., 1990). A defined expression level of Oct4 seems essential in determining the fate of ESCs and deprivation of Oct4 leads to spontaneous differentiation in murine and human ESCs (Yamanaka, 2007; Niwa et al., 2000; Zaehres et al., 2005).

#### 1.2.3.2. Sox-2

Sox-2 or Sox2, also known as SRY (sex-determining region Y)-box 2, is a member of the SRY-related HMG-box (high-mobility group) transcription factor family and encoded by an intronless gene. It is highly involved in embryonic development and governing cell fate and therefore expressed in the ICM, epiblast, ESCs, germ cells, as well as in stem and precursor cells of the CNS (Li et al., 1998) (Pevny et al., 1998; Yuan et al., 1995; Pevny et al., 2005; Avilion et al., 2003; Masui et al., 2007). Furthermore, multipotent cells of the extraembryonic ectoderm also express Sox2 (Avilion et al., 2003). Like Oct4, Sox2 is employed as a pluripotent marker of early mouse development. Sox2 null mouse embryos fail to develop an epiblast resulting in early death before implantation (Avilion et al., 2003) and in ESCs deletion of Sox2 in ESCs leads to trophectoderm differentiation (Masui et al., 2007). These results suggest an important role for Sox2 in maintaining pluripotency.

In ESCs, Sox2 forms a heterodimer with Oct3/4 in order to regulate the transcription of downstream targets such as UTF1 (Nishimoto et al., 2003), Fgf4 (Yuan et al., 1995), and Fbx15 (Tokuzawa et al., 2003). The two transcription factors together with Nanog share various target genes in both human and mouse

ESCs. These target genes comprise actively expressed pluripotency genes as well as transiently silenced genes for developmental and lineage specification functions, activated in later stages of development (Boyer et al., 2005; Chambers et al., 2003; Nichols et al., 1998; Loh et al., 2006).

Furthermore, Oct4, Sox2, and Nanog seem to be able to coregulate themselves as well as each other by binding their own promoters (Kuroda et al., 2005; Chew et al., 2005; Tomioka et al., 2002; Boyer et al., 2005). This evidence strongly suggests that Oct4, Sox2, and Nanog are fundamental in regulating early embryonic development as well as maintenance of pluripotency in vitro.

#### 1.2.3.3. C-Myc

Contrarily, c-Myc, another factor found to be important for cellular reprogramming, is a proto-oncogene first discovered in human cancers (Dalla-Favera et al., 1982). Besides its major role as a transcription factor upon dimerization with its partner protein Max to bind to DNA enhancer box sequences (E-boxes), c-Myc also regulates global chromatin structures by binding histone acetyltransferases (HATs). Early-stage c-Myc null mouse embryos appear to develop normally but die 9-10 days after gestation, showing abnormalities in heart, pericardium, and neural tube and having extensive defects in erythropoiesis and vasculogenesis (Davis et al., 1993; Baudino et al., 2002). Surprisingly, deletion of c-Myc in murine ESCs does not seem to have an effect on proliferation or self-renewal capabilities but rather induces differentiation (Cartwright et al., 2005).

#### 1.2.3.4. Klf4

Klf4, a zinc-finger protein belonging to the family of Krueppel-like factors (KLFs), functions both as a tumor suppressor and an oncogene. Klf4 is vastly expressed in differentiated and postmitotic epithelial cells of the GI tract and skin as well as in fibroblasts such as MEFs and NIH3T3 (Shields et al., 1996). Forced expression of Klf4 in cultured cells leads to a blockage in cell cycle progression and DNA synthesis. Together with the fact that Klf4 null embryos show normal development but die within hours after birth due to faulty differentiation of skin (Segre et al., 1999) and colon (Katz et al., 2005), Klf4 seems to play a crucial role in the shift between proliferation and differentiation. Furthermore, p21, one of its downstream targets, seems to function as a switch between the oncogenic and the tumor suppressor properties, as the deletion of p21 reverses the cytostatic force of Klf4 (Rowland et al., 2005; Rowland et al., 2006).

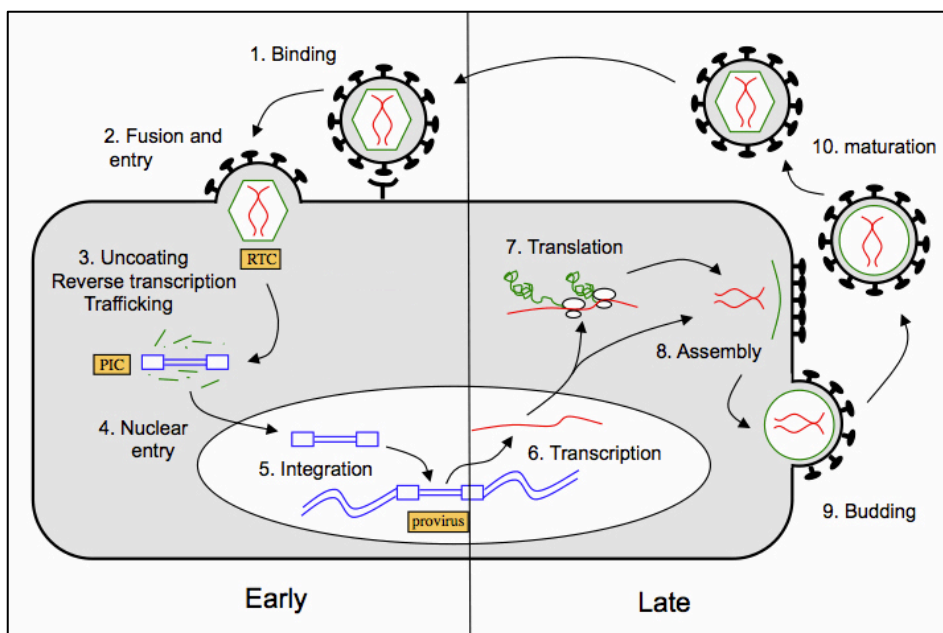
### 1.2.4. Reprogramming Strategies

Various approaches for reprogramming of somatic cells have been proposed and studied intensively so far. The choice of the right reprogramming strategy greatly depends on the given cell type and the intended use of the iPSCs to be generated. The different methods can be grouped based on whether or not the transgenes integrate into the host genome. Integrative approaches generally provide higher reprogramming efficiencies (0.01% - 1%), as the transgenes get stably integrated into the host cell genome and are expressed at high levels. However, the major problem with these techniques is the fact that the virus is randomly integrated into the genome and as a result could alter the gene expression of crucial genes such as oncogenes or tumor suppressor genes. This is the main roadblock for excluding these techniques for intended clinically relevant applications. Original methods employ retro- and lentiviral systems as well as excisable transposons and floxed lentiviruses. Recent techniques are based on integration-free delivering systems such as adenoviruses or plasmids or DNA-free methods like proteins or RNAs.

#### 1.2.4.1. Integrative Methods

##### 1.2.4.1.1 *Retroviral Vectors*

The first approaches for reprogramming of somatic cells by factor delivery employed constitutively active retroviral vectors that integrate in a stably manner into the genome of the host cell. When entering a cell, the retroviral RNA is reversely transcribed into DNA, which is then capable of integrating into the host cell genome. Integrated viral DNA is furthermore transcribed into mRNA, eventually leading to expression of the viral transgenes (Figure). Retroviruses have been used widely for gene therapy and basic biomedical research as their characteristics are well known and they provide high transduction efficiencies.



**Fig. 4 The life cycle of a retrovirus.**

The life cycle of a retrovirus is divided into early and late phase with different stages shown above. Many interactions between viral factors and the host cell are still partly unknown. Retroviruses are commonly used in molecular biology to insert genetic material such as DNA into a cell, a process called transduction (Bishop, 2011).

Yamanaka and colleagues described the very first retroviral approach for cellular reprogramming. Each of the four monocistronic retroviral vectors used each carries one transgene that was described to be detrimental in changing a somatic cell into a pluripotent-like cell, namely Oct4, Klf4, Sox2, and c-Myc (Takahashi et al., 2007; Takahashi et al., 2006). This original technique uses retroviral vector systems such as pMX (Takahashi et al., 2006; Kitamura et al., 2003) or pMIG (Hawley et al., 1994; Aasen et al., 2008; Park et al., 2008b), based on the Moloney murine leukaemia virus (MMLV). For these vectors, the transgene expression is driven by the 5' MMLV long terminal repeat (LTR) promoter, which is generally silenced in embryonic stem cells. Transgene silencing is required to get fully reprogrammed iPS cells that should eventually upregulate their endogenous pluripotency factors (Hotta et al., 2008). One transgene encoding for one specific transcription factor needed for reprogramming is incorporated in the viral backbone (Takahashi et al., 2006), replacing the viral proteins gag, pol, and env (Figure retro lentiviral vectors, shao). In addition, these vectors are replication-defective, meaning the genes for viral replication and virion packaging are deleted. Thus, they deliver the transgenes into the host cell and avoid triggering a lytic pathway that would eventually result in cell death.

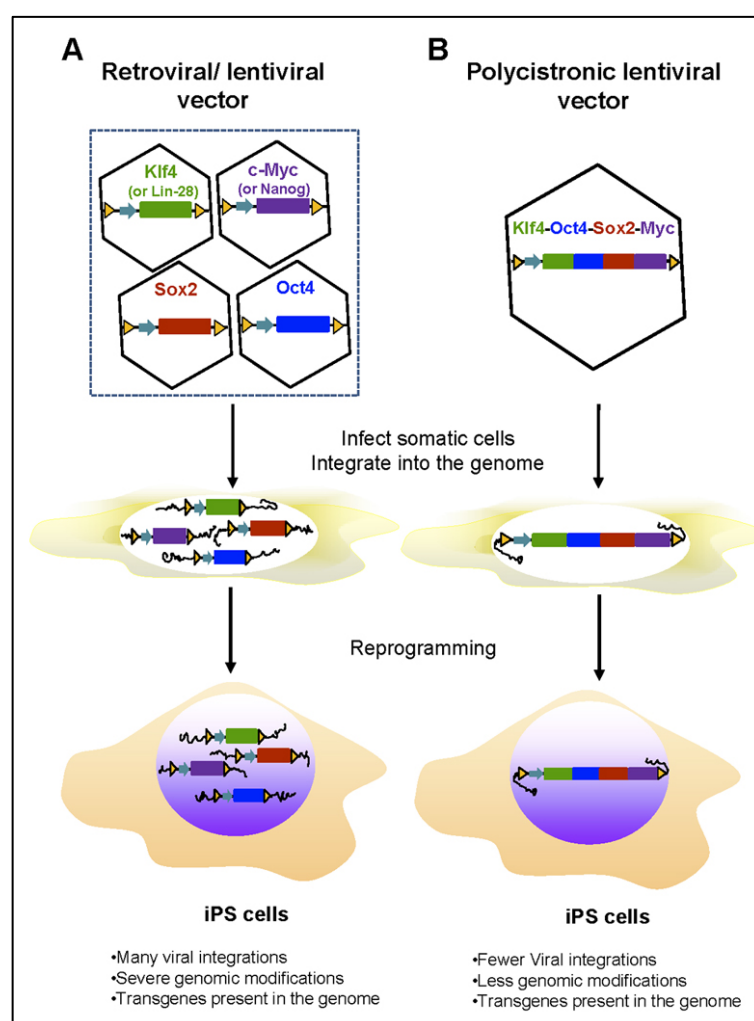
Fully reprogrammed iPS cells are selected based on various methods. The first approach by Yamanaka and colleagues used the mouse ESC-specific Fbx15 locus with an integrated dormant drug resistance allele, activated upon the expression of the pluripotent transcription factors and leading to the formation of drug-resistant iPS colonies (Takahashi et al., 2006). As this method also selected partially reprogrammed cells, soon new methods for selecting iPS colonies were published. By selecting for the reactivation of pluripotency genes such as Oct4 and Nanog, iPS colonies closely resembling ESCs could be established (Okita et al., 2007; Maherali et al., 2007). The selection technique applied for generating retrovirally transduced iPS cells in the HSCI iPS Core Facility uses the pMIG vector systems containing a green fluorescent protein (GFP) inserted after an IRES sequence. Expression of GFP by the transduced cells is an indication for successful transduction of all three or four transcription factors. Silencing of GFP expression occurring 2-3 weeks post transduction is an indication for successful reprogramming and marks the colonies that can be selected for further expansion and characterization.

Major drawbacks of this technique are the integrative character of the retrovirus, leading to genomic insertions, persistent expression of transgenes or transgene reactivation and therefore causing alterations in gene functions and insertional mutagenesis (Kustikova et al., 2005; Nakagawa et al., 2008). These major roadblocks exclude the resulting iPSCs for clinically relevant applications in humans (Yamanaka, 2009). Despite this, the cells to be transduced need to be highly proliferating in order to achieve adequate transduction efficiencies (Shao et al.). Another limitation of the retrovirus system is the fact that only dividing cells can be transduced (Shao et al., 2009).

#### *1.2.4.1.2 Lentiviral Vectors*

Shortly after the first reports using retroviruses, new methods using HIV-based lentiviral vectors were published (Yu et al., 2007; Bielesch et al., 2007). Lentiviruses represent a subclass of retroviruses and therefore also integrate into the host genome but are capable of infecting both dividing and non-dividing cells. For this approach, the inserted transgenes are driven by the EF1 $\alpha$  promoter. As this promoter is known to be potent in embryonic stem cells, a lack of transgene silencing in reprogrammed iPSCs may be observed. Besides this, as with the retrovirus, genomic integrations and insertional mutagenesis in these iPSCs generated present a major risk for applications in humans (Yu et al., 2007).

One main advantage in employing lentiviral vectors was the introduction of polycistronic vectors, carrying all four transgenes in one or two open reading frame (ORF) on one viral vector (Figure...). By using self-cleaving 2A peptides, efficient release of each factor was provided but at the same time reducing the number of viral insertions and therefore the risk of mutations and genomic instability. This lentiviral approach significantly increased the reprogramming efficiency up to 100-fold as well as the transgene silencing in reprogrammed iPS cells (Shao et al., 2009). Shortly after lentiviruses, polycistronic vectors were also used for retroviral systems for cellular reprogramming.



**Fig. 5 Schematic design of mono- and polycistronic vectors for cellular reprogramming.**

Originally, monocistronic vectors carrying only one transgene were employed, which results in low transduction efficiencies and partly reprogrammed cell. The introduction of polycistronic vectors, carrying all transgenes on one vector, improved these hurdles (Shao et al., 2010)

Recent advances led to the construction of inducible lentiviral vectors, that allow temporary control over the factor expression by use of small molecules such as doxycycline and therefore establish a better system to study the molecular and biochemical events taking place during the reprogramming phase (Brambrink et al., 2008; Stadtfeld et al., 2008b).

#### 1.2.4.1.3 *Excisable Vector Systems*

One alternative to integrative methods is provided by excisable factor delivery systems such as the piggyBac (PB) transposons. Transposons are mobile genetic elements capable of moving in between various positions within the genome of a cell (McClintock, 1950). This process, also called transposition, is known to cause mutations and genomic instability and is facilitated by the enzyme transposase. Normally, active transposase enzymes cut the transposon and insert it into a different position in the genome. The failing of re-insertion, leaving the genome with a seamless excision, is used to facilitate generation of genetically clean iPS cells (Woltjen et al., 2009; Kaji et al., 2009). Despite reasonable efficiencies of 0.1% and the obtention of transgene-free iPS lines, laborious screening of successfully excised lines and possible gene duplications by repeated transpositions (Mermer et al., 1987) make this approach less favourable.

Another excisable technique for generating transgene-free iPSCs is a lentiviral system using flox sites. Jaenisch and colleagues constructed a vector with transgenes flanked with loxP sites in both LTRs, which can be excised upon Cre-recombinase expression in these cells (Soldner et al., 2009). Despite the later use of a polycistronic excisable construct, which greatly increased the overall transduction efficiency, the generated iPS cells still harboured insertion sites due to multiple transgene excisions (Chang et al., 2009) and are thus not suitable for the clinic.

#### 1.2.4.2. Non-Integrative Methods

In order to overcome safety issues raised by the use of integrating reprogramming techniques such as retro- or lentiviral systems, transiently transfected circular plasmids were employed to generate murine iPSCs (Okita et al., 2008; Gonzalez et al., 2009). Despite the obtention of iPS with high similarities to ES cells, low efficiencies and the necessity of multiple rounds of transfections are major drawbacks for this method.



Another approach for the generation of iPSCs without integration into the genome was reported by Yu and colleagues (Yu et al., 2009). Using episomal vectors based on the oriP/Epstein–Barr nuclear antigen-1 (EBNA1), they generated human iPS cells from fibroblasts, entirely free of vectors and transgene sequences. Episomal plasmids replicate extrachromosomally and only once per cell cycle. Moreover, they don't require viral packaging. Selection of iPS clones that had spontaneously lost the episomal vectors, which are gradually lost in proliferating cells, was performed. These clones possessed typical human ES cell morphology as well as similar gene expression profiles and were able to form all three germ layers in teratomas. Although this method offers a novel non-integrative method leaving no traces of vectors in the generated iPS cells, low efficiencies (0.0006%) were reported.

Another non-integrative reprogramming technique uses adenoviral vectors, which do not integrate into the host genome. Despite a high infection efficiency for many cell types and rare insertion events during the reprogramming process, the overall transduction efficiency was very low (0.001% - 0.0001%) except for fetal liver cells, making this process not suitable for large-scale production of iPS cells (Stadtfield et al., 2008c; Zhou et al., 2009b).

#### *1.2.4.2.1 Sendai Virus-based Vectors*

The second approach used for reprogramming in the course of this thesis is based on a non-integrative Sendai virus (SeV) vector system (Fusaki et al., 2009). This system makes use of a single-stranded, negative-sense RNA virus, which lacks its F protein and is therefore non transmissible. Selection of successfully reprogrammed cells is mainly based on morphological criteria as well as alkaline phosphatase staining (Fusaki et al., 2009). SeV vectors have repeatedly been used for studying various diseases (Masaki et al., 2001; Ferrari et al., 2007) due to their well controllable exogenous gene expression capabilities (Tokusumi et al., 2002), the non-integrative character due to their RNA nature (Lamb et al., 2001) and their moderate cytotoxicity (Li et al., 2000). These advantages make the Sendai vector an appealing and efficient method for generating integration-free iPSCs. The only drawbacks this relatively young method brings along is the fact that these viral vectors are somewhat difficult to eliminate from the infected cells and it can take up to 20 or more passages to eliminate various viral transgenes entirely, promoting the likelihood of generating aneuploid iPS cell lines due to prolonged expression of c-Myc (Fusaki et al., 2009).

#### 1.2.4.3. DNA-Free Methods

Most recent approaches for somatic cell reprogramming try to avoid the use of viral vectors or plasmids and hence DNA entirely. Warren and colleagues developed a technique that makes use of synthetic mRNAs to reprogram human somatic cells. By using modified mRNAs, they were able to achieve efficiencies of 2-4% by using five factors (OKSM + Lin28) as well as low-oxygen (5%) culture conditions. In addition, colonies appeared earlier compared to conventional viral-mediated methods. Despite its efficiency, bottlenecks of this technique include the need for daily transfections for a set amount of time resulting in high dosages of reprogramming factors and therefore possible oncogenic effects on the cells (Warren et al., 2010).

A second technique avoiding the use of DNA employs the four reprogramming factors in form of proteins fused with peptides. Recombinant modified proteins were expressed in *E.coli* inclusion bodies, refolded and purified. So far, mouse and human cell lines have been successfully reprogrammed with this technique. However, protein-mediated reprogramming shows low efficiencies as well as very slow kinetics and may therefore not be the method of choice for generating clinically relevant iPSCs (Zhou et al., 2009a; Kim et al., 2009a).

#### 1.2.5. Cell Type of Origin

Concerning the donor cell type used for reprogramming, various studies have shown that the origin of iPSCs plays an important role in the efficiency and kinetics of the reprogramming process (Hochedlinger et al., 2008; Gonzalez et al., 2011). Variability in reprogramming efficiency and factor requirement is associated with the varying levels of endogenous reprogramming factors expressed in different donor populations. For instance, neural progenitor cells express endogenous Sox2 and reprogramming of these cells into iPSCs could be achieved in the absence of a Sox2 transgene (Eminli et al., 2008) and even with Oct4 as a single factor (Kim et al., 2008). Similarly, cord blood CD133+ cells, which can easily be made available via cell banks, only need exogenous Oct4 and Sox2 expression to be reprogrammed into iPSCs (Giorgetti et al., 2009).

In general, the most commonly used cell type, which is also fairly accessible by means of skin biopsies, are adult and neonatal fibroblasts. Fibroblasts were the first cell population to be reprogrammed by defined factors and various factor cocktails have successfully been used in both, mouse and human cells (Takahashi et al., 2007; Yu et al., 2007; Nakagawa et al., 2008; Takahashi et al., 2006).

Disease-specific fibroblasts can be obtained from cell repositories around the world. So far, various mouse cell types such as liver cells (Stadtfeld et al., 2008c), pancreatic cells (Stadtfeld et al., 2008a), lymphocytes (Hanna et al., 2008) and neural progenitor cells (Eminli et al., 2008), as well as human cord blood cells, keratinocytes (Aasen et al., 2008), adipose-derived stem cells (Aoki et al., 2010), and peripheral blood cells (Loh et al., 2010; Staerk et al., 2010) have been reprogrammed.

Another important aspect influencing the efficiency of the reprogramming process is the differentiation state of the original cell type. Compared to terminally differentiated B and T cells, reprogramming of haematopoietic stem and progenitor cells was achieved at 300 times higher efficiency (Eminli et al., 2009). This is consistent with the notion that in the course of reprogramming, the epigenetic state of a somatic cell is reset to a state similar or identical to pluripotency (Hochedlinger et al., 2006). Logically, the more differentiated a cell type is, the greater is the barrier that needs to be overcome to epigenetically remodel the genome.

Besides differences in efficiency, the quality of the generated iPS cells can also vary between cell populations. Upon transplantation of mouse neurospheres differentiated from iPS cells into brains of immunodeficient mice, Miura and colleagues observed various extents of teratoma formation depending on the origin of these iPSCs. The researchers pointed out that teratoma formation capabilities can also be affected by other variables such as the reprogramming and differentiation techniques, the location of transplantation and other factors (Miura et al., 2009). A different study showed that three-factor (without c-Myc) derived murine iPS cells were able to produce viable chimeras that survived a time period of 100 days, whereas a minor portion of chimeras generated by four-factor derived iPS cells died of tumors within this period (Nakagawa et al., 2008). These results are likely to be a reflection of the quality of the generated stem cells.

Addition of specific chemical compounds altering histone modifications or DNA methylation has been shown to facilitate the overall reprogramming success as well as promote the transition of partially reprogrammed cells into fully reprogrammed iPSCs. Such compounds include the G9a histone methyltransferase (G9a HMTase) inhibitor (Shi et al., 2008), valproic acid (VPA), a histone deacetylase inhibitor (Huangfu et al., 2008) and 5' azacytidine (Looijenga et al.), a DNA methyltransferase inhibitor (Mikkelsen et al., 2008). Furthermore,

vitamin C has been proven to increase reprogramming efficiency in murine fibroblasts by preventing senescence (Esteban et al., 2010) and influencing DNA methylation (Chung et al., 2010). Despite their positive effects, some compounds have been shown to have negative effects on the generated iPSCs as they could have the potential to cause mutations. Addition of small molecules may therefore be a possible but partly unpredictable solution towards achieving a higher reprogramming efficiency.

#### **1.2.6. Cultivation of iPSCs**

Parameters such as culture and derivation conditions, supporting feeder cells as well as medium composition are crucial components when working with induced pluripotent stem cells. These factors have the ability to regulate the entire reprogramming process and influence its efficiency.

Murine and human iPS cells are cultured under conditions used for the maintenance of their counterparts, mouse or human embryonic stem cells. These conditions support growth and self-renewal as well as the maintenance of a pluripotent state (Akutsu et al., 2005; Cowan et al., 2004; Lerou et al., 2007).

Embryonic stem cells, especially human ESCs, require fibroblast-derived factors to maintain their pluripotent state and steady proliferation rate (Dravid et al.; Xu et al., 2005). Mouse ESCs have been shown to grow even in the absence of feeder cells and fibroblasts growth factors (Ying et al., 2008) and mouse iPSCs can similarly be derived and maintained under feeder-free conditions (Wernig et al., 2008a; Stadtfeld et al., 2008b). Typical culture components such as fetal bovine serum (FBS), bovine serum albumin (BSA), animal-derived enzymes such as trypsin or irradiated mouse embryonic fibroblast feeder cells make the thereof generated stem cells unsuitable for clinical applications (Ahrlund-Richter et al., 2009). Great attention needs to be paid in defining methods for xeno- and feeder-free derivation and maintenance of human iPS cells in order to establish a way for potential clinical applications and at the same time reaching out towards fulfilling mandatory good manufacturing practice (GMP) standards (Unger et al., 2008).

Culturing iPS cells in a hypoxic environment of 5% O<sub>2</sub> instead of atmospheric 21% O<sub>2</sub> has been shown to influence the cellular reprogramming by increasing the efficiency fourfold (Yoshida et al., 2009). Furthermore, addition of valproic acid (VPA), a histone deacetylase inhibitor, can increase the reprogramming efficiency even further (Huangfu et al., 2008) and addition of Rho-associated kinase (ROCK)

inhibitor has been shown to facilitate culturing of iPSCs (Park et al., 2008a; Watanabe et al., 2007).

Therefore, appropriate culture conditions need to be specified before selecting a specific reprogramming method and specific donor cells and in addition appropriate culture conditions need to be defined tailored to meet the needs of both, donor cells and arising iPS cells.

### **1.2.7. Characterization of iPSCs**

In order to ensure the generation of fully reprogrammed iPS cells, various criteria have been discussed and established by the scientific community (Hochedlinger et al., 2008; Daley et al., 2009; Ellis et al., 2009). The assays used to determine unique features associated with pluripotency include morphological, molecular, as well as functional characteristics. Characterization assays used for this thesis consist of an array of methods determined by the Harvard Stem Cell Institute iPS Core Facility to be sufficient to ensure pluripotent iPS cells have been generated.

Morphologically, iPS cells must present identical features to ES cells such as round colonies, defined borders and transparent appearance. In addition they need to demonstrate unlimited self-renewal. Concerning molecular features, iPSCs must show ESC-indistinguishable gene expression profiles including expression of key pluripotency genes such as Nanog, Oct4 and others as well as pluripotency-associated surface and antigens such as SSEA-3/-4 and Tra-1-60. In regards to functional criteria, iPS cells must have the ability to differentiate into lineages from all three germ layers, which is assessed in here by in-vitro differentiation into embryoid bodies (EBs).

#### **1.2.7.1. G-Band Karyotyping Analysis**

In addition to ensuring a pluripotent state, any genetic abnormalities in the resulting iPSCs need to be excluded. As reported by various groups, somatic cell reprogramming as well as prolonged culturing of cells can cause genetic aberrations, such as point mutations or chromosomal translocations in the genome of resulting iPS cells (Gore et al., 2011; Lister et al., 2011). These findings correlate with previous reports about the tendency of ES cells to harvest genetic abnormalities (Lerou et al., 2007).

Thus, iPSC lines are tested for genetic lesions to ensure a normal and healthy genetic composition by means of G-band karyotyping analysis.

#### 1.2.7.2. Alkaline Phosphatase Staining

The undifferentiated state of pluripotent ESCs and iPSCs is assessed by positive staining for the enzyme alkaline phosphatase (AP) and loss of AP expression has been proposed as an early indicator for differentiation (Palmqvist et al., 2005). Alkaline phosphatase is a hydrolase enzyme known to dephosphorylate proteins, nucleotides, as well as alkaloids. The enzyme functions under alkaline conditions and exists within all body tissues. It catalyzes the hydrolysis of p-nitrophenylphosphate into phosphate and p-nitrophenol, a yellow colored molecule. The amount of p-nitrophenol produced is proportional to the amount of alkaline phosphatase present within the reaction and can therefore be used for quantitative as well as qualitative analysis.

This assay provides a first indication for pluripotentiality as fixed iPSC colonies appear in a purple to red tone while differentiated cells do not stain.

#### 1.2.7.3. Immunocytochemistry for Pluripotent Stem Cell Markers

On a molecular level, iPS cells need to display the expression of proteins associated with pluripotency and thus also expressed in ES cells.

For this purpose, these markers were chosen: Oct4 and Nanog, two transcription factors, and SSEA-3, SSEA-4 and Tra-1-60, cell surface antigens. In order to visualize marker expression on resulting iPS cells, immunocytochemistry combined with immunofluorescence was employed. For this method, Paraformaldehyde (PFA)-fixed cells are stained with a primary antibody specific for a certain antigen. In order to visualize the antigen-antibody interaction, a secondary fluorescent antibody is employed, which is specific for the primary antibody used. This technique is also called indirect immunofluorescence.

This assay provides a relatively fast and qualitative determination of pluripotent cells and is easy and inexpensive to perform. Variability within this technique can be introduced through the fluctuating sensitivity and specificity of the diluted antibodies used.

#### 1.2.7.4. Qualitative Analysis for the Expression of Pluripotency Genes

Another step to determine the molecular criteria for pluripotency is by characterizing the gene expression profiles of iPS cells. This is achieved by assessing the gene expression of key pluripotency genes such as Nanog, Oct4, Dnmt3b, hTERT, Rex1, and Sox2.

For this purpose, polymerase chain reactions (PCR) with specific primer pairs are set up in order to exponentially amplify specific genes. In order to accomplish this, cDNA is synthesized from RNA and added to a PCR mixture containing forward and reverse primers as well as a Taq Polymerase and dinucleotriphosphates (dNTPs). In a series of defined consecutive cycles of various temperatures, specific cDNA gets amplified, which can then be qualitatively analyzed via agarose gel electrophoresis.

#### 1.2.7.5. In-vitro Differentiation and Qualitative Analysis of Differentiation Markers

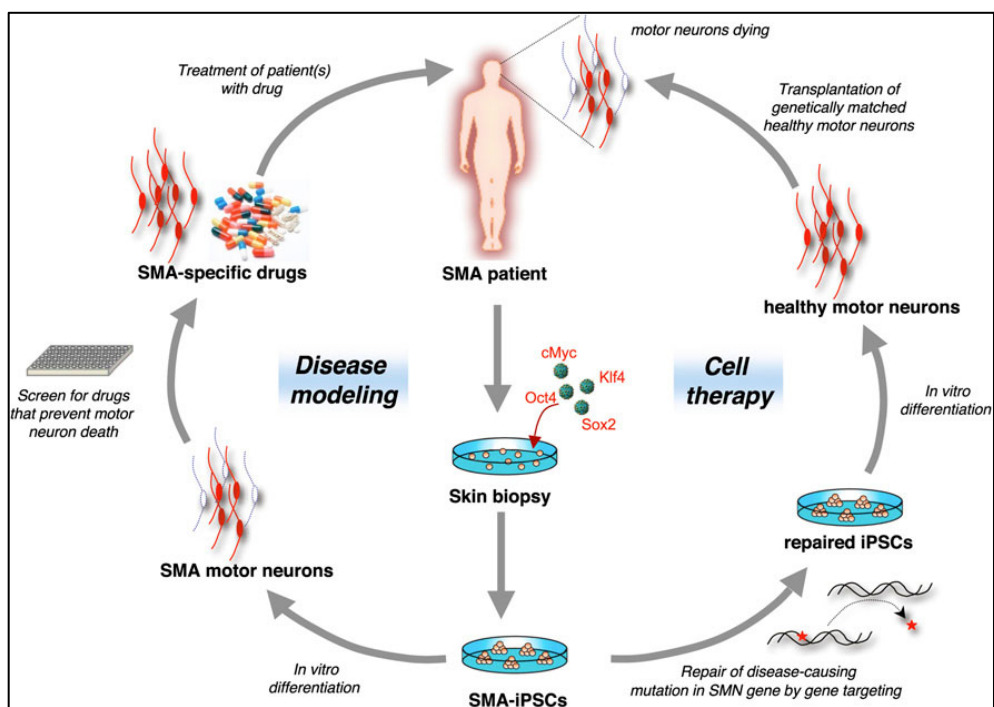
In order to assess the pluripotent state of iPSCs on a functional level, in-vitro differentiation of embryoid bodies and subsequent analysis for differentiation markers of all three embryonic germ layers is performed. This constitutes the second most stringent assay for determining pluripotency in human iPS cells besides testing for teratoma formation in immunodeficient mice.

iPS cells are first plated onto non-attaching tissue culture plates to initiate EB formation and EBs are then further cultured to promote differentiation into cells of all three embryonic germ layers.

Differentiation capability identical to ESCs is confirmed by subsequent analysis of differentiation markers such as Flk1 and GATA2 for mesoderm origin, AFP and GATA4 for endoderm origin, and NCAM and Pax6 for ectoderm origin. As for analyzing pluripotency markers, PCRs with specific primer pairs are set up and qualitative gene expression is analyzed via agarose gel electrophoresis.

#### 1.2.8. Applications

One major application of somatic cell reprogramming generating human induced pluripotent stem cells is the ability to provide human disease models (Figure 5). Such models will greatly facilitate research on multiple genetic and degenerative disorders by recapitulating the development, pathology as well as the action of drugs via drug and toxicology screenings.



**Fig. 6 Potential future applications of iPSCs.**

Shown are the potential applications of iPSC technology for disease modeling and cell therapy describing SMA (Spinal Muscular Atrophy) as an example. SMA leads to degeneration of motor neurons, causing devastating symptoms in patients suffering from the disease. Potential therapies for the future could be established by deriving iPSCs from SMA patients, differentiating them into motor neurons in vitro to generate a culture model of the disease and using this model to screen for novel drugs.

One alternative would be to repair a known disease-specific mutation in iPSCs by gene targeting prior to their differentiation, resulting in healthy differentiated cells usable for cell therapy (Stadtfield et al., 2010).

Shortly after human iPSCs were generated for the first time, scientists developed the first model of a human disease with the help of these cells, a model of amyotrophic lateral sclerosis (ALS) (Dimos et al., 2008). ALS is a neurodegenerative disease causing major loss of motor neurons in the spinal cord. Dimos and colleagues were able to generate human iPS cells from relatively old patients and furthermore succeeded in differentiating these cells into functional motor neurons, in this case without recapitulating the diseased phenotype.

Another group of researchers generated hiPSCs from patients with spinal muscular atrophy (SMA), a disease also causing degeneration in motor neurons (Ebert et al., 2009). The researchers were able to demonstrate a gradual degeneration when they differentiated SMA-derived iPSCs into motor neurons. In addition, these neurons were responsive to treatment with various drugs, providing a perfect proof of principle (Figure 6). Similarly, iPS cells derived from patients with



Parkinson's disease and differentiated into dopamine-producing neurons contributed to an increase in dopamine upon transplantation into brains of Parkinsonian rats (Hargus et al., 2010).

Scientists made an even more striking discovery in 2009 when they derived human iPS cells from patients with Fanconi anaemia disease and differentiated them into cells of the hematopoietic lineage (Raya et al., 2009). This is a process that is impaired in the course of Fanconi's disease, causing bone marrow failure and decline of hematopoietic stem cells in such patients. Differentiating the disease-derived cells was only possible after genetically correcting the disease by introducing the original FA-related gene. Likewise, Kazuki and colleagues corrected iPS cells derived from patients with Duchenne muscular atrophy (DMA) by using human artificial chromosomes (Kazuki et al., 2010).

So far, disease models have amongst others also been created from iPS cells derived from Familial dysautonomia (FD) (Lee et al., 2009), Rett syndrome (RTT) (Marchetto et al., 2010), as well as Long QT syndrome type 1 (Moretti et al., 2010).

As far as cell- and tissue-based therapy is concerned, human iPS cells are still far from being used in the clinic. Even though various genetic diseases have been corrected in vitro and it would theoretically be possible to treat these patients with iPS cells in vivo, little is known about the behavior of human iPS cells in vivo. Many iPS cell lines being tumorigenic due to their generation process are far more dangerous than human adult stem cells or embryonic stem cells. One option of using iPSCs in humans in the near future would only be justifiable if it was the patient's best or only option of treatment. In addition, regulatory frameworks for stem cell therapy would need to be revised or adapted for the use of iPSCs in many countries. Nevertheless, safer ways for producing integration- and mutation-free iPS lines derived under xeno- and feeder-free conditions need to be investigated before making attempts to move towards clinical applications for a broader population.

Considering the evidence, iPS cells clearly provide a solid base for the development of disease models for studying human diseases, drug development and drug screening as well as a diagnostic tool.

### 1.2.9. ESCs and iPSCs – Identical, Similar, or Different?

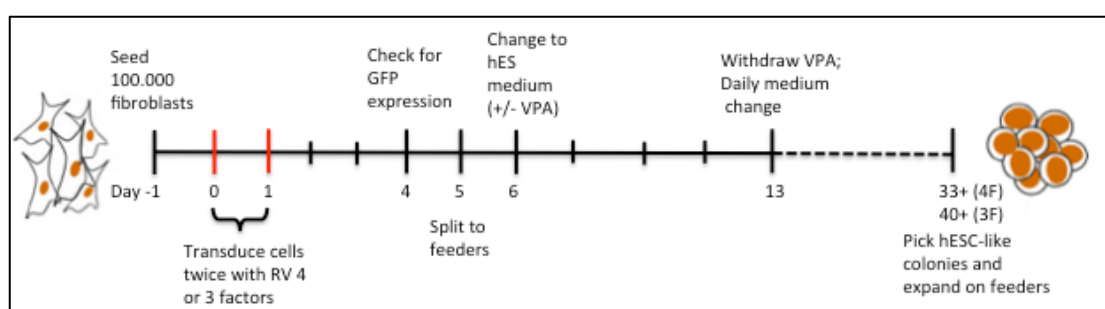
Criteria used to compare ESCs and iPSCs are biological assays testing for developmental potency and molecular assays comparing gene expression profiles and epigenetic characteristics. Assays determining the developmental capacity are considered crucial for claiming that iPSCs are pluripotent. In mice, chimera formation and germline transmission are commonly used to confirm developmental potential of pluripotent iPSCs. In humans however, due to ethical reasons, less stern assays are performed such as in vitro differentiation into EBs and teratoma formation in mice. In contrast to biological assays, which mostly have a qualitative nature, molecular assays allow a more quantitative comparison of ESCs and iPSCs. Numerous studies claimed iPSCs to be similar or almost indistinguishable from ESCs by means of morphology, expression of pluripotency and differentiation markers, global gene expression, the state of X chromosome inactivation, and DNA methylation profiles (Mikkelsen et al., 2008) + others. Despite this, in the last couple of years, more studies were published pointing out slight but important differences between iPSCs and ESCs. Chin and colleagues (Chin et al., 2009) showed obvious differences in global gene expression analyses between these two stem cell types and proposed that iPSCs illustrate a unique subtype of pluripotent cells. Nevertheless, repeated analyses of extensive data on histone modification and gene expression patterns revealed that variations between iPSCs and ESCs detected in past studies most likely portrayed experimental noise and not consistent differences between these two cell types (Guenther et al., 2010; Newman et al., 2010). Furthermore, a different study compared global DNA methylation patterns as well as in vitro differentiation of iPSCs at early passages to human ESCs. The researchers concluded that using transcription factors for reprogramming, compared to nuclear transfer or cell fusion, can leave epigenetic marks in the generated iPSCs pointing towards the cell type of origin (Kim et al., 2010). Contrarily, such patterns were not seen when ESCs were derived by means of nuclear transfer, suggesting that a full reset of the epigenetic memory of a somatic cells may only be achieved by nuclear transfer. However, one possible reason for these results is that human ESCs but not iPSCs were derived in the presence of ERK inhibitors, known to facilitate full reprogramming (Silva et al., 2008b; Ying et al., 2008). In addition, the iPSCs used in this study were derived from fibroblasts and showed a partially demethylated endogenous Nanog promoter, a fact known to cause incomplete reprogramming (Kim et al., 2010).

However, it was shown that cloned mice give birth to viable offspring while showing abnormal and variable imprinting in their genome (Humpherys et al., 2001). Even though these findings indicate that deregulation of genes still allows normal development, clear criteria and necessary requirements need to be established allowing the generation of safe iPSCs that can be used in disease research and clinical applications for humans.

## 2 MATERIALS AND METHODS

### 2.1. Somatic Cell Reprogramming Using a Retroviral Approach

This part describes the generation of iPS cells from human diseased fibroblasts by means of a retroviral reprogramming approach originating from a protocol described by Yamanaka and colleagues in 2007 (Takahashi et al., 2007) and further adapted by the HSCI iPS Core Facility, Cambridge MA, USA (Figure 7).



**Fig. 7 Schematic timeline of the reprogramming process using retrovirus.**

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

The cell lines used were derived from patients with a diseased state known as constitutional mosaic trisomy 8 and the reprogramming process was conducted as part of a contract work between an external investigator and the HSCI iPS Core Facility in Cambridge Massachusetts, USA.

#### 2.1.1. Isolation of Human Fibroblasts from a Skin Biopsy

A fresh human skin biopsy was obtained with the help of a dermatologist and stored in a 1.5 mL pre-warmed cryovial (VWR, Cat. No. 82050-180) containing 1 mL of DMEM 10% FBS (450 mL DMEM (Invitrogen, Cat. No. 11995-073), 50 mL inactivated FBS (Invitrogen, Cat. No. 16000044), 5 mL P/S (Invitrogen, Cat. No. 15140-155) and 5 mL L-Glutamine (Invitrogen, Cat. No. 25030-156) per 500 mL, filtered with a 0.22  $\mu$ m filter bottle (Fisher Scientific, Cat. No. 09761102)). The skin sample was stored on ice at all times.

Two washing steps were performed by transferring the skin biopsy with a sterile forceps from the cryovial into a 15 mL conical tube containing dPBS with P/S (1:100) and from there into a second 15 mL conical tube containing dPBS with

P/S (1:100). Afterwards, the biopsy was transferred into a 6-cm cell culture dish and one drop of DMEM 10% FBS was added on top of the tissue. By using a sterile scalpel, the biopsy was cut into small fragments of <1mm length. The pieces were transferred into a second 6-cm cell culture dish into which a grid had been cut in the middle beforehand. Several drops of DMEM 10% FBS were added on top of the tissue fragments to keep it moist and the fragments were carefully pressed into the grid cut in the dish in order to attach firmly. In addition, 0.5 mL of DMEM 10% FBS were added to the edge of the dish to avoid flooding the tissue in the middle. The dish was incubated at 37°C (5% CO<sub>2</sub>) and after several hours, 0.5 mL DMEM 10% FBS were added to the dish. This was repeated up to a final volume of 4 mL of DMEM 10% FBS. Medium was changed once a week until fibroblast growth could be observed in the dish, after that cells were fed every three days. Upon 80% confluency of the primary fibroblasts, cells were trypsinized using 5-7 mL Trypsin + EDTA (Invitrogen, Cat. No. 25300-062).

Cat. No. 25300-062) for 5 minutes, neutralized with 5-7 mL DMEM 10% FBS, spun down at 1200 rpm for 5 minutes and the pellet was resuspended in 2-4 mL fresh DMEM 10% FBS and transferred into one or two wells of a 6-well plate (VWR, Cat. No. 82050-842) depending on the size of the cell pellet. When approximately 80% confluency was reached the cells were passaged as described above and transferred into a T25 culture flask (VWR, Cat. No. 82051-074) for expansion (final volume 7 mL). Passaging henceforward was performed at a ratio of 1:3 (one T25 into three T25 flasks or one T75 with a final volume of 15 mL).

The tissue pieces in the culturing dish were continuously fed with fresh DMEM 10% FBS until 80% confluency was reached for the second time. Cells were trypsinized as described above but this time a cell scraper (VWR, Cat. No. 29442-200) was used in order to detach all tissue fragments from the plate. Isolated fibroblasts were cultured as previously described until expansion into a T75 culture flask (VWR, Cat. No. 82050-856).

### **2.1.2. Production of Non-Replicating Retrovirus in HEK293T Host Cells**

In order to assemble the viral components for transduction of fibroblasts, human embryonic kidney cells (HEK293T) were used. At day 4 before transfection, a cryovial of HEK293T cells was thawed by shortly placing it in a 37°C water bath and mixing it with 9 mL of pre-warmed Dulbecco's modified Eagle medium (DMEM, Gibco) 10% FBS with addition of antibiotics penicillin and streptomycin

(WPS). The cell suspension was spun down at 1200 RPM for 5 minutes and resuspended in fresh, pre-warmed DMEM 10% and transferred to a T75 cell culture flask to a final volume of 15 mL. The HEK293T cells were incubated in a standard cell incubator at standard cell culture conditions of 37°C with 5% CO<sub>2</sub>.

On the day before transfection, the HEK293T cell culture flask was washed with dPBS (Gibco) once and cells were collected by using 5-7 mL Trypsin for 5 minutes at 37°C. The cell suspension was then centrifuged at 1200 RPM for 5 minutes and the pellet was resuspended in fresh DMEM 10% FBS without antibiotics, plated onto six 10-cm cell dishes (VWR, Cat. No. 82050-916) with a final volume of 10 mL and incubated under standard cell culture conditions.

On the day of transfection (D-2), four out of the six 10-cm dishes were chosen based on cell density, which should be 90-95% confluent. The two other plates were trypsinized at standard conditions and frozen down at a ratio of 1:10 in a freezing suspension containing DMSO (Sigma-Aldrich, Cat. No. D2650) and DMEM 10% FBS and stored in liquid nitrogen at -128°C.

For the transfection, a previously established protocol using Lipofectamine® (Invitrogen, Cat. No. 11668019) was used. For generation of retroviral particles, the following plasmid vectors and concentrations were being used: 10.5 µg GAG-POL for structural viral proteins, 1.5 µg VSV-G (vesicular stomatitis virus G) for envelope proteins as well as 12.5 µg of one of the pMIG vectors containing Oct4, Sox2, Klf4, or c-Myc. Four 15 mL conical tubes were prepared for the four transcription factors and filled up to 1 mL with opti-MEM Reduced Serum medium (Invitrogen, Cat. No. 11058-021). In addition, one 15 mL conical tube containing 75 µL Lipofectamine per tube of transcription factor (=4) and opti-MEM up to 4 mL was prepared and left at room temperature (RT) for 5 minutes. Then, 1 mL of Lipofectamine/opti-MEM mix was added to each tube of transcription factors and incubated at RT for 20 minutes. At last, 2 mL of the plasmid/Lipofectamine mixture was added dropwise to one 10-cm dish of HEK293T cells each and incubated under standard cell culture conditions.

From D-1 onwards until D2, any handling of cells in contact with VSV-G pseudotyped retrovirus was performed under BL2+ biosafety conditions. Any equipment used in the laminar flow hood in direct contact with the virus or viral supernatant was inactivated by treating with Vesphene Ilse Non-sterile Disinfectant Cleaner (Steris, Cat. No. 6461) before disposal.

The next day (D-1) early morning, medium was changed in each of the four 10-cm plates to fresh, pre-warmed 10 mL DMEM 10% FBS by manually aspirating the medium, inactivating it with vesphene and discarding it into a dedicated virus waste container.

On D0 in the early morning, viral supernatant from each 10-cm plate was collected individually for the first time in a 50 mL conical centrifuge tube and vacuum-filtered using a Steriflip 0.45 µm filter (Fisher Scientific, Cat. No. SE1M003M00). The four different viral supernatants were stored at 4°C after sealing with parafilm until further use. Fresh, pre-warmed 10 mL DMEM 10% FBS was added to the 10-cm plates for a second collection of viral supernatant on the following day. This procedure was repeated on D1 and finally the 10-cm plates were discarded after treating with vesphene for 20 minutes. Viral supernatants can be stored up to one week at 4°C but should ideally be used for transduction of fibroblasts immediately after collection.

### **2.1.3. Transduction of Isolated Fibroblasts with Retrovirus**

All human fibroblast samples to be transduced were tested for the presence of mycoplasma prior to transduction. This was performed by testing 24-hour old medium from a cell culture flask by using the MycoAlert<sup>®</sup> Mycoplasma Detection Kit (Lonza, Cat. No. LT 07-418). Fibroblasts contaminated with mycoplasma would be discarded.

On the day before transduction (D-1), previously isolated and expanded fibroblasts were trypsinized for 5 minutes, spun down at 1200 RPM for 5 minutes and resuspended in fresh, pre-warmed DMEM 10% FBS in order to determine the cell number by means of a hemocytometer (T10 Automated Cell Counter, Bio-Rad Laboratories, CA, USA). For the reprogramming protocol, for each sample of fibroblasts 100.000 cells were plated into one well of a 6-well plate. The wells were previously coated with 0.1% gelatin and incubated for an hour at 37°C. To test the viral supernatants individually for their potency, four wells of a 6-well plate with 100.000 cells per well were plated.

On D0 of transduction, the fibroblasts should be 70-80% confluent. Medium was removed from all wells and the following compounds were added to each well of fibroblasts, depending on the (reprogramming setup and) number of transcription factors used:

**Table 1. Transduction mixture prepared for transducing fibroblasts by retroviral means.**

<b>4 Factors (Oct4, Sox2, Klf4, c-Myc)</b>		<b>3 Factors (Oct4, Sox2, Klf4)</b>	
1 mL	Oct4 viral supernatant (VS)	1 mL	Oct4 viral supernatant (VS)
1 mL	Sox2 VS	1 mL	Sox2 VS
1 mL	Klf4 VS	1 mL	Klf4 VS
200 $\mu$ L	c-Myc VS	1 $\mu$ L	Polybrene (Final C.: 2.5 $\mu$ g/mL)
1 $\mu$ L	Polybrene (Final C.: 2.5 $\mu$ g/mL)	2 mL	DMEM 10% FBS
1 mL	DMEM 10% FBS		
<i>4,2 mL</i>	<i>Final Volume</i>	<i>4 mL</i>	<i>Final Volume</i>

For the four wells on the control plate, 1 mL of one of the four viral supernatants and 1 mL of DMEM 10% FBS together with 0.5  $\mu$ L Polybrene (Millipore, Cat. No. TR-1003-G) were added to each plate. Transduction was carried out for 6-8 hours under standard cell culture conditions. Afterwards, viral supernatants were removed, the wells were washed with dPBS once, fresh, pre-warmed DMEM 10% FBS was added and the cells were incubated under standard cell culture conditions.

On the next day (D1), a second transduction identical to D0 was performed. After 6-8 hours of transduction, the wells were washed with dPBS twice, fresh, pre-warmed DMEM 10% FBS was added and the cells were incubated under standard cell culture conditions throughout D2 and D3.

On D4, transduction efficiency was assessed by expression of GFP in the four wells containing one single transcription factor and in all individual wells containing fibroblast samples by fluorescence imaging. Upon GFP expression, plates with a MEF-feeder-layer were prepared. For this, irradiated CF1 mouse embryonic fibroblasts (MEFs 2 Million, GlobalStem, Cat. No. GSC-6201G) stored in liquid nitrogen were thawed by shortly placing the bottom of the cryovial in a water bath (37°C) and transferring the cells to fresh, pre-warmed DMEM 10% FBS. The cells were spun down, resuspended in DMEM 10% FBS and plated onto 0.1% gelatin-coated 10-cm plates at a density of  $1 \times 10^6$  cells per dish.



One 10-cm dish per well of human fibroblasts was prepared and incubated under standard cell culture conditions.

The next day (D5), transduced human fibroblasts were replated onto MEF-feeder 10-cm plates by washing the cells with dPBS once, trypsinizing for 5 minutes and resuspending in fresh, pre-warmed DMEM 10% FBS.

On D6, the medium was changed from DMEM 10% FBS to hESC medium in order to promote the formation of iPS colonies, containing 400 mL DMEM/F12 (Invitrogen, Cat. No. 11330-057), 100 mL KOSR (Invitrogen, Cat. No. 10828-028), 5 mL P/S (Invitrogen, Cat. No. 15140-122), 5 mL L-Glutamine (Invitrogen, Cat. No. 25030-081), 5 mL MEM-NEAA (Invitrogen, Cat. No. 11140-050) and 500  $\mu$ L 2-Mercaptoethanol (Invitrogen, Cat. No. 21985023) per 500 mL. The medium was filtered with a 0.22  $\mu$ m filter prior to use and in addition 10ng/mL  $\beta$ FGF (Invitrogen, Cat. No. PHG0261) was added right before using the medium. For each human fibroblast sample, VPA (1000X, EMD Biosciences, Cat. No. 676380-5GM) was added at a final concentration of 50  $\mu$ M to one 10-cm plate per human sample only. For the first week from D6 onwards, medium was changed every other day. From D13 onwards, no more VPA was added and hES medium was changed daily.

#### **2.1.4. Cultivation and Maintenance of iPSCs**

Visible, morphological changes were observed as early as day 10 after transduction (D0). The culture dishes were observed regularly for the appearance of iPS colonies that silenced GFP expression, which reflects the accomplishment of a fully reprogrammed state in the cells. For each emerged colony of reasonable size, a feeder layer of irradiated MEFs was plated into one well of a 6-well plate the day before picking of the cells as described in 4.1.5.

The day after plating the feeder cells, fully reprogrammed cells based on morphology and negative GFP expression were manually transferred to the MEF-coated 6-well plate by means of a drawn-out glass Pasteur pipette performed under a dissection microscope located in a laminar flow hood. Differentiated areas within a colony appear white and “bulky”, while undifferentiated colonies are opaque or transparent. Differentiated areas were carefully excised and discarded while the remaining undifferentiated colonies were scored into a grid-like pattern with a fire drawn glass pipette to create small, floating pieces, before lifting them off the dish with a P200 pipette and transferring the colony pieces to a

new MEF plate. The picked colonies were labeled as passage 0 and cultivated using hESC medium with the unique addition of 10 $\mu$ M ROCK Inhibitor (500X, Y-27632, EMD Chemicals Inc., Cat. No. 688001-500UG) at standard cell culture conditions. Each colony picked was considered as one distinct cell line of the transduced fibroblasts.

This procedure was repeated for several undifferentiated iPSC colonies from the initial 10-cm dish. Feeding was omitted the day after picking to ensure compact attachment of the new colony.

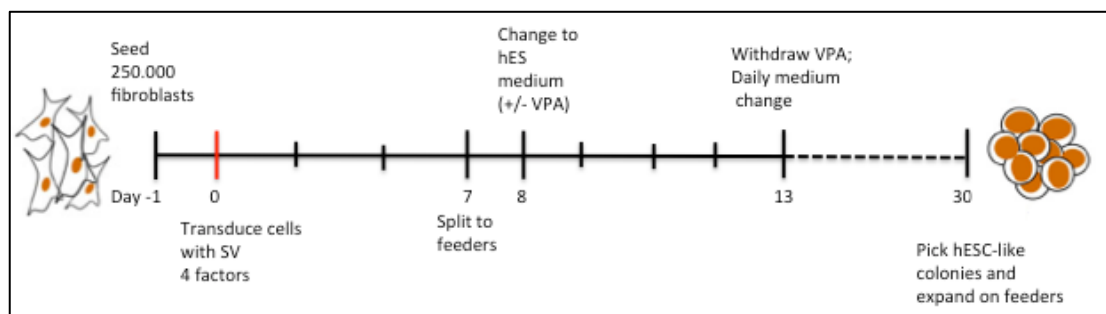
Subsequently, iPSCs were fed daily with fresh, pre-warmed hESC medium for 7 to 10 days. The cells were then split at a ratio of 1:1 to 1:3 depending on cell density onto a new layer of irradiated MEFs in 6-well plates. iPSCs were split by aspirating the hESC medium, washing with dPBS once and incubating with 1 mL Collagenase IV (1mg/mL, Stem Cell Technologies, Cat. No. 07909) per well of a 6-well plate for approximately 10 minutes at 37°C. As soon as visible curling of the edges of the colonies appeared, collagenase IV was aspirated and 1 mL of pre-warmed hESC medium (no bFGF) was added before lifting the cells using a cell scraper (Corning, Cat. No. 3008). The cell suspension was transferred to a 15 mL conical tube and the wells were washed twice with hESC medium (no bFGF). The tubes were centrifuged for 5 minutes at 1200 rpm before resuspending the cell pellets in 1 mL per one well of a 6-well plate intending to plate of fresh, pre-warmed hESC. The cell pellets were triturated carefully in order to obtain medium-sized cell fragments for an increased survival outcome.

After 2-3 weeks of expansion, a small portion of cells was frozen down for means of backup. For the freezing procedure, identical steps as for splitting cells were carried out. The cell pellet from one well of a 6-well plate was transferred to one cryovial by carefully resuspending the pellet in 250  $\mu$ L hESC medium and 250  $\mu$ L 2X freezing medium (2 mL DMSO, 8mL defined FBS, kept on ice) to keep large cell fragments. The cryovials were stored in an isopropanol-containing Nalgene® Cryo 1°C “Mr. Frosty freezing container (VWR, Cat. No. 55710-200) at -80°C for 24-48 hours and subsequently transferred to a liquid nitrogen tank at -128°C and left there for long-term storage or until further use.

## **2.2. Somatic Cell Reprogramming using a Sendai Virus**

This part describes the generation of iPSC cells from human fibroblasts with the help of a reprogramming protocol using a Sendai viral vector originating from a

protocol described by Fusaki and colleagues in 2009 (Fusaki et al., 2009), commercially sold by Life Technologies Corporation and further adapted by the HSCI iPS Core Facility, Cambridge MA, USA (Figure 8).



**Fig. 8 Schematic timeline of the reprogramming process using Sendai virus**

Timeline of cellular reprogramming using retroviruses involving preparation of donor fibroblasts, viral transduction, selection of reprogrammed cells and subsequent cultivation of generated iPS cells.

The fibroblasts cell lines used were derived from patients suffering from bipolar symptoms or relatives showing no symptoms (yet) as a control and the reprogramming process was conducted as part of a contract work between an external investigator and the HSCI iPS Core Facility in Cambridge, MA, USA.

### 2.2.1. Transduction of Isolated Fibroblasts with Sendai Virus

The fibroblasts samples used were obtained from a cell repository (Coriell Institute, NJ, USA). All human fibroblast samples to be transduced were tested for the presence of mycoplasma prior to transduction. This was performed by testing 24-hour old medium from a cell culture flask by using the MycoAlert<sup>®</sup> Mycoplasma Detection Kit (Lonza, Cat. No. LT 07-418).

One day before transduction (D-1), fibroblasts were trypsinized for 5 minutes, spun down at 1200 RPM for 5 minutes and resuspended in fresh, pre-warmed DMEM 10% FBS in order to determine the cell number by means of a hemocytometer. For each sample of fibroblasts, 250,000 cells were plated into one well of a 6-well plate. The wells had been previously coated with 0.1% gelatin and incubated for an hour at 37°C. The cells were then incubated under standard cell culture conditions overnight.

On the day of transduction (D0), the fibroblasts should be 90% up to entirely confluent. In the early morning, medium was removed and replaced with 2 mL DMEM 10% FBS without Pen/Strep.

The Sendai viruses used, encoding the four transcription factors (Oct4, Sox2, Klf4, c-Myc) required for reprogramming, were provided in the CytoTune™-iPS Reprogramming Kit (Invitrogen, Cat. No. A-1378002). Two mL of DMEM 10% without Pen/Strep for each two wells to be transduced were pre-warmed. The four tubes containing the different viral vectors were thawed one tube at a time by immersing the bottom of the tube in a 37°C water bath for 10 seconds. The tubes were then allowed to thaw at RT and were quickly centrifuged before placing them on ice. The multiplicity of infection was set at 3 and therefore  $7.5 \times 10^5$  colony-infecting units (CIU) were added from each viral vector tube to the 2 mL of DMEM medium. After that, medium was aspirated from all wells of fibroblasts and 1 mL of viral vector solution in DMEM was added per well. The cells were then incubated under standard cell culture conditions overnight. Virus waste and pipettes in touch with viral solution were treated with vesphene and discarded accordingly.

The next day (D1), preferably after 24 hours, medium was replaced with fresh DMEM 10% FBS. From then on, medium was replaced every other day.

On day 6 after transduction, one day before passaging the transduced fibroblasts onto feeder cells, 10cm-cell culture dishes were plated with irradiated MEFs with a density of 1 million cells per dish as described in 4.1.5.

The next day (D7), transduced fibroblasts were ready to harvest and replate onto feeder cells. Cells were washed once with PBS and 1 mL of Accutase (Invitrogen, A11105-01) was added per well and incubated at 37°C for 4 minutes or until the cells have rounded up. The accutased wells were neutralized with 3 mL of DMEM 10% and the cells were collected into a 15 mL conical tube. In order to detach all cells, 3 mL of DMEM 10% were added to each well and a cell scraper was used to detach any remaining cells. The cell suspensions were spun down at 1200 rpm for 5 minutes and the pellets were resuspended in 1 mL DMEM 10%. At this stage, two cell lines with very high proliferation rates were split onto two 10-cm dishes, whereas the other four cell lines were transferred onto one new feeder dish each.

The next day, medium was removed and switched to hESC medium. For the lines with two 10-cm dishes, one was treated additionally with VPA for one week. From then on, feeding was performed daily until formation of colonies was observed (around D25-30).

### **2.2.2. Cultivation and Maintenance of Sendai Virus-derived iPSCs**

Visible changes in morphology were observed as early as day 10-15 after transduction, but picking of colonies for expansion and characterization was not performed until day 31-37. Due to the lack of a reporter gene in the Sendai viral vectors, selection of fully reprogrammed cells was based on morphology only.

For each emerged colony of reasonable size, a feeder layer of irradiated MEFs was plated into one well of a 6-well plate the day before picking of the cells as described in 4.1.5.

The day after plating the feeder cells, fully reprogrammed cells, based on morphological criteria, were manually transferred to the MEF-coated 6-well plate by means of a drawn-out glass Pasteur pipette performed under a dissection microscope located in a laminar flow hood. The picked colonies were labeled as passage 0 and cultivated using hESC medium with the unique addition of 10 $\mu$ M ROCK Inhibitor (500X, Y-27632, EMD Chemicals Inc., Cat. No. 688001-500UG) at standard cell culture conditions. Each colony picked was considered as one distinct cell line of the transduced fibroblasts.

This procedure was repeated for several undifferentiated iPS colonies to be transferred from the initial 10-cm dish. Feeding was omitted the day after picking to ensure compact attachment of the new colony.

### **2.3. Characterization of Reprogrammed Cell Lines**

In order to fully evaluate the identity of reprogrammed stem cells, thorough characterization of these cell lines is required. Commonly accepted assays for determining the cellular gene expression, chromosomal abnormalities, expression of antigens and differentiation capabilities were employed and the results were analyzed carefully.

#### **2.3.1. Alkaline Phosphatase Treatment**

An Alkaline Phosphatase (AP) assay was performed using the Alkaline Phosphatase Detection Kit (Millipore, Cat. No. SCR004) and carried out according to the manufacturer's protocol.

Approximately six days prior to the start of the AP assay, 1-2 wells per cell line to be analyzed of a 12-well plate were prepared by plating a layer of irradiated MEFs in DMEM 10% FBS medium (approximately 80.000 cells per well). On the following day, medium was changed to hESC medium and 5-7 iPSC colonies for

each line to be analyzed were picked and transferred to one well of a 12-well plate. The plates were incubated under standard cell culture conditions and fed every day with hESC medium except the day after picking.

Six days after picking of colonies, the following solutions were prepared for the AP assay:

- ❖ 4% Paraformaldehyde (PFA) in PBS
- ❖ 1X Rinse Buffer
  - 50 mL PBS + 25  $\mu$ L TWEEN®20 (Sigma-Aldrich, Cat. No. P1379)
- ❖ Staining Solution
  - 2 parts Fast Red Violet Solution (Millipore)
  - 1 part Naphtol AS-BI phosphate (Millipore)
  - 1 part Ultra-Pure™ Dnase-RNase-Free Distilled Water (Invitrogen, Cat. No. 10977)

First, hESC medium was aspirated and cells were fixed with 500  $\mu$ L 4% PFA for 1-2 minutes under a chemical fume hood. The PFA was removed and discarded into a dedicated PFA waste container. The cells were washed once with 500  $\mu$ L 1X Rinse Buffer and 500  $\mu$ L Staining Solution were added to each well and incubated for 15 minutes at RT in the dark. Afterwards, the wells were washed with 1X Rinse Buffer once and left in 1 mL PBS. Each well was examined under a microscope at 4x/10x magnification for positive alkaline phosphatase expression and photos were taken for documentation. The plates were sealed with Parafilm, wrapped in aluminum foil and stored for 1-2 weeks at 4°C for second examination if necessary.

### **2.3.2. Immunocytochemistry**

Five to seven days prior to performing the Immunocytochemistry assay (ICC), 6 wells per cell line to be analyzed of a 48-well plate were prepared by plating a layer of irradiated MEFs in DMEM 10% FBS medium (approximately 30.000 cells per well). On the following day, medium was changed to hESC medium and 3-5 iPSC colonies for each line to be analyzed were picked and transferred to one well of a 48-well plate. The plates were incubated under standard cell culture conditions and fed daily with hESC medium except the day after picking.

Five to seven days after picking, once the colonies had reached a reasonable size, the following solutions were prepared for the ICC assay:

- ❖ PBS / 0.05% TWEEN®20
  - 50 mL PBS + 25 µL TWEEN®20:
- ❖ PBS / 0.1% Triton® X-100
  - 10 mL PBS + 10 µL Triton® X-100 (Sigma-Aldrich, Cat. No. X100-100mL)
- ❖ 4% Paraformaldehyde (PFA) in PBS
- ❖ DAPI
  - 1 µL DAPI (1mg/mL) in 10 mL PBS
- ❖ 4% Donkey Serum
  - 15 mL PBS + 600 µL Donkey Serum (Fisher Scientific, Cat. No. NC9624464)

First, hESC medium was aspirated and cells were washed with 500 µL PBS three times. The cells were fixed with 4% PFA for 20 minutes, washed with PBS/0.05% TWEEN®20 solution three times and permeabilized by using 500 µL of PBS/0.1% Triton® X-100 for 15 minutes at RT.

Following permeabilization, cells were washed with PBS/0.05% TWEEN®20 solution three times and unspecific binding sites were blocked by adding 500 µL of 4% Donkey Serum per well. The plates were wrapped in Parafilm and stored at 4°C overnight.

The following day, the following primary antibodies were prepared by diluting in 4% Donkey Serum accordingly:

**Table 2. Primary antibodies and dilution factors used for immunocytochemistry; all diluted in 4% donkey serum.**

<b>Primary Antibodies</b>	<b>Dilution</b>
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

Before adding the primary antibodies, donkey serum was aspirated and 100  $\mu$ L of the primary antibody dilution was added to one well per cell line each. One well, serving as a control, was treated with 4% Donkey Serum only. The plates were then incubated at RT for 1 hour.



Secondary antibodies for immunocytochemistry were prepared by diluting in PBS accordingly:

**Table 3. Primary antibodies and according secondary antibodies with dilution and excitation and emission spectra (in nm).**

All Secondary Antibodies were diluted in PBS. Secondary antibodies were purchased from Invitrogen.

<b>Primary AB</b>	<b>Secondary AB</b>	<b>Dilution</b>	<b>Excitation [nm]</b>	<b>Emission [nm]</b>
<b>Oct4</b>	Alexa Fluor® 488 donkey anti-rabbit IgG (Cat. No. A21206)	1:500	495	519
<b>Nanog</b>	Alexa Fluor® 488 donkey anti-rabbit IgG (Cat. No. A21206)	1:500	495	519
<b>SSEA-3</b>	Alexa Fluor® 594 goat anti—rat IgM (Cat. No. A21213)	1:500	590	617
<b>SSEA-4</b>	Alexa Fluor® 488 goat anti—mouse IgG (Cat. No. A21121)	1:500	495	519
<b>Tra-1-60</b>	Alexa Fluor® 555 goat anti—mouse IgM (Cat. No. A21426)	1:500	555	565

Before adding the secondary antibodies, three washing steps with PBS/0.05% TWEEN®20 solution were performed. 100 µL of according secondary antibody was added to one well per cell line each. The negative control was treated with PBS only and the plates were then incubated at RT for 1 hour in the dark.

Afterwards, secondary antibody got aspirated and the plates were washed with PBS/0.05% TWEEN®20 solution three times. Counterstaining was performed with 100 µL of DAPI solution for up to 1 minute and washed once with PBS before examining the plates by means of a fluorescence microscope for the

expression of pluripotency markers. When wrapped in parafilm and aluminum foil, plates can be stored at 4°C for up to one week for further investigation.

### 2.3.3. Karyotyping

For karyotyping, one well of a 6-well plate of the cell line to be characterized was split into in a gelatin-coated T25 cell culture flask on a layer of irradiated MEF feeder cells under standard cell culture conditions. Cells were fed with hESC for 5-7 days until approximately 70% confluency was reached. At that stage, the flasks were sent to an external laboratory for karyotyping analysis (Cell Line Genetics; 510 Charmany Drive, Suite 254; Madison, WI 53719) and results were usually received within one to three weeks after placing the order.

### 2.3.4. Testing the Pluripotency Markers

In order to confirm the pluripotent state of the reprogrammed induced pluripotent stem cells, the following pluripotent markers and specific primer sequences were employed:

**Table 4. Primer sequences for detection of pluripotency-specific gene products by means of PCR.**

The PCR program was set up with a holding step at 94°C for 5 minutes, a repeated number of cycles and with an extended elongation period at 72°C and a final holding step at 4°C. Oct4 and Sox2 primers were selected to target the endogenous expression of the genes, unlike other primers, which target the exogenous gene products.

Primer	Forward Sequence	Reverse Sequence
<b>Nanog</b>	TCCAACATCCTGAACCTCAG	GACTGGATGTTCTGGGTCTG
<b>Oct4</b>	GTGGAGGAAGCTGACAACAA	CAGGTTTTCTTTCCCTAGCT
<b>Sox2</b>	TTGTCCGAGACGGAGAGCG	TGACCACCGAACCCATGGAG
<b>Rex1</b>	TGGACACGTCTGTGCTCTTC	GTCTTGGCGTCTTCTCGAAC
<b>Dnmt3b</b>	ATAAGTCGAAGGTGCGTCGT	GGCAACATCTGAAGCCATTT
<b>hTERT</b>	TGTGCACCAACATCTACAAG	GCGTTCTTGGCTTTCAGGAT

In order to perform PCR analyses, cDNA (complimentary DNA) was generated from RNA previously extracted from cells growing on feeders.

#### 2.3.4.1. Extracting RNA

RNA was extracted by using the Qiagen RNeasy Mini Kit (Qiagen, Cat. No. 74106) together with using the RNase-free DNase Kit (Qiagen, Cat. No. 79254). The two kits provided all reagents except 70% ethanol.

The previously recovered cell pellet was resuspended in 350  $\mu$ L of RLT Buffer and vortexed for one minute. Afterwards, 350  $\mu$ L of 70% ethanol were added and pipetted up and down slightly. The total volume was transferred into a Rneasy spin column and centrifuged at 10.000 rpm for 15 seconds. The flow-through was discarded and 350  $\mu$ L of RW1 Buffer were added to the spin column before centrifuging again at 10.000 rpm for 15 seconds and discarding the flow-through. In a separate microcentrifuge tube (VWR, Cat. No. 14231-062), 10  $\mu$ L of DNase and 70  $\mu$ L of HDD Buffer (both RNase-free DNase Set, Qiagen, Cat. No. 79254) were mixed. Subsequently, 80  $\mu$ L of DNase mixture were added to the spin column and incubated for 15 minutes at RT. 350  $\mu$ L of RW1 Buffer was then added and the column was centrifuged at 10.000 rpm for 15 minutes. After the flow-through was discarded, 500  $\mu$ L of RPE Buffer were added followed by a 2-minute centrifugation step at 10.000 rpm. Flow-through was discarded and once more, 500  $\mu$ L of RPE Buffer were added to the column and centrifuged like before. This time, the flow-through together with the collection tube was discarded and the spin column was placed in a fresh collection tube before spinning it down at 12.000 rpm for 1 minute. Afterwards, the spin column was transferred to a fresh 1.5 mL collection tube and 30  $\mu$ L of RNase-free water were added on top of the column and centrifuged at 10.000 for 1 minute. This time, the spin column was discarded and the collection tube containing extracted RNA was kept. RNA concentration was determined by means of a spectrophotometer (NanoVue, GE Healthcare, Buckinghamshire, GB) and recorded. The collection tubes were labeled accordingly and stored at  $-80^{\circ}\text{C}$  for further analysis or for use in cDNA synthesis.

#### 2.3.4.2. cDNA Synthesis

Complimentary DNA (cDNA) was generated by using the SuperScript® III Reverse Transcriptase Kit (Invitrogen, Cat. No. 18080-044). All reagents needed for cDNA synthesis were thawed on ice except reverse transcriptase (RT), which was kept at  $-20^{\circ}\text{C}$ . One  $\mu$ g of previously extracted RNA was added to two PCR tubes (VWR, Cat. No. 82050-976 for tubes, Cat. No. 82050-416 for caps) each and the tubes were labeled “+” and “-”.

Subsequently, 1  $\mu\text{L}$  of dNTP Mix (10 mM), 1  $\mu\text{L}$  of random hexamers (50ng/ $\mu\text{L}$ ), and DEPC-treated Water (Ambion, Cat. No. 9915G) adding up to a total volume of 13  $\mu\text{L}$  were added to each tube. The PCR tubes were then heat-inactivated at 65°C for 5 minutes and quickly incubated on ice for at least 1 minute. Afterwards, 4  $\mu\text{L}$  of 5X First Strand Buffer, and 1  $\mu\text{L}$  of DTT (0.1M), 1  $\mu\text{L}$  of RNase OUT™ Recombinant RNase Inhibitor (Invitrogen, Cat. No. 10777019) were added to each tube. One  $\mu\text{L}$  of RT was added to the “+” tube only and for means of a control 1  $\mu\text{L}$  of DEPC-treated water was added to the “-“ tube. The PCR tubes were spun down shortly and placed in the PCR machine running a preset RT-PCR program including a 5 minute holding step at 25°C, a 45 minute holding step at 50°C, a 15 minute holding step at 70°C and a final holding step at 4°C.

Finally, the newly synthesized cDNA was transferred to microcentrifuge tubes, labeled accordingly and stored at -20°C for further use.

#### 2.3.4.3. Polymerase Chain Reaction (PCR)

In order to analyze the pluripotency markers by means of agarose gel electrophoresis, PCR reactions were performed to amplify the given samples. For this purpose, cDNA previously reversely transcribed from extracted RNA was used.

For the PCR setup, cDNA samples, adequate volumes of Platinum® Blue PCR SuperMix (Invitrogen, Cat. No. 12580-023) as well as forward and reverse primers of the PCR to be performed were thawed on ice. A PCR master mix containing 23  $\mu\text{L}$  of Platinum® Blue PCR SuperMix, 0.5  $\mu\text{L}$  of forward primer, and 0.5  $\mu\text{L}$  of reverse primer was prepared. Finally, 24  $\mu\text{L}$  of master mix as well as 1  $\mu\text{L}$  of according cDNA were added to each PCR tube and labeled accordingly. The tubes were briefly spun down before placing them into the PCR machine (C1000 Thermal Cycler, Bio-Rad Laboratories, NJ, USA) and running a preset PCR protocol adapted to the specific primers employed. After the amplification, samples were either analyzed by means of agarose gel electrophoresis immediately or stored at 4°C for further use.

#### 2.3.4.4. Agarose Gel Electrophoresis

Qualitative agarose gel electrophoresis was performed in order to verify the presence of pluripotency markers and subsequently also the presence of differentiation markers in the generated iPSCs.

In general, a 1.5% agarose gel was prepared by dissolving 0.75 g of Agarose powder (Fisher Scientific, Cat. No. BP1356-500) in 50 mL 1X Tris-acetate-EDTA (TAE) Buffer (diluted from a 50X stock, Invitrogen, Cat. No. 24710030). The solution was heated up in the microwave for approximately 2 minutes or until all the agarose was dissolved and was then left in a chemical fume hood to cool down. One  $\mu\text{L}$  of 1% Ethidium Bromide solution (Fisher Scientific, Cat. No. 1302-10) was added to the agarose solution before pouring it into a prepared gel carrier of 8x10cm. Combs were inserted and the gel was left to solidify for approximately 20 minutes in the fume hood. Finally, the gel carrier was placed into the plexiglass electrophoresis gel apparatus and 1X TAE Buffer was added until the marked level of the apparatus. For each sample, 10  $\mu\text{L}$  were loaded per slot and in addition 10  $\mu\text{L}$  of 1 Kb Plus DNA Ladder (Invitrogen, Cat. No. 10787-026), diluted in 10X BlueJuice™ Gel Loading Buffer (Invitrogen, Cat. No. 10816-015) and Ultra-Pure Water (Invitrogen, Cat. No. 10977) in a ratio of 1:1:8 was added to serve as a size marker control. The voltage was set at 80 – 95 volts and the gel was analyzed under UV light after approximately 20-30 minutes.

### **2.3.5. Testing for Differentiation Markers by in-vitro Differentiation of Embryoid Bodies**

To test for the full differentiation potential of the cultured iPS cells, embryoid bodies (EBs) were formed in culture and expression of distinct germ layer markers was subsequently analyzed by PCR.

#### **2.3.5.1. In-vitro Embryoid Body Formation**

In order to establish EBs in culture, one well of a 6-well plate with a preferably high density was chosen per line. The well was washed with PBS once and collagenase was added and incubated at 37°C for approximately 10 minutes or until the edges of the colonies lifted up slightly under the microscope. Collagenase was aspirated, hESC medium was added and a cell scraper was employed to detach all cells. After spinning the cell suspension at 1200 rpm for 5 minutes, the cell pellet was resuspended in 2 mL of fresh and pre-warmed hESC medium without  $\beta\text{FGF}$  and the cell clumps in suspension were transferred to one well of a low-attachment 6-well plate (Fisher Scientific, Cat. No. 3471). The cells were incubated under standard cell culture conditions and fed with hESC medium without  $\beta\text{FGF}$  every three days for one week. Subsequently, the cell aggregates were spun down and resuspended in fresh 2 mL of DMEM 10% FBS, before transferring them into one well of a regular 6-well plate coated with 0.1% gelatin.

The embryoid bodies were incubated under standard cell culture conditions and fed every three days with DMEM 10% FBS for another week. The aggregates were then trypsinized for 5 minutes at 37°C, spun down at 1200 rpm for 5 minutes and the pellet was washed with PBS once before it was either directly used for RNA extraction or frozen at -80°C for further use.

#### 2.3.5.2. PCR for Testing of Differentiation Markers

For the detection of molecular markers specific for the three embryonic germ layers, PCRs were performed. Six differentiation markers (Table 5) known to be representative for endoderm, mesoderm, and ectodermal lineages were employed. All procedures like extraction of RNA, cDNA synthesis, PCR and final agarose gel electrophoresis were performed as described before (see chapter 2.3.4.2 – 2.3.4.5).

**Table 5. Primer sequences for detection of differentiation-specific gene products by means of PCR.**

The PCR program was set up with a holding step at 94°C for 5 minutes, a repeated number of cycles and with an extended elongation period at 72°C and a final holding step at 4°C. The primers indicated were the ones most commonly used for assessing the three specific germ lineages.

<b>Primer</b>	<b>Lineage</b>	<b>Forward Sequence</b>	<b>Reverse Sequence</b>
<b>AFP</b>	Endoderm	AGCTTGGTGGTGGATGAAA C	CCCTCTTCAGCAAAGCAG AC
<b>GATA4</b>	Endoderm	CTAGACCGTGGGTTTTGCAT	TGGGTTAAGTGCCCCTGT AG
<b>FLK1</b>	Mesoderm	AGTGATCGGAAATGACACT GGA	GCACAAAGTGACACGTTG AGAT
<b>GATA2</b>	Mesoderm	GCAACCCCTACTATGCCAA CC	CAGTGGCGTCTTGGAGA AG
<b>NCAM</b>	Ectoderm	ATGGAAAACCTCATTAAAGTG AACCTG	TAGACCTCATACTCAGCA TTCCAGT
<b>PAX6</b>	Ectoderm	TCTAATCGAAGGGCCAAAT G	TGTGAGGGCTGTGTCTGT TC

### **3 RESULTS**

The experiments underlying this scientific thesis were conducted as part of a contract work between external investigators and the HSCI iPSC Core Facility in Cambridge, Massachusetts, USA. Therefore, this section will only describe the experiments focusing on the iPSC generation using Sendai virus and will only contain characterization assays commissioned by the external investigators.

#### **3.1. Generation of Human Fibroblasts with Sendai Virus**

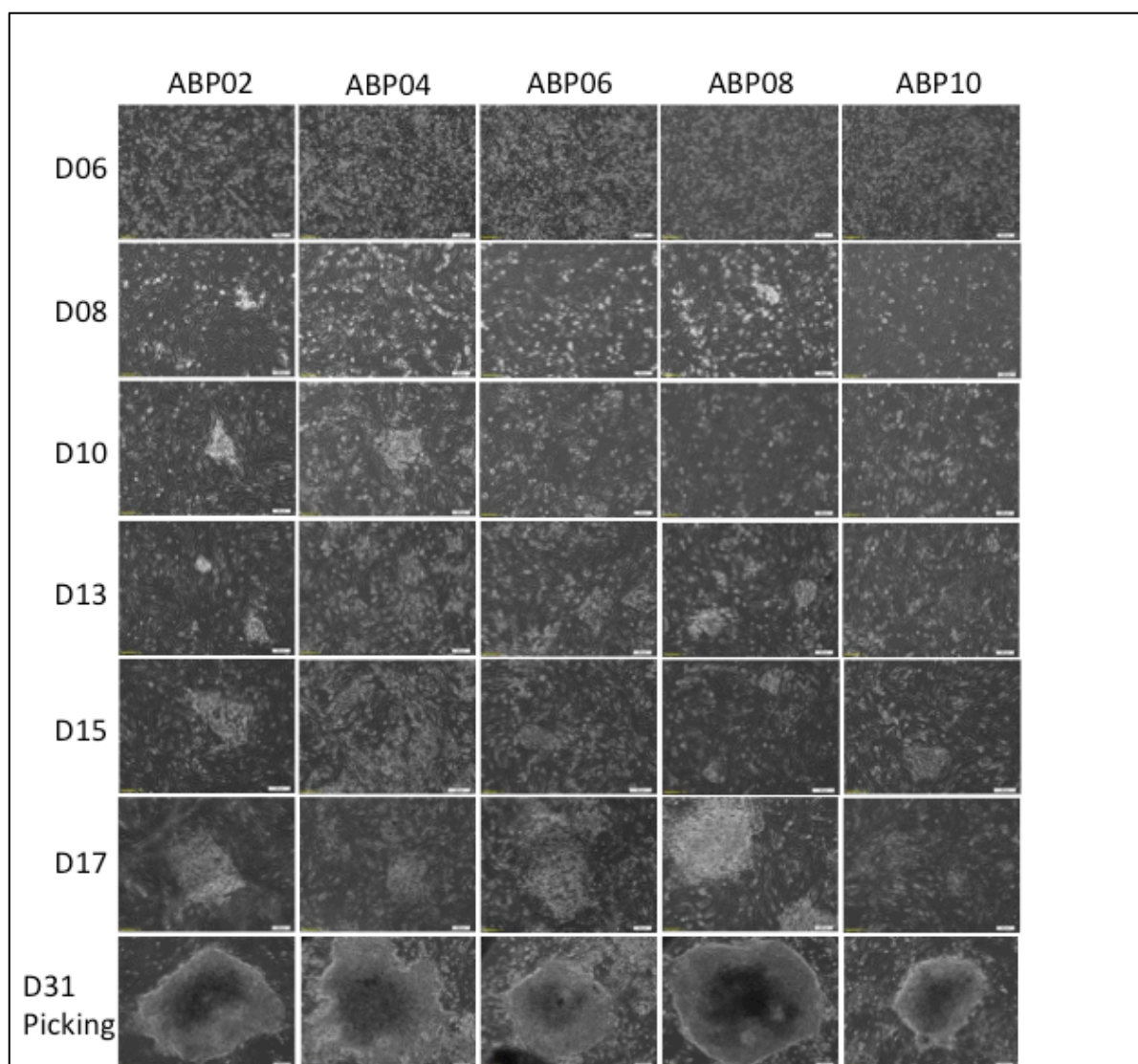
Primary human fibroblasts seeded at a density of 250.000 cells per well of a 6-well plate were transduced using the CytoTune™-iPS Reprogramming Kit (Invitrogen, Cat. No. A-1378002). The cells were transduced with the four Yamanaka factors Oct-4, Sox2, Klf4, and c-Myc at a MOI of 3.

The transduced fibroblasts were replated onto a MEF feeder layer seven days post transduction and medium was changed from DMEM 10% FBS to hES medium containing bFGF. In addition, VPA was added to indicated samples for the duration of one week.

First morphological changes were observed at around day 8-10 after transduction and based on morphological criteria colonies were picked approximately at day 31.

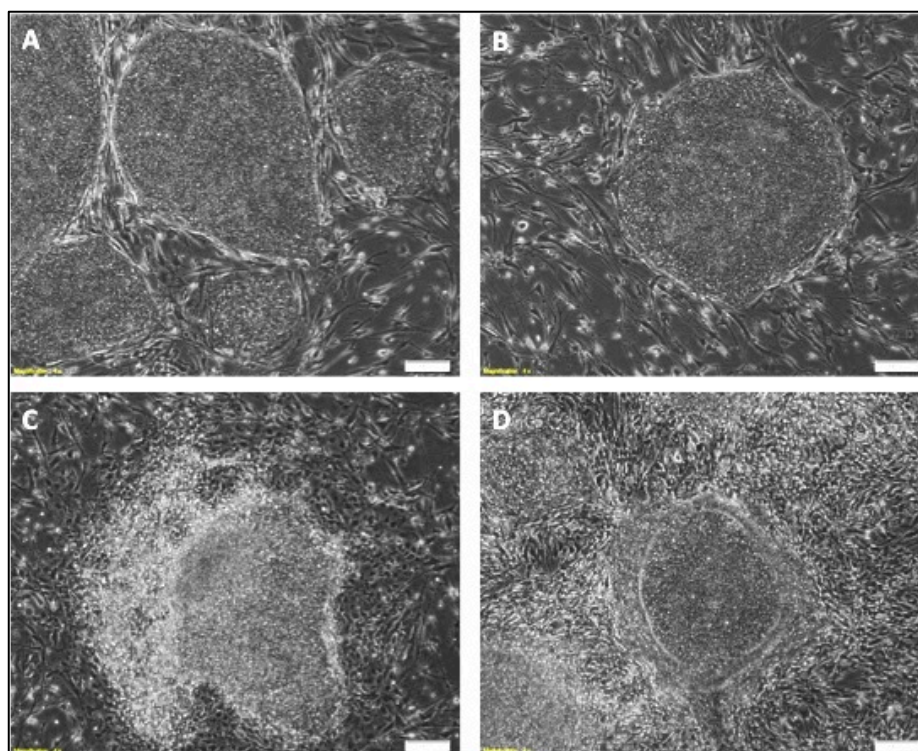
A timeline of morphological changes occurring after transduction gives an understanding of the kinetics of the reprogramming process (Figure 9).





**Fig. 9 A timeline of morphological changes following the transduction of human fibroblasts.** First visible changes in morphology after transduction of 250.000 fibroblasts with Sendai virus were observed around 8-10 days after transduction. Images shown are representative for the different patient samples ABP02 to ABP10. Colonies were picked on day 31 after transduction for further expansion and characterization. Scale bar = 200 $\mu$ m.

After colonies were picked from the initial 10-cm cell culture dish and further expanded, a clear iPS morphology could be observed, indicated by round colonies, defined borders and rather transparent appearance (Figure 10A-B). Differentiation in general in iPS colonies can be observed frequently for individual cell lines (Figure 10C-D) but should be removed under the picking hood before passaging the cells.



**Fig. 10 Morphology of fully established iPS colonies.**

(A, B) iPS colonies at passage 7 showing normal morphology indicated by a round shape, clear appearance and defined borders; (C, D) iPS colonies at passage 9 showing differentiation; Scale bar = 200 $\mu$ m.

For each transduced patient sample, 7-10 hESC-like clones were picked, labeled as individual cell lines from 1 to 10 and further expanded in 6-well plate formats. Three cell lines per patient sample were chosen for detailed characterization (Table 6).

**Table 6. Patient fibroblast samples and established iPS cell lines selected for further characterization.**

Three iPS lines per patient fibroblast sample were chosen for detailed characterization; Use of VPA during transduction as indicated.

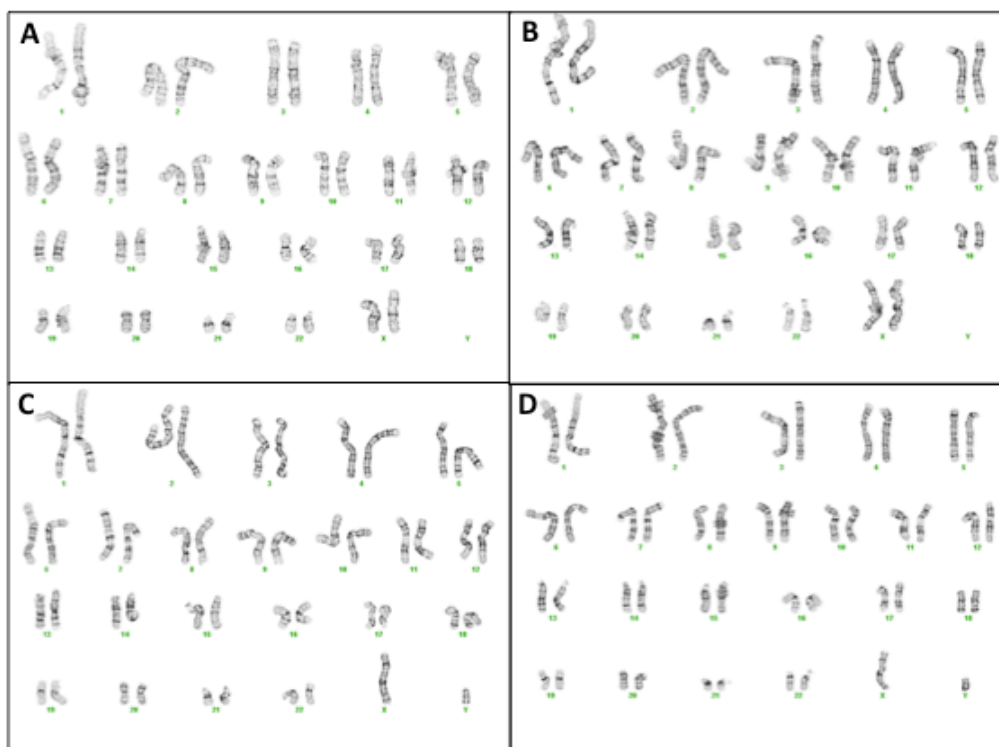
<b>Patient Sample</b>	<b>Cell Lines established</b>	<b>VPA (Y/N)</b>
<b>ABP02</b>	ABP02-1	N
	ABP02-3	N
	ABP02-7	N
<b>ABP04</b>	ABP04-1	N
	ABP04-2	N
	ABP04-3	N
<b>ABP06</b>	ABP06-1	N
	ABP06-2	N
	ABP06-4	Y
<b>ABP08</b>	ABP08-1	N
	ABP08-2	N
	ABP08-3	N
<b>ABP10</b>	ABP10-5	N
	ABP10-6	Y
	ABP10-7	Y

To give an appropriate overview and due to a restricted outline, one cell line out of four different patients will be described in the following sections only.

## 3.2. Characterization of Sendai-reprogrammed iPSCs

### 3.2.1. Karyotyping Analysis

In order to ensure a pluripotent state for the iPSC cell lines generated, any genetic abnormalities had to be excluded. Thus, each iPSC line to be characterized was tested for genetic lesions to ensure a normal and healthy genetic composition by means of G-band karyotyping analysis (Figure 11).



**Fig. 11 G-band karyotyping analysis of ABP iPSC cell lines.**

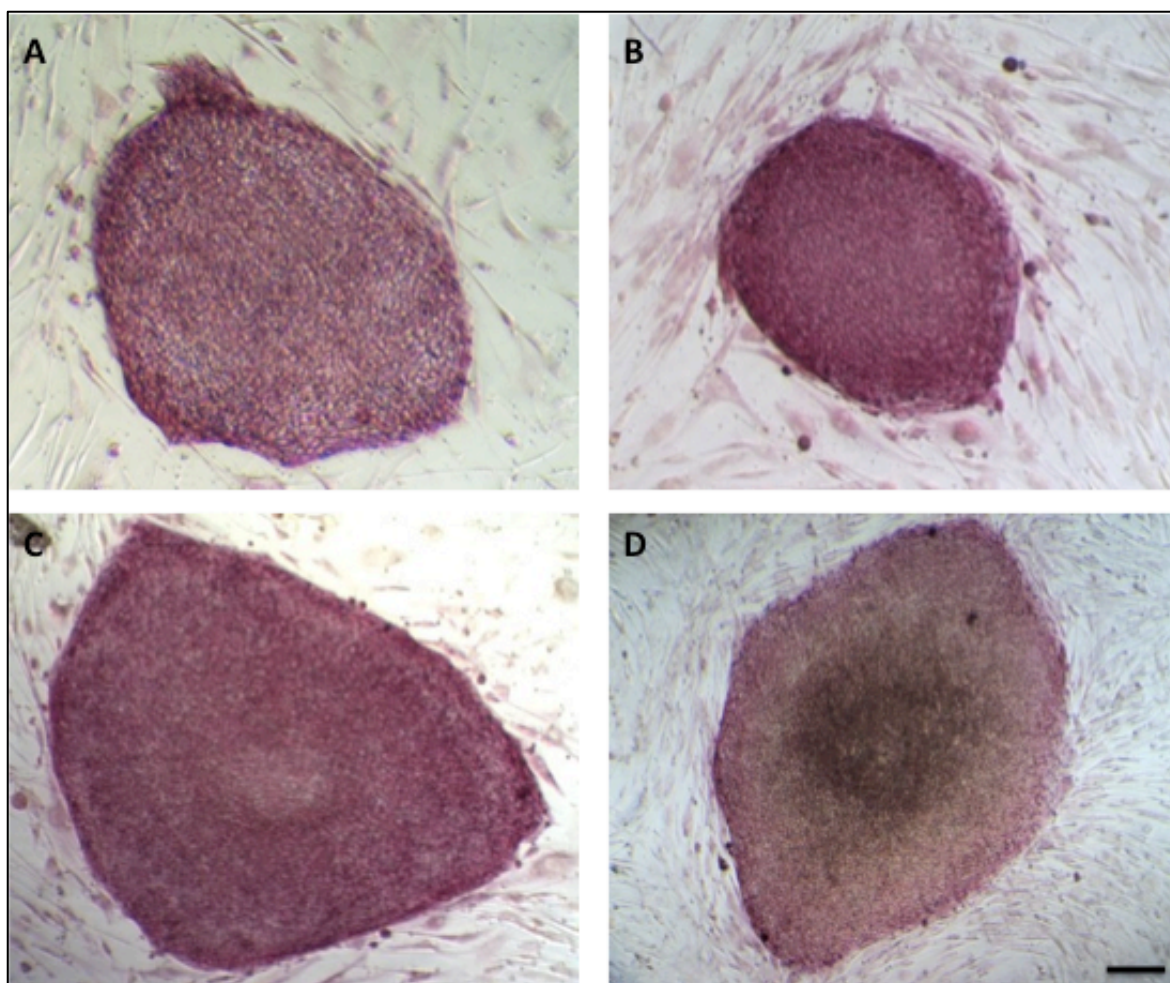
Karyotyping analysis for ABP02-7 (A), ABP04-2 (B), ABP06-4 (C), ABP08-1 (D). All karyotypes appear normal and represent a healthy genetic composition. A and B have a female, C and D a male chromosomal composition and karyotyping was performed at passage numbers 2 to 6. Karyotyping was performed by Cell Line Genetics, Madison WI, USA.

### 3.2.2. Alkaline Phosphatase Staining

As an initial, quick, as well as reliable assay for assessing the pluripotent state of the generated ABP iPSC cell lines, alkaline phosphatase staining was performed using the Alkaline Phosphatase Detection Kit (Millipore, Cat. No. SCR004) (Figure 12).

This assay provides a fast and reliable proof for pluripotentiality as fixed iPSC colonies appear in a purple to red tone while differentiated parts or whole differentiated colonies do not stain. AP Staining was performed



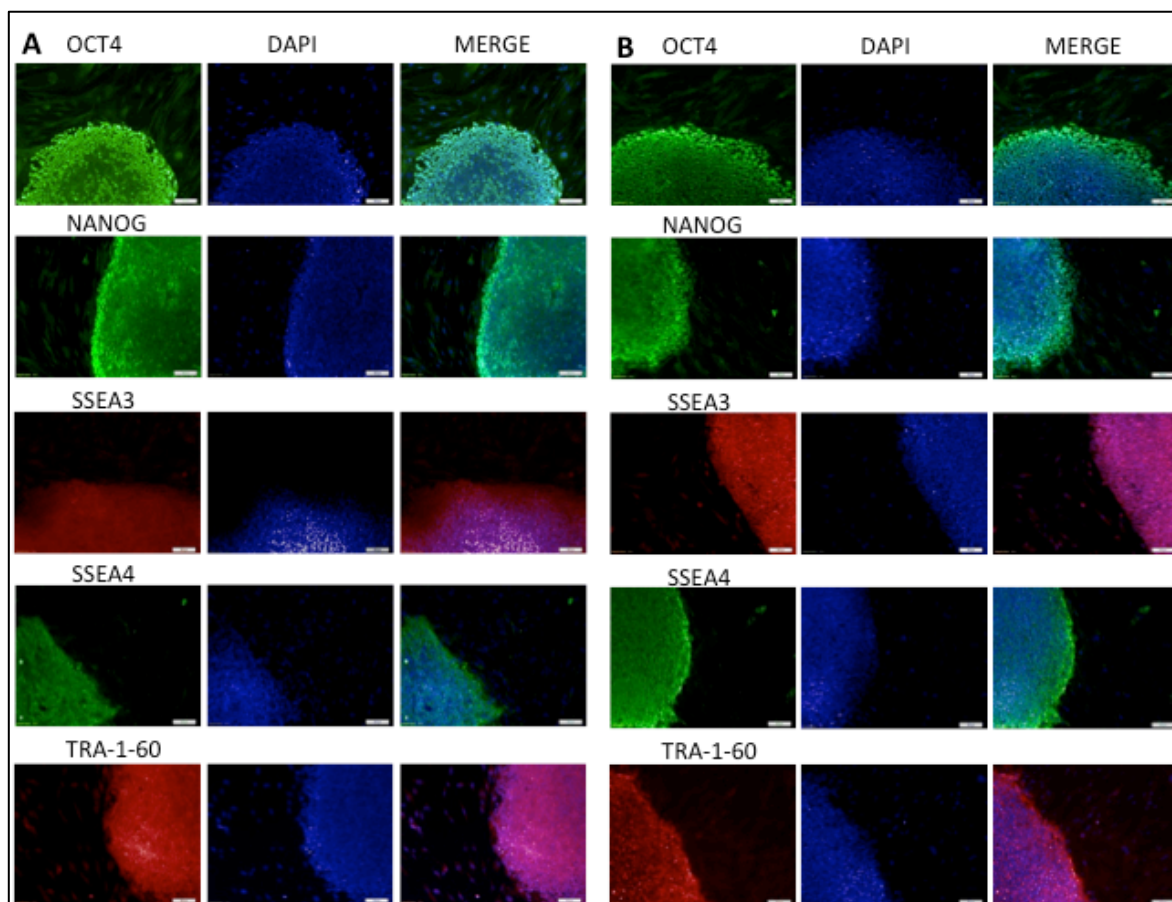


**Fig. 12 Alkaline Phosphatase staining of human iPS cell lines.**

AP staining of iPS cell lines was performed to assess their pluripotent character. Shown are ABP02-7 (A), ABP04-2 (B), ABP06-4 (C), ABP08-1 (D). Scale bar = 200 $\mu$ m.

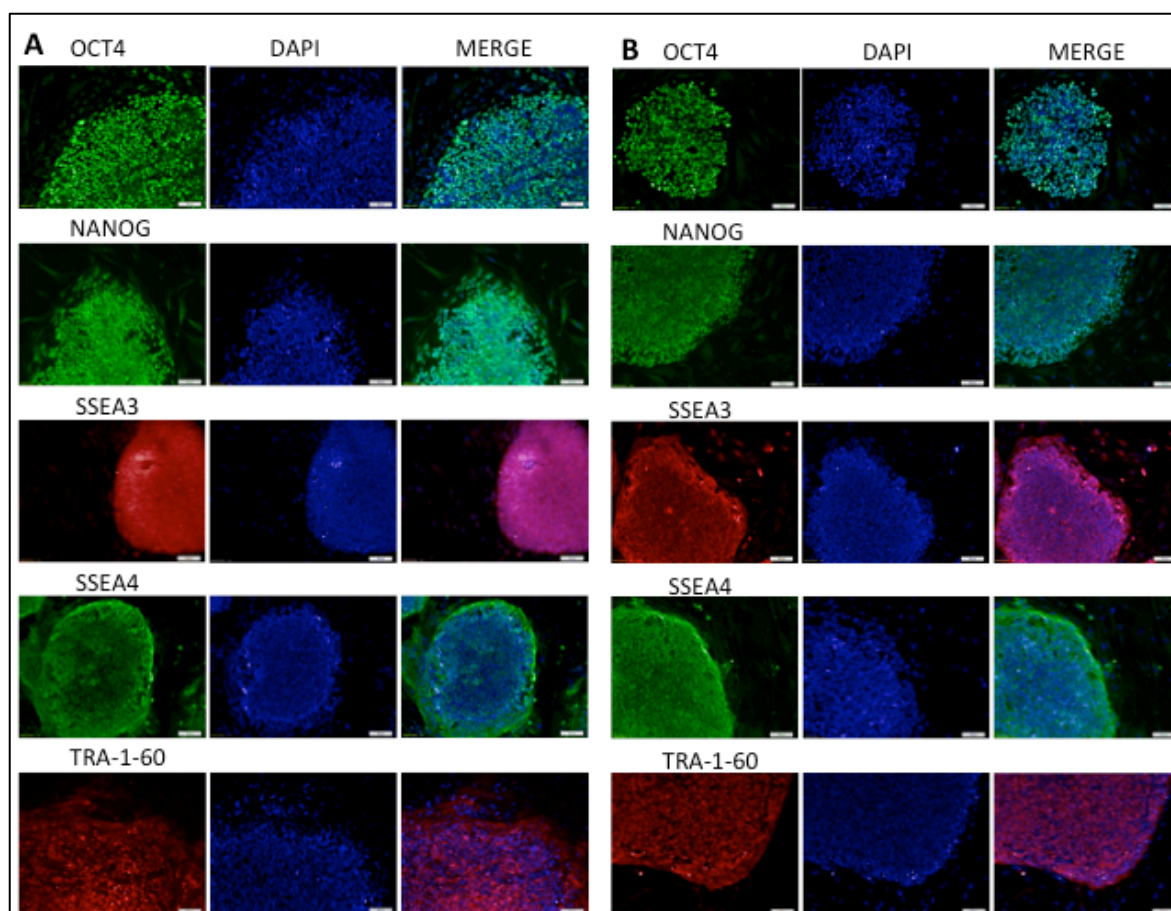
### 3.2.3. Immunocytochemistry for Pluripotent Markers

As the next assay, immunocytochemistry was performed in order to verify the expression of stem cell markers associated with pluripotency in the generated iPS cell lines. The primary antibodies chosen were Oct4 and Nanog for nuclear localization and SSEA-3, SSEA-4 and Tra-1-60 for cell surface localization. Marker expression was detected by means of fluorescence microscopy (Figures 13-14).



**Fig. 13 Immunocytochemistry for pluripotent stem cell markers.**

Shown are the primary antibody, the DAPI nuclear counterstain and the merged image with DAPI for the iPS lines *ABP02-7* (A), *ABP04-2* (B). The following markers were tested: Oct4 and Nanog (nuclear) as well as SSEA-3, SSEA-4 and Tra-1-60 (cell surface). Scale bar = 100 $\mu$ m.



**Fig. 14 Immunocytochemistry for pluripotent stem cell markers.**

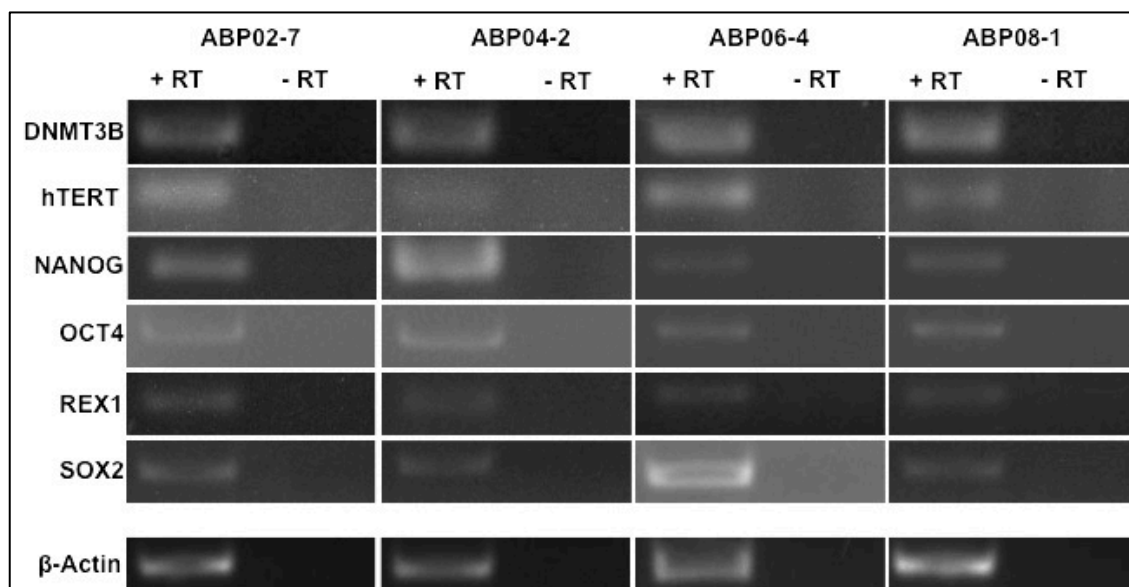
Shown are the primary antibody, the DAPI nuclear counterstain and the merged image with DAPI for the iPS lines *ABP06-4* (A), *ABP08-1* (B). The following markers were tested: Oct4 and Nanog (nuclear) as well as SSEA-3, SSEA-4 and Tra-1-60 (cell surface). Scale bar = 100 $\mu$ m.

All iPS cell lines tested indicate a clear pluripotent character based on the positive expression of pluripotent stem cell markers in both, the nucleus and the cell surface. Therefore, these results are in conformity with the previous AP staining results and thus with the assumption that the cell lines generated are pluripotent.

### 3.2.4. Qualitative Analysis for the Expression of Pluripotency Genes

The next step to assess the molecular criteria for pluripotency was taken by characterizing the gene expression profiles of the iPS cells. This was achieved by determining the gene expression of key pluripotency genes such as Nanog, Oct4 (endogenous), Dnmt3b, hTERT, Rex1, and Sox2 (endogenous), and  $\beta$ -Actin as a housekeeping gene (Figure 15).

Gene expression was determined by extracting RNA from iPS cells on feeder layers from passage numbers four to ten, subsequent conversion to cDNA, and PCR assays for the specific pluripotent primers.



**Fig 15. Pluripotent gene expression profiles of iPS cell lines.**

Analysis of the gene expression profiles for key pluripotent genes for the iPS cell lines ABP02-7, ABP04-2, ABP06-4, ABP08-1. CDNA was analyzed for key pluripotency genes such as Nanog, Oct4, Dnmt3b, hTERT, Rex1, and Sox2. As a housekeeping gene  $\beta$ -Actin was used.

All iPS cell lines analyzed exhibit a distinct pluripotent character based on the expression of key pluripotent genes (Figure 15). These results confirm the assumption based on previous experiments that the generated cell lines exhibit a pluripotent character on a molecular level.

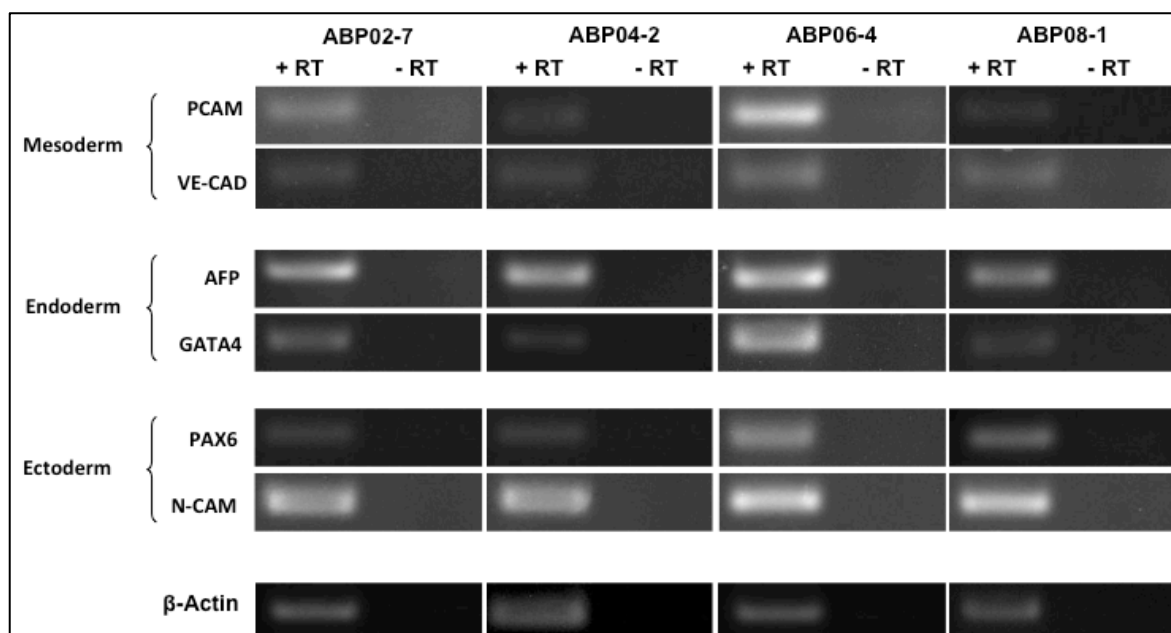
### 3.2.5. Qualitative Analysis for Differentiation-associated Genes

To assess the pluripotent state of iPSCs on a basic functional level, in-vitro differentiation of embryoid bodies and subsequent analysis for differentiation markers of all three embryonic germ layers was performed. This represents the second most stringent assay for determining pluripotency in human iPS cells besides testing for teratoma formation in immunodeficient mice.

For this purpose, iPS cells were plated onto dedicated tissue culture plates to initiate EB formation and EBs were then further cultured for two weeks to promote differentiation into cells of all three embryonic germ layers. Finally, RNA was extracted, converted to cDNA and PCR assays for the individual differentiation primers were performed (Figure 16).



Differentiation capability was determined by analysis of differentiation gene expression such as Flk1 or Pcam and GATA2 or Ve-cad for mesoderm origin, AFP and GATA4 for endoderm origin, and NCAM and Pax6 for ectoderm origin (Figure 16).



**Fig 16. Differentiation-associated gene expression of iPS cell lines.**

Analysis of the gene expression profiles for key differentiation-associated genes for the iPS cell lines ABP02-7, ABP04-2, ABP06-4, ABP08-1. CDNA was analyzed for key differentiation genes such as Flk1/Pcam, GATA2/Ve-cad, AFP, GATA4, NCAM, and Pax6. As a housekeeping gene  $\beta$ -Actin was used.

As with the pluripotent gene expression profile, all iPS cell lines analyzed exhibit a distinct gene expression of various germ layer-associated genes (Figure 16). These results manifest the assumption that all generated iPS cell lines exhibit a pluripotent character on a functional level.

## 4 DISCUSSION

Based on the original technique developed by Japanese scientist Shinya Yamanaka and colleagues in 2006 (Takahashi et al., 2006), we were able to reproduce the protocol to successfully generate disease-specific iPSC cell lines from human fibroblasts with the help of Sendai virus-mediated transduction. The Sendai viral vectors used were provided by a commercial kit (Invitrogen, Cat. No. A-1378002).

The transduction process and subsequent cultivation of generated iPSC lines was carried out as expected and for all five ABP patient samples (ABP02, ABP04, ABP06, ABP08, ABP10) seven to ten individual iPSC cell lines could be established. As requested by the external investigators, three cell lines per patient were selected to be characterized in detail (Table 6). For means of simplification and due to restricted outline, one iPSC cell lines out of four patients was described in the context of this paper: ABP02-7, ABP04-2, ABP06-4, ABP08-1.

Standard characterization of iPSC cell lines in the HSCI iPSC Core Facility includes karyotyping analysis, alkaline phosphatase staining, immunocytochemistry for pluripotent stem cell markers, qualitative analysis of pluripotency genes, as well as in-vitro differentiation and qualitative analysis of differentiation markers.

As reported by different sources, somatic cell reprogramming as well as prolonged culturing of cells can cause genetic abnormalities, such as point mutations or chromosomal translocations in the genome of resulting iPSC cells (Gore et al., 2011; Lister et al., 2011). These findings also correlate with previous reports about the tendency of embryonic stem cells to harvest genetic aberrations (Lerou et al., 2007). In order to assure that a normal, healthy karyotype was maintained during transducing the cells with viral vectors, G-band karyotyping analysis was performed. All iPSC lines tested show a normal, healthy karyotype result (Figure 11).

The first assay for determining whether transduction of the fibroblasts with Sendai viral vectors carrying the four transcription factors Oct4, Sox2, Klf4 and c-Myc was successful in establishing pluripotent stem cells, alkaline phosphatase staining was performed. Undifferentiated, pluripotent stem cells are known to have elevated AP levels (Palmqvist et al., 2005) thus this assay is used as a first

indication for pluripotentiality. The staining for the selected iPSC lines was positive, indicated by the appearance of red colonies (Figure 12).

The next assay performed was immunocytochemistry to assure that all iPS cell lines established expressed stem cell-related proteins on a molecular level, which are also expressed in their embryonic counterparts. For this purpose, Oct4 and Nanog, two transcription factors, were chosen as nuclear markers and SSEA-3, SSEA-4 and Tra-1-60 as cell surface antigens. In order to visualize marker expression on resulting iPS cells, immunocytochemistry on fixed cells combined with immunofluorescence was employed. All iPS cell lines tested showed a clear expression of the specified stem cell markers (Figure 13-14) and are therefore in accordance with the AP staining results, assuming that the cells are pluripotent. Slight variability within this technique as indicated by different expression intensities can occur due to fluctuating sensitivity and specificity of the diluted antibodies. Nevertheless, according to the obtained results, a pluripotent character of the stained iPSC lines can be presumed.

Another assay to confirm the pluripotent state on a molecular level was the determination of the gene expression profiles of the generated iPS cell lines for key pluripotency genes such as Nanog, Oct4, Dnmt3b, hTERT, Rex1, and Sox2. For this purpose, polymerase chain reactions (PCR) with specific primer pairs were set up in order to exponentially amplify specific genes. In a series of defined consecutive cycles of various temperatures, specific cDNA got amplified, which was then further qualitatively analyzed via agarose gel electrophoresis. As anticipated by the previous assays performed, expression of key pluripotency genes was observed (Figure 15) for all iPSC lines selected. Variation in intensity of the bands can be explained in various ways. One reason could be the variation in the gene expression itself, either due to actual low expression in the cells, which is why a gene profile for six different pluripotency genes is generated. Another reason could be manually introduced deviations during the setup of the PCR or in general variation during the PCR process or during gel electrophoresis.

As a last assay in-vitro differentiation of embryoid bodies and subsequent analysis for differentiation markers of all three embryonic germ layers was performed, assessing the pluripotentiality on a functional level. This assay constitutes the second most stringent assay for determining pluripotency in human iPS cells besides testing for teratoma formation in immunodeficient mice.

For this mean, IPS cells were plated onto non-attaching tissue culture plates to initiate embryoid body formation, which were then further cultured to promote the differentiation into cells of all three embryonic germ layers. Differentiation capability similar to ESCs was confirmed by subsequent analysis of differentiation-associated markers such as Flk1 or Pcam and GATA2 or Ve-cad for mesoderm origin, AFP and GATA4 for endoderm origin, and NCAM and Pax6 for ectoderm origin. As for analyzing pluripotency markers, PCRs with specific primer pairs were set up and qualitative gene expression was analyzed via agarose gel electrophoresis. As already anticipated by all previously performed assays, expression of differentiation-associated genes was observed for all iPSC lines selected (Figure 16). Again, variation in intensity of the bands could be due to low gene expression or operator handling, which is why at least two markers for each germ line were tested.

In addition to confirming a pluripotent state, the unlimited self-renewing capacity of the generated iPSC lines was assessed by culturing cells for at least 20 passages (not shown). No morphological changes were observed, indicating that these cells possess the self-renewing capacity seen in their embryonic counterparts, in ESCs (Thomson et al., 1998).

## 5 CONCLUSION

Looking at the presented data in this paper, the generation of disease-specific induced pluripotent stem cells from human fibroblasts was a success. The characterized iPSC lines show all signs of pluripotency assessed by standard tests set to determine pluripotency in stem cells by the HSCI iPS Core Facility.

All things considered, the advancements made in the past years in stem cell and regenerative biology hold many promises. Generating autologous, pluripotent cells from human cells in the course of several weeks to months opens new opportunities in term of cell therapy or organ repair.

Despite these promising hopes, obstacles such as viral transduction methods, low efficiencies and relatively expensive characterization protocols need to be modified or overcome before thinking about moving iPS technologies towards the clinic in a commercial manner.

First clinical trials using iPS cells have yet to be initiated. The Food and Drug Administration (FDA) has approved two studies so far for testing human embryonic stem cell-derived differentiated retinal pigment epithelial (RPE) cells in patients with Stargardt's macular dystrophy. First results from one trial published only recently raise the hopes for future approvals of iPS-derived cells for clinical trials. However, many more years will be needed to completely assess all the molecular and functional properties of iPS cells in order to aim at safely using them for human applications.

For now, the use of iPS cells may be restricted to develop functional disease models and provide useful and patient-specific platforms for drug screenings. Nevertheless, iPS cells hold great promises in replacing or at least becoming equivalent to ethically controversial human embryonic stem cells. Therefore, in order to achieve a progress in regenerative medicine, a more profound understanding of cellular reprogramming, the molecular basis of pluripotency as well as lineage-specific differentiation mechanisms of iPS cell lines is needed.

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