Induced Pluripotent Stem Cells

The Reprogramming of Skin Fibroblasts into iPS Cells by overexpression of the transcription factors Oct4, Sox2, Klf4 and c-Myc

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Theresa FÜGENSCHUH

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Internal Supervisor: Dr. Barbara Entler

External Supervisor: Laurence Daheron, PhD

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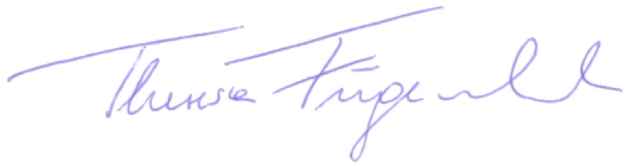
Statutory declaration

I declare in lieu of an oath that I have written this research paper 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 research paper has not been submitted elsewhere for examination purposes.

Klagenfurt, February 11, 2011



Theresa Fügenschuh, Marshall-Plan Stipendiatin

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2. List of Abbreviations

AD	Alzheimer's Disease
ADA-SCID	Adenosinedeaminase Severe Immunodeficiency
AFP	Alpha-fetoprotein
ALS	Amyotrophic Lateral Sclerosis
AP	Alkaline Phosphatase
BMD	Becker-Duchenne Muscular Dystrophy
BP	Bipolar Disorder
bp	Base pairs
cDNA	Complementary DNA
cm	Centimeter
c-Myc	Cellular Myc
CO_2	Carbon dioxide
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	Deoxyribonucleic acid
Dnmt3b	DNA (cytosine-5-)-methyltransferase 3 beta
dNTP	Deoxynucleotide Triphosphate
DPBS	Dulbecco's Phosphate-buffered saline
DS	Down Syndrome
DTT	Dithiothreitol
EB	Embryoid Body
E.Coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetic acid
ES	Embryonic Stem

ESC	Embryonic Stem Cell
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
Flk1	Fetal Liver Kinase 1
FRV	Fast Red Violet
GAG	Group-specific antigen
GFP	Green Fluorescence Protein
HD	Huntington Disease
HDAC	Histone Deacetylase
HEK	Human Embryonic Kidney
hESC	Human Embryonic Stem Cells
HSCI	Harvard Stem Cell Institute
hTERT	Human Telomerase Reverse Transcriptase
ICC	Immunocytochemistry
iPSC	Induced Pluripotent Stem Cells
IRES	Internal Ribosomal Entry Site
JDM	Juvenile Diabetes Mellitus
kbp	Kilo-base pair
Klf4	Krüppel-like factor 4
KOSR	Knockout™ Serum Replacement
LTR	Long Terminal Repeat
MD	Doctor of Medicine
MEF	Mouse Embryonic Fibroblast
MEM-NEAA	Modified Eagle Medium with Non-Essential Amino Acids
MIG	MSCV-IRES-GFP
mg	Milligram
mL	Milliliter
mm	Millimeter

mM	Millimolar
mRNA	Messenger RNA
MSCV	Murine Stem Cell Virus
µm	Micrometer
µL	Microliter
NCAM	Neural Cell Adhesion Molecule
NIH	National Institutes of Health
ng	Nanogram
nt-ESC	Nuclear Transfer Embryonic Stem Cell
Oct4	Octamer binding transcription factor 4
Pax6	Paired Box Gene 6
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PFA	Paraformaldehyde
P/S	Penicillin/Streptomycin
RiPSC	RNA induced Pluripotent Stem Cells
ROCK	Rho-associated Protein Kinase
rpm	Rotations per minute
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
Sox2	Sex determining region Y Box 2
SSEA-3	Stage-specific embryonic antigen 3
SSEA-4	Stage-specific embryonic antigen 4
TAE	Tris base, acetic acid, EDTA
US	United States
UV	Ultraviolet
VPA	Valproic Acid
VSV	Vesicular Stomatitis Virus

3. Introduction

In 1998, James A. Thomson (University of Wisconsin) demonstrated that human embryonic stem cells (hESC) could be derived from blastocysts and that they possessed the ability to differentiate into any cell type in the human body. He predicted that these cells could be used to recreate in-vitro versions of human tissues and be used in future applications, such as therapeutic purposes and drug discoveries [1]. Thomson's paper posed as a significant breakthrough in stem cell technology and opened the door for many other research areas.

However, in 2001, US President George W. Bush signed an executive order that restricted federal funding of stem cell research to hESC lines already in existence at this point in time, which slowed down research essentially. Additionally, the voices against the moral issues of using fertilized egg cells for research became louder and the ethic question was debated thoroughly by both political and religious leaders making embryonic stem cells a highly controversial subject.

In an effort to avoid moral debates, researchers have set their focus on generating pluripotent stem cells from sources other than embryos. A series of methods to reprogram somatic cells into pluripotent stem cells have been investigated. So far, three approaches to nuclear reprogramming have been determined: nuclear transfer, cell fusion and transcription factor transduction. During nuclear transfer, also known as cloning, a nucleus from a differentiated somatic cell is transplanted into an enucleated oocyte. Nuclear reprogramming is initiated and a genetic clone of the original somatic cell is developed. The animals, fully cloned from the one oocyte, however, show many abnormalities, such as impaired immune systems, obesity in adults, increased cancer susceptibility and premature death. Embryonic stem cells can be derived from these cloned blastocysts and the derived nt-ESCs are very similar to ESC. Although nt-ESCs have been generated from many species (mice, rat, cows, non-Human primate), so far this technique has not been successful for human cells. It is technically challenging and the

collection of a huge number of human oocytes would be necessary to overcome these challenges.[2]

When a somatic cell is fused to a pluripotent stem cell, the resulting cell is pluripotent. The fused cells have one of two fates: they either form hybrids or heterokaryons. While the hybrids proliferate and the nuclei of the cells fuse together, heterokaryons cannot divide and contain multiple nuclei. This technique is useful in the observation of the genome and the impact of one genome on another. The heterokaryons have been extremely useful in the outlining of the molecular mechanisms required to start reprogramming. However, these fused cells have limited applications. [2]

Transcription-factor transduction, the method first elucidated by the Japanese researchers Kazutoshi Takahashi and Shinya Yamanaka in 2006, involves the overexpression of the four transcription-factor-encoding genes Oct4, Klf4, Sox2 and c-Myc, causing the cells to reprogram into a pluripotent state. The factors, transduced via retroviral vectors, caused a reprogramming of adult mouse fibroblasts to a pluripotent state. The same technique was, two years later in 2008, implemented in the use in human adult fibroblasts to obtain human pluripotent stem cells by the same researchers and showed remarkable results. [2]

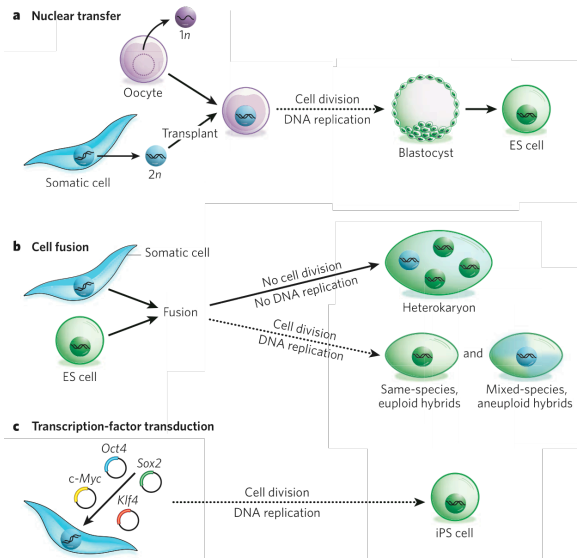

Fig. 1: Three approaches to nuclear reprogramming: a) nuclear transfer, involving the transplant of the genome of a somatic cell into an enucleated oocyte; b) cell fusion, the fusion of two cells of the same species creating a heterokaryon and a hybrid nucleus,

which has the ability to divide – the genome of both cells is integrated and expressed, c) transcription-factor transduction, which involves the viral integration of the growth factors Oct4, Klf4, Sox2 and c-Myc into a somatic cell, causing it to reprogram itself into a pluripotent state called iPS [2].

In 2009, US President Barack Obama reopened the debate on embryonic stem cells and lifted the ban established by George W. Bush. Federal funding could from then on be used for research on all hESC lines included in the NIH registry. The moral issue, however, remains, making the use of reprogrammed cells an ethical alternative to early embryos.

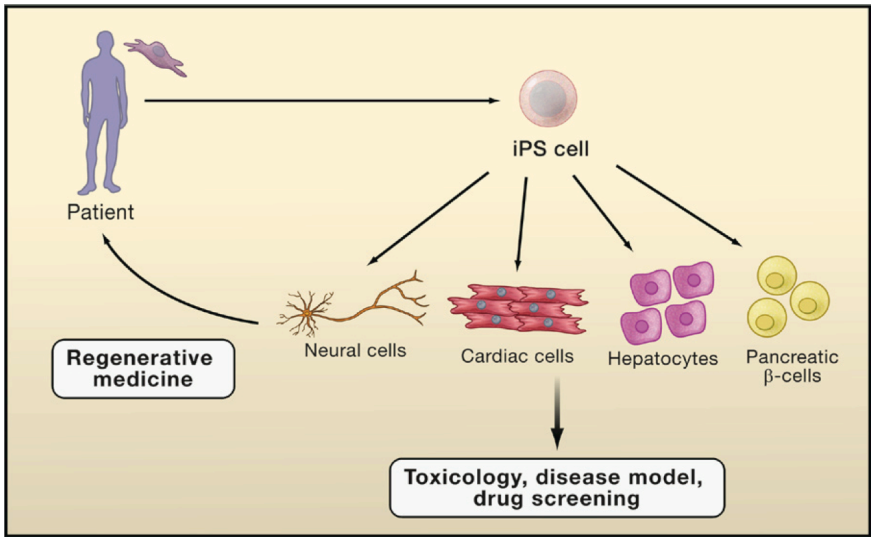

Fig. 2: The use of iPS cells is greatly established in the medical field. Patient-specific fibroblasts are reprogrammed into a pluripotent state and then re-differentiated into cell types of the ecto-, meso- and endodermal germ layers. These cells can then be used for toxicology tests, disease models, drug screenings, etc. So far, their use in regenerative medicine is not established, however, clinical trials have been launched. [3]

The iPS Core Facility of the Harvard Stem Cell Institute in Cambridge, Massachusetts has adopted the technique demonstrated by Takahashi and Yamanaka and has made it their prime target to derive induced pluripotent stem cells from isolated fibroblasts, obtained either in culture or through skin biopsies. The cell lines are, after their reprogramming into iPS cells, fully characterized in a series of experiments to prove their pluripotency, as well as their potential to derive into any of the three germ layers found in embryos. These experiments include the testing for the pluripotency markers Dnmt3, hTERT, Nanog, Oct4, Rex1 and Sox2 in undifferentiated iPS cells, as well as the testing for the differentiation markers AFP, GATA4, Flk1, GATA2, NCAM and Pax6, where each group of two represents a germ layer (endoderm,

mesoderm and ectoderm, respectively) in in-vitro differentiated cell clusters, known as embryoid bodies (EBs). Furthermore, the derived iPS cells are tested for the presence of Alkaline Phosphatase, an enzyme definitely, but not exclusively found in stem cells to prove their pluripotent state and colonies are picked for immunocytochemistry to show expression of the markers Oct4 and Nanog in the nucleus of the cells, as well as SSEA3, SSEA4 and Tra-1-60 on the cell surface. A karyotype analysis eliminates the use of potentially harmful cell lines due to chromosomal damage.

4. Steps to facilitate reprogramming into iPS

4.1 Isolation of fibroblasts from a skin biopsy

4.1.1 General information

The question on which cell type to use in reprogramming has been a topic of constant discussion since the cultivation of iPS cells became possible. For the first reprogramming attempts (both in mouse and human), fibroblasts were used, as their acquisition is fairly non-invasive, their derivation is technically simple [4] and their attributes, such as their compatibility with ESC culture conditions, make them an excellent starting cell population. Since the success of fibroblast transduction, a number of other cell types have been reprogrammed in mice, namely stomach and liver cells [5], pancreatic β cells [6], lymphocytes [7] and neural progenitor cells [8]. In humans, keratinocytes have been proven to be reprogrammable [9]. The experiments showed that there is a large difference between cell types in their reprogramming efficiency and in the effort needed for them to reprogram correctly. A number of factors influencing the optimal cell type for the application at hand have been elaborated: (1) the convenience of factor delivery, (2) the availability and ease of derivation of the cell type and (3) the age and source of the cells being used [10]. Fibroblasts, easily acquired through a skin biopsy, have proven to be reprogrammed most efficiently and are therefore used in the experiments at hand.

The skin biopsies, patient- and disease-specific, are acquired as punch biopsy with the help of a dermatologist and transported to the laboratory immediately

after their removal. The attained tissue is then prepared and cultured in the lab according to the undermentioned techniques.

The skin samples have, so far, been taken from several genetic disease groups, including Alzheimer's Disease (AD), Amyotrophic Lateral Sclerosis (ALS), Huntington Disease (HD), Bipolar Disease (BP), Autism, Down Syndrome (DS), Severe Combined Immunodeficiency (ADA-SCID), Becker-Duchenne Muscular Dystrophy (BMD), Duchenne Muscular Dystrophy (DMD), Juvenile Diabetes Mellitus (JDM) and Parkinson's Disease (PD)

4.1.2 Procedure

Prior to the acquisition of the skin biopsy, one 1.5 mL cryovial (VWR, Cat.-Nr. 82050-180) per skin biopsy is filled with 1 mL of DMEM 10% containing 450 mL DMEM (Invitrogen, Cat. Nr. 11995-073), 50 mL inactivated FBS (Invitrogen, Cat. Nr. 16000044), 5 mL P/S (Invitrogen, Cat. Nr. 15140-155) and 5 mL L-Glutamine (Invitrogen, Cat.-Nr. 25030-156) that was filtered through a 0.22 µm filter bottle (Fisher Scientific, Cat.-Nr. 09761102). The tube is placed in a 50 mL conical tube (VWR, Cat.-Nr. 21008-940) and sealed with Parafilm (1x125ft, VWR, Cat.-Nr. 52858-000) for the transport to and from the site of the operation. Should the transport of the samples from the site of skin biopsy removal to the lab take longer than 30 minutes, the sample needs to be kept on ice. Upon return to the lab, two 15 mL conical tubes (VWR, Cat.-Nr. 21008-936) are prepared, containing a DPBS (Invitrogen, Cat.-Nr. 14190-250)-P/S solution (the concentration of P/S in solution is 1:100) and labeled "Wash 1" and "Wash 2", respectively. Furthermore, a 6-cm dish (VWR, Cat.-Nr. 82050-546) for biopsy preparation, as well as a 6-cm dish for biopsy culturing are put out. All materials needed are transferred under a tissue culture hood for handling. Before handling the skin biopsy, a grid is cut into the dish intended for culturing using a sterile scalpel (Fisher Scientific, Cat.-Nr. NC9714653).

A 5-mL pipette (VWR, Cat.-Nr. 53300-421) is needed to transfer the skin biopsy from the cryovial with DMEM 10% into the tube labeled "Wash 1". The amount of medium carried over to the washing tube is to be kept at a minimum. The skin sample is washed briefly in the first tube and then

transferred to the tube labeled “Wash 2” with the help of another 5-mL pipette. After washing, the biopsy is transferred to the preparation dish and a drop of DMEM 10% is added to make handling of the biopsy easier.

The punched out skin sample is cut into small pieces with a diameter of <1mm using a fresh sterile scalpel. Depending on the size of the skin biopsy, a number of ten or more pieces of tissue should be reached. Using sterile forceps, the tissue shreds are transferred to the dry culturing dish with the previously cut grid with each piece lying in the cross of two lines. Once all pieces are set in place, excess medium is aspirated using a pipette tip. One mL of DMEM 10% is added around the edge of the dish, deliberately not touching the skin pieces in the grid. The culturing dish is placed in the incubator at 37°C, 5% CO_2 for 20 minutes to let the pieces firmly attach to the plate. After 20 minutes, 4 mL of DMEM 10% are slowly added to the dish to cover all skin pieces making sure to keep them from detaching. The cells are from then on fed once a week until fibroblast growth becomes visible. After that, the cells are fed with pre-warmed DMEM 10% every three days.

Once fibroblasts have reached a confluence of 80%, the dish is washed with 2 mL of DPBS, ensuring that all traces of DMEM 10% are removed. After aspiration of the DPBS, 2 mL of Trypsin + EDTA (Invitrogen, Cat.-Nr. 25300-062) are added and the covered dish is incubated for 4 minutes at 37°C. After the incubation period, the Trypsin is neutralized with 2 mL of DMEM 10%. The dish is lightly tapped against a hard surface to release the cells into suspension. The cells are sucked up with the medium and transferred to a 15 mL conical tube. To remove all fibroblasts from the culturing dish, the plate is washed with another 2 mL of DMEM 10%, which are sucked up and added to the 4 mL already collected. The collected cells are spun down for 4 minutes at 1200 rpm (200g) and the supernatant is aspirated. The collected cells are resuspended in 2 mL of DMEM 10% and plated in one well of a 6-well plate (VWR, Cat.-Nr. 82050-842). The day after the cells were passaged, feeding is skipped to allow the cells to firmly attach to the plate. From then on, the fibroblasts are fed with DMEM 10% every three days until they have become 80% confluent again. The cells are then trypsinized for four minutes, neutralized with DMEM 10%, spun down at 1200 rpm and resuspended in 3

mL of fresh DMEM 10%. The cells are transferred to a T25 (VWR, Cat.-Nr. 82051-074) already containing 4 mL of fresh DMEM 10%. The T25 is then placed back in the incubator and cultured until a further passaging is necessary. All passaging steps from this moment on are carried out in a ratio of 1:3 (one T25 is split into three T25 flasks or one T75).

The tissue pieces, still firmly attached to the culturing plate, are fed with fresh DMEM 10% and cultured for another round of fibroblast growth. After confluence of 80% has been reached again, the cells are trypsinized as above. The trypsin is neutralized with DMEM 10% and the cells, including the skin biopsy pieces, are scraped off the plate with a cell lifter (VWR, Cat.-Nr. 29442-200). To remove the skin pieces, a cell strainer is used (Fisher Scientific, Cat.-Nr. 08-771-2). The cells are spun down and then plated in a ratio of 1:3 on three wells of a 6-well plate with 2 mL of DMEM 10% per well. Daily feeding is resumed two days after passaging. Further passaging steps occur in T25 flasks and T75 flasks (VWR, Cat.-Nr. 82050-856) respectively.

4.1.3 Critical Steps

In this procedure, it is vital to cut the skin biopsy into as many small pieces as possible. Their size should be kept at roughly one mm or less to ensure their proper sticking to the bottom of the culturing dish, as well as their ability to grow new pericytes and keratinocytes, which, in turn, lead to the growth of fibroblasts. The skin biopsy is to be cut into pieces with single, determined strokes and “sawing” the pieces apart with forward and backward motions is to be avoided at all costs.

The medium should be reliably changed every seven days until fibroblasts start appearing and every three days after that to ascertain the steady growth of cells.

Furthermore, the use of trypsin should be watched closely. To facilitate the use of trypsin, all medium has to be washed off the cells, as the serum, as well as the Calcium and Magnesium within inhibit trypsinization. After the trypsin has been added to the cells, a period of no longer than *5 minutes* is to be allowed to pass before neutralization of the trypsin with medium takes place, as a further trypsinization is likely to cause large amounts of cell death.

4.1.4 Fresh skin biopsy vs. already cultivated fibroblasts

As mentioned in the protocol by Nimet Maherali et al. [10], the age of the sample at hand plays an important role in the efficiency of reprogramming. Though not scientifically proven, the iPS Core has found that fresh skin biopsies have a better chance of successful reprogramming. These cultures show earlier changes after reprogramming and the first iPS colonies appear faster than in later passages. Additionally, the amount of colonies counted has a tendency of being much higher compared to cultured fibroblasts.
From experience, it can be said that the older the fibroblast sample and the later the passage of the line, the more difficult and less efficient transduction will be.

4.2 Production of non-replicating retrovirus in HEK293 host cells

4.2.1 General principle

The choice of the right expression vector is one of great importance in the reprogramming of fibroblasts into iPS cells. A whole range of methods has been discovered and the selection of the right method should be carefully considered. The first choice to be made is the one between transient and stable expression. In transient expression, the plasmid is not integrated into the genome and expression of introduced factors only takes place for three to five days. In stable expression, the plasmid is integrated into the genome permanently, which establishes clones that express the protein of interest at a high level.

During transient transfection, the permeability of the cells is increased either through a specific treatment, such as Calcium Phosphate or commercially available cationic lipid transfection reagents, e.g. Lipofectamine™ 2000 (Invitrogen), FuGENE® (Roche) and ExGen 500 (Fermentas), or electric pulses (Electroporation or Nucleofection).

In gene transduction, a method used for stable expression, the plasmid is integrated into the genome using a retro-, or lentivirus. The advantage of this method is its highest efficiency compared to other techniques. However, a series of limitations, i.e. the size limit for the transgene and the random integration into the genome, which can cause serious mutations if an essential

gene is interrupted, needs to be taken into consideration when choosing this method.

Since the induction of pluripotency in human fibroblasts is shown to work very well with the retroviral method discovered by Takahashi and Yamanaka [11], it is still widely used as the main method for reprogramming.

Retroviruses, single-stranded RNA viruses, contain long terminal repeats (LTRs) at both ends of the retroviral genome, as well as gag, pol and env sites. The LTRs comprise the viral promoter and enhancers and are required in Cis for the viral integration into the host genome. The gag region, located at the 5' end of the genome, encodes for the major structural proteins of the viral capsid. The pol region, which can only be found in fusion with a gag site due to a -1 ribosomal frame shift that occurs in the region where gag and pol overlap, is responsible for the encoding of the reverse transcriptase, the protease and the integrase. Gag binds to the 5' cap structures of cellular mRNAs, a process necessary for the expression of viral information, while pol has an N-terminal necessary for the packaging of viral positive strands [12]. The env encodes for the envelope of the virus, which determines its host range.

Fig. 3: Schematic drawing of a retroviral vector with a long terminal repeat (LTR) region containing U3, R and U5 on the non-coding 5' end, the gag, pol and env region and an LTR at the 3' end. The white spaces between pol and env, as well as between env and the 3' LTR are areas where other proteins, specific to the retrovirus, are encoded. [13]

The retroviral vectors used for reprogramming at the HSCI iPS core facility are MIG-Oct4-IRES-GFP, MIG-Sox2-IRES-GFP, MIG-Klf4-IRES-GFP and MIG-Myc-IRES-GFP, where the gag-, pol- and env region of the retroviral vector were replaced by the genes of interest.

The MIG (MSCV-IRES-GFP) vector, sequenced by AddGene, has a total length of 6573 bp, with the MSCV backbone taking up a length of 5900 bp. This backbone is previously assembled and includes the IRES site. The addition of an IRES site, a translation stimulator that allows the translation of mRNA regions that would normally be deemed as untranslated by overriding

the stop-codon of the mRNA sequence, enables the addition of genes to the translation regions [14]. A gene encoding for a green fluorescent protein (GFP) to facilitate the monitoring of the transduction is added to the translated region of the vector with the help of such an IRES site. The GFP insert has a length of 700 bp.

The plasmid is assembled and grown in the Stbl3™ E.Coli strain, a trademark strain by Invitrogen (Cat.-Nr. C737303), which is used for the cloning of unstable inserts, such as lentiviral DNA containing direct repeats.

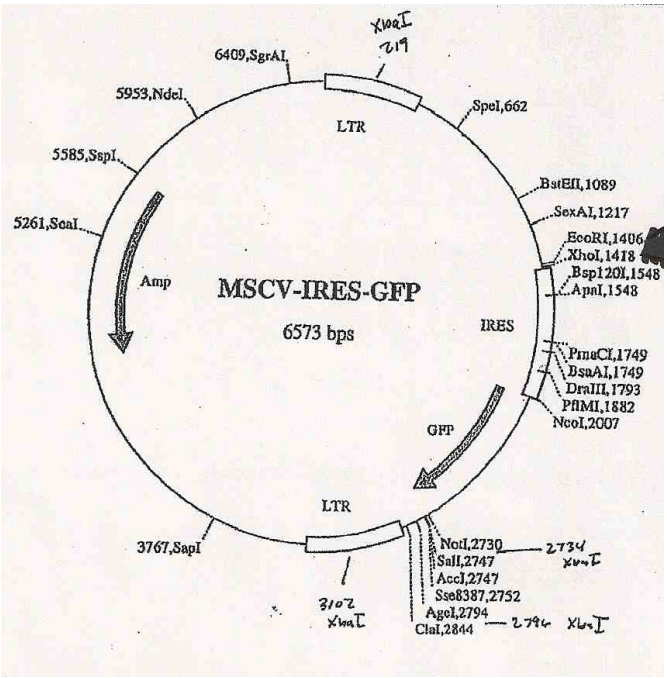

Fig. 4: Addgene (http://www.addgene.org), plasmid 20672 (MSCV-IRES-GFP) vector containing an IRES site between the two LTR sites. The IRES site allows the insertion of the GFP encoding gene.

In order to produce a fully functioning, yet non-replicating virus, the retroviral vector is co-transfected with the “packaging” plasmids (GAG-POL and ENV) in HEK293T host cells. The viruses, transduced into the cells with the help of reagents known to promote infection, such as hexadimethrine bromide (Polybrene) [15], reverse transcribe into DNA, which integrate into the host cell’s genome to form the proviruses. The proteins, produced through transcription of the proviral sequences, then accumulate in the host cell.

The packaging signal Ψ however, which would normally be located between the U3-r and U5 LTR of the retroviral vector and promote the budding of the virus and thereby its further spreading [16] is not added to the mixture. The virus is therefore fully able to produce proteins, however, it does not have the ability to infect other non-replicating cells.

The produced viral proteins are in a later step used to transduce the targeted fibroblasts in order to facilitate their reprogramming into iPS cells.

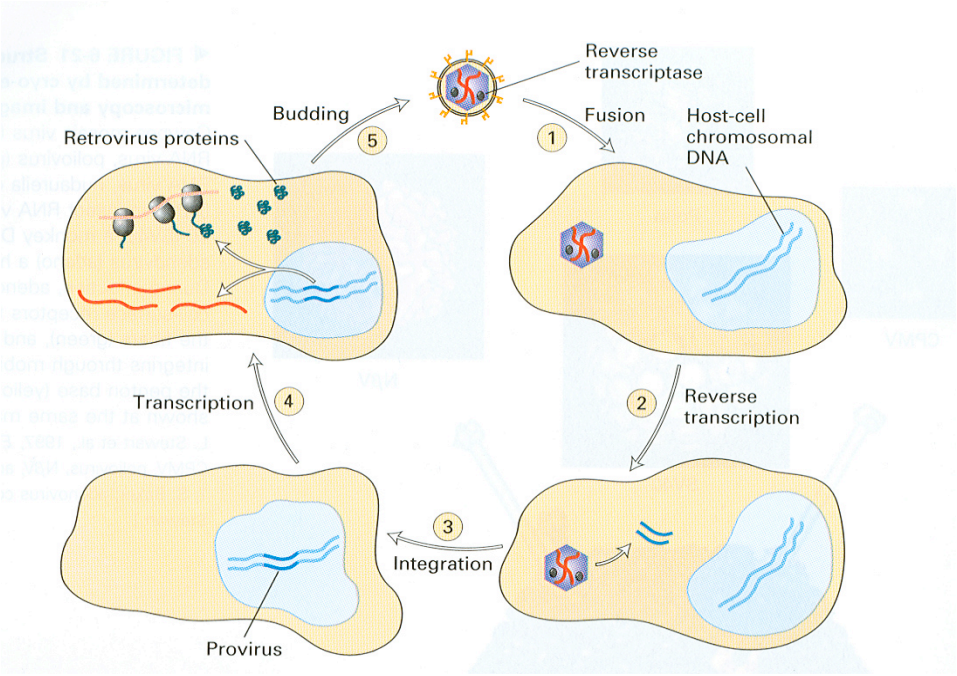

Fig. 5: Retroviral life cycle: The virus molecules enter the cell and reverse transcriptase is released to reverse transcribe the RNA contained in the nucleus of the virus into the DNA that then integrates into the genome as provirus. After integration, the viral genome gets transcribed into RNA again, which, with the retroviral proteins forms a new virus particle. In the regular retroviral cycle, the particle would then separate itself from the cell and bud to form a complete particle, ready to infect a new cell and reproduce. [17]

4.2.2 Procedure

Three days before transfection is to take place, one cryovial of previously frozen HEK293T cells is thawed by swirling the vial in a 37°C waterbath until the contents are partially unfrozen (a small core of ice should remain). The vial is sprayed with ethanol and placed in a tissue culture hood where all further steps take place. One mL of pre-warmed DMEM 10% is gently added to the vial of HEK293T cells to completely thaw it and the solution is

transferred to a 15 mL conical tube containing 9 mL of DMEM 10%. The tube is centrifuged at 1200 rpm for four minutes and the supernatant is aspirated. The cell pellet is resuspended in 5 mL of DMEM 10%. The cells are then plated in a T75 flask containing 10 mL of medium, bringing the final volume in the T75 to 15 mL. The T75 is incubated at 37°C, 5% CO_2.

The day before transfection, the T75 containing the HEK293T cells is washed with DPBS once and trypsinized for 4 minutes at 37°C. The Trypsin is neutralized with an equal amount of DMEM 10% and the cells are collected and spun down for 4 minutes at 1200 rpm. The supernatant is aspirated and the cells are resuspended and plated onto six 10-cm plates (VWR, Cat.-Nr. 82050-916) in DMEM 10% *without* antibiotics. Only four of the plates are used for transfection (depending on the amount of samples to be transduced, four plates are the required minimum. If more than four samples and the control plate are to be transduced, the amount of HEK293T cells used has to be adjusted). The other two plates may either be cultivated to the amount of a T75 and frozen again or, if they are not needed, discarded.

The following day, the mixture for transfection is prepared. For transfection of one 10-cm plate, 12 µg MIG, 10.5 µg GAG-POL and 1.5 µg VSV (encodes for ENV) are mixed in a 15 mL conical tube. Depending on the concentrations of the components in solution, the rest up to 1000 µL is filled up with opti-MEM Reduced Serum Medium (Invitrogen, Cat.-Nr. 11058-021). Four mixtures, each one containing a different virus (Oct4, Sox2, Klf4 and c-Myc), are prepared.

An additional mixture containing 75 µL of Lipofectamine® (Invitrogen, Cat.-Nr. 11668019) and 925 µL of opti-MEM is prepared for each of the four plates.

After mixing the virus components with the opti-MEM and a waiting period of 5 minutes at room temperature, 1 mL of the Lipofectamine-opti-MEM mixture is added to each tube containing the virus-mix. After another waiting period of 20 minutes at room temperature, the 2 mL of solution are added dropwise to one plate of HEK293T cells and the plate is labeled with the according virus.

In the early morning of the day after transfection, the medium in the 10-cm plates is aspirated manually with a 10-mL pipette (VWR, Cat.-Nr. 53300-523) and transferred to a special virus waste container. In order to eliminate all

traces of virus, Vesphene IIse Non-sterile Disinfectant Cleaner (Steris, Cat.-Nr. 6461), previously poured into a 50 mL conical tube and placed in the tissue culture hood, is then pipetted up and down and the pipette is placed in the biowaste. The plates are then fed with 10 mL of DMEM 10% *with* antibiotics and incubated overnight at 37°C, 5% CO_2. The pipette used to add the medium is treated with Vesphene.

The following morning, the medium of the plates containing the virus is collected in a 50 mL conical tube for each virus and labeled accordingly. The tube is screwed on top of a Steriflip 0.45 µm filter (Fisher Scientific, Cat.-Nr. SE1M003M00) and the medium is vacuum-filtered to remove all dead or detached cells. After all medium has passed the filter, the tube containing the virus medium is unscrewed from the filter and closed tightly and sealed with Parafilm. About 2 mL of Vesphene are added to the top of the filter, which is left to sit at room temperature. After a waiting period of about 10 to 20 minutes, the filters can be discarded in the regular biowaste.

The HEK293T plates are carefully fed with fresh DMEM 10% and the pipette used to feed the plates is treated with Vesphene before disposal. The plates are placed back in the incubator at 37°C, 5% CO_2.

A second round of virus harvesting can be done the next morning if the cells look healthy (no excessive peeling of the cells from the plate or large amounts of cell death).

After the second virus harvest, the HEK293T plates are discarded by pouring about 10 mL of Vesphene into each plate and letting them sit for 10 to 20 minutes at room temperature before their regular disposal in the biowaste. Afterwards, the Vesphene is collected and transferred to the viral waste container. The plates can then be disposed of in the regular biowaste.

The collected virus from both the first and the second day of virus harvest is used immediately to transduce the fibroblasts or alternatively can be stored for few days at 4°C. The top of the 50 mL conical tube used after filtration should be sealed with Parafilm to avoid contamination of other substances stored in the fridge.

4.2.3 Critical Steps

In order to allow maximum transfection efficiency, the waiting periods described in the protocol need to be complied with as stated. This assures the correct assembly of the virus before it is added to the cells.

During this procedure, it is important that precautions are taken to ensure safety of the user. Therefore, the use of laboratory sleeves (VWR, Cat.-Nr. 94001-106) pulled over the regular lab coat is necessary, as well as the use of two pairs of gloves. After every contact with the infected plates or the liquid used to feed or wash them, the used pipettes have to be cleaned with Vesphene to inactivate the virus in them.

After all virus-related steps have been concluded, the surface of the used tissue culture hood has to be thoroughly cleaned with a Vesphene-water mixture (5 mL Vesphene + 45 mL water) and sprayed with 70% Ethanol. The hood has to be closed and the UV light has to be turned on for *at least* 20 minutes before regular cell culture work can be continued.

4.3 Transduction of isolated fibroblasts with retrovirus

4.3.1 General principle

After the merging of the retrovirus parts in HEK293T host cells, the assembled, non-replicating virus is added to the fibroblasts intended for reprogramming. Each growth factor to be expressed is added with a separate virus, meaning that four viruses for Oct4, Klf4, Sox2 and c-Myc have to be added to start proper four-factor-reprogramming. Each virus expresses GFP.

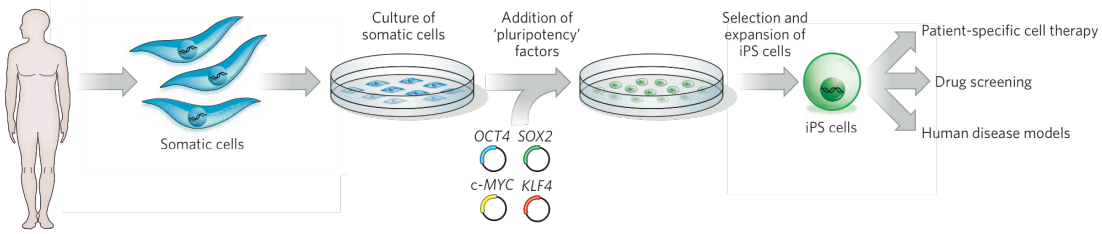

Fig. 6: General depiction of the transduction of somatic cells with the retroviral vectors containing Oct4, Sox2, Klf4 and c-Myc. After transduction, the cells are left to differentiate into iPS colonies before they are further processed through picking, expanding and characterization. [2]

Although the exact events happening in the cell during reprogramming are not fully searched out, it is known that once cells have reached a state of pluripotency, the expression of the integrated viruses is inactivated, which can be checked under UV light, as also the GFP will be inactivated in iPS cells. It is speculated that the inactivation of the retroviral promoter may be due to epigenetic modifications, e.g. histone methylation. [18].

In a quest to enhance the efficiency of reprogramming, a series of small molecules has been tested, such as DNA methyltransferase inhibitors and histone deacetylase inhibitors. VPA, an HDAC, is the most popular chemical molecule added to the cells. It is known to enhance the reprogramming efficiency of four-factor-reprogramming and restores the efficiency in the case of three-factor-reprogramming (without c-Myc) in mouse fibroblasts [19].

Tbl. 1: Small molecules identified to be helpful to the induction of human adult fibroblasts into pluripotent stem cells and their role in reprogramming. [10]

Molecule/Factor	Target/Mode of Action	Role in Reprogramming	References
Valproic Acid	histone deacetylase inhibitor	enhances reprogramming efficiency with four factors (O/S/M/K) in mouse fibroblasts	Huangfu et al., 2008a, 2008b
		restores reprogramming efficiency in mouse fibroblasts without c-Myc (O/S/K only)	
		permits reprogramming of human fibroblasts treated with OCT4 and SOX2, though at extremely low efficiency	
5-azacytidine; shRNA against Dnmt1	DNA demethylating agent	~4-fold enhancement of reprogramming efficiency with four factors (O/S/M/K) in mouse fibroblasts	Mikkelsen et al., 2008
		no effect if applied too early; toxic to differentiated cells	
BIX01294	histone methyltransferase inhibitor	restores reprogramming efficiency in mouse neural progenitor cells with Oct4/Klf4 to four-factor level (O/S/M/K)	Shi et al., 2008b
		permits reprogramming of mouse neural progenitor cells in the absence of Oct4, though at extremely low efficiency and requires the presence of the other three factors (S/M/K)	
BayK8644	L-type calcium channel agonist	cooperates with BIX01294 to enable reprogramming of mouse embryonic fibroblasts with Oct4/Klf4	Shi et al., 2008a
Wnt3a	cell signaling molecule; transcriptional activation of multiple downstream targets, including c-Myc	1.2-fold enhancement of reprogramming efficiency with four factors (O/S/M/K) in mouse fibroblasts	Marson et al., 2008
		~20-fold enhancement with three factors (O/S/K); partially restores efficiency in the absence of c-Myc	
siRNA against p53 and Utf1 cDNA	tumor suppressor; ESC-specific cofactor, respectively	modest increase in four-factor (O/S/M/K) reprogramming efficiency of human fibroblasts with individual factors (4 + p53siRNA or 4 + UTF1)	Zhao et al., 2008
		~100-fold enhancement with the combined factors (O/S/M/K + p53siRNA + UTF1); further slight enhancement by the exclusion of c-MYC (O/S/K + p53siRNA/UTF1)	

O, Oct4; S, Sox2; M, c-Myc; K, Klf4; Dnmt1, DNA methyltransferase 1.

4.3.2 Procedure

The day before transduction, the previously isolated and in DMEM 10% expanded fibroblasts are trypsinized for 4 minutes. After neutralizing the Trypsin, the cells are spun down for 4 minutes at 1200 rpm and then resuspended in 1 mL of medium. An aliquot of 10 µL is transferred to a 1.5 mL microcentrifuge tube and 10 µL of Trypan Blue (Fisher Scientific, Cat.-Nr. MT-25-900-CI) are added. After thorough mixing, a 10-µL aliquot is transferred to a hemocytometer and the cells are counted. The cell count is adjusted to 50,000 cells/mL and 100,000 cells are plated in one well of a six-well plate bringing the total volume in the well to 2 mL. Two wells of each cell line have to be plated for transduction. If more than one sample is supposed to be transduced, this procedure has to be repeated for each sample. In addition to the samples to be transduced, a control plate containing four wells of 100,000 fibroblasts/well, usually taken from the fibroblast line that shows the best growth, has to be prepared. The cells are incubated at 37°C, 5% CO_2 overnight.

The following morning, the virus harvested after the first day of transfection is taken out of the 4°C fridge and the Parafilm seal is removed. The fibroblasts for transduction and the control plate are placed in the tissue culture hood and the DMEM 10% is aspirated from the cells. For the fibroblasts to be transduced, 1 mL of fresh DMEM 10% is added to the wells. To start transduction, 1 mL of each of the media containing the assembled virus for Oct 4, Sox2 and Klf4 and 200 µL of the medium containing the virus for c-Myc are *dropwise* added to each well, bringing the final volume per well to 4.2 mL. To increase the transduction efficiency, 1 µL of Polybrene (Millipore, Cat.-Nr. TR-1003-G) is added to each well.

For the control plate, the DMEM 10% is removed from the wells and 1 mL of fresh DMEM 10% is added. In contrast to the plates of fibroblasts used for transduction, 1 mL of only one virus per well is added. In the end, the plate should have one well for each Oct4, Sox2, Klf4 and c-Myc. Because the volume in the wells only consists of 2 mL, only 0.5 µL of Polybrene are added for increased transduction efficiency.

As with all viral activities, the materials used to transfer the virus to the well have to be cleaned with Vesphene. The plates are incubated for 6 to 8 hours at 37°C, 5% CO_2 in an isolated incubator only used for transduction.

After 6 to 8 hours, the viral medium is removed from the wells with a pipette. The medium is disposed in a special virus waste and the pipette is treated with Vesphene. The wells are washed with DPBS once, repeating the procedure of manually pipetting the solution out of the wells, disposing of it in the virus waste and treating the pipette itself with Vesphene afterwards. Subsequently, 2 mL of fresh DMEM 10% are added to the wells and the fibroblasts are left to rest in the incubator at 37°C, 5% CO_2 overnight.

The following morning, the DMEM 10% is removed from the cells and discarded in the special virus waste and the pipette is treated with Vesphene. The cells are transduced again using either the viral medium from the first day or second day of virus harvest. After 6 to 8 hours of incubation at 37°C, 5% CO_2, the virus is removed and the cells are washed twice with DPBS, taking the same virus precautions into consideration as in the first transduction cycle. Two mL of fresh DMEM 10% are added to the wells and the cells are incubated at 37°C, 5% CO_2. The pipette is treated with Vesphene. The same procedure is carried out in both the actual transduced fibroblasts and the control plate.

Two days after the second transduction, the cells are checked under a microscope with UV light. If the cells of all the wells of the control plate show a GFP signal, it can be assumed that each virus is expressed in the cells and the transduction was successful.

For the wells showing GFP signal a layer of irradiated CF1 mouse embryonic fibroblasts (MEFs) is plated in 10-cm plates. The control plate is not taken into consideration and is treated with Vesphene for 10-20 minutes and disposed of in regular biowaste.

The plates are prepared for the layer by adding 0.1% gelatin in sterile water (Millipore, Cat. Nr. ES-006-B) and letting it sit for 20-30 minutes at room temperature under a tissue culture hood. During this waiting period, 15 mL of

DMEM 10% are poured into a 50 mL conical tube. The MEFs, available in 2 million cells/vial and 4.5 million cells/vial (GlobalStem, 2M – Cat. Nr. GSC-6201G, 4-5M – Cat. Nr. GSC-6001G), are retrieved from the liquid nitrogen tank and held into a 37°C waterbath until they are partially thawed. Depending on the amount of dishes necessary, it should be taken into account that for one 10-cm plate one million cells are necessary. The cryovial is sprayed with 70% ethanol and placed under the tissue culture hood. From the 15 mL of prepared DMEM 10%, 1 mL is taken up with a pipette and slowly added to the half-frozen mixture of MEFs in the cryovial, thereby completely thawing it. The mixture is removed from the cryovial and transferred to the 14 mL of prepared DMEM 10%. The tube is spun down at 1200 rpm for four minutes. During this time, the gelatin in the dishes is aspirated and 5 mL of DMEM 10% are added to the plates. The supernatant in the tube, after it has finished spinning down, is aspirated and 5 mL of fresh DMEM 10% are added for each dish. 5 mL of the cell mixture is added to each of the plates, bringing the final volume to 10 mL per dish. To spread the MEFs evenly in the plates, they are moved in an up-and-down and side-to-side motion and placed in the incubator at 37°C, 5% CO_2 for a minimum of 8 hours, but preferably overnight.

The following day, the transduced fibroblasts are taken out of the incubator and placed in the tissue culture hood. The DMEM 10% in the wells is aspirated (no more virus precautions are necessary at this point) and the cells are washed once with DPBS to remove all traces of medium. The cells are trypsinized with 1 mL of Trypsin for 4 minutes at 37°C. Afterwards, the Trypsin is neutralized with an equal amount of DMEM 10%. A cell lifter is used to completely remove the cells from the bottom of the well. The cells are then centrifuged in a 15 mL conical tube (one tube for each well) and resuspended in 5 mL of fresh DMEM 10%.

The MEFs that were plated the previous day are taken out of the incubator during the centrifugation of the transduced fibroblasts and the DMEM 10% on the plates is aspirated and replaced with 5 mL of fresh DMEM 10%. The 5 mL of cell solution per 15 mL tube are added to the MEF plate and the plate is labeled with the according cell line.

The day after the plating of the fibroblasts on MEF, the medium of the plates is changed to hESC medium containing 400 mL DMEM/F12 (Invitrogen, Cat.-Nr. 11330-057), 100 mL KOSR (Invitrogen, Cat.-Nr. 10828-028), 5 mL P/S (Invitrogen, Cat.-Nr. 15140-122), 5 mL L-Glutamine (Invitrogen, Cat.-Nr. 25030-081), 5 mL MEM-NEAA (Invitrogen, Cat.-Nr. 11140-050) and 500 µL 2-Mercaptoethanol (Invitrogen, Cat.-Nr. 21985023). Additionally, βFGF (Invitrogen, Cat.-Nr. PHG0261) is added (1 µL per 10 mL, final concentration 10ng/mL) right before use. Furthermore, to one of the two plates per transduced fibroblast line 10 µL of VPA 1000X (EMD Biosciences, Cat.-Nr. 676380-5GM, dissolved in DPBS and filtered at 0.22 µm to a final concentration of 0.5 mM) is added. The plate is marked accordingly.

4.3.3 Critical steps

As with all virus-related steps in the aforementioned transfection, special precautions have to be taken to ensure the safety of the person transducing the fibroblasts. Lab sleeves have to be worn over the lab coat, making sure that no skin is left in the open where the virus could infect cells outside the culturing dish. A second pair of gloves needs to be worn over the first. All surfaces and instruments that came in contact with the plates or liquid in the plates need to be cleaned with Vesphene to inactivate the virus. The hood used for this procedure step needs to be cleaned with a Vesphene-water mixture (see mixture in Step 4.2.3) and has to be, after all virus-work is finished, closed with the UV light turned on for at least 20 minutes.

4.3.4 Alternatives, Pros and Cons

The retroviral vectors first used to reprogram mouse and human fibroblasts [11], as well as the inducible lentiviruses of later generations [20] fall in the category of stable expression and have been criticized for their permanent and random integration into the host genome. Alternative methods include the omittance of c-Myc, the gene known to be a very active oncogene, however, this leads to significantly lower efficiency and longer waiting periods before colonies appear [21].

Non-integrating approaches have so far been tested and adenoviral delivery and transient transfection have been deemed as successful in the reprogramming of mouse cells [22].

Another approach that limits the integration sites in the genome foresees the use of one vector that includes all four reprogramming factor genes and, additionally, an IRES or 2A self-cleavage peptide. The integration takes place with the help of a lentivirus system containing a LoxP sequence. The expression of Cre recombinase cuts out the vector, leaving behind only an LTR in the iPS genome [23].

Transposon systems have also been used for reprogramming purposes. The plasmid-based transposon is integrated into the genome with the help of a transposase and induces reprogramming. Once iPS colonies have been established, the transposase is re-expressed and the integrated vector is excised from the genome, leaving, in most cases, no trace behind. [24]

The most recent and probably the most groundbreaking new technique was published in November 2010. Luigi Warren et al. discovered a method of reprogramming cells based on the integration of synthetic mRNA that was modified to overcome innate antiviral responses. The mRNA is repeatedly administrated and transcribes for the proteins of the canonical four Yamanaka transcription factors. Since there is no permanent integration into the genome, this method is safe for cell therapy. Additionally, in test cultures of BJ cells, its efficiency proved to be much higher than that of virus transduction [25]. Of course, as this method is still very new, the technique has to be tested extensively before it can be implemented in patient therapies.

4.4 Cultivation of iPS cells from transduced fibroblasts

4.4.1 General information

There are three types of human iPS cells, which can be differentiated based on their expression profiles of cell surface markers, as well as their retroviral silencing [26]. The most well reprogrammed type tests positive for the pluripotency markers SSEA-4 and TRA-1-60 during immunocytochemistry. They are the only ones to be able to form teratomas that contain cells of all

three germ layers (ectoderm, mesoderm and endoderm). The two other types are partially reprogrammed cells that are morphologically similar to correctly reprogrammed iPS cells [27], however, test negative for SSEA-4 and TRA-1-60 and still express retroviral factors, e.g. the inserted GFP gene, which can be tested for under UV light.

Within 14 days of transduction of the fibroblasts, changes in the form of colony formation should be visible on the plate. The white cell clusters, the precursors of fully reprogrammable iPS colonies, will be visible with the naked eye and, under UV light, will still show expression of GFP.

After three to four weeks of daily feeding, fully reprogrammed iPS colonies will be visible under the microscope. The morphology of the cells is easily separated from the underlying MEFs, as iPS colonies have clearly defined edges. Under the UV light no GFP expression should be visible.

4.4.2 Procedure

For the first week after transduction, the 10-cm plates are fed with 10 mL of pre-warmed hESC medium, supplemented with 1 µL of βFGF per 10 mL of medium every other day. To the plates labeled to receive VPA, 10 µL are added with the hESC medium. After the first week, the VPA is no longer added and the plates are from then on fed every day with fresh hESC medium + βFGF.

Once changes are visible, the plates are regularly checked under a UV light microscope to evaluate their state of reprogramming. When the cell clusters stop expressing GFP, they are considered potential iPS colonies and marked. After the cell clusters that were labeled and counted have grown to a reasonable size, a layer of irradiated MEFs is plated in an according number of wells of a 6-well plate. For all 6 wells of a 6-well plate, one million MEFs are needed and a minimum of two plates has to be plated to accommodate the two million cells per vial. The MEFs are thawed as described during the transduction of the cells and, after being spun down, resuspended in 12 mL, if 2 million MEFs/vial are used, or 24 mL if 4.5 million MEFs/vial are used. The wells, coated with 0.1% gelatin, are covered with 1 mL of DMEM 10% per well and 1 mL of cell solution is added on top. After placing them in the incubator,

the plates are slid forwards and backwards, as well as side to side to ensure proper distribution of the MEFs in solution. The cells are incubated at 37°C, 5% CO_2 overnight.

The following day, the DMEM 10% on the MEFs is replaced with 2 mL of hESC medium under the addition of βFGF (1 µL per 10 mL), as well as 4 µL of ROCK inhibitor Y-27632 in solution (EMD Chemicals Inc., Cat.-Nr. 688001-500UG) per well.

The colonies to be transferred to wells are picked manually with a glass-pipette picking tool under a picking microscope. The cluster is isolated from the rest of the cells by removing the MEFs in a circle around it. The colony is then scraped in a grid-like manner and removed from the plate with a circular motion of the picking tool. The pieces, which should all be floating, but located around the same area, are then sucked up and transferred to one well of the 6-well plate with a P200 pipette. The well is marked with the line, if the cells are VPA positive and the passage (the passage from the 10-cm plate to the first 6-well plate is known as passage 0) and the colony is then considered as one line of iPS from the specific patient sample. The procedure is repeated for every colony treated as iPS. Feeding of the new colonies is suspended the first day after picking to allow the colony pieces to attach to the plate. Afterwards, the lines are fed with 2 mL of hESC medium + βFGF every day until the colonies in the wells have reached a reasonable size and number (the well should not be completely covered and little to no cell differentiation should be visible). After 10 days at the latest, the colonies are transferred to a new layer of MEFs, which needs to be changed every 7 to 10 days. The splitting ratio largely depends on the amount of colonies in the well. Most often the ratio from passage 0 to 1 is kept at 1:1, although if the number of colonies is very high, a passage of 1:2 is possible and recommended.

Depending on the number and size of colonies, further passages are made using Collagenase IV (1mg/mL, Stem Cell, Cat.-Nr. 07909). The procedure is highly similar to trypsinization: the hESC medium is aspirated and the cells are washed with 1 mL of DPBS before 1 mL of pre-warmed Collagenase IV is added to the wells to be split. The cells are incubated at 37°C for 10 minutes.

The Collagenase is neutralized with hESC medium without βFGF and the cells are lifted off the plate with a cell lifter. They are collected and spun down at 1200 rpm for 4 minutes. The hESC medium is aspirated and the cells are resuspended as many mL of hESC medium + βFGF as there are wells to be plated. One mL of hESC medium + βFGF is added to the wells with MEFs before the cells are transferred. Since the cell clusters have a tendency to move up the pipette, the cell solution has to be resuspended before the transfer to each well and no more than one mL should be pipetted at once.

After passaging of the cells has reached a total of six wells, a part of the cells can be frozen for stocking purposes. For each line, six wells of MEF are plated the previous day. Two of the iPS wells are then split in a ratio of 1:3 using Collagenase IV. They are resuspended in 6 mL of hESC medium + βFGF and portioned into the six wells of MEF, each of which already contains 1 mL of hESC medium + βFGF. The other four wells are treated with Collagenase IV and collected in one 15 mL conical tube per line. To remove all cells from the wells, they are each washed with 1 mL of hESC medium, which is also collected in the tube. The cells are then spun down at 1200 rpm for four minutes, during which time a tub of ice is prepared to keep the 2X freezing medium containing 8 mL FBS and 2 mL DMSO (Sigma, Cat.-Nr. D2650) and the cryovials cool. After the cells have finished spinning down, the supernatant is aspirated. The cell pellet is resuspended in half hESC medium without βFGF and half freezing medium. One mL of the cells is then transferred to a 1.5 mL cryovial labeled with the cell line, the number of wells frozen into one vial, the passage number, initials of the freezer and the date. The cryovials are transferred to a Nalgene Cryo 1°C “Mr. Frosty” Freezing Container (VWR, Cat.-Nr. 55710-200) and stored at -80°C at least overnight. The next day, the cells are moved to the liquid nitrogen tank (-130°C) for long-term storage.

4.4.3 Critical steps

The culturing of iPS cells takes practice and a close culture observation to get a feel for the culture and its replication cycle is important to ensure that the

growth of the cells is not impaired by lack of space or nutrients, as this would lead to the differentiation and merging of the cells.

The passaging of the colonies has to be planned beforehand, as a layer of MEFs is needed for the iPS colonies to be maintained in an undifferentiated state. These MEFs are preferably to be plated the day (or at least 8 hours) before the splitting of the iPS cells is started and should be observed under the microscope to see if they cover the entire surface of the plate. Passaging usually takes place in a ratio of 1:3, however, if the MEFs have reached an age between 7 to 10 days, the cells need to be split onto a fresh layer of MEFs and the ratio might differ, depending on the speed of cell growth.

Feeding has to take place every day and cannot be skipped. The addition of βFGF to the pre-warmed hESC medium is essential to keep the cells from differentiating.

When cells are picked manually, ROCK Inhibitor has to be added to the wells with hESC medium + βFGF *before* the colonies are transferred to the wells, as this permits the survival of the dissociated colonies and ensures cell survival. The passages that follow the manual picking are not under the addition of ROCK Inhibitor, unless they are also manual pickings.

Before cells can be frozen, a contamination with Mycoplasma, a bacterium that is resistant to most antibiotics that target the cell wall synthesis (e.g. Penicillin) due to the lack of such and therefore very dangerous organism, has to be excluded. The bacteria do not cause the typical symptoms of contamination, such as milky medium and cell debris in the wells and can go undetected for months. However, if contaminated, the colonies in the wells are under the constant pressure of nutrient competition with the bacterium, which causes a constant deterioration of cell growth. The infection with the bacterium makes all cells on the plate, as well as in the incubator and in the used cell culture hood unusable, so a thorough testing of the iPS colonies before long-term storage is essential. The test is performed using the Lonza MycoAlert Kit Mycoplasma Detection Assay (Lonza, Cat.-Nr. LT 07-418) by collecting 200 µL of the one day old medium on one of the wells per line, spinning the sample down for 5 minutes at 10,000 rpm to remove all traces of

cells from the medium. Furthermore, a negative control of fresh medium is taken. Afterwards, 60 mL of the cell medium is pipetted into the well of a 96-well plate per sample. The negative, as well as a positive control (Lonza, Cat.-Nr. LT 07-518) are added. The reagent, provided in the kit and up to then stored at -20°C, is thawed and 60 µL of it are added to each well. After a 5 minute incubation period at room temperature, the plate is measured and the values are recorded (A-value). The plate is taken out of the measuring device and 60 µL of the substrate, also provided in the kit and normally stored at -20°C, are added, followed by another 10 minute incubation period at room temperature. The plate is then measured again (B-value). If the ratio between the A-value and the B-value is below 1.0, it can be assumed that the samples are Mycoplasma negative and can be frozen for long-time storage. The Mycoplasma test should be performed at regular intervals during cell culturing.

5. Characterization of reprogrammed cells

5.1 Alkaline Phosphatase Treatment

5.1.1 General Information

The discrimination between differentiated and reprogrammed cells is at times a challenging task. Though the characteristic shape of an iPS colony with defined edges and homogeneous center is a relatively easy indicator, the need for a foolproof method is very much needed. In murine organisms, the stem cell state of reprogrammed cells can be established through their ability to contribute to the germ line of chimeric mice, i.e. mice composed of more than one gene pool. A respective method for hES cells is not available, as the use of embryos is highly unethical and would most likely not produce very accurate results. Teratoma formation in mice to test the undifferentiated pluripotency of iPS cells has also been used to establish results. However, this method not only takes prolonged periods time (up to 12 weeks) before a full analysis is possible, but is also a relatively insensitive and nonquantitative method. Furthermore, large numbers of cells are necessary to facilitate the

growth of the teratoma. The method has therefore not proven to be very effective for analytical purposes [28].

Studies have shown that cultures used to derive undifferentiated murine ESCs contain cells that can form single cell-derived colonies of alkaline phosphatase positive cells [29] and that the loss of the ability to produce AP is one of the earliest indicators of differentiation [30].

Alkaline phosphatase, an enzyme that is responsible for the dephosphorylation of molecules, such as nucleotides, proteins and alkaloids under alkaline conditions has the ability to stain fixed hES and iPS cells. The undifferentiated cell clusters exhibit a red to purple color, while differentiated cells do not stain and remain colorless.

This assay therefore allows for a quick, accurate and quantitatively relevant way to determine the differentiation state of hES or iPS cells.

5.1.2 Procedure

The Alkaline Phosphatase assay is performed according to the protocol provided by the manufacturer of the Alkaline Phosphatase Detection Kit (Millipore, Cat.-Nr. SCR004).

About six days before the AP assay can be performed, a layer of MEFs needs to be plated in the wells of a 12-well plate with one well for each line to be analyzed. The number of cells per well is to be set at about 83,000 (24 wells of a 12-well plate can be plated with one vial of 2 million MEFs). The plate is then incubated at 37°C, 5% CO_2. The following day, the MEF layer is washed with DPBS and the DMEM 10% medium is replaced with 1 mL of hESC medium containing βFGF (1µL per 10mL). The cells for the AP assay are picked, pipetted into the wells of the 12-well plate and labeled. About 5 to 7 colonies, depending on their size, should be picked per well and line. The plate is then again incubated at 37°C, 5% CO_2. Feeding is suspended the day after picking to allow proper attachment of the colonies and daily feeding with hESC medium + βFGF is continued thereafter. Five days after picking, the iPS colonies are ready for the AP assay to be performed.

Prior to beginning the AP assay, a 1X Rinse Buffer out of 50 mL DPBS and 25 µL TWEEN®20 (Sigma-Aldrich, Cat.-Nr. P1379) needs to be prepared. Furthermore, a solution of 4% Paraformaldehyde in PBS has to be obtained and is stored at 4°C until needed. At the iPS Core, the 4% Paraformaldehyde is prepared by the nearby Histology Core.

To start the assay, the medium in the wells of the 12-well plate is aspirated. The aspirator tip needs to be changed after every well to avoid contamination of the separate lines with one another. The cells are then transferred under a chemical hood and the 4% PFA is added to the wells (500 µL per well) to fix the cells. After 1 to 2 minutes, the PFA can be taken off the cells and is pipetted into a special waste container. After fixing, the aspirator tip no longer has to be changed when switching between cell lines. The cells are then rinsed with the 1X Rinse buffer. While the buffer stays in the wells, the staining solution is prepared. The solution contains Fast Red Violet Solution and the Naphtol AS-BI phosphate, both provided in the Alkaline Phosphatase Detection Kit (Millipore, Cat.-Nr. SCR004). They are mixed with Ultra-Pure™ DNase/RNase-Free Distilled Water (Invitrogen, Cat.-Nr. 10977) in a ratio of 2:1:1 (FRV:Naphtol:water). The solution exploits a bright yellow color if mixed correctly.

After preparation of the staining solution, the Rinse Buffer is aspirated and the staining solution is added (500 µL per well). The plate is incubated for 15 minutes in a dark space at room temperature. After the incubation period, the wells are washed with the 1X Rinse Buffer and, after aspirating, covered in 1mL of DPBS to prevent a drying of the wells. The red color of the colonies should be visible to the naked eye.

The plate is then observed under a microscope. A picture of each well is taken and stored for documentation purposes. The plate is, after the pictures are taken, discarded or, for further analysis, wrapped in Parafilm, covered in aluminum foil and stored at 4°C.

5.1.3 Critical Steps

The use of PFA underlies strict precautions. It is classified as a suspended carcinogen and therefore proper care has to be taken while handling. It is only

to be pipetted under a chemical fume hood only to prevent harmful gases from escaping and gloves need to be worn at all times. PFA waste needs to be collected in separate tubes and is disposed of by professionals. The wearing of gloves during the whole procedure is absolutely mandatory to ensure the safety of the lab personnel.

5.2 Immunocytochemistry

5.2.1 General Information

In order to determine the full extent of reprogramming apart from the already established morphology, iPS colonies need to undergo a more thorough assay that allows for the detection of five stem cell markers that are also found in hES cells.

Specifically, the stem cell markers Oct4 and Nanog, located in the stem cell's nucleus, and SSEA3, SSEA4 and Tra-1-60, located on the cell surface, are targeted. These markers are known to be expressed in human ES cells and, if the iPS cells are fully reprogrammed, should also be expressed by iPS colonies.

Immunocytochemistry, better known as immunofluorescence, is a common and well-known technique in biology and is used in the determination of biomolecule distribution in samples. Two methods of immunofluorescence can be distinguished: the direct, or primary method and the indirect, or secondary, method. The direct method calls for the use of a fluorescent primary antibody that binds directly to the target molecule, which can be visualized with UV light after only one incubation period. The indirect method more commonly used on iPS colonies calls for the use of primary antibodies that bind to antigens in the sample's structure. The antibody-antigen interaction can then be visualized with the use of secondary fluorescent antibodies that are targeted at the primary antibodies.

Immunofluorescence has both advantages and disadvantages, the main of which were discussed by Stuart J. Schnitt M.D. from the Department of Pathology at the Beth Israel Deaconess Medical Center, as well as the Harvard Medical School. Immunofluorescence can be performed in most

laboratories, as, next to a chemical fume hood, no special equipment is necessary. Furthermore, it is an inexpensive method that gives clear results about the pathology of the examined samples. However, immunofluorescence relies on the use of antibodies that, depending on the sample, fluctuate in sensitivity and specificity. It is a non-quantitative method and requires clear results, as there is no percentage-system for positivity installed [33].

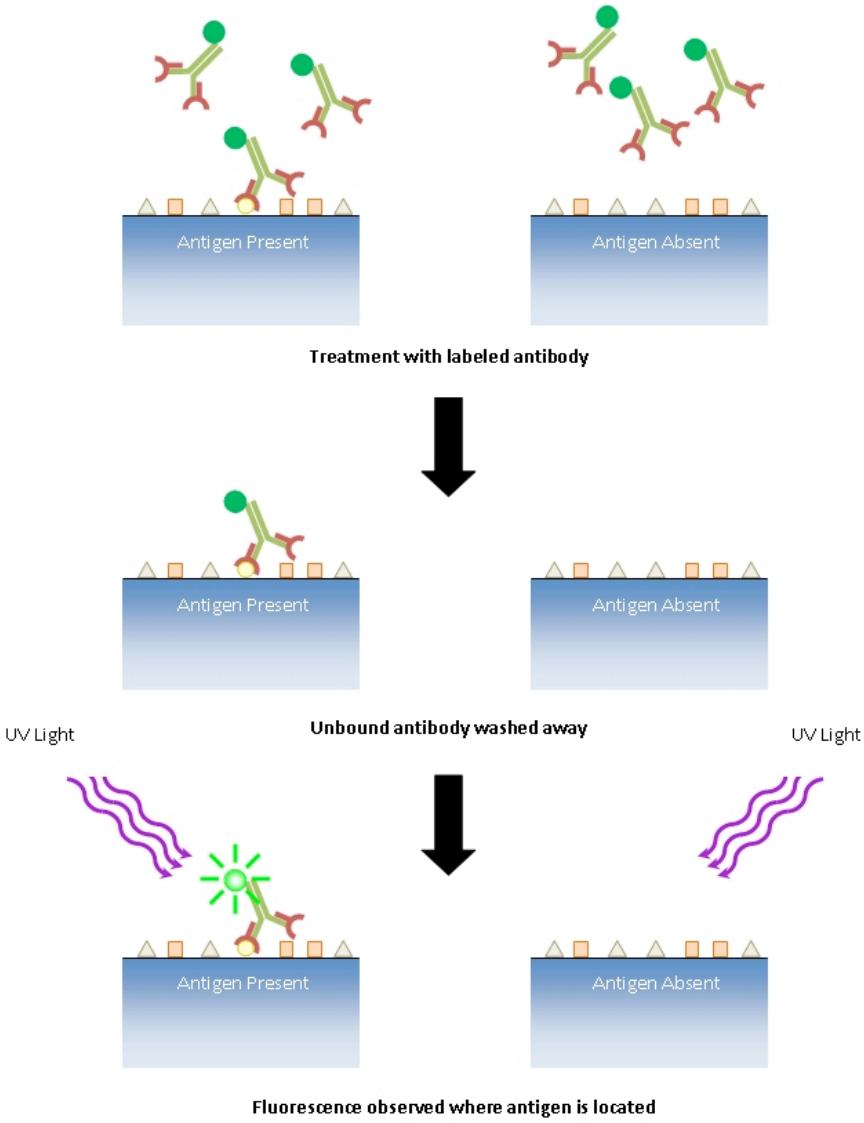

Fig. 7 Direct immunofluorescence: The antibody, previously labeled with fluorescence dye is added to the mix and binds directly to the antigen. The unbound antibody is washed off after an incubation period and the sample is exposed to UV light. If the antigen is present, fluorescence values will be visible. [31]

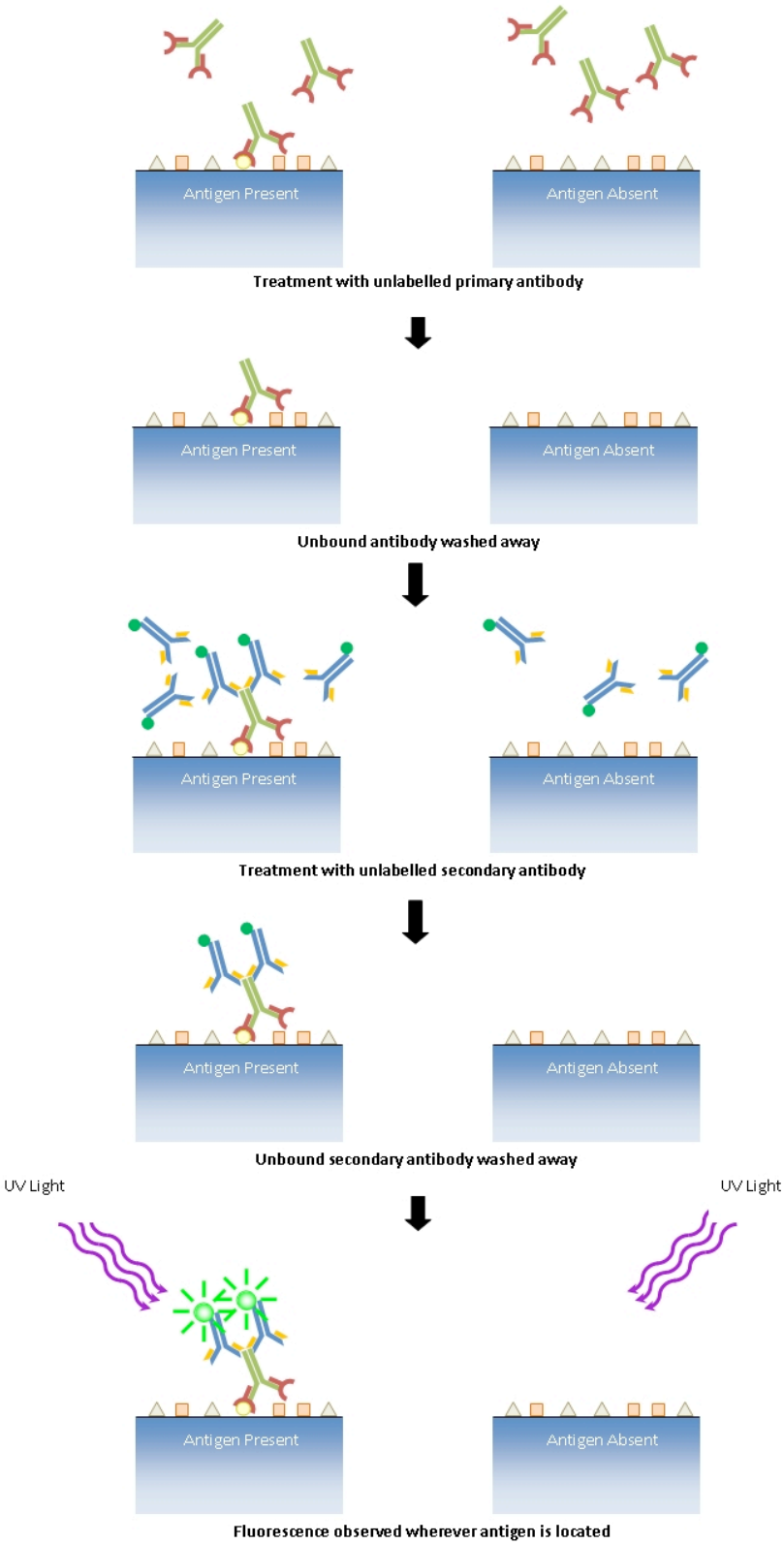

Fig. 8: Indirect Immunofluorescence is performed using a specific primary antibody that targets antigens present in the cell's structure. A secondary fluorescent antibody binds to the primary antibody and can be visualized under UV light. If the primary antibody does not bind, no fluorescence will be visible. [32]

5.2.2 Procedure

About 6 to 8 days before beginning the immunocytochemistry assay, a layer of MEFs is plated in as many wells of a 48-well plate as needed, with a total of 6 wells per line necessary for the assay. The amount of cells per well is set at about 30,000 cells, which can best be achieved by resuspending a 2-million vial in 12 mL of DMEM 10%. One mL is then aliquoted and mixed with 5 mL of DMEM 10%. The 6 mL of cell solution are then plated in 6 wells of the 48-well plate. After plating, cells are incubated at 37°C, 5% CO_2 overnight.

The following day, the colonies for the immunocytochemistry assay are picked into the wells that were previously washed with DPBS and the DMEM 10% was replaced with hESC medium + βFGF. For each line, 6 wells with each 3 to 4 colonies need to be picked and labeled. The plate is incubated at 37°C, 5% CO_2 after the picking and feeding is suspended the following day to assure proper attachment of the colonies. Afterwards, the plate is fed every day with hESC medium + βFGF. Once the cells reached a reasonable size, the assay can be started and the necessary reagents are prepared. Said ingredients include:

- ***DPBS/0.05% TWEEN®20:*** 50 mL DPBS mixed with 25 µL TWEEN®20
- ***DPBS/0.1% Triton® X-100:*** 10 mL DPBS mixed with 10 µL Triton® X-100 (Sigma Aldrich, Cat.-Nr. X100-100 mL)
- ***4% Donkey Serum:*** 15 mL DPBS mixed with 600 µL Donkey Serum (Fisher Scientific, Cat.-Nr. NC9624464), prepared during the permeabilization period

To start, the medium in the wells of the plate is aspirated. For each line, a different pipette tip needs to be used to avoid contamination. The wells are then washed three times with DPBS (500 µL per well) and, under the chemical fume hood, fixed with 4% PFA for 20 minutes. The PFA is collected in a special waste container and the wells are washed 3 times with the DPBS/0.05% TWEEN®20 solution (500 µL per well). The cells are then permeabilized with DPBS/Triton® X-100 for 15 minutes at room temperature (500 µL per well) and in the waiting period, the 4% donkey serum mixture used for blocking is prepared. After permeabilization is completed, the cells

are washed three times with DPBS/0.05% TWEEN®20 three times and blocked with 500 µL of the donkey serum mixture per well. The plate is then wrapped in Parafilm and stored at 4°C overnight.

The following day, the primary antibodies, diluted in 4% donkey serum, are prepared according to the dilutions in Tbl.2.

Tbl. 2 Immunocytochemistry: Primary Antibodies with Catalog-Numbers and final dilutions for proper use in ICC. All dilutions for primary antibodies are prepared with 4% donkey serum.

Primary Antibody	**Dilution**
Oct4 (AbCam, Cat.-Nr. ab19857)	1:100
Nanog (AbCam, Cat.-Nr. ab21624)	1:50
SSEA3 (Millipore, Cat.-Nr. MAB4303)	1:200
SSEA4 (Millipore, Cat.-Nr. MAB4304)	1:200
Tra-1-60 (Millipore, Cat.-Nr. MAB4360)	1:200

The donkey serum in the wells is aspirated and the wells are washed once with DPBS. 100 µL of the primary antibody is added to one well of each line, so that for each line, one well of each of the five antibodies is prepared. The sixth well is treated as a negative control and only 4% donkey serum is added to substitute the primary antibody. The plate is then incubated for one hour at room temperature, during which time the secondary fluorescent antibodies are prepared in DPBS according to Tbl. 3.

Tbl. 3 Immunocytochemistry: Primary antibodies and their according secondary antibodies in a dilution of 1:500 in DPBS. The fluorescence color of the secondary antibody can be seen under certain filters – a blue filter for green fluorescence and a green filter for red fluorescence.

Primary Antibody	**Secondary Antibody**	**Dilution**	**Fluorescence color**
Oct4	Rabbit (Invitrogen, Cat.-Nr. A21206)	1:500	Green
Nanog	Rabbit (Invitrogen, Cat.-Nr. A21206)	1:500	Green
SSEA3	Rat (Invitrogen, Cat-Nr. A21213)	1:500	Red
SSEA4	Mouse IgG (Invitrogen, Cat.-Nr. A21121)	1:500	Green
Tra-1-60	Mouse IgM (Invitrogen, Cat.-Nr. A21426)	1:500	Red

After the incubation hour, the wells are washed with DPBS/0.05% TWEEN®20 three times and 100 µL of the according secondary antibody are added to the wells. The negative control is treated with only DPBS. The plate is then covered in tinfoil and incubated in the dark for one hour at room temperature. Afterwards, the wells are washed three times with DPBS/0.05% TWEEN®20 and counterstained with DAPI for 10 to 20 seconds (100 µL per well).

Pictures of the same iPS colony are taken with both the DAPI filter and the filter of the corresponding fluorescent antibody (blue filter for green fluorescence, green filter for red fluorescence). The pictures are later colored in using a photo-editing program.

5.2.3 Critical Steps

Immunocytochemistry highly relies on the permeabilization of the cells. Therefore, the Triton® X-100 used for said purpose needs to be completely dissolved in the DPBS before it is added to the wells.

Paraformaldehyde is a suspended carcinogen and needs to be handled with special care and only under a chemical fume hood to ensure the proper safety of the lab staff. The waste collected after fixing the cells needs to be stored in a separate waste container and is to be discarded only by professionals.

During the whole procedure, the wearing of gloves is obligatory.

5.3 Testing for Pluripotency Markers

5.3.1 General Information

The full pluripotency state of the generated iPS colonies is a vital attribute to guarantee their full functionality. Tests have shown that already a small amount of undifferentiated cells causes the formation of germ cell tumors comprised of more than one cell type called teratomas [34]. The formation of said tumors prevents their use in transplantations and should be avoided. Furthermore, the use of inchoately reprogrammed cells can result in the inability of full differentiation and increases the risk of immature teratoma formation. A thorough testing of the cell's gene expression before iPS

colonies can be used for therapeutic purposes is therefore absolutely necessary.

Pluripotency markers, the ones tested namely being Dnmt3b, hTERT, Nanog, Oct4, Rex1 and Sox2, are only expressed if the cell has fully reached the desired pluripotent state and are ready for further purpose.

To test for the pluripotency genes the widely known technique of polymerase chain reaction (PCR) is used. The method uses cycles of thermal levels to induce the exponential reproduction of DNA fragments. This allows for the amplification of signals in the targeted DNA sequence. The method starts with the use of primers of the complementary sequence to that on the cDNA, earlier synthesized from collected mRNA samples.

The cDNA is added to a mixture of Taq Polymerase, dNTPs, a buffer solution, a Forward and a Reverse primer, a loading buffer and water. This solution can either be prepared in a Master Mix where all components are combined without the DNA before they are distributed amongst the samples or, alternatively, a pre-prepared mixture that contains all the ingredients can be bought. After addition of the cDNA and a heating up to 94°C for 5 minutes in the preheated PCR machine, the samples continue on into repetitive cycles of denaturation at 94°C for 30 seconds, annealing at a temperature defined by the base pair sequence of the primers (normally between 56 and 62°C) for 30 seconds and elongation at 72°C for 30 seconds. After 30 to 35 consecutive cycles, a final elongation step of 5 minutes at 72°C is initiated before the sample is cooled down to 4°C for storage.

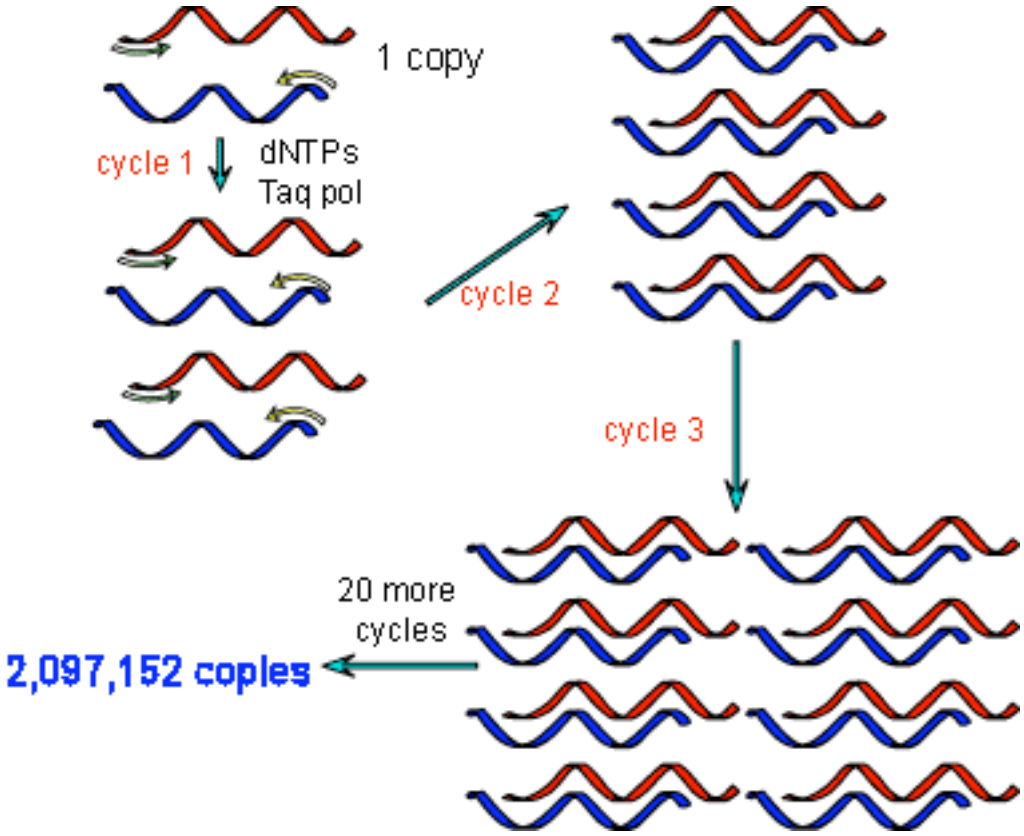

Fig. 9 Diagram of a typical PCR program. The mixture containing the dNTPs, the Taq Polymerase and the primers attaches to the annealed strands of cDNA and form new strands of DNA. Already after 3 consecutive cycles of denaturation, annealing and elongation, the amount of DNA in the sample has quadrupled from 2 strands to 8 strands. Exponential growth leads to over two million copies after 23 cycles. [35]

For the testing for pluripotency markers, the following primer sequences are used:

Tbl. 4 PCR Pluripotency primer sequences with according annealing temperatures and cycle numbers. The general program consists of a 94°C hold for 5 minutes and then an according number of cycles at the regular PCR program before a 5 minute extended elongation period at 72°C and a final hold at 4°C until the samples are taken out of the machine. The Oct4 and Sox2 primers, unlike the others, target the endogenous expression of the genes.

Primer	Sequence Forward	Sequence Reverse	Annealing Temperature [°C]	Cycle Number
Dnmt3b	ATAAGTCGAAGGTGCGTCGT	GGCAACATCTGAAGCCATTT	56	30
hTERT	TGTGCACCAACATCTACAAG	GCGTTCTTGGCTTTCAGGAT	57	33
Nanog	TCCAACATCCTGAACCTCAG	GACTGGATGTTCTGGGTCTG	58	30

Oct4 (endogenous)	GTGGAGGA AGCTGACA ACAA	CAGGTTTTC TTTCCCTAG CT	56	30
Rex1	TGGACACG TCTGTGCTC TTC	GTCTTGGC GTCTTCTCG AAC	60	30
Sox2 (endogenous)	AGCTACAG CATGATGC AGGA	GGTCATGG AGTTGTACT GCA	58	30

5.3.2 Procedure

Before PCR can be started, cDNA – the complementary DNA strand to mRNA – needs to be produced from pure cell colonies, meaning no contamination from other cell types is allowed. A layer of Matrigel therefore substitutes the MEF layer on which the iPS colonies are normally grown on.

5.3.2.1 Growing cells on Matrigel

A week prior to cell harvesting for RNA extraction, the cells for PCR and DNA Extraction, are transferred onto a layer of Matrigel. For each cell line, two wells of Matrigel are needed – one for RNA extraction, the other one for DNA extraction (not needed by the iPS Core, but handed over to investigators at the time of cell collection). The transfer ratio is set at 1:1, i.e. one well of cells grown on MEFs is transferred onto one well of Matrigel, as the different environment causes a large strain on the cells and can lead to large amounts of cell death right after the transfer.

To start, the BD HESC Qualified Matrigel (Fisher Scientific, Cat.-Nr. 354277), stored at -80°C, is slowly thawed overnight in a tub of ice at 4°C. The following day, 10 mL of cold DMEM/F12 are poured into a 15-mL conical tube and 100 µL of Matrigel are added. The rest of the Matrigel aliquot is placed back at -80°C. The mixture is plated in wells of a 6-well plate with an intended 1 mL per well, meaning a total of 10 wells can be plated with one aliquot. The Matrigel is left to set for one hour at room temperature. After the resting period, the medium in the wells to be transferred onto Matrigel is aspirated. The cells are washed with DPBS and Collagenase IV is added. The cells are detached, spun down, the medium is aspirated and then replaced with mTeSR®1 medium (Stem Cell, Cat.-Nr. 05850). During the spinning down,

the DMEM/F12 in the Matrigel wells is aspirated and replaced with 1 mL of mTeSR®1 per well. No washing with DPBS is necessary for this change of medium. After the plating of the colonies (for each well, the pellets are resuspended in 1 mL of mTeSR®1), the cells are incubated at 37°C, 5% CO_2. Feeding is skipped the following day and then carried on every day. After seven days, the cells are trypsinized for four minutes and each well is transferred to a separate tube. The cell pellets are washed with DPBS once to remove all traces of medium. This is especially important for DNA Extraction. The well containing more cells, i.e. the larger pellet, is used for RNA extraction; DNA extraction is done with the other well. If extraction does not take place immediately after the cell pellet collection, the pellet can be stored at -80°C until further use. All DPBS should be removed from the tube before the freezing process.

5.3.2.2 RNA Extraction

RNA Extraction is performed using the Qiagen RNeasy Mini Kit (Qiagen, Cat.-Nr. 74106). Buffers RLT, RW1 and RPE, as well as RNase-free water are provided in the kit. A 70% Ethanol mixture has to be prepared ahead of time. The RNase-free DNase Kit needs to be bought separately (Qiagen, Cat.-Nr. 79254). The cell pellet needs to be taken out of the freezer and thawed before the extraction can take place.

First, 350 µL of Buffer RLT are added to the thawed cell pellet and the solution is vortexed for one minute. Another 350 µL of 70% ethanol solution are added and the mixture is pipetted up and down to mix it. The 700 µL are transferred to an RNeasy spin column provided in the kit and centrifuged for 15 seconds at 10,000 rpm. The flow-through is discarded in the biowaste (the collection tube is used again for the next step) and 350 µL of Buffer RW1 are added to the column before another spinning down for 15 seconds at 10,000 rpm. In a separate Eppendorf tube (VWR, Cat.-Nr. 14231-062), 10 µL of DNase and 70 µL of Buffer HDD, provided in the RNase-free DNase Kit, are mixed for each column. 80 µL of the mixture are transferred directly onto the spin column membrane after the flow-through of the samples has been discarded. After a 15-minute waiting period, 350 µL of Buffer RW1 are added to the spin column and the column is centrifuged for 15 seconds at 10,000

rpm. The flow-through is discarded and 500 µL of Buffer RPE are added to the spin column. After another centrifugation of 15 seconds at 10,000 rpm, the flow-through is discarded and another 500 µL of Buffer RPE are added to the column. The column is centrifuged for 2 minutes at 10,000 rpm. The 2 mL collection tube is discarded along with the flow-through and the column is placed in a fresh collection tube before another centrifugation of 1 minute at 12,000 rpm. The spin column is then transferred to a 1.5 mL collection tube. The collection tubes provided in the kit should be used, as it is guaranteed that these tubes are RNase-free. 30 µL of RNase-free water are added directly to the spin column membrane and the column is centrifuged for 1 minute at 10,000 rpm. The column can now be discarded and the liquid in the collection tube is kept. The concentration of the RNA in the sample is measured and recorded. The sample is then stored at -80°C until further use in cDNA preparation.

5.3.2.3 *cDNA*

To produce cDNA, all ingredients (except RT) are thawed on ice. To determine the amount of RNA needed, the concentration of the collected RNA is needed and 1000 is divided by said concentration (in ng/µL) to determine the amount needed. The amount of µL per RNA sample that result in the calculation are added to two PCR tubes (VWR, Cat.-Nr. 82050-976 for tubes, VWR, Cat.-Nr. 82050-416 for caps), labeled “+” and “-“. Furthermore, 1 µL of dNTPs (10 mM) and 1 µL of Random Hexamers (50ng/µL) provided in the SuperScript® III Reverse Transcriptase Kit (Invitrogen, Cat.-Nr. 18080-044), as well as DEPC water (Ambion, Cat.-Nr. 9915G), adding up to 13 µL total volume are added to each tube. This procedure is repeated for each RNA sample. The tubes are placed in a PCR machine and heat inactivated for 5 minutes at 65°C and then quickly cooled down on ice for one minute. To the “+” tube(s), 4 µL of 5X First Strand Buffer, 1 µL of DTT (0.1 M), 1 µL of RNase OUT™ (Invitrogen, Cat.-Nr. 10777019) and 1 µL of RT (up to now kept at -20°C) (200 U/µL, Invitrogen, Cat.-Nr. 18080-044) are added. In the “-“ tube, the RT is replaced with 1 µL of DEPC water. The mixtures in the tubes are spun down for a few seconds and then placed back in the PCR machine where the RT-PCR program, consisting of a 5 minute hold at 25°C, a 45

minute hold at 50°C and a 15 minute hold at 70°C before a final, extended hold at 4°C, is run. After the program has reached the 4°C hold, the samples are transferred to separate autoclaved Eppendorf tubes and labeled with date, name of cell line and either "+" or "-". The cDNA is then stored at -20°C.

5.3.2.4 PCR for pluripotency markers

To start PCR, all cDNA samples – "+" and "-" – are put on ice to thaw, along with enough Platinum® Blue PCR SuperMix (Invitrogen, Cat.-Nr. 12580-023) to guarantee 23 µL per sample. One extra tube is generally calculated in to even out small pipetting errors. Furthermore, the Forward and Reverse Primer for the PCR to be performed are thawed. The primers are provided in a concentrated solution that needs to be mixed in a ratio of 60:40 (RNase/DNase-free water:primer) before use for PCR (usually an aliquot of 100 µL is prepared and frozen if there is left-over product). All PCR primers for pluripotency can be found in Tbl. 4. A master mix, containing 23 µL of Platinum Blue PCR Mix, 0.5 µL Forward Primer and 0.5 µL Reverse Primer per sample is prepared. 24 µL of the mix are then added to each PCR tube and 1 µL of cDNA is added to each tube. The pipette tip between each transfer of cDNA to the tubes is changed to avoid contamination of samples with one another and with the negative controls. Preferably, the "+" cDNA of one sample is placed next to the "-" sample of the same cDNA to avoid mistakes in the marking of the tubes and to allow a proper documentation of the positive and negative sample.

After completion of the mixing in the tubes, the samples are spun down briefly, placed in a PCR machine (preferably pre-warmed) and the according program, determined by the primers, can be started.

After the PCR program is finished, the samples can be taken out and run on a 1.5% Agarose gel or stored at 4°C until further use. The procedure is repeated for each pluripotency primer and Actin, which, as a housekeeping-gene, is present in all RT positive samples and acts as a positive control.

5.3.2.5 *Running of an Agarose Gel*

Before preparation of the Agarose gel, the number of samples has to be taken into consideration. If more than 10 samples are tested at once, a large gel

needs to be prepared; sample numbers under 10 can be run on a small gel. For small gels, 0.75g of Agarose Powder (Fisher Scientific, Cat.-Nr. BP1356-500) are immersed in 50 mL of 1X TAE Buffer, prepared from a 50X stock (Invitrogen, Cat.-Nr. 24710030). The mixture is heated in a microwave for 2 to 3 minutes and should be stirred occasionally during the heating. Once no more particles are visible in the solution, the mix can be left to cool for 5 to 10 minutes, after which period 1 µL of 1% Ethidium Bromide solution (Fisher Scientific, Cat.-Nr. 1302-10) is added and the gel is poured into a previously prepared small gel chamber. A comb is inserted into the mix and the gel is left to harden for about 20 minutes. After the gel has hardened, it is transferred to the gel apparatus and can be loaded with the samples. For large gels, which can hold up to 26 samples, excluding the marker lane (for each row, one marker is required), the above mixture is doubled. Heating and hardening times vary accordingly.

A 1 Kb Plus DNA Ladder (Invitrogen, Cat.-Nr. 10787-026) in a mixture of 1:1:8 with 10X Blue Juice™ Gel Loading Buffer (Invitrogen, Cat.-Nr. 10816-015) and Ultra-Pure water is used as marker. The gel is then run at about 80 to 95V for about 30 minutes the gel is transferred under the UV light. If no bands are visible, the gel can be placed back on the gel rack and run for a longer time period. Once bands become visible under UV light, a picture of the gel is taken for documentation purposes.

5.3.3 Critical Steps

During RNA Extraction and cDNA synthesis, a contamination with RNase is to be avoided at all costs, as a degradation of the RNA would lead to negative results. Therefore, the use of gloves during all steps of the RNA Extraction and cDNA synthesis are obligatory.

Ethidium Bromide is a fluorescing agent that binds to double stranded DNA. Due to its intercalating feature, EtBr is considered to be a major health risk and a very potent mutagen that affects the natural mechanisms involved in DNA replication. The handling of EtBr therefore underlies strict rules. Liquid agarose gel samples have to be transferred to a chemical fume hood before the EtBr is added to the gel in order to avoid both the contact and the fumes

of the hot chemical. Furthermore, the use of gloves during the handling of the gel before, during and after the addition of EtBr is mandatory.

After the run of the gel has ended, the picture of the gel has to be taken within a short period of time after the run was stopped, as a diffusion of the DNA into the surrounding TAE Buffer is possible and alters results.

5.4 Testing for Differentiation Markers

5.4.1 General Information

One of embryonic stem cells' main and most coveted attributes is their high potential to differentiate into the cell types of the body under constant self-renewal. Since one of the intended applications for iPS colonies is to mimic ES cells in their natural environment in the body in disease models of all kinds, their potential of the aforementioned attribute needs to be confirmed to allow a proper and full differentiation into the needed cell type.

A common method to check for differentiation markers is the in-vitro formation of EBs (embryoid bodies), cell clusters grown in suspension that develop the three germ layers also found in the early embryonic stages before major cell differentiation takes place. The layers are divided into endo-, meso- and ectoderm. The endoderm layer, also known as internal layer, develops into most epithelial cells and provides, amongst others, the lining for the intestinal tract, as well as the cells of the liver, pancreas, thymus, the thyroid and parathyroid gland. The mesoderm layer, also known as middle layer, builds the cells of the muscular system (skeletal, smooth and cardiac), the reproductive system and the adrenal complex, as well as the dermis of the skin. The ectoderm layer forms the outer body shell, including the epidermis of the skin and its derivatives (hair follicles, sweat glands, etc.) and the nervous system, as well as the cornea and lens of the eye and the tooth enamel.

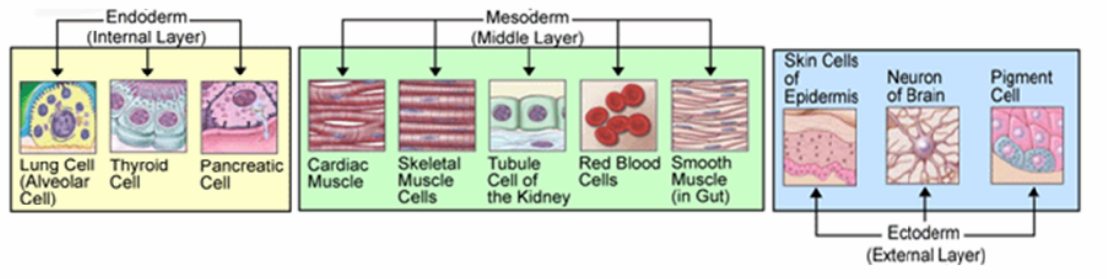

Fig. 10 Germ layers of the human body developed in the embryonic stadium. The cell layers are divided into endo-, meso- and ectoderm with each layer developing into cell types of different regions of the human body, i.e. inner, middle and outer layer. [36]

To test for the full differentiation potential of the harvested cells, EBs are formed PCR is performed with two markers per layer.

Tbl. 5 PCR Differentiation primer sequences and according annealing temperatures and cycle numbers.

Primer	Germ layer	Sequence Forward	Sequence Reverse	Annealing Temperature [°C]	Cycle Number
AFP	Endoderm	AGCTTGGTG GTGGATGAA AC	CCCTCTTCA GCAAAGCA GAC	58	30
GATA4	Endoderm	CTAGACCGT GGGTTTTGC AT	TGGGTTAA GTGCCCCT GTAG	61	30
Flk1	Mesoderm	AGTGATCGG AAATGACACT GGA	GCACAAAG TGACACGTT GAGAT	63	32
GATA2	Mesoderm	GCAACCCCT ACTATGCCAA CC	CAGTGGCG TCTTGGAG AAG	58	35
NCAM	Ectoderm	ATGGAAAACT CATTAAAGTG AACCTG	TAGACCTCA TACTCAGCA TTCCAGT	58	33
Pax6	Ectoderm	TCTAATCGAA GGGCCAAAT G	TGTGAGGG CTGTGTCT GTTC	57	35

5.4.2 Procedure

In order to produce the cDNA needed for the PCR for the detection of differentiation markers, the preparation of embryoid bodies (EBs) is required.

5.4.2.1 In-vitro differentiation of EBs

For one well of EBs, one well of iPS colonies, preferably a well with high confluence is washed with DPBS and Collagenase IV is added. After 10 minutes, the cells are neutralized, spun down and then resuspended in 2 mL of hESC medium *without* βFGF. The cells are transferred to one well of a low-attachment 6-well plate (Fisher Scientific, Cat.-Nr. 3471) and incubated at 37°C, 5% CO_2. The wells are fed every three days for one week with hES *without* βFGF. The medium can either be aspirated carefully, making sure that no cell clusters are taken along, or new medium can be added on top without removing the old medium first.

After one week, the cell clusters are transferred onto regular 6-well plates whose wells have been covered in 0.1% gelatin. To transfer the EBs, the clusters are pipetted into 15-mL tubes and spun down for 4 minutes. Afterwards, the hESC medium is aspirated and the cells are resuspended in 2 mL of DMEM 10% and transferred into the well. The EBs are incubated at 37°C, 5% CO_2 and fed every three days with DMEM 10%.

Another week later, the cells are ready for RNA extraction and trypsinized to release them from the well. Similarly to the way regular iPS colonies are handled, they are spun down and the pellet is washed with DPBS to remove all traces of medium. After aspiration of the DPBS, the pellet can either be frozen at -80°C or RNA can be extracted right away.

For EBs, only RNA is needed for further analysis, meaning only one well of iPS colonies is needed.

5.4.2.2 PCR

After RNA Extraction and cDNA preparation, executed in the same way as in the PCR for pluripotent markers, PCR to detect the differentiation primers, listed in Tbl. 5 can be performed. PCR products are run through 1.5% Agarose gels with Ethidium Bromide to detect the markers and pictures of the gels are taken for documentation purposes.

5.4.3 Critical Steps

For the RNA Extraction, cDNA synthesis and PCR analysis of differentiated cells, the same precautions need to be taken as for the same steps of pluripotent PCR analysis.

Additonally, the need for special care during the feeding of the EBs in suspension is very high. While the EBs are visible to the naked eye, it is essential that as many cell clusters as possible stay in the well when the medium is replaced. Therefore, the change of medium is best done under a picking microscope to ensure that all particles stay in the well.

5.5 Karyotyping

5.5.1 General Information

Through experimental data published in 2010, it has come to attention that, similar to regular ES cells, also iPS colonies are subject to chromosomal abnormalities, which most likely occur during the reprogramming process or during the prolonged periods of time in culture. One of the most common abnormalities found in iPS colonies is a trisomy of chromosome 12, meaning a third copy of the 12^{th} chromosome is present, rather than the two copies expected [37]. Not only does this specific aberration, much like any other chromosomal abnormality, cause difficulties in the differentiation potential of the iPS lines, but the duplication of chromosomes also indicates a disrupted division system of the cells. This loss of control of cell division causes tumorogenesis and cancer. Therefore, a thorough testing of the cells' karyotype becomes necessary to ensure their safety in further testing.

5.5.2 Procedure

To start, the bottom of a T25 flask is coated with gelatin and a layer of MEFs is plated on top. The surface area of said T25 flask is estimated to be about as big as 3 wells of a 6-well plate, meaning about 3 mL of a 2-million MEF vial suspended in 12 mL of medium should be used to adequately cover the bottom of the flask. The rest of the 5-mL holding limit is provided by an added 2 mL of fresh DMEM 10%. The following day, one well of the cell line needed for karyotyping is scraped off the plate after Collagenase IV was used for 10

minutes. The cells are spun down and resuspended in 5 mL of fresh hESC medium + βFGF. The DMEM 10% in the T25 is washed off and the cells are added to the T25. The flask is then closed tightly and the cells are incubated at 37°C, 5% CO_2. Feeding is suspended the next day to allow a proper attachment of the cells. Afterwards, feeding is continued every day with fresh hESC medium + βFGF. After about 5 to 7 days, the flask's bottom should be covered to about 70% with colonies. The afternoon of the send-off to the external lab for analysis, the medium in the flask is aspirated and replaced with fresh hESC medium + βFGF. However, unlike on other feeding days, the flask is filled completely to the brim (ca. 35 mL) and an extra tube with about 20 mL of hESC medium (no βFGF required) per cell line is prepared. The tops of both the T25 flask, as well as the 50-mL tube, are wrapped tightly with Parafilm to ensure that no spillage can occur on the trip to the karyotyping facility (Cell Line Genetics; 510 Charmany Drive, Suite 254; Madison, WI 53719). The flask and tube are furthermore wrapped in paper towels, placed in zip-lock bags and wrapped in bubble wrap before they are packed into a padded envelope for send-off. The package is to be sent with next-day delivery and as close to the sending deadline as possible to allow the cells' medium temperature to be as warm as possible during the whole trip.

5.5.3 Critical Steps

The drop-off of the cells at the latest possible point in time on the day of the sending is very important to allow for maximum heat retention of the cell medium during transport. Furthermore, all spillage is to be avoided, as the medium is counted as biowaste and the precautions that can be taken during the trip might not be adequate for the containment of the spill.

6. Results

The HEK293T cells were transfected and the virus was harvested. On the day of the virus harvest, two fibroblast samples, previously cultured by the investigators, were transduced with the Yamanaka Factors (Oct4, Sox2, Klf4 and c-Myc) in the virus medium. The procedure was repeated the next day. Two days after the second round of transduction of the fibroblasts, the plates

containing the samples, as well as the control plate, also transduced on both days, were examined under a UV-light microscope. A brightfield image was taken of each well. The same area of the well was then examined under UV light.

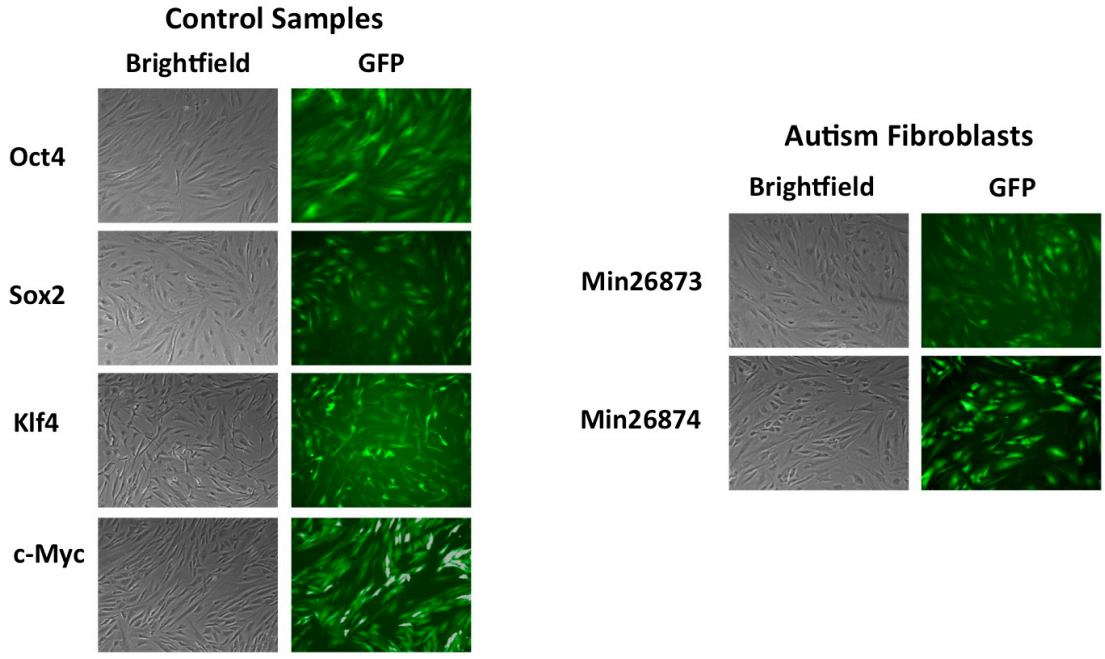

Fig. 11 Expression of GFP two days after transduction: Brightfield image and UV-light image of the control plate containing one well for each of the transcription factors (left) and Brightfield and UV-light image of the Autism patient fibroblast samples (right).

As Fig. 11 shows, both the control plate with the wells only containing one virus per well and the two Autism patient fibroblast samples show a bright and definite expression of GFP. The transduction can therefore be assumed to have been successful and the MEFs for the coming step were plated accordingly (Four 10-cm plates).

After about three weeks of culturing the cells on the 10-cm dishes (one plate of each line with VPA, the other one without), colonies began to appear, which were picked and cultivated as separate lines. For sample Min26873 (also known as 185F, meaning the sample was taken from the father of the Autism patient), 11 lines were isolated, picked and labeled with letters from A to J. Lines Min26873 F and Min26873 H did not contain VPA. For Min26874 (also known as 185B, meaning the sample was taken from the brother of the Autism patient), 5 lines were isolated and labeled from A to E. All of the lines isolated for Min26874 contained VPA. After all samples had been cultivated

for about another two weeks, the lines for characterization were determined to be Min26873 B, Min26873 G and Min26873 J, as well as Min26874 A, Min26874 B and Min 26874 D. The rest of the lines were completely frozen down after a Mycoplasma Test that determined their negativity to the contamination with the bacterium.

The lines to be characterized were expanded to 10 wells per line and a few vials were frozen down before full characterization started. The Alkaline Phosphatase Assay was the first test performed.

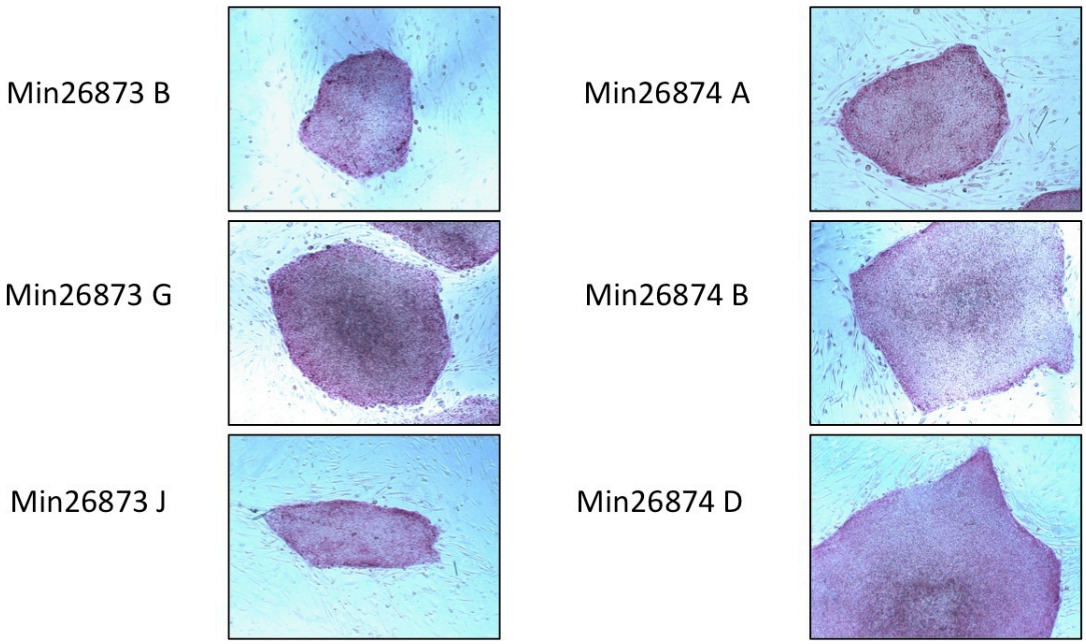

Fig. 12 Alkaline Phosphatase Assay of Min26973 B, Min26873 G and Min26873 J (left), as well as Min26874 A, Min26874 B and Min26874 D (right). All cell lines express the vivid red color expected in pluripotent stem cells.

The assay, completed after 5 days of culturing and summarized in Fig.12, showed that all wells tested gave way to cell colonies expressing the characteristic red color expected in pluripotent stem cell colonies. The assay was concluded to be positive and the immunocytochemistry assay was started with the plating of the colonies in a 48-well plate. Five to seven days after culturing was started, the cells were fixed and blocked overnight with Donkey serum. The primary antibodies and secondary fluorescent antibodies were added the next day and the cells were counterstained with DAPI.

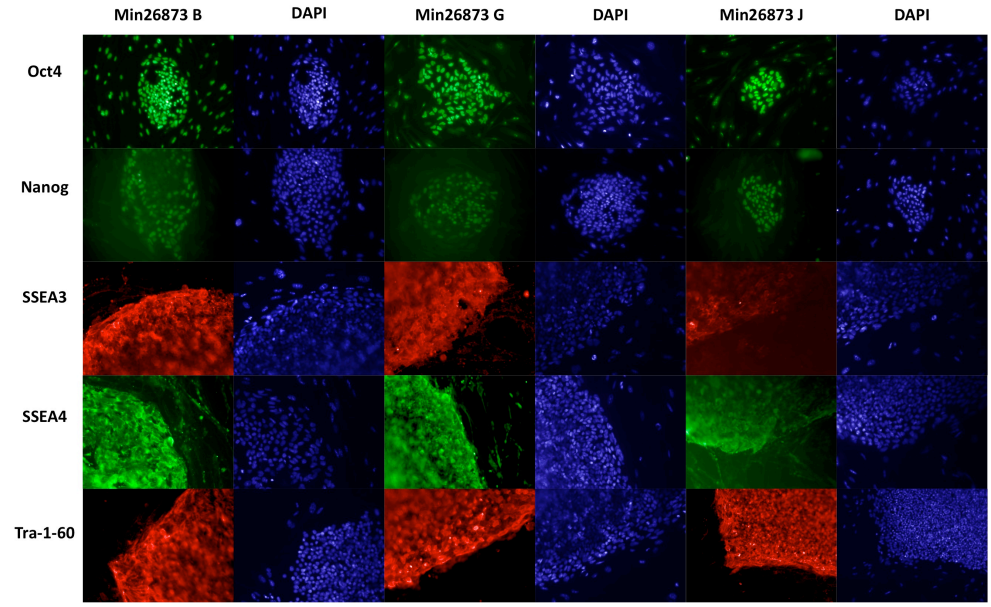

Fig. 13 Immunocytochemistry Assay of Min26873 B, Min26873 G and Min26873 J respectively. The cells are shown with the fluorescent antibody on the left and the DAPI nucleus counterstain on the right.

Testing for the markers Oct4, Nanog, SSEA3, SSEA4 and Tra-1-60 and a counterstain of the nucleus with DAPI showed that in both the characterized Min26873 lines, see Fig.13, and the Min26874 lines (Fig.14) the secondary fluorescent antibody bound to the areas of preferred binding. Oct4 and Nanog relocated in the cells' nucleus, while SSEA3, SSEA4 and Tra-1-60 relocated to the cells' outer surface.

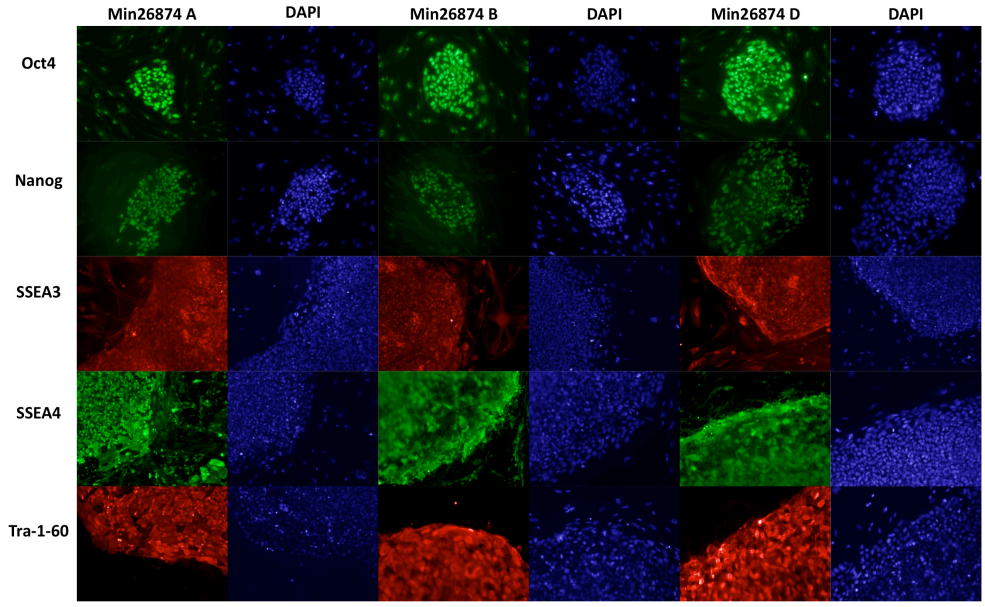

Fig. 14 Immunocytochemistry Assay of Min26874 A, Min26874 B and Min26874 D, respectively. The cells are shown with the secondary fluorescent antibody on the left and the according DAPI nucleus counterstain on the right.

Prior to PCR analysis, cell line samples were sent off for karyotyping, as an abnormal karyotype would have excluded that cell line from further analysis and proper send-off to investigators. The cells were cultured in T25 flasks for a period between 5 and 7 days and then sent off to Cell Line Genetics in Madison, Wisconsin, where analysis took place. The karyotype results were summarized by Cell Line Genetics in a report, as well as an image of the chromosomal line-up provided via e-mail 7 to 10 business days after the flask's arrival, provided cell growth was sufficient. As summarized by the institute, all cell lines appeared to have a normal karyotype with no aberrations. With this data in mind, the PCR assays were started.

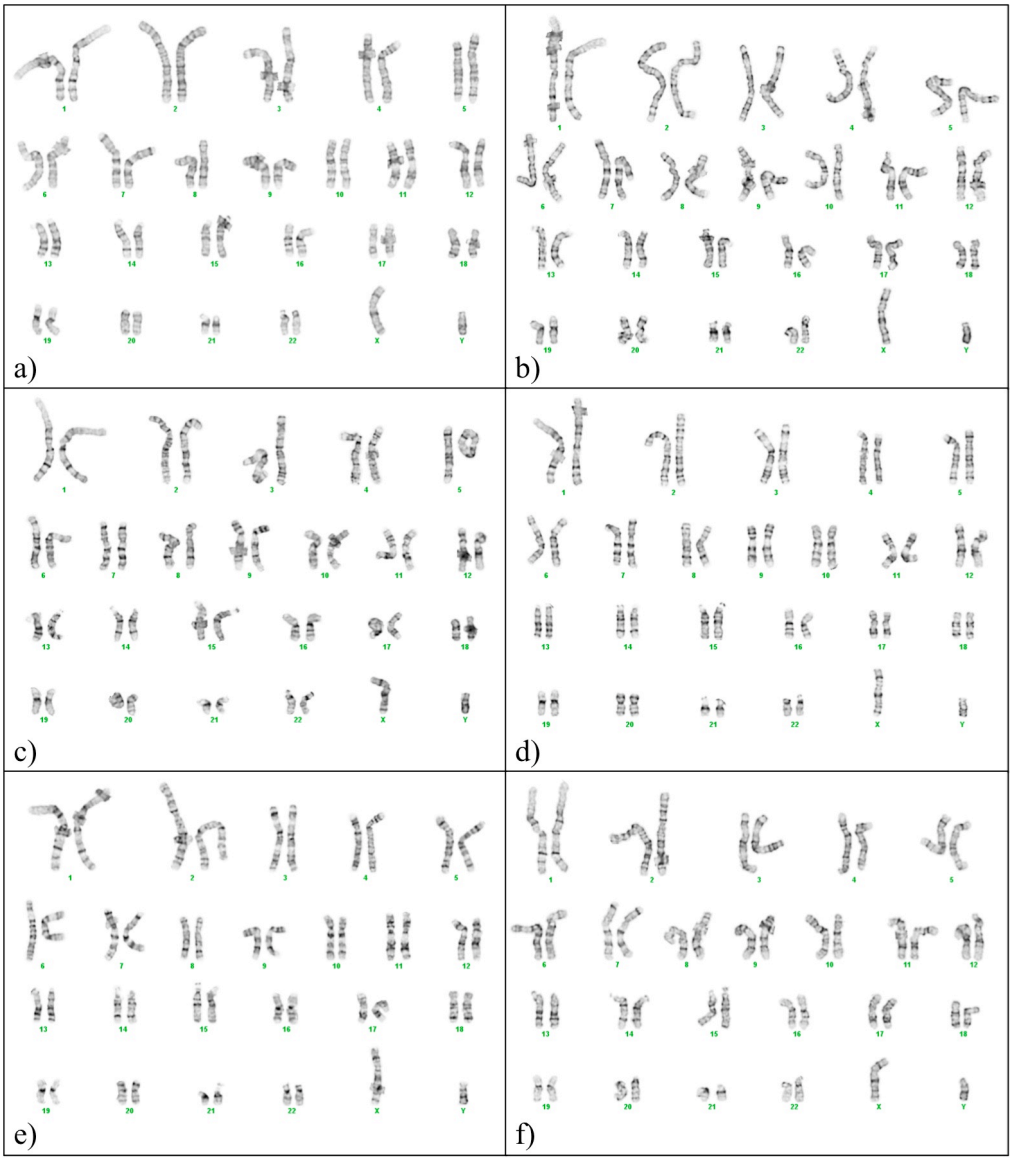

Fig. 15 Karyotype of Min26873 B (a), Min26873 G (b), Min26873 J (c), Min26874 A (d), Min26874 B (e) and Min26874 D (f) by Cell Line Genetics; no chromosomal abnormalities were detected; all six karyotpyes were male.

After the conclusion of the cell-culturing assays and the karyotyping, the cells were plated on Matrigel for a period between 5 and 7 days and RNA was collected. cDNA was synthesized and the PCR for the pluripotency markers Dnmt3b, hTERT, Nanog, endogenous Oct4, Rex1, endogenous Sox2 and Actin, used as a positive control, was performed.

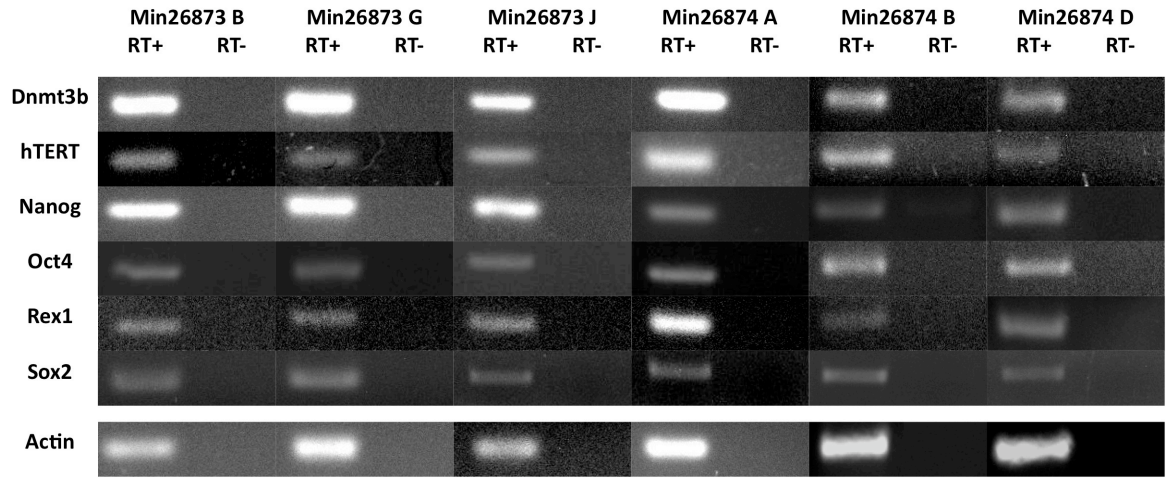

Fig. 16 PCR for Pluripotency Markers – the cell lines were analysed and express the markers Dnmt3b, hTERT, Nanog, Oct4, Rex1 and Sox2. Both Oct4 and Sox2 are endogenous markers to allow the differentiation between the regular and the virus-version of the markers. Actin, used as a positive control was expressed clearly by all cell lines.

In Fig.16, the results are summarized after the pictures taken of the gels were cut up and aligned according to their cell line. Actin was, as expected, expressed clearly by all Reverse Transcriptase positive samples. All pluripotency markers showed a definite expression band in the samples tested and concluded the pluripotency assay as positive.

Next, the RNA of the embryoid bodies grown in suspension for a week and then on gelatin for another was extracted and cDNA was synthesized using the same program as for the synthesis of cDNA of the pluripotency markers. The six markers for differentiation, Flk1 and GATA2 for mesoderm, AFP and GATA4 for endoderm and Pax6 and NCAM for ectoderm, as well as Actin, again used as a positive control, were tested.

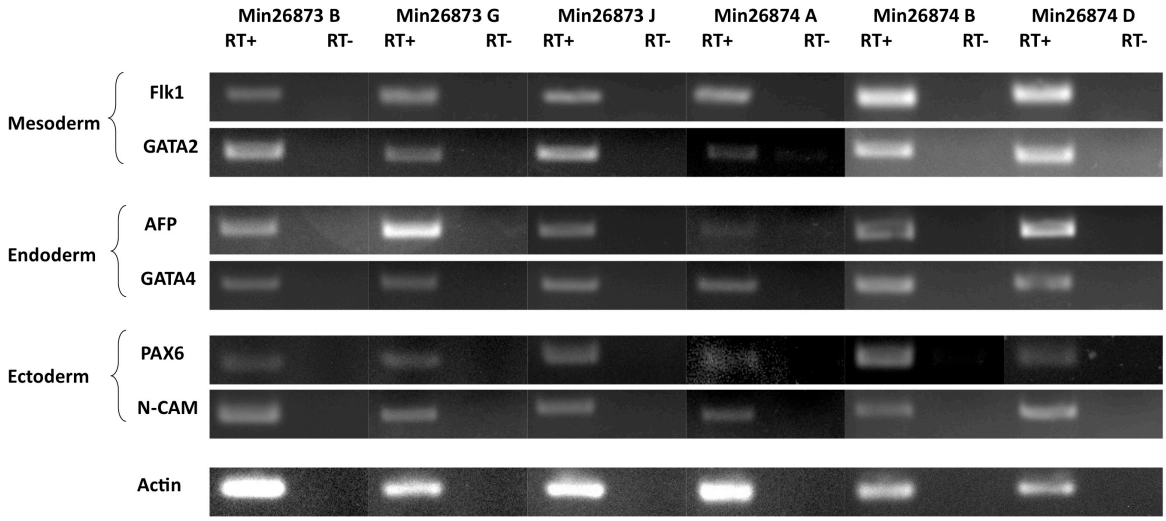

Fig. 17 PCR for Differentiation Markers – the embryoid body cell lines tested all expressed the markers that differentiate between the three germ layers. Actin, used as a positive control, was also clearly expressed by all cell lines.

The pictures of the bands were, again, cut up and aligned with the other markers according to the cell line (Fig.17). Actin was expressed by all Reverse Transcriptase positive samples in a clear manner. The expression of the other factors varied in intensity depending on the sample. However, a band was always visible for each line and the clear expression of the in each case other marker for the affected germ layer ensured the existence of the tested layer and the overall result of the assay was positive.

7. Summary

The fibroblast samples' derived iPS colonies lead, in the case of Min26873 to an efficiency of 0.011% and in the case of Min26874 to an efficiency of 0.005%, which is a relatively good quote in the derivation of induced pluripotent stem cell colonies.

Alkaline Phosphatase Treatment allowed for the first look at the state of the colonies' reprogramming status and the vibrant red color of the treated colonies gives insight on the pluripotency assumption of said cell lines. The immunocytochemistry assay shows the clear differentiation between the surface-located and the intranuclear stem cell markers and allows for the further conclusion that the tested cell lines express said markers in a definite and localized manner.

The cell lines all expressed normal male karyotypes and all pluripotency markers were expressed clearly. The tested differentiation markers assured the definite presence of all three germ layers. Though some markers were expressed less well than others, the other clearly expressed marker for the affected germ layer allows to presume the presence of the aforementioned layer. The mRNA development of the differentiated cell clusters at the point of collection show signs of variable transcription sequences, which may cause said irregularities in the expression of certain markers.

These results in mind, the experiments can be concluded with a positive result. The fibroblast samples' fully characterized cell lines show all signs of pluripotency, meaning their reprogramming into induced pluripotent stem cells was successful. Their differentiation potential was further on proven in the in-vitro differentiation assays, making them great targets for future projects. A handing-over of the cell lines to investigators is now possible.

All things considered, the discoveries and advances in recent years in the field of stem cell technology have by far been the most important. The development of iPS colonies from a skin biopsy in a rather simple method is a great way to introduce the technique into the medical world. However, the protocol to reprogram cells in this manner is time intensive and the efficiency, though higher than in other techniques, is very low. Furthermore, the full reprogramming and characterization of the cells is a rather high-priced task.

All in all, the need for alternatives is still given as the viral integration into the genome makes these cells unsafe for cell therapy. The recent developments in the field of RNA transfection derived iPS (RiPS) have led to a large step ahead and although the results of the tests so far are still a subject of controversy, the article gives insight into the work of the future.

For now, the use of induced pluripotent stem cells derived from skin biopsies, as developed by the iPS Core, offer a great alternative to embryonic stem cells in their disease-modeling abilities and, although their therapeutic use is still not possible, research in the field of stem cell technology is as important as ever to ensure the process to further equivalence of iPS cells to ES cells.

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