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induced pluripotent stem cell (iPSC) generation“

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1 Abbreviations

μg	Microgram
μl	Microliter
μM	Micromol
AP	Alkaline phosphatase
APC	Adenomatous polyposis coli
APC	Fluorochrome APC, Alexa Fluor 647
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate buffered Saline
Dsh	Dishevelled
FBS	Fetal bovine serum
Fz or Fzd7	Frizzled 7
GSK-3	Glycogen Synthase Kinase 3
hESC	Human embryonic stem cell
HUES9	Human embryonic stem cells (9)
iPSC	Induced pluripotent stem cell
IWP	Inhibitor of Wnt Production (= IWP-1)
IWR	Inhibitor of Wnt Response (= IWR-1-exo)
KMOS	Klf4, c-Myc, Oct4, Sox2 (4 Yamanaka factors)

KOS or w/o c-myc	Klf4, Oct4, Sox2 (3 Yamanaka factors)
KSR	Knock out serum
MCM	Condition media
MEF	Mouse embryonic fibroblasts
NEAA	Non-essential amino acid
PEI	Polyethyleneimine
ROR2	Receptor tyrosine kinase-like orphan receptor
Rpm	Rounds per minute
VPA	Valproic Acid Salt

2 Introduction

In the process of cellular differentiation an unspecialized cell becomes a more specialized cell type. Differentiation occurs multiple times during development of a multicellular organism. An undifferentiated cell which is able to divide and give rise to all cell types of an adult organism is known as pluripotent. In mammals, these pluripotent cells are called embryonic stem cells. Some years ago scientists wondered whether differentiation is terminal or can be reversed. This debate was put to rest when differentiated cells were reprogrammed to pluripotent cells. Several methods were established, some less or more efficient:

2.1 Reprogramming of somatic cells:

Recent breakthrough studies demonstrated that somatic cells can be reprogrammed to an embryonic stem cell-like, or induced pluripotent stem cell (iPSC) state (Yamanaka et al. 2006). This technology holds great promise for development of patient-specific cell populations suitable for cell replacement therapies. Reprogramming of somatic cells opened a broad field of scientific research and eliminated many of the ethical issues associated with embryonic stem cells.

There are several different methods to reprogram a somatic cell. The first and most useable methods are cell fusion, somatic nuclear cell transfer (SNCT) and the most common method for iPSC generation, the transduction of a defined set of transcription factors in somatic cells (King and Briggs 1953; Yamanaka et al. 2006 and Yu et al. 2007)

2.1.1 Somatic nuclear cell transfer (SNCT):

Somatic nuclear cell transfer is a laboratory technique to create a clonal embryo using an enucleated oocyte and a diploid donor nucleus. This technique is used in embryonic stem cell research and regenerative medicine referred to as therapeutic cloning. The donor nucleus of a somatic cell is removed and then inserted into a denucleated oocyte. The first example of a cloned model organism, the northern leopard frog, was by King and Briggs (King and Briggs 1953). They described the method of transferring a nucleus

from an embryonic cell into an enucleated and unfertilized egg of the same species (*Rana pipiens*). A couple of years later Gurdon (Gurdon JB et al. 1958) extended this postulate by cloning *Xenopus laevis* and modifying the technique. More recently, this technique of SCNT has been used to clone many other animals, including the sheep named Dolly (Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997). "Viable offspring derived from fetal and adult mammalian cells". *Nature* **385** (6619): 810–3).

2.1.2 Breakthrough - Generation of induced pluripotent stem cells by Yamanaka:

Induced pluripotent stem (iPS) cells can be created by reprogramming somatic cells with retroviral transduction of four transcription factors Oct4, Sox2, Klf4, and c-myc (Yamanaka et al. 2006) or Oct4, Sox2, Nanog and Lin28 (Yu et al. 2007).

Yamanaka selected 24 genes as candidate factors for iPSC generation (Figure 1) by narrowing them down to 10 and finally to the 4 genes listed above. It turned out, that the 4 factor transduction showed the most colonies and the best colony formation. (Yamanaka et al. 2006)

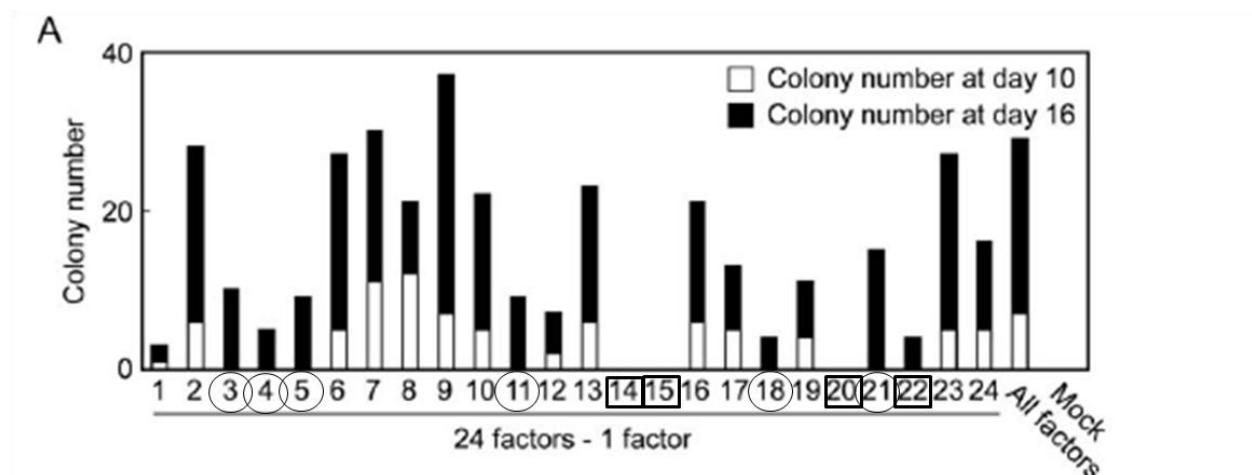


Figure 1: Effect of the removal of individual factors from the pool of 24 transduced factors (Yamanaka et al. 2006), marked are the 10 factors, which were used for further investigations. Rectangles: 4 factors, essentially used for general iPSC generation (Oct4, Sox2, Klf4, c-Myc); Circles: 6 factors which were in the short list, including β -catenin.

These iPS cells are thought to hold therapeutic potential, as somatic cells (mouse embryonic fibroblasts, MEF and adult mouse tail tip Fibroblasts, TTF) from an individual

could be reprogrammed and utilized to generate patient-specific cell types suitable for transplantation. For instance, it has been postulated that iPSC technology could be used to produce tissue for patients with retinal degeneration (Comyn et al. 2010). However, current methods of reprogramming require integration of retroviruses, which have been demonstrated to increase the risk of tumorigenesis (Okita et al. 2007). These methods suffer from the burden to potentially alter the recipient genome by gene insertion (Ho et al. 2010). However, depending on the cell type to be reprogrammed, some of these factors can be eliminated or replaced with small molecules. These alternative methods of generating iPS cells, without modifying the genome need to be established in order to improve the prospect of therapeutic applications of iPS cells, for instance protein-mediated (Kim et al. 2009) or RNA-mediated iPS cell production (Warren et al. 2010). A potential alternative method to achieve the iPS cell state is through manipulation of the culture conditions, or the extracellular microenvironment. Many developmental studies have demonstrated that differentiation and regenerative processes are regulated by multiple developmental signaling pathways. One such developmental signaling pathway is regulated by Wnt genes.

2.1.3 Method of reprogramming – Retroviral transduction:

This method was established by Yamanaka et al. 2006 and is described extensively in chapter 3.2.1.4 and the used materials in chapter 3.1.3.

First the transfection or virus production was performed. The retrovirus of 4 factors (Oct4, Klf4, Sox2 and c-Myc) was produced on HEK 293T cells, which are easy to handle and transfect. To get a high titer virus, it was recommended, to transfect several plates per vector. Each factor had a packaging (CMV-GP) and an envelope plasmid (VSVG, see Table 27). After transfecting 5-6 hours with transfection reagent PEI, the media was changed and the 72 hour transfection for virus collection was started. After the condition media was collected, the virus purification was started by centrifugation with an ultracentrifuge. The virus pellet was resuspended over night and used for transduction on the next day.

Second of all, the transduction into somatic cells or also virus infection was accomplished. The concentrated virus was used to infect adult human foreskin fibroblasts to be reprogrammed. The small amount of virus (usually around 200µl) was added to the normal culture media of fibroblasts and changed after 5-6 hours infection. Between 8 and 12 days post-transduction induced pluripotent stem cell colonies emerge.

2.2 Consideration of Wnt signaling in reprogramming process

Currently, 19 distinct WNT (*Wingless* and *INT-1*) genes have been identified that code for secreted lipid-modified glycoproteins (see the “Wnt homepage” at <http://www.stanford.edu/group/nusselab/cgi-bin/wnt/>; Nusse et al.2009). Wnt signaling is involved in embryonic development and is required for tissue homeostasis of adult organs (Clevers et al. 2006). Germline mutations in the Wnt pathway cause several hereditary diseases, and somatic mutations are associated with cancer of the intestine and a variety of other tissues (Clevers et al. 2006).

Mutations in WNT genes or disruptions in the Wnt signaling pathway can catastrophically alter human embryonic development (Logan et al. 2004) and can contribute to disease, most notably cancer (Clevers et al. 2006, Kinzler et al. 1996, Klaus et al. 2008). For example loss of WNT3 function has been shown to lead to tetra-amelia, a rare and fatal genetic defect (Niemann et al. 2004). Additionally, the Wnt signaling cascade has been demonstrated to exert significant effects on adult stem cells of the intestine (Gregorieff et al. 2005), skin (Gat et al. 1998, Ito et al. 2007), brain (Chenn et al. 2002, Kalani et al. 2008, Lie et al. 2005, Soen et al. 2006) and blood (Reya et al. 2003, Willert et al.2003, Luis et al.2009).

More recent studies also suggest that Wnt signaling influences the efficiency of induction of the pluripotent state, even in the absence of c-Myc (Marson et al. 2008). This raises the possibility of manipulating Wnt signaling as a means of inducing pluripotency without the use of viral transduction of transcription factor genes, which carry the risk of oncogenic mutations.

Several lines of evidence indicate that Wnt signalling affects iPSC generation (Yamanaka et al. 2006, Marson et al. 2008). However, it is not known whether Wnt signaling is essential to the reprogramming process. β -Catenin was one of the 24 genes which were used as candidate factors (Figure 1). As far back as Yamanaka's breakthrough, he showed the importance of β -catenin and the Wnt pathway involvement in iPSC generation.

The role of β -catenin in Wnt signaling is indispensable. Wnt/ β -catenin signaling is called 'The Canonical Wnt Signaling Pathway' (Figure 2). In the absence of Wnt signaling, Glycogen synthase kinase (GSK-3) constitutively phosphorylates the β -catenin protein. β -catenin is associated with Axin, a scaffold-protein, complexed with GSK-3 and adenomatosis polyposis coli (APC). Phosphorylated β -catenin is subsequently degraded, so that its steady state levels are low. Wnt signaling is initiated when Wnt binds its receptor, frizzled (Fz), and dishevelled (Dsh/Dvl) is recruited to the membrane. GSK-3 is inhibited by the activation of Dvl. Accordingly, β -catenin accumulates and can be subsequently translocated into the nucleus where Wnt target genes are activated (Figure 2). Thus β -catenin functions as key component in canonical Wnt signaling. Additionally, β -catenin is also part of the complex which forms adherens junctions for cell-cell-adhesion.

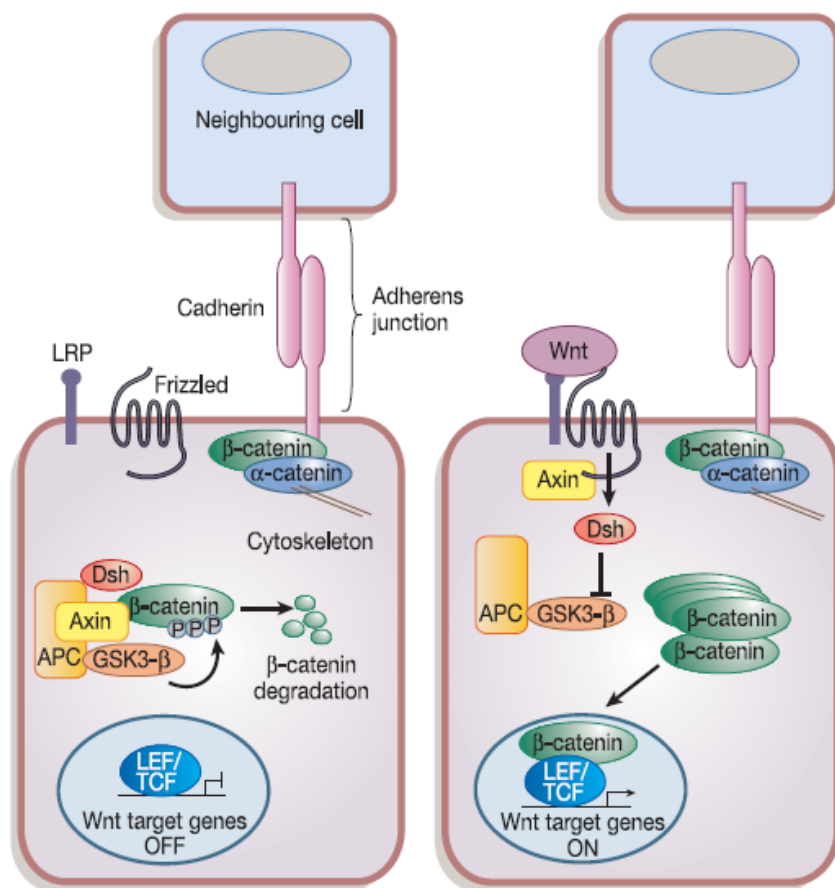


Figure 2: Canonical Wnt Signaling (Reya et al. 2005)

2.3 Use of Wnt signaling pathway inhibitors

Wnt signaling proteins are small secreted proteins that are active in embryonic development, tissue homeostasis, and tumorigenesis. Wnt proteins bind to receptors on the cell surface, initiating a signaling cascade that leads to β-catenin activation of gene transcription. Chen B. et al. 2009 identified two classes of small molecules that disrupt Wnt pathway responses. Whereas one class inhibits the activity of porcupine the other abrogates destruction of Axin proteins suppressors of the Wnt pathway activity.

- The first class is IWP compounds, which are inhibitors of Wnt production. IWP compounds target the acyltransferase porcupine and inactivate it. Porcupine is a member of the membrane-bound O-acyltransferase (MBOAT) family and usually adds a

palmitoyl group to Wnt proteins that is essential for the production of Wnt proteins and their signaling ability. IWP action is that it inactivates Porcupine function, either by directly inhibiting the porcupine or a porcupine regulator.

- The second class is IWR compounds, which are inhibitors of Wnt response. Through the addition of IWR compounds, Axin protein levels rise, accompanied by elevated levels of β -catenin phosphorylation which lead to a proteasome-mediated destruction of β -catenin. IWR compounds induce stabilization of Axin proteins via direct interaction that could be used to control levels of Wnt pathway response.

Another suppressor is GSK3-inhibitor (factor XV) which is a highly bioreactive complex that acts as an inhibitor by binding to the ATP binding site through an induced fit mechanism. In addition this complex induces the Wnt signaling pathway in mammalian cells.

2.4 Objectives of my work

The goal of this project is to study the influence of Wnt signaling on the regulation of induced pluripotency. We hypothesize that Wnt signaling is a necessary component of the reprogramming process capable of inducing and maintaining the pluripotent state. The results of this project should provide significant insight into the possibility of utilizing Wnt proteins as reagents to generate and maintain iPS cells. We will perform standard reprogramming of human fibroblasts using this method while perturbing Wnt signaling in a number of ways. The used modulators are IWP-1, IWR-1-exo, GSK3-inhibitor (factor XV), Wnt3a and Wnt5a. IWP-1 and IWR-1-exo should suppress Wnt signaling (see chapter 2.3). GSK3-inhibitor inhibits Wnt signaling in a double negative way; therefore Wnt signaling will be activated. Wnt3a activates the canonical pathway and Wnt5a activates the non-canonical one but inhibits the canonical Wnt signaling. Axin and β -catenin are two genes, which will be overexpressed, and Frizzled 7 and Receptor tyrosine kinase-like orphan receptor (ROR2) knocked down. Adult human foreskin fibroblasts were used as somatic cells for the reprogramming process. For further used cell types see chapter 3.1.2.

A long-term goal of our studies is to generate iPS cells with soluble factors, including Wnt proteins, rather than the four retroviral factors, which need to be transduced into the cells, thereby permanently and potentially adversely altering the genomic make-up of the reprogrammed cell.

We established the basic methodology for generating iPS cells using the factors from the Yamanaka laboratory (Yamanaka et al. 2006, Takahashi et al. 2007). Willert's lab studies the role of Wnt signaling in embryonic stem cells and this project on iPS cell generation is a natural extension of this work.

3 Materials and Methods

3.1 Materials:

3.1.1 Equipment:

Table 1: Used equipment

device	device name	supplier
Beckman centrifuge tube	Cat.nr. 358126	Beckman
cell culture plates (15cm,10cm,6-well, 12-well)		Coring
cell Strainer (40µm)		BD Falcon
Cellomics microarray	Cellomics VTI High Content Screening System	Thermo Fisher Scientific Inc.
centrifuge	5810R 15amp version	Eppendorf
centrifuge	Avanti J-E Centrifuge	Beckman Coulter
conical tubes (15ml, 50ml)		Sarstedt, Corning
cryo 1°C mMr. Frosty freezing container with isopropanol		NUNC
cryo-vials		Nunc
EVOS dissection micorscope	EVOS	AMG
FACS tube		BD Falcon
flow cytometer	BD FACSCanto II	BD Biosciences
freezer -20°C		Kenmore
freezer -80°C/-150°C		Sanyo- VIP Series
fridge 4°C		Sanyo
gelelectrophoresis device		Biorad
gene pulser cuvette (0.2cm)		Biorad

genepulser		Biorad
imager (gels, plates)		Alpha Innotech
incubator	Sanyo Co ₂ Incubator	Sanyo Scientific
incubator shaker	Max-4000, A-Class	Barnstead-Lab Line
isopropanol		Sigma Aldrich
maxiprep kit	NucleoBond	Macherey-Nagel
microscope	Axiovert 40, CFL	Zeiss
nanospectrometer	ND-1000	Nanodrop
PCR machine		Eppendorf
qPCR machine	7900 HT Fast Real-Time PCR System	Applied Biosciences
qPCR plate		Sigma Aldrich
RNA isolation Kit	NucleoSpin RNA II	Macherey-Nagel
scale	S-4001	Denver Instrument
sterile filter (0.22µm)		Millipore
sterile one-way pipettes		Costar
syringe Luer-Lok Tip (5ml, 10ml)		BD Falcon
tissue culture hoods	Class II Biohazard Safety Cabinet	ESCO
ultracentrifuge	Optima L-80 XP Ultracentrifuge	Beckman Coulter
waterbath	Aquabath	Barnstead-Lab Line

3.1.2 Cell types

- *Human embryonic Kidney (HEK) 293T Cells:* (America Type Culture Collection - <http://www.atcc.org>). HEK 293T cells are easy to culture and transfect. The transfectability by various techniques is about 100% and they have a high expansion rate.

In my work, these cells were used for lentiviral and retroviral production.

- *Primary mouse embryonic fibroblasts:* Millipore-Speciality Media, Embryomax ® PMEF-P3, Strain CF-1, Cat# PMEF-CF, Lot# 90927-4, Store -80°C 00004)
PMEF feeders are treated with mitomycin-C and can be directly plated. These cells are from passage 3. $5-6 \times 10^6$ cells will produce the appropriate density feeder layer.

In my iPSC protocol a monolayer of primary mouse embryo fibroblast (PMEF or MEF) was used as feeder cells. They perform two important roles in stem cell culture: they secrete several important growth factors into the medium, which help maintain pluripotency, and they provide a cellular matrix for iPS cells to grow.

- *Adult human foreskin Fibroblasts (WT83)*
Cassiano Carromeu from the Muotri Lab at UCSD established this cell line. The cell line has a low passage number and its abbreviation is WT83.
- *Human embryonic stem cells:*

The human embryonic stem cell lines are pluripotent and were previously derived from human blastocysts (Cowan et al N Engl J Med. 2004 Mar 25;350(13):1353-6; Thomson et al. Science. 1998 Nov 6;282(5391):1145-7).

In my work I used the HUES9 (Harvard University, HUES cell facility) line mainly as positive control for pluripotency. Pluripotency antibody markers are SSEA-3, SSEA-4, Tra 1-60, Tra 1-81, Oct-4 and staining for alkaline phosphatase.

(<http://www.mcb.harvard.edu/melton/HuES/>, <http://stemcells.nih.gov>)

3.1.3 Materials for cell culture

3.1.3.1 Reagents, chemicals, small molecules, buffers:

Table 2: List of reagents, chemicals, small molecules and buffers used for cell culture

product	supplier
2-mercaptoethanol (55μM final) 55mM stock	GIBCO-Invitrogen
Accutase	Millipore
bFGF, stock: 100ng/μl	Millipore
Collagenase	GIBCO-Invitrogen
DMEM (different types)	Cellgro
DMSO	Sigma Aldrich
FBS (fetal bovine serum)	GIBCO-Invitrogen
Gluta-MAX	GIBCO-Invitrogen
GSK3-inhibitor (Factor XV) 10mM	Calbiochem; EMD4 Biosciences Santa Cruz
HY Clone-DPBS/Modified	Thermo Scientific
IWP; Inhibitor of Porcupine (IWP-1) 5mM	Lawrence Lum lab; University of Texas, Southwestern Medical Center Dallas
IWR; Inhibitor of Axin (IWR-1-exo) 10mM	Lawrence Lum lab; University of Texas, Southwestern Medical Center Dallas
Knock out serum (KSR)	GIBCO-Invitrogen
Matrigel	BD
NEAA	Cellgro

Penicillin/Streptomycin	GIBCO-Invitrogen
Plasmanate	Talecris Biotherapeutics
TrypLE™ Express	GIBCO-Invitrogen
Valproic acid sodium salt (VPA) 10mM dissolved in DPBS	Sigma Aldrich
WNT protein buffer: 1%CHAPS, 1x PBS, 1M NaCl	1%CHAPS, 1x PBS, 1M NaCl; various vendors; e.g. BIOTANG
CHAPS	
3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate	
Wnt3a, stock: 2.5µM	Willert lab; UCSD
Wnt5a, stock: 2.5µM	Willert lab; UCSD

3.1.3.2 Growth Media:

Table 3: Composition of growth media for 293T cell culture, foreskin fibroblast cell culture and MEF cells culture.

Ingredient	Amount (specifications for 550ml-560ml)
DMEM 1x	500ml
FBS (10%)	50ml
Penicillin/Streptomycin (1%) (optional)	5ml
Glutamine (optional)	5ml

Table 4: Composition of growth media for 293T cell culture used after transfection

Ingredient/supplier	Amount (specifications for 520ml-530ml)
DMEM 1x	500ml
FBS (4%)	20ml
Penicillin/Streptomycin (1%) (optional)	5ml
Glutamine (1%) (optional)	5ml

Table 5: Composition of growth media for human embryonic stem cell (HUES9) culture.

Ingredient /supplier	Amount (specifications for 500ml)
Knockout DMEM	385ml
Knockout Serum	50ml
Plasmanate	50ml
NEAA	5ml
Penicillin/Streptomycin	5ml
Gluta-MAX	5ml
2-mercaptoethanol (55µM final) 55mM stock	0.5ml

HESCs (HUES9) require bFGF for undifferentiated growth. For best results it is recommended to add bFGF immediately prior to use (final concentration of 20ng/ml)

Table 6: Composition of growth media for induced pluripotent stem cell generation.

Ingredient/supplier	Amount (specifications for 200ml)
DMEM/F-12,50/50, 1x	158ml
Knockout Serum	40ml
NEAA	2ml
2-mercaptoethanol (55µM final) 55mM stock	0.4ml

Table 7: Composition of freezing media.

Ingredient/supplier	Amount (specifications for 50ml)
DMSO (10%)	5ml
FBS (90%)	45ml

3.1.4 Materials for microbiological methods

3.1.4.1 Microorganisms:

E.Coli DH5 α cloning strain was used for all transformations. The bacteria were transformed with high efficiency. Successful DNA transformation was ensured by antibiotic selection. All plasmids carried the gene that confers ampicillin resistance.

3.1.4.2 Growth media:

Table 8: Chemical composition of Terrific Broth (Mediatech-VWR-Cellgro).

ingredient	amount (specifications for 1000ml)
Casein Peptone	12 g
KH ₂ PO ₄	2.31g
K ₂ HPO ₄	12.54g
UF Yeast Powder	24
Glycerol	4ml

Table 9: Chemical composition of Miller's Luria Batani (LB) Broth (Mediatech, Inc)).

ingredient	amount (specifications for 1000ml)
Casein Peptone	10g
NaCl	10g
UF yeast powder	5g

3.1.4.3 DNA-plasmids:

Table 10: List of DNA plasmids used for retroviral production of the four Yamanaka factors (www.addgene.org)

name (retroviral)	concentration ng/ μ l	ratio 260/280
pMX-hc-Myc	2513.10	1.88
pMX-hKlf4	2853.21	1.86
pMX-hSox2	3548.31	1.85

pMX-hOct4	3468.25	1.85
CMV-GP (packaging vector)	1928.67	1.89
VSVG (envelope vector)	4910.81	1.57

Table 11: List of DNA plasmid constructs used for lentiviral production to knockout or overexpress Wnt pathway compounds (www.addgene.org)

name (lentiviral)	concentration ng/μl	ratio 260/280
β-catenin-HIV-IRE	4059.48	1.79
Axin-HIV-IRE	1631.02	1.84
Fzd7(5)shRNA	4684.21	1.67
ROR2shRNA-625	2972.04	1.86
ROR2shRNA- 1490	2196.50	1.88
Lentiviral GFP reporter	1494.86	1.89
psPAX2 (packaging vector)	3554.25	1.84
VSVG (envelope vector)	4536.86	1.71

Table 12: Concentration and pH of the retroviral and lentiviral transfection reagent polyethyleneimine (PEI)

PEI concentration: 1μg/μl	1 x PBS pH 4.45
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3.1.5 Materials for molecular biological methods

3.1.5.1 Kits

Table 13: List of kits used for molecular biological methods.

Product	cat. no.	Supplier
RNA isolation	740955	Nucleospin RNAII; Macherey-Nagel
Plasmid DNA Purification (Maxiprep)	12162 740414	• Qiagen • NucleoBond; Macherey-Nagel
Alkaline Phosphatase (AP) Staining Kit	000009	StemGent

3.1.5.2 Buffers

Table 14: List of buffers used for molecular biological methods.

Product	supplier
DPBS	Mediatech
50x TAE Buffer (Tris Base, Acetic acid, EDTA)	242g TrisBase, 57.1ml glacial acetic acid, 18,6g EDTA for 1000ml

Table 15: Buffers and reagents for Maxiprep (NucleoBond; Macherey-Nagel)

product	amount
Buffer RES	150ml
Buffer LYS	150ml
Buffer NEU	150ml
Buffer EQU	500ml
Buffer WASH	300ml
Buffer ELU	180ml
Rnase A (lyophilized)*	10mg

70% Ethanol (Sigma-Aldrich)	50ml
Isopropanol (Sigma-Aldrich)	50ml
1x TE-Buffer (pH: 8.0)	50ml

* Rnase A was dissolved by the addition 1ml buffer RES. After dissolving, the Rnase A solution was transferred back to the bottle containing buffer RES. The buffers final concentration of Rnase A is 60µg/ml.

Table 16: Buffers and reagents for RNA isolation (Nucleospin RNAII; Macherey-Nagel)

product	amount
Lysis Buffer RA1	25ml
Wash Buffer RA2	15ml
Wash Buffer RA3	12.5ml
Membrane Desalting Buffer (MDB)	25ml
Reaction Buffer for rDNase	7ml
rDNase (Rnase-free, lyophilized)*	1 vial
Rnase-free H ₂ O	15ml
2-Mercaptoethanol (GIBCO)	50ml
70% Ethanol (Sigma Aldrich)	50ml

*540µl of Rnase-free H₂O was added to the rDNase vial and incubated for 1 minute at room-temperature. The Dnase was completely dissolved and aliquoted 10µl per tube for storage at -20°C.

3.1.5.3 Reagents, chemical, primers and enzymes:

Table 17: List of reagents and chemicals used for molecular biological methods

product	supplier
UltraPure Agarose	Invitrogen
1kbDNA ladder O'GeneRuler	Fermentas
0.1µg/ml	

6x Loading dye	Fermentas
Sybr Safe DNA gel stain (10,000x concentration in DMSO)	Invitrogen
qScript cDNA Supermix	Quanta; Biosciences Inc.
TaqMan ® Mastermix	Gene Expression Assays – Applied Biosciences
TaqMan ® Probes (18S, Nanog, Pou5F1)	Gene Expression Assays – Applied Biosciences

Table 18: List of primer sequences used for molecular biological methods to verify pluripotency of established iPS cell lines

name	sequence
Nanog	TCACACGGAGACTGTCTCTCCTCTT
Pou5f1	GTGAATGACATTTGTGGGTAGGTTA

Table 19: List of restriction endonucleases and restriction endonuclease buffers (www.neb.com) to verify the purified plasmids

restriction enzymes & buffers	supplier
EcoRI (NEB3 or EcoRI buffer) 20U/μl	New England Biolabs (NEB)
XhoI (NEB4) 20U/μl	New England Biolabs (NEB)
NotI (NEB3)10U/μl	New England Biolabs (NEB)
PvuII (NEB2) 10U/μl	New England Biolabs (NEB)
HindIII (NEB2) 10U/μl	New England Biolabs (NEB)
10x NEBuffer 2, 100x BSA	New England Biolabs (NEB)
10x NEBuffer 3, 100x BSA	New England Biolabs (NEB)
10x NEBuffer 4, 100x BSA	New England Biolabs (NEB)
10x EcoRI buffer , 100x BSA	New England Biolabs (NEB)

3.1.6 Materials for analytical methods

3.1.6.1 Chemicals, buffers, reagents

Table 20: List of chemicals used for analytical methods

product	supplier
Trypan Blue Stain 0.4%	Gibco-Invitrogen
Albumin from bovine serum (BSA)	Sigma
DAPI	Sigma Aldrich
Glycine Solution (2.6M) in PBS	J.T. Baker

Table 21: Chemical composition of flow cytometry buffer (FACS-Buffer)

ingredients	amount (specifications for 510ml)
DPBS	500ml
BSA (1%)	0.5g
EDTA (10mM)	10ml of 0.5M EDTA

3.1.6.2 Antibodies

Table 22: List of antibodies used for analytical methods

product	supplier
Alexa Fluor 647 mouse anti-human Tra1-81 IgM (conjugated) 5µl/1million cells	Biolegend
Isotype Control: Alexa Fluor 647 mouse IgM,κ 5µl/1million cells	Biolegend
PE mouse anti-human SSEA-4 (MC-813-70 Ig G3 (conjugated) 5µl/1million cells	Biolegend
Isotype Control PE mouse Ig G3, κ	Biolegend

3.1.6.3 Alkaline phosphatase staining kit (StemGent)

Table 23: Alkaline phosphatase staining kit used for iPSC generation quantification

product	amount
PBST (0.05% Tween)	500ml
Fix Solution	25ml
Solution A	10ml
Solution B	10ml

3.1.7 Software programs for bioinformatic methods

Table 24: Software programs and databases used for bioinformatic methods

software program/database	supplier/website
Addgene	http://www.addgene.org
Pubmed	http://www.ncbi.nlm.nih.gov/pubmed
National Center for Biotechnological Information	http://www.ncbi.nlm.nih.gov/
Cell culture information	http://www.tpp.ch/technical_information/product_information/Cell_cultivation.htm
Flow Jo	http://www.flowjo.com/
Cellomics Software (data not shown)	http://www.cellomics.com ArrayScan® VTI (500 Series)Version 5.1.10-0.63x (Build 6027)
Microsoft Office	http://office.microsoft.com/
Photoshop CS5	http://www.photoshop.com
The Global Bioresource Center	http://www.atcc.org
HUES Cell Collection	http://www.mcb.harvard.edu/melton/HuES/
Stem Cell Information	http://stemcells.nih.gov

3.2 Methods:

3.2.1 Cell culture methods

3.2.1.1 General procedures:

All cell culturing was performed in the UCSD Human Embryonic Stem Cell Core Facility, under sterile conditions, using the Class II Biohazard Cabinet, unless otherwise stated. Sterile one way materials were used. Lab coats and gloves were always worn. Media or other reagents were warmed up to 37°C and hands were washed with 70% ethanol before working or touching cell dishes. Cells were cultured on 15cm, 10cm or 6-well plates and stored at 37°C with 5% CO₂. The cell confluency was checked under the light microscope (Zeiss)

Table 25: List of reagent volumes according to dish sizes

dish size	growth media vol.	DPSB vol.	dissociation vol.	bFGF vol.
15cm dish	20ml	15ml	TrypLE 3ml	5µl
10cm dish	10ml	10ml	Accutase 3ml, TrypLE 2ml	3µl
6-well	3ml	3ml	TrypLE 1ml, Collagenase 1ml	1µl
12-well	1.5ml	1.5ml	Tryple 0.5ml	0.5µl

3.2.1.2 Culture of HEK 293T cells

HEK 293T cells were always cultured in a 15cm plate with DMEM 1x + 10%FBS, except for production of retro- or lentivirus when DMEM 1x + 4% FBS was used.

- Cell thawing:

A vial of cells was thawed in the waterbath at 37°C. 9ml of pre-warmed media were added to a 10cm dish, and after entirely thawing the cell suspension, cells were spread evenly over the plate and gently mixed.

- Cell passaging:

Cells were washed gently with DPBS and TrypLE was added (amounts of reagents to their according dish size, see Table 25). The plate was incubated at 37°C for 5 minutes. The dissociated cells were easily removed by adding Media. For better dispersion, the cell suspension was pipetted up and down 5 times. Depending on the requested density, the appropriate amount of cell suspension was transferred into the next culturing plate and gently mixed to spread them out. Usually cells were split 1:5 every other day.

- Cell freezing:

Until cell dissociation the procedure was done as already described in chapter 3.2.1.2—cell passaging. Afterwards the cell suspension was transferred into a 15ml conical tube and centrifuged at 200 x g for 4 minutes. The liquid was aspirated and the cell pellet was resuspended in 1ml freezing media per 12-16 x 10⁶ cells (Table 28). The cells were transferred immediately into a cryo-tube, inserted into a Mr. Frosty (Nalgene Labware) containing Isopropanol. Mr. Frosty was stored at -80°C overnight allowing the cells to slowly freeze (approximately 1°C decrease per minute). After one day at -80°C the tubes can be transferred into the -150°C freezer.

3.2.1.3 Transfection for lentivirus (2nd Generation):

Transfection

Lentivirus was always produced by transfecting HEK 293T cells. The cell dish was 70% confluent, or ~11 x 10⁶ cells per 15cm dish. To increase the amount of virus it was

recommended to transfect at least two plates per virus. Specific amounts of DNA were used (see Table 26).

Table 26: Amount of plasmid DNA for lentiviral transfection in one 15cm plate

vector	amount (µg)	molar ratio	size (kb)
Transfer vector	22.5	2	8-9
psPAX2	14.6	1	10.7
VSVG	7.9	1	5.8

The plasmid mix (see Table 26) was added to 1ml of DMEM 1x, filtered, and 4 times the amount of DNA, of PEI (1µg/µl) was added. The tube was vortexed and incubated for 5 minutes until PEI neutralized the anionic colloidal charge. The 1ml was spread out over the plate. Mixed briefly and incubated at 37°C at 5% CO₂. The media was changed 5-6 hours post-transfection.

Virus collection:

The first virus collection was performed 48 hours post-transfection. The supernatant was filtered (0.22µm filter unit) and stored at 4°C. 72 hours post-transfection, a second media collection was done. First and second virus collections were pooled and processed as follows.

Virus purification:

For purifying the virus, the Beckman Coulter Optima L-80 XP Ultracentrifuge with the SW 32 Ti rotor was used, which fits 6 centrifuge capsules. Before loading the virus, the capsule was placed into a tube holder on a laboratory scale. An adapter and the Beckman centrifuge tube were inserted and the scale was tared. The ultracentrifuge requires that all samples are perfectly balanced against each other. The amount of 28g of liquid virus was not exceeded and the two capsules towards each other were supposed to have a difference not more than 0.05g. If compensation was needed in any tube, DMEM F-12 was added.

If the tubes were not filled with the correct amount of liquid, they would have collapsed during centrifugation. These special tubes need an adapter to properly fit in the capsules.

After loading virus and capsules, the ultracentrifuge was set up at 19400 rpm and 4°C. Two hours later the centrifuge tubes were carefully removed from the capsules. On the bottom of the tube a small pellet should be visible. The supernatant was discarded into 10% bleach and the centrifuge tubes inserted into 50ml conical tubes. 100µl of DMEM-High Glucose was added onto the cell pellet and not-mechanically resuspended overnight at 4°C. On the next day fibroblasts were infected with 150-200µl of virus. (see chapter 3.2.1.10)

3.2.1.4 Transfection for retrovirus: Yamanaka cocktail (Oct4, Sox2, Klf4, c-Myc)

The method for generating iPS cells has been described (Yamanaka et al. 2006, see chapter 2.1.2). In this thesis the factors to induce the pluripotent state are called “Yamanaka factors”, “four factors” or “KMOS”, the abbreviation for Oct4, Sox2, Klf4, c-Myc.

The retrovirus was produced on HEK 293T cells as well. The cell dish was 80-100% confluent, or 16×10^6 – 20×10^6 cells per 15cm dish. To achieve a high titer virus, it was recommended to transfect at least 6 plates per vector. To generate all four viruses carrying the Yamanaka factors 24 plates were used. For specific amounts of transfected DNA, see table 27.

Table 27: Amount of plasmid DNA for retroviral transfection in one 15cm plate

vector	amount (µg)	size (kb)
Transfer vector (factor)	13.8	5.5-6
CMV-GP	9.2	~12
VSVG	4.6	5.8

The DNA-mix (see Table 27) was added to 1ml of DMEM 1x, filtered and 4 times the amount of DNA, of PEI (1µg/µl) was added. The tube was vortexed and incubated for 5

minutes until PEI neutralized the anionic colloidal charge. The 1ml was spread out over the plate. Mixed briefly and incubated at 37°C at 5% CO₂. 5-6 hours post-transfection the media was replaced to 16ml DMEM 1x with 4% FBS to avoid cell overgrowth.

Virus collection:

The supernatant was collected only once after 72 hours post-transfection and all factors were pooled to simplify the virus purification. The virus collection was performed as already described in chapter 3.2.1.3.

Virus purification:

To harvest the virus, the description in chapter 3.2.1.3 was followed. Usually two centrifugations were set up for 24 plates containing 16ml of virus.

On the next day, the virus was pooled, pelleted at 200 x g for 4 minutes and one 6-well of fibroblasts was infected with concentrated 200µl of virus-supernatant.

3.2.1.5 Transfection for retrovirus: 3 Yamanaka factors without c-Myc (Oct4, Sox2, Klf4)

The Yamanaka factors without c-Myc are called “three factors” or “KOS”, the abbreviation for Oct4, Klf4, Sox2.

To set up a 3 factor retrovirus, 18 plates were used and the virus production was performed as described in chapter 3.2.1.4 without transfection of the transfer vector c-myc.

3.2.1.6 Culture of adult human foreskin fibroblasts

Early passage human foreskin fibroblasts were cultured in 10 cm dishes using DMEM 1x + 10%FBS.

- Cell thawing, passaging, freezing:

The thawing, passaging and freezing procedures of human foreskin fibroblasts were done as already described in chapter 3.2.1.2 using the right amount of volumes (Table 25) fibroblasts needed longer for their dissociation and additionally could be used a cell scraper to completely detach all cells.

3.2.1.7 Culture of MEFs

Mouse embryonic fibroblasts (MEFs) were always cultured in DMEM 1x with 10% FBS. This cell type was previously mitotically inactivated by radiation. As a result MEFs were never passaged. After thawing, the cells were useable for up to one week.

- Cell thawing and preparation of MEF plates:

One vial of MEFs was used for eight 10cm dishes or other plate sizes with equal surface size.

The thawing procedure of mouse embryonic fibroblasts was done as already described in chapter 3.2.1.2 using the right amount of volumes (Table 25).

- MEF plates used for condition media (MCM):

A day after preparing MEF plates (10cm) the media was changed to 15ml of HUES9 or iPSC media (see chapters 3.2.1.8 and 0). This step was performed every 24 hours for 7 days, and the condition media was collected by filtering. The MCM was useable for two weeks.

- MEF plates used for feeder cells:

Depending on the experiment, the size of dishes was chosen. For iPSC generation MEF cells were prepared on 6-well plates, one day before transferring other cells onto feeders.

3.2.1.8 Culture of HUES9

HUES9 cells were always cultured on MEF plates or matrigel-coated plates.

- Preparation of matrigel plates:

A vial of matrigel (BD) was thawed on ice for 30 minutes and resuspended in 25ml of DMEM-F12. The right amount of matrigel suspension was added to cover the plate, followed by 15 minutes incubation at 37°C. During the incubation time, the matrigel proteins were self-assembling, producing a thin film which covered the surface of the cell dish. After incubation the plates could be used immediately or stored at 4°C.

- Production of HUES MCM:

HUES MCM was produced as already described in chapter 3.2.1.7

- Cell passaging:

To passage, HUES9 cells were gently washed with DPBS and incubated with accutase (amount of volumes see table 25) for 4 minutes. The plate was agitated to help the colonies detach. When HUES9 colonies were dislodged, DMEM-F12 was added, the cells collected and pelleted at 200 x g for 4 minutes to get rid of the accutase. The right amount of cell signaling32 was taken and HUES-MCM and bFGF (100ng/μl) were added. Usually cells were passaged every 2 days 1:10.

- Maintenance of HUES cells in a pluripotent state:

HUES9 cells could either grow on feeder cells (MEF) or on matrigel coated plates.

Extracellular matrix components of Matrigel or feeder cells were necessary to maintain the pluripotent state. Additionally, the HUES9-MCM media had to be changed every day and the growth factor bFGF (100ng/μl) was added every day as well.

3.2.1.9 Culture of iPS cells

- Production of iPSC-MCM:

iPSC-MCM was produced as already described in chapter 3.2.1.7.

- Cell passaging (mechanically):

Single iPSC colonies were isolated under the EVOS microscope.

The media was aspirated and 1ml new iPSC-MCM was added. The chosen colonies were dissected from the cell layer using a 26 gauge needle attached to a syringe. This layer was built up between MEFs, non-infected fibroblasts and iPSC colonies. Since the cell layer was sliced, the colonies could be easily removed by scratching them off with a 20ul pipet tip. The 1ml media and floating colonies were collected and transferred to a matrigel plate (production of matrigel plates, see chapter 3.2.1.8), where additional 2ml of iPSC-MCM and growth factor bFGF (100ng/μl) was added.

- Pluripotent maintenance:

iPSC colonies could either grow on feeder cells (MEF) or on Matrigel-coated plates.

Extracellular matrix components of matrigel or feeder cells were necessary to maintain the pluripotent state. Additionally, the iPSC-MCM had to be changed every day and the growth factor bFGF (100ng/μl) was added every day as well.

3.2.1.10 Generation of induced pluripotent stem cells

Each independent iPS cell generation experiment required between 12 and 30 days and precise planning was critical. To start the iPS cell generation, the retroviruses containing the Yamanaka factors (KMOS or KOS) were produced as described in chapter 3.2.1.4 and 3.2.1.5. On the same day of the retroviral transfection, one vial of frozen human fibroblasts was thawed and cultured as described in chapter 0 in a 10cm dish, to obtain 100% confluency two days later.

Day 0 – Preparing fibroblasts

One day before infection, fibroblasts were transferred and plated. Different densities were obtained by plating the cells as described in figure 3. After dissociation of a 10cm plate the cell suspension contains 10ml of media. Different amounts of cells were plated as shown in figure 3. Various cell densities were used to ensure that the proper cell density (50% confluency) for the retroviral infection would be achieved 24 hours later.

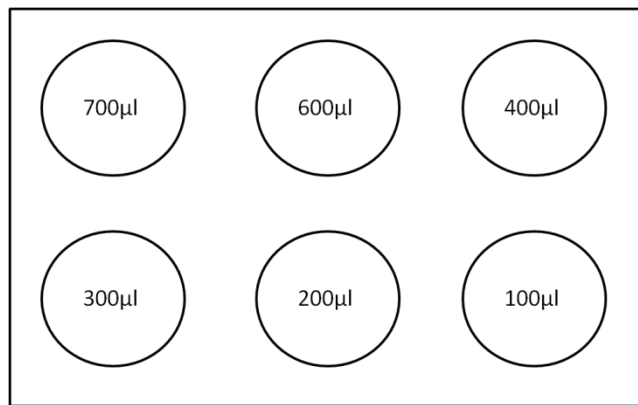


Figure 3: Preparation of WT83 fibroblasts on day 0. Various used volumes (μl) of fibroblast cell suspension are indicated on the 6-wells. Additionally 3ml media was added.

Day 1 – Fibroblast infection:

A well of 50% confluency (see table 28) was chosen, the existing media reduced and 200μl of the pooled Yamanaka cocktail (preparation see chapter 3.2.1.4) were added per 6-well. The cells were incubated for 5-6 hours at 37°C and media changed afterwards.

Day 2 – Preparing feeder cells:

The infected cells were left untouched in their well. If there was a lentiviral infection involved in the experiment, the Yamanaka transduced cells were transduced again that day (see chapter 3.2.1.3).

Feeder cell plates were prepared as already described in chapter 3.2.1.7.

Day 3 – Cell transfer

The protocol of chapter 3.2.1.2 was used to dissociate the infected fibroblasts and additionally, a cell scraper was used to detach all cells. They were transferred onto feeder cells in a proper density.

Table 28: Seeding densities, dilutions and cell numbers

Cell density	Cell number	Seeding dilution on day 3	Cell number/6-well after cell transfer, according to seeding dilution	Seeded cell number/cm ²
100% confluency	1.1×10^6			
50% confluency (recommended for transduction)	5.5×10^5	1:6	9.2×10^4	10,222
		1:12	4.5×10^4	5000
		1:15 (recommended)	3.6×10^4	4000
		1:30 (recommended)	1.8×10^4	2000
		1:60	9.1×10^3	1011
		1:120	4.6×10^3	511

Day 4 – Changing into iPSC media

The iPSC media was prepared by filtering all ingredients as listed in table 5 through 0.2microns. 35signali addition, a fresh and sterile stock of 10mM valproic acid (VPA) was prepared. The media was changed as described in 0 and additionally, cells were treated 5 days (from day 4 until day 8) with VPA using a 1:1000 dilution (final VPA concentration = 10μM). VPA is a histone deacetylase inhibitor (HDAC inhibitor), which enhances the reprogramming process (Huangfu et al. 2008)

The iPSC media replacement at 72 hours post-transduction was essential. If there was small molecule treatment involved in the experiment, treatment was started on day 4. Different small molecule treatments are described in chapter 3.2.1.11

Day 5 – Day 10 – Cell feeding and treating every day:

The next days, the media was replaced 0 and treated as described in chapter 3.2.1.11. Day 8 was the last day of VPA treatment.

Day 11 – Colony formation:

From day 11 onward cells were treated as before except iPSC-MCM was used for feeding. iPSC-MCM production was described in chapter 0.

3.2.1.11 Perturbing Wnt signaling pathway:

- Through adding small molecules:

In some experiments iPSC were treated with small molecules. Cells were treated every day at the same concentration until the cells were analyzed. The different small compounds were Wnt3a (stock = 2.5 μ M), Wnt5a (stock = 2.5 μ M) (both diluted 1:400), IWP (stock = 5mM, IWR (stock = 10mM) and the GSK3-inhibitor (Factor XV, stock = 10mM) used at various concentrations.

- Through lentiviral overexpression or knock down:

In other experiments lentivirus-mediated knock-down or overexpression of important Wnt pathway components were done. The lentiviral knock downs of Frizzled 7, ROR2 were performed and overexpressions of lenti-GFP, β -catenin and Axin. (for lentiviral constructs see table 11)

3.2.2 Microbiological and biological methods

The preparation of buffers, chemicals, reagents and equipment is shown in chapter 3.1.

3.2.2.1 Transformation: (chemical transformation and electroporation)

Production of electro-competent bacteria (DH5α):

E.Coli DH5α (35μl) in 10ml LB Media were inoculated overnight on 37°C. The next day inoculations with 1/100th Volume of the overnight culture were performed into twice 500ml of LB Media. The 2 cultures were incubated on a shaker at 37°C until the OD₆₀₀ between 0.5 and 1.0 was reached. The cultures were centrifuged at 4000 x g for 15 minutes at 4°C. Afterwards, their supernatant was poured off and the bacterial pellets resuspended in 500ml ice-cold distilled sterile water. This procedure was repeated twice but the centrifuge time was reduced to 10 minutes and at the 3rd wash the volume was reduced to 250ml per pellet.

After the 3rd wash the pellets were resuspended in 20ml ice cold sterile 10% glycerol and pooled. Again the bacteria were centrifuged at 4000 x g for 10 minutes at 37°C, the supernatant aspirated and the pellet resuspended in 2-3ml ice cold sterile 10% glycerol. To store small aliquots for experimental use, the bacteria were aliquoted 50μl per tube and frozen at -80°C.

Electroporation of plasmid DNA into electro-competent E.Coli DH5αe

The Gene Pulser Cuvette (Biorad, 0.2cm) was chilled on ice before starting the transformation and the electro-competent bacteria were thawed on ice. After approximately 10 minutes, 50μl of bacteria and 1μl of Plasmid DNA were pooled, transferred into the cuvette and incubated on ice for 30 minutes. The Gene Pulser was set on “bacteria”. The cuvette was placed immediately in the holder and pulse button was pressed. The pulsed bacteria were removed from the holder, 1ml LB media was added and the suspension transferred into a sterile culture tube. The culture tube was incubated on the shaker 30 minutes at 37°C to allow the bacteria to recover and

express the antibiotic resistance marker encoded by the plasmid. While incubating, ampicillin plates were warmed up at room-temperature. For plating the right amount of bacteria, the transformed bacteria were spun. The pelleted bacteria were resuspended with 100µl LB Media. 20µl of the cell suspension was transferred and plated onto plates containing the appropriate antibiotics. The plates were sitting at room temperature until the liquid has been absorbed. The plate was inverted and incubated at 37°C over night. On the next day, transformed single colonies appeared on the plate. These colonies had the right DNA insert and were picked for bacterial cultures used for DNA proliferation and DNA purification (see chapter 3.2.3.1).

Production of chemical-competent bacteria E.Coli DH5α:

The production of the chemical-competent bacteria was performed by Willert lab, UCSD.

Chemical transformation of plasmid DNA into chemical-competent E.Coli DH5α:

A chemical transformation is generally less efficient than the electroporation.

One vial of chemical competent bacteria was thawed on ice. After thawing for 10 minutes, 1µl Plasmid DNA was added to about 30µl of bacteria. The mixture was incubated on ice for 30 minutes, followed by a heatshock at 42°C for 30 seconds and placed on ice again for 1-2 minutes. After the transformation procedure 500µl LB Media was added and incubated on the shaker at 37°C for 45-60 minutes to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid. The following steps were performed as described in chapter 3.2.2.1(Electroporation).

3.2.3 Molecular biological methods:

The preparation of the buffers, chemicals, reagents, equipment and kits is showed in chapter 3.1.

3.2.3.1 DNA plasmid purification (Maxiprep):

The NucleoBond Xtra Kit was used to purify DNA Plasmid.

- *Preparation of a starter culture:*

A 3-5ml starter culture of LB or Terrific Broth with a single colony picked from a freshly streaked agar plate was inoculated and incubated at 37°C and 300rpm for 5 hours.

- *Preparation of a large overnight culture:*

Inoculations of overnight cultures were performed by diluting the starter culture 1/1000 into the 300ml LB or Terrific Broth also containing the appropriate, selective antibiotic. The cultures were grown overnight at 37°C and 300rpm for 12-16 hours.

- *Harvesting of bacterial cells:*

The cell culture OD₆₀₀ was measured and the recommended volume was determined, according to the formula: $V \text{ [ml]} = 1200/\text{OD}_{600}$

The chosen volume of cell culture was centrifuged at 6,000 x g for 10 minutes at 4°C and afterwards, the supernatant discarded completely.

- *Resuspension, cell lysis, equilibration, neutralization, loading and washing:*

The pellet was resuspended in 12ml of resuspension buffer RES, already including Rnase A. After complete dissociation, 12ml of buffer LYS buffer were added to the suspension and the tube was gently mixed by inverting the tube 5 times and incubated for 5 minutes. To equilibrate the NucleoBond Xtra Column 25 ml of buffer EQU were applied to the rim of the column filter.

After cell lysis the suspension was neutralized by 12 ml of buffer NEU and mixed by inverting the tube 10-15 times. The lysate was loaded immediately afterwards onto the freshly equilibrated NucleoBond Xtra Column Filter and was emptied by gravity flow. The filter and column was washed once by addition of 15ml buffer

EQU. After the liquid was filtered through, the filter was removed from the column. Another washing step was performed by adding buffer WASH onto the column. The purified DNA elution was done by adding 15ml of buffer ELU onto the column and collecting the liquid in a 50ml conical tube.

- *Precipitation:*

For proper precipitation 10.5ml room-temperature isopropanol was added, vortexed and incubated for 2 minutes. Afterwards, the tube was centrifuged at 15,000 x g for 30 minutes at 4°C. The supernatant was removed and 70% room-temperature ethanol was added and the pellet resuspended. Another centrifugation was started at 15,000 x g for 5 minutes at 4°C. Ethanol was removed carefully from the tube with a pipette tip and the pellet was dried at room temperature for 10-20 minutes. Finally the pellet was resuspended in 200ul TE Buffer (pH 8.0) and the DNA concentration was determined on the nanospectrometer.

- *Measurement:* see 3.2.4.1

3.2.3.2 RNA purification kit

The Nucleospin RNAII kit was used to isolate RNA. The cells were washed with 1x PBS, scraped off the plate, collected in 1ml of 1x PBS and spun at 14,000 x g for 1 minute. The pellet was lysed by 350µl buffer RA1 and 3.5µl 2-mercaptoethanol. The lysate was filtered by adding the viscous suspension onto the NucleoSpin filter and centrifuged at 11,000 x g for 1 minute. The filter was discarded, 350µl of 70% ethanol were added and mixed to adjust the RNA binding conditions. To bind the RNA the suspension was loaded onto NucleoSpin RNA II column and spun for 30 seconds at 11,000 x g. 350µl of the membrane desalting buffer were added to the membrane and again centrifuged at 11,000 x g for 1 minute to dry the membrane.

The preparation of Dnase reaction mixture was prepared as follows:

- 10µl reconstituted rDNase
- 90µl reaction buffer for rDNase

95µl of the mixture was applied directly onto the center of the silica membrane of the column and incubated for 15 minutes at room-temperature. Three membrane washes were performed by adding 200µl of RA2, 600µl of RA3 and spinning for 30 seconds at 11,000 x g. This procedure was repeated one more time by adding 250µl RA3 and spinning for 2 minutes at the same speed. Finally, the RNA was eluted in 60µl Rnase-free H₂O and centrifuged for 1 minute at the usual speed. For measurement of RNA concentration see the chapter analytical methods.

3.2.3.3 PCR – cDNA synthesis

To successful synthesize cDNA, 1µg of RNA, 4µl of cDNA Supermix were mixed with ddH₂O a final volume of 20µl. The cDNA was reverse transcribed on the PCR machine for 1 hour. The synthesized cDNA was stored at 4°C.

3.2.3.4 Real-time quantitative PCR analysis

qPCR of iPSC line containing all four factors and three factors was performed on the 7900 HT Fast Real-Time PCR System (Applied Biosciences) using TaqMan® Mastermix (Gene Expression Assays – Applied Biosciences) and their lineage-specific gene primers. In this experiment the primers 18S (control), Nanog, Pou5f1 were used (Applied Biosciences). Mastermix, probes and dH₂O were mixed as described in table 29 and added onto the qPCR plate. At the end 200ng of DNA template were added in each well.

The used cycle parameters for this specific qPCR are described in table 30.

Table 29: Preparation of mastermix, probe and dH₂O per sample

ingredient	amount (x1)	amount (x7)
TaqMan® Mastermix	10µl	70µl
TaqMan® Probe	1µl	7µl
dH ₂ O	9µl	56µl

Table 30: Program of the cycle parameters for the real-time qPCR.

step	°C	duration
Initial Denaturation	50°C	2 min
Enzyme Activation	95°C	10 min
Denaturation	95°C	15 sec (40 cycles)
Extension and primer annealing	60°C	1 min (40 cycles)

3.2.3.5 DNA gel electrophoresis

The success of the DNA purification 3.2.2.1 was confirmed by electrophoresis on a 1% agarose gel and the DNA concentration was measured on the nanospectrometer. For the agarose gel 1% agarose was dissolved in 1x TAE buffer, melted in a microwave, 10% sybr safe (10,000x concentration in DMSO) added and poured into the gel chamber. A comb was used to create wells in the gel. After 15-20 minutes the gel was solidified and transferred in the running-equipment, which was then filled with 1x TAE buffer to cover the gel. The samples (for additional digestion see chapter 3.2.3.6) about 0.3µg DNA were mixed with loading dye (1/5 of the total amount) and ddH₂O (70% of the total amount) and were loaded on the gel. 6µl of the 1kb DNA ladder was loaded on the gel as well. The gel then was run for about 25 minutes at 100V. The gel was photographed using the gel documentation system. The isolated DNA was then used for a retroviral transfection, see chapter 3.2.1.4.

3.2.3.6 Restriction digestion:

Further improvement of successful DNA purification was given through a restriction digestion. Each plasmid DNA had restriction sites where specific restriction enzymes cut the strands. Every enzyme had their specific restriction enzyme buffer and BSA was added depending on the prescriptions. The samples were mixed with ddH₂O (70% of the total amount) and enzyme mastermix. (10% of the total amount)

Enzyme mastermix:

- 10% Restriction enzyme
- 90% Restriction enzyme Buffer

To achieve a digestion, the samples were incubated on 37°C for 1 hour. After the digestion, 20% loading dye was added to each sample and the DNA gelelectrophoresis performed (see chapter 3.2.3.5).

3.2.4 Analytical methods

The preparation of the buffers, chemicals, reagents, equipment and kits is showed in chapter 3.1.

3.2.4.1 Nanospectroscopy

The concentration of purified plasmid DNA or RNA was detected on the nanospectrometer.

Measurement of DNA concentration: For equilibration and blank TE Buffer (pH 8.0) was required. Ratios of 260:280 were recommended between 1.7 and 1.9 and concentrations usually about 2-4 µg/µl.

Measurement of RNA concentration: The same technique was used as for DNA measurement, just the blank was RNase-free H₂O.

Between day 7 and day 23 different analytical methods were performed to find the best method with low background and clear read out, regarding iPSC generation efficiency:

3.2.4.2 Alkaline phosphatase (AP) staining in situ and colony counting using Adobe Photoshop or Cellomics Arrayscan Vti.

The culture medium of the iPSC plates was aspirated and washed twice with 1x PBST. To fix them, 1ml of fixing solution (Paraformaldehyde solution) was added and incubated for 1-2 minutes. The cells should not be over-fixed, which would result in the loss of AP activity. Afterwards the fixing solution was aspirated, washed once with 1x PBST and the staining solution (solution A: sodium nitrate solution; solution B: fast red violet solution - substrate: naphthol AS-BI phosphat) was prepared at a ratio of 1:1 right before the staining process. Accordingly, 500µl freshly prepared staining solution were added and incubated 5-15 minutes in the dark. The color change was closely monitored every 2-3 minutes to avoid non-specific staining and the reaction was stopped when the

color turned bright by aspirating the staining solution and washing 3 times with 1x PBS. AP expression produces a purple stain, while the absence of AP expression showed no stain. For storage, the well was covered by 500µl 1x PBS to prevent drying and stored at 4°C.

Every well was photographed to quantify colonies, the counting function of Adobe Photoshop was used, and purple stained colonies were counted.

3.2.4.3 Flow cytometry and staining for Tra1-81 or SSEA-4

Cell dissociation:

The cells (= induced pluripotent stem cells growing on MEF feeder cells and some non-reprogrammed fibroblasts) were washed with DPBS Buffer and for dissociation filter-sterilized 1mg/ml collagenase IV (1ml/6well, 5ml/10cm) was added. After 1 hour the cells were coming off their feeder cells. For better dissociation they were pipetted up and down and transferred to a 15ml conical tube. The wells were washed with 3ml of DMEM-F12, also added into the tube and centrifuged at 200 x g for 4 minutes. Afterwards, the supernatant was aspirated, and 5ml of FACS Buffer have been added to the cells. For all further steps has been worked on ice. To achieve single cell suspension the cells have been transferred through a cell strainer and counted.

Cell counting:

Preparation for cell counting:

- 10µl of single cell suspension
- 10µl of Trypan Blue

10µl of the preparation was counted using the haemocytometer.

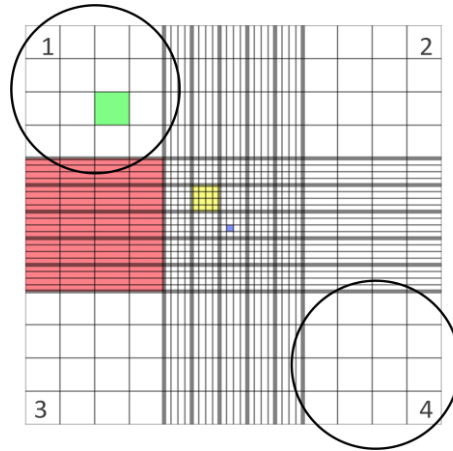


Figure 4: Haemocytometer (http://static.flex.com/pictures/3/1/0/t/Haemocytometer_Grid.png)

The cells located in chamber 1 and 4 (marked in figure 4) were counted. If trypan blue entered in the cells, these cells were considered dead and were not counted. The counted number of cells was divided by two, multiplied by 10,000, the dilution factor and the total volume, see formula below.

$$\text{Total number of cells} = \text{number of cells (counted)} \times 10,000 \times \text{dilution factor} \times \text{total volume of sample}$$

The chosen cells were transferred into a FACS tube and incubated with the appropriate antibody.

Antibody staining:

Preparation mix for antibody staining per sample:

- 100µl FACS buffer
- 2.5µl antibody for 500.000 cells

The desired number of cells were centrifuged at 200 x g for 4 minutes, the supernatant aspirated and the pellet mixed with 102.5µl of preparation mix and incubated 10 to 30 minutes at 4°C in the dark.

Cell washing:

After the staining procedure the cells were washed properly 3 times. 1ml of FACS buffer was added, pellet resuspended and centrifuged again at 200 x g for 4 minutes. After the last wash, 350µl of FACS buffer was added into each FACS tube.

Flow cytometry on BD FACS Cantoll (BD Biosciences):

To set up the machine proper controls were always required. HUES9 cells with equal preparation and antibody staining as all the samples, functioned as positive control and an Isotype Control was used as negative control. Fluorochromes used in this analysis are listed in table 31.

Table 31: Fluorochromes and wave lengths according to
(http://www.bdbiosciences.com/documents/Multicolor_Fluorochrome_Specs.pdf)

	Fluorescence Emission Color	Ex-Max(nm)	Excitation Laser Line(nm)	Em-Max(nm)
Alexa Flour 647 (APC)	red	650	595,633,635,640,647	668
PerCP-Cy5.5	far red	482	488,532	695
FITC	green	494	488	519

After running the samples the flow cytometer was cleaned with bleach and ddH₂O.

3.2.4.4 Flow cytometry and staining for alkaline phosphatase (AP) expression

Cell dissociation and cell counting were done as in chapter 3.2.4.3.

Alkaline phosphatase (AP) staining:

The cells were centrifuged at 200 x g for 3 minutes and fixed 1-2 minutes with 400µl of the AP kits (Stemgent, see table 13) fixing solution. By adding 20µl of 2.6M glycine solution the fixing process was quenched and washed twice by 1x PBST; 1ml has been

added, pellet resuspended and centrifuged again at 200 x g for 4 minutes. The cells were stained with 200µl of AP staining solution, prepared at a ratio 1:1 containing solution A and B (see chapter 3.2.4.2), for 1-2 minutes and then immediately washed to avoid non-specific staining.

Cell washing and flow cytometry:

The washing procedure and flow cytometry was performed as already described in chapter 3.2.4.3.

4 Results

4.1 Method for induction of pluripotency

4.1.1 Method

The goal of the experiment was to generate iPS cells and evaluate their efficiency under different conditions related to the Wnt Pathway. The experimental procedure is precisely presented in chapter 3.

Summarizing chapter 2, the method of iPS cell generation was invented 2006 by Yamanaka (Yamanaka et al. 2006). 4 factors (Oct4, Klf4, Sox2 and c-Myc) were retrovirally transduced into adult human foreskin fibroblasts. Changing the cell culture conditions (changing the microenvironment and equal treatment as human embryonic stem cells) on day 3 post-transduction causes induced pluripotent stem cell colonies emergence approximately one week later.

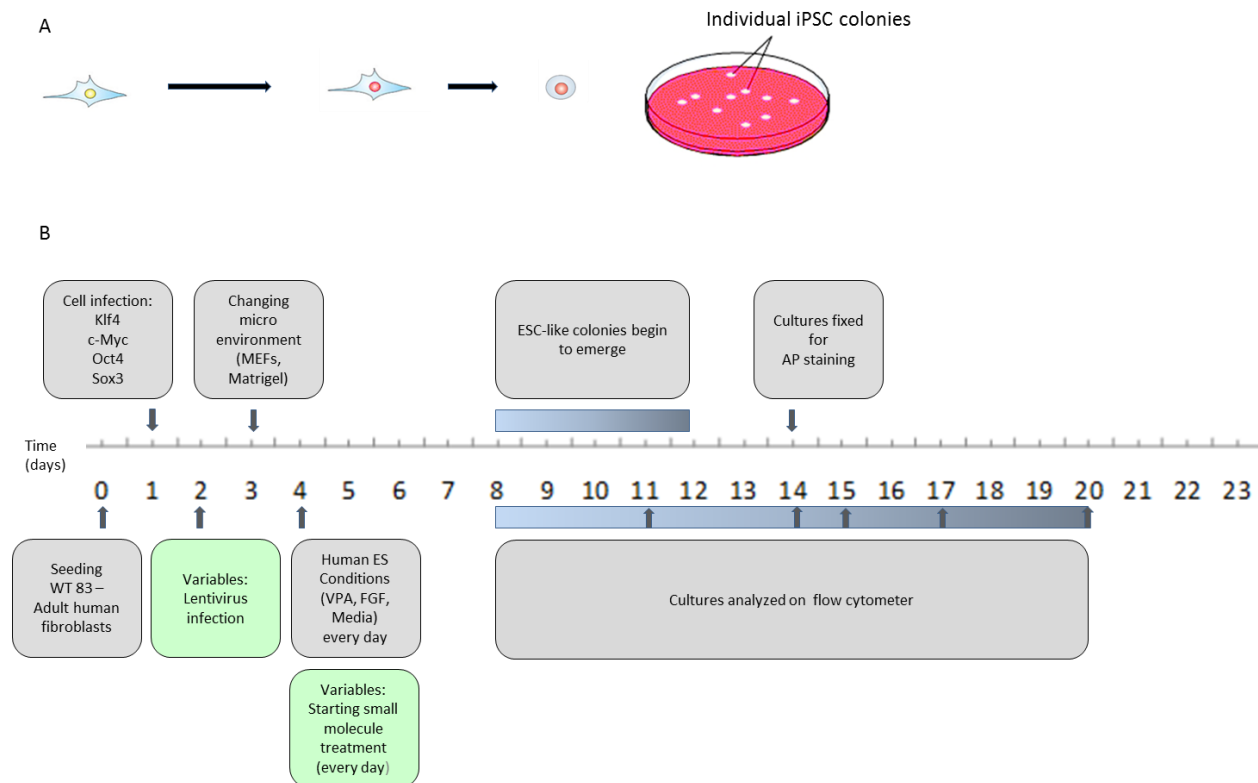


Figure 5: Schematic timeline of general iPSC colony formation with 4 factors (Oct4, Klf4, Sox2, c-Myc)
A, General description of the reprogramming process. 1, fibroblast cell with yellow nucleus = untransduced; 2, fibroblast with red nucleus = after transduction; 3, induced pluripotent stem cell; 4, plate with individual induced pluripotent stem cell colonies; B, Exact time schedule; in light green: either starting small molecule treatment or lentiviral overexpression/knockdown; in grey: indication of fibroblast seeding, cell infection, microenvironment change and human embryonal stem cell conditions in cell culture; emerging of ESC-like colonies; two analysis methods: alkaline phosphatase staining and analysis on flow cytometer

Figure 5A depicts a wildtype (WT83) adult human fibroblast cell (origin see chapter 3.1.2), a cell transduced with the reprogramming factors (red nucleus), and the reprogrammed cell and individual iPSC colonies in a dish. Figure 5B shows a more detailed timecourse. Day 0 the WT83 – adult human fibroblasts were seeded in the right (50% confluent, see figure 3) density. The next day four Yamanaka factors (KMOS) were transduced retrovirally into those fibroblasts. The microenvironment was changed on day 4. The cells were transferred onto MEF feeder cells (see chapter 3.1.2) and on day 5, the media was changed into human embryonic stem cell-like condition (Figure 5B). Additionally, for perturbing the Wnt signaling pathway (see chapter 3.2.1.11) the lentiviral infection was performed on day 2 and small molecule treatment with soluble Wnt components was started on day 4.

The first ESC-like colonies emerge between day 8 and day 12, depending on the titer of the virus and on the transduction efficiency. In the case of an alkaline phosphatase staining, colonies were fixed and stained on day 14, otherwise the cultures were analyzed on the flow cytometer (see chapters 3.2.4.3 and 3.2.4.4) between day 8 and day 20. Furthermore, the whole reprogramming process was alternatively performed with only three factors (without c-myc, see chapter 3.2.1.5)

4.1.2 Gelelectrophoresis

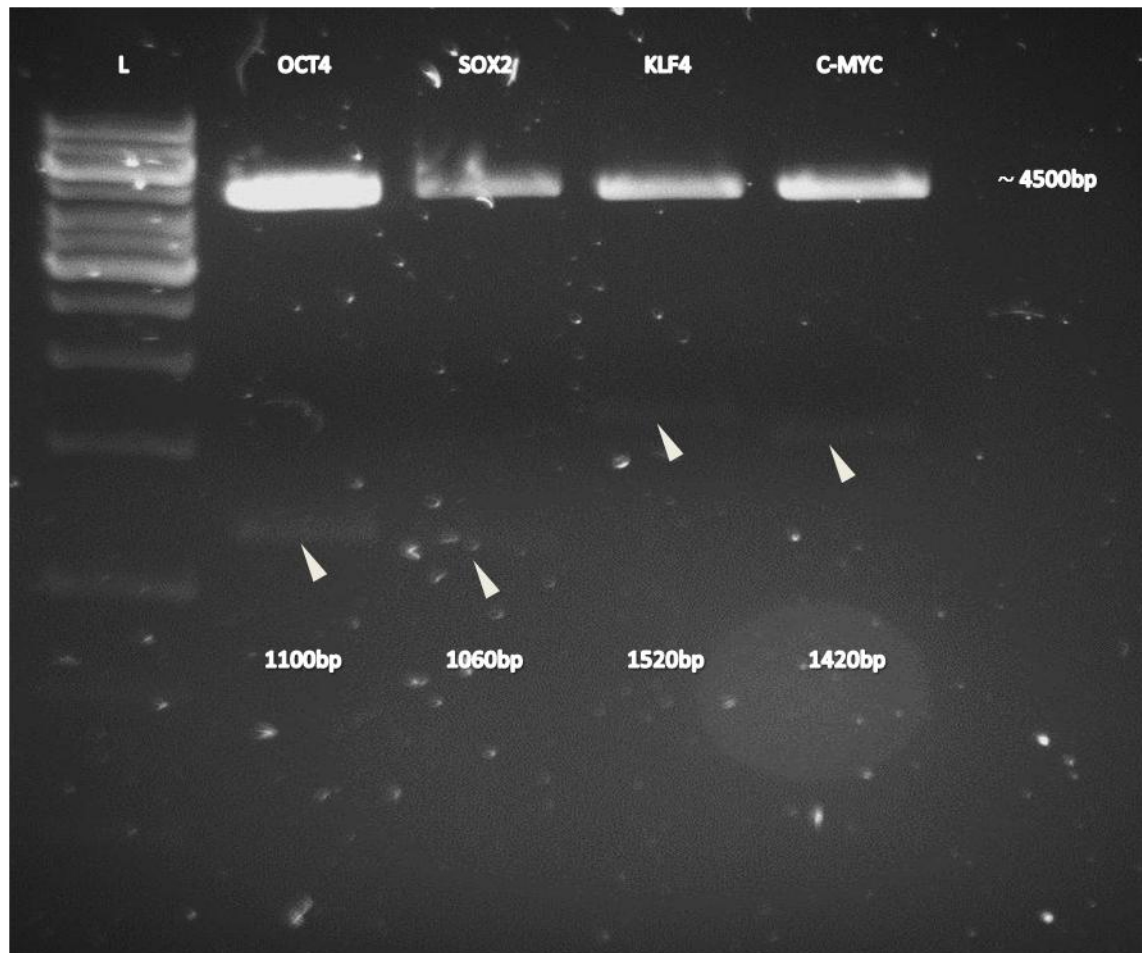


Figure 6: Restriction digestion of plasmids carrying the four reprogramming factors (Oct4, Sox2, Klf4, c-Myc). L, 1kb DNA ladder O'Gene Ruler, 250-10.000 bp; Arrowheads are indicating the small DNA fragments

Figure 6 illustrates a 1% agarose gel verifying the plasmid DNA of the four reprogramming factors. The DNA isolation was done using the NucleoBond Kit – Macherey-Nagel and 0.3µg DNA were digested with Not I (Sox2, Klf4, c-Myc) and EcoRI (Oct4) and then loaded on the gel. The concentration of the isolated DNA was determined using the nanospectrometer.

4.1.3 Assaying iPSC generation

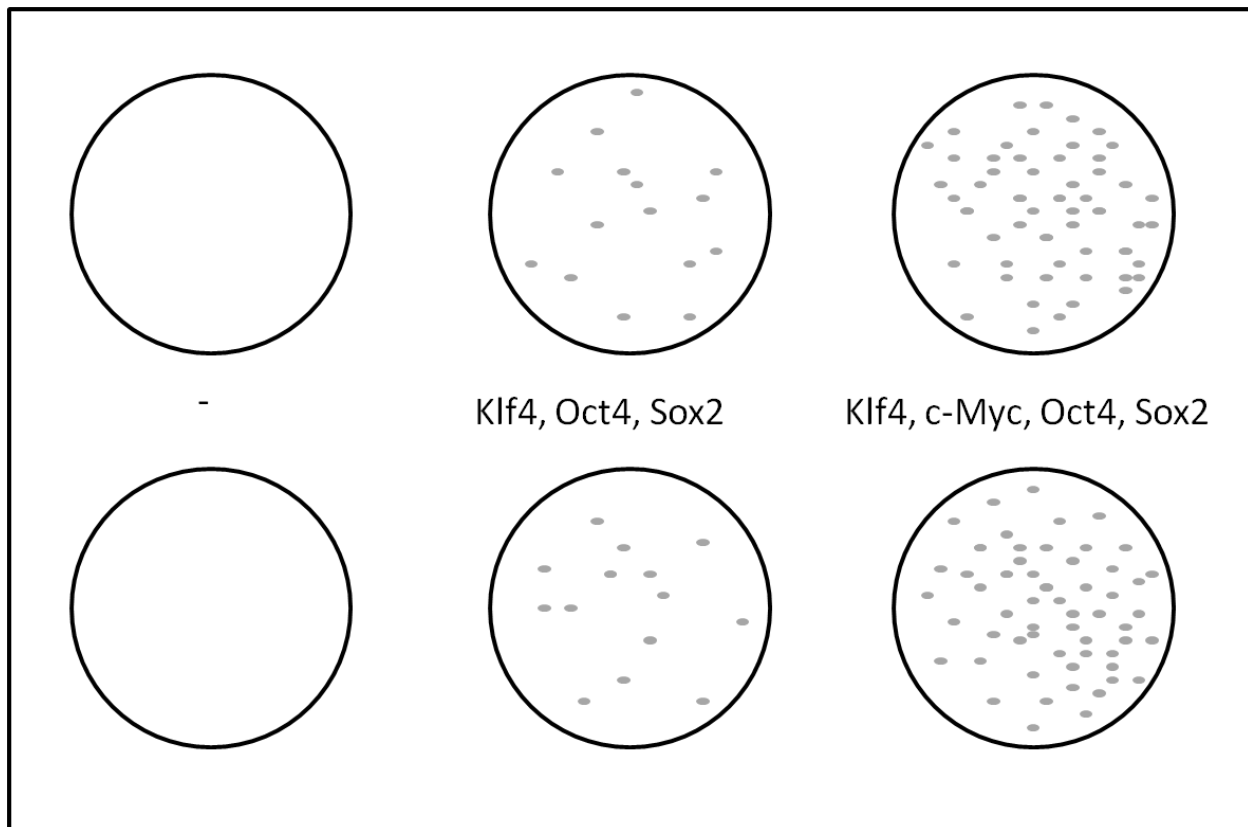


Figure 7: Schematic of iPSC colony generation in 6-well

The two wells on the left in Figure 7 show non-transduced WT83 fibroblasts. Cells in the middle wells were transduced with 3 factors and the wells on the right were transduced with 4 factors (Oct4, Klf4, Sox2, c-Myc).

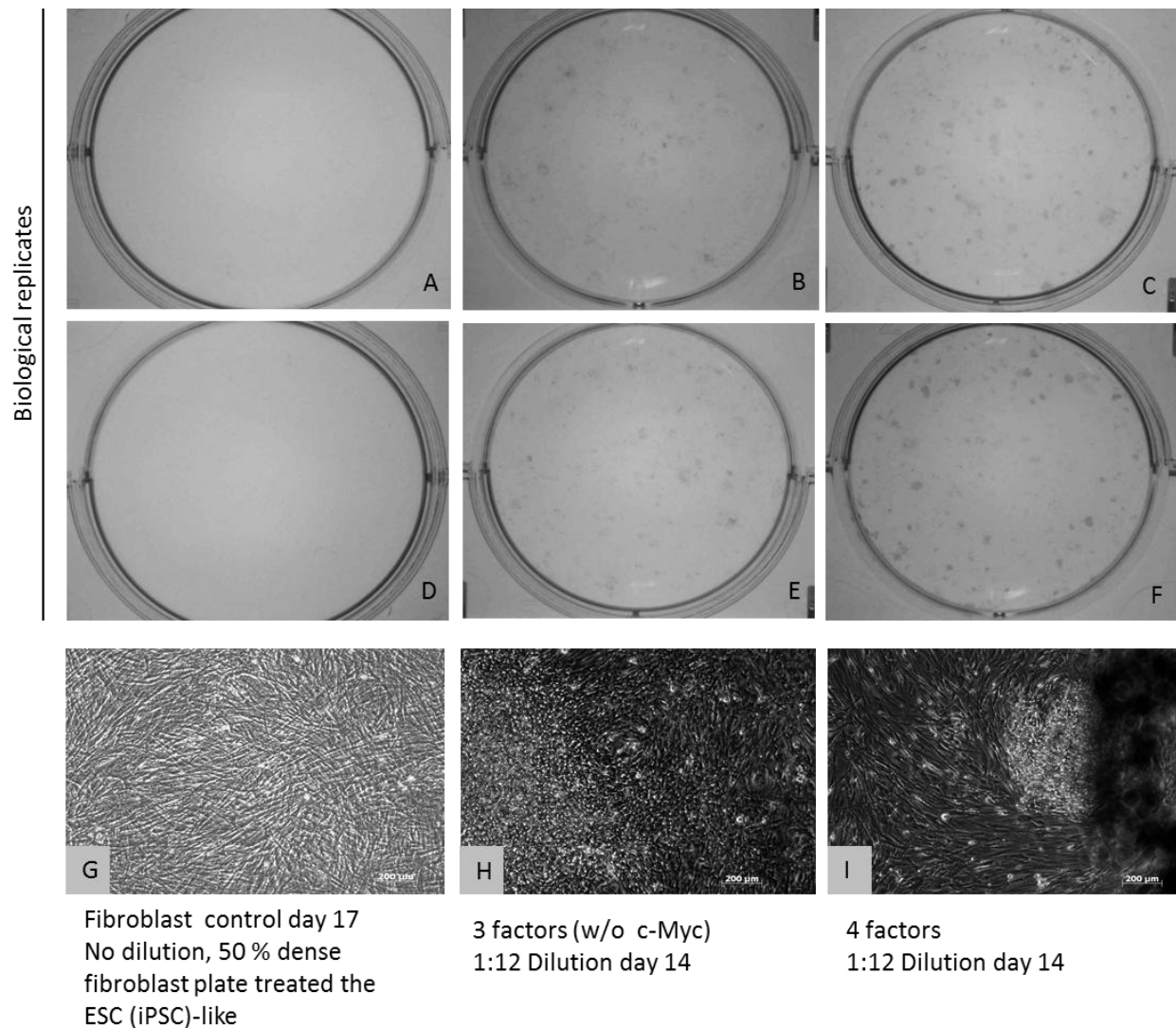


Figure 8: Assaying iPSC generation; A-F, biological replicates of negative control, 3 factor and 4 factor iPS cell generation in a 6-well plate (labeled below); G-I, bright field images

Figures 8A-F show the number of alkaline phosphatase-positive iPSC colonies on day 14 growing on MEF feeder cells. Cells were transduced with 0 (A and D), three (w/o c-myc, B and E) and four factors (C and F), according to the schematic in Figure 7. A and D, B and E, and C and F are biological replicates. On day 3 post-infection cells were passaged at 1:12 from the plate of infected fibroblasts. Thereafter, cells were not passaged. According to table 28 (see chapter 3.2), around 5000 cells were seeded per cm^2 . Non-transduced WT83 fibroblasts (Fig. 8 A and D) were treated in the same media conditions as transduced cells and served as negative controls.

Figure 8G, H and I are bright field images of WT83-adult human fibroblasts (passage 14), three factor and four factor transduced WT83 fibroblasts. In Figure 8 H and I colony formation is visible on day 14. A black round-shaped mark used to observe an emerging colony on a daily basis is visible on the right side of the picture in Figure 8I.

As a separate experiment, I tried to establish two iPS cell lines by myself (bright field images, see figure 15 and 16), which were analyzed in this assay. A qPCR assay was done, to show the differences of Nanog and Oct4 expression in two established cell lines: an iPS cell line, which was transduced with 3 factors and another one transduced with 4 factors.

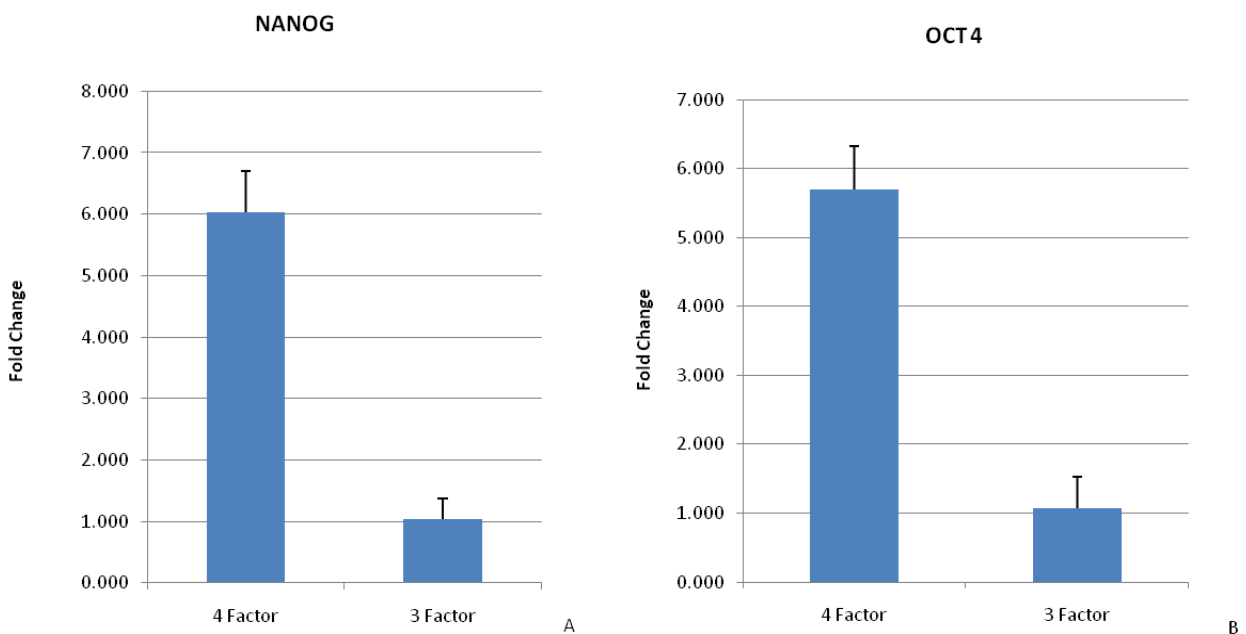


Figure 9: Quantitative real-time PCR analysis of putative iPSC clones (two clones). The analysis was done in triplicates (errorbars). The bars show the difference of specific gene expression in 4 factor and 3 factor transduced iPS cells (data does not include controls).

Figure 9 shows Nanog (A) and Oct4 (B) expression in iPS cells transduced with three factors compared to iPS cells transduced with four factors. Primers for Oct4 were specific for the endogenous gene and do not amplify the transgene transcript. qPCR for

Oct4 and Nanog were performed on two clones which were manually passaged 7 times (chapter 3.2.1.8). The first passage was done on day 15 post-infection onto matrigel and afterwards cultured until day 53 (7 passages) and fed every day (Media, bFGF).

4.1.4 Efficiency assays

The goal of this project was to study the influence of Wnt signaling on iPSC generation. We utilized several approaches to perturb Wnt signaling during iPSC generation and tested several assays to quantify reprogramming efficiencies.

- Flow cytometry:
 - Alkaline phosphatase staining (for experimental procedure, see chapter 3.2.4.4)
 - Antibody 55signaling (data not shown)
 - Tra1-81 and SSEA-4 staining (for experimental procedure, see chapter 3.2.4.3)
- Alkaline phosphatase staining with colony counting (for experimental procedure, see chapter 3.2.4.2)

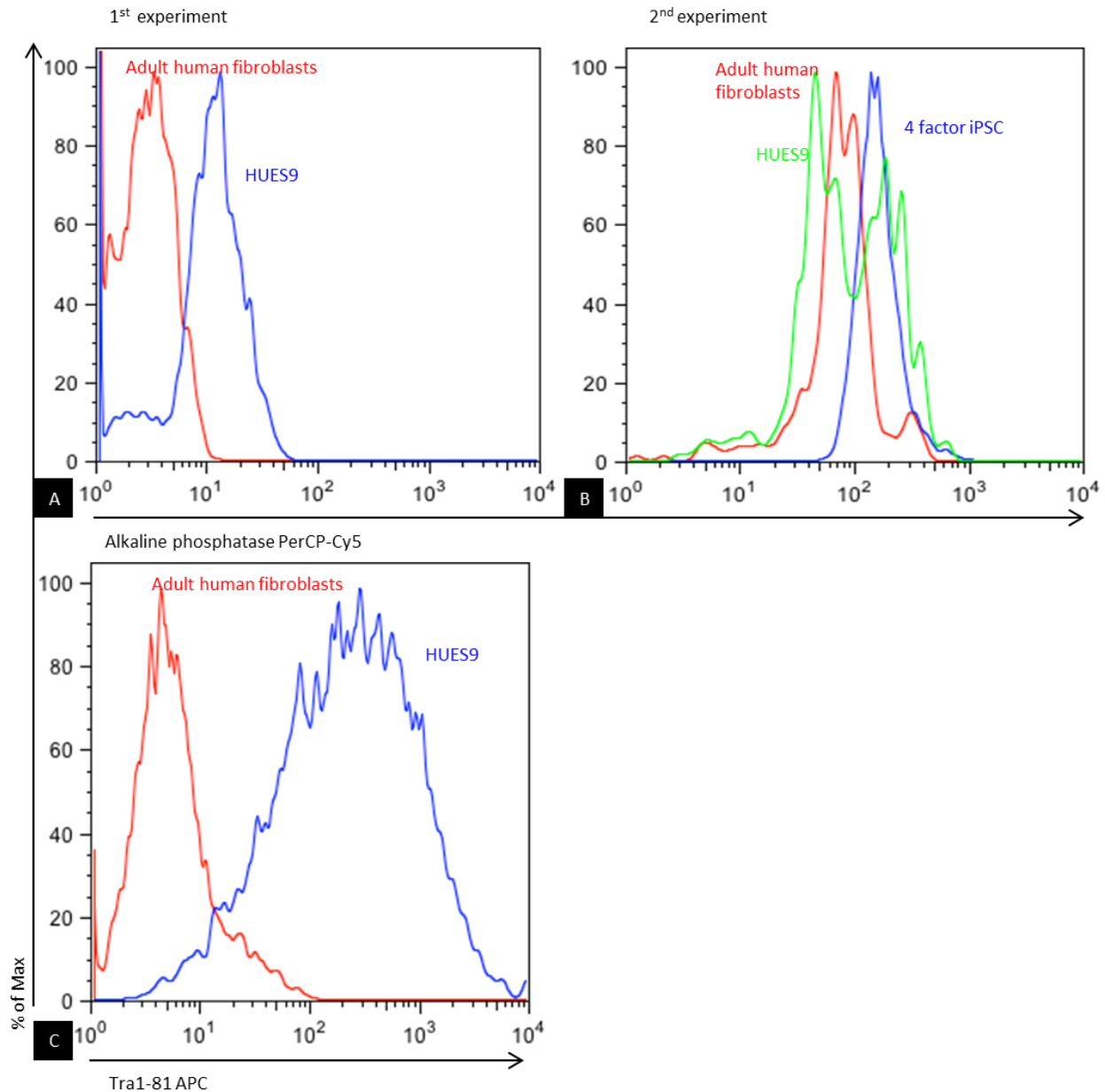


Figure 10: Flow cytometry analysis of human pluripotent stem cells. Red: adult human fibroblasts (negative control); human embryonic stem cells = HUES9 (positive control);

Figure 10A and B show fixed alkaline phosphatase-stained cells detected by flow cytometry with the fluorochrome PerCP-Cy5, according to table 31 (see chapters 3.2.4.3 and 3.2.4.4). The PerCP-Cy5 settings of the BD FACS Cantoll were assumedly used to detect the alkaline phosphatase stained cells.

Flow cytometry of two control cell lines (adult human fibroblasts and HUES9 cells) yields two distinct peaks, demonstrating that hESCs have higher AP activity than fibroblasts. As shown in Figure 10B, the difference is detectable in several cell lines using flow cytometry. However, we decided that these differences were not robust enough to allow us to apply this AP staining assay as a quantitative assay to detect iPSC generation efficiencies. In contrast, cells stained with a pluripotent stem cell surface marker Tra1-81 (Figure 10C) conjugated with fluorochrome APC (see table 31) showed a strong separation in signal strength between adult human fibroblast and human embryonic stem cell. We concluded that this difference in Tra1-81 staining on hESCs and fibroblasts was sufficiently large that this stain would serve as a robust assay to quantify iPSC generation by flow cytometry (see results figure 30).

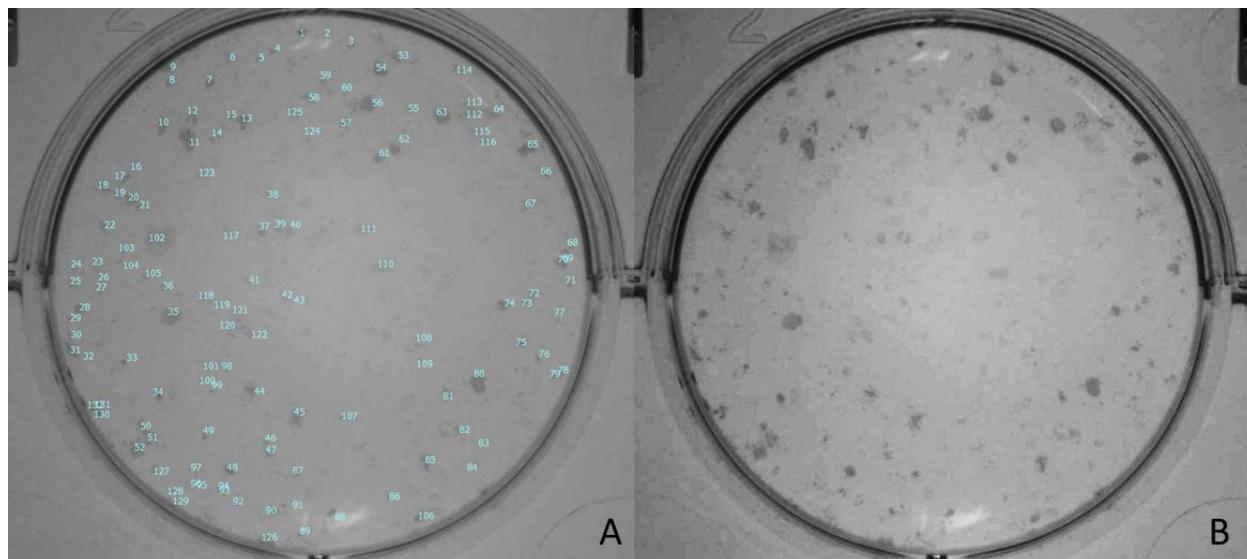


Figure 11: Method of counting colonies by Adobe Photoshop CS5. A, B are one equal 6-well with iPS cells (KMOS) stained for alkaline phosphatase on day 14; A, shows the counts additional to the stained colonies; B, just the AP staining for comparison

Figure 11A and B illustrate the same well of iPSC colonies, where A shows colony counts. In this experiment iPS cells were transduced with four factors (KMOS) and grown on MEF feeder cells. Colonies were fixed and stained for AP on day 14. Adobe Photoshop CS5 was used for analyzing the colony numbers. All visible and stained

colonies were counted using the counting tool. After every count the number appeared on the colony (see figure 11), to simplify the time-consuming evaluation process. Colony counts were performed in a blinded fashion by several lab members.

4.1.4.1 Pictures of general iPSC generation

This chapter contains images of all used cell lines. Their origin was described in chapter 3.1.2 and their culturing methods were described in chapter 3.2.1.

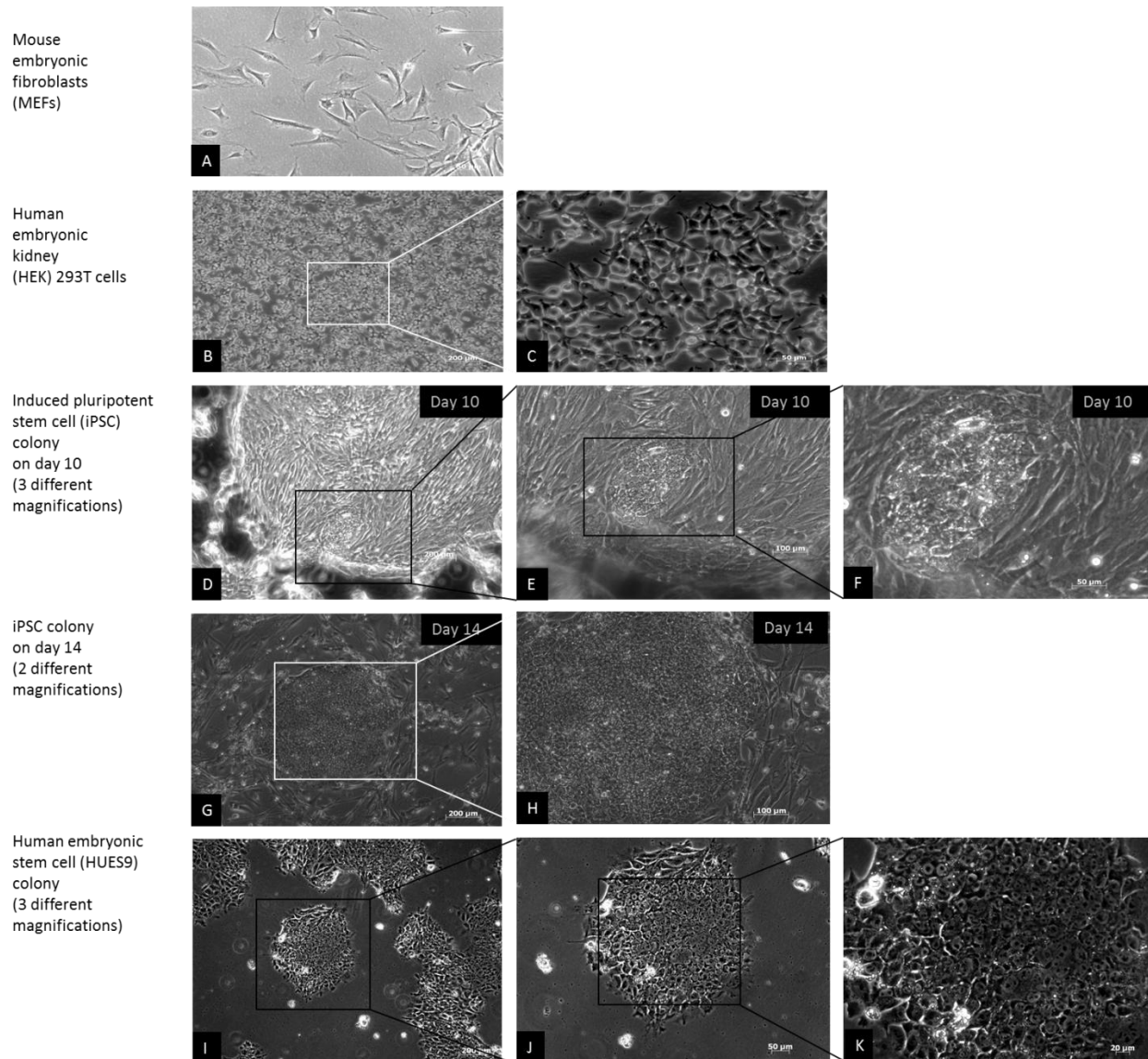


Figure 12: Phase contrast microscopy images of cell lines used in these studies. (bars with proper size in the right corner of each figure)

Irradiated mouse embryonic fibroblasts (MEF, Figure 12 A, also see chapter 3.1.2) serve as feeder cells for hESCs and secrete several important growth factors and provide a cellular matrix for hESCs and iPS cells. Human embryonic kidney cells (HEK 293T, Figure 12 B and C) were used to generate the viral vectors for the infection of

human fibroblasts. Figure 12D, E and F demonstrate one iPSC colony on day 10 at three different magnifications and G and H another iPSC colony on day 14. These iPSC colonies were derived from adult human fibroblasts, called WT83 and were grown on irradiated MEF feeder cells. The iPSC colony in Figure 12G exhibits several characteristics of ES cells, including defined borders, round shape, large nucleoli and scant cytoplasm for each cell. HUES9 cells (Figure 12I, J and K), a human embryonic stem cell line, were included in these studies as positive controls. The images shown in Figure 12 I-K illustrate slightly differentiated hESCs as evidenced by the rough borders. Figure 12D and E show a black round-shaped labeling on the right side of the picture, which was used to mark the colony and observe the same colony every day.

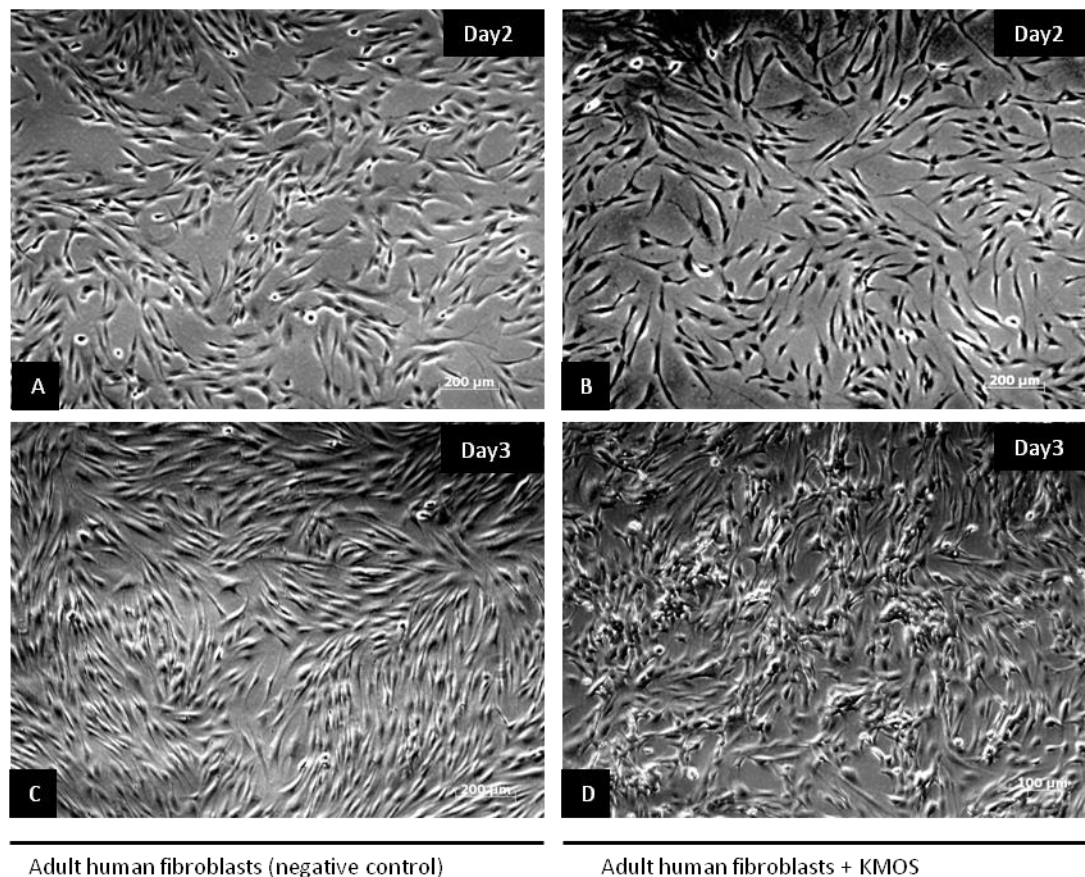


Figure 13: Phase contrast images of human fibroblasts in an early stage of iPSC generation (bars with proper size in the right corner of each figure).

Figure 13A-D illustrate infected and uninfected human fibroblasts at day 2 and 3 post-infection prior to passaging them onto MEF feeder cells. Day 2 or 3 post-transduction, cells were still in normal fibroblast culture conditions. Figure 13A and C show non-infected fibroblasts at day 2 and 3, and B and D infected ones. Cells in Figure 13D show a drastic difference compared to cells in Figure 13B which can be attributed to the transduction of the reprogramming factors KMOS. This timeline is being continued in Figure 14.

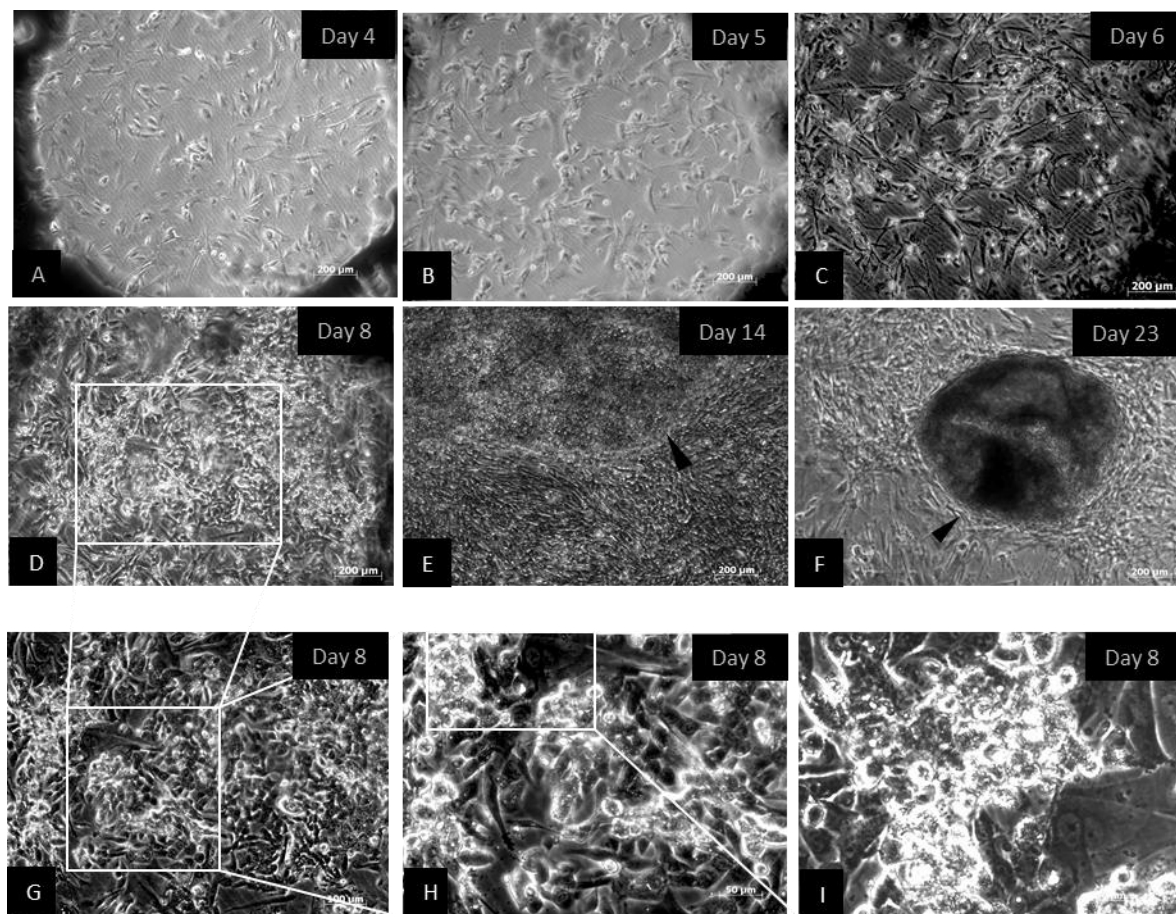


Figure 14: Timeline of iPSC generation (bars with proper size in the right corner of each figure)

Cells on days 4-23 were cultured on irradiated MEF feeder cells and fed every day. In Figure 14A-F cells were photographed on different days as they acquire iPSC properties. Images in Figure 14G-I are at a higher magnification. In Figure 14A-D the same field of cells (as marked with a colony marker) was photographed to monitor the

progression of iPSC generation. On days 14 to 23 (Figure 14E and F) defined borders characteristic of human pluripotent stem cells become visible.

4.1.4.2 Isolation of iPSC clones

Colonies with characteristics of iPSCs (see Figure 14E) were manually isolated and passaged over 34 days (Figure 15). The isolated iPSC line was used to confirm that cells were reprogrammed and acquired a pluripotent state. For the experimental procedure see chapter 3.2.1.8. As already described in chapter 4.1.3 (figure 9) this experiment was done as separate assay to examine the possibility of establishing an iPS cell line. The clones were analyzed by qPCR on day 53.

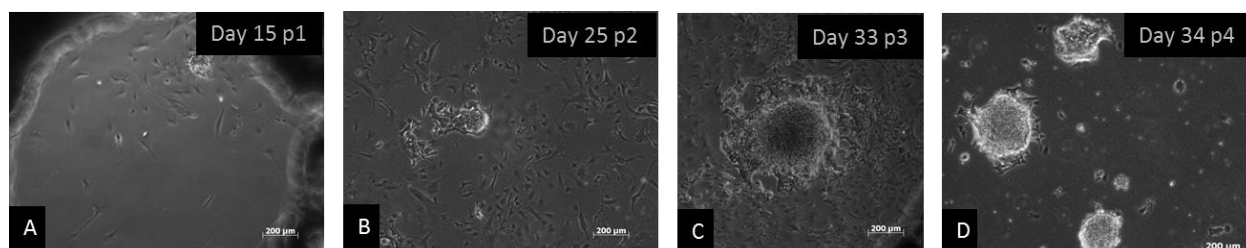


Figure 15: Isolation of iPSC clones (w/o C-MYC). A, after the 1st passage cells still do not appear ESC-like; only KOS transduced, colonies need longer until appearing ESC-like (B); B,C, cells around the iPSC colonies are either not infected adult human fibroblasts, differentiated iPS cells or unintended transferred MEF feeder cells; D, three well defined iPSC colonies with distinct borders (bars with proper size in the right corner of each figure)

Figure 15A-D show manually isolated iPSC clones (three factors) growing on matrigel on day 15-34. Cell passaging (see chapter 3.2.1.9) was performed on day 14 post-transduction using the EVOS microscope. Two clones were transferred and Figure 15A shows one of those clones. They underwent 3 more passages (Figure 15B and C) until day 34 (Figure 15D), where they appeared more like iPSC colonies. Figures 15B and C show plenty of fibroblasts, which is a consequence of the manual isolation technique. These cells were growing until day 53 (three additional passages) and then analyzed by qPCR.

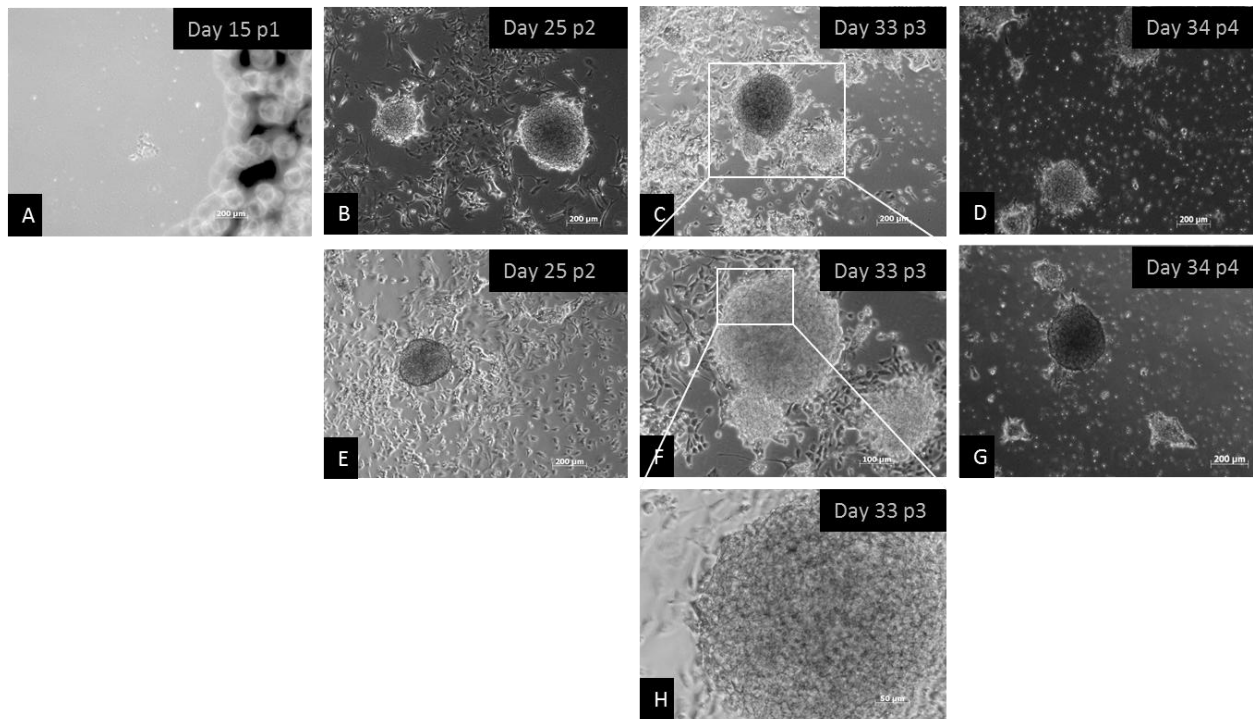


Figure 16: Isolation of iPSC clones (KMOS). A, after 1st passage cells still do not appear ESC-like; B,E well defined iPSC colonies with distinct borders; Generally, the cells around the iPSC colonies are either not infected adult human fibroblasts, differentiated iPS cells or unintended transferred MEF feeder cells; C,F,H in the center: iPS cells, on the rim: adult human fibroblasts; D,G iPSC colonies which have unusual morphology; they grow planar and also for 3D cell aggregates (dark colony in G) (bars with proper size in the right corner of each figure)

Figure 16A-H show manually isolated iPSC clones (four factors) grown on matrigel (BD, see chapter 3.2.1.8) on day 15-34. The iPSC colonies were isolated from human fibroblasts transduced with the four Yamanaka factors KMOS on day 1. Two colonies were manually passaged on day 14 post-transduction, underwent four passages until day 34 (Figure 16D and G). Figure 16C, F and H show the same colony after three passages in different magnifications on day 33. The colonies have defined borders, but reveal unusual iPSC morphology, as they were growing in three dimensions (dark colony Figure 16G). White 3D cell aggregates or tumular colonies were detected by the unaided eye. This was an unusual discovery for the iPSC generation process. Probably a tumor formation occurred (data not shown).

After the first passage on day 15 (Figure 16A), the colony was hardly visible and still did not appear fully reprogrammed. After several additional passages (Figure 16B, E, C, D and G) cells started to exhibit defined borders by day 34. However, there was no

observation of an iPSC colony confluent well. The cells were cultured three additional passages until day 53 and analyzed by qPCR.

4.1.4.3 Virus control

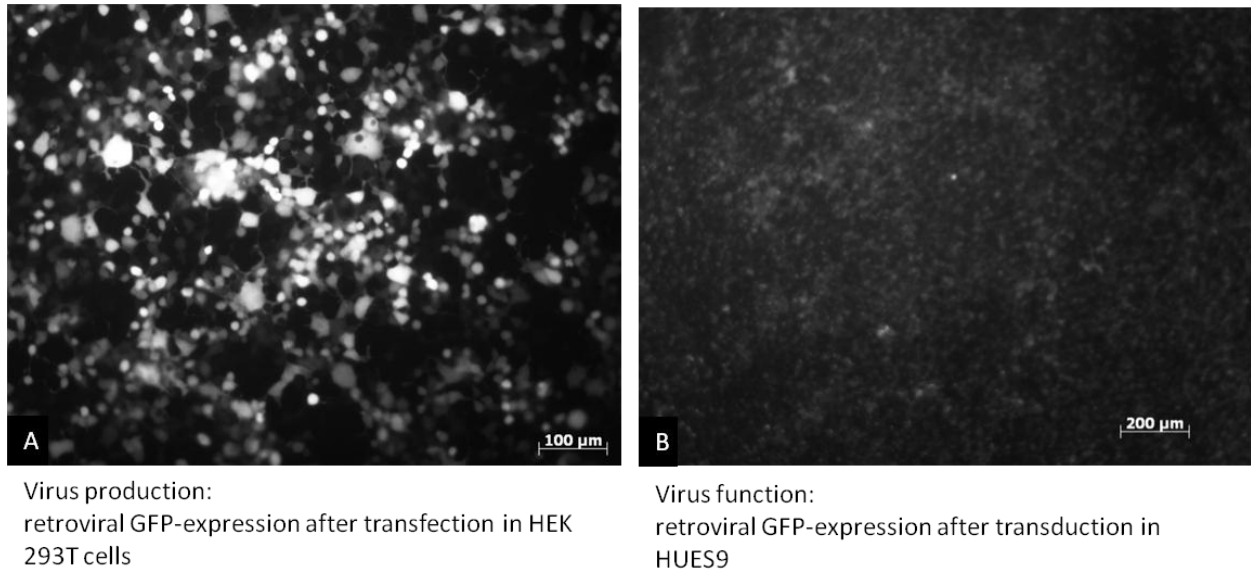


Figure 17: Fluorescence microscopy of GFP-expression after retroviral transfection (A) and virus transduction/infection (B); (bars with proper size in the right corner of each figure)

HEK 293T cells transfected with a retrovirus (for retroviral transfection, see chapter 3.2.1.4 and also background in chapter 2.1.3) carrying a constitutively expressed GFP were expressing GFP as evidenced by the fluorescence signal. Infection of hESC (HUES9) with the virus obtained from the transfected HEK 293T cells yielded GFP expressing cells as expected. Untransduced cells showed no GFP-expression (data not shown). The virus titer was not determined.

4.2 Effect of Wnt 3a and Wnt5a on iPSC generation

General procedures were performed as described in chapter 3.2.1.4 and Figure 5. In the next chapters, flowcharts are presented to illustrate how and when the Wnt signaling pathway was perturbed.

The attention was always focused on comparable conditions and consistent iPSC generation while treating with small molecules or overexpressing and knocking down genes. The efficiency of iPSC generation was only scored and compared within individual experiments because slight difference in conditions, such as viral titer and seeding density, can drastically alter the number of iPSC colonies per dish.

The following data contains three different batches of iPS cell generation with their treatments, which were diversified for optimal read-out. In the first set, presented in chapter 4.5, the infected adult human fibroblasts were transferred into wells without dilutions (1:1) and then analyzed on the flow cytometer. In the second one (presented in chapter 4.4), we tried to find out the best cell density to grow and reprogram to optimize our comparison. Therefore we compared four different dilutions. We plated infected adult human fibroblasts at dilutions of 1:6, 1:12, 1:60, 1:120 into 6-wells and stained with alkaline phosphatase. The results of the last and most significant iPSC generation are recorded in this chapter (4.2) and also chapter 4.3. We decided to use two dilution numbers, which, in the last batch (4.4), resulted to be the most meaningful cell colony numbers to compare and used 1:15 and 1:30 dilutions.

4.2.1 Time course for Wnt3a and Wnt5a small molecule treatment

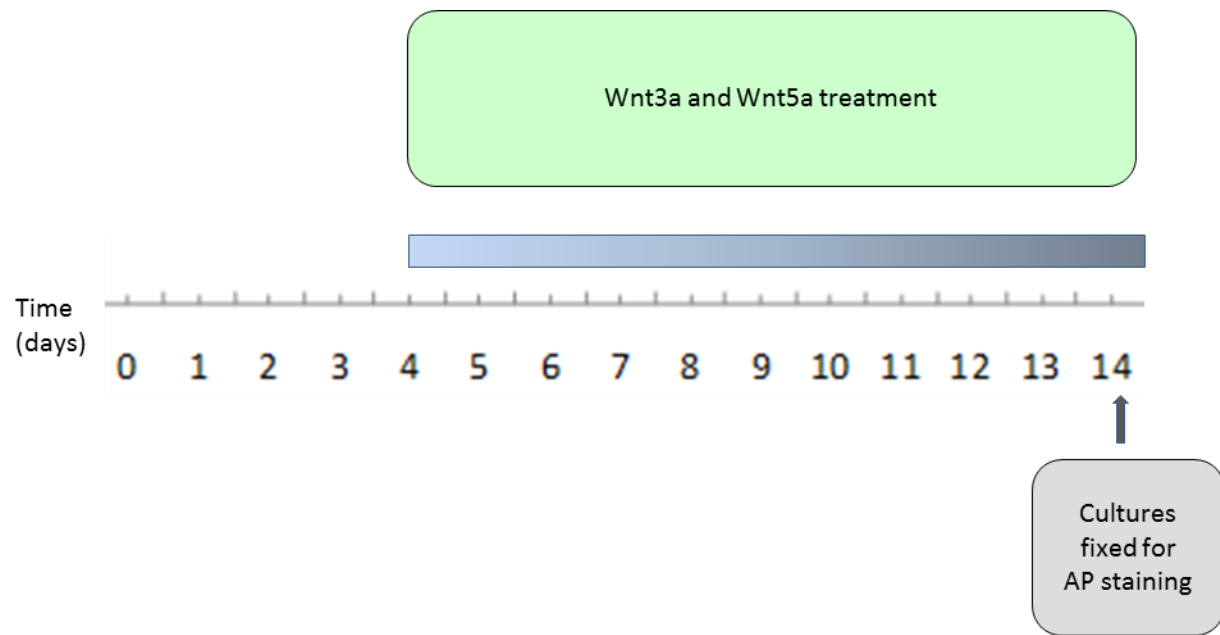


Figure 18: Time course for Wnt3a and Wnt5a small molecule treatment.

The timeframe of treating with Wnt proteins (Wnt3a or 5a) is illustrated in Figure 18 and the day of fixing and staining with alkaline phosphatase is indicated.

4.2.2 Alkaline phosphatase stain of Wnt3a and Wnt5a treated cells

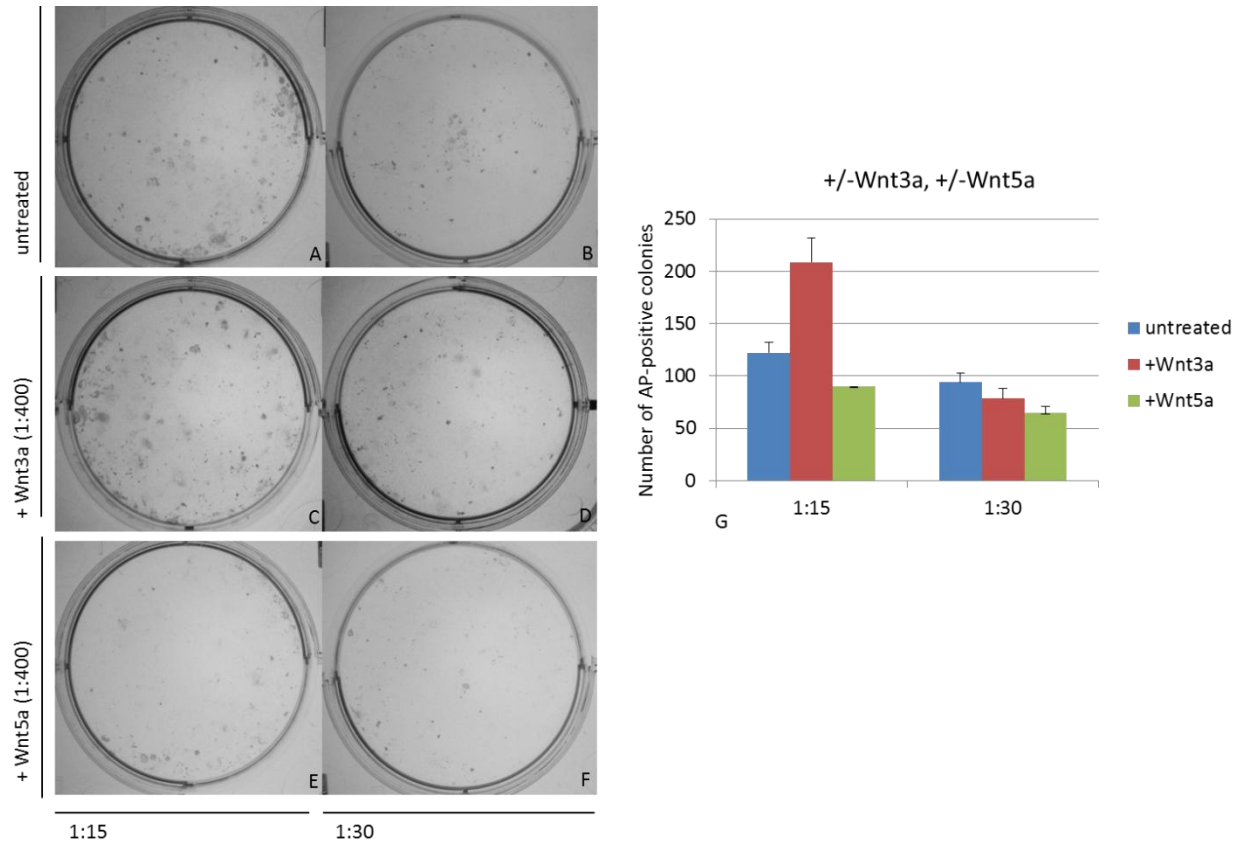


Figure 19: iPSC colonies (KMOS transduced) with Wnt3a and Wnt5a treatment stained for alkaline phosphatase; A-F, AP stained colonies in 6-wells; G, graph of untreated, +Wnt3a, +Wnt5a iPSC colonies in 1:15 and 1:30 dilution; generally, all experiments were done in duplicates (errorbars)

Cells transduced with the reprogramming factors were not treated (Figure 19A and B), treated with Wnt3a (C and D) or treated with Wnt5a (E and F) and then stained for AP activity in 6-well dishes. In Figure 19C and D standard iPSC generation was performed with Wnt3a treatment, and Figure 19E and F with Wnt5a treatment. The number of colonies was counted and illustrated in a chart format in Figure 19G. After KMOS transduction cells were either seeded at 1:15 (A,C and E) or at 1:30 (B, D, and F). The effects of Wnt3a and 5a treatment were most pronounced in the 1:15 dilution. The 1:30 dilution refused significant varieties.

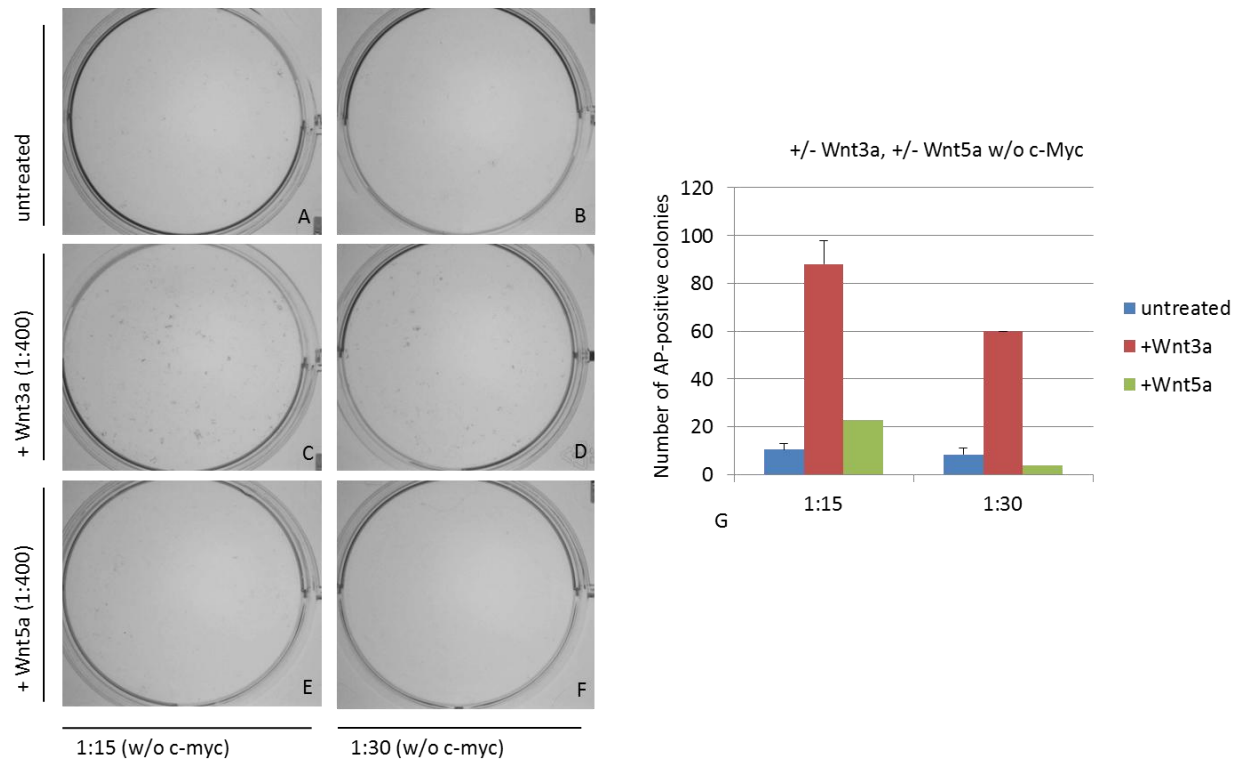


Figure 20: iPSC colonies without c-myc (KOS transduced) with Wnt3a and Wnt5a treatment stained for alkaline phosphatase; ; A-F, AP stained colonies in 6-wells; G, graph of untreated, +Wnt3a, +Wnt5a iPSC colonies in 1:15 and 1:30 dilution; generally, all experiments were done in duplicates (errorbars)

Figure 20A and B were AP stained 6-wells of standard iPSC generation KOS transduced without specific treatment. In Figure 20C and D three factor standard iPSC generation was performed with Wnt3a treatment or in Figure 20E and F with Wnt5a treatment. The chart in Figure 20G illustrates the number of alkaline phosphatase positive-counted colonies compared within the three different conditions. The relations 1:15 and 1:30 represent the dilution of seeded infected fibroblasts on day 3.

Therefore, the efficiency of iPSC generation in cells transduced with KOS was significantly increased in the presence of Wnt3a, while Wnt5a had an insignificant effect.

4.3 Effect of small molecule Wnt pathway agonists and antagonists on iPSC generation

To investigate the effect of ectopic Wnt signal activation, cells undergoing reprogramming were treated with small molecules (IWP, IWR and GSK3-inhibitor in various concentrations).

4.3.1 Time course for small molecule treatment of Wnt pathway agonists and antagonists

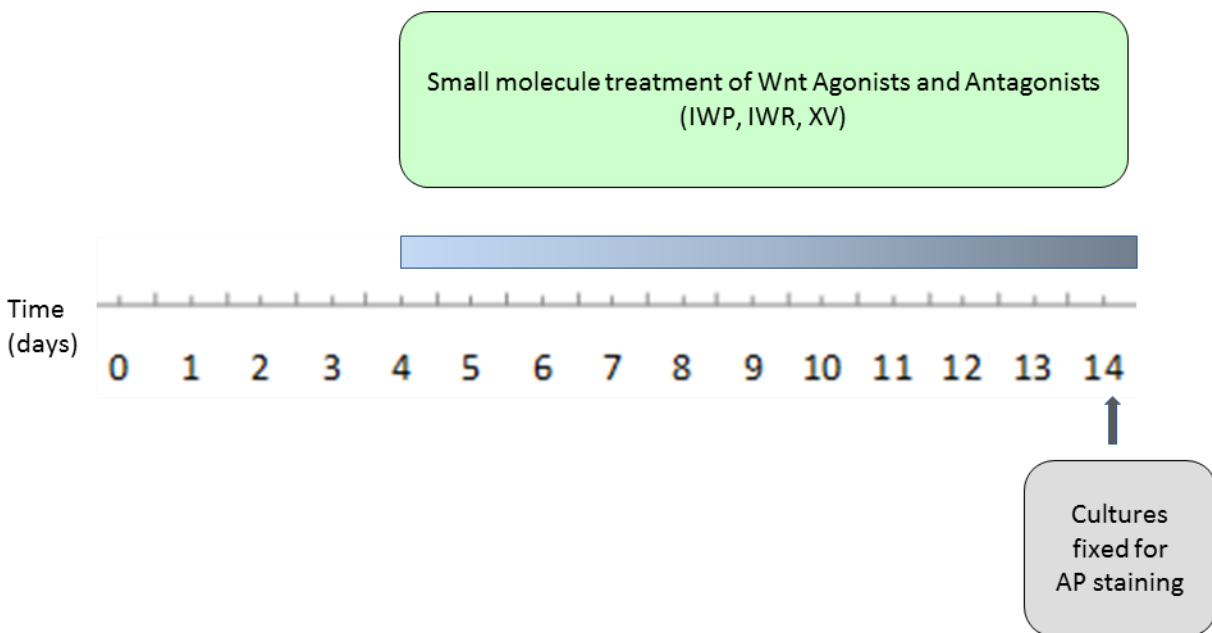


Figure 21: Time course for small molecule treatment of Wnt pathway agonists and antagonists

The timeframe of small molecule treatment, which act as Wnt agonists and antagonists is illustrated in Figure 21 and the day of fixing and staining for alkaline phosphatase is indicated. For the whole iPSC generation process see Figure 5 and Figure 14.

4.3.2 Alkaline phosphatase stain of GSK3-inhibitor, IWP and IWR treated cells

4.3.2.1 Small molecule treatment with GSK3-inhibitor (Factor XV)

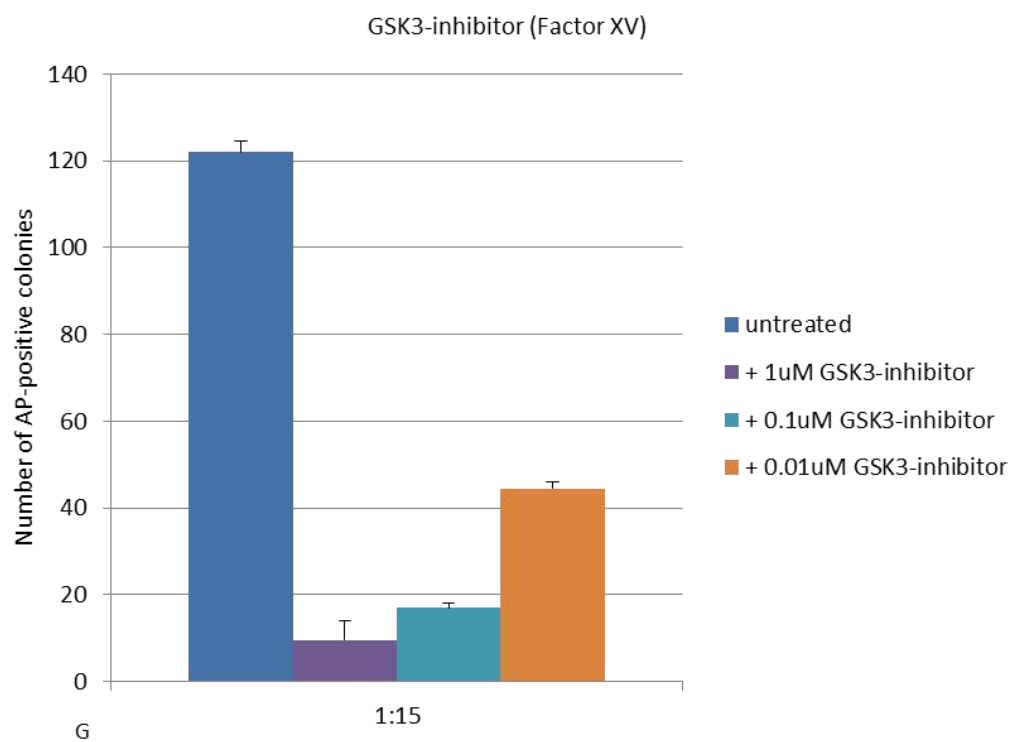
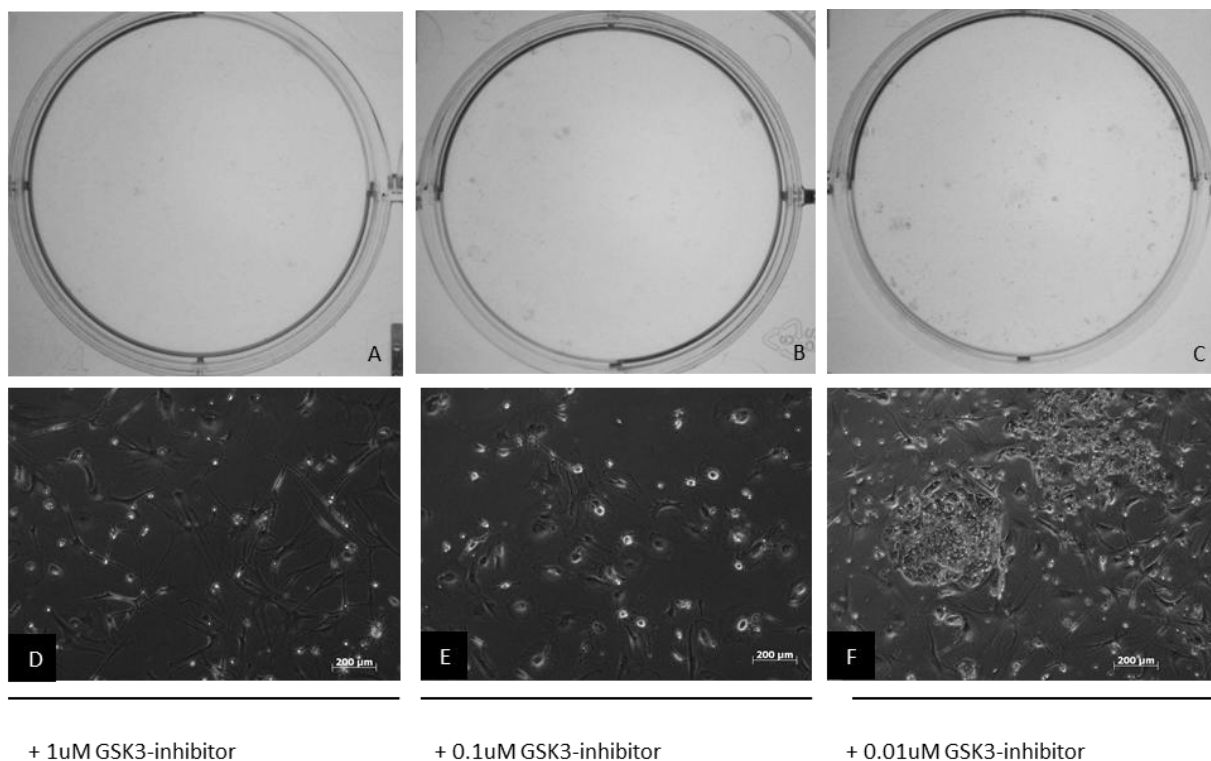


Figure 22: iPSC colonies (KMOS transduced) with GSK3-inhibitor treatment stained for alkaline phosphatase; generally, all experiments were done in duplicates (errorbars); uM=μM

Cells, which were transduced with four factors in Figure 22A-F, were treated with different doses of GSK3-inhibitor (1 μ M, 0.1 μ M, 0.01 μ M). Figure 22A-C show AP stained 6-well plates of the three dose treatments and Figure 22D-F bright field microscopy images according to the AP picture above. Figure 22G illustrates the numbers of counted colonies compared to different culture conditions. The relation 1:15 represents the dilution of seeded infected fibroblasts on day 3. GSK3-inhibitor treatment was toxic to the cells regardless of whether low or high concentration.

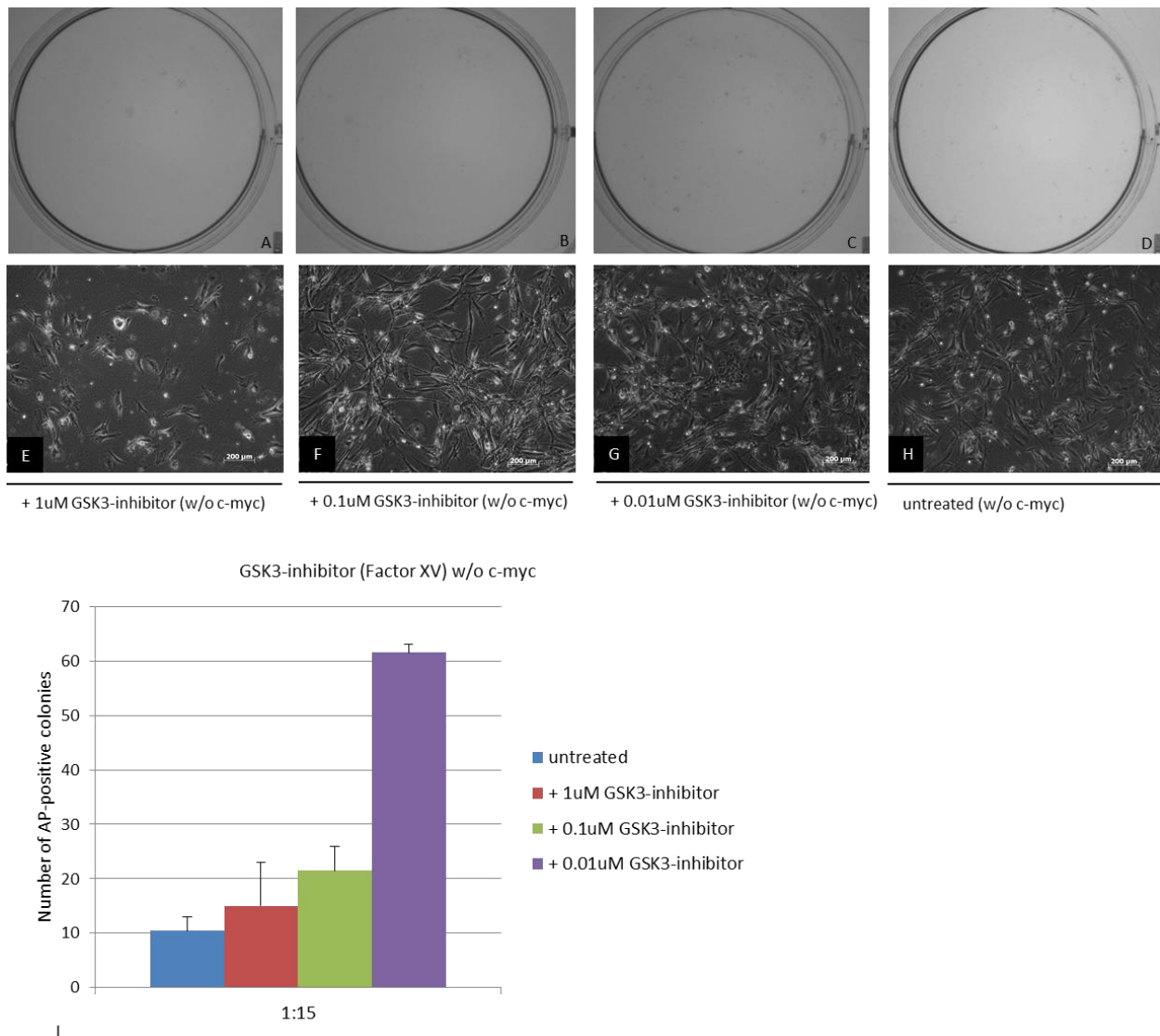


Figure 23: iPSC colonies without c-Myc (KOS transduced) treated with GSK3-inhibitor stained for alkaline phosphatase; generally, all experiments were done in duplicates (errorbars); μ M= μ M

Compared to Figure 22, cells in Figure 23 were transduced with KOS. Figure 23A-C and E-G were treated with three different doses of GSK3-inhibitor. Figure 23A-D show AP stained 6-well plates and Figure 23 E-H bright field microscopy images of the three dose treatments and one untreated control. The chart (Figure 23I) compares four different culture conditions. The relation 1:15 represents the dilution of seeded infected fibroblasts on day 3.

Regarding the untreated iPSC colonies in Figure 22, a 12-fold higher bar was detected. Certainly, a three factor transduction, led to less colony production. Though, the 0.01 μ M treatment with GSK3-inhibitor enhanced the iPSC colony formation 6-fold in KOS transduced iPSC colonies. Through GSK3-inhibitor adding at the lowest dose, Wnt target genes were activated and colony formation was enhanced, especially lacking c-myc.

4.3.2.2 Small molecule treatment with inhibitor of Wnt production (IWP)

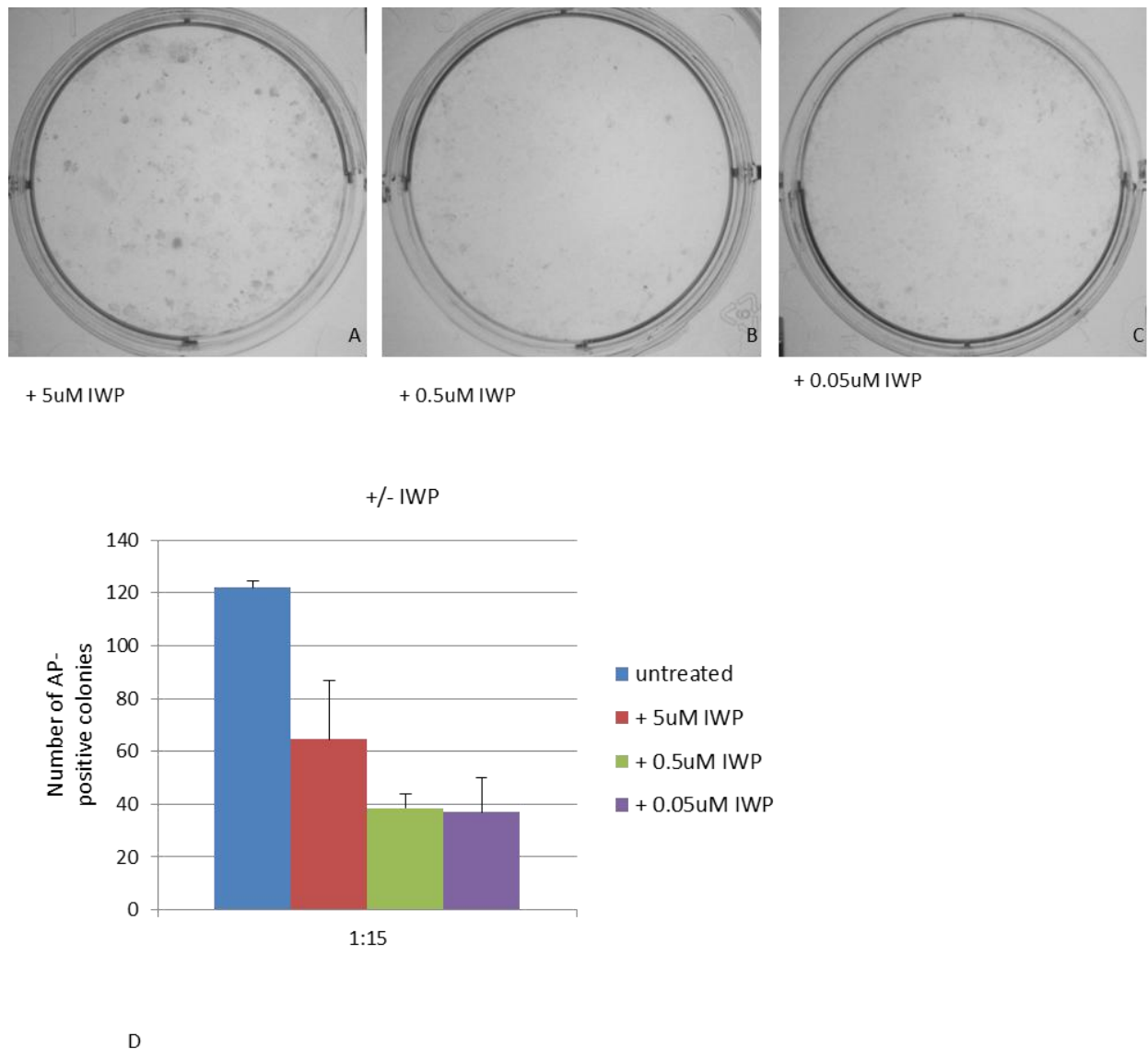


Figure 24: iPSC colonies (KMOS transduced) with three different doses of IWP concentration; generally, all experiments were done in duplicates (errorbars); uM= μ M

Cells were transduced with KMOS (Figure 24A-C) and treated with different doses of IWP (5 μ M, 0.5 μ M, 0.05 μ M). The images demonstrate AP stained 6-well plates of the three dose treatments. Figure 23D illustrates the number of counted colonies compared to different culture conditions in a chart. The relations 1:15 represent the dilution of seeded infected fibroblasts on day 3. Treating with IWP resulted into approximately 2-fold colony reduction. iPSC colonies without c-myc transduction did not show any

positive stained colonies (data not shown). The inhibition through IWP and the lack of c-myc led to a total inhibition of iPSC production.

4.3.2.3 Small molecule treatment with inhibitor of Wnt response (IWR)

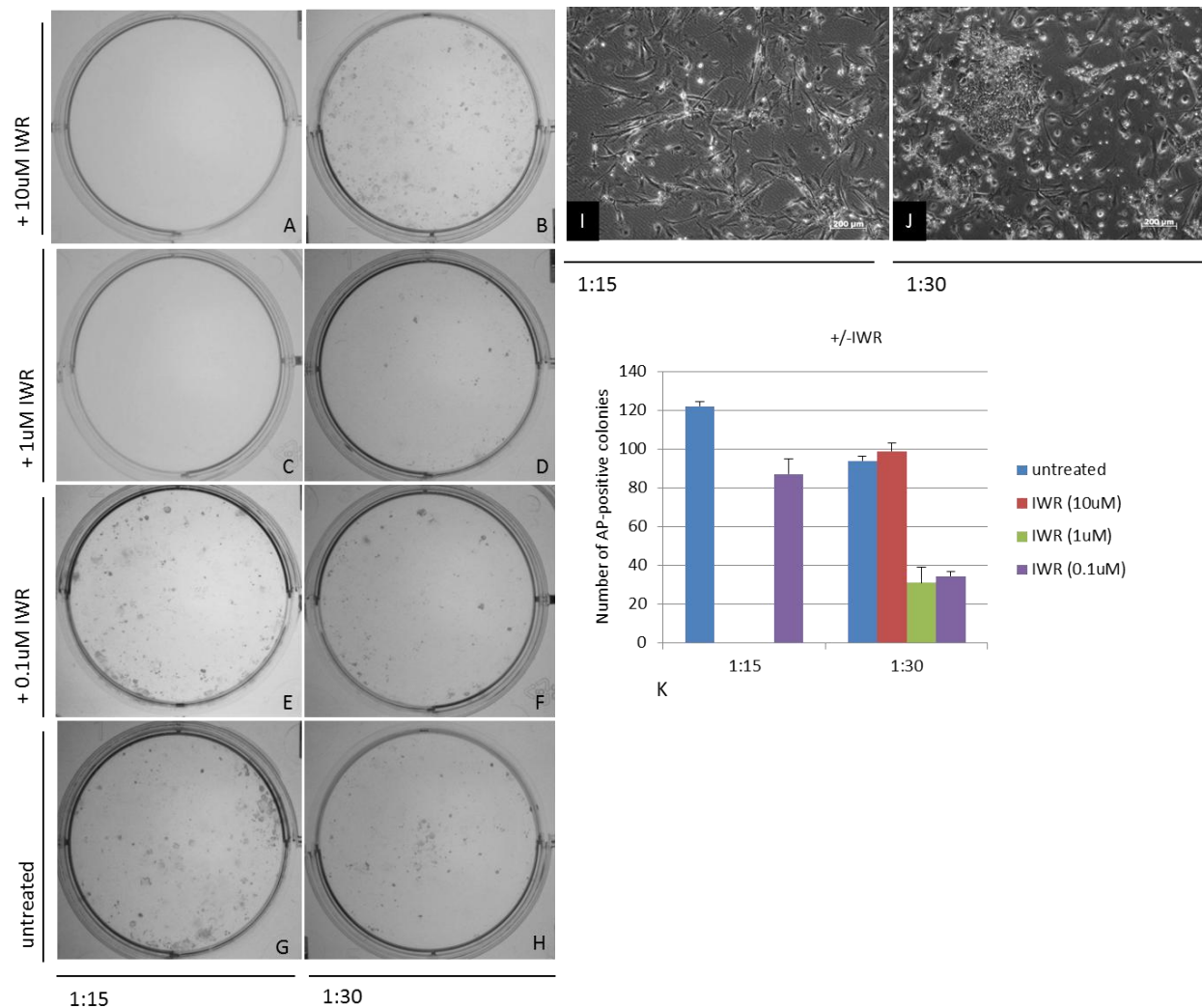


Figure 25: iPSC colonies (KMOS transduced) with three different doses of IWR concentration; generally, all experiments were done in duplicates (errorbars); A,C show no iPSC colony formation at all, probably a mistake occurred; I, J bright field images according to A and B; K graph illustrates 1:15 and 1:30 dilution to get a more conclusive chart; uM=µM

Cells undergoing reprogramming with KMOS were treated with different concentrations of IWR. The images (Figure 25A-F) demonstrate AP stained 6-well plates with different treatments labeled in the figure. For comparison, standard reprogramming mechanism without IWR addition was displayed in Figure 25G and H. Figure 25 I and J show bright field microscopy according to the AP stained 6-wells (Figure 25A and B). The chart in

Figure 25K compares the three dose treatments and the number of AP-positive counted colonies. The relations 1:15 and 1:30 represent the dilution of seeded infected fibroblasts on day 3. 1:15 dilution result did not show a convincing effect. However, the 1:30 dilution did direct into a contradictory result. The highest concentration of IWR doses led to approximately the same colony number as the untreated control. Whereas, the 1 μ M and 0.1 μ M concentrations lowered the colony formation process. iPSC colonies without c-myc transduction did not show any positive stained colonies (data not shown). The interaction of the inhibiting deed of IWR and the absence of c-myc reveal into total inhibition of iPSC production.

4.4 Effect of overexpression of Wnt pathway components Axin and β -catenin on iPSC generation

The experiment of overexpressing Axin and β -catenin in cells undergoing the reprogramming mechanism was performed in a separate batch apart from the previous ones. Different amounts of infected adult human fibroblasts at dilutions of 1:6, 1:12, 1:60, 1:120 were plated, stained with alkaline phosphatase and compared in 6-wells (1:60 and 1:120 data not shown).

All general procedures were performed as above (chapter 3.2.1.4 and Figure 5).

4.4.1 Time course for overexpression of Wnt pathway components

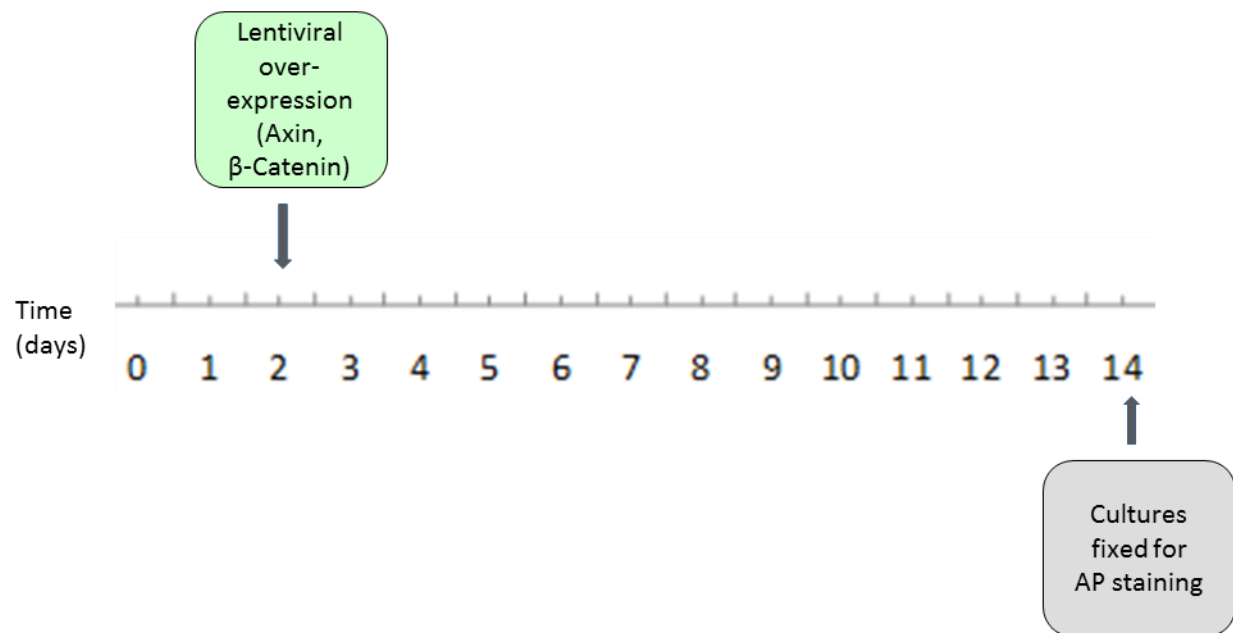


Figure 26: Time course for overexpression of Wnt pathway components

Cells were transduced with KMOS or KOS, as already described in Figure 5. The timepoint of Wnt pathway component overexpression was indicated in the image above (Figure 26) as well as the day of evaluating the experiment by alkaline phosphatase staining.

4.4.2 Alkaline phosphatase stain

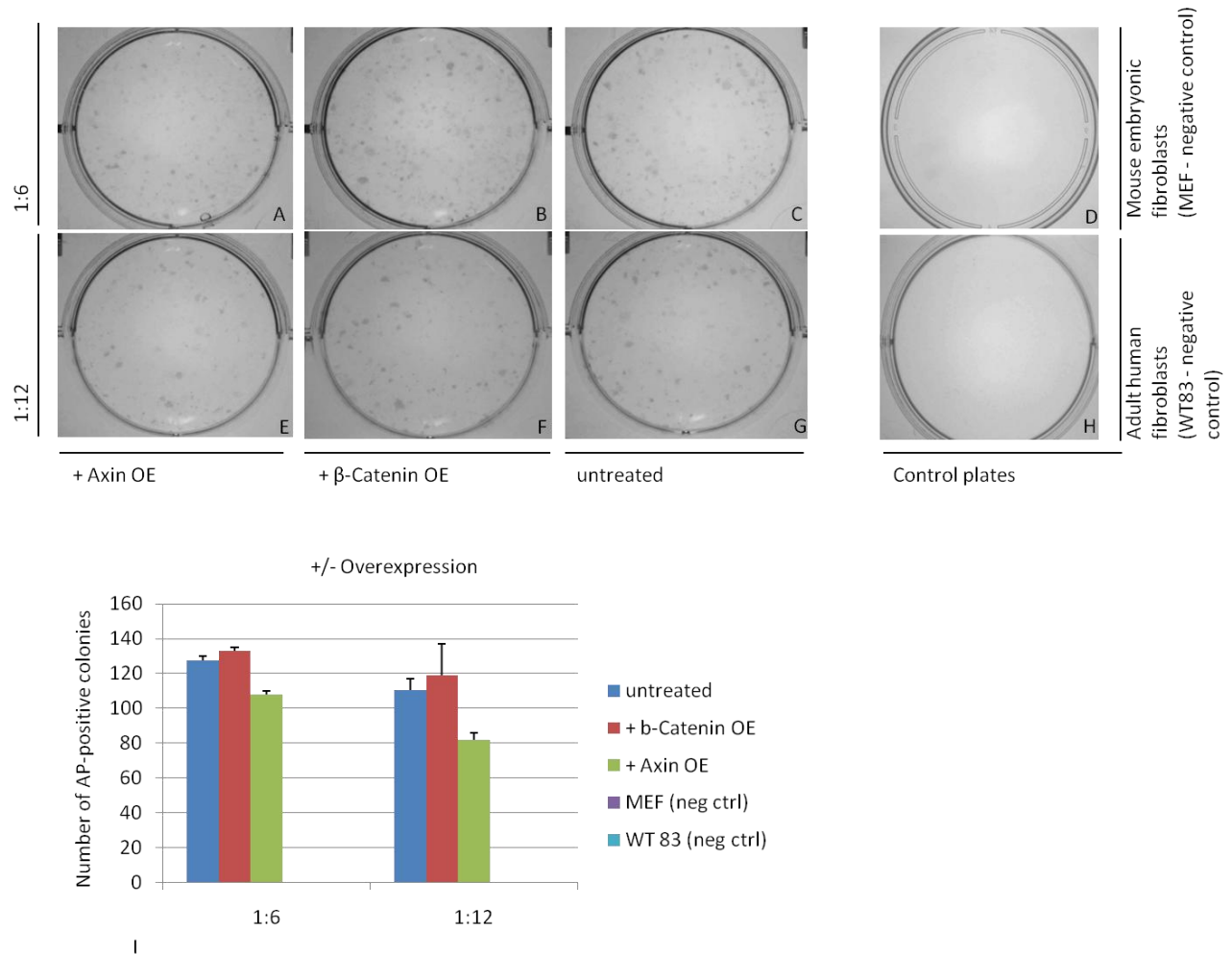


Figure 27: iPSC colonies (KMOS transduced) with overexpression of Axin and β -Catenin; A-C,E-G iPSC colony formation under different conditions; D,H two control plates; I shows twice almost the same pattern in two separate diluted (1:6, 1:12) experiments; generally, all experiments were done in duplicates (errorbars);

Standardly produced iPSC colonies (transduced with KMOS) were used as positive controls (Figure 27C and G) and Figure 27D and H shows two different negative controls. In Figure 27A,B and E,F the iPSC colony generation was affected by overexpression of two important Wnt pathway components, Axin and β -Catenin. The chart in Figure 27I illustrates AP-positive colonies of three variously treated iPSC generations and two controls. The relations 1:6 and 1:12 represent the dilution of seeded infected fibroblasts on day 3 and OE signifies overexpression. The blue and the

red bars in Figure 27I gave just a slight upregulation of the β -catenin treated cells, which is not very significant and has to be repeated in future. Another proof for lacking significance is the high errorbar on the red column in the 1:12 dilution experiment. Whereas, Axin showed a more consistent colony reduction.

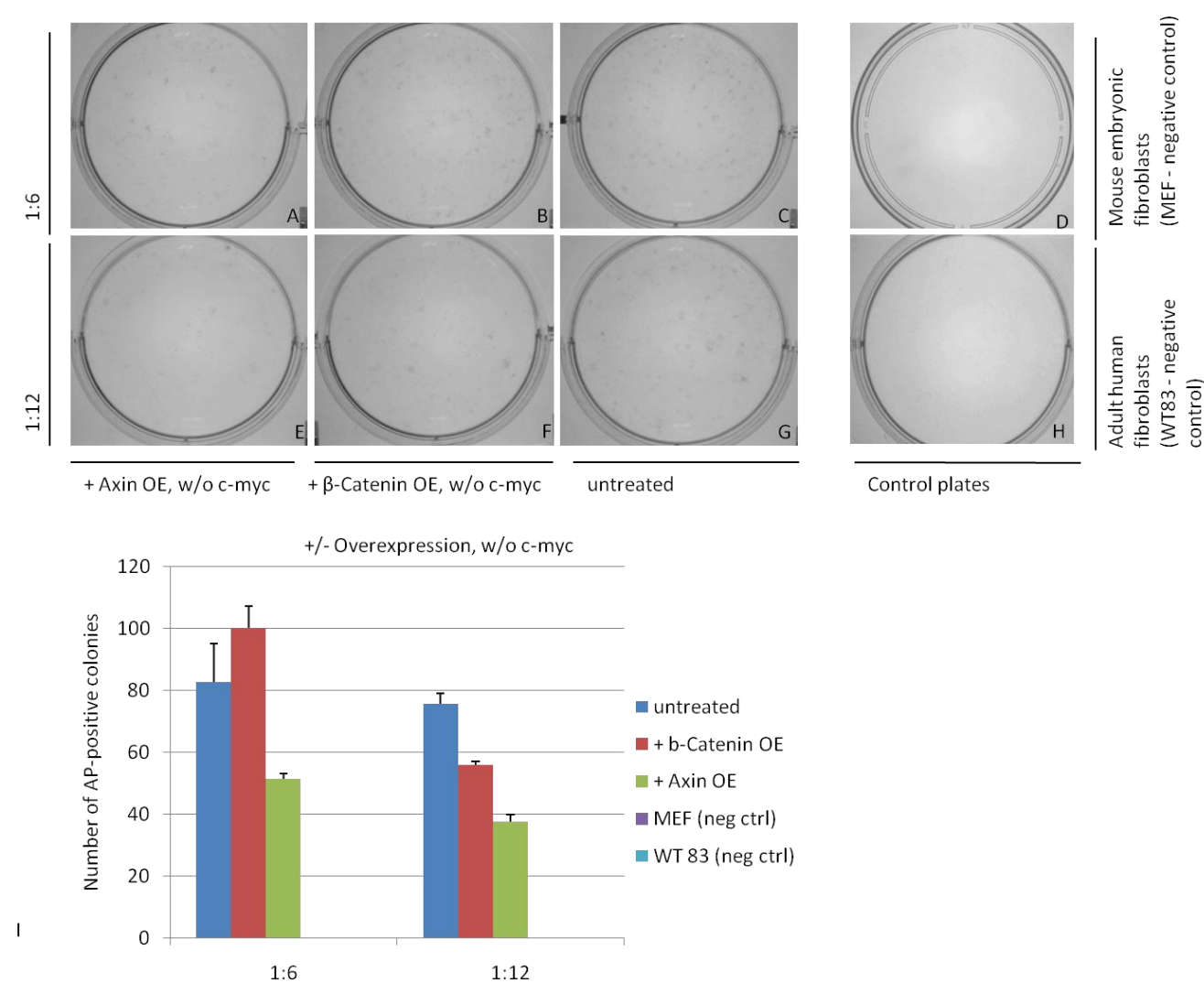


Figure 28: iPSC colonies without c-myc (KOS transduced) with overexpression of Axin and β -catenin; A-C,E-G iPSC colony formation under different conditions; D,H two control plates; I chart, showing experiments in two different dilutions; generally, all experiments were done in duplicates (errorbars)

Standard iPSC colony formation with KOS transduced cells affected by overexpression of two Wnt pathway components shown in images 28A, B and E, F. Figure 28C and G were iPSC colonies performed standardly with three factors used as positive control and

Figure 28D and H were used as two different negative controls. The chart in Figure 28I illustrates AP-positive colonies of three variously treated iPSC generations without c-myc and two controls. The relations 1:6 and 1:12 represent the dilution of seeded infected fibroblasts on day 3 and OE means overexpression. Regarding figure 28I and the red bars (β -catenin), lacks consistency. Whereas, the green bar (+ Axin) showed a consistent reduction compared to the blue bar. Moreover, the reduction concerning blue and green bars was also regressing according to their dilutions.

4.5 Effect of shRNA-mediated knockdown of Wnt pathway components FDZ7, ROR2 on iPSC generation

In this experiment, cells underwent standard reprogramming while lentiviral shRNA-mediated knockdown of Wnt pathway components and also other Wnt dependent treatments. As it was the first set we tried, the cells were not diluted, and all the growing cell densities were 1:1. Flow cytometry was used for the evaluation process, which was not significant enough. All general procedures were performed as above (chapter 3.2.1.4 and Figure 5 and 14).

4.5.1 Time course for knockdown of Wnt pathway components

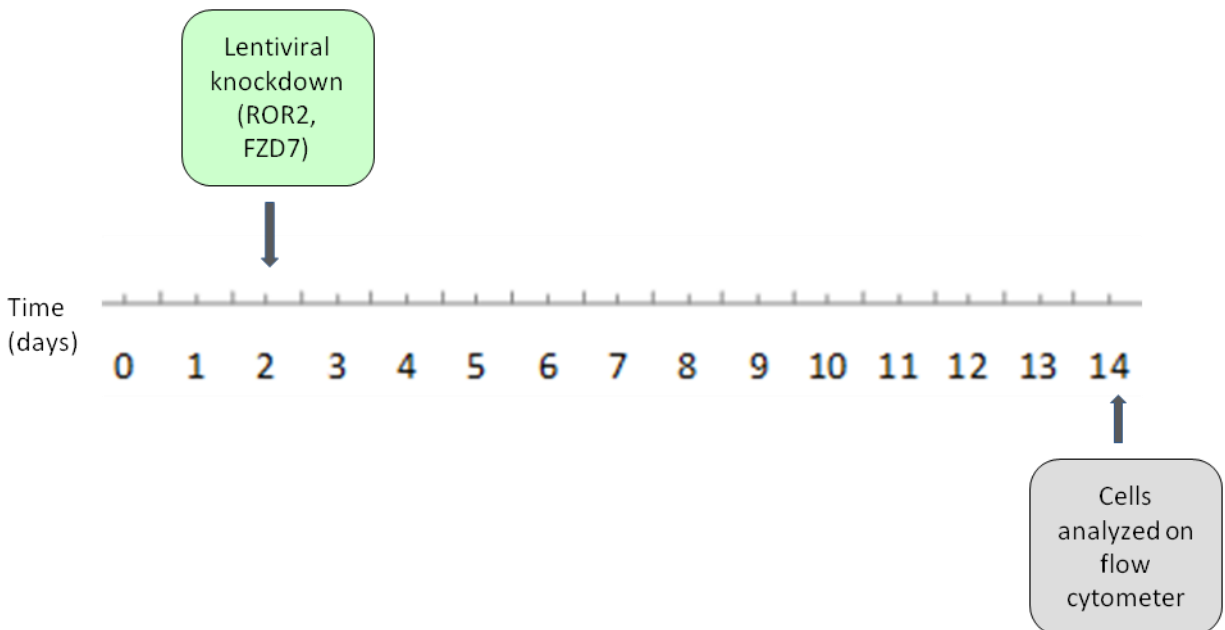


Figure 29: Time course for knockdown of Wnt pathway components

Figure 29 represents the timepoints of lentiviral shRNA-mediated knockdown of important Wnt pathway components and the day of evaluating pluripotent cells by flow cytometry. Timepoints of other Wnt dependent treatments see Figure 26, 21 and 18.

4.5.2 Flow cytometry analysis

In the first set, the infected adult human fibroblasts were transferred into wells without dilutions (1:1) and then analyzed on the flow cytometer.

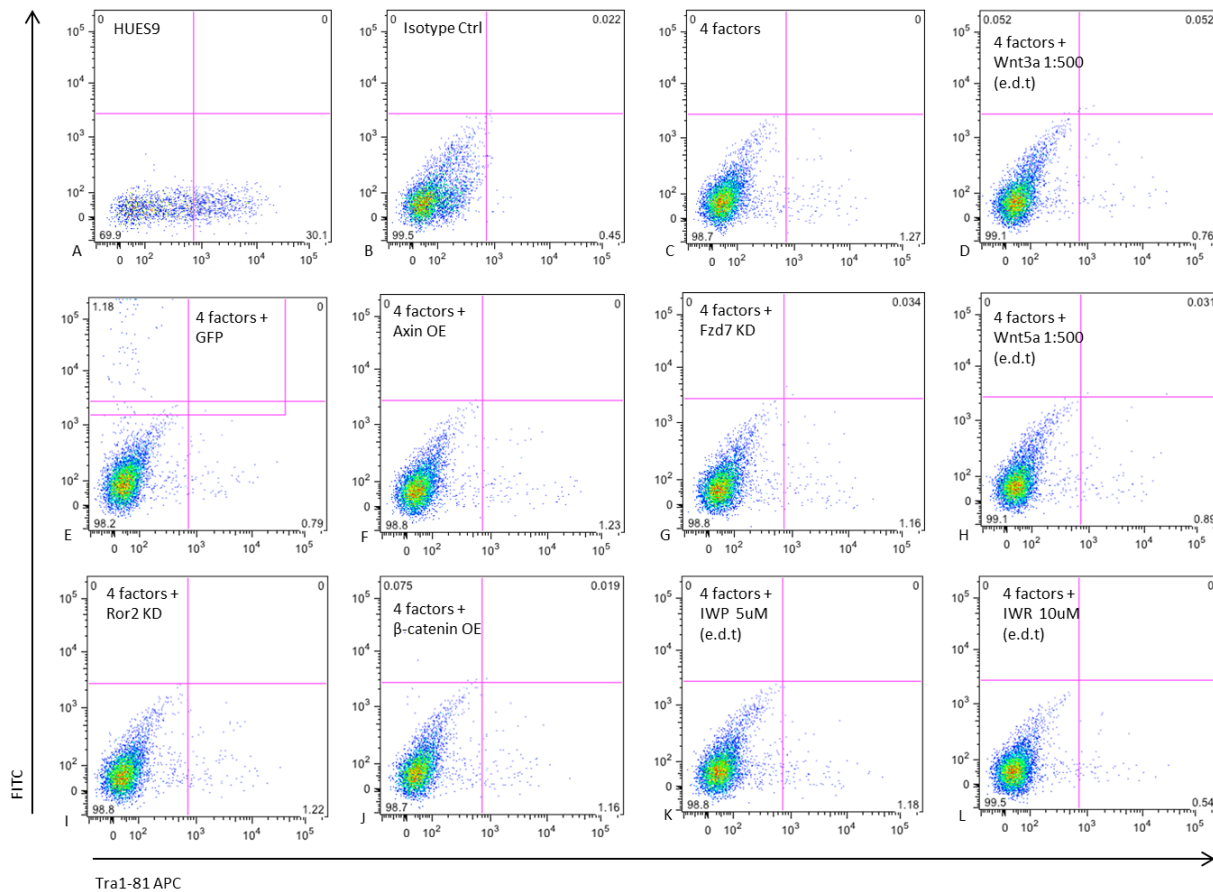


Figure 30: Scattered-plots of flow cytometry expression analysis of the human cell surface marker Tra1-81 on iPS cells transduced with KMOS with different treatments; FITC = control; E, FITC positive results, because of GFP expression; D,H 1:500 signifies the dilution; D,H,K,L e.d.t = every day treatment; F,J OE = overexpression; G,I KD = knock down;

This assay was performed on day 14 post-transduction of KMOS by counting 10.000 cells on the flow cytometer (BD FACS Canto II). The scattered-plots compare the APC versus the FITC intensity. They illustrate all cells and show how many of those 10.000 cells were stained for Tra1-81, which is pointed out by the number remarked in the

lower right corner of each plot. The iPSC generation was perturbed with many different Wnt pathway-dependent treatments, included the shRNA-mediated knock down of Frizzled 7 (Figure 30G) and ROR2 (Figure 30I). Figure 30A shows one positive control (HUES9), B, one negative control (Isotype control) and C, standardly reprogrammed cells transduced with KMOS. Figures 30D-L are four factor transduced iPS cell formations with different treatments, labeled in the figure.

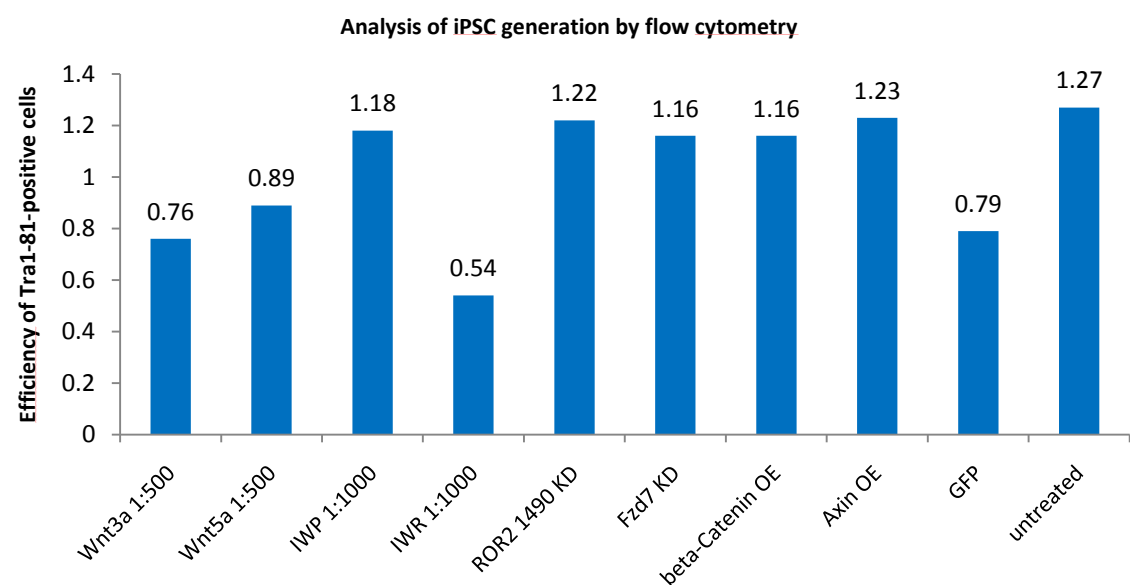


Figure 31: Efficiency numbers indicated of counting 10.000 cells (see Figure 30)

This chart exemplifies the efficiency of Tra1-81 positive cells after standard reprogramming (KMOS) adding Wnt pathway-dependent treatments, overexpressions (OE) or knockdowns (KD) by flow cytometer analysis referring to Figure 30 and released an unsignificant result.

As the flow cytometer analysis was unable to detect the various differences, we followed the alkaline phosphatase protocol and did not accomplish the shRNA-mediated knockdown along these lines.

5 Discussion

The main objective of this work was the comparison and evaluation of reprogramming efficiency while perturbing the Wnt signaling pathway in a number of ways. A proper assay with consistent parameters played an important role. A long period of time was invested to figure out the most efficient, reliable and consistent assay for comparison.

5.1 Perturbing Wnt signaling through small molecule treatment:

5.1.1 Wnt3a and Wnt5a treatments on cells transduced with Klf4, c-Myc, Oct4 and Sox2:

According to Figure 19G where Wnt3a and Wnt5a had various effects regarding different seeding densities (1:15 and 1:30). The influence of Wnt3a and Wnt5a treatments were dependent on the number of present cells. Seeding dilution 1:15 gave the most reliable result which corroborates our hypothesis. Wnt3a increased and Wnt5a decreased the number of iPSC colonies transduced with four factors (KMOS). However, seeding dilution 1:30 provided totally different results. Both treatments, Wnt3a and Wnt5a, slightly decreased the colony number compared to the untreated control. To this end, no significance could be concluded from the 1:30 experiment, but the number of seeded cells and their density could be a critical point.

5.1.2 Wnt3a and Wnt5a treatments on cells transduced with Klf4, Oct4 and Sox2:

Regarding Figure 20A-F the generation of iPSC transduced with KOS was slower and less effective, whereas the Wnt specific treatments had determining effects. Considering the result in Figure 20G Wnt3a treated cells gave a 9-fold increase in iPSC colonies, in both seeding dilutions (1:15 and 1:30). This outcome suggested a compensation of Wnt3a for the lack of c-myc. The regulation and compensation of Wnt3a in three factor transduced cells (KOS) was already described by Marson et al. 2008. Wnt3a treatment had a vast effect on three factor induced cells. Wnt3a treated KMOS induced cells did not show the same effect on colony increase, compared to chapter 5.1.1. Treatments of Wnt5a did not show any severe effects.

5.1.3 GSK3-inhibitor (Factor XV) treatment on cells transduced with Klf4, c-Myc, Oct4 and Sox2:

The GSK3-inhibitor treating shows a double negative effect on the cells. Therefore, Wnt target genes are activated and as a result iPSC generation increased. As illustrated in Figure 22G all three doses of GSK3-inhibitor show a decreasing effect, whereby colony formation increases with declining concentration. Though, no significant distinction could be detected. In Figure 22D-F the cell toxicity was visible in bright field images. The least toxic effect was given at the lowest concentration. Down dilution of GSK3-inhibitor is important to obtain any crucial effect. In summary, protein kinase inhibitors are toxic to cells.

5.1.4 GSK3-inhibitor (Factor XV) treatment on cells transduced with Klf4, Oct4 and Sox2:

The same toxicity problems occurred in that assay (Figure 23E-G), as already discussed in chapter 5.1.3. Nevertheless KOS transduced cells showed a significant raise (6-fold) of iPSC colony formation at the lowest dose and a slight increase of 1 μ M and 0.1 μ M concentrations (Figure 23I). Assumingly, GSK3-inhibitor and thus the activation of the Wnt signaling pathway were contributing to iPSC colony formation, especially in the absence of c-myc. Consequently, that experiment supported the hypothesis of GSK3-inhibitor activating Wnt target genes and therefore increasing colony formation number in iPSC generation.

Probably, the effect of GSK3-inhibitor treatment in the experiment lacking c-myc was higher, due to the fact that, generally KOS transduced iPSC generation functions 12-fold less sufficient than the KMOS transduced iPSC formation (see Figure 22G and 23I).

5.1.5 IWP treatment on cells transduced with Klf4, c-Myc, Oct4 and Sox2:

iPSC colony formation with various treatment of IWP doses (Figure 24), gives less colony numbers at all three doses compared to the untreated iPSC production control. IWP treatment revealed a constant decrease whether at higher or lower dose. To

conclude, using inhibitor of Wnt production was not dose dependent. A tiny addition of any IWP concentration decreased colony formation 2 to 3 fold.

5.1.6 IWR treatment on cells transduced with Klf4, c-Myc, Oct4 and Sox2:

According to Figure 25, inhibitor of Wnt response treatment outcomes were cell density dependent. The two experiments (1:15 and 1:30) did not concur. Bright field images in Figure 25I and J show two different cell densities (1:15 I and 1:30 J) with the same amount of IWR treatment. The seeding dilution 1:15 and addition of IWR concentrations 10 μ M and 1 μ M inhibited iPSC generation one hundred percent, whereas the lowest concentration (0.1 μ M) affected the cells only in a tiny decrease. Alternatively, the IWR treatment of 1:30 diluted cells did not show the same effect. 10 μ M IWR treatment had about the same colony number as the untreated control. And the lower doses decreased the number about 3-fold. No correlation to the other seeding density was detected.

In summary, both experiments of IWR treatment decrease the iPSC process. Though, some contradictory results were concluded. The 10 μ M concentration of IWP inhibits the iPSC generation completely (in 1:15), whereas in the 1:30 experiments, seems to keep the same cell colony number as the untreated control. The 0.1 μ M concentration of IWP shows the other way around. This experiment has no clear read out, and should be repeated. Probably, an error occurred while the reprogramming process took place.

Another interpretation would be a highly sensitive correlation of cell densities and IWR concentrations. A slight alteration in one of those two components would change the effect completely.

5.2 Perturbing Wnt signaling through overexpression or knock down

5.2.1 β -catenin overexpression on cells transduced with KMOS and KOS:

Already Yamanaka et al. 2006 suggested β -catenin as one of his narrowed-down 10 target genes (Figure 1) for his first attempt of reprogramming cells in 2006. The experiment (Figure 27I) did not show a striking effect between the untreated iPSC control and the β -catenin treated cells. Assumingly, the titer of the β -catenin lentivirus

could not reach the point to increase the iPSC production. Unfortunately, the titer of the virus was not measured.

Another problem occurred for KOS transduced cells. B-catenin was enhancing the reprogramming process at 1:6 cell density while at 1:12 cell density iPSC production was scaling down. As already mentioned in KMOS transduced cells, the β -catenin lentivirus infected cell number was not detected and the titer was not calculated.

5.2.2 Axin overexpression on cells transduced with KMOS and KOS:

Axin overexpression consistently lowered the iPSC production efficiency in KMOS and KOS transduced cells about 1.5-fold (Figure 27I and Figure 28I). This finding is consistent with our hypothesis, as Axin overexpression enhances β -catenin degradation.

Up to my knowledge this is the first report that Axin overexpression partly inhibits iPSC colony production.

5.2.3 Wnt pathway dependent knockdown on cells transduced with Klf4, c-Myc, Oct4 and Sox2:

The set of experiments in chapter 4.5 of shRNA-mediated knocking down of Frizzled7 and ROR2 was performed in parallel with the other already mentioned Wnt signaling pathway dependent treatments. The analyses were done through flow cytometry. The different efficiencies were indicating a small range between 0.54 and 1.27%. Concerning the numbers of GFP-overexpressing (0.79) and untreated cells (1.27), both show a high background. Technically, those two efficiencies should show an equal value, however compared to the other efficiencies in Figure 31, that noise (see also chapter 4.1.4) reveal limited sensitivity. Finally, flow cytometry analysis was not used as a sensitive assay to measure crucial differences.

Further on, all analyses were done by alkaline phosphatase staining and colony counting, which was determined as the most reliable assay.

5.3 Future plans

Currently, human pluripotent stem cell research has an exciting fast development. The induction and generation of iPS cells is one of the most promising research fields in molecular medicine. Therefore, a broad range of future issues exists. In this project, I would continue to repeat all experiments again and focus on Axin and β -catenin overexpression in iPSC formation. Furthermore, another evaluation process should be figured out and the flow cytometry analysis improved. Generally, to get more consistent results, infected adult human fibroblasts should be counted, before they get transferred on day 3, to achieve always the same number of colony formation. Besides, the retroviral three factor transduction should be optimized.

Further improvement would be, working without retroviral transduction. These days, other mechanisms as far as RNA-mediated or protein-mediated iPSC generation are possible.

5.4 Summary

Overall, the majority of the findings were dose and cell density dependent, on the supposition that always the same cell number was examined. The results suggest that Wnt3a tend to improve, while Wnt5a tend to decrease iPSC generation efficiency (Figure 19 and 20). More alkaline phosphatase-positive colonies were observed with faster rates of growth, significantly with the addition of Wnt3a. Wnt3a significantly compensates the lack of c-myc in KOS transduced iPSC.

Furthermore, Axin overexpression appeared to reduce the number of alkaline phosphatase-positive colonies compared to the negative control (Figure 27 and 28). This finding is consistent with our hypothesis, as Axin overexpression enhances β -catenin degradation. Interestingly, no observation of a significant increase in reprogramming efficiency with β -catenin overexpression was obtained (Figure 27 and 28). Possibly, the titer of the β -catenin lentivirus was too low to create a significant effect. This idea was further suggested by a Wnt-promoter luciferase reporter assay that demonstrated a two-fold increase in Wnt activity with β -catenin overexpression (data not shown).

In summary, the data suggests that activation of Wnt signaling increases the efficiency of iPS cell generation while inhibition of Wnt signaling decreases the efficiency of iPS cell generation.

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7 Abstract

Adult human somatic cells are capable of being reprogrammed to induced pluripotent stem cells (iPSC) by retroviral transduction of several transcription factors (Oct4, Sox2 and either Klf4, Yamanaka et al. 2006, and c-Myc or Lin28 and Nanog, Yu et al. 2007). While iPS cells hold therapeutic potential for the future of regenerative medicine, current methods of induction and maintenance of the pluripotent state are extremely inefficient (with reported efficiencies of <0.01%) and pose significant oncogenic risk (Okita et al. 2007). Several studies have demonstrated that the efficiency of reprogramming can be significantly enhanced by modifying culture conditions through the addition of small molecules or growth factors (Marson et al. 2008). Here we proposed a set of experiments to determine whether Wnt proteins, a class of signaling molecules with potent stem cell activities, and their signaling pathways regulate the acquisition of the pluripotent state. The goal of this project was to study the impact of Wnt signaling on the reprogramming and the regulation of the induced pluripotent state. This work provided insight into the possibility of utilizing Wnt proteins as reagents to generate iPS cells in the absence of viral gene transduction. Additionally, the induction of pluripotency was performed with only three factors (without the factor c-myc).

In summary, Wnt3a increases efficiency of iPS cell number of four and three factor transduced iPS cells. Especially in the absence of c-myc, Wnt3a seems to compensate the lack of one factor. Another essential finding was the double negative effect of GSK3-inhibitor, especially in the three factor transduced iPS cells. In three factor transduced iPS cells the inhibitor acted as Wnt pathway activator and contributed to the iPSC formation, while in the four factor transduced cells, it was toxic to the cells.

Wnt5a and Axin overexpression partly inhibited iPSC colony production consistently in three and four factor iPS cell production.

The results suggest that Wnt signaling definitely supports iPSC generation, some of those molecules or overexpressed genes indicate an up- or down regulation of induced pluripotent stem cell formation.

8 Zusammenfassung

Adulte humane somatische Zellen haben die Fähigkeit zur Reprogrammierung in pluripotente Stammzellen durch retrovirale Transduktion von diversen Faktoren (Oct4, Sox2, Klf4, c-Myc; Yamanaka et al. 2006 oder Oct4, Sox2, Lin28, Nanog; Yu et al. 2007). Obwohl die Reprogrammierung der Zelle in der Zukunft für die erneuerbarer Medizin viel Potenzial hat, sind die heutigen Methoden der Induzierung und Aufrechterhaltung des pluripotenten Zustandes äußerst ineffizient (berichtete Effizienz <0.01%) und es besteht ein onkogenes Risiko (Okita et al. 2007). Diverse Studien zeigen wie durch Änderung der Zellkulturbedingungen die Reprogrammierungseffizienz erheblich erhöht werden kann. Dies geschieht durch Zugabe von kleinen Molekülen oder Wachstumsfaktoren ins Zellkulturmedium (Marson et al. 2008). Wir führten eine Reihe an Experimenten durch, um festzustellen, ob Wnt Proteine, welche Signalmoleküle mit wirkungsvollen Stammzellaktivitäten sind, und deren Signalwege die Aneignung des pluripotenten Zustandes regulieren. Das Ziel dieses Projektes war die Auswirkung des Wnt Signalweges auf die Reprogrammierung der Zelle und dadurch die Regulation des induziert pluripotenten Zustandes zu studieren. Diese Arbeit gewährt Einblick in eine mögliche Methode, die zulässt, Wnt Proteine als Reagenzien zu verwenden, die induzierte pluripotente Stammzellen ohne virale Gentransduktion generieren. Außerdem wurde die Induktion der Pluripotenz auch durch drei Faktoren gewährleistet (ohne c-myc). Schlussendlich wurde festgestellt, dass Wnt3a die Effizienz der induzierten pluripotenten Stammzellen verbessert und speziell ohne c-myc, scheint Wnt3a das Fehlen des vierten Faktors zu kompensieren. Auf die Zellen, die mit drei Faktoren induziert wurden, wirkt der GSK3-inhibitor mit doppelt negativem Effekt als Wnt Signalwegaktivator und fördert die induziert pluripotente Stammzellenherstellung, während er in den mit vier Faktoren transduzierten Zellen nur toxisch wirkt. Wnt5a und Axinüberexpression inhibieren zum Teil den induziert pluripotenten Herstellungsvorgang nach Induzierung von drei und auch vier Faktoren. Die Ergebnisse dieses Projekts weisen auf eine definitive Unterstützung des Wnt Signalweges auf die Reprogrammierungseffizienz hin.

9 Curriculum Vitae

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Since 2003	Student at the University of Vienna, Molecular Biology Relevant courses: Specialization on 3 areas – Molecular Medicine, Cell Biology, Immunology
2002-2003	Student at the Technical University of Vienna, Technical Mathematics
1994-2002	Sports High School in Maria Enzersdorf, Austria (Graduation June, 1 st 2002)

Professional Experience:

Feb – Nov 2010	Diploma Thesis in Molecular Medicine (Karl Willert PhD, Ass. Prof. and ao. Univ.-Prof. Mag. Dr. Georg Weitzer) “Studying the Role of Wnt Signaling in iPSC generation” <i>Cellular and Molecular Medicine Departement, University of California San Diego</i>
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