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1 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 into 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. Differentiated cells can be reprogrammed into pluripotent cells. Several mechanisms were established, some less or more efficient:

1.1.1 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 turns away ethical problems.

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 (Yamanaka et al. 2006 + Yu et al. 2007)

1.1.2 Somatic nuclear cell transfer (SNCT):

Somatic nuclear cell transfer is a laboratory technique to create a clonal embryo using an oozyte and a 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 was removed and then inserted into a denucleated oozyte. First incident of a cloned model organism 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.

1.1.3 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 then 4, 3 and 2 factors, and compared the differences. It turned out, that the 4 factor transduction showed the most colonies and the best colony formation. (Yamanaka et al. 2006)

These iPS cells are thought to hold therapeutic potential, as somatic cells 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). Though, 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.

1.2 Consideration of Wnt Signaling in Reprogramming Process

Currently, 19 distinct WNT (W*ingless* and *I*NT-*1*) genes have been identified that code for secreted lipid-modified glycoproteins (Nuss et al.2009). Wnt signaling is involved in embryonic development and also controls homeostatic self-renewal in a number of adult tissues. (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 Yamanakas breakthrough, he showed the importance of β-catenin and the Wnt pathway involvement in iPSC generation.

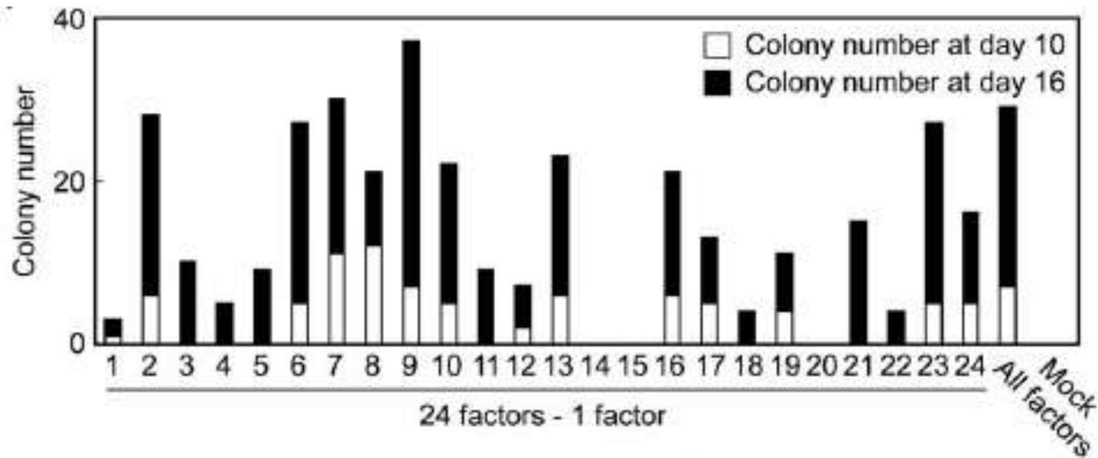


Figure 1: Effect of the removal of individual factors from the pool of 24 transduced factors (Yamanaka et al. 2006)

The role of β-catenin in Wnt signaling is indispensable. Wnt/β-catenin signaling is called 'The Canonical Wnt Signaling Pathway' (Figure 2). The absence of Wnt insists the Glycogen synthase kinase (GSK-3) to constitutively phosphorylate 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 degraded, therefore its level will not reach a sufficient significance. The presence of Wnt leads Wnt to bind its receptor, frizzled (Fz), and dishevelled (Dsh) is recruited to the membrane. GSK-3 is inhibited by the activation of Dsh. Accordingly, β-catenin accumulates and can be subsequently translocated into the nucleus where Wnt target genes were activated (Figure 2). This β-catenin works as key component. Additionally, β-catenin is also part of the complex which forms adherens junctions for cell-cell-adhesion.

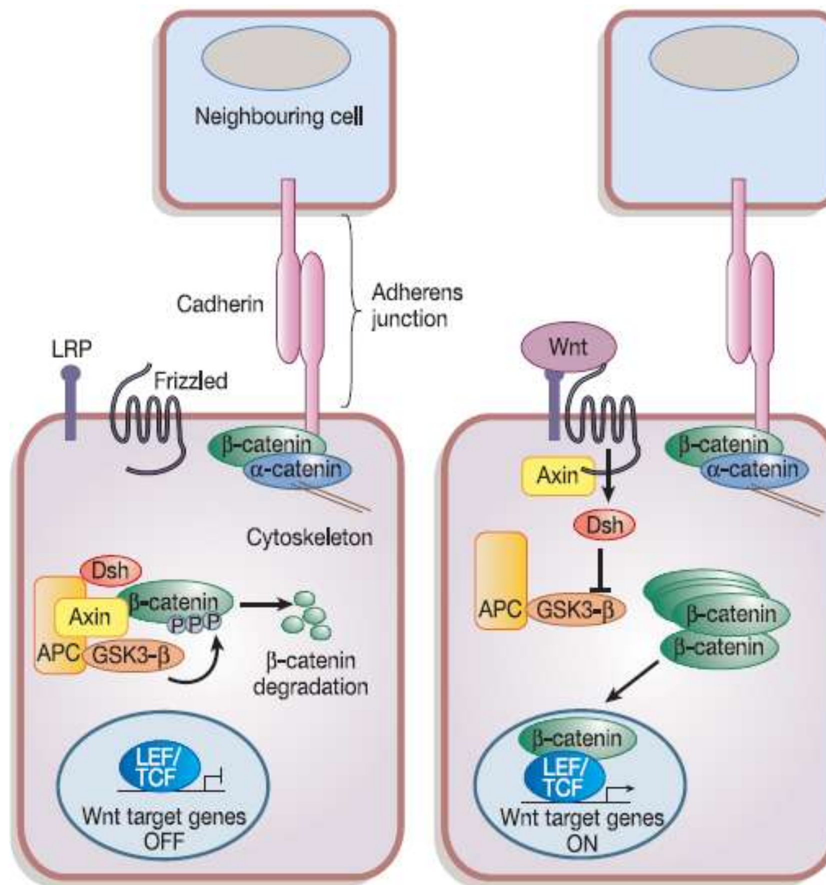


Figure 2: Canonical Wnt Signaling (Reya et al. 2005)

1.3 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.

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.

2 Materials and Methods

2.1 Materials:

2.1.1 Equipment:

Table 1: Used equipment

Device	device name	supplier
flow cytometer	BD FACSCanto II	BD Biosciences
centrifuge	5810R 15amp version	Eppendorf
centrifuge	Avanti J-E Centrifuge	Beckman Coulter
ultracentrifuge	Optima L-80 XP Ultracentrifuge	Beckman Coulter
tissue culture hoods	Class II Biohazard Safety Cabinet	ESCO
Cellomics microarray	Cellomics VTI High Content Screening System	Thermo Fisher Scientific Inc.
fridge 4°C		Sanyo
freezer -20°C		Kenmore
freezer -80°C/-150°C		Sanyo- VIP Series
incubator	Sanyo Co_2 Incubator	Sanyo Scientific
incubator shaker	Max-4000, A-Class	Barnstead-Lab Line
waterbath	Aquabath	Barnstead-Lab Line
maxiprep kit	NucleoBond	Macherey-Nagel
RNA isolation Kit	NucleoSpin RNA II	Macherey-Nagel
microscope	Axiovert 40, CFL	Zeiss
nanospectrometer	ND-1000	Nanodrop
scale	S-4001	Denver Instrument
EVOS		AMG
imager (gels, plates)		Alpha Innotech
genepulser		Biorad
sterile one-way pipettes		Costar
sterile filter (0.22um)		Millipore

cell Strainer (40um)		BD Falcon
syringe Luer-Lok Tip (5ml, 10ml)		BD Falcon
FACS tube		BD Falcon
cell culture plates (15cm,10cm,6-well, 12-well)		Coring
gene pulser cuvette (0.2cm)		Biorad
gelelectrophoresis device		Biorad
Mr. Frosty with isopropanol		NUNC
cryo-vials		Nunc
Beckman centrifuge tube	Cat.nr. 358126	Beckman
conical tubes (15ml, 50ml)		Sarstedt, Corning
qPCR plate		Sigma Aldrich
PCR machine		Eppendorf
qPCR machine	7900 HT Fast Real-Time PCR System	Applied Biosciences

2.1.2 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 they were used for lentiviral and retroviral transfection.

- *Primary mouse embryonic fibroblasts:*
  Millipore-Speciality Media, Embryomax ® PMEF-P3, Strain CF-1, 5 x 10^6 PMEF cells MITO-C treated, Cat# PMEF-CF, Lot# 90927-4, Store -80C 00004)

  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 maintaining pluripotency, and they provide a cellular matrix for iPS cells to grow.

- *Adult human foreskin Fibroblasts (WT83)*
  Cassiano Carromeu from 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 consist of 17 different stem cell lines. They are all pluripotent and were derived from human blastocysts.
  My work was focused on the HUES9 (Harvard University, HUES cell facility) line, and especially in this project HUES9 cells were used mainly as positive control. HUES 9 antibody markers are SSEA-1, 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)

2.1.3 Materials for cell culture

Growth Media:

Table 2: chemical composition of growth media for 293T cell culture, foreskin fibroblast cell culture and MEF cells culture.

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

Table 3: Chemical composition of growth media for 293T cell culture used after transfection

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

Table 4: Chemical composition of growth media for human embryonic stem cell (HUES9) culture.

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

Important for HUES9 culture to add bFGF (recommended 2ul stock/10ml media) immediately prior to use (final concentration of 20ng/ml)

Table 5: Chemical composition of growth media for induced pluripotent stem cell generation.

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

Table 6: Chemical composition of freezing media.

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

Reagents, chemicals, small molecules, buffers :

Table 7: List of reagents, chemicals, small molecules and buffers used for cell culture

product	supplier
TrypLE™ Express	GIBCO-Invitrogen
HY Clone-DPBS/Modified	Thermo Scientific
Collagenase	GIBCO
Matrigel	BD
Accutase	Millipore
bFGF 100ng/ul	Millipore
Valproic acid sodium salt (VPA) 10mM dissolved in PBS	Sigma Aldrich
Wnt3a	Willert lab
Wnt5a	Willert lab
IWP 5mM	Lum lab
IWR 10mM	Lum lab
GSK3-Inhibitor (Factor XV) 10mM	Calbiochem
CHAPS 3-[(3-cholamidopropyl) dimethylammonio]-1-	1%CHAPS, 1x PBS, 1M NaCl

propanesulfonate

2.1.4 Materials for microbiological methods

2.1.4.1 Microorganisms:

E.Coli DH5α cloning strain was used for all transformations. The bacteria were transformed with high efficiency. The successful DNA transformation was controlled by the appropriate antibiotic. All used plasmids were ampicillin or carbomycin resistant.

2.1.4.2 Growth Media:

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

Ingredient/supplier	**Amount (specifications for 1000ml)**
Casein Peptone	12 g
KH2PO4	2.31g
K2HPO4	12.54g
UF Yeast Powder	24
Glycerol	4ml

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

Ingredient/supplier	**Amount (specifications for 1000ml)**
Casein Peptone	10g
NaCl	10g
UF yeast powder	5g

2.1.4.3 DNA-Plasmids:

Table 10: List of DNA plasmids used for retroviral transfection of the four Yamanaka factors

name	**Concentration ng/ul**	**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	1928.67	1.89

VSVG	4910.81	1.57

Table 11: List of DNA plasmid constructs used for lentiviral transfection.

name	Concentration ng/ul	Ratio 260/280
B-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
Lenti –GFP	1494.86	1.89
psPAX2	3554.25	1.84
VSVG	4536.86	1.71

Table 12: Concentration and pH of the transfection reagent Polyethyleneimine (PEI).

PEI conc: 1ug/ul	1 x PBS pH 4.45

2.1.5 Materials for molecular biological methods

2.1.5.1 Kits

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

product	cat. No.	supplier
RNA isolation		Nucleospin RNAII; Macherey-Nagel
Plasmid DNA Purification (Maxiprep)		• Qiagen • NucleoBond; Macherey-Nagel
Alkaline Phosphatase (AP) Staining Kit	00-0009	StemGent

2.1.5.2 Buffers

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

product	supplier
DBPS	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 dissolvation the RNase A solution was transferred back to the bottle containing buffer RES. The buffers final concentration of RNase A is 60ug/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_2O	15ml
2-Mercaptoethanol (GIBCO)	50ml
70% Ethanol (Sigma Aldrich)	50ml

*540ul of RNase-free H_2O was added to the rDNase vial and incubated for 1 minute at room-temperature. The DNase was completely dissolved and aliquoted 10ul per tube for storage at -20°C.

2.1.5.3 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 0.1ug/ml	Fermentas
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

name	sequence
Nanog	
Pou5f1	GTGAATGACATTTGTGGGTAGGTTA

Table 19: List of Restriction endonucleases and restriction endonuclease buffers (www.neb.com)

restriction enzymes	supplier
EcoRI (NEB3 or EcoRI buffer) 20U/ul	New England Biolabs (NEB)
XhoI (NEB4) 20U/ul	New England Biolabs (NEB)
NotI (NEB3)10U/ul	New England Biolabs (NEB)
PvuII (NEB2) 10U/ul	New England Biolabs (NEB)
HindIII (NEB2) 10U/ul	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)

2.1.6 Materials for analytical Methods

2.1.6.1 Chemicals, buffers, reagents

Table 20: List of chemicals used for analytical methods

product	supplier
Tryphan 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)

Ingredient/supplier	Amount (specifications for 510ml)
DPBS	500ml
BSA (1%)	0.5g
EDTA (10mM)	10ml of 0.5M EDTA

2.1.6.2 Antibodies

Table 22: List of antibodies used for analytical methods

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

2.1.6.3 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

2.1.7 Software programs for bioinformatic methods

Table 24: Software programs and databases used for bioinformatic methods

software program/database	supplier/website
Addgene	http://ww.addgene.org
Pubmed	http://www.ncbi.nlm.nih.gov/pubmed
National Center for Biotechnological Information	http://www.ncbi.nlm.nih.gov/
Cell cultivation 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

2.2 Methods:

2.2.1 Cell Culture Methods

2.2.1.1 General Procedures:

All cell culturing was performed in the human embryonic stem cell core facility, under sterile conditions, using the Class II Biohazard Carbinet, unless otherwise stated. Steril 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% CO_2. 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	5ul
10cm dish	10ml	10ml	Accutase 3ml, TrypLE 2ml	3ul
6-well	3ml	3ml	TrypLE 1ml, Collagenase 1ml	1ul
12-well	1.5ml	1.5ml	Tryple 0.5ml	0.5ul

2.2.1.2 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 was used DMEM 1x + 4% FBS.

- Cell thawing:

A vial of cells was thawed in the waterbath at 37°C. The cell plate and 9ml of media was yet prepared, considering the toxic DMSO of thawed cells. After entirely thawing the suspension, cells were spread consistently all 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) and removed by pipetting after 10 seconds. The plate was incubated at 37°C for 5 minutes. Afterwards, the cells have been dissociated and could easily be 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 2.2.1.2–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 10^6 cells (Table 28) the cells were transferred immediately into a cryo-tube, inserted into a Mr. Frosty containing Isopropanol. Mr. Frosty was stored at -80°C and froze the cells slowly by decreasing the temperature 1°C per minute.
  After one day the tubes can be transferred into the -150°C freezer.

2.2.1.3 Transfection for Lentivirus (2nd Generation):

<u>Transfection</u>

Lentivirus was always produced on HEK 293T cells. The cell dish was 70% confluent, which means about 11 x 10^6 cells were growing. To increase the titer and the

transduction efficiency 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 (ug)	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 was added to 1ml of DMEM 1x, filtered, and 4 times the amount of DNA, of PEI (1ug/ul) 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% CO_2. 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 (0.22um filter unit) and stored at 4°C. 72 hours post-transfection, the second media collection was done and the purification step was initialized.

Virus Purification:

For purifying the virus, the Bleckman Coulter Optima L-80 XP Ultracentrifuge with the SW32i 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 necessarily, the scale was tared. The ultracentrifuge requires a precisely and sensitive balance of the opposite rotor capsules. The amount of 28g of 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 of the capsules. On the bottom of the tube a small pellet should be appeared. The supernatant was discarded into 10% bleach and the centrifuge tubes inserted into 50ml conical tubes. 100ul of DMEM-High Glucose was added onto the cell pellet and not-mechanically resuspended over night at 4°C. On the next day fibroblasts got infected with 150-200ul of virus. (see chapter 2.2.1.10)

2.2.1.4 Transfection for Retrovirus: Yamanaka Cocktail (Oct4, Sox2, Klf4, c-Myc)

The Yamanaka factors are referring to his paper (Yamanaka et al. 2006, see chapter 1.1.3) and his first induction of pluripotent stem cells. In this thesis they are called “Yamanaka factors”, “four factors” or “KMOS”, the abbreviation for OCT4, KLF4, SOX2, C-MYC.

The retrovirus was produced on HEK 293T cells as well. The cell dish was 80-100% confluent, which means about 16×10^6 – 20×10^6 cells were growing. To achieve a high titer and iPS cell generation efficiency, it was recommended to transfect at least 6 plates per vector. For the whole cocktail 24 plates were used. For specific amounts of transfected DNA, see table 27.

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

vector	Amount (ug)	Size (kb)
Transfer vector (factor)	13.8	5.5-6
CMV-GP	9.2	~12
VSVG	4.6	5.8

The DNA-mix was added to 1ml of DMEM 1x, filtered and 4 times the amount of DNA, of PEI (1ug/ul) 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% CO_2. 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 and all factors were pooled to simplify the virus purification. The virus collection was performed as already described in chapter 2.2.1.3.

Virus Purification:

To harvest the virus, the description in chapter 2.2.1.3 was followed. Usually two centrifugations were set up for 24 plates containing 16ml of virus.
On the next day, the virus was pooled, spinned down at 200 x g for 4 minutes and one 6-well of fibroblasts was infected by 200ul of virus-supernatant.

2.2.1.5 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 2.2.1.4 without transfection of the transfer vector C-MYC.

2.2.1.6 Culture of adult human foreskin fibroblasts

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 2.2.1.2 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.

2.2.1.7 Culture of MEFs

Mouse embryonic fibroblasts (MEFs) were always cultured in DMEM 1x with 10% FBS. This cell type was never passaged, and after cell thawing useable for one week.

- Cell thawing and preparation of MEF plates:
  One vial of MEFs was supposed to be 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 2.2.1.2 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 2.2.1.8 and 2.2.1.9) 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.

2.2.1.8 Culture of HUES9

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

- Preparation of matrigel plates:
  A vial of matrigel 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 2.2.1.7

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

- Pluripotent maintenance:
  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 was added every day as well.

2.2.1.9 Culture of iPS Cells

- Production of iPSC-MCM:
  iPSC-MCM was produced as already described in chapter 2.2.1.7.

- Cell passaging:
  To passage single iPSC colonies has been working under the EVOS microscope.

The media was aspirated and 1ml new iPSC-MCM was added. The chosen colonies were cut off the cell layer with 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 2.2.1.8), where additional 2ml of iPSC-MCM and growth factor bFGF 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 was added every day as well.

2.2.1.10 Generation of induced pluripotent stem cells

The whole process in my project took at least between 12 and 30 days and precisely planning and planning ahead was indispensable. To start the whole iPS cell generation, the retrovirus containing the Yamanaka factors (four or three) was produced as described in chapter 2.2.1.4 and 2.2.1.5. On the same day of the retroviral transfection, one vial of frozen human fibroblasts were thawed and cultured as described in chapter 2.2.1.6 in a 10cm dish, to obtain 100% confluency two days later.

Day 0 – Preparing fibroblasts

One day before fibroblast infection, they were transferred and plated in different confluences on 6-wells. The cell suspension after dissociation contains 10ml of media. The different confluences were plated as shown in figure 3. Different cell densities were used to reach the right cell density 24 hours later for the retroviral infection.

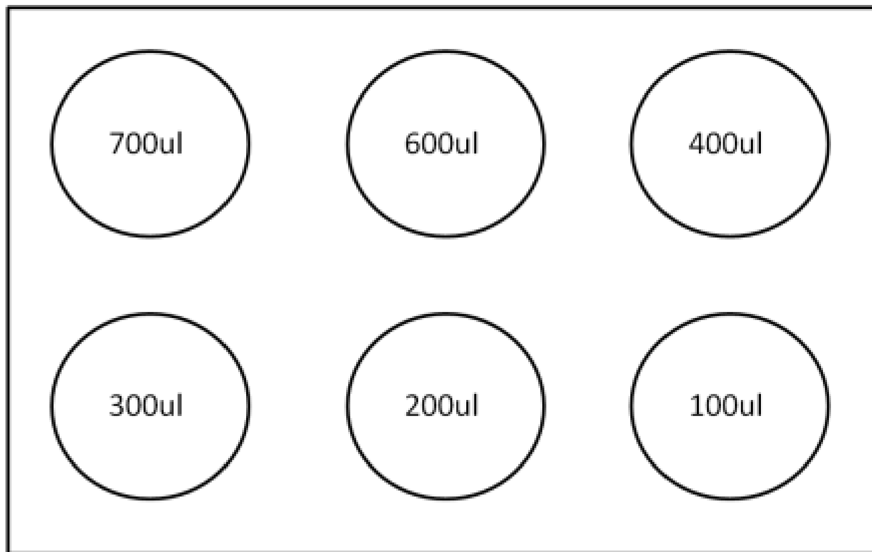


Figure 3: Preparation of WT83 fibroblasts on day 0
*ul of cell suspension in 3ml of Media

Day 1 – Fibroblast infection:

The well 50% confluent well (see table 28) was chosen, the existing media reduced and 200ul of the pooled Yamanaka cocktail (preparation see chapter 2.2.1.4) were transduced 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 by proper lentivirus (see chapter 2.2.1.3).

Feeder cell plates were prepared as already described in chapter 2.2.1.7.

Day 3 – Cell transfer

The protocol of chapter 2.2.1.2 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 x 10^6			
50% confluency (recommended for transduction)	5.5 x 10^5	1:6	9.2 x 10^4	10,222
		1:12	4.5 x 10^4	5000
		1:15 (recommended)	3.6 x 10^4	4000
		1:30 (recommended)	1.8 x 10^4	2000
		1:60	9.1 x 10^3	1011
		1:120	4.6 x 10^3	511

Day 4 – Changing into iPSC media

The iPSC media was prepared by filtering all ingredients as listed in table 5 and also a fresh stock of 10mM VPA which was sterilized. The media was changed as described in 2.2.1.9 and additionally, cells were treated 5 days (from day 4 until day 8) with valproic acid using a 1:1000 dilution. VPA is a histone deacetylase inhibitor (HDAC inhibitor), which enhanced the reprogramming process.

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 2.2.1.11

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

The next days, the media was replaced 2.2.1.9 and treated as described in chapter 2.2.1.11. Day 8 was the last day of VPA treatment.

Day 11 – Colony formation:

Since day 11 the same steps as day 10 were done but using iPSC-MCM. iPSC-MCM production was described in chapter 2.2.1.9

2.2.1.11 Perturbing Wnt Signaling Pathway:

- Through adding small molecules:
  In some experiments iPSC were treated with small molecules. Treatment was added every day in the same concentration until the cells were analyzed. The different small compounds were Wnt3a, Wnt5a, IWP, IWR and the GSK3-inhibitor - Factor XV.

- Through lentiviral overexpression or knock down:
  In other experiments lentivirus knock-downs or overexpressions 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.

2.2.2 Microbiological and biological methods

The preparation of buffers, chemicals, reagents and equipment is shown in chapter 2.1.

2.2.2.1 Transformation: (Chemical transformation and electroporation)

Production of electro-competent bacteria (DH5αe):

E.Coli DH5α (35ul) in 10ml LB Media were inoculated overnight on 37°C. The next day inoculations with $1/100^{th}$ Volume of the overnight culture were performed into twice 500ml of LB Media. The 2 cultures were shaked at 37°C until the OD_{600} 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 was 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 50ul 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 was thawed on ice. After approximately 10 minutes, 50ul of bacteria and 1ul 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 was pressed. The pulsed bacteria were removed from the holder, 1ml LB media was added and the suspension transferred into an O_2 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, carbomycin or ampicillin plates were warmed up at room-temperature. For plating the right amount of bacteria, the transformed bacteria was spinned and 100ul LB Media was added to the pellet. 20ul of the cell suspension was transferred and plated onto plates containing the appropriate antibiotics, either carbomycin or ampicillin. 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 2.2.3.1).

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 maybe less efficient than the electroporation.

One vial of chemical competent bacteria was thawed on ice. After thawing for 10 minutes, 1ul Plasmid DNA was added in about 30ul 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 500ul 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 2.2.2.1 – Electroporation.

2.2.3 Molecular biological methods:

The preparation of the buffers, chemicals, reagents, equipment and kits is showed in chapter 2.1.

2.2.3.1 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 OD_{600} was measured and the recommended volume was determined, according to the formula: V [ml] = 1200/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 completely 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 concial tube.

- *Precipitation:*
  For proper precipitation 10.5ml room-temperature isopropanol were 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 measured on the nanospectrometer.
- *Measurement*: see 2.2.4.1

2.2.3.2 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 spinned 14,000 x g for 1 minute. The pellet was lysed by 350ul buffer RA1 and 3.5ul 2-mercaptoethanol. The lysate was filtered by adding the viscose suspension onto the NucleoSpin filter and centrifuged at 11,000 x g for 1 minute. The filter was discarded, 350ul 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 spinned 30 seconds at 11,000 x g. 350ul 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:

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

95ul 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 200ul of RA2, 600ul of RA3 and spinning for 30 seconds at 11,000 x g. This procedure was repeated one more time by adding 250ul RA3 and spinning for 2 minutes at the same speed. Finally, the RNA was eluted in 60ul RNase-free H_2O and centrifuged for 1 minute at the usual speed. For RNA measurement concentration see chapter analytical methods.

2.2.3.3 PCR - cDNA synthesis

To successful synthesize cDNA, 1ug of RNA, 4ul of cDNA Supermix and the rest ddH_2O were mixed to obtain a total volume of 20ul. The cDNA was reverse transcribed on the PCR machine for 1 hour. The synthesized cDNA was stored at 4°C.

2.2.3.4 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) A mixture of mastermix, probes and dH_2O was performed 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 dH_2O per sample

ingredient	amount (x1)	amount (x7)
TaqMan® Mastermix	10ul	70ul
TaqMan® Probe	1ul	7ul
dH_2O	9ul	56ul

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)

2.2.3.5 DNA gelelectrophoresis

The success of the DNA purification 2.2.2.1 was controlled 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 added and poured into the gel chamber. The comb was attached onto the apparatus. After 15-20 minutes the gel was solidified and transferred in the running-equipment, which was then filled up to the max-mark with 1x TAE buffer. The samples (for additional digestion see chapter 2.2.3.6) about 0.3ug DNA were mixed with loading dye (1/5 of the total amount) and 70% ddH2O and were loaded on the gel. 6µl of the 1kb DNA ladder was loaded on the gel as well. The equipment then was run for about 25 minutes at 100V. The gel was then photographed using the gel documentation system. The isolated DNA was then used for a retroviral transfection, see chapter 2.2.1.4.

2.2.3.6 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 70% ddH2O and 10% enzyme mastermix.

Enzymemastermix:

- 10% Restrictionenzyme
- 90% Restrictionenzyme 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 2.2.3.5).

2.2.4 Analytical Methods

The preparation of the buffers, chemicals, reagents, equipment and kits is showed in chapter 2.1.

2.2.4.1 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 ug/ul.

Measurement of RNA concentration: The same technique was used as for DNA measurement, just the blank was RNase-free H_2O.

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

2.2.4.2 Alkaline Phosphatase (AP) staining in situ and colony counting on photoshop or cellomics

The culture medium of the iPSC plates was aspirated and washed twice with 1x PBST. To fix them, 1ml of fixing 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 was prepared at a ratio of 1:1 right before the staining process. Accordingly, 500ul 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 would result in a purple stain,

while the absence of AP expression showed no stain. Eventually, the well was covered by 500ul 1x PBS to prevent drying and stored at 4°C.

2.2.4.3 Flow Cytometry and staining for Tra1-81 or SSEA-4

Cell dissociation:

The cells have been washed with DPBS Buffer and for their dissociation have been added filter-sterilized 1mg/ml collagenase IV (1ml/6well, 5ml/10cm). After 1 hour the cells were coming off their feeder cells. For better dissociation they have been pipetted up and down and transferred to a 15ml conical tube. The wells have been 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:

- 10ul of single cell suspension
- 10ul of Tryphan Blue

10ul of the preparation was taken and added onto the hemocytometer.

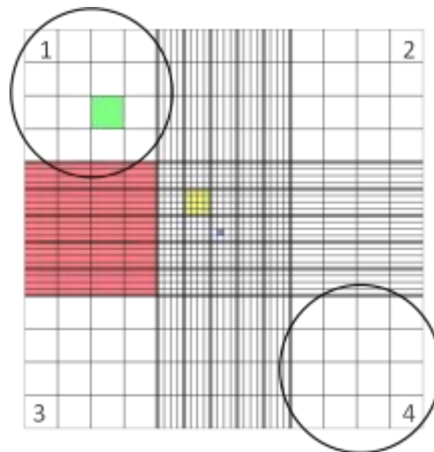


Figure 4: Haemocytometer (http://static.flex.com/pictures/3/1/0/t/Haemocytometer_Grid.png)

The cells, located in chamber 1 und 4 (marked in Figure 4), were counted, without regarding the blue stained cells. If tryphan blue entered in the cells, these cells were considered dead. The counted number of cells was divided by two, multiplied by 10,000, the dilution factor and the total volume, see formula below.

Total number of cells = number of cells (counted) x 10,000 x dilution factor x total volume of sample

The chosen cells were transferred into a FACS tube and stained for the right antibody.

Antibody staining:

Preparation mix for antibody staining per sample:

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

The chosen aliquot of cells was centrifuged 200 x g for 4 minutes, the supernatant aspirated and the pellet treated with 102.5ul 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, 350ul of FACS buffer was pipetted into each FACS tube.

Flowcytometry on BD FACS CantoII (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. All used fluorochromes are listed in table 31.

Table 31: Flourochromes 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	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 machine was cleaned by bleach and ddH_2O.

2.2.4.4 Flow cytometry and staining for alkaline phosphatase expression

Cell dissociation and cell counting were done as in chapter 2.2.4.3.

Alkaline phosphatase staining:

The cells were centrifuged at 200 x g for 3 minutes and fixed 1-2 minutes with 400ul of the AP kits (Stemgent) fixing solution. By adding 20ul 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 200ul of AP staining solution (see chapter 2.2.4.2) 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 2.2.4.3.

3 Results

3.1 Method for induction of pluripotency

3.1.1 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 presented in chapter 2.

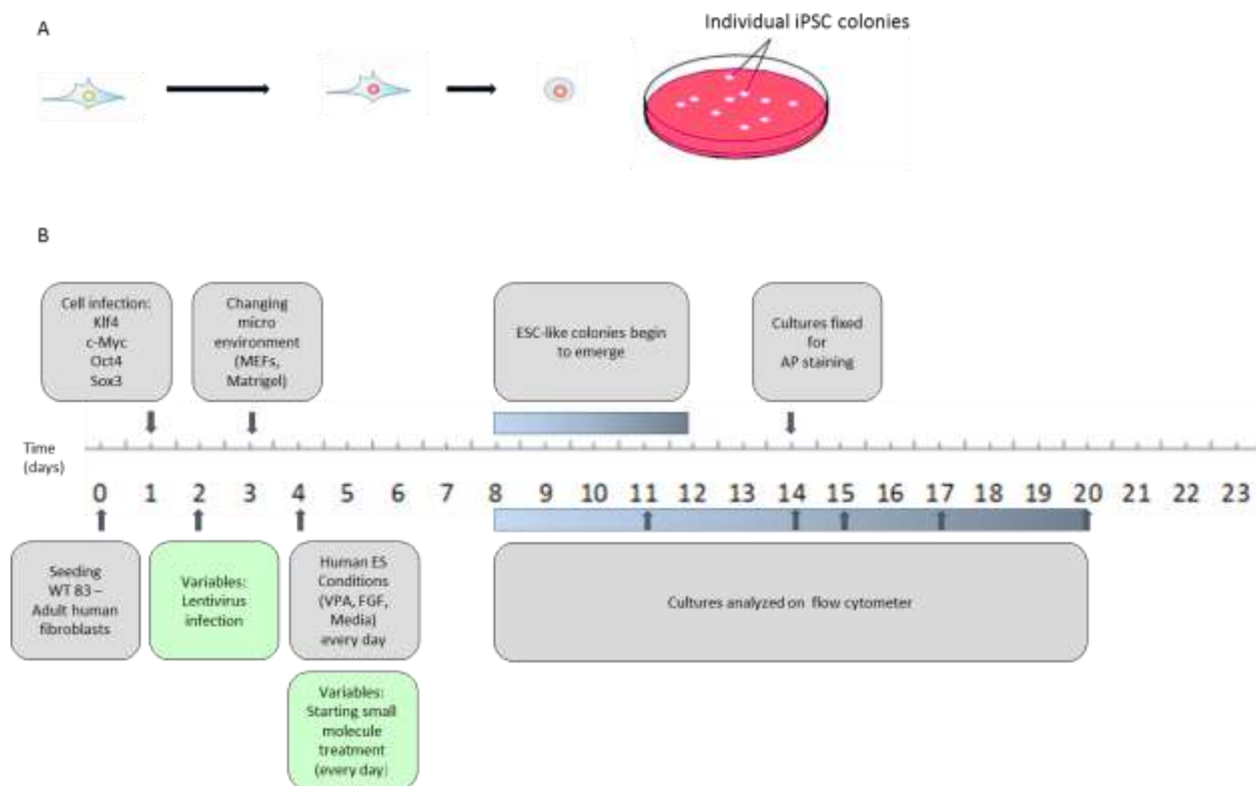


Figure 5: Schematic timeline of general iPSC colony formation with 4 factors (Oct4, Klf4, Sox2, c-Myc) A, General description; B, Exact timecourse, additionally indicated the day of starting small molecule treatment or lentiviral overexpression/knockdown in light green.

Figure 5A shows a general description of the untouched WT83 adult human fibroblast cell (origin see chapter 2.1.2), its altered genome (red nucleus), 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 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 2.1.2) 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 2.2.1.11) 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 chapter 2.2.4.3 and 2.2.4.4) between day 8 and day 20. Furthermore, the whole reprogramming process was alternatively performed with only three factors (without c-myc, see chapter 2.2.1.5)

3.1.2 Gelelectrophoresis

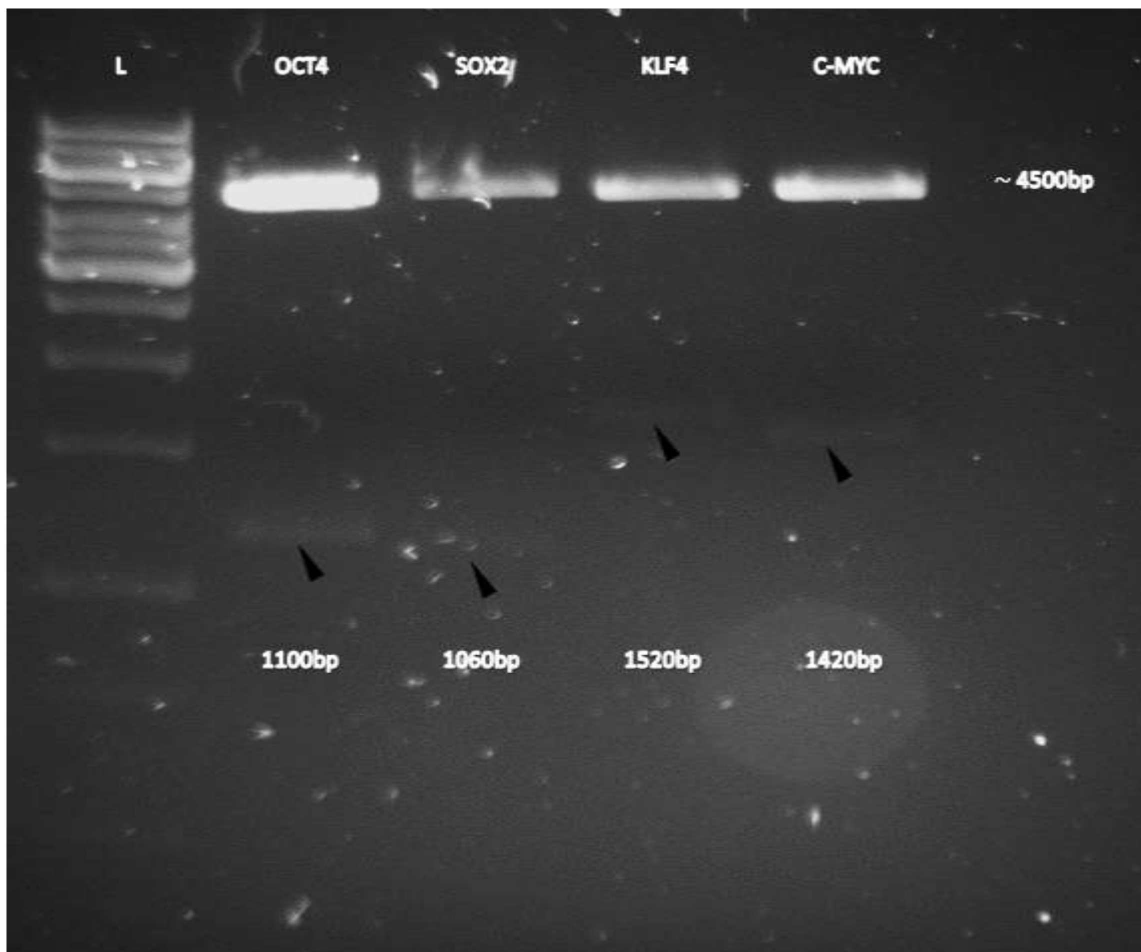


Figure 6: Restriction digestion of plasmids carrying the four reprogramming factors.

Figure 6 shows control of the DNA purification on a 1% agarose gel. The DNA isolation was done using the NucleoBond Kit -Macherey-Nagel and 0.3ug DNA were digested by their proper restriction enzymes, which was Not I, besides for Oct4 was used EcoRI and then loaded on the gel. The concentration of the isolated DNA was determined using the nanospectrometer.

3.1.3 Assaying iPSC generation

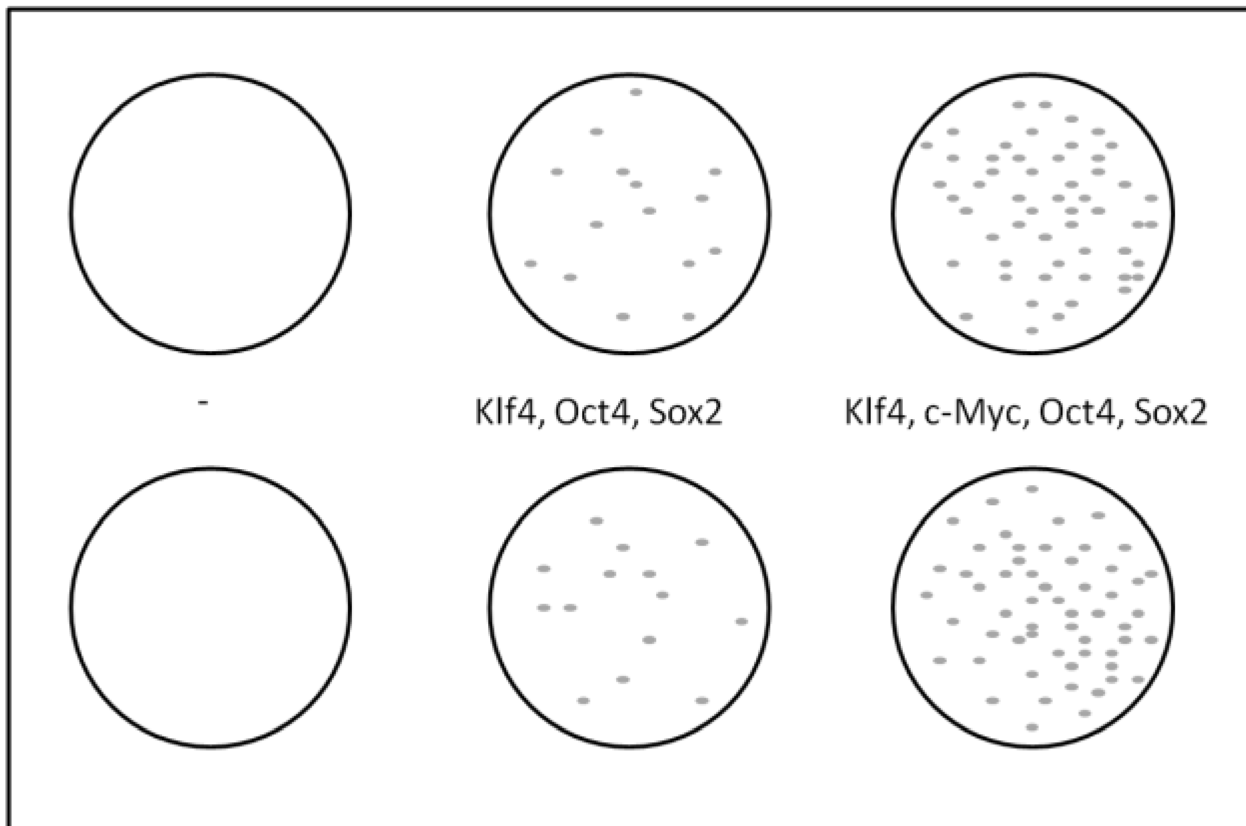


Figure 7: Schematic of iPSC colony generation in 6-well

The two 6-wells on the left in Figure 7 show non-transduced WT83 fibroblasts, the middle ones three factor transduced WT83 fibroblasts and the 6-wells on the right four factor transduced iPSC colonies (Oct4, Klf4, Sox2, c-Myc).

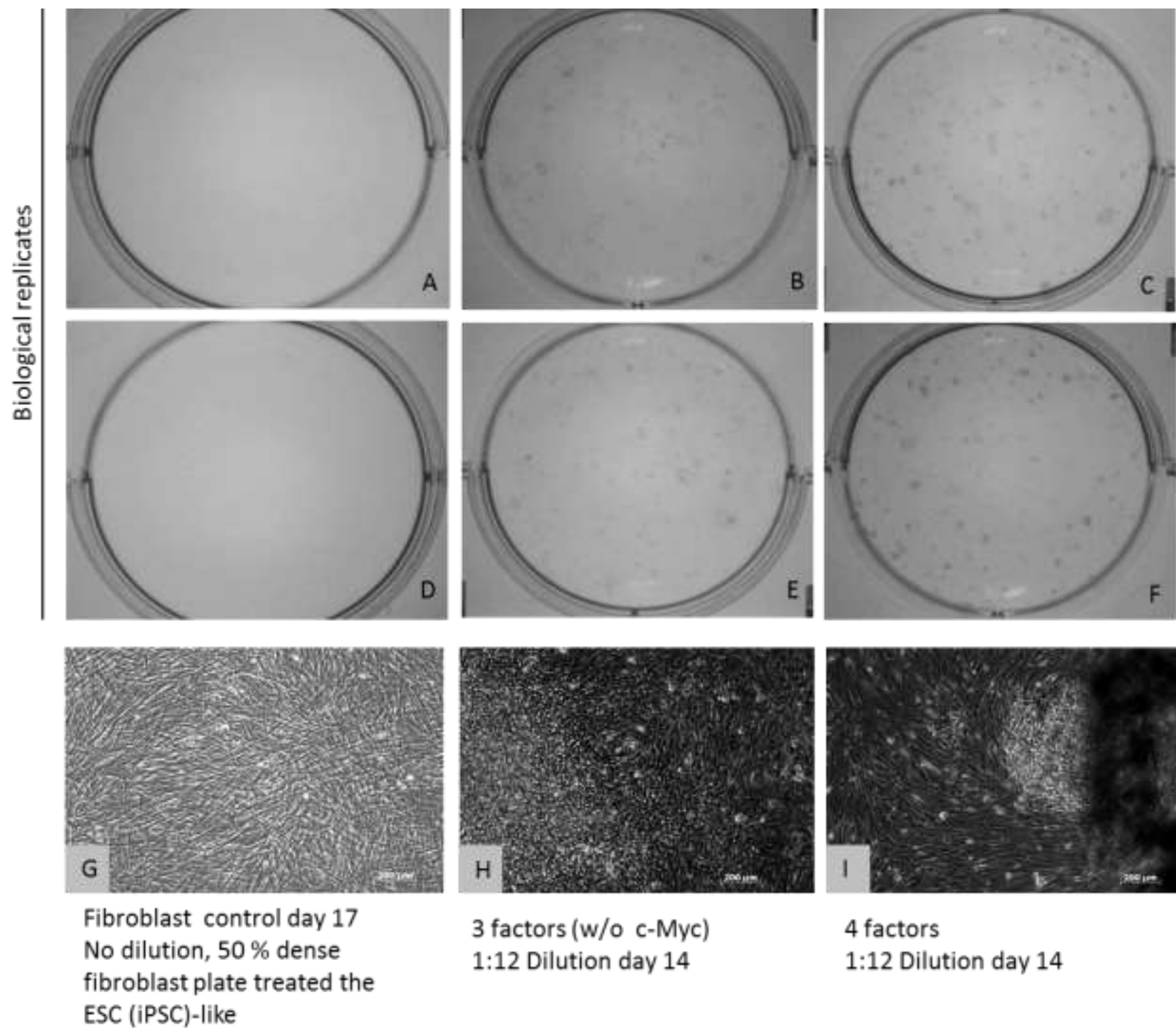


Figure 8: Assaying iPSC generation

Figure 8A-F shows the number of alkaline phosphatase-positive iPSC colonies on day 14 growing on MEF feeder cells. They 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. These cultures underwent no passages and were seeded at a 1:12 dilution of infected fibroblasts on day 3. According to table 28, around 5000 cells were seeded per cm^2. In Figure 8A and D WT83 fibroblasts without transduction were treated in the same media conditions as transduced cells, which is used as negative control.

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. Figure 8I shows 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.

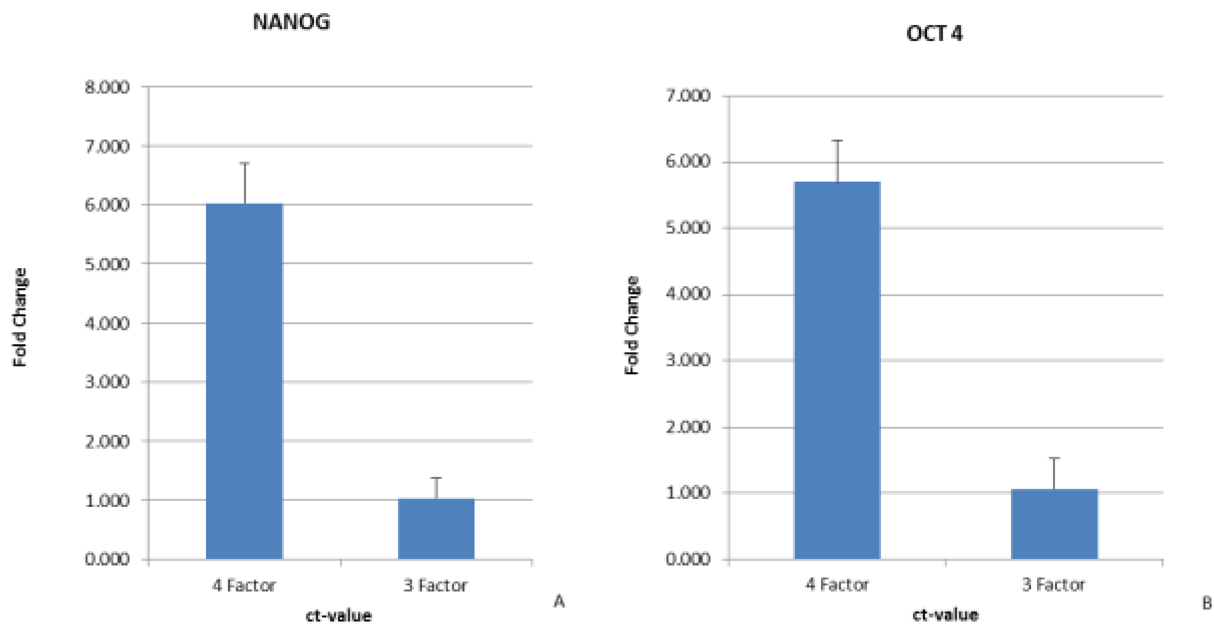


Figure 9: Quantitative reverse transcription-PCR analysis of putative iPSC clones

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 both genes were endogenous genes. The data indicates qPCR of two clones which were manually passaged 7 times (chapter 2.2.1.8). The first passage was done on day 15 onto matrigel and afterwards cultured until day 53 (7 passages) and fed every day (Media, bFGF).

3.1.3.1 Efficiency assays

The goal of this project was to study the influence of Wnt signaling on iPSC generation. We examined different assays to investigate the efficiencies by perturbing the Wnt pathway in a number of different ways.

- Flow cytometry:
    - Alkaline phosphatase staining (for experimental procedure, see chapter 2.2.4.4)
    - Antibody titering (data not shown)
    - Tra1-81 and SSEA-4 staining (for experimental procedure, see chapter 2.2.4.3)

- Alkaline Phosphatase staining with colony counting (for experimental procedure, see chapter 2.2.4.2)

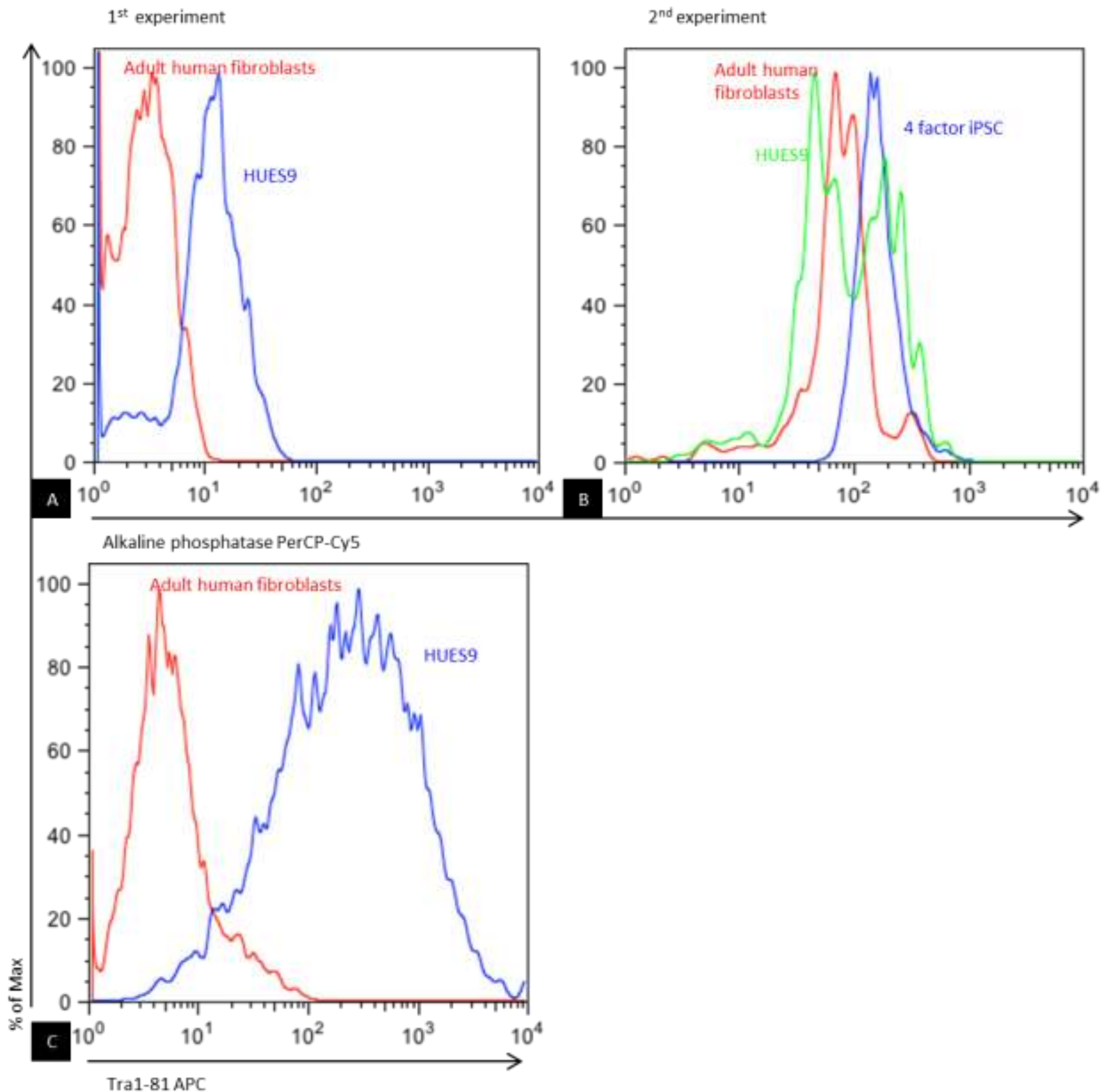


Figure 10: Flow cytometry analysis of human pluripotent stem cells

Figure 10A and B show fixed alkaline phosphatase-stained cells detected by flow cytometer with the fluorochrome PerCP-Cy5, according to Table 31.

The two control cell lines (adult human fibroblasts and HUES9 cells) should show two very distinct peaks. Indicated in Figure 10A those peaks do not show a big variation. In Figure 10B the peaks of all three cell types are overlapping. The peak of the HUES9 cells is overlapping with the fibroblast and the iPSC peak, which shows a wide range of

noise. Alternatively, cells were stained with a pluripotent stem cell surface marker Tra1-81 (Figure 10C) with conjugated fluorochrome APC (Table 31). The difference of adult human fibroblast and human embryonic stem cell peaks was sufficient enough to use the assay.

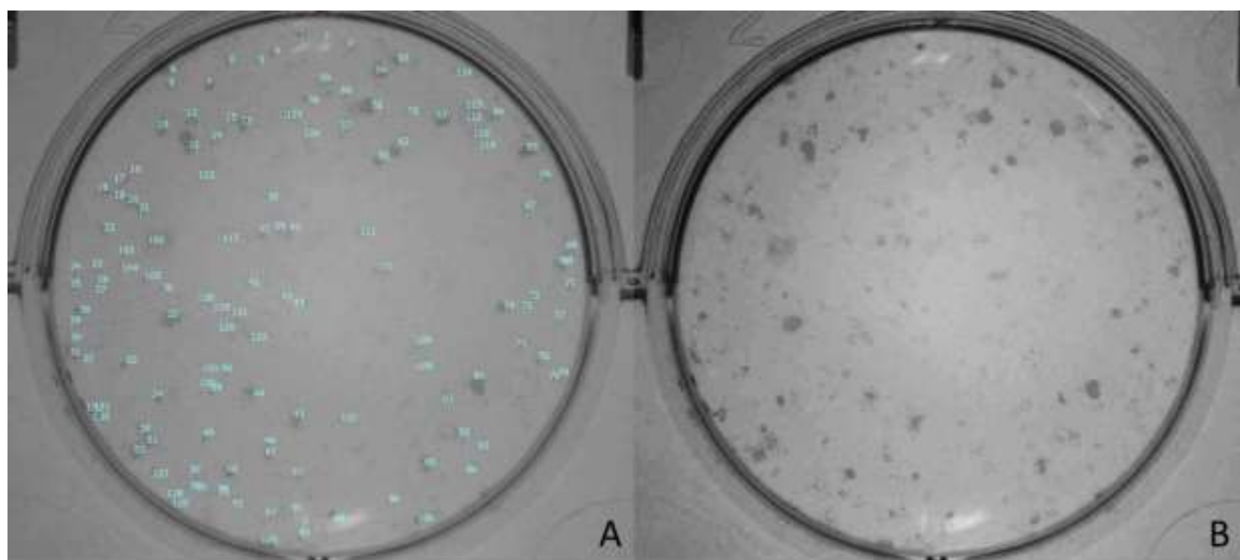


Figure 11: Method of counting colonies by Adobe Photoshop CS5

Figure 11A and B are one single well with similar alkaline phosphatase-stained colonies, where A shows colony counts and B stained colonies without numbers. In this 6-well iPS cells were transduced by four factors (KMOS), growing on MEF feeder cells and the colonies were fixed and stained on day 14.

Adobe Photoshop CS5 was used for analyzing the colony numbers and adjusting pictures. Generally, all visible, as stained colony appearing, colonies were counted. To count colonies constantly other lab members were involved in data analysis and were able to blind-count colonies.

3.1.3.2 Pictures of general iPSC generation

This chapter contains images of all used cell lines. Their origin was described in chapter 2.1.2 and their culturing methods were described in chapter 2.2.1.

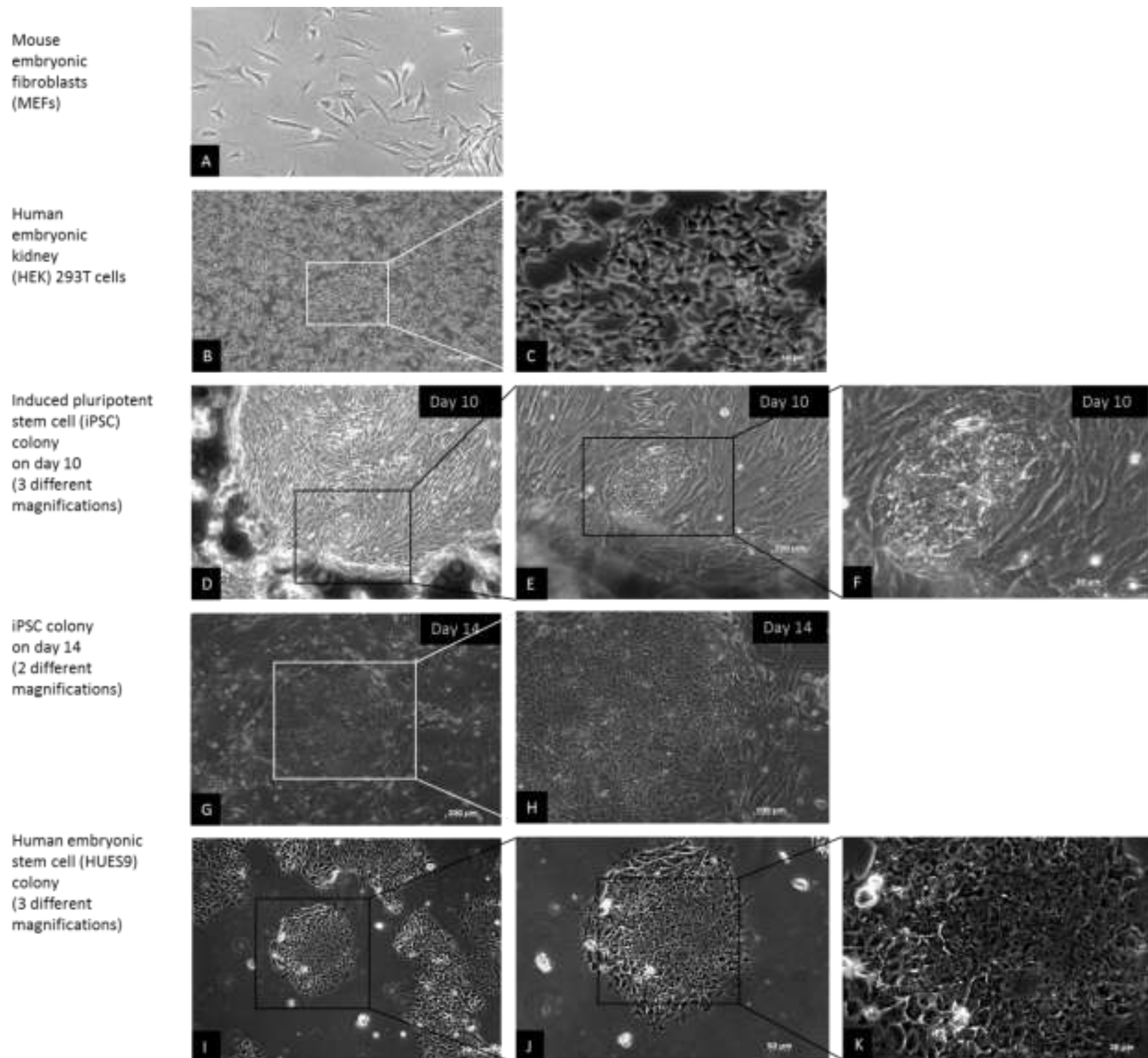


Figure 12: Brightfield microscopy images of multiple cell lines

In Figure 12A MEF feeder cells are illustrated which secrete several important growth factors into the medium and provide a cellular matrix for HUES9 cells or iPS cells. Figure 12B and C show HEK 293T cells in two different magnifications which are easy to culture and efficient to transfect. Figure 12D, E and F demonstrate one iPSC colony

on day 10 in 3 different magnifications and G and H another iPSC colony on day 14, which look healthier. There are more live cells in the colony than in Figure D, detectable through not growing on top of each other. These iPSC colonies were derived from adult human fibroblasts, called WT83 and growing on MEF feeder cells. The iPSC colony in Figure 12G holds many ES-like pluripotent morphology characteristics, as far as a defined borders, round shape, large nucleoli and scant cytoplasm for each cell. HUES9 cells (Figure12I, J and K) are human embryonic stem cells and also included in the research as positive control in diverse experiments. The borders of the HUES9 colony were already differentiated through wrong or unusual treatment and handling.

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.

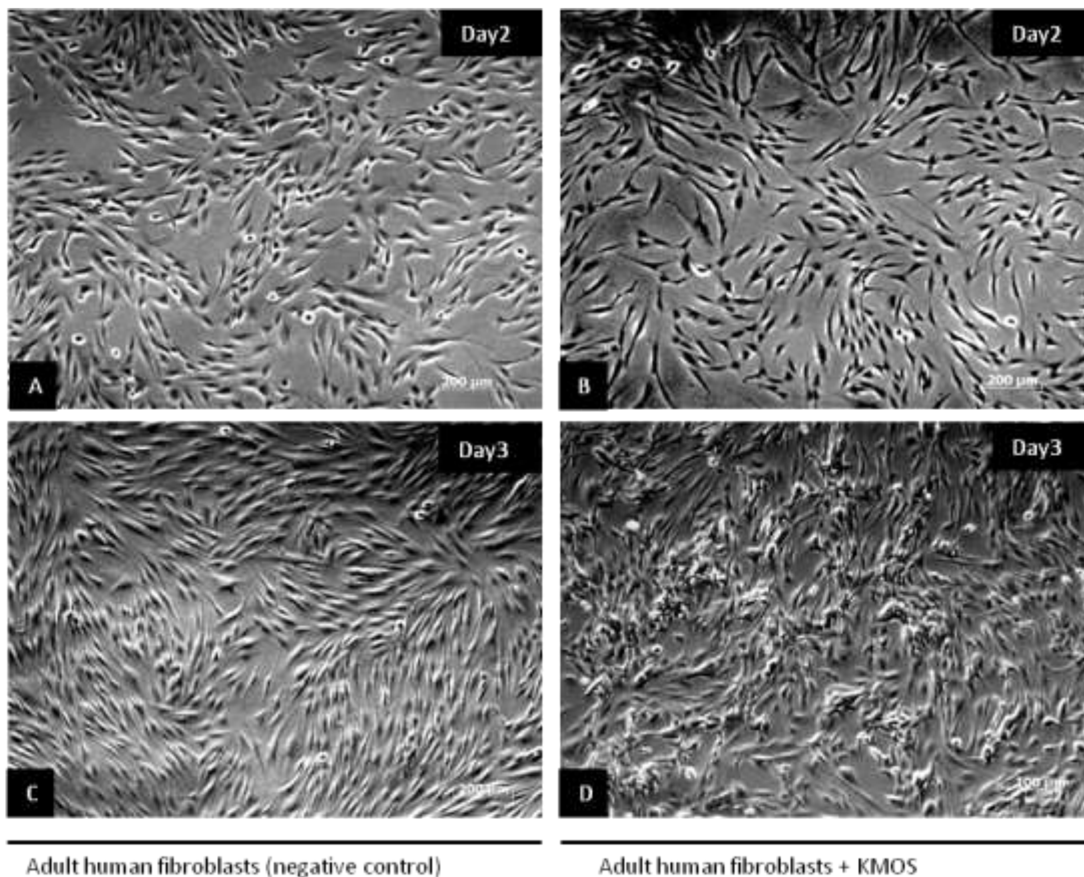


Figure 13: Phase contrast images of human fibroblasts in an early stage of iPSC generation.

Figure 13A-D are day 2 and 3 post-infected cells of standard iPSC generation before they were transferred 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. Figure 13D shows a drastic difference compared to Figure 13B which occurred in only one day. This timeline is being continued in Figure 14.

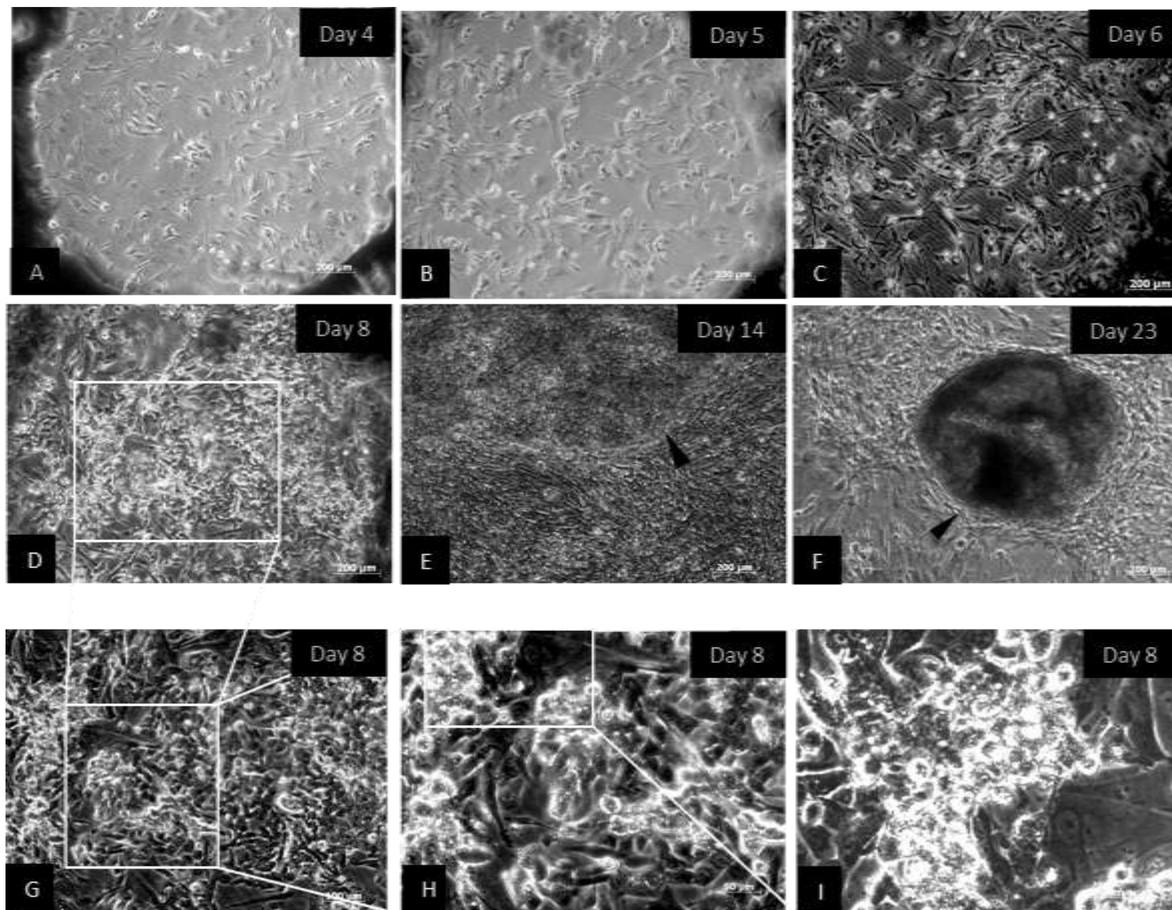


Figure 14: Timeline of iPSC generation

Cells on days 4-23 were cultured on MEF feeder cells and fed every day. In Figure 14A-F iPSC formation photographed on different days and Figure 14G-I are images of higher magnifications of Figure 14D on day 8. Figure 14A-D were the same cells spot on different days. Figure 14E and F were imaged on day 14 and 23. At this time point defined colony borders are visible. Figure 14A, B and C show a black round-shaped

labeling on the right side of the picture which was used to mark the colony and follow the same colony every day.

3.1.3.3 Isolation of iPSC clones

These experiments, of manually passaging colonies over a long period of time, were done to generate a cell line and identify this cell line by qPCR. For the experimental procedure see chapter 2.2.1.8.

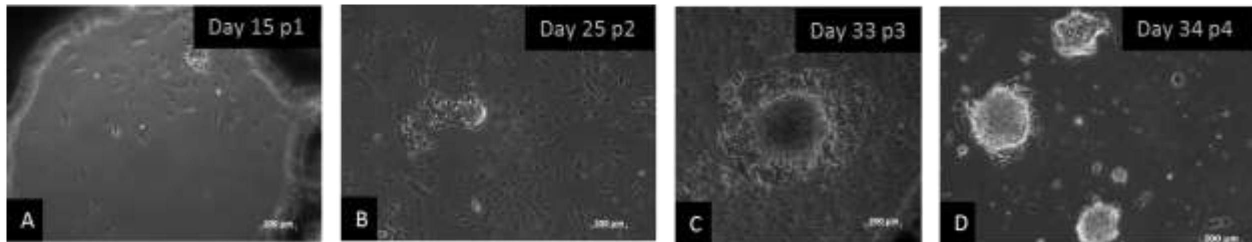


Figure 15: Isolation of iPSC clones (w/o C-MYC)

Figure 15A-D show manually isolated iPSC clones (three factors) growing on matrigel on day 15-34. Cell passaging was performed on day 14 post-transduction using the EVOS microscope. Two clones were passaged and Figure 15A shows one of those clones. They underwent 3 more passages (Figure 15B and C) until day 34 (Figure 15D), were they appeared more like iPSC colonies. Figure 15B and C show still plenty of fibroblasts, because of the passaging technique. iPSC colonies were not passaged exclusively without fibroblasts, were not 100% transduced or slower reprogrammed. These cells were growing until day 53 (three additional passages) and then analyzed by qPCR.

Figure 14A, B and C show a black round-shaped labeling on the right side of the picture which was used to mark the colony and follow the same colony every day.

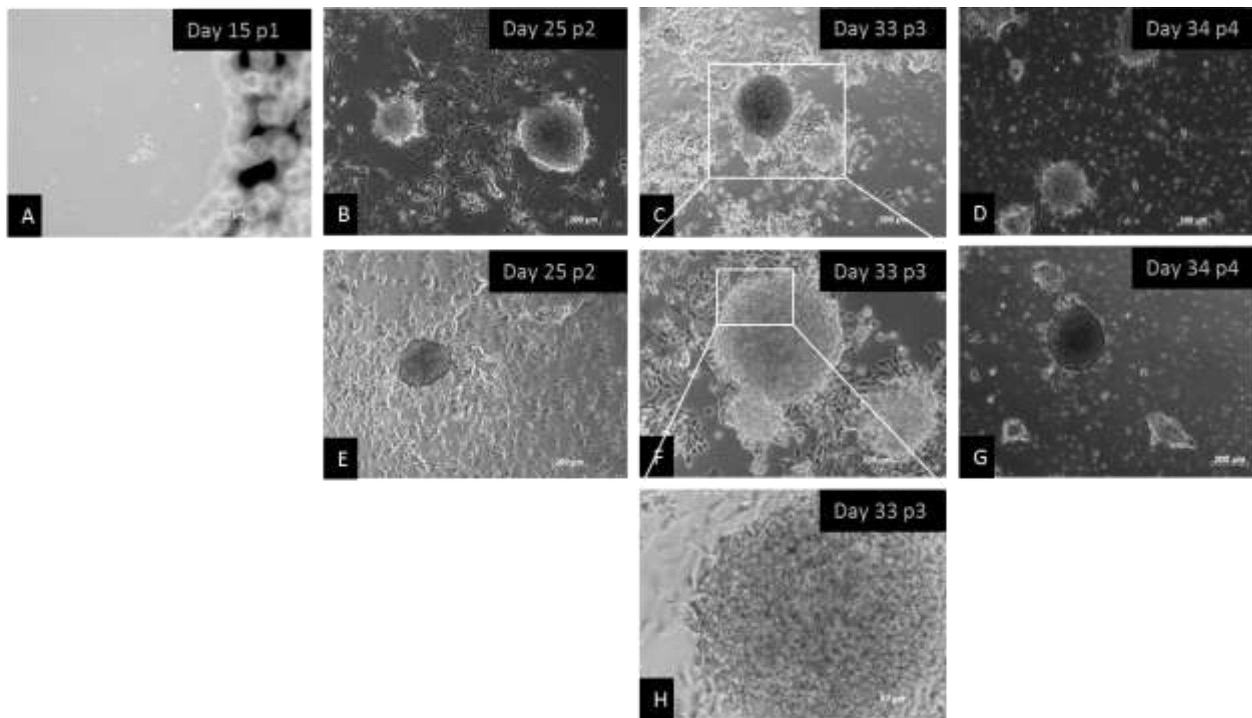


Figure 16: Isolation of iPSC clones

Figure 16A-H show manually isolated iPSC clones (four factors) growing on matrigel on day 15-34. The iPSC colonies were generally transduced with the four Yamanaka factors 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. After the first passage on day 15 (Figure 16A) the colony was hardly visible and still did not appeared fully reprogrammed. The cells underwent more passages (Figure 16B, E, C, D and G) and showed defined borders at day 34. These cells were cultured three additional passages until day 53 and analyzed by qPCR.

Figure 16A show a black round-shaped labeling on the right side of the picture which was used to mark the colony and follow the same colony every day.

3.1.3.4 Virus Control

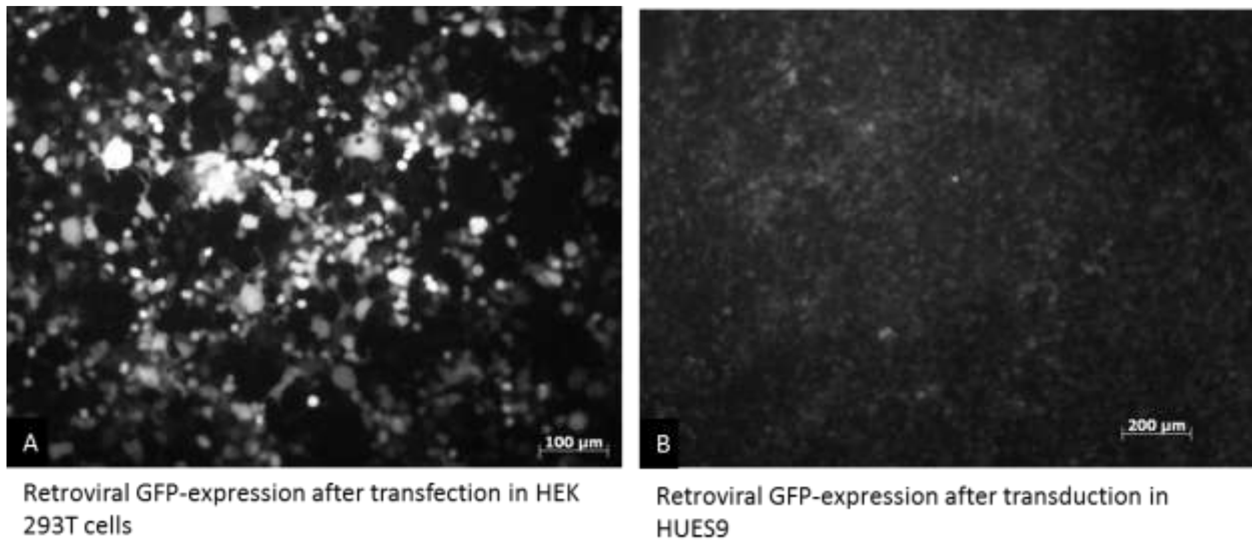


Figure 17: Fluorescence microscopy of GFP-expression after retroviral transfection (A) and transduction (B).

Figure 17A proves the function of the Yamanka retroviruses. HEK 293T cells showed GFP-expression one day after transfection which gives evidence of proper technique. The transfection efficiency is still unclear. Figure17B shows cell fluorescence of transduced HUES9 cells one day after transduction. Untransduced cells showed no GFP-expression (data not shown). The virus titer could not be referenced.

3.2 Effect of Wnt 3a and Wnt5a on iPSC generation

General procedures were performed as described in chapter 2.2.1.4 and Figure 5. In the next chapters, single timecharts are presented for each way Wnt Signaling 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 conditions have been evaluated almost always in one similar batch of iPSC generation, because of virustiter-dependent iPSC colony formation.

3.2.1 Time course for Wnt3a and Wnt5a small molecule treatment

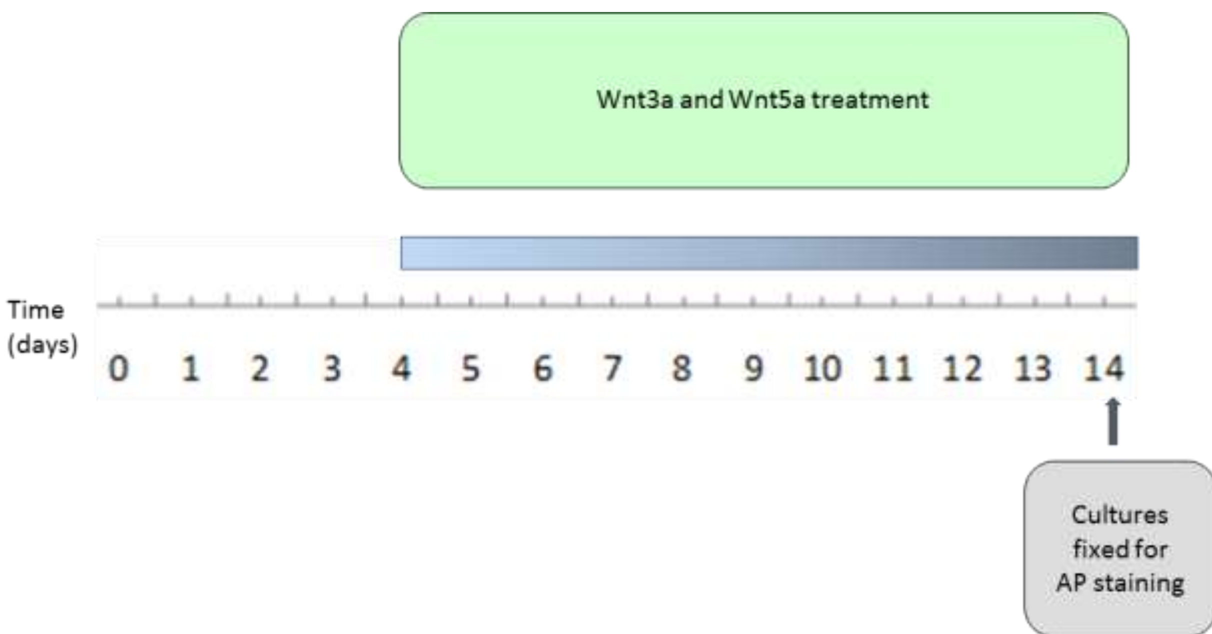


Figure 18: Time course for Wnt3a and Wnt5a small molecule treatment.

The timeframe of treating with small molecules is illustrated in Figure 18 and the day of fixing and staining with alkaline phosphatase is indicated.

3.2.2 Alkaline phosphatase stain of Wnt3a and Wnt5a treated cells

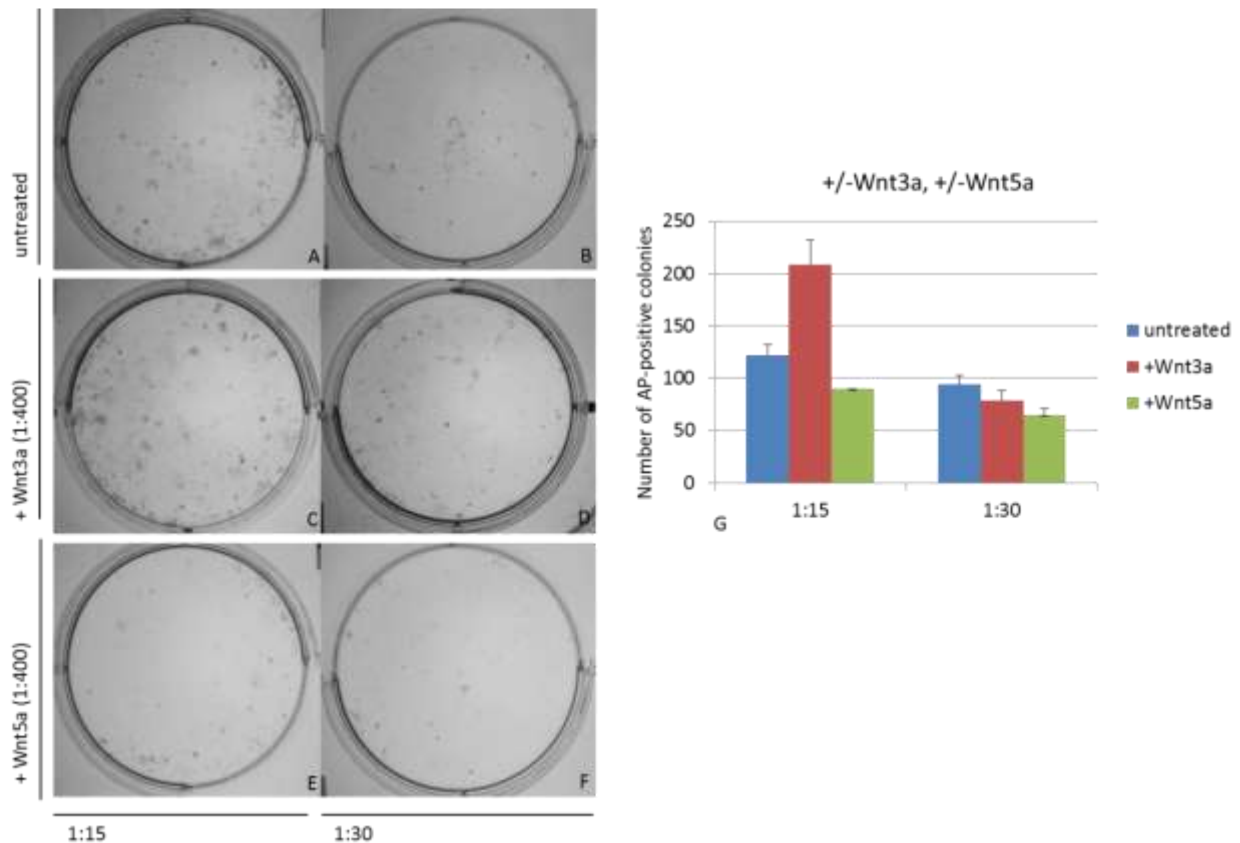


Figure 19: iPSC colonies with Wnt3a and Wnt5a treatment stained for alkaline phosphatase

Figure 19A and B were AP stained 6-wells of standard iPSC generation without specific treatment. In Figure 19C and D standard iPSC generation was performed with Wnt3a treatment, and Figure 19E and F with Wnt5a treatment. The chart in Figure 19G illustrates the number of alkaline phosphatase positive-counted colonies compared to different treatments. The relations 1:15 and 1:30 represent the dilution of seeded infected fibroblasts on day 3.

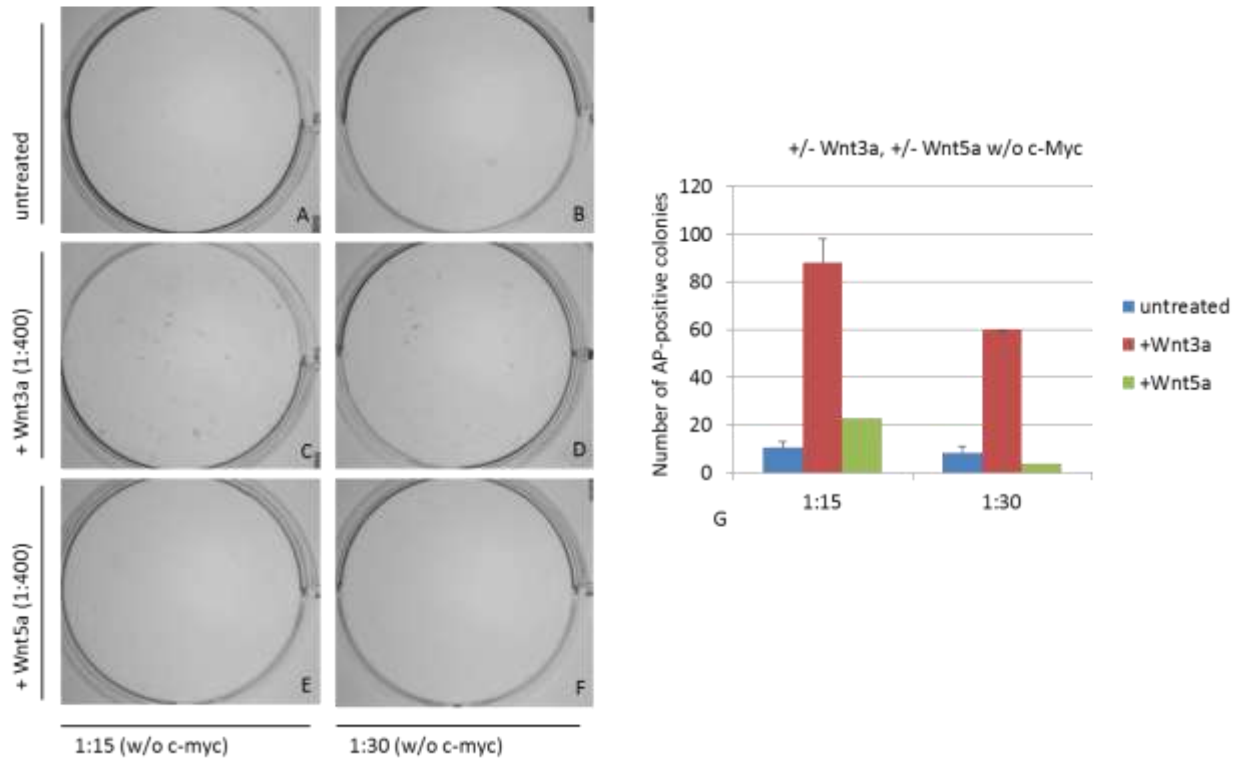


Figure 20: iPSC colonies without c-myc with Wnt3a and Wnt5a treatment stained for alkaline phosphatase

Figure 20A and B were AP stained 6-wells of standard iPSC generation with (KOS) 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 to the three different conditions. The relations 1:15 and 1:30 represent the dilution of seeded infected fibroblasts on day 3.

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

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

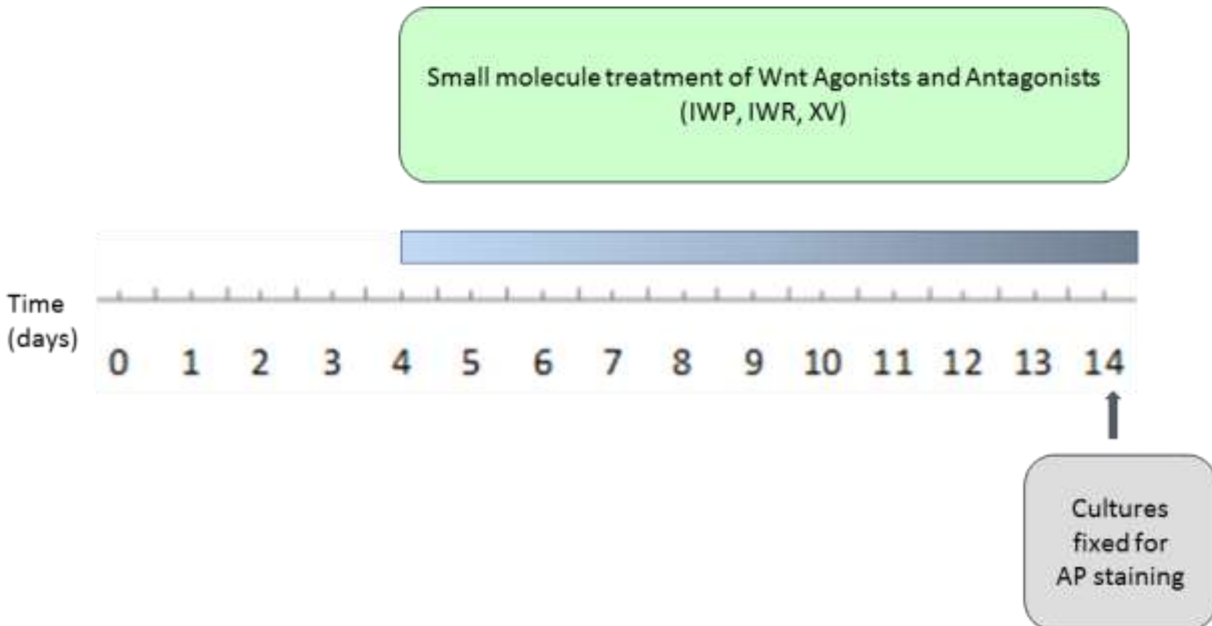
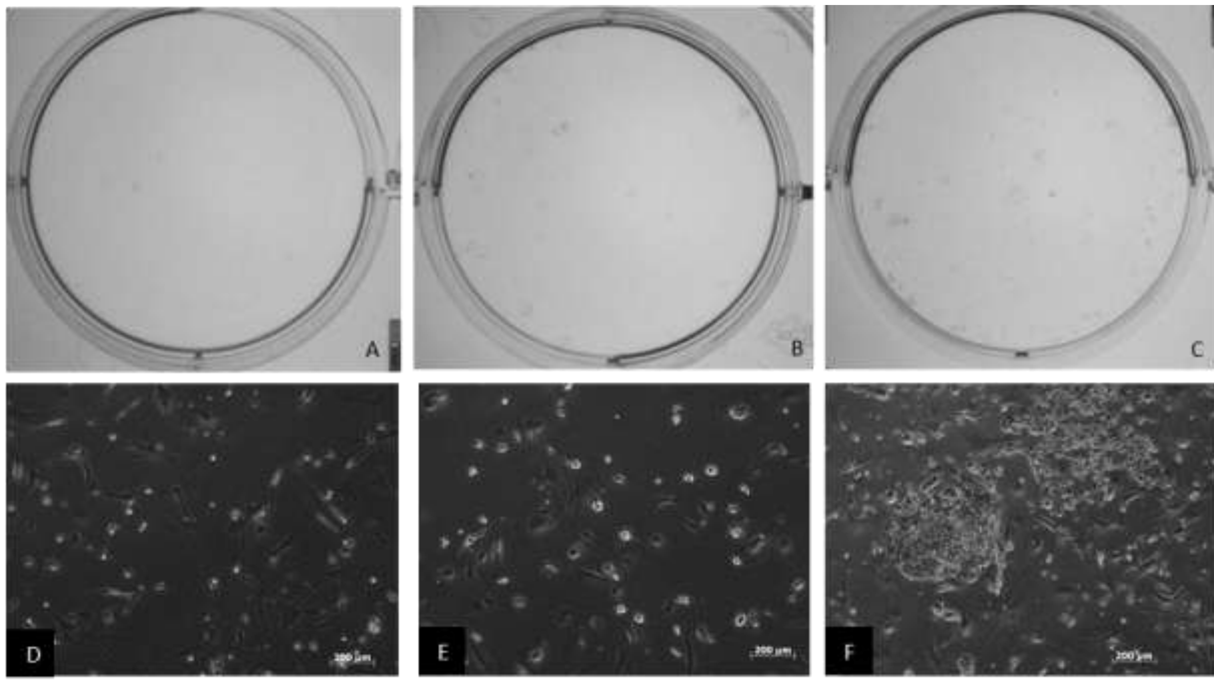


Figure 21: Time course for small molecule treatment of Wnt pathway agonists and antagonists

The timeframe of treating with small molecules, 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 14.

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

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



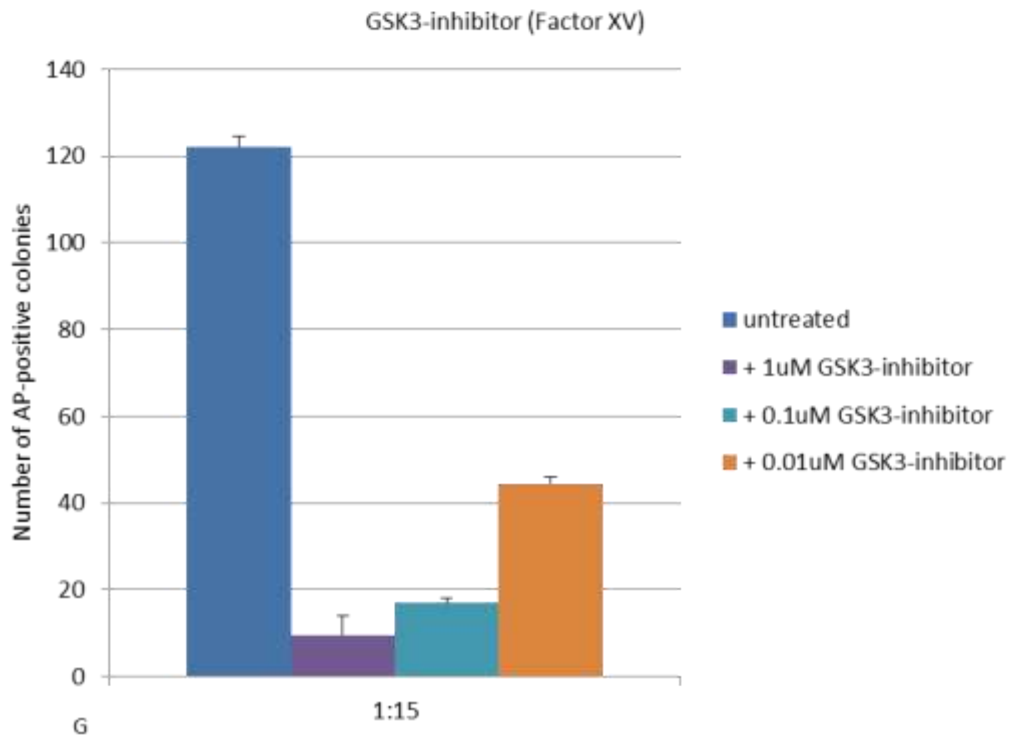
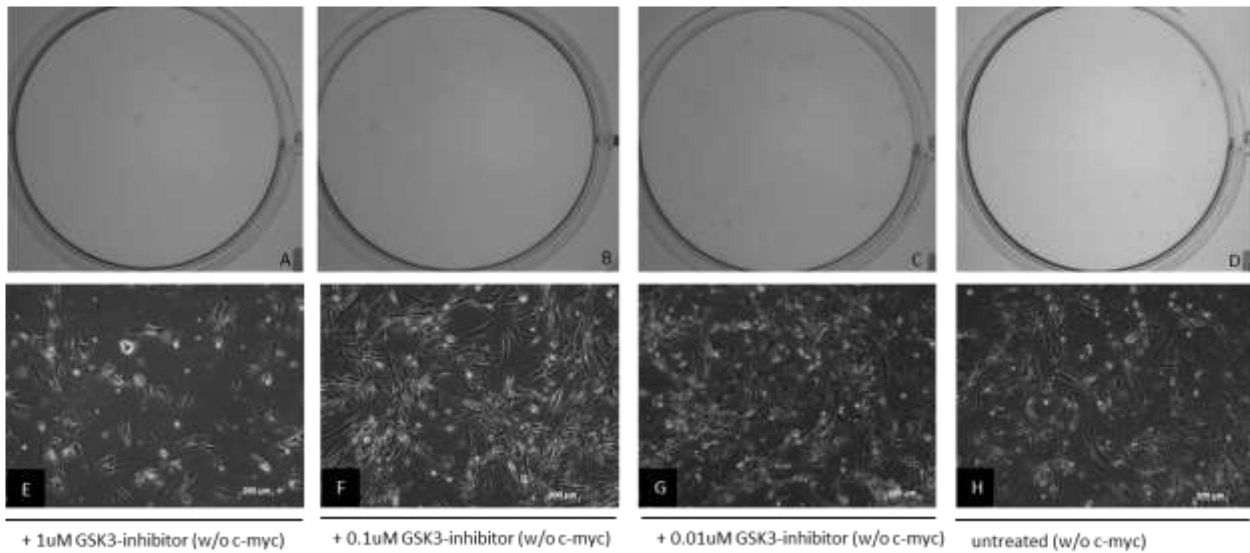


Figure 22: iPSC colonies with GSK3-inhibitor treatment stained for alkaline phosphatase

Figure 22A-F were treated with different doses of GSK3-inhibitor (1uM, 0.1uM, 0.01uM) 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.



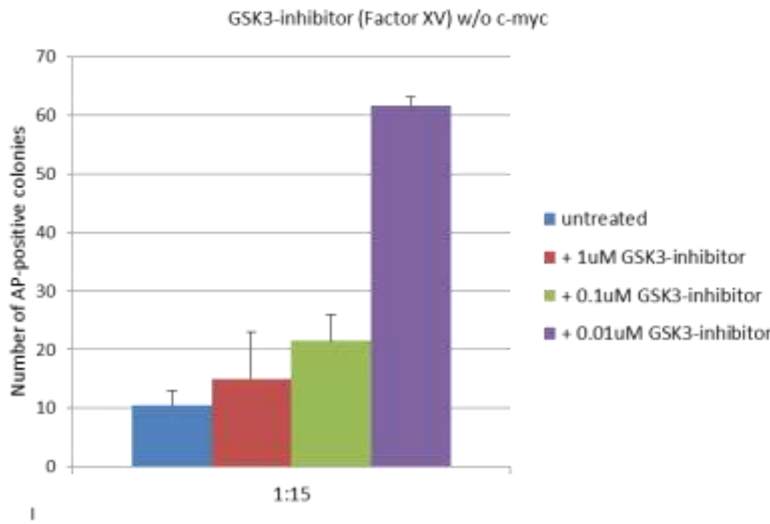


Figure 23: iPSC colonies without c-Myc treated with GSK3-inhibitor stained for alkaline phosphatase

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 in Figure 23I compares four different culture conditions. The relation 1:15 represents the dilution of seeded infected fibroblasts on day 3.

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

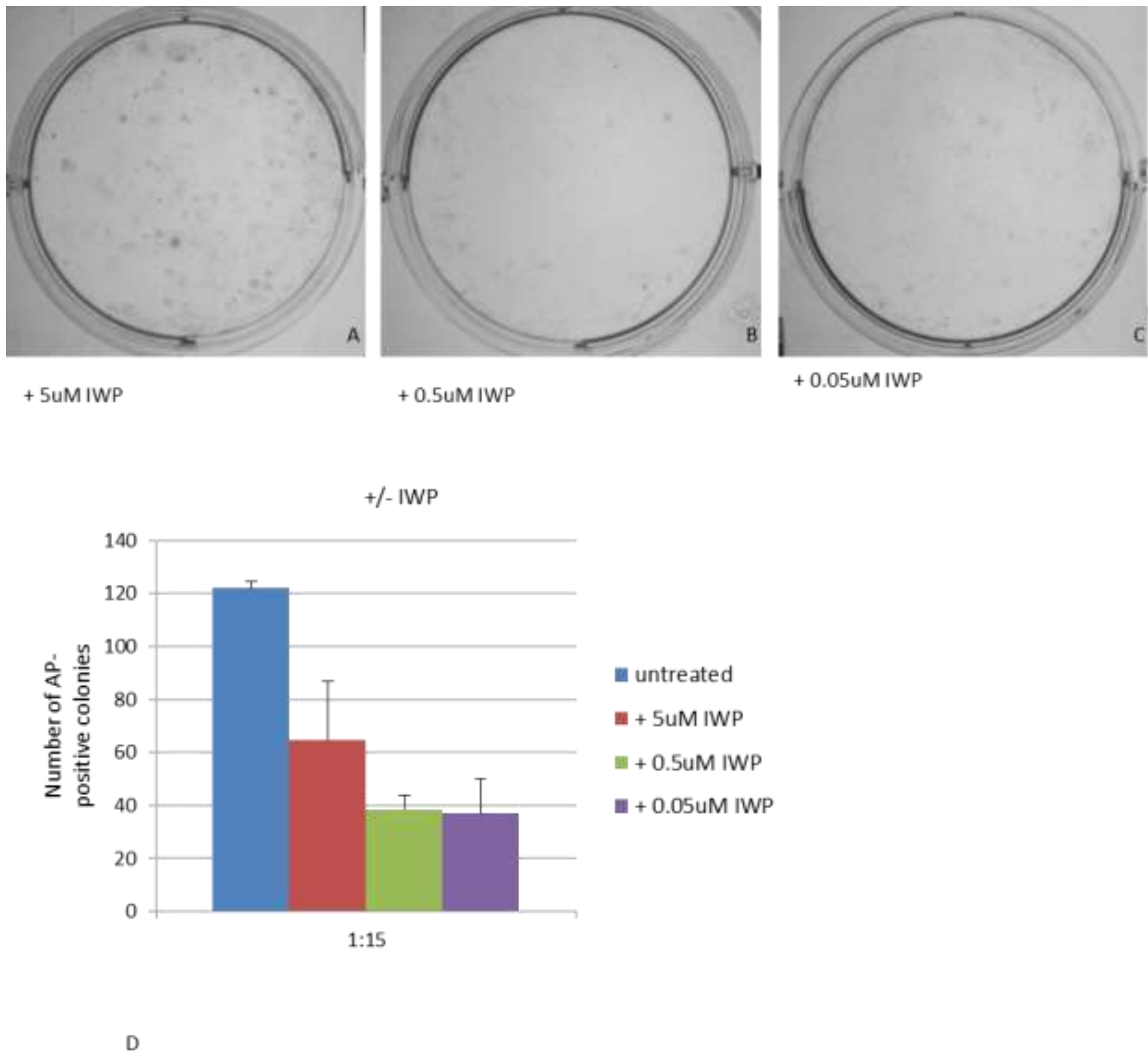


Figure 24: iPSC colonies with three different doses of IWP concentration

Figure 23A-C were treated with different doses of IWP (5uM, 0.5mM, 0.05uM) The figures show AP stained 6-well plates of the three dose treatments. Figure 23D

illustrates the number of counted colonies compared to different culture conditions. The relations 1:15 represent the dilution of seeded infected fibroblasts on day 3. iPSC colonies without c-myc transduction did not show any positive stained colonies (data not shown).

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

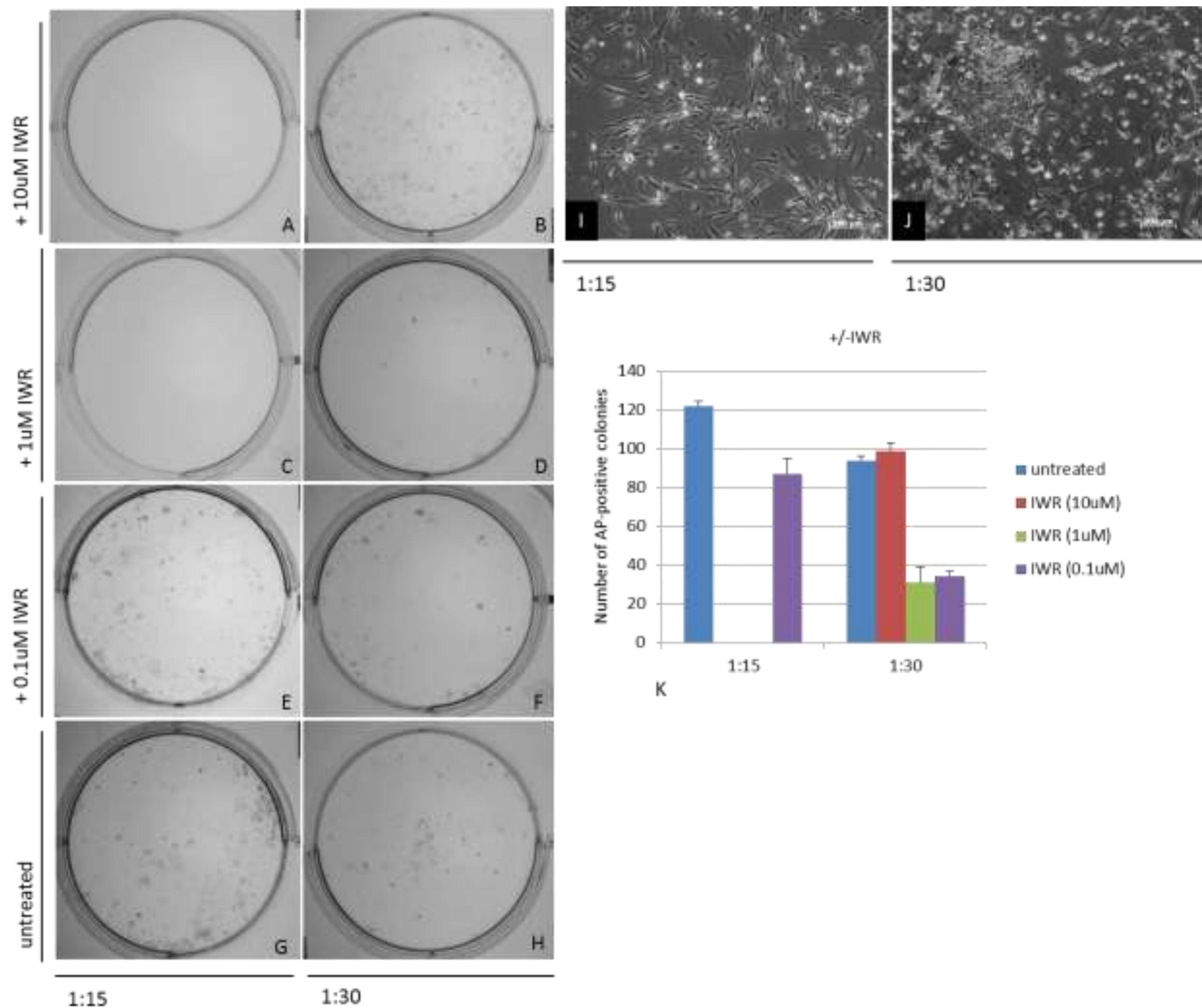


Figure 25: iPSC colonies with three different doses of IWR concentration

Figure 25A-F show AP stained 6-well plates which were treated with three different doses of IWR. The treatments were labeled in the figure. Figure 25G and H were performed with standard iPSC generation. 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. iPSC colonies without c-myc transduction did not show any positive stained colonies (data not shown).

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

The experiment of overexpressing Axin and β-catenin was performed in a separate batch apart from the previous ones. All general procedures were performed as above (chapter 2.2.1.4 and Figure 5).

3.4.1 Time course for overexpression of Wnt pathway components

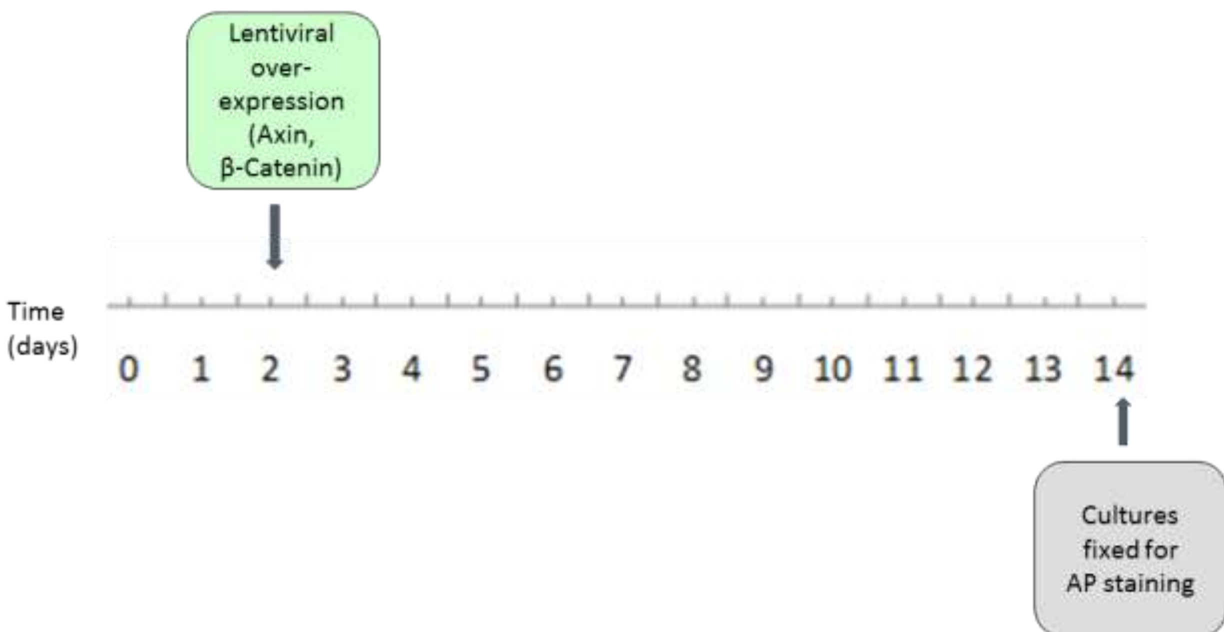


Figure 26: Time course for overexpression of Wnt Pathway components

Figure 26 shows the timepoint of overexpression of Wnt pathway components, while iPSC generation was taking place (see Figure 14), and the day of evaluating the experiment by alkaline phosphatase staining.

3.4.2 Alkaline phosphatase stain

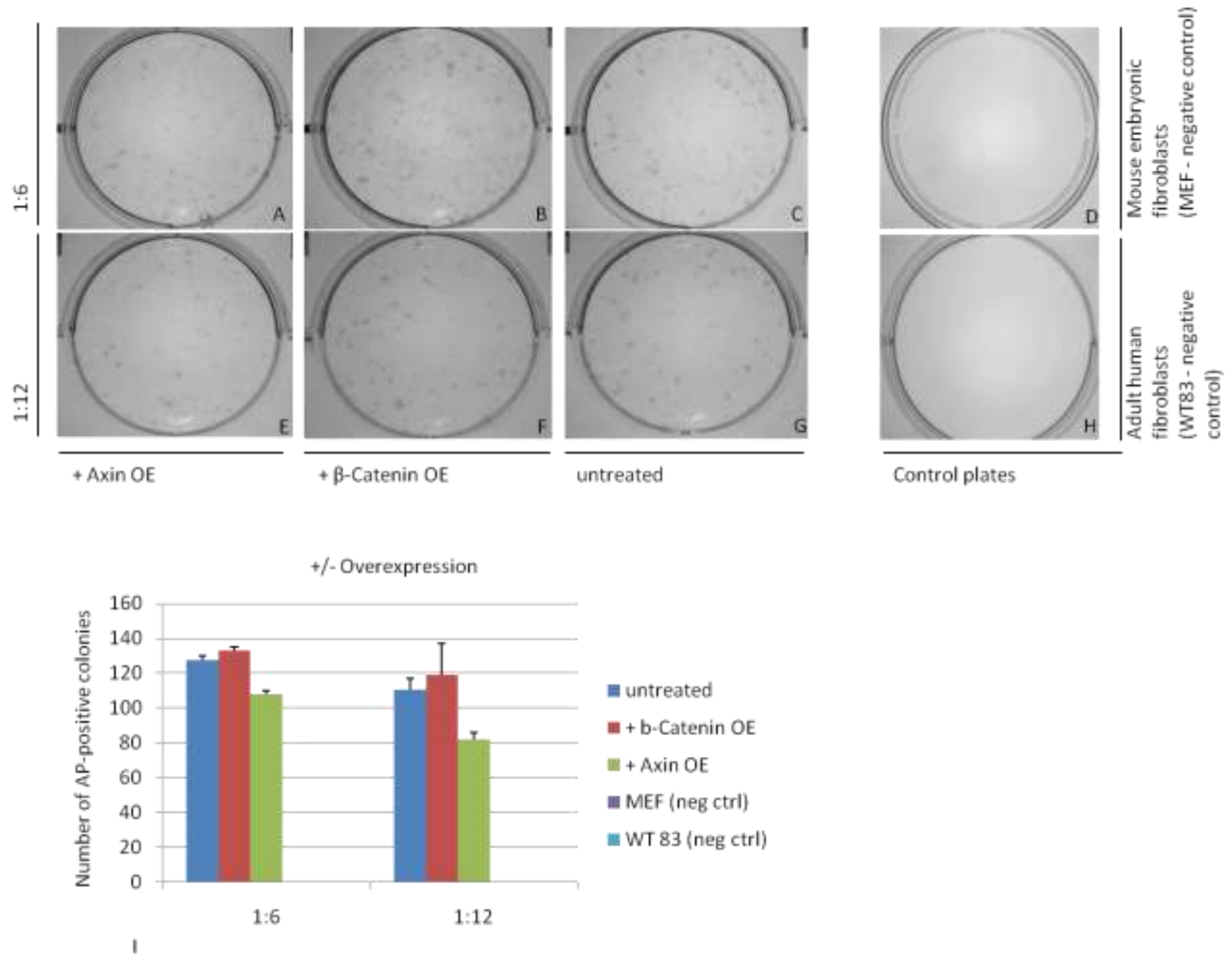
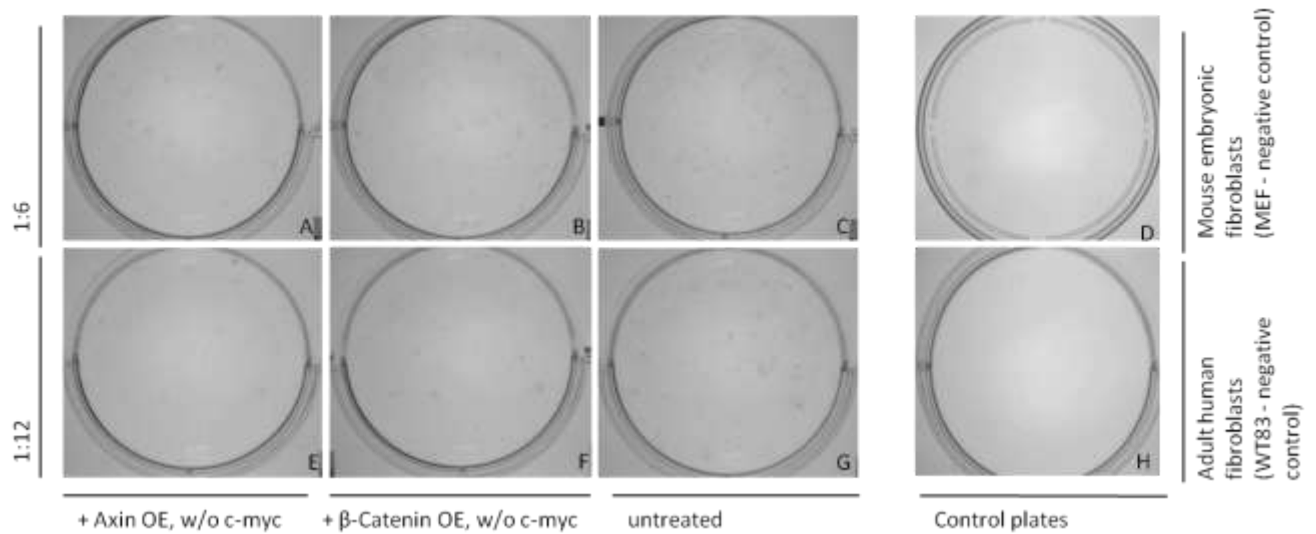


Figure 27: iPSC colonies with overexpression of Axin and β-Catenin

Figure 27C and G were standardly produced iPSC colonies used as positive control and Figure 27D and H were 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 means overexpression.



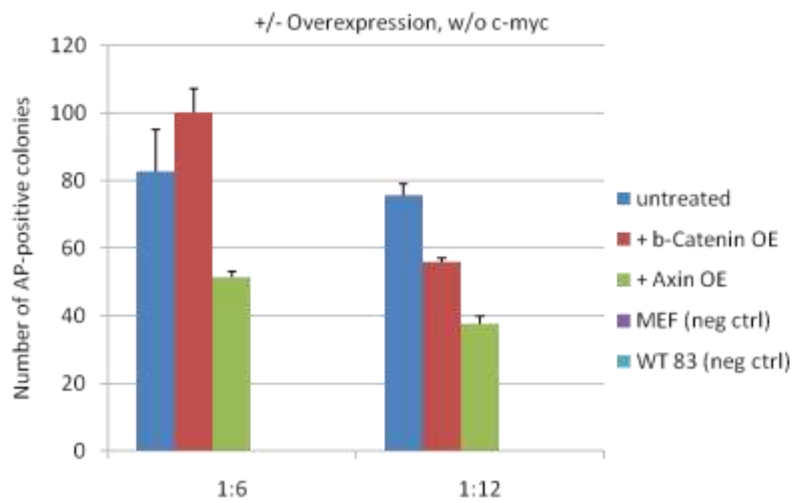


Figure 28: iPSC colonies with overexpression of Axin and β-Catenin without C-MYC

Figure 28A, B and E, F show standard iPSC colony formation without c-myc affected by overexpression of two Wnt pathway components. Figure 28C and G were iPSC colonies performed standardly with three factors used as positive control and Figure 28D and H were 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.

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

3.5.1 Time course for knockdown of Wnt pathway components

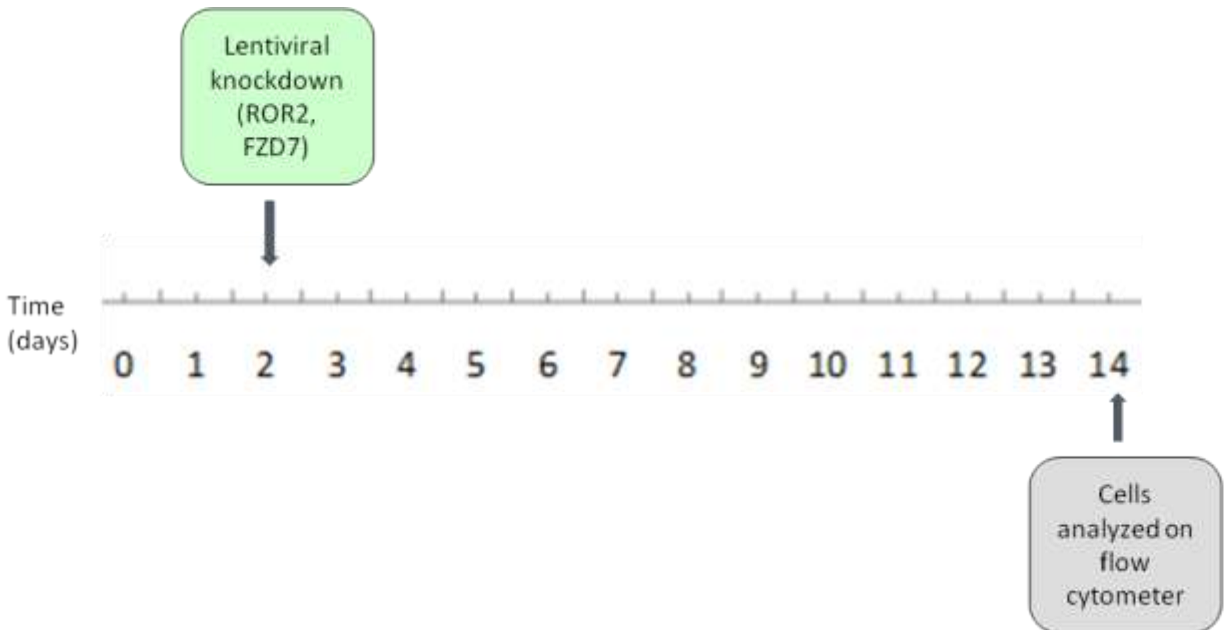


Figure 29: Time course for knockdown of Wnt Pathway components

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.

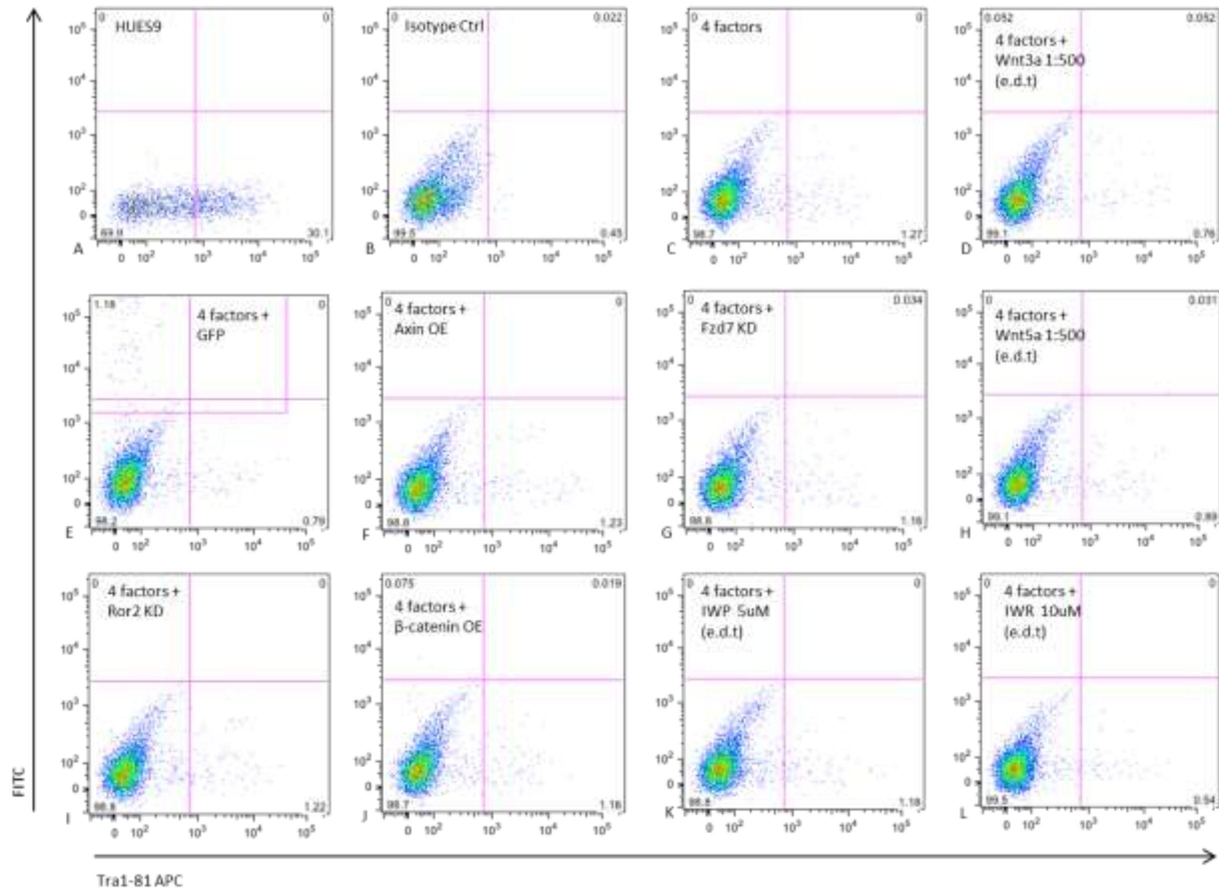


Figure 30: Flow cytometry expression analysis of the human cell surface marker Tra1-81 on iPS cells with different small molecule treatments

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 show one positive control (HUES9), B, one negative control (Isotype control) and C, general non-Wnt specific treated iPS cells transduced with KMOS. Figures 30D-L are four factor transduced iPS cell formations with different treatments, labeled in the figure. E.d.t. means ‘every day treatment’, OE/KD overexpression and knock down and the relations 1:500 and 1:1000 represent the dilution of the stock solutions.

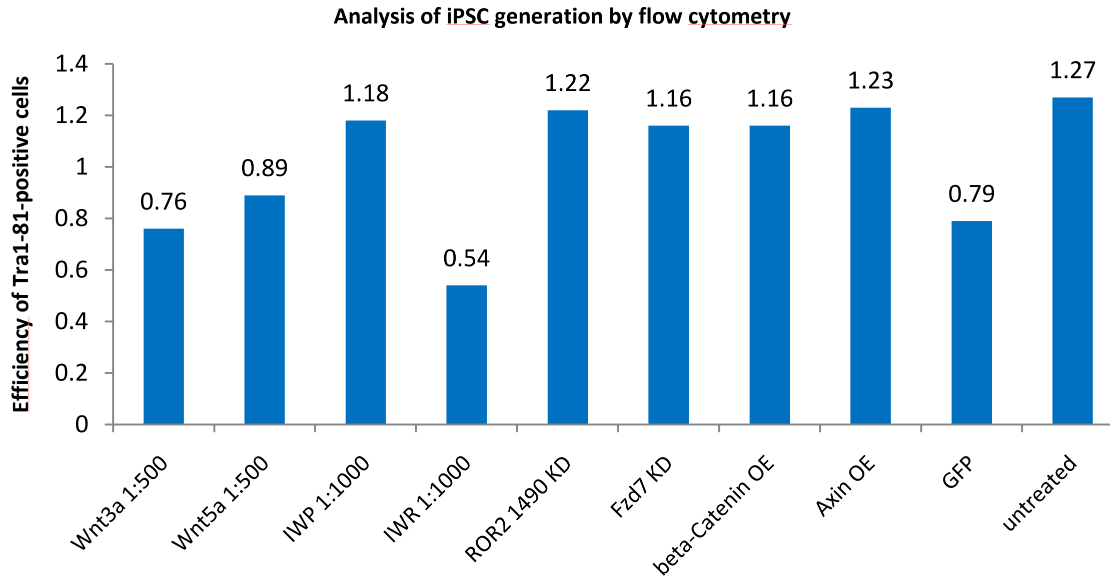


Figure 31: Efficiency numbers indicated of counting 10.000 cells (summary of Figure 30)

This chart exemplifies the efficiency of Tra1-81 positive cells after different Wnt pathway-dependent treatment by the flow cytometer analysis referring to Figure 30. The relations 1:500 and 1:1000 represent the dilution of the stock solutions.

4 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.

4.1 Perturbing Wnt signaling through small molecule treatment:

4.1.1 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, decreased the colony number compared to the untreated control. To this end, the number of seeded cells and their density was a critical point.

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

Regarding Figure 20A-F iPSC generation 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 4.1.1. Treatments of Wnt5a did not show any severe effects.

4.1.3 GSK3-inhibitor treatment on cells transduced with Klf4, c-Myc, Oct4 and Sox2:

The hypothesis shows a double negative effect of treating with GSK3-inhibitor cells which activates Wnt target genes and as a result increases iPSC generation. As shown in Figure 22G all three doses of GSK3-inhibitor show a decreasing effect, whereby colony formation increases with declining concentration but no significant distinction. 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.

4.1.4 GSK3-inhibitor 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 4.1.3. Nevertheless KOS transduced cells showed a significant raise (6-fold) of iPSC colony formation at the lowest dose and a slight increase of 1uM and 0.1uM 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-inhibtior activating Wnt target genes and therefore increasing colony formation number in iPSC generation.

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

iPSC colony formation with various treatment of IWP doses (Figure 24), gave less colony formation 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.

4.1.6 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. 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 10uM and 1uM inhibited iPSC generation one hundred percent, whereas the lowest concentration (0.1uM) affected the cells only in a tiny decrease. Alternatively, the IWR treatment of 1:30 diluted cells seeded did not show the same effect. 10uM 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, no influence of IWR treatment on cells seeded in low (1:30) densities was given. In contrast, at the higher seeding density (1:15) IWR was extremely decreasing iPSC formation and no colonies were generated, which would confirm that Wnt signaling is a necessary component in iPSC formation. To this end, only the right cell densities were contributing to a determining result.

4.2 Perturbing Wnt signaling through overexpression or knock down

4.2.1 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. β-catenin was enhancing the reprogramming process at 1:6 cell density while at 1:12 cell density iPSC production was scaling down. As already described in KMOS transduced cells, the titer of the β-catenin lentivirus was not detected.

4.2.2 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).

Up to my knowledge this is the first report of Axin overexpression for partly inhibiting iPSC colony production.

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

shRNA mediated knocking down of Frizzled7 and ROR2 was performed in parallel with all the other, already discussed, 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 3.1.3.1) reveal limited sensitivity. Finally, flow cytometry analysis was not used as a sensitive assay to measure crucial differences.

Plenty of analyses were done by alkaline phosphatase staining and colony counting, which was investigated as the most reliable assay.

4.3 Summary

Overall, the majority of the findings were treatment dose and cell density dependent. The results suggest that Wnt3a tend to improve, while Wnt5a tend to decrease iPSC generation efficiency or remain constantly (Figure 19 and 20). More alkaline phosphatase-positive colonies were observed with faster rates of growth, significantly with the addition of Wnt3a. 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).

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6 Abbreviations

AP	Alkaline phosphatase
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco’s Phosphate buffered Saline
Rpm	Rounds per minute
ul	Microliter
ug	Microgram
uM	Micromol
VPA	Valproic Acid Salt
KMOS	Klf4, c-Myc, Oct4, Sox2
	(4 Yamanaka factors)
KOS	Klf4, Oct4, Sox2
	(3 Yamanaka factors)
iPSC	Induced pluripotent stem cell
hESC	Human embryonic stem cell
IWP	Inhibitor of Wnt Production
IWR	Inhibitor of Wnt Response
APC	Adenomatous polyposis coli
Dsh	Dishevelled
Fz or Fzd7	Frizzled 7
ROR2	Receptor tyrosine kinase-like orphan receptor
GSK-3	Glycogen Synthase Kinase 3
MEF	Mouse embryonic fibroblasts
HUES9	Human embryonic stem cells (9)
FBS	Fetal bovine serum
MCM	Condition media

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.
The results suggest that Wnt signaling definitely supports iPSC efficiencies, 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. Die Ergebnisse dieses Projekts zeigen auf eine definitive Unterstützung des Wnt Signalweges auf die Reprogrammierungseffizienz hin. Einige der Wnt Signalwegproteine oder überexprimierten Gene weisen eine Hinauf- oder Hinunterregulierung der induziert pluripotenten Stammzellerzeugung auf.

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