Cellular reprogramming of human mesenchymal stem cells derived from young and old individuals using viral and non-viral approaches

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Matthias Megges

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Abbreviations

ACTN2	sarcomeric actinin, alpha 2
AFP	alpha-fetoprotein
ALCAM, CD166	activated leukocyte cell adhesion molecule, cluster of differentiation 166
ALK4	activin receptor like kinase receptor 4
ALK5	activin receptor like kinase receptor 5
ALK7	activin receptor like kinase receptor 7
BMPR2	bone morphogenetic protein receptor, type II
BUB1	budding uninhibited by benzimidazoles 1 homolog
CCNA2	cyclin A2
CCNB1	cyclin B1
CCNB2	cyclin B2
CD14	cluster of differentiation 14, myeloid cell-specific leucine-rich glycoprotein
CD34	cluster of differentiation 34, hematopoietic progenitor cell antigen CD34
CD44	cluster of differentiation 44
PTPRC, CD45	protein tyrosine phosphatase, cluster of differentiation 45
CDC20	cell-division cycle protein 20
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
CDKN2D	cyclin-dependent kinase inhibitor 2D
CHEK1	dual specificity protein kinase
DPPA4	developmental pluripotency associated 4
E2F	E2F transcription factor
E2F2	E2F transcription factor 2
EBNA-1	Epstein-Barr nuclear antigen-1
ENG / CD105	endoglin / cluster of differentiation 105
ERK	extracellular signal-regulated kinase
FGF2	fibroblast growth factor 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF3	growth differentiation factor 3
GFP	green fluorescent protein
GTSE1	G-2 and S-phase expressed 1
hTERT	telomerase reverse transcriptase
KLF4	krueppel-like factor 4
LIN28	lin-28 homolog A (C. elegans)
MAX	c-Myc associated factor X
MCM2	minichromosome maintenance complex component 2
MCM3	minichromosome maintenance complex component 3
MCM5	minichromosome maintenance complex component 5
MCM6	minichromosome maintenance complex component 6
MEK	mitogen-activated protein kinase 1

c-Myc	v-c-Myc myelocytomatosis viral oncogene homolog
MYT1	myelin transcription factor 1
NANOG	Nanog homeobox
NT5E / CD73	5'-nucleotidase, ecto / cluster of differentiation 73
OCT4 / POU5F1	octamer-binding protein 4 / POU class 5 homeobox 1
p21 / CDKN1A	cyclin-dependent kinase inhibitor 1A
p53	tumor protein p53
PKMYT1	protein kinase, membrane associated tyrosine/threonine 1
PLK1	polo-like kinase 1
pRB	retinoblastoma-associated protein
PTTG1	pituitary tumor-transforming 1
RRM2	ribonucleotide reductase M2
RRM2B	ribonucleotide reductase M2 B (TP53 inducible)
α-SMA	alpha-smooth muscle actin
SOX17	SRY (sex determining region Y)-box 17
SOX2	SRY (sex determining region Y)-box 2
SSEA-1	stage specific antigene 1
SSEA-4	stage specific antigene 4
SV40LT	simian virus 40 large T-antigen
TGFβ	transforming growth factor β
THY1 / CD90	Thy-1 cell surface antigen / cluster of differentiation 90
TRA-1-60	tumour related antigen -1-60
TRA-1-81	tumour related antigen -1-81
TRA-2-49	tumour related antigen -2-49, alkaline phosphatase
TTK	TTK protein kinase
TUJ1	neuron-specific class III β-tubulin
TβR-II	transforming growth factor, beta receptor II

1 Introduction

This study is about the generation of induced pluripotent stem cells (iPSCs) from human mesenchymal stem cells (hMSCs). iPSCs are very similar to embryonic stem cells (ESCs). Viral and non viral methods will be applied and the effect of the inhibition of MEK, ALK4/5 and 7 as well as p53 during the reprogramming process will be analysed. The following section will provide information about all three named cell types, the applied techniques to generate iPSCs and inhibitor mediated effects as well as the role that age-related changes in hMSCs could have in cellular reprogramming.

1.1 Pluripotency compared to multipotency and unipotency

Pluripotency

Pluripotency is the property of cells to give rise to all cell lineages of the three embryonic germ layers endoderm (e.g. cells of the gastrointestinal tract), ectoderm (e.g. cells of the nervous system), mesoderm (e.g. cells of bone and muscle) and germ cells. Cells that are pluripotent are promising candidates for the application in tissue replacement therapies and for the generation of disease models.
The pluripotent state is regulated by transcriptional and epigenetic mechanisms (Chen and Daley, 2008). Key players in the induction of the pluripotency are the genes *OCT4*, *SOX2*, and either *KLF4* and *c-Myc*; *NANOG* and *LIN28* (Takahashi et al., 2007; Yu et al., 2009).

Multipotency

Multipotency is the capacity of a cell to give only rise to a limited number of cell lineages that mostly are already part of the tissue of origin of the multipotent cell. An example for multipotent cells are mesenchymal stem cells that can differentiate into osteoblasts, adipocytes, chondrocytes (Roobrouck et al., 2008).

Unipotency

In addition, cells that have the capacity to give rise to one type of terminally differentiated cells are unipotent. These cells are already committed to only one cell fate direction (Young et al., 2004).

1.2 Self-renwal and cellular senescence

Self-renwal is the ability of stem cells to create new stem cells, which are in the same undifferentiated state like the parental cells. Symmetric and asymmetric cell division and the regulation of these play an important role in the maintenance of self-renewal within a stem cell population. Symmetrical cell division means that one parental stem cell gives rise to two identical daughter cells, which are both identical to the parental cell. Asymmetric cell division means that one cell divides into an identical daughter cell and a daughter cell that is already committed to a cell lineage (Kuhn and Tuan; 2010).

In embryonic stem cells self-renewal is promoted by TGFβ signaling and FGF2 signaling pathways (Greber et al., 2007).
Cellular senescence is a process that restricts the *in vitro* expansion of a cell line to a certain number of population doublings before the cells start to degenerate. The cause of this phenomenon is the response of the cells to not reparable damage of the DNA that affects the telomers or the non telomeric regions of the genome. The DNA damage is due to the amount of reactive oxygen species in the cells which increases with the number of population doublings (Ksiazek, 2009).

1.3 Human mesenchymal stem cells

Mesenchymal stem cells (MSCs) were discovered as a colony forming adherend cell type in the bone marrow with a fibroblast morphology and the potential to differentiate into osteoblasts (Friedenstein et al., 1968). The term "mesenchymal stem cells" was introduced in 1991. Later it was found that hMSCs have the potential to differentiate into several mesodermal lineages like chondrocytes, adipocytes and stromal cells of the connective tissue in addition to osteoblasts (Pittenger et al., 1999).
Cells with similar features like bone marrow derived mesenchymal stem cells were detected in many different tissues such as fat, muscle, placenta, cartilage and others (Figure 1) (Kuhn and Tuan, 2010). Furthermore, it has been reported that hMSC populations contain subpopulations of cells that are capable to generate cells of the three germinal layers (Roobrouck et al., 2008). In addition, bone marrow derived hMSCs are able to differentiate into ectodermal neuronal cells and skin cells as well as hepatocytes which is an endodermal lineage (Kuhn and Tuan, 2010; Stock et al., 2010). Despite this, mesenchymal stem cells are defined as non pluripotent but as multipoint cells.
The surface marker profile that is used to identify hMSCs is currently still a matter of debate. The most commonly used markers for hMSCs are the expression of NT5E (CD73), THY1 (CD90), ENG(CD105), ALCAM (CD166), CD44 and the absence of CD34, PTPRC (CD45) and CD14 (Satija et al., 2007). A factor that complicates the definition of surface markers for hMSCs is that their expression depends on various extracellular conditions. In addition, the interaction of hMSCs with their environment determinates their cell fate towards self-renewal or differentiation (Kuhn and Tuan, 2010).

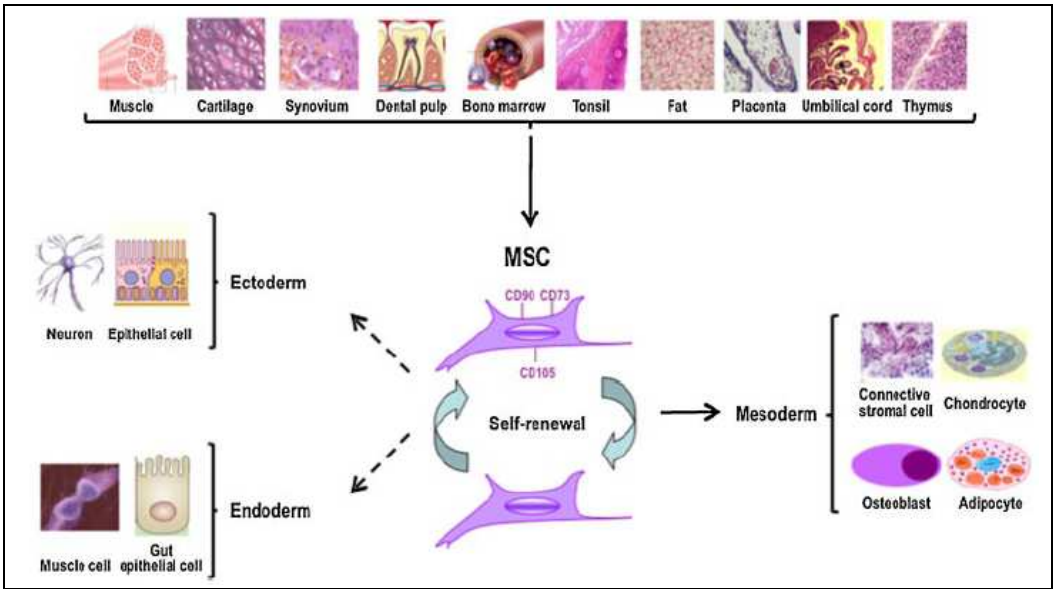

Figure 1 Illustration of features and occurrence of mesenchymal stem cells. hMSCs have been detected in many different tissues. The most common surface markers of hMSCs are CD90, CD73, and CD105. hMSCs can differentiate in various mesodermal lineages and it is still unclear, whether hMSCs are able to differentiate into ectodermal and endodermal lineages. Adapted from Kuhn and Tuan, 2010.

Human bone marrow derived mesenchymal stem cells are capable of self-renewal. However, unlike embryonic stem cells which have no limit in their capacity of self-renewal, MSCs progressively loose the ability to replicate *in vitro* which eventually leads to senescence (Ksiazek, 2009).

Due to their multilineage potential and the fact that research on MSCs does not raise ethical concerns, MSCs have become a promising tool for tissue engineering and cell therapies (Chen et al., 2008). However, the applications of mesenchymal stem cells are limited by insufficient *in vitro* expansion due to senescence associated short life span and donor age-related decrease in self-renewal and differentiation potential (Roobrouck et al., 2008).

Bone marrow derived hMSCs are isolated by density gradient centrifugation and subsequent seeding on a plastic surface to isolate them as adherent cells. However, hMSC populations isolated with this method are described to be heterogeneous and to probably contain few true stem or progenitor cells (Roobrouck et al., 2008).

1.3.1 Possible effects of donor age-related differences on iPSC generation from hMSCs

hMSCs from older donors have a slower proliferation rate, are flatter and have a bigger size compared to hMSCs from younger donors. In addition, they show signs of senescence from early passages *in vitro* (Baxter et al., 2004). The process of senescence in hMSCs is triggered by damage to telomeric DNA due to progressive telomere shortening in hMSCs from older donors leading to the activation of p53 and the arrest of the cell cycle (Ksiazek, 2009). As hMSCs derived from older donors have a bigger subpopulation that is senescent than hMSCs from younger donors, it is likely that it is easierto reprogramm the hMSCs of younger donors. This conclusion is underlined by the finding that p53 associated signaling plays a major role

in limiting the generation of iPSCs (Banito et al., 2009). Therefore, it will be tested whether blocking of p53 signaling will have an effect on the expected age-related difference in the efficiency of iPSC generation from hMSCs.

1.3.2 In vitro differentiation of hMSCs to adipocytes and osteoblasts

The analysis of the differentiation potential of hMSCs is a tool to confirm their multilineage potential.
In vitro, confluent hMSCs can be induced to differentiate into adipocytes by exposure to fetal bovine serum, dexamethasone, isobuthylmethylxanthine and insulin (Farmer, 2006). Furthermore, hMSCs can be differentiated to osteoblasts by culturing them in the presence of ascorbic acid and β-glycerophosphate (Di Benedetto et al., 2010).

1.4 Molecular markers of smooth muscle cell and cardiomyocyte differentiation

A commonly used marker for the differentiation of hMSCs to vascular smooth muscle cells is the actin isoform alpha-smooth muscle actin (Skalli et al., 1989). Alpha-smooth muscle actin interacts with other proteins to allow the contraction of smooth muscle cells (Guo et al., 2007).
Sarcomeric alpha actinin or actinin alpha 2 is an actin binding protein expressed in cardiomyocytes. There, it anchors the myofibrillar actin filaments and binds to cardiac ion channels (Maruoka et al., 2000).

1.5 Pluripotent cells and cell lines

1.5.1 Embryonic stem cells

The first differentiation step in the development of the mammalian embryo happens during the formation of the blastocyst when cells of the morula develop into trophopblast cells and cells of the inner cell mass. The inner cell mass consists of embryonic stem cells (Rossant, 2001).
Human embryonic stem cells (hESCs) are characterised by two features. The first feature is pluripotency. hESCs are therefore able to give rise to all lineages of the three germ layers endoderm, ectoderm and mesoderm both *in vitro* (formation of embryoid bodies) and *in vivo* (teratoma formation in immunodeficienct mice). The second feature is capability of self-renewal. hESCs are therefore able to escape senescence and can be expanded indefinitely in culture. Furthermore, embryonic stem cells express the surface markers SSEA-4, TRA-l-60, TRA-1-81, and TRA-2-49 (alkaline phosphatase) (Thomson et al., 1998).
The first successful culture of pluripotent mouse embryonic stem cell lines was achieved in 1981. At this time mouse embryonic stem cells were successfully cultured on a feeder layer of mouse embryonic fibroblasts (Evans and Kaufman, 1981).
The techniques used to culture and isolate mouse ESCs were later used for the establishment of human embryonic stem cell lines from pre-implanted embryos that were produced by *in vitro* fertilization (Thomson et al., 1998). Today over 400 hESC lines are available but few of them are characterised well (Jensen et al., 2009). One of the best characterised hESC lines is

H1, the stem cell line used in this work. H1 was derived by James Thomson and colleagues (Thomson et al., 1998).
During *in vitro* cultivation the pluripotent status of hESCs can be maintained by TGFβ and FGF2 mediated signaling pathways as these help to promote NANOG expression, a key regulator of pluripotency (Greber et al., 2007; Vallier et al., 2005). Therefore, the addition of FGF2 to the culture media is part of the protocol of hESC and iPS cell culture.
Since the first human embryonic stem cell lines were characterised, hopes have risen to use these cells as renewable source of human cells for the applications in cell therapies and for *in vitro* tests (Améen et al., 2008). However, the use of hESCs in research and medicine has limitations. As the isolation of hESCs requires the destruction of human embryos, ethical and moral concerns are high. Moreover, the derivation of lineage restricted cells from hESCs as well as the immune rejection of hESCs derived tissues are still problematic (Rao and Condic, 2008).

1.5.2 Induced pluripotent stem cells

Induced pluripotent cells (iPSCs) are cells that are reprogrammed into a state which is almost identical to ESCs. In 2006 induced pluripotent stem cells were first successfully generated from mouse fibroblasts through retroviral transduction and forced expression of transcription factors expressed in embryonic stem cells (Takahashi and Yamanaka, 2006). Shortly after this, two groups successfully achieved the reprogramming of human adult fibroblasts by viral transfection and overexpression of the four transcription factors OCT4, SOX2, KLF4 and c-Myc (Takahashi et al., 2007) or OCT4, SOX2, NANOG and LIN28 (Yu et al., 2007).
Induced pluripotent stem cells and hESCs share the features of pluripotency and self-renewal, as well as the expression of the surface markers SSEA-4, TRA-l-60, TRA-1-81, TRA-2-49 (alkaline phosphatase) and the expression of marker genes for hESCs such as NANOG, OCT4, SOX2, KLF4, DPPA4, GDF3 and hTERT. Furthermore, they have the same morphology, the promoters of ES cell specific genes are active in both cell types and they both form a teratoma when injected into immunodeficient mice.
Both iPSCs and ESCs form ball shaped structures, so called embryoid bodies, when cultured in low attachment dishes. When embryoid bodies of hESCs and iPSCs are seeded on gelatine coated dishes, both differentiate in endodermal lineages (expressing the marker genes SOX17 and AFP), mesodermal lineages (expressing the marker α-SMA) and ectodermal lineages (expressing the maker genes TUJ1 and NESTIN) (Takahashi et al., 2007).
However, despite this close similarity there are specific differences in the gene expression between hESCs and iPSCs as shown by comparative global gene expression analysis (Chin et al., 2009). Therefore, further clarification towards the degree of similarity between hESCs and iPSCs is needed.
Nevertheless, due to their close similarity to ESCs and their derivation methods, which do not include the use of pre-implantation embryos, iPSCs are interesting targets for the research on complex human disorders and for cell therapy (Amabile and Meissner, 2009).

iPSC generation from different cell types

Until today it has been achievedto reprogrammm many different cell types to induced pluripotent stem cells mainly with the initially used method of retroviral transduction of *OCT4*, *SOX2*, *KLF4* and *c-Myc* (Takahashi et al., 2007). So far several human somatic cell types have been reprogrammed such as fibroblasts, keratinocytes, peripheral blood cells and adipose stem cells. Mesenchymal stem cells were reprogrammed to iPSCs by the retroviral expression of the factors OCT4, SOX2, KLF4, c-Myc, SV40LT and hTERT (Park et al., 2008). Yet, the efficiency of iPSC generation was very low.
Moreover, integrated pro-viruses in the genome of iPSCs represent a risk for their clinical application (Chen and Liu, 2009).

Obstacles in the reprogramming process

The inhibition of p53 signaling has been shown to significantly increase the efficiency of inducing pluripotency in murine and human somatic cells (Hong et al., 2009). Consistently, cellular senescence and associated p53 up-regulation due to DNA damage and oxidative stress was described as an obstacle during the reprogramming process. In addition, somatic cells of older donors are reported to be more difficultto reprogrammm into iPSCs due to higher numbers of senescent cells (Banito et al., 2009; Prigione et al., 2010).

Partially reprogrammed cells

Several groups have observed the appearance of cells with an altered morphology which is different from the parental and from ESCs. These cells were described as partially reprogrammed cells (Park et al., 2008; Takahashi et al., 2007). The intermediate state of these cells between the parental cell and iPSCs is due to impaired targeting of the reprogramming factors OCT4, SOX2 and KLF4. Moreover it is possible to drive the cells into an pluripotent state with small molecules (Sridharan et al., 2009).

1.6 Surface marker genes for embryonic stem cells and induced pluripotent stem cells

Human embryonic stem cells and induced pluripotent stem cells are defined by the presence of the cell surface epitopes SSEA-4, TRA-1-60 and TRA-1-81 as well as by the absence of SSEA-1. In addition, the pluripotent state of embryonic stem cells and induced pluripotent stem cells is marked by the expression of TRA-2-49 (tissue non specific alkaline phosphatase) (Takahashi et al., 2007).
SSEA-4 (stage specific antigene 4) and SSEA-1 (stage specific antigene 1) are glycolipid antigens. SSEA-4 is mainly expressed in embryonic carcinoma cells and embryonic stem cells in contrast to SSEA-1. SSEA-1 expression rises in differentiating embryonic stem cells (Adewumi et al., 2007).
TRA-1-60 (tumour related antigen-1-60) and TRA-1-81 (tumour related antigen-1-81) are keratan sulfate antigens. Both were first discovered in embryonic carcinoma cells. TRA-1-60 and TRA-1-81 are highly expressed in undifferentiated embryonic stem cells. Their expression cannot be detected in differentiated cells (Adewumi et al., 2007).

A further maker of pluripotent stem cells is the expression of tissue non specific isoform of the enzyme alkaline phosphatase. The tumour related antigen 2-49 (TRA-2-49) epitope which is part of the tissue non specific alkaline phosphatase has been detected in embryonic carcinoma cells and later in embryonic stem cells (Adewumi et al., 2007). Alkaline phosphatases hydrolyse monophosphate esters as glycoproteins bound to the cell membrane. There are four different isoforms of that are expressed in different tissues (Fedde and Whyte, 1990).

1.7 Reprogramming with defined factors

The most commonly used factors to generate induced pluripotent stem cells are OCT4, SOX2, KLF4, c-Myc. In some cases also NANOG, LIN28 and SV40LT were used in addition. Next to viral methods iPSCs generation with non viral approaches was reported (Kiskinis and Eggan, 2010).

1.7.1 Factors used for the generation of induced pluripotent stem cells

OCT4 was identified in embryonic stem cells, early embryos and germ cells as part of the OCT family of transcription factors. The protein OCT4 contains the POU domain which is a 150 amino acid long sequence that binds to the octamer sequence (ATTA/TGCAT) on the DNA to regulate gene expression (Okamoto et al., 1990). OCT4 is commonly used as pluripotency marker because it regulates the expression of genes which play an important role in the maintenance of pluripotency but also in the regulation of differentiation. OCT4 forms heterodimers with SOX2 (Yamanaka, 2007).

SOX2 belongs to the SOX (SRY-related HMG box) family of transcription factors and was first detected in embryonic carcinoma cells (Yuan et al., 1995). The high mobility group (HMG) DNA binding domain of the SOX2 protein binds to the motif A/TA/TCAAA/TG on the DNA and thereby regulates gene expression. SOX2 is expressed in embryonic stem cells and germ cells but also in precursor cells of the central nervous system (Zappone et al., 2000). The absence of SOX2 in embryonic stem cells leads to differentiation highlighting the importance of SOX2 for the maintenance of pluripotency (Masui et al., 2007). SOX2 and OCT4 form heterodimers that regulate gene expression synergistically. SOX2 and OCT4 coregulate the expression of themselves and the expression of NANOG (Yamanaka, 2007).

c-Myc is one of the first proto-oncogenes detected to be involved in cancer (Dalla-Favera et al., 1982). The C-terminus of the protein has a basic region/helixloop-helix/leucine zipper (BR/HLH/LZ) domain which binds to the protein MAX. Both bind as dimer to the DNA sequence (CACA/GTG). Moreover, the C-terminus of the c-Myc protein plays a role in the transactivation by binding to proteins with histone acetylase activities. In contrast to that, the N terminus of c-Myc binds to proteins, which are part of acetyl transferase complexes (Davis et al., 1993). When *c-Myc* is deleted in embryonic stem cells they proliferate normally and stay in the undifferentiated state (Davis et al., 1993). c-Myc has more than 25.000 binding sites and may modify the chromatin structure by binding to these sites. In addition, c-Myc is involved in the transcriptional control of genes that regulate the cell growth and proliferation (Knoepfler et al., 2006).

KLF4 is a zinc-finger protein and part of the Krueppel-like factor (KLF) protein family. It contains protein domains resembling those of the Drosophila embryonic pattern regulator Krueppel (Schuh et al., 1986). KLF4 is expressed in human embryonic stem cells as well as skin cells (Yamanaka, 2007). Moreover, KLF4 can act as a tumour suppressor and as oncogene and was found to inhibit DNA synthesis and cell cycle progression on cultured cells (Chen et al., 2001). However, when p21, one of its target genes, is absent KLF4 down-regulates p53 and thereby promotes cell proliferation (Rowland et al., 2005). Furthermore, KLF4 is involved in self-renewal of embryonic stem cells and is a pluripotency marker (Li et al., 2005).

NANOG is a transcription factor containing a homeodomain, that binds DNA in a sequence specific manner to regulate the expression of genes involved in the maintenance of pluripotency in embryonic stem cells and induced pluripotent stem cells. NANOG plays an important role in the embryonic development and forced expression of NANOG has been shown to sustain self-renewal of undifferentiated embryonic stem cells. In addition, NANOG facilitates iPSC generation from human cells and serves as marker of the pluripotent state to isolate fully reprogrammed induced pluripotent stem cells (Silva et al., 2009).

SV40LT antigen is an oncoprotein that is capable of transforming various cell lines and to induce tumours in various animal models. It is one of two proteins that are expressed in a cell that is infected by the simvian virus 40.
The capability of SV40LT to transform cells is due to its manipulation of the cell cycle regulatory proteins such as the tumour suppressor p53 and members of the retinoblastoma family of proteins such as pRB. In particular SV40LT interacts with pRB and relieves the bound E2F transcription factor that controls the expression of genes in the G1-S-phase transition of the cell cycle.
Furthermore, SV40LT inhibits p53 expression upon DNA damage and the associated expression of p21 and its inhibition of CDK4/6 and CDK2. Therefore, the cell cycle is not stopped in the G1-phase and the cells enter the S-phase even when DNA is damaged (Ali and DeCaprio, 2001).

Role of the reprogramming factors in iPSC generation

It has been reported that the generation of iPSCs requires the transformation by KLF4 and c-Myc. c-Myc causes p53 dependent apoptosis in fibroblasts which is most likely repressed by KLF4. In contrast to that, KLF4 activates p21 and impairs proliferation. According to this the balance of KLF4 and c-Myc during the reprogramming process is critical.
In addition to its transforming effects c-Myc recruits histone acetylase complexes that facilitate the expression of embryonic stem cell specific genes. Only the forced expression of both c-Myc and KLF4 would lead to tumour formation therefore probably OCT4 directs the cell fate from tumour cells to induced pluripotent cells.
SOX2 and OCT4 both synergistically activate target genes in the reprogramming process. KLF4 thereby may function as cofactor. Yet, the amount of SOX2 required for iPSC generation is unknown (Yamanaka, 2007).
The low efficiency of iPSC generation (>1 %) might be explained by the requirement of specific amount of the four factors that can only be realised in a small amount of transfected

cells. Moreover, depending on the cell type other factors may be necessary for more efficient reprogramming (Yamanaka, 2007).

1.7.2 Optimisation of iPSC generation

Numerous different approaches to derive iPSC cells and to optimise the generation of these cells have been reported since the first generation of iPS cells. An overview of the techniques is illustrated in Figure 2.

Reprogramming using viruses

In the first reported derivation of iPSCs from human fibroblasts retroviruses were used to transduce *OCT4*, *SOX2*, *KLF4* and *c-Myc* yielding 0,02 % reprogrammed cell colonies (Yamanaka, 2007). At the same time, human fibroblasts were successfully reprogrammed via transduction of lentiviral constructs carrying *OCT4*, *SOX2*, *NANOG* and *LIN28* with an efficiency of 0,02- 0,0095 % (Yu et al., 2007). These protocols could be reproduced by numerous groups confirming the robustness of these methods. However, the generated iPSCs are far from the point where they can be used in medicine because of still unresolved problems.
The major problems of the viral methods are the random integration of proviruses into the genome of the reprogrammed cells and the fact that KLF4 and c-Myc are oncogenes that could be reactivated. These conditions cause a high probability of genomic instability and mutagenesis in iPSCs (Wang et al., 2010).
Therefore, reprogramming with fewer factors was tried to make their medical application safer. In this context, iPSCs were generated from fibroblasts with OCT4, SOX2, KLF4 but without c-Myc (Nakagawa et al., 2008). Subsequently, many studies reported the generation of iPSCs from specialised cells with reduced factors. For instance, neural stem cells have been reprogrammed only with OCT4 (Kim et al., 2009).
A further problem is the low efficiency of the viral reprogramming method. To solve it, additional factors were used. For instance, the addition of the viral oncogene SV40LT enhances the efficiency of iPSC generation from fibroblasts. Moreover, the inhibition of p53 has been demonstrated to enhance the iPSC generation from human fibroblasts up to 100-fold (Zhao et al., 2008).

Non viral methods of iPSC generation

Many studies have shown that it is possible to derive iPSCs from various human somatic cells by means of non viral methods. By using non viral methods problems like mutagenesis through provirus integration and reactivation of oncogenic transgenes can be circumvented. However, most non viral methods have the disadvantage that the efficiency of iPSC generation is even lower compared to the retroviral methods (Chen and Liu, 2009).
Most methods use non viral based vectors that do not integrate into the genome of the transfected cell.
Referring to this, induced pluripotent stem cells were generated from murine somatic cells by transfection of two plasmids, one carrying the transgenes OCT4, KLF4 and SOX2 and the other vector encoding c-Myc (Okita et al., 2008).

A further non viral methods used a piggyBac (PB) based vector system consisting of a vector and a transposon for doxycyclin inducible expression of the reprogramming factors (Woltjen et al., 2009). Moreover, iPS cells were successfully generated by transfection of somatic cells with OCT4, SOX2, KLF4 and c-Myc proteins. This method resulted in completely vector free iPSCs, however the efficiency was low and the delivery into the cells is problematic (Kim et al., 2009).

Non viral reprogramming with episomal plasmids

Recently it was shown, that it is possible to generate iPS cells completely free of vector of transgene sequences with a different non viral method. In this method, somatic cells were nucleofected with oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-based episomal vectors expressing reprogramming factors.
oriP/EBNA1 vectors are derived from the Epstein-Barr virus and are a suitable tool to introduce reprogramming factors into somatic cells because transfection of the vector is possible without viral packaging. The episomal vectors can be removed from the cells subsequently simply by culturing them as they are gradually lost with a rate of 5 % per cell division due to defective plamid synthesis. Moreover, oriP/EBNA1-based episomal vectors have the ability of extra chromosomal replication through the cis-acting oriP element on the vector and the EBNA-1 gene which encodes a replication initiation factor (Yates et al., 1984). The generation of induced pluripotent stem cells from fibroblasts with this method has been reported with an efficiency of 0,003-0,006 %. The generated iPS cells showed ES cell characteristics and the episomal vectors were lost after 9-10 passages in two established iPSC cell lines. Subsequent analysis of whole-genome expression profiles revealed that the generated iPSCs are most similar to ESCs than iPSCs generated with other viral and non viral methods (Yu et al., 2009).
In this work, human bone marrow derived mesenchymal stem cells will be reprogrammed to induced pluripotent stem cells with this method for vector free generation of iPSCs.

Small molecule inhibitors enhance the reprogramming of somatic cells

As depicted in Figure 2 various chemicals that enhance the efficiency of iPSC generation or even replace one or two of the reprogramming factors were identified. These chemicals either influence epigenetic factors or the signal transduction during the process of cellular reprogramming (Feng et al., 2009).
A recently reported approach to enhance the efficiency of iPSC generation from fibroblasts over 100-fold involves the inactivation of the TGFβ and the MEK-ERK pathways using a combination of the small molecule inhibitors SB431542 and PD0325901. In addition, the iPSC generation was faster than without the inhibitors (Lin et al., 2009).
The TGFβ family of growth factors regulate a wide variety of cellular processes in many different cell types. In TGFβ signaling ligands like TGFβ1 cause that Type I and Type II receptors, both serin/threonin kinases, are brought together. The Type II receptor activates the Type I receptor which results in a signaling cascade that leads, depending on the ligand, to induction or repression of gene expression. Among the Type II receptors are TβR-II, BMPR2 and three others. The group of the Type I receptors comprises seven members, ALK (Activin receptor like kinase receptor) 1-7. The small molecule inhibitor SB431542 specifically inhibits the Type I receptors ALK4, ALK5 and ALK7 (Inman et al., 2002).

Furthermore, TGFβ is involved in the induction of Epithelial to Mesenchymal Transition, which is an important process in the embryonic development and has implications for iPSC generation (Lin et al., 2009). The induction of pluripotency is favoured by Mesenchymal to Epithelial Transition (Wang et al., 2010).
The chemical compound PD0325901 is an inhibitor of the MAP/ERK kinase MEK. MEK is activated by extracellular signals through the MAP kinase pathway. This pathway plays a major role in the regulation of growth and differentiation of cells. PD0325901 inhibits the signal transduction by inhibiting the phosphorylation of ERK by MEK (Barrett et al., 2008).
As the combined use of SB431542 and PD0325901 drives partially reprogrammed fibroblasts to a fully pluripotent state (Lin et al., 2009), both inhibitors will be used to enhance reprogramming efficiency of human mesenchymal stem cells in this work.
A further inhibitor used in this study is Pifithrin α. Pifithrin α blocks the transcriptional activity of the tumour suppressor p53 and has been shown to protect several cell types from DNA damage induced apoptosis independent from p53 through a mechanism that involves cyclin D1 (Sohn et al., 2009).
p53 has been suggested to play a major role in the senescence related inhibition of iPSC generation (Banito et al., 2009). Furthermore, the inhibition of p53 by short interfering RNA (siRNA) has been shown to enhance the efficiency of iPSC generation (Zhao et al., 2008). Therefore, it will be tested in this study whether it is possible to enhance the iPSC generation from human mesenchymal stem cells by inhibition p53 activity with Pifithrin α.

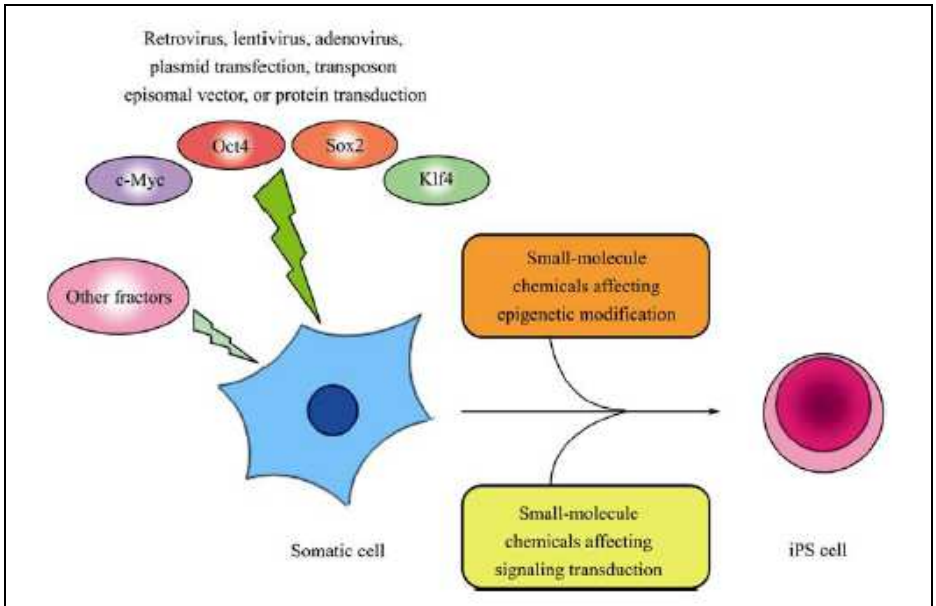

Figure 2 Different methods of iPSC generation. Viral methods comprise over expression of for example the factors OCT4, SOX2, KLF4, and c-Myc using retroviruses, lentiviruses or adenoviruses. Non viral reprogramming methods use protein transduction, plasmid transfection or episomal vectors. Numerous small molecules have been described that improve iPSC generation. Some of them are chemicals that alter the epigenetic status during reprogramming. Other chemicals inhibit signaling pathways to promote iPSC generation (Chen and Liu, 2009)

2 Aim of the work

The *in vitro* expansion and application possibilities of primary human mesenchymal stem cells in regenerative medicine are limited by their short life span in culture and restricted differentiation potential. These two features have been altered in human fibroblasts and other somatic cells by reprogramming them to induced pluripotent stem cells by the ectopic expression of the four transcription factors OCT4, SOX2, KLF4, and c-Myc. iPSCs have very similar features compared to embryonic stem cells and acquire self-renewal properties and pluripotency. Therefore iPSC generation from hMSCs will broaden their application potential.

Currently, most reprogramming techniques to generate iPSCs from somatic cells are using integrative retroviruses. This approach results in a low reprogramming efficiency, which can be increased using the chemicals SB431542 (inhibitor of ALK4/5 and 7) and PD325901 (inhibitor of MEK) and by inhibition of p53 during the reprogramming of fibroblasts.

hMSCs have been reprogrammed to iPSCs by retroviral expression of the six factors OCT4, SOX2, KLF4, c-Myc, hTERT and SV40LT.

To increase the low efficiency of iPSC generation and determine the possibility of replacing reprogramming factors with small molecule inhibitors, hMSCs will be reprogrammed with retroviruses only carrying *OCT4*, *SOX2*, *KLF4*, and *c-Myc* and additional treatment with the inhibitors of ALK4/5 and 7, MEK and p53. Moreover, the putative effect of donor age on the quality of iPSC generation from hMSCs will be analysed by applying this reprogramming protocol to hMSC lines derived both from a 17 and a 74 year old donor. The age-related transcriptional differences will be analysed with a microarray-based global gene expression of the two hMSC lines.

Insertion mutations, a disadvantage of the retroviral approach, can be avoided by reprogramming fibroblasts with oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-based episomal vectors.

To obtain iPSCs without residual viral sequences, hMSCs will be reprogrammed using the episomal plasmids pEP4 E02S ET2K and pEP4 E02S EM2K which carry different combinations of reprogramming factors. By additional application of inhibitors (SB431542 and PD325901) the usual low efficiency of this method will be enhanced.

Finally, biochemical and genetic methods will be used to compare the similarity of hMSC-derived iPSCs (viral/non viral approach) with human embryonic stem cells and the parental hMSCs.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

2-Propanol (Merck, 109634)
Acetic Acid (Merck)
Alizarin Red S (Sigma)
Ammoniumhydroxide (Merck)
Ascorbic Acid (Sigma)
Bromphenol Blue (AppliChem GmbH)
Dexamethasone (Sigma, D1756-25MG)
Dimethylformamide
DMSO (Sigma)
Ethanol ≥99,8 % (Roth, 5054.2)
Ethidium bromide (Sigma, E1510)
HCL (Roth)
3-Isobutyl-1-Methylxanthine (Sigma, I5879)
Insulin from Bovine Pancreas (Sigma, I6634)
NaCl (Fluka)
NaOH (Fluka)
Oil Red O (Sigma)
Oil Red O (Sigma, O-0625)
Paraformaldhyde (Science Services)
PD0325901 (Stemgent, 04-0006)
Pifithrin α (Stemgent, 04-0038)
Propylene glycol
Proteinase K (Sigma)
SB431542(Sigma, S4317)
Triton X-100 (Sigma)
Triton-X-100 (Sigma, T9284)
Y-27632 (Stemgent, 04-0029)
β-Glycerophosphate (Sigma)
β-Mercaptoethanol (Sigma, M6250)

3.1.2 Consumables

0,2 ml Thermo-Strip (ABgene, AB0266)
1,8 ml Cryo Tube™ vials (Nunc)
BD Microlance™ 3 injection needle (Becton Dickinson)
Cell scraper and spatula (TPP)
Filter for cell culture media 0,22 µM pore size (Corning, PAS)
Filter for cell culture supplements pore size 0,22 µm Filtropur S 0.2 (Sarstedt)
Flasks, dishes and 6 well, 12 well and 24 well plates for cell culture (TPP)

Micro tube 1,5 ml EASY CAP (Sarstedt, 72.690.550)
Safe Seal Tips 10 µl, 20 µl, 200 µl, 1.000 µl (Biozym)
Serological pipettes 1ml, 5 ml, 10 ml, 25 ml, 50 ml (TPP)

3.1.3 Laboratory devices

ABI Prism 7700 (Applied Biosystems)
Agarose gel electrophoresis equipment (Amersham)
Camera for microscopy model AxioCam ICc3
Clean bench type HeraSafe (Hereaus Instruments)
Clean bench type HeraSafe (Hereaus Instruments)
Centrifuge 5415D (Eppendorf)
Heatable water bath
HERAguard R Clean Bench (Heraeus, Thermo Fischer Scientific Inc.)
Incubator (INNOVA CO-170 Incubator, New Brunswick Scientific)
Incubator type Hereaus 6.000 (Hereaus Instruments)
Inverted microscope model CK2 (Olympus)
Leica MZ 95 Stereo Microscope (Leica)
Mobile pipettor PIPETBOY acu
Neubauer Counting Chamber (Roth)
Objective 10xCplanF 10x/0,25 pHC (Olympus)
Objective 4xUplanF 4x/0,13 pHL (Olympus)
Pipettes (Gilson)
SPD131DDA SpeedVac concentrator
Spectrophotometer type NanoDrop® ND-1.000
Thermo Block type DRI Block DB2A (Techne)
Thermocycler PTC100 (MJ Research Inc)
Thermomixer (Eppendorf)
Zeiss, LSM 510 Meta confocal microscope

3.1.4 Cell lines

All human mesenchymal stem cells (hMSCs) were obtained from the research group "Zelltherapie" at Julius Wolff Institute, Berlin, where they were isolated from bone marrow aspirates that were taken during a hip operation.

Obtained cell lines

MSC17: human mesenchymal stem cells derived from a 17 year old patient, male
MSC23: human mesenchymal stem cells derived from a 23 year old patient. male
MSC74: human mesenchymal stem cells derived from a 74 year old patient, male

H1 (WA01): embryonic stem cell line, registered cell line in the National Institute of Health, purchased from WiCell Research Institute (Madison, WI, USA), Lot Number H1-MCB-1, isolated before 19th November 2006
Further information:
http://www.wicell.org/index.php?products_id=113&mCoA=2&id=208&option=com_oscommerce&osMod=product_info&Itemid=192&osCsid=8e401f7936ff95dce8a9b4df08fe951 (13.07.2010)

v-iPSCs: iPSCs that were derived from MSC74 cells by infection with retroviruses encoding *OCT4*, *SOX2*, *KLF4* and *c-Myc* and additional treatment with the inhibitors PD0325901, SB431542 and Pifithrin α

p-iPSC 1-4: are iPSC cell lines that were derived from MSC74 by nucleofection with the episomal plasmid pEP4 E02S ET2K and additional treatment with the inhibitors PD0325901 and SB431542

HEK293T: transformed Human Embryonic Kidney cell line (MPI, Berlin)

MEFs: Mouse Embryonic Fibroblasts (MEFs) The cells were isolated from pregnant female mice (CF-1, Harlan, USA) after they were sacrificed. MEFs were obtained from Ph.D. Raed Abu-Dawud and from Ph.D. Alessandro Prigione.

3.1.5 Cell culture

3.1.5.1 Media

Media for hMSCs and MEFs

Components to make 500 ml of media
445 ml of DMEM (High Glucose, Gibco, Invitrogen)
50 ml of FBS (10 %, Biochrom AG, Berlin)
5 ml of Penicillin-Streptomycin (1/100, Gibco, Invitrogen)
Mix all components and filter with a 500 ml Vacuum Filter/Storage Bottle System, 0,22 µm Pore 19,6 cm² Membrane (Corning, 430756)

hESC media (unconditioned media)

400 ml of Knockout ™ DMEM (Gibco, Invitrogen)
100 ml of Knockout ™ Serum Replacement (20 %, Gibco, Invitrogen)
5 ml of 200 mM L-glutamine (1/100, Gibco, Invitrogen)
5 ml of Penicillin-Streptomycin (1/100, Gibco, Invitrogen)
5 ml of Non-Essential Amino Acids (1/100, Gibco, Invitrogen)
Mix all components and filter with a 500 ml Vacuum Filter/Storage Bottle System, 0,22 µm Pore 19,6 cm² Membrane (Corning, 430756)

After filtering addition of:

-35 µl of 0,1,4 M β-Mercaptoethanol (Sigma)

-respective volume of 10 µg/ml FGF2 stock solution when the media is used to a final concentration of 4 ng/ml FGF2

β-Mercaptoethanol solution for hESC media

14,3 M β -Mercaptoethanol (Sigma) was diluted 1:10 in PBS, filtered and stored at −20 °C in 40 µl aliquots. Aliquots were thawed and used immediately.

10 µg/ml FGF2 stock solution

Reconstitute 50 µg recombinant human basic fibroblast growth factor (FGF2, Peprotech, 100-18B) in 5 ml of PBS with 0,2 % BSA (Bovine Serum Albumin, Fraction V, 99 % purity, Sigma, A9418). Only BSA solution was filtered with a 22 µm pore size syringe filter (Corning, 431225) as the FGF2 solution is very sticky. Aliquots were stored at -20 °C.

3.1.5.2 Passaging of the cells

Trypsin/EDTA(1x) (PAA The cell Culture Company, L11-004)

PBS without $Ca^{2+}Mg^{2+}$ (Gibco, Invitrogen)

3.1.5.3 Cryopreservation

Freezing media for hMSCs

90 % FBS (Sigma, F7524) 10 % DMSO HYBRY Max, sterile filtered (Sigma, D26509).

Freezing media for hESCs, v-iPSCs and p-iPSCs

10 % of DMSO (Sigma)
40 % of Knockout ^{TM} DMEM (Gibco, Invitrogen)
50 % of Knockout ^{TM} Serum Replacement (Gibco, Invitrogen)

Freezing media for MEFs

10 % of DMSO (Sigma)
40 % of DMEM (High Glucose, Gibco, Invitrogen)
50 % of FBS (Biochrom AG, Berlin)

Freezing container (Nalgene)

3.1.5.4 Culture on feeder cell coated dishes

Mitomycin C (Roche Applied Science)

Mitomycin C stock solution:

1 mg/ml in PBS filter sterilise with 0,22 µm pore size filter

Media for the inactivation of MEFs

2 ml Mitomycin C stock solution
200 ml media for MEFs
filter, store at 4 °C and use within four weeks

1 % gelatine stock solution:

for 40 ml:
0,4 g gelatine (porcine)
40 ml $_{dd}H_2O$ mix well and autoclave

0,1 % gelatine solution for coating dishes

for 200 ml:
20 ml 1 % gelatine solution
180 ml PBS for cell culture (Gibco, Invtrogen)

3.1.5.5 Feeder cell free culture

Growth Factor-Reduced Matrigel® (Becton Dickinson)

3.1.5.6 Thawing of the cells

10 mM Rho-associated kinase (ROCK) inhibitor Y-27632 stock solution
(applied in a 1:1.000 dilution in the culture media after hESCs and iPSCs were thawed)

Reconstitute 2 mg Y-27632 in 526 µl DMSO.
Filter with 0,22 µm pore size filter. Store aliquots at -20 °C. The solution has to be kept in dark.

3.1.6 In vitro differentiation

3.1.6.1 Adipocyte differentiation

Insulin from bovine pancreas (Sigma, I6634): 2 mg/ml stock solution in $_{dd}H_2O$, pH was adjusted using 50 mM acetic acid until the solution was clear.
Dexamethasone (Sigma, D1756-25MG): 10^{-3} M stock solution in $_{dd}H_2O$.
3-Isobuthyl-1-Methylxanthine (Sigma, I5879): 50 mM in DMSO (Sigma)

Adipocyte differentiation media

Culture media for hMSCs and MEFs
Supplemented with:
5 µg/ml Insulin (1:400 dilution of 2 mg/ml stock solution)
10 µM Dexamethasone (1:1.000 dilution of 10^{-3} M stock solution)
500 µM 3-Isobuthyl-1-Methylxanthine (1:100 dilution of 50 mM stock solution)

Control media

Culture media for hMSCs and MEFs
Supplemented with:
1 % DMSO (as 3-Isobuthyl-1-Methylxanthine was diluted in DMSO)

3.1.6.2 Osteoblast differentiation

β-glycerophosphate (Sigma): 10 mM stock solution in $_{dd}H_2O$, has to be kept in the dark
ascorbic acid (Sigma): 1 mg/ml stock solution in $_{dd}H_2O$

Osteoblast differentiation media

Culture media for hMSCs and MEFs
Supplemented with:

10 µg/ml ascorbic acid (1:100 dilution of stock solution)
500 µM β-glycerophosphate (1:20 dilution of stock solution)

Control media

Culture media for hMSCs and MEFs without additives

3.1.7 Staining to detect osteoblast and adipocyte differentiation

3.1.7.1 Oil Red O staining

Fixing solution
1x PBS containing 4 % paraformaldehyde (PFA) (Science services)

2 ml 16 % PFA were topped up with 6 ml 1x PBS and mixed.

0,5 % Oil Red O solution:

0,5 g Oil Red O (Sigma)
100 ml 2-Propanol (Merck)

The solution has to be freshly prepared before use. Furthermore, it has to be heated for several minutes at 95 ºC and filtered.

filter paper (Schleicher & Schüll)

fresh preparation of Oil red O for immediate application

6 parts of 0,5 % Oil Red O in 2-Propanol
4 parts of distilled water
The solution was mixed and filtered using filter paper (Schleicher & Schüll).

3.1.7.2 Alizarin Red S staining

Fixing solution

See Oil Red O staining

2 % Alizarin Red S staining solution

2 g Alizarin Red S
100 ml distilled water

The solution was mixed well and the pH was adjusted to 4,2 using 10 % ammonium hydroxide

3.1.8 Analysis of Nucleic Acids

3.1.8.1 Isolation of genomic DNA

FlexiGene DNA Kit (Qiagen)

Protease (Quiagen)
Centrifuge 5415D (Eppendorf)
70 % ethanol in $_{dd}H_2O$

3.1.8.2 Polymerase Chain Reaction

Taq polymerase, dNTPs and $MgCl_2$ solution were purchased from Invitrogen.
PFU polymerase was purchased from Promega.

10x-buffer

500 mM Tris-Cl pH 8,8
200 mM $(NH_4)_2SO_4$
15 mM $MgCl_2$
0,1 % Tween 20

Thermocycler PTC100 (MJ Research Inc)

3.1.8.3 Agarose gel electrophoresis

Ethidium Bromide (10 mg/ml; Invitrogen)

Perfect Plus 1 kb DNA ladder (Roboklon, E3131-01)
5 µl (500 ng DNA) of the marker were used for agarose gel electrophoresis

6x DNA loading buffer

0,2 % Bromophenol Blue
60 % Glycerol
60 mM EDTA

Agarose (Invitrogen, 15510-027)

50x TAE Buffer (Tris-Acetate-EDTA)

242 g Tris base
57,1 ml Acetic acid
100 ml 0,5 M EDTA
Add $_{dd}$H2O to 1.000 ml and adjust pH to 8,5.

AlphaImager™ (Alpha Innotech)

3.1.8.4 RNA isolation

RNeasy® Mini Kit (Qiagen)
DNAse I (RNase-free DNase set, Qiagen)

β-Mercaptoethanol (Sigma)

Spectrophotometer NanoDrop® ND-1.000
Savant SPD131DDA SpeedVac

3.1.8.5 Transcription of RNA to cDNA

Oligo dT Primer (Invitek)
5X-reaction buffer (Promega)
dNTP-Mix (Invitrogen)
MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (200 U/µl, USB)

Thermocycler PTC100 (MJ Research Inc).
Transcription of RNA to cDNA

3.1.8.6 Real time PCR

96-well Optical Reaction Plates (Applied Biosystems).
SYBR® Green PCR Master Mix (Applied Biosystems)
ABI PRISM 7900HT Sequence Detection System (Applied Biosystems)
SDS 2 software (Applied Biosystems)
MS Excel (Microsoft)

3.1.8.7 Primer

Primers were ordered from Eurofins MWG operon
The primers were diluted in $_{dd}H_2O$ to a stock solution of 100 µM, which was applied undiluted in the PCR and diluted 1:20 in $_{dd}H_2O$ in the real time PCR.

Primers for PCR:

Table 1 Primer used for PCR analysis of oriP and for DNA fingerprinting (D10S1214: microsatellite which is highly variable in the general population based on highly-variable number of tandem repeats (VNTR)

Name		*Sequence (5´-3´)*
D10S1214	forward	ATT GCC CCA AAA CTT TTT TG
	reverse	TTG AG ACC AGT CTG GGA AG
oriP	forward	TTCCACGAGGGTAGTGAACC
	reverse	TCGGGGGTGTTAGAGACAAC

Primers for real time PCR:

Table 2 Primer used for real time PCR

Gene		*Sequence (5´-3´)*
OCT4	forward	GTGGAGGAAGCTGACAACAA
	reverse	ATTCTCCAGGTTGCCTCTCA
SOX2	forward	TGGCGAACCATCTCTGTGGT
	reverse	TGGCGAACCATCTCTGTGGT
NANOG	forward	CCTGTGATTTGTGGGCCTG
	reverse	GACAGTCTCCGTGTGAGGCAT
KLF4	forward	TGGCTCTCCTCAAGCGTATT
	reverse	GTGGAGAAAGATGGGAGCAG
c-Myc	forward	TCAAGAGGCGAACACACAAC
	reverse	AGGAACTGCTTCCTTCACGA
LIN28	forward	TGGCTCTCCTCAAGCGTATT
	reverse	GCAAACTGCTGGTTGGACAC
GAPDH	forward	GTGGACCTGACCTGCCGTCT
	reverse	GGAGGAGTGGGTGTCGCTGT

3.1.8.8 Microarray based gene expression analysis

Illumina BeadChip hybridisation

Linear amplification kit (Ambion)
BeadStation 500 platform (Illumina)
HumanHT-12 v3 Expression BeadChips (Illumina)

Analysis of the gene expression data

Software:
BeadStudio (Illumina)
MS Excel (Microsoft)

Online tools:
VENNY (Olivero, 2007, http://bioinfogp.cnb.csic.es/tools/venny/index.html)
DAVID, annotation, visualization and integrated discovery (http://david.abcc.ncifcrf.gov)

3.1.9 Viral reprogramming

3.1.9.1 Retrovirus production

hMSCs media (see section cell culture)

HEK293T media

for 500 ml media:
445 ml Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Invitrogen)
50 ml of FBS (10 %, Biochrom AG, Berlin)
5 ml of Penicillin-Streptomycin (1/100, Gibco, Invitrogen)
5 ml of 200 mM L-glutamine (1/100, Gibco, Invitrogen)
Mix all components and filter with a 500 ml Vacuum Filter/Storage Bottle System, 0,22 µm Pore 19,6 cm^2 Membrane (Corning, 430756)

Plasmids for retrovirus production

pCMV-VSV-G (Addgene, plamid 8454)
pUMVC3-gag-pol (Addgene, plasmid 8449)

Retroviral vectors

pMXs-hOCT3/4 (Addgene, plasmid 17217)
pMXs-hSOX2 (Addgene, plasmid 17218)
pMXs-hKLF4 (Addgene, plasmid 17219)
pMXs-hc-Myc (Addgene, plasmid 17220)
pBABE GFP (Addgene, plasmid 10668)

The pMXs vectors containing *OCT4*, *SOX2*, *KLF4* and *c-Myc* transgenes were created by Yamanaka et al.

2,5 M $CaCl_2$ (Sigma, tissue culture grade), filtered with 0,22 µM pore size filter

2x HBS: 281 mM NaCl
100 mM HEPES
1,5 mM Na_2HPO_4
pH 7,12
filtered with 0,22 µM pore size filter

0,45 µm pore size syringe driven filter (Durapore)
Polyallomer centrifugation tubes (Beckman)

Beckman L7 ultracentrifuge, rotor type SW-28 (Beckman)

3.1.9.2 Retrovirus infection

Polybrene® (Sigma, 107689-10G)

3.1.9.3 Reprogramming with retroviruses

10 µg/ml FGF2 stock solution (see section cell culture)
hMSC media (see section cell culture)
HESC media (see section cell culture)
Conditioned media (see section preparation of conditioned media in the methods part)

10 mM PD-0325901 stock solution (applied 1:20.000 in reprogramming experiments)

Reconstitute 2 mg PD0325901 in 415 µl of DMSO.
Filter with 0,22 µm pore size filter. Store aliquots at -20 °C.

10 mM SB-431542 stock solution (applied 1:5.000 in reprogramming experiments)

Reconstitute 5 mg SB-431542 in 1301 µl of DMSO.
Filter with 0,22 µm pore size filter. Store aliquots at -20 °C. Protect from light.

10 mM Pifithrin α stock solution (applied 1:1.000 in reprogramming experiments)

Reconstitute 5 mg of Pifithrin α in 1361 µl of DMSO.
Filter with 0,22 µm pore size filter. Store aliquots at -20 °C.

3.1.10 Non viral reprogramming

3.1.10.1 Nucleofection of hMSCs with episomal plasmids

Human MSC (Mesenchymal Stem Cell) Nucleofector® Kit (Lonza Cologne AG, VPE-1001)

Nucleofector® Device (Lonza Cologne AG)

plasmid pMAX GFP, used as control for nucleofection, part of the Human MSC (Mesenchymal Stem Cell) Nucleofector® Kit (Lonza Cologne AG, VPE-1001)

Episomal plasmids

pEP4 E02S ET2KEM2K (Addgene, plasmid 20927)
pEP4 E02S EM2K (Addgene, plasmid 20923)

Both plasmids were originally generated by James A. Thomson et al.

3.1.10.2 Non viral reprogramming

Inhibitor stock solutions for reprogramming and media: see section reprogramming with retroviruses

3.1.11 Isolation of reprogrammed cells and derivation of cell lines

HERAguard R Clean Bench (Heraeus, Thermo Fischer Scientific Inc.)
Leica MZ 95 Stereo Microscope (Leica)
BD Microlance™ 3 injection needle (Becton Dickinson)

3.1.12 Characterisation of reprogrammed cells

3.1.12.1 Alkaline phosphatase staining

Both are solutions part of ES cell characterisation kit (Millipore, SCR001):

Fast Red Violet solution (0,8 g/l stock)
Napthol AS-BI phosphate solution (4 mg/ml) in AMPD (2-amino-methyl-1,3-propanediol) buffer (2 M/l), pH 9,5

Not part of the kit:
1x Rinse Buffer: TBST: 20 mM Tris-HCl, pH 7,4, 0,15 NaCl, 0,05 % Tween-20

3.1.12.2 Imunofluorescence staining

Fixing solution:

4 % paraformaldehyde (Science Services), 16 % stock solution diluted in PBS

Solution to permeabilise the cells:

1 % Triton X-100 in PBS

Blocking solution used to dilute the antibodies as well

10 % chicken serum, 0,1 % Triton X-100 in PBS

Primary antibodies

Pluripotency associated factors

Anti OCT4 (C-10) mouse polyclonal antibody (Santa Cruz, sc-5279), applied in a 1:100 dilution
Anti SOX2 (Y-17) goat polyclonal antibody (Santa Cruz, sc-17320), applied in a 1:100 dilution
Anti NANOG mouse monoclonal antibody (Abcam, ab62734), applied in a 1:100 dilution
Anti c-Myc (N-262) rabbit polyclonal antibody (Santa Cruz, sc-764), applied in a 1:100 dilution

Antibodies used from the ES cell characterization tool (Millipore, SCR001):

Anti SSEA-1 mouse monoclonal antibody, applied in a 1:100 dilution
Anti SSEA-4 mouse monoclonal antibody, applied in a 1:100 dilution
Anti TRA-1-60 mouse monoclonal antibody, applied in a 1:100 dilution
Anti TRA-1-81 mouse monoclonal antibody, applied in a 1:100 dilution

Antibodies used to analyse embryoid body based differentiation

Anti SOX17 goat polyclonal antibody (R&D, AF1924), applied in a 1:50 dilution
Anti β-Tubulin III (TUJ1) mouse monoclonal antibody (Sigma, T8660), applied in a 1:1.000 dilution
Anti Nestin mouse monoclonal antibody (Chemicon, MAB5326), applied in a 1:200
Anti Smooth Muscle Actin mouse monoclonal antibody (Dako Cytomation, M0851), applied in a 1:100 dilution

Cardiomyocyte specific staining

Anti α-Actinin (Sarcomeric) mouse monoclonal antibody (EA-53) (Sigma, A7811), applied in a 1:100 dilution

Secondary antibodies

All secondary antibodies were applied in a 1:300 dilution

Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Invitrogen, A10667)
Alexa Fluor 594 conjugated goat anti-mouse IgG (H+L) (Invitrogen, A11013)
Alexa Fluor 488 conjugated chicken anti-goat IgG (H+L) (Invitrogen, A11006)
Alexa Fluor 594 conjugated chicken anti-goat IgG (H+L) (Invitrogen, A11013)
Alexa Fluor 488 conjugated donkey anti-rabbit IgG (H+L) (Invitrogen, A11015)

DAPI Stock solution:

10 mg of DAPI (Molecular Probes, D-1306) in 2 ml of Dimethylformamide (DMF). Aliquots were stored at –20 ºC.

Working Solution (100 ng/ml in PBS):

2 µl of DAPI stock solution in 100 ml of PBS
The solution has to be stored at 4 ºC protected from light.

3.1.12.3 Embryoid body based differentiation

BD Microlance™ 3 injection needle (Becton Dickinson)
60 mm ultra low attachment culture dish (Corning)

Antibodies (see section Immunofluorescence staining)
Media and supplements as well as gelatine coating of dishes see section cell culture

3.2 Methods

3.2.1 Isolation of human mesenchymal stem cells

The human mesenchymal stem cells (hMSCs) used in the experiments of this work were obtained from the research group "Zelltherapie" at Julius Wolff Institut, Berlin. The cells were isolated from bone marrow aspirates of patients of different ages. The bone marrow aspirates were taken from the hip bone in an operation. The constituent parts of the bone marrow aspirates were separated by density gradient centrifugation. The part containing mononuclear cells was then seeded on cell culture dishes. Adhering cells obtained by this procedure were used in the experiments of this work.

3.2.2 Characterisation of hMSCs

hMSCs of three different male patients that were isolated as described were used in this work. Differences in the gene expression pattern of MSC17, MSC23 and MSC74 were determined by microarray-based gene expression analysis using an Illumina HumanHT-12 v3 Expression BeadChip. Experiments aimingto reprogramm hMSCs to induce the generation of pluripotent stem cells with retroviruses were carried out with MSC17 and MSC74. Reprogramming experiments using non viral episomal plasmids were performed with MSC74. The differentiation potential regarding adipogenesis and osteogenesis of MSC17 and MSC74 was analysed in *in vitro* differentiation experiments prior to the reprogramming experiments. Information about the origin of the hMSCs and the procedure carried out are summarised in Table 3.

Table 3 General information about the hMSC lines and the experimental procedures the cell were used for

Cell line	***Conducted experiments***
MSC17	Microarray-based gene expression analysis, *in vitro* differentiation, viral reprogramming
MSC23	Microarray-based gene expression analysis
MSC74	Microarray-based gene expression analysis, *in vitro* differentiation, viral and non viral reprogramming

3.2.3 Cell culture

All used cells and cell lines were cultured at 37 °C and 5 % CO_2 in an incubator (INNOVA CO-170 Incubator, New Brunswick Scientific) under humidified atmosphere. All treatments and maintenance procedures were carried out using a clean bench type HeraSafe (Haereus Instruments).

3.2.3.1 Culture and media change

Culture of human mesenchymal stem cells and mouse embryonic fibroblasts

The media of hMSCs and MEFs was replaced every other day. Both were cultured in 150 cm^2 tissue culture dishes (TPP) filled with 10 ml of the respective media.

Culture of human embryonic stem cells, v-iPSCs and p-iPSCs on MEF feeder cells

The human embryonic stem cell lines H1, v-iPSCs and p-iPSCs were cultured on mitotically inactivated mouse embryonic fibroblasts seeded the day before ($2{,}5x10^5$ inactivated MEFs per well of a 6-well plate seeded on gelatine coated culture dishes). The media was replaced every day and 4 ng/ml FGF2 was added every time. Human embryonic stem cells, v-iPSCs and p-iPSCs were cultured in 6-well tissue culture plates (TPP). The cells were cultured in 2,5 ml media per well of a 6-well tissue culture plate.

Inactivation of MEFs and preparation of feeder cell coated dishes

MEFs were cultured in 150 cm^2 tissue culture dishes until 80 % confluency was reached. The number of dishes depended on the necessary number of MEFs for the planned number of coated dishes.
The desired number of 6-well cell culture dishes were coated with 0,1 % gelatine (10-fold dilution of 1 % stock solution in PBS) and incubated at RT for at least 2 h.
Meanwhile, mitomycin C (Roche) was diluted in PBS (1 mg/ml) and filtered. The media from MEFs cultured in 150 cm^2 tissue culture dishes was aspirated. Subsequently, MEFs were washed with PBS. After that, 20 ml of MEF media, containing 10 µg/ml of mitomycin C was added to the MEFs. After 2 h incubation at 37 °C mitomycin C containing media was removed and cells were washed twice with PBS, trypsinised, centrifuged, counted and resuspended in warm media to plate them as feeder layer on the gelatine coated dishes or to resuspend them in freezing media for storage. Another possibility is to thaw already inactivated, cryopreserved MEFs and to seed these as feeder cells.

Culture of human embryonic stem cells under feeder-free conditions

hESCs were cultured on Matrigel®-coated plates in conditioned media using the same times for media change, culture dishes and media volumina. 4 ng/ml FGF2 was added when the conditioned media was changed leading to a total concentration of 8 ng/ml FGF2 in the media.

Preparation of Matrigel®-coated plates

Matrigel® (Becton Dickinson) was slowly thawed overnight at 4 °C (on ice) to avoid gel formation. On the next day, an adequate volume of cold Knockout™ DMEM was added to the 10 ml of Matrigel® and mixed well. Bottles containing Matrigel® and falcon tubes were kept on ice 1 ml aliquots were pipetted into each pre-chilled tube and stored at -20 °C. Before coating, 1 ml of Matrigel® aliquot was diluted in 14 ml of cold Knockout™ DMEM. 1,5 ml

of diluted Matrigel® solution was added to a single well of a 6-well plate and 1 ml was added to a single well of a 12 well-plate. Afterwards the plates were wrapped with parafilm and left overnight at 4 °C. The Matrigel® solution was removed immediately before plating human embryonic stem cells.

Preparation of conditioned media

Inactivated MEFs were plated at a density of $5{,}6x10^4$ cells/cm^2 and MEF media was replaced with hESC media (0,5 ml/cm^2) supplemented freshly with 4 ng/ml of FGF2. Conditioned media was collected from feeder flasks after 24 h of incubation and fresh hESC media containing 4 ng/ml of FGF2 was added to the feeder cells. This procedure was repeated for the next 6 days. Each day, collected conditioned media was placed at -20 °C. After 6 days the whole collected media was mixed together and filtered (Corning, 0,22 μm pore size). Subsequently, 50 ml aliquots were made and stored at -80 °C. Conditioned media was supplemented with additional 4 ng/ml of FGF2 before it was added to hESCs grown on Matrigel® or to hMSCs during reprogramming.

3.2.3.2 Passaging of the cells

Passaging of human mesenchymal stem cells and mouse embryonic fibroblasts

hMSCs and MEFs were split when subconfluency could be observed. Doing this, the cells were washed with 5-10 ml PBS depending on the size of the cell culture dish. 2,5-5 ml Trypsin/EDTA (1x) were added subsequently. This was followed by incubation at 37 °C and 5 % CO_2 until all cells detached. 2,5-5 ml culture media was put to neutralise the Trypsin. Subsequently, the cell suspension was transferred to a centrifuge tube with a conic end (cells of same genotype and passage coming from different dishes were merged). The cell suspension was spun down for 5 min at 500 g. The supernatant was taken off and the pellet was resuspended in an appropriated volume of culture media depending on the split ratio of the cell number.
hMSCs were split with a ratio of 1:3. MEFs were split in a ratio of 1:6.

Passaging of human embryonic stem cells, v-iPSCs and p-iPSCs on MEF feeder cells or cultured in feeder free conditions

Removal of differentiated cells and splitting were carried out under a HERAguard Clean Bench (Heraeus, Thermo Fischer Scientific Inc.) with a Leica MZ 95 Stereo Microscope placed in it.
One day prior to passaging, Matrigel®-coated plates or feeder cell coated plates were prepared to plate cells after splitting. In the feeder free condition the Matrigel® solution was removed and 2 ml of conditioned media (supplemented with FGF2) were added and plates were put into the incubator to warm. In case of feeder cell coated dishes the MEFs media was removed, the cells were washed with PBS and new ES cell media (supplemented with FGF2) was added. 80-100 % confluent hESCs, v-iPSCs or p-iPSCs colonies were cut manually into pieces of uniform size with a BD Microlance™ 3 injection needle (Becton Dickinson). Next, the media was aspirated and 1,5 ml of hESC media or conditioned media (supplemented with FGF2) were added to the cells. The cut pieces were gently removed with a cell scraper and

the cell clumps were resuspended. Each 500 µl of the suspension were transferred to a coated well filled with the respective fresh media. The plates were returned into the incubator and slowly agitated left to right and back to front to obtain an even distribution of cells. The day after seeding, the undifferentiated cells were visible as small colonies, with subsequent culture these colonies became large and compact. In this way, the cells were split with a ratio of 1:3 once per week.

Morphological distinction between hESCs and differentiated cells

Due to high nuclear cytoplasmic ratio, the hESC colonies form ordered, flat, tight colonies with sharp borders, with low light scattering properties while differentiated colonies are irregular with uneven edges or transparent centers and exhibit high light-scattering property. The differentiated cells are bigger than undifferentiated cells due to a reduced nuclear-cytoplasmic ratio.

3.2.3.3 Freezing of cells

Freezing of human mesenchymal stem cells and mouse embryonic fibroblasts

hMSCs or MEFs were washed with 5-10 ml PBS according to the size of the culture dish. The cells were trypsinised as previously described. After the centrifugation step, the pellet was resuspended in hMSC or MEF freezing media. Afterwards, the suspension was shared to different cryovials, put into a freezing container (Nalgene) and placed immediately at -80 °C overnight. The next day, vials were transferred to liquid nitrogen for long-term storage.

Freezing of human embryonic stem cells, v-iPSCs and p-iPSCs

To freeze human embryonic stem cells, v-iPSCs and p-iPSCs colonies were manually cut as in the splitting procedure, gently scraped and resuspended. The suspension was spun down for 5 min at 500 g. The pellet was resuspended in PBS and spun down. Subsequently, the pelleted cell clumps were resuspended in hESC freezing media and transferred into a cryovial. The cryovials were put into a freezing container (Nalgene) and placed immediately at -80 °C overnight. The next day vials were transferred to liquid nitrogen for long-term storage.

3.2.3.4 Thawing of cells

Thawing of human mesenchymal stem cells and mouse embryonic fibroblasts

The vial containing frozen cells was removed from the liquid nitrogen and quickly defrosted using a 37 °C warm water bath. Afterwards, 70 % ethanol was used to sterilise the cryovial and the vial was allowed to air dry before opening. The content of the vial was transferred into 10 ml pre-warmed hMSCs media in a 15 ml centrifuge tube. Subsequently, the suspension was spun down at 500 g, 4 ºC, 5 min. The supernatant was removed and cell pellet was resuspended in the respective amount of media to split the cell number with a ratio of 1:6 in the case of MEFs or to split with a ratio of 1:3 in the case of hMSCs. Finally, the appropriate

part of the cell suspension was transferred to a 150 cm^2 tissue culture dish containing fresh media.

Thawing of human embryonic stem cells, v-iPSCs and p-iPSCs

A feeder cell coated plate was prepared the day before thawing. Doing this, $2{,}5x10^5$ inactivated MEFs per well of 6-well plate were seeded on gelatine coated dishes and allowed to attach and grow until the morning of the next day. Then, the MEF media was replaced with ES cell media supplemented with 4 ng/ml of FGF2.
The cryovial containing the cells was removed from the liquid nitrogen storage tank and thawed quickly in a 37° C water bath. The cells were added into a 15 ml centrifuge tube containing 10 ml of pre-warmed ES cell media. The cells were briefly centrifuged, resuspended in 2 ml of warm ES cell media and added gently to a prepared feeder cell coated cell culture plate. The content of one cryovial was plated into one well of a 6-well plate. To support the survival of the thawed cells 10 µM Rho-associated kinase (ROCK) inhibitor Y-27632 (1:1.000 of 10 mM stock solution) was added to the media (Watanabe et al., 2007).

3.2.4 in vitro adipocyte differentiation

$1x10^5$ hMSCs were plated per well of a 6-well tissue culture plate. Three wells of a 6-well plate were used per experimental condition "treated" and for the condition "control" to analyse a triplicate. hMSCs were cultured in 2,5 ml culture media for mesenchymal stem cells until confluency could be observed. The day of confluency was defined as day zero of the differentiation experiment. From day zero the cells of three wells were cultured in adipocyte differentiation media. hMSCs of three wells of the culture dish were grown in control media. On day 21 of the treatment the cells were fixed and Oil Red O staining was performed.

3.2.5 in vitro osteoblast differentiation

Differentiation of hMSCs to osteoblasts was achieved by plating $1x10^5$ hMSCs per well of a 6-well tissue culture plate with uncoated surface. Like in the *in vitro* adipocyte differentiation protocol, three wells of a 6-well plate were plated per experimental condition to analyse a triplicate. hMSCs were cultured in 2,5 ml culture media for mesenchymal stem cells until the cells were confluent. From the day of confluency the cells of three wells were cultured in osteoblast differentiation media. Confluent hMSCs in the other three wells of the culture dish were grown in control media. On day 21 of the treatment Alizarin Red S staining was performed.

3.2.6 Staining to detect adipocyte and osteoblast differentiation

3.2.6.1 Oil Red O staining

Oil Red O is a lipophilic dye that stains the triglycerides and lipides in the fat vacuoles of adipocytes in a red colour. It was used to visualise the differentiated adipocytes after *in vitro* adipocyte differentiation was completed.
First, the culture media was removed and the cells were washed twice in PBS. The cells were left to dry for 30 min at RT and were fixed in PBS containing 4 % PFA over night at 4 ºC.

Afterwards, the staining solution was aspirated and 2 ml 100 % propylene glycol were added and left on the fixed cells for 5 min.
Meanwhile, the staining solution was prepared. Subsequently propylene glycol was poured off and 3 ml of filtered Oil Red O solution diluted in water was added to each well of the 6 wells tissue culture plate. This was followed by an incubation of 4 h at RT and 5 min incubation in 85 % propylene glycol after the staining solution was poured off. In the last step, the stained cells were washed carefully with distilled water. The stained tissue culture plates were left to dry and stored at 4 ºC.

3.2.6.2 Alizarin Red S staining

After the *in vitro* experiments, differentiated Osteoblasts were visualised with Alizarin Red S, a dye that stains the mineralised bone matrix in red.
The cells were washed with PBS and subsequently fixed overnight using 4 % paraformaldehyde in PBS. The fixed cells were washed several times with PBS before 1 ml of a 2 % Alizarin Red S staining solution was added to the cells for 5 min. After this, excess dye was washed away gently with water. Finally, 1 ml of PBS was added per well of a 6-well plate and the stained cells were kept at 4° C.

3.2.6.3 Documentation of the staining

After Oil Red O or Alizarin Red S staining were performed, pictures were taken using a Zeiss, LSM 510 Meta microscope with a connected camera for microscopy model AxioCam ICc3 and the software Axiovision 4.6. The pictures were taken at bright field conditions under halogen light with a magnification of 5- to 10-fold.
Pictures of Alizarin Red S staining were taken partly using an inverted microscope model CK2 (Olympus). The pictures (4.000 x 3.000 pixel; 180 dpi) were taken through the ocular of the microscope at a 4-fold magnification. With a digital camera (Canon model Power shot A650IS).

3.2.7 Analysis of Nucleic Acids

3.2.7.1 Isolation of genomic DNA

Genomic DNA was isolated using the FlexiGene DNA Kit (Qiagen) according to the protocol for isolation of genomic DNA from cultured cells provided by the manufacturer.
To isolate genomic DNA 300 µl of buffer FG2 (denaturation buffer) were mixed with 3 µl of Quiagen protease one hour before use. The hMSC were trypsinised as described in the section cell culture and pelleted at low speed with a centrifuge. The pellet was resuspended and the cell number was determined using Neubauer Counting Chamber. The appropriate amount of volume for $2x10^6$ cells was transferred into a separate 1,5 ml micro tube and pelleted at 300 g. The supernatant was removed carefully. Subsequently, 300 µl buffer FG1 were used to resuspend the pellet. Afterwards, 300 µl buffer FG2/QIAGEN Protease preheated to 65 °C were added to the suspension and the mix was incubated in a heating block at 65°C for

10 min. After this step, 600 µl 2-propanol (100 %) were added and the solution was mixed thoroughly by inversion until the DNA precipitated and became visible. The solution was centrifuged for 3 min at 10.000 x g. The supernatant was removed and the tube was inverted and put onto a piece of absorbent paper. The DNA could be seen as a small white pellet at this step. At this point, 600 µl 70 % ethanol were added and the mix was vortexed for 5 s followed by a centrifugation for 3 min at 10.000 x g. The supernatant was discarded and the tube was left to dry on a clean piece of absorbent paper for at least 5 min. In the next step 200 µl buffer FG3 were added to the pellet and the suspension was vortexed for 5 s at low speed followed by an incubation for 30 min at 65 °C in a heating block. The solution containing the dissolved DNA was stored at -80 °C. The DNA quality and content was measured using a spectrophotometer type NanoDrop® ND-1.000.

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3.2.7.2 Polymerase Chain Reaction

PCR reactions were used to perform DNA fingerprinting of p-iPSCs and to detect the oriP sequence of the episomal plasmid pEP4 E02S ET2K.

Reaction mix for one DNA sample:

2,5 µl	10x-buffer (see below)
0,2 µl	dNTP-Mix (dATP, dCTP, dGTP, dTTP each 25 mM)
2,5 µl	25mM $MgCl_2$
0,25 µl	100µM forward primer
0,25 µl	100µM reverse primer
0,4 µl	Taq DNA Polymerase (Invitrogen)
0,1 µl	PFU Polymerase (Promega)
1 µl	DNA
ad 25 µl	$_{dd}H_2O$

10x-buffer	500 mM Tris-Cl pH 8,8
	200 mM $(NH_4)_2SO_4$
	15 mM $MgCl_2$
	0,1 % Tween 20

The DNA fragments amplified by PCR using the primer for oriP have the size 544 bp.
The DNA fragments amplified by PCR using the primer for D10S1214 for the DNA fingerprint have a size below 250 bp depending on the variable number of tandem repeat in the genome of the analysed cells.

Table 4 Thermocycler programmes used for DNA fingerprinting and oriP PCR

PCR to analyse	DNA fingerprint (D10S1214)	oriP
Initial denaturation	94 °C for 2 min 30 s	94 °C for 5 min
Cycles	35	32
Denaturation	94 °C 30 s	94 °C for 15 s
Primer annealing	56 °C 45 s	55 °C for 30 s
Primer extension	65 °C for 2,5 min	68 °C for 1 min
Final extension	65 °C for 10 min	68 °C for 7 min
Final hold	4 °C	4 °C

The PCR was carried out using a Thermocycler type PTC100 (MJ Research Inc).

3.2.7.3 Agarose gel electrophoresis

Gel preparation

For the preparation of an agarose-gel (1 to 3 % gels were used in this work) the appropriate amount of agarose was dissolved in 200 ml 1xTAE buffer. The solution was boiled using a microwave until it got clear. Subsequently, the solution was left to cool for several minutes and 5 µl ethidium bromide were added. The solution was mixed and subsequently poured into an agarose gel tray. Finally, gel combs with the appropriated number of teeth and size of the teeth were put into the liquid gel solution.

Electrophoresis and gel documentation

All PCR products were analysed by agarose gel electrophoresis as well as the isolated RNA to examine the RNA quality.
6x loading buffer was added to each sample and the agarose gel was loaded with the sample mix. 1x TAE buffer was used as separation buffer per gel tank. The length of the amplicons was specified according to the Perfect Plus 1 kb DNA ladder (Roboklon). The gels were run at 90 V for 30 min or longer depending on the size of the amplicon and the pore size of the agarose gel. The nucleic acids were visualised with UV light using the AlphaImager™ (Alpha Innotech).

3.2.7.4 RNA isolation

RNA isolation was performed using the RNeasy® Mini Kit (Qiagen) following the manufacturer's instructions, including the optional step of DNase I treatment of the samples on the

column using the RNase-Free DNAse Set (Quiagen) to get rid of trace amounts of genomic DNA.
To isolate the RNA, the cells were briefly rinsed with pre-warmed PBS (37 °C). RLT lysis buffer (highly denaturing guanidine-thiocyanate-containing buffer) to which β-mercaptoethanol was added (10 µl per 1 ml RLT buffer) prior to use was pipetted on the cells. After this, the cells were scraped out with a cell scraper and collected in a 1,5 ml microcentrifuge tube, followed by vortexing for 1 minute to ensure proper cell lysis. For further homogenisation the lysate was passed through a 20-gauge (0,9 mm) BD Microlance™ 3 injection needle (Becton Dickinson) attached to a sterile plastic syringe for 10 times. Subsequently, the lysates were transferred to the RNeasy®-columns. 80 µl DNAse I incubation mix (70 µl Buffer RDD mixed with 10 µl DNAse I stock solution) from the RNase-Free DNAse Set were added directly to the RNeasy® spin column membrane. The solution was incubated for 15 min at RT. Further RNA isolation was carried out according to the manufacturer's instructions. Finally, the RNA was eluted into the RNAse free tubes provided with the kit using two elutions of 15 µl of RNAse-free sterile water out of which 1 µl was used for quality check by agarose gel electrophoresis and photometric quantification with a spectrophotometer type NanoDrop® ND-1.000.

3.2.7.5 Measurement of DNA and RNA concentration

The quantity and quality of RNA and genomic DNA was analysed with a spectrophotometer type NanoDrop® ND-1.000. 1 µl of the respective sample was applied to the device and measured. If the concentrations are outside measurable values the samples were either concentrated by speedVac centrifugation using a Savant SPD131DDA SpeedVac concentrator or diluted by adding RNAse-free water.

3.2.7.6 Transcription of RNA to cDNA

2 µg of RNA and Oligo dT Primer (1 µg/µl; Invitek) were mixed and topped up with $_{dd}H_2O$ to a volume of 15 µl. This solution was incubated for 3 min at 70 °C, cooled on ice and supplemented with a master mix consisting of following components:

5 µl	5x-reaction buffer (Promega)
0,5 µl	dNTP-Mix (dATP, dCTP, dGTP, dTTP each 25 mM)
0,1 µl	MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (200 U/µl, USB)
9,4 µl	$_{dd}H_2O$

After 1 hour incubation at 42 °C the reaction was stopped at 65 °C for 10 min.
The incubation was carried out using a Thermocycler PTC100 (MJ Research Inc).

3.2.7.7 Real time PCR

The cDNA prepared from 2 µg of total RNA was diluted at the rate of 1:8 with $_{dd}H_2O$ and used for real time PCR, which was performed in 96-well Optical Reaction Plates (Applied Biosystems).

Reaction mix for one cDNA sample:

5 µl	SYBR®Green PCR Master Mix (Applied Biosystems)
1 µl	each of the forward and reverse primers (5 pmol/µl)
3 µl	diluted cDNA

The amplifications were carried out in triplicates per gene with three wells as negative controls without template. *GAPDH* was amplified along with the target genes as endogenous control for normalization. The PCR reaction was carried out on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using the following program:

stage 1: 50 °C for 2 min
stage 2: 95 °C for 10 min
stage 3: 95 °C for 15 s and 60 °C for 1 min, for 40 cycles
stage 4: 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s

The last heating step in stage 4 was performed with a ramp rate of 2 % in order to generate a dissociation curve of the PCR product. The output data generated by the SDS 2 software (Applied Biosystems) were transferred to MS Excel (Microsoft) for analysis. The differential mRNA expression of each gene was normalised against the *GAPDH* mRNA expression in the respective samples and calculated using the comparative C_t (threshold cycle) method ($\Delta\Delta C_t$ method). The fold change of the measured genes in hMSCs over the control (embryonic stem cell line H1) was calculated as follows:

$\Delta CT = CT_{gene} - CT_{GAPDH}$
$\Delta\Delta CT = \Delta C_{hMSC} - \Delta CT_{control}$
Fold Change = $2^{-\Delta\Delta CT}$

3.2.7.8 Microarray based gene expression analysis

The Illumina HumanHT-12 v3 Expression BeadChip was used for microarray-based gene expression analysis of MSC17, MSC23 and MSC74. This chip consists of 12 arrays that contain more than 48.000 probes targeting mRNAs of more than 25.000 genes. The probes were generated using the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) and UniGene databases. The transcription of genes of the whole genome can be detected with a high coverage due to the design of the chip. The probes are fixed to the surface of beads in cavities of the array surface on the chip. The probes, are short DNA sequences immobilised along with a high number of probes with the same sequence. The analysed RNA is first transcribed into cRNA followed by incorporation of biotin-labeled nucleotides in the next step before it is hybridised on the chip. The fluorescence emission of Cy3 is

quantitatively detected and the average signal for each gene associated probe is used for further gene expression analysis.

Illumina BeadChip hybridisation

The hybridisaton of the RNA to the chip was performed by Aydah Sabah in the Automation group of the Max Planck Institute for Molecular Genetics.
To analyse the RNA samples with the BeadChip, biotin-labelled cRNA was produced by means of a linear amplification kit (Ambion) using 500 ng of quality-checked total RNA as input. Chip hybridisations, washing, Cy3-streptavidin staining, and scanning were performed on an Illumina BeadStation 500 platform (Illumina) using reagents and following protocols supplied by the manufacturer. cRNA samples of hMSCs were hybridised on Illumina HumanHT-12 v3 Expression BeadChips. One sample of each hMSC line (MSC17, MSC23 and MSC74) was hybridised on the chip and analysed.

Analysis of the gene expression data

Basic expression data analysis

All basic expression data analysis was carried out using the manufacturer's software BeadStudio (Illumina). Raw data was background-subtracted and normalised. Normalised data were then filtered for significant expression on the basis of negative control beads. Gene lists with the average signals measured by the Illumina BeadStation 500 platform as well as detection p-values were transferred to an MS Excel table using BeadStudio. The cells of this table were conditionally formatted. All genes with a detection p-value below 0,1 were considered as expressed. All cells containing an expression p-value below 0,01 were formatted to appear in red to distinguish the genes that are expressed from those that are not detected as expressed. Genes with a detection p-value, greater than 0,01 (not detected) in all samples were removed.
The selection for differentially expressed genes was performed on the basis of chosen thresholds for fold changes plus statistical significance according to an Illumina custom model (Kuhn et al., 2004). All genes with a differential p-value above 0,05 were not considered as differentially expressed. In order to mark the differentially expressed genes in the MS Excel table, conditional formatting was performed in this case as well. All cells with differential p-values (calculated by the software BeadStudio for the respective gene in the sample) below 0,01 were marked in dark blue and those with a differential p-value between 0,01 and 0,05 marked in light blue. According to this colour code significantly expressed genes that have a differential p-value below 0,05 could be selected and the data of these genes could be analysed further.

Analysis of global gene expression similarities

In order to test for global gene expression similarities within the hybridised hMSC samples, pair wise Pearson correlation coefficients were calculated and based on this a cluster analysis was performed with the software BeadStudio.
Moreover, dot plot graphs depicting the correlation of the gene expression of MSC17 and MSC23, MSC17 and MSC74 as well as MSC23 and MSC74 were generated with BeadStu-

dio. Furthermore, gene lists containing only genes with a detection p-value below 0,01 for all three analysed hMSC RNA samples were uploaded to an online tool for generation of Venn diagrams called VENNY. In this way a Venn diagram was generated depicting the number of overlapping genes between all combinations of two samples, between the three samples and numbers of genes that are only expressed in one of the samples.

Analysis of expression of MSC marker genes in all three samples

A list of genes that are commonly expressed in all hMSC lines was generated with the software BeadStudio. Using this list, it was determined, whether the MSC related marker genes *NT5E (CD73), THY1(CD90), ENG (CD105), ALCAM (CD166)* and *CD44* are contained.

Analysis of up- and down-regulated genes

The average signals measured for the genes of two different samples were compared and the ratio of both gave the fold change of up- or down-regulation. The ratio or fold change was calculated with MS Excel. The cells in the table were formatted in order to generate lists of up- or down-regulated genes. Doing this, all cells with a ratio above 1,5, meaning 1,5-fold up-regulation, were marked red. All cells with a ration between 1,5 and 0,66 were marked grey. The associated genes are considered as not regulated. Finally, cells with a ratio below 0,66 are marked green, visualizing down-regulated genes. This colour code was used to extract lists of genes that are up- or down-regulated with age in hMSCs comparing MSC17 and MSC74.

Analysis of age dependend up-or down-regulated pathways

The lists of genes that are up-or down-regulated were entered into the DAVID functional annotation tool with the official gene symbol as input. DAVID stands for annotation, visualization and integrated discovery (http://david.abcc.ncifcrf.gov).
Groups of genes associated with specific pathways, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and on the BIOCARTA annotation of genes, were analysed together to assess age dependent pathway regulation in hMSCs. The pathway annotations cell cycle and p53 signaling were chosen and analysed further by mapping the regulated genes to the respective assigned KEGG pathways and by plotting the log2 ratio (MSC74/MSC17) of the average signals of the detected age regulated genes. In the plots genes with a differentiation p-value below 0,01 were marked with an asterisk.

3.2.8 Reprogramming of human mesenchymal stem cells

3.2.8.1 Course of events during the reprogramming experiments

An overview of the course of events during the reprogramming experiments is depicted in Figure 3.
To reprogramm hMSCs retroviruses were produced and subsequently tested in preliminary experiments before retroviral reprogramming. For viral reprogramming MSC17 and MSC74

cells were infected with the produced retroviruses and additionally treated with and without inhibitors. The resulting reprogrammed cells were called v-iPSCs.
To reprogramm hMSCs without viruses, episomal plasmids carrying reprogramming factors were used. The protein expression of the transgenes on the episomal plasmid pEP4 E02S ET2K was analysed in a preliminary experiment. The reprogramming was started with nucleofection of the episomal plasmids into the MSC74. During non viral reprogramming the nucleofected cells were additionally treated with and without inhibitors. The isolated reprogrammed cells were called p-iPSCs.
Both v-iPSCs and p-iPSCs were characterised towards their morphological similarity to the embryonic stem cell line H1 and towards their expression of alkaline phosphatase and *NANOG*. Moreover, p-iPSCs were characterised towards their expression of pluripotency associated factors such as *OCT4*, *SOX2*, *KLF4*, *c-Myc* as well as towards their expression of pluripotency associated surface antigens such as SSEA-4, SSEA-1, TRA-1-60 and TRA-1-81. Further characterisation included DNA fingerprinting, analysis and assessment of pluripotency by embryoid body based differentiation in cells of endoderm, ectoderm and mesoderm. Furthermore, a PCR for oriP on the episomal plasmid ET2K was performed to confirm deprivation of the plasmid. Finally, the ability of p-iPSCs to differentiate into adipocytes and osteoblasts was assessed with *in vitro* differentiation experiments and Oil red O as well as Alizarin Red S staining.

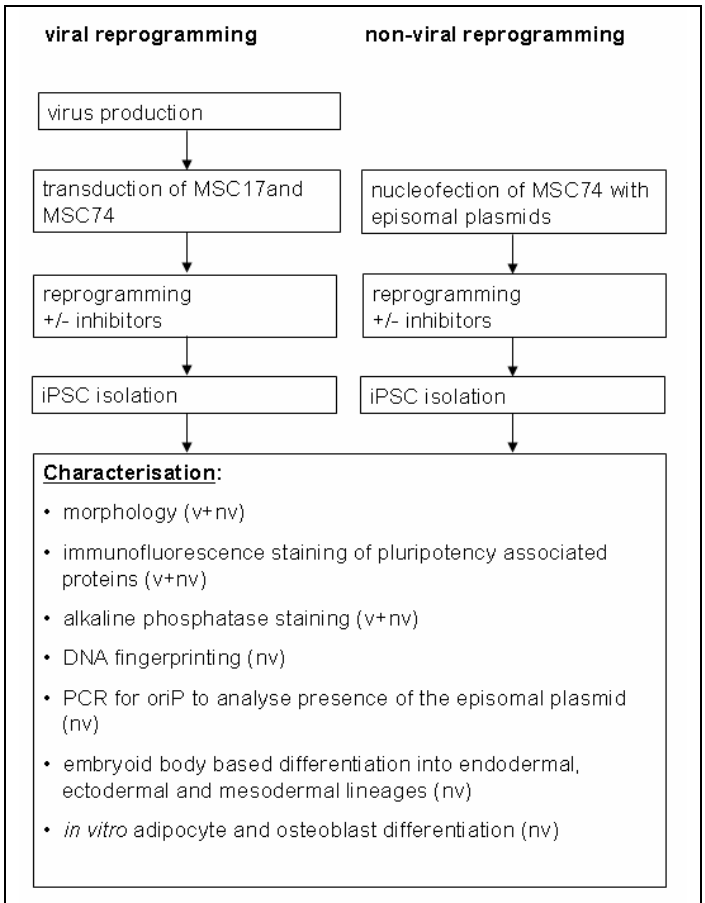


Figure 3 Overview of procedures performed in the viral and non viral reprogramming experiments as well as for the characterisation of the reprogrammed cells. v: cells reprogrammed with viruses, nv: cells reprogrammed with episomal plasmids

3.2.8.2 Retrovirus production

The virus production was performed in a separate laboratory of the security level S2.
HEK293T cells were plated at a density of $8x10^6$ cells per 150 mm tissue culture dish 24 h before the transfection. To expand HEK293T cells it is important that the cells are split when 70 % confluency is reached. The cells were cultured in hMSC media. Two hours before the transfection the media was changed to HEK293T media.
The plasmid DNA mix to transfect the cells was prepared by adding

9 µg of plasmid pCMV-VSV-G (encoding proteins of the virus envelope)
20 µg plasmid pUMVC3-gag-pol (encoding proteins for viral packaging)
32 µg of plasmid pMXs-hOCT3/4, pMXs-hSOX2, pMXs-hKLF4, pMXs-hc-Myc or pBABE GFP.

Subsequently the plasmid solution was made up to a final volume of 1125 µl in $_{dd}H_2O$. At this point 125 µl of 2,5 M $CaCl_2$ were added to each mix followed by an incubation at RT for 5 min. To form precipitates for transfection 1250 µl of 2x HBS solution was added drop wise to the 1250 µl plasmid–$CaCl_2$ mixture while vortexing at full speed. Following the addition of 2x HBS, the mixture was added to the HEK293T cells immediately. The calcium phosphate precipitated plasmid DNA was allowed to stay on the cells for 14 h after which the media was replaced with fresh HEK293T media. The cell supernatant was collected 24 h and 48 h after the media change followed by filtering with a 0,45 µm pore size syringe driven filter (Durapore). The filtered supernatant was transferred into Polyallomer centrifugation tubes (Beckman). Subsequently the cell supernatant was centrifuged at 20.000 rpm for 2 h at 4 °C at vacuum in a Beckman L7 Ultracentrifuge with a rotor type SW-28. Afterwards, the supernatant was aspirated and the pellets were left to dry on tissue paper for 20 min. During this time the rotor and holders were bleached. The dried pellets were resuspended in Knockout™ DMEM adding 400 µl to bigger pellets and 200 µl to smaller pellets. After the addition of Knockout™ DMEM the solution was not vortexed but incubated at 4 °C overnight covered with Parafilm. On the next day the suspension was pipetted slowly up and down followed by the preparation of aliquots which were stored at -80 °C.

3.2.8.3 Reprogramming using retroviruses

Retrovirus infection

To reprogram them, hMSCs were infected with retroviruses containing plasmids encoding *OCT4*, *SOX2*, *KLF4* or *c-Myc*. The retroviral infection was performed in a separate laboratory of the security level S2.
First, hMSCs were seeded at a density of $2,5x10^5$ cells per well of a 6-well plate one day prior to the infection. The cells were cultured over night in hMSC media. To infect the cells 10 µl each of an aliquot of the retroviruses encoding *OCT4*, *SOX2*, *KLF4* and *c-Myc* were added to 1 ml of hMSC media. Afterwards, 4 µg/ml Polybrene were added to the mix (1:1.000 dilution of 4 mg/ml stock solution). The cells were incubated at 37 °C in the virus containing mix followed by another infection after 24 h. The amount of virus used for the

next infection depended on the appearance of the infected cells. When the cells were in an acceptable state, another 10 µl virus aliquot were used, otherwise half of the amount of virus was used. For MSC74 half the amount was used for the second infection. Following the second infection, the cells were again incubated for 24 h and split with a ratio of 1:3 using trypsin as previously described. The third part of the infected cells of one well was seeded each on feeder cell coated well of a 6-well plate and cultured further in hESC media.
Retroviral infection and reprogramming was carried out with the hMSC lines MSC17 and MSC74 in the same way. However, MSC17 were seeded in addition to the described density of $2{,}5x10^5$ at a density of $4x10^5$ to determine the effect of the plating density prior to the infection.

Monitoring of the infection efficiency

MSC17 and MSC74 were infected in the described way with retroviruses containing the plasmid pBABE-GFP to estimate the efficiency of the retroviral infections during retroviral reprogramming. The cells were infected once and the expression of GFP was detected using a Zeiss, LSM 510 Meta confocal microscope on the next day.

Verification of the transgene expression after retroviral infection

MSC17 cells were infected with retroviruses carrying either *OCT4*, *SOX2*, *KLF4* or *c-Myc* as described. One day after infection the media was changed and the proteins expressed by the transgenes were visualised using immunofluorescence and a Zeiss, LSM 510 Meta confocal microscope.

Reprogramming of the cells

The chronological course of the procedures during retroviral reprogramming is depicted in Figure 4.
The day the infected hMSCs were seeded on feeder cell coated plates was defined as day zero of the reprogramming experiment. The cells were cultured until day seven of the experiment in hESC media with 4 ng/ml FGF2 and from day seven conditioned media was used and supplemented with 4 ng/ml FGF2 to a total of 8 ng/ml FGF2 prior to media change. During reprogramming the media was changed every day. 2 ml media were used for one well of a 6-well plate. Colonies of putative reprogrammed cells were isolated at day 40 in both reprogramming experiments.
During the retroviral reprogramming experiments it was tested to enhance the efficiency of the iPSC derivation from hMSCs by adding the MEK inhibitor PD325901 and an inhibitor of the TGFβ receptors ALK4, ALK 5 and ALK 7 SB431542 in combination (experimental condition 2i) or the p53 inhibitor Pifithrin α (experimental condition p53) to the culture media. A combination of PD325901, SB431542 and Pifithrin α was tested as well (experimental condition p53+2i). Both inhibition of p53 and the combination of PD325901 and SB431542 are known to enhance the efficiency of iPSC generation from human fibroblasts (Hong et al., 2009; Lin et al., 2009).
The inhibitors were applied in the following concentrations: PD325901: 0,5 µM (1:20.000 dilution of 10 mM stock solution), SB431542: 2 µM (1:5.000 dilution of 10 mM stock solu-

tion) and Pifithrin α: 10 µM (1:1.000 dilution of the 10 mM stock solution). The inhibitors were stored at -20 °C as aliquots and thawed directly before use.

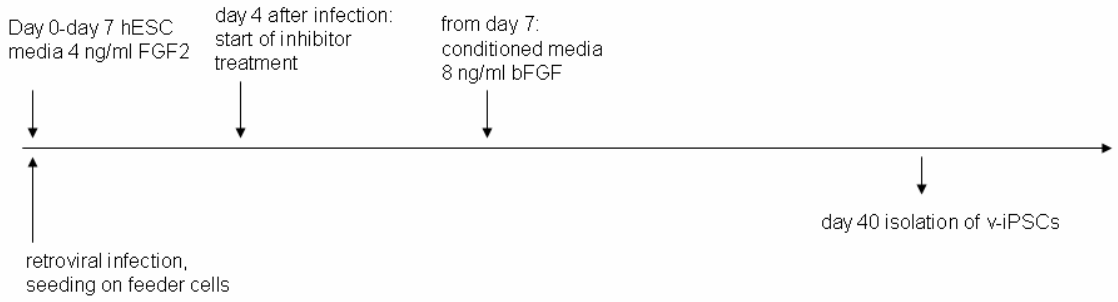


Figure 4 Course of events during retroviral infection

3.2.8.4 Non viral reprogramming

Nucleofection of hMSCs with episomal plasmids

The episomal plasmids were delivered to MSC74 cells using the Human MSC (Mesenchymal Stem Cell) Nucleofector® Kit (Lonza Cologne AG) and the Nucleofector® Device (Lonza Cologne AG) according to the manufacturer's protocol.
This transfection method delivers DNA into cells by electroporation.
The MSC74 cell line was in passage four when the reprogramming experiments were performed. MSC74 cells were expanded until four 150 cm^2 cell culture dishes were 80 % confluent.The cells were trypsinised. The cell pellet was resuspended in 1 ml of hMSC media and counted using a Neubauer Counting Chamber. The plasmid DNA and cells were mixed before nucleofection by combining

-10 µg of the episomal plasmid in case only one plasmid was used, or 5 µg each when two episomal plasmids were used.
-100 µl Human MSC Nucleofactor solution (solution has to be at room temperature)
-the respective amount of volume containing $1x10^6$ MSC74 cells

The solutions were mixed in a 1,5 ml reaction tube and transferred into an Amaxa certified cuvette with a plastic pipette. It is important that the cells do not stay more than 15 min in the human MSC nucleofactor solution and that no air bubbles appear between the two electrodes in the cuvette during the transfer.
The cuvette was closed and placed into the Nucleofector® device. For nucleofection of the episomal plasmids into the hMSC the program C-17 for high cell survival was chosen on the Nucleofector® device and the start button was pressed. After the nucleofection, the mix in the cuvette was immediately transferred to a previously prepared 150 cm^2 tissue culture dish. The dishes were either uncoated, coated with Matrigel® or coated with feeder cells. The culture dishes contained 15 ml media. Depending on the coating of the dish, conditioned media, ES cell media or hMSC media was used. The cuvette was rinsed with culture media to obtain the maximum amount of nucleofected cells.

Monitoring of the nucleofection efficiency

To monitor the nucleofection efficiency, 1 million MSC74 cells were transfected with the supplied plasmid pmaxGFP which encodes green fluorescent protein. The respective volume for 5 µg plasmid was used and nucleofected into the cells as described. The expression of GFP was monitored using the Zeiss, LSM 510 Meta confocal microscope.

Verification of transgene expression on plasmid pEP4 E02S ET2K

$1x10^6$ MSC74 cells were nucleofected with the episomal plasmid as described. After the nucleofection, $1x10^5$ cells were seeded per well of a 12 well plate to be able to stain them separately. The protein expression of the transgenes *OCT4*, *SOX2* and *KLF4* on the plasmid was assessed by immunofluorescence using the respective antibodies and the Zeiss, LSM 510 Meta confocal microscope.

Non viral reprogramming of the cells

$1x10^6$ MSC74 cells were nucleofected with a total amount of 10 µg of either the plasmid pEP4 E02S ET2K (ET2K), pEP4 E02S EM2K (EM2K) or a combination of both. ET2K carries the genes *OCT4*, *KLF4*, *SOX2* and *SV40LT*. EM2K is harbouring the same genes whereas *SV40LT* is replaced by *c-Myc*.
Eight different conditions were used in the non viral reprogramming experiments using different parameters for dish coating, episomal plasmids and associated transgenes as well as culture in the presence of inhibitors. The different experimental conditions are summarised in Table 5.
The course of events for the non viral reprogramming experiments is depicted in Figure 5. Nucleofected hMSCs were seeded on feeder cells, on Matrigel® or on an uncoated surface in 150 mm^2 cell culture dishes. The nucleofected cells which were seeded on an uncoated surface were trypsinised after seven days and subsequently seeded on a feeder cell coated dish. When the cells were seeded on feeder cells, hESC media with 4 ng/ml FGF2 was used for seven days for culture. After seven days the media type was switched to conditioned media supplemented with 8 ng/ml FGF2 as previously described. In the experimental conditions where the nucleofected cells were plated on Matrigel® coated plates, the cell culture was immediately carried out with conditioned media. Two inhibitors, SB431542 and PD325901, were applied which were described to enhance the efficiency of iPSC generation from fibroblasts (Lin et al., 2009). The inhibitors were applied in the same concentrations as for viral reprogramming. The nucleofected cells were treated with the combination of both inhibitors. During the reprogramming experiment the media was changed every other day.

Table 5 Experimental conditions of reprogramming experiment with episomal plasmids carried out with MSC74. ET2K: pEP4 E02S ET2K, EM2K: pEP4 E02S EM2K, O: *OCT4*, S: *SOX2*, K: *KLF4*, M: c-Myc; 2i: treatment with 2 µM SB431542 (inhibitor of ALK 4/5 and 7) and 0,5 µM PD325901 (inhibitor of MEK)

Condition	*Plasmid name*	*Genes on the plasmid*	*Amount of plasmid used in µg*	*2i*	*Seeded on*
1	ET2K+EM2K	O, K, S, SV40LT+O, S, M, K	5 each	+	Feeder cells
2	ET2K	O, K, S, SV40LT	10	+	Feedercells
3	ET2K	O, K, S, SV40LT	10	-	Matrigel®
4	EM2K	O, S, M, K	10	-	Matrigel®
5	ET2K	O, K, S, SV40LT	10	+	Matrigel®
6	ET2K	O, K, S, SV40LT	10	-	Matrigel®
7	ET2K	O, K, S, SV40LT	10	-	uncoated (replated on feeder cells after 7 days)
8	ET2K	O, K, S, SV40LT	10	+	uncoated (replated on feeder cells after 7 days)

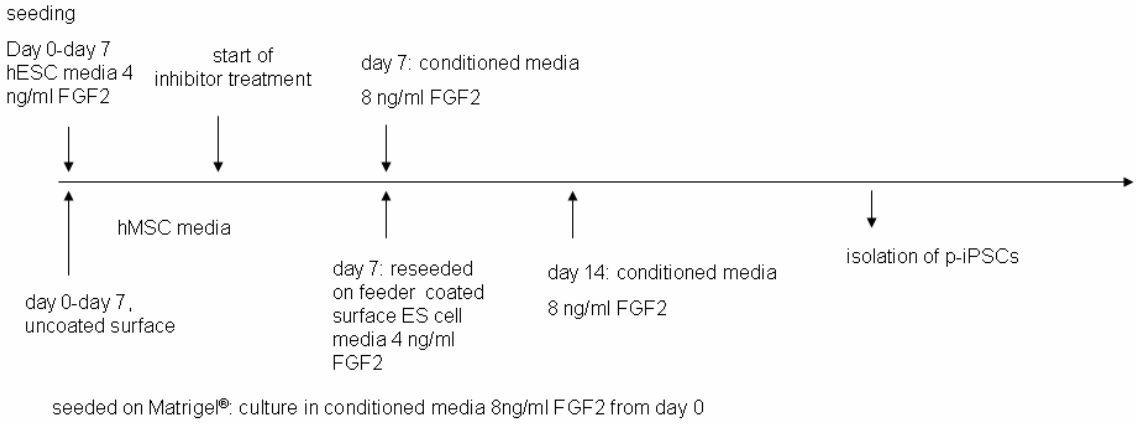


Figure 5 Course of events during non viral reprogramming experiments conducted with MSC74. Above the horizontal arrow: events when nucleofected cells were seeded on feeder cells. Below the horizontal arrow: when nucelofected cells were seeded on uncoated dishes. Inhibitor treatment was started on day four after the nucleofection.

3.2.8.5 Isolation of reprogrammed cells and derivation of cell lines

During the course of the reprogramming experiments the morphology of the cells was monitored. Colonies consisting of cells with a different morphology than the parental hMSCs, like high nucleus cytoplasm ratio, were isolated.

The isolation was carried out under a HERAguard R Clean Bench (Heraeus, Thermo Fischer Scientific Inc.) with a Leica MZ 95 Stereo Microscope placed in it. Similar to splitting of human embryonic stem cells, 6-well plates coated with feeder cells were prepared the day before isolation. To isolate the colonies of reprogrammed cells, a circle has been cut around the cell colony with a BD Microlance™ 3 injection needle (Becton Dickinson). Bigger colo-

nies were cut in four pieces. The colony pieces were transferred into one well of a 6-well of the new prepared feeder cell coated plate. The plate was put gently into the incubator and the cells were left to attach. Attachment could be observed already on the next day. Attached colony pieces were cultured further and split every week as described for hESCs. The colonies of v-iPCS and p-iPSCs were cultured until passage six to obtain stable cell lines.

3.2.9 Characterisation of the reprogrammed cells

3.2.9.1 DNA fingerprinting analysis

DNA fingerprinting was performed to analyse the relatedness of the generated p-iPSC cell lines to the parental MSC74 cells and to rule out the possibility that p-iPSCs are the product of a contamination with the embryonic stem cell lines H1 and H9 as well as the fibroblast line HFF.
The DNA fingerprint was generated by isolating the genomic DNA of the cells (see section Isolation of genomic DNA) and performing a PCR with the primer set D10S1214. Thereby the primers amplify highly variable numbers of tandem repeats (VNTR) across the whole genome. According to the number of tandem repeats, amplicons with different sizes or only with one size are generated. These were visualised by agarose gel electrophoresis with a 2 % and a 3% agarose gel.

3.2.9.2 oriP PCR

The genomic DNA of all four derived p-iPSC lines as well as of MSC74 and the genomic DNA of two positive controls was isolated using the FlexiGene DNA Kit (Qiagen). The positive controls were MSC74 and MSC17 cells which were nucleofected with the episomal plasmid ET2K as previously described in section non viral reprogramming. The genomic DNA of the positive control was isolated four days after the nucleofection.
The PCR product was visualised using a 1,5 % agarose gel and the size marker Perfect Plus 1 kb DNA ladder (Roboklon).

3.2.9.3 Alkaline phosphatase staining

The undifferentiated state of embryonic stem cells and induced pluripotent cells is characterised by the expression of high levels of alkaline phosphatase. The staining procedure to visualise alkaline phosphatase was performed using the ES cell characterization tool following the manufacturer's instructions.
The analysed cells were fixed with 4 % paraformaldehyde in PBS for 2 min at RT. The fixative was subsequently removed and the fixed cells were rinsed several times with 1x Rinse Buffer. Afterwards, a Naphthol/Fast Red Violet solution was prepared by mixing Fast Red Violet (FRV) with Naphthol AS-BI phosphate solution and water in a 2:1:1 ratio (Fast Red Violet:Naphthol:water). The stained cells were covered with this solution (1 ml per well of a 6-well plate) followed by incubation at RT in the dark for 15 min. Subsequently the staining solution was aspirated and the cells were washed using 1x Rinse Buffer. Finally, the cells were covered with PBS and the result was analysed with a microscope. Cells that express alkaline phosphatase were stained purple with this staining procedure. The staining was

documented using a digital camera and an inverted microscope as well as the Zeiss, LSM 510 Meta microscope with a connected camera for microscopy model AxioCam ICc3 and the software Axiovision 4.6 as described for the documentation of Alizarin Red S staining.

3.2.9.4 Imunofluorescence staining

The cells were fixed with 4 % paraformaldehyde for 20 min at RT and washed two times with PBS. Subsequently, the cells were permeabilised with 1 % Triton X-100 in PBS for 10 min. Next the cells were blocked with a solution consisting of 10% chicken serum, 0,1 % Triton X-100 in PBS for 45 min at RT. Subsequently, the cells were washed with PBS two times for 5 min at RT. The primary antibody was diluted in 10 % chicken serum, 0,1 % Triton X-100 in PBS as described in the materials section. The cells were covered with primary antibody solution using 200 µl per well of a 24 well culture plate. The primary antibody was left on the cells for 1 h at RT. After this step, the cells were washed twice with PBS for 5 min at RT. The secondary antibody against the species in which the first antibody was produced, was diluted in the same solution as the primary antibody. The dilution rate of the secondary antibodies can be found in the materials section. The secondary antibody was either conjugated to the dye Alexa Fluor 488 for green fluorescence or to Alexa Fluor 594 for red fluorescence. The diluted secondary antibody solution was added to the cells using the same volume like for the primary antibody and was left on the cells for 1 h at RT in the dark followed by two washing steps with PBS for 5 min at RT. The nuclei of the cells were counterstained with 4′,6-Diamidin-2-phenylindol (DAPI). Doing this, 200 µl of DAPI solution (see section Materials) was added on the cells for 20 min at RT. Finally, the cells were covered with PBS to keep them moist. The fluorophores on the secondary antibodies were visualised using a Zeiss, LSM 510 Meta confocal microscope with a connected camera for microscopy model AxioCam ICc3 and the software Axiovision 4.6.

3.2.9.5 Analysis of pluripotency by embryoid body based differentiation

The colonies of three confluent wells of the respective p-iPSC line p-iPSC 1, p-iPSC 2, p-iPSC 3 or p-iPSC 4 were cut manually into pieces of the same size with a BD Microlance^{TM} 3 injection needle (Becton Dickinson). Subsequently, the cells were scraped with a cell scraper. The cell clumps were transferred to a 60 mm ultra low attachment culture dish (Corning) containing hESC without FGF2. The cells were cultured in 5 ml of media per cell culture dish.
After overnight culture in suspension, p-iPSCs formed spheres called embryoid bodies (EBs). The media was changed every other day by transfer of the EBs into a 15 ml tube and sedimentation of the EBs by leaving them to settle for 5 min at 37 °C in a water bath.
After removal of the supernatant, fresh media was added. Finally, the EBs were transferred to the low attachment plates for further culture. The suspension culture was carried out for seven days. Then the EB were seeded on 24 well cell culture dishes coated with 0,1 % gelatine for attachment and induction of differentiation. The EBs were cultured in ES cell media in this condition for 14 days. To demonstrate their ability to differentiate into lineages of the three germ layers, the EBs were fixed with 4 % paraformaldehyde after 14 days followed by immunofluorescence staining of marker proteins of the three germ layers. SOX17 was stained to detect endoderm differentiation. α-SMA was stained to detect differentiation into

mesodermal lineages. TUJ1 and Nestin were stained to detect differentiation in the direction of ectoderm.

4 Results

In this section the following nomenclature will be applied:
- hMSCs: human mesenchymal stem cells
- iPSCs: induced pluripotent stem cells
-hESCs: human embryonic stem cells
-MSC17: human mesenchymal stem cells derived from a 17 year old patient
-MSC23: human mesenchymal stem cells derived from a 23 year old patient
-MSC74: human mesenchymal stem cells derived from a 74 year old patient
-v-iPSC: reprogrammed cells derived from viral reprogramming of MSC74
- p-iPSC 1, p-iPSC 2, p-iPSC 3 and p-iPSC 4: four partially reprogrammed iPSC cell lines derived from non viral reprogramming of MSC74

4.1 Characterisation of hMSC lines before cellular reprogramming

4.1.1 Different morphology and growth properties of MSC17 and MSC74

hMSCs of the cell lines MSC17 and MSC74 were used in reprogramming experiments in this work in order to derive induced pluripotent stem cells. During culture of the cells, it could be observed that MSC17 cells grow faster than MSC74 cells. MSC17 had to be split with a ratio of 1:3 after four days whereas MSC74 had to be split after eight days when both cell lines were seeded at the same density. Furthermore, MSC17 were smaller than MSC74. However, MSC17 contained some fibroblast like, elongated cells with a morphology similar to MSC74. All MSC74 cells had an elongated fibroblast like morphology. Moreover, MSC74 cells were wider compared to MSC17. (Figure 6)

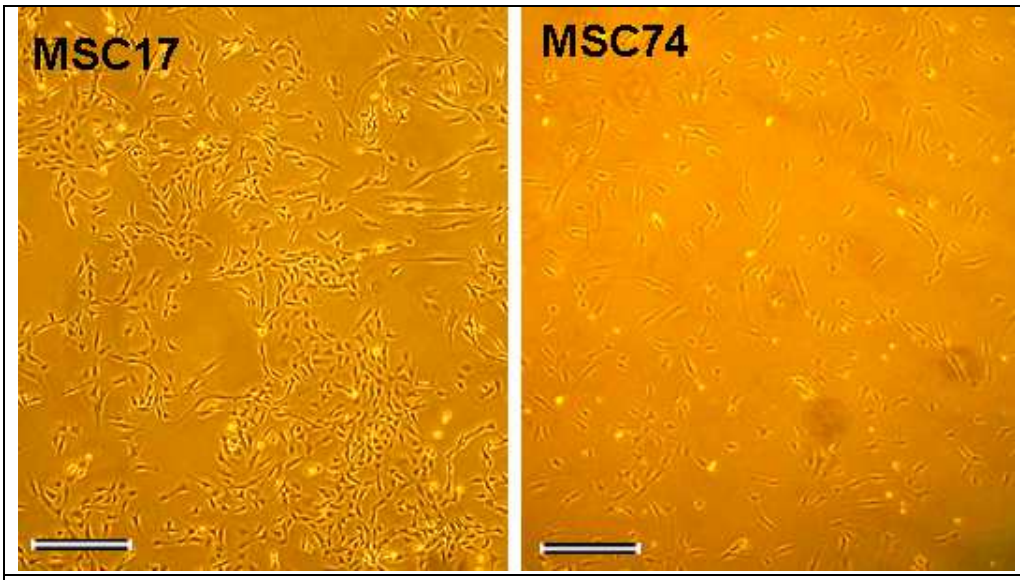


Figure 6 Different morphology of hMSCs used for cellular reprogramming. left: bright field picture of MSC17 passage 3, right: bright field picture of MSC74 passage 4. Bright field microscopy, 4-fold magnification, scale bar: 50 µm.

4.1.2 Differentiation efficiencies of hMSCs change depending on donor age

Before MSC17 cells and MSC74 cells were used for the reprogramming experiments, it was necessary to verify that they are multipotent and can differentiate into adipocytes and osteoblasts to be sure that the applied cells are mesenchymal stem cells. Therefore, *in vitro* differentiation experiments were performed as described in the Methods section.

After 21 days of culture in adipocyte differentiation media an estimated 2 % of all treated MSC17 cells differentiated into Oil Red O positive adipocytes. The detected Oil Red O positive cells were smaller than Oil Red O positive cells derived from MSC74 under the same conditions. MSC17 culture in control media did not result in Oil Red O positive cells. In addition to that, MSC17 cells arranged themselves after 10 days of culture in clusters as depicted in Figure 7 (treatment). The cell clusters were formed only when MSC17 were cultured in adipocyte differentiation media and could not be observed in the control.

In contrast to that, an estimated 20 % of MSC74 cultured in adipocye differentiation media differentiated into Oil Red O positive cells after 21 days cultured in adipocyte differentiation media. No Oil red O positive cell could be observed in the control. The results of these experiments suggest that MSC17 and MSC74 can differentiate into adipocytes whereas MSC17 differentiated with a five times lower efficiency into adipocytes compared to MSC74. (Figure 7)

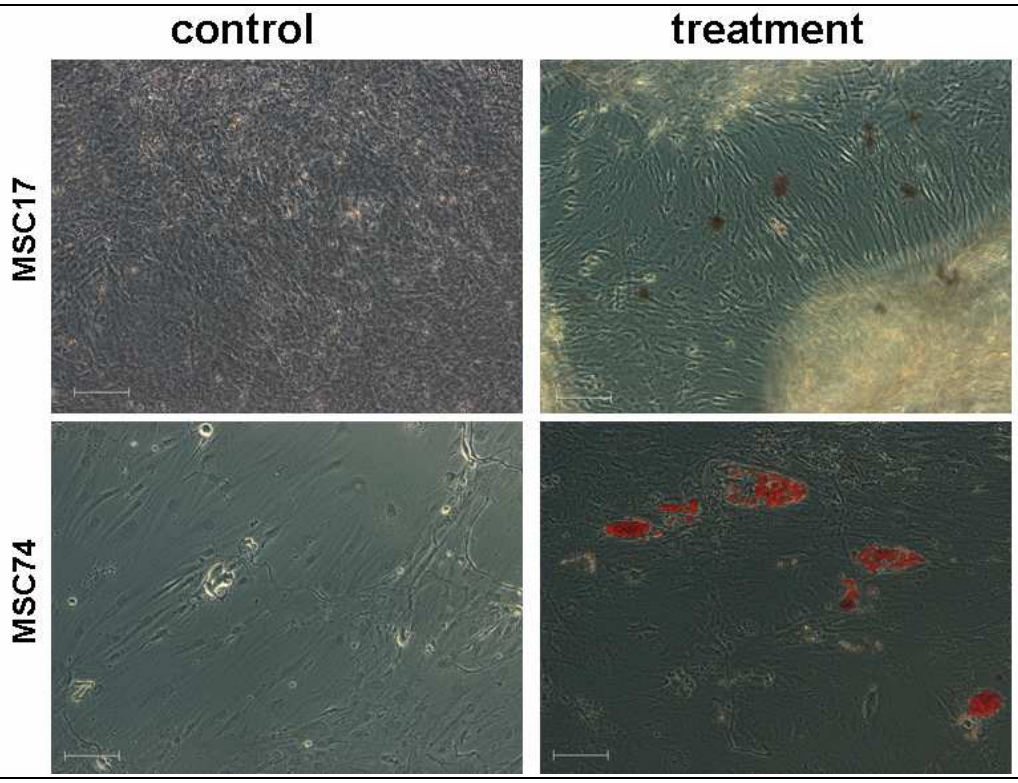


Figure 7 Decreased number of Oil red O positive cells in MSC17 (upper panel) compared to MSC74 (lower panel) after 21 days of culture in adipocyte differentiation media (treatment) or in maintenance media supplemented with DMSO (control). Bright field microscopy, 10-fold magnification, scale bar: 100 µm.

When *in vitro* osteoblast differentiation experiments were conducted, an estimated 10 % of MSC17 cells differentiated into Alizarin Red S positive cells when cultured 21 days in osteoblast differentiation media. No Alizarin Red S positive cells could be detected when MSC17 cells were cultured in control media. (Figure 8 B)

Concerning MSC74, an estimated 50 % of the cells differentiated into mineralised Alizarin Red S positive cells. (Figure 8 A and B) No Alizarin Red S positive MSC74 cells could be detected when the cells were cultured in control media. According to these results MSC17 and MSC74 are able to differentiate into osteoblasts. However, osteoblast differentiation of MSC17 was less efficient compared to MSC74. The performed differentiation experiments revealed that MSC17 cells have a lower capability to differentiate into osteoblasts and adipocytes compared to MSC74.

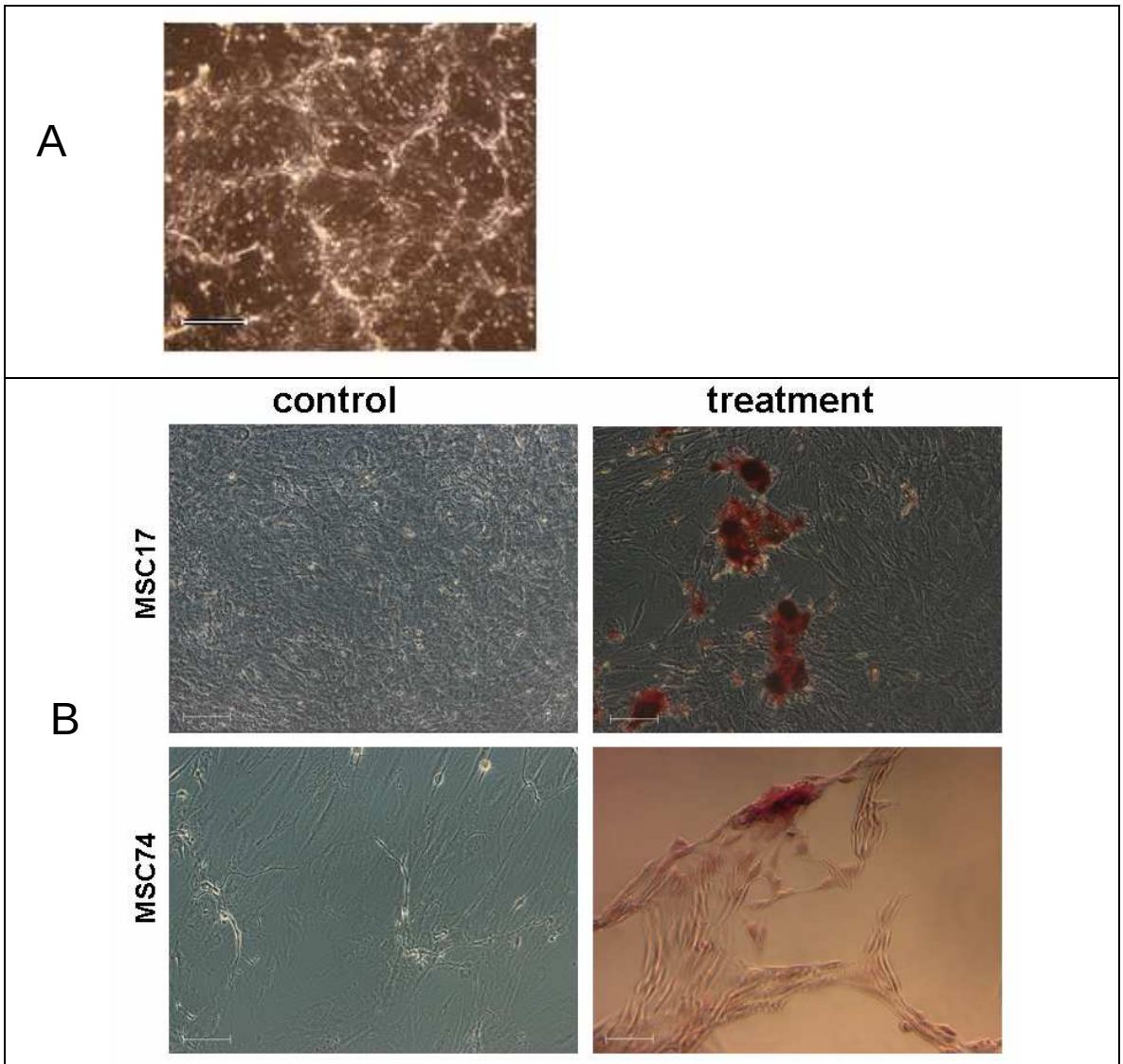


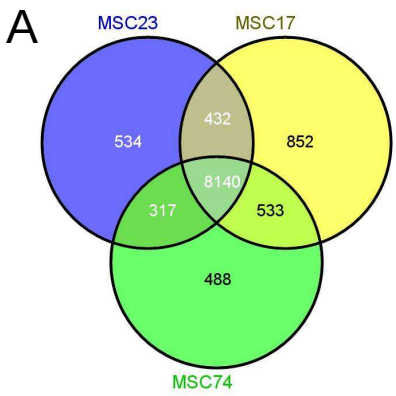
Figure 8 hMSCs used for cellular reprogramming can be differentiated into osteoblasts. A: MSC74 passage 5 after 21 days of culture in osteoblast differentiation media, phase contrast microscopy, picture taken with a digital camera, 4-fold magnification, scale bar: 100 µm. B: MSC17 passage 4 (upper panel) and MSC74 passage 5 (lower panel) differentiate into Alizarin Red S positive osteoblasts after 21 days of culture in osteoblast differentiation media (treatment) compared to culture in maintenance media (control). Bright field microscopy, 10-fold magnification, scale bar: 100 µm.

4.1.3 Changes and similarities in the gene expression comparing hMSCs of young and old donors

In order to characterise transcriptional differences between MSC17 and MSC74 a microarray-base gene expression analysis was carried out with RNA samples of the cell lines. To get a more complete picture, a RNA sample of MSC23 was used in this analysis as additional sample.

4.1.3.1 The MSC-related transcriptional signature is conserved in all hMSC lines

For a general overview on differences and similarities between the gene expression patterns of MSC17, MSC23 and MSC74, lists of genes detected as expressed were generated as described in the Methods section. Using these gene lists, a Venn diagram was originated.
According to the Venn diagram 8140 genes are expressed in all three hMSC lines. 543 genes are only expressed in MSC23, 852 genes are only expressed in MSC17 and 488 genes are only expressed in MSC74. 432 genes are expressed in MSC23 and MSC17 whereas MSC17 and MSC74 have with 533 genes the highest number of common genes. The lowest number of common expressed genes could be found in MSC23 and MSC74. (Figure 9 A)
Subsequently, a list of the 8140 genes that are commonly expressed in all hMSC lines was generated using the data analysis software BeadStudio. Using this list, it was determined, whether MSC related marker genes are contained. Figure 9 B shows the average signals of all detected hMSC related marker genes that could be found in the gene list.
Mesenchymal stem cells are distinguished from hematopoetic stem cells by their lack of *CD34*, *CD45* and *CD14*. *CD34* and *CD45* were not found to be expressed in all three hMSC lines whereas *CD14* expression could be detected. Genes expressed particularly in hMSCs such as *NT5E (CD73)*, *THY1 (CD90)*, *ENG (CD105)*, *ALCAM (CD166)* and *CD44* were detected to be expressed at a similar level in all three analysed hMSC lines. According to these results the majority of expressed genes is equal in all three MSC samples and is not affected by the age of the patient the cells were derived from. Among the conserved expressed genes there are those that are particularly expressed in hMSCs confirming that all three cell lines are hMSCs.



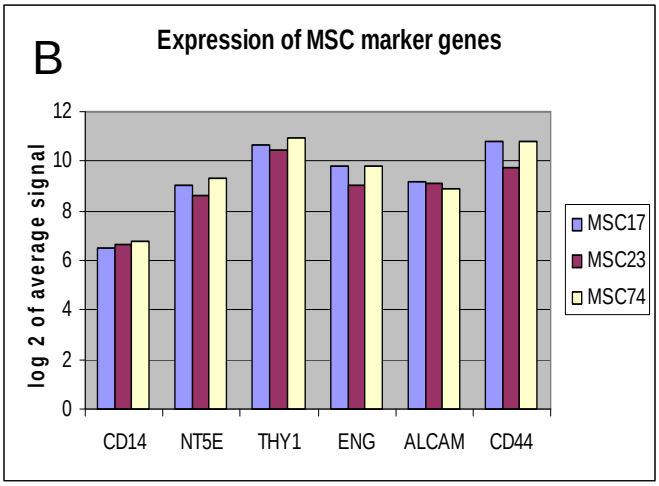


Figure 9 Similarities in the global gene expression of hMSCs derived from two young (17 and 23 years) and one old (74 years) patient and the conservation of hMSC related transcriptional signature in these cells. A: Venn diagram depicting the numbers of consistently expressed genes. Generation of the diagram: see Methods section. B: Log 2 ratio of the average signal of hMSC marker genes expressed by the hMSCs of all three patients. Measurement and data processing to generate the diagram: see Methods section.

4.1.3.2 Donor age-related changes of the gene expression in hMSCs

The data obtained by global gene expression analysis with the Illumina HumanHT-12 v3 Expression BeadChip was processed with the software BeadStudio in order to analyse the correlation between the expression patterns of MSC17, MSC23 and MSC74.
Figure 10 A shows that the global gene expression of MSC17 and MSC74 cluster together, whereas the global gene expression pattern of MSC23 does not cluster together with MSC17 and MSC74. The reason for this might be that the RNA sample of MSC23 was obtained from another laboratory. However, it was isolated with the same method like MSC17 and MSC74.
Furthermore, the gene expression data of all combinations of the three hMSC samples was depicted in dot plot diagrams generated by the software BeadStudio. According to this data analysis the correlation coefficient between the global gene expression of MSC17 and MSC23 is 0,9269, between global gene expression of MSC17 and MSC74 0,9648 and between the gene expression data of MSC23 and MSC74 0,9331. Considering these results the least similar gene expression patterns among the analysed samples belong to MSC23 and MSC17. A difference which was not expected but could also be due to the fact that the RNA sample was not isolated with the other two samples together. The most similar gene expression patterns among the analysed samples are the expression patterns of MSC74 and MSC17. (Figure 10 B, C, D)

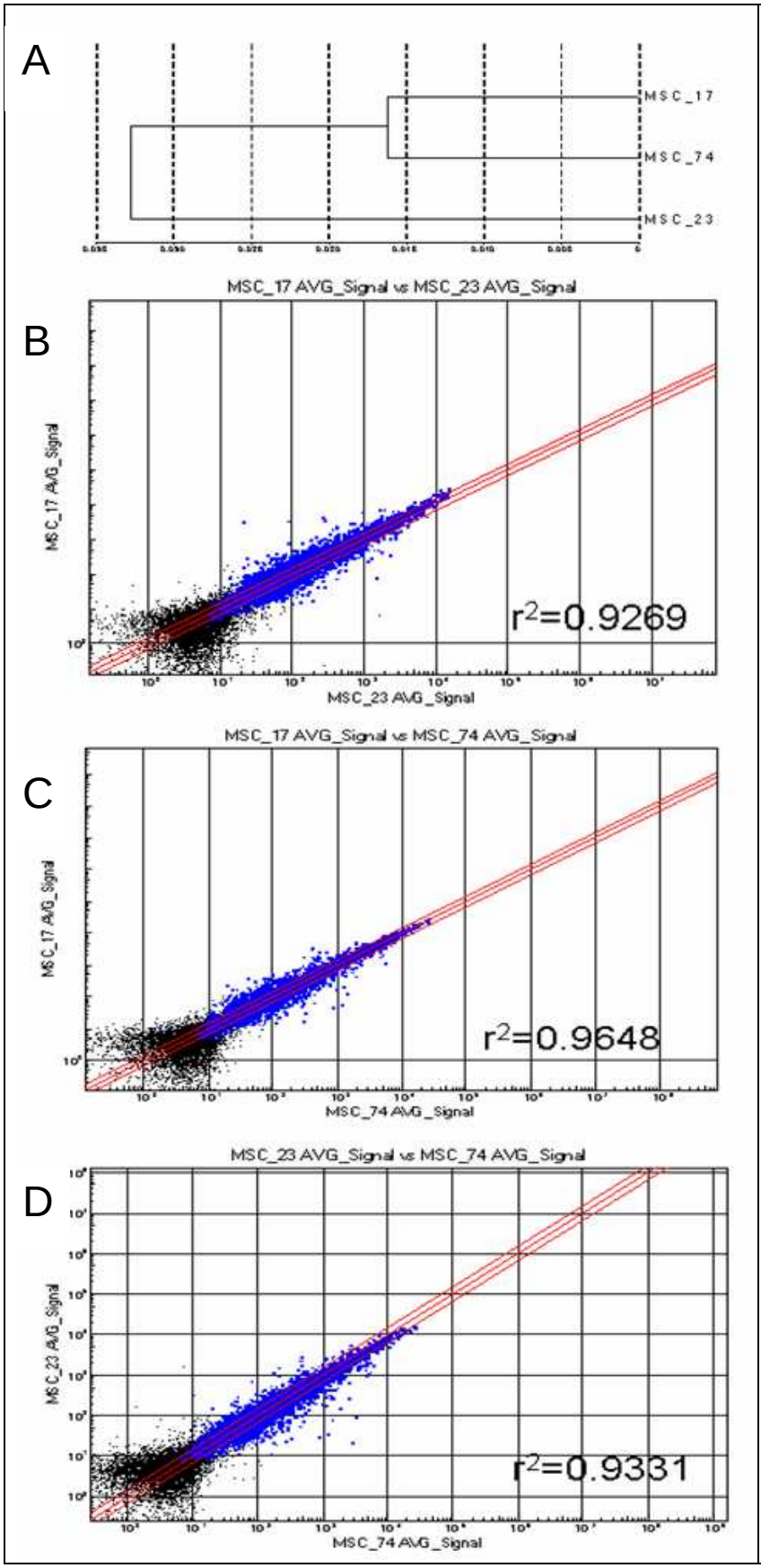


Figure 10 Comparison of the global gene expression in MSC17, MSC23 and MSC74. A: Pearson correlation diagram depicting the similarity of the gene expression according to the correlation coefficient. B, C, D: dot plot diagrams depicting the correlation of the average signals (AVG_Signal) measured for MSC17 (MSC_17), MSC23 (MSC_23) and MSC74 (MSC_74). Comparison of MSC17 to MSC23 (B) MSC17 to MSC74 (C) and MSC23 to MSC74 (D). Black dots: genes with a detection p-value below 0,01 (considered as not detected). Blue dots: genes with and detection p-value above 0,01 (detected genes). All genes represented by blue dots above the upper or below the lower red line are considered as differentially expressed genes compared to the other sample. Upper red line: represent a differential p-value of 0,01 as upper limit. All blue dots above the line are genes that are up-regulated. Lower red line: represent a differential p-value of 0,01 as lower limit. All blue dots above the line are genes that are down-regulated. r^2:correlation coefficient.

4.1.3.3 Pathways involved in age-related differences in hMSCs

The data obtained from the microarray-based gene expression analysis comparing the transcriptome of MSC17, MSC23 and MSC74 was used to create lists of genes that are up- and down-regulated with age in hMSCs. Regarding these results, the question arose, which pathways may be affected by the age-related up- or down-regulation of the analysed genes. To answer the question the generated age-related gene lists (corresponding gene symbol as input) were further analysed using the gene annotation tool DAVID to identify altered pathways.

The gene lists generated by relation of MSC74 to MSC17 were analysed. The detected KEGG annotated and BIOCARTA annotated pathways that changed significantly with age in the analysed hMSCs are shown in Table 6.

Several of the shown KEGG and BIOCARTA annotated pathways in Table 6 refer to pathways which are not expected to be present in hMSCs. The reason for that is that the genes preferably traced back to a certain pathway by the annotation tool even though they are involved in other pathways that are more likely found in hMSCs.

Table 6 shows a number of different pathways that very likely are up- or down-regulated with donor age in MSC74 compared to MSC17. As p53 and other genes associated with cell cycle regulation were shown to be involved in lowering the efficiency of iPSC generation (Hong et al., 2009), the data analysis focused on this aspect of age-related changes in hMSCs.

The pathway with the KEGG annotation cell cycle was found to be significantly down-regulated with a p-value of 0,000003 comparing MSC74 and MSC17. The assigned down-regulated genes are: *E2F2*, *PKMYT1*, *TTK*, *CHEK1*, *CDC20*, *MCM2*, *PTTG1*, *MCM3*, *CDK2*, *MCM5*, *MCM6*, *CCNB1*, *CCNB2*, *PLK1*, *CDKN2D*, *BUB1* and *CCNA2*. (Figure 11, top) Moreover, the pathway with the KEGG term p53 pathway was detected to be down-regulated in MSC74 cells compared to MSC17 cells. The genes *CCNB1*, *CCNB2*, *RRM2*, *CHEK1*, *GTSE1*, *CDK2* (Figure 11, bottom) were assigned to this pathway with a p-value of 0,042.

The down-regulated genes *CCNB1*, *PLK1*, *CDKN2D*, *CHEK1* were assigned to the BIOCARTA term cell cycle as well. In addition, cell cycle was the only pathway detected as down-regulated using the BIOCARTA annotation with a p-value of 0,026.

Table 6 Pathways possibly affected by age-related up- or down-regulation of genes in hMSCs. Data analysis procedure to obtain pathway annotations: see Methods section. 1: Number of genes from the target input genes, annotated for the corresponding term, 2. Percentage of annotated target genes compared to the overall number of input, 3: p-values < 0,05 were counted as significant, but the whole output is shown.

Down in MSC74 compared to MSC17				**Up in MSC74 compared to MSC17**			
KEGG_PATHWAY-Term	Count[1]	%[2]	p-value[3]	*KEGG_PATHWAY-Term*	Count[1]	%[2]	p-value[3]
Cell cycle	17	4	0,000003	Complement and coagulation cascades	6	2,3	0,0059
Oocyte meiosis	12	2,8	0,00023	ECM-receptor interaction	6	2,3	0,0059
DNA replication	7	1,6	0,00043	Cell adhesion molecules (CAMs)	6	2,3	0,0059
Pyrimidine metabolism	8	1,9	0,017	Glycine, serine and threonine metabolism	3	1,2	0,0059
Progesterone-mediated oocyte maturation	7	1,6	0,033				
Folate biosynthesis	3	0,7	0,037				
p53 signaling pathway	6	1,4	0,042				
ECM-receptor interaction	6	1,4	0,087				
BIOCARTA-Term	Count[1]	%[2]	p-value[3]	*BIOCARTA-Term*	Count[1]	%[2]	p-value[3]
Cell Cycle	4	0,9	0,026	Ghrelin	4	1,6	0,0013
				Acute Myocardial Infarction	3	1,2	0,0013

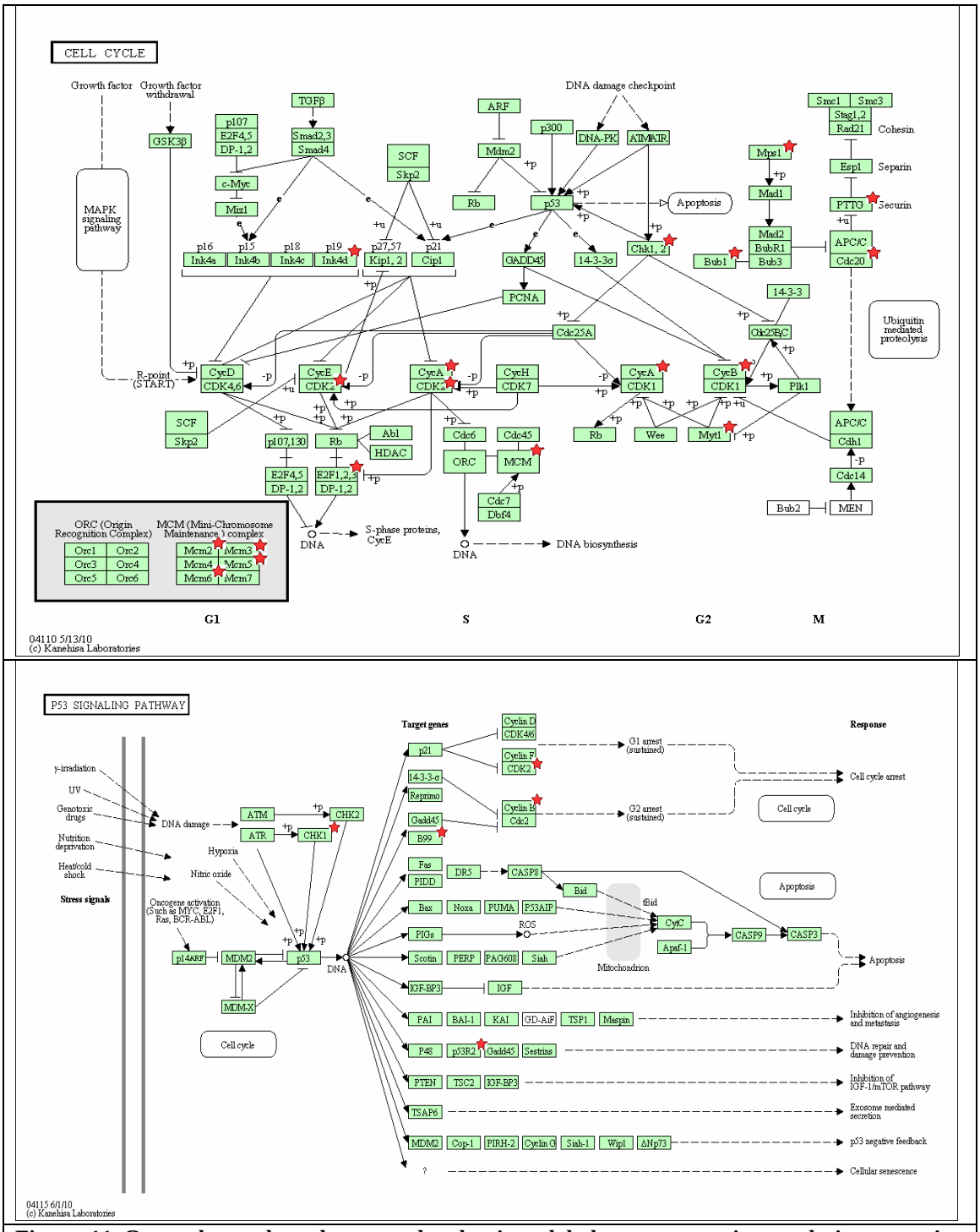


Figure 11 Genes detected as down-regulated using global gene expression analysis comparing MSC74 to MSC17 are involved in cell cycle regulation (top) and p53 signaling (bottom). Down-regulated genes represented by the green boxes containing the official gene symbol are marked with red stars. The pictures were obtained using the gene annotation tool DAVID by entering lists of the official gene symbol of the down-regulated genes in MSC74 compared to MSC17 and by mapping the genes in the annotated pathway term by KEGG. (world wide web, http://david.abcc.ncifcrf.gov, 20.07.2010)

Among the genes detected as down-regulated in MSC74 relative to MSC17 that are part of cell cycle associated pathways, there are six genes whose average signals differed with a p-value below 0,01. These genes are *CCNB2*, *CHEK1*, *PKMYT1*, *TTK*, *PTTG1*, *MCM5*,

MCM6, *BUB1* and *CCNA2*. The down-regulated genes associated with the KEGG term p53 signaling pathway contain genes whose measured average signals differ with a p-value below 0,01, as well. These genes are *CCNB1*, *CCNB2*, *CHEK1* and *GTSE1*. However, *p53* was detected as up-regulated in MSC74 as the measured average signal was higher in MSC74 than in MSC17. (Figure 12)

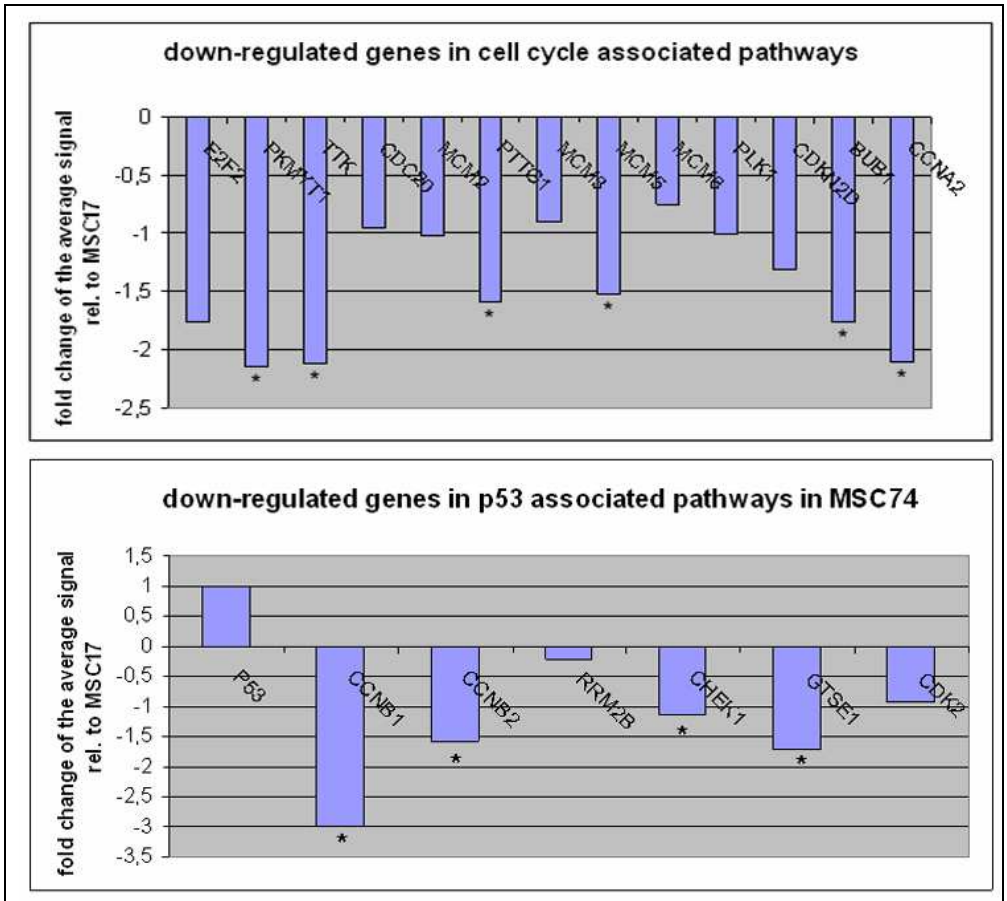


Figure 12 Fold change of the average signal of genes found to be down-regulated in MSC74 relative to the average signal measured for MSC17. Average signals of genes in MSC74 differing from those in MSC17 with a differential p-value below 0,01 are marked with an asterisk. The average signals of genes that are not marked with an asterisk differ with a p-value below 0,05. The list of genes of which the average signal is depicted was generated as described in the Methods section.

4.1.4 hMSCs express pluripotency-associated factors KLF4 and c-Myc

Before the reprogramming experiments were started, the expression of the factors *OCT4*, *NANOG*, *SOX2*, *KLF4* and *c-Myc* in hMSCs was analysed at the RNA and protein level. The results were used to determine, whether any reprogramming factors could be left out in the reprogramming experiments using retroviruses or episomal plasmids.
Human mesenchymal stem cells express similar levels of *c-Myc* and *KLF4* compared to the embryonic stem cell line H1. *OCT4*, *NANOG*, *SOX2* and *LIN28* were found to be expressed at much lower levels compared to H1. This was suggested by the results of the real time PCR analysis of the RNA of MSC23. (Figure 13 B) The expression values of the samples were

measured in this analysis in triplicate. The standard deviation of the triplicate was low in all triplicates of the respective measured gene as depicted in Figure 13 A.

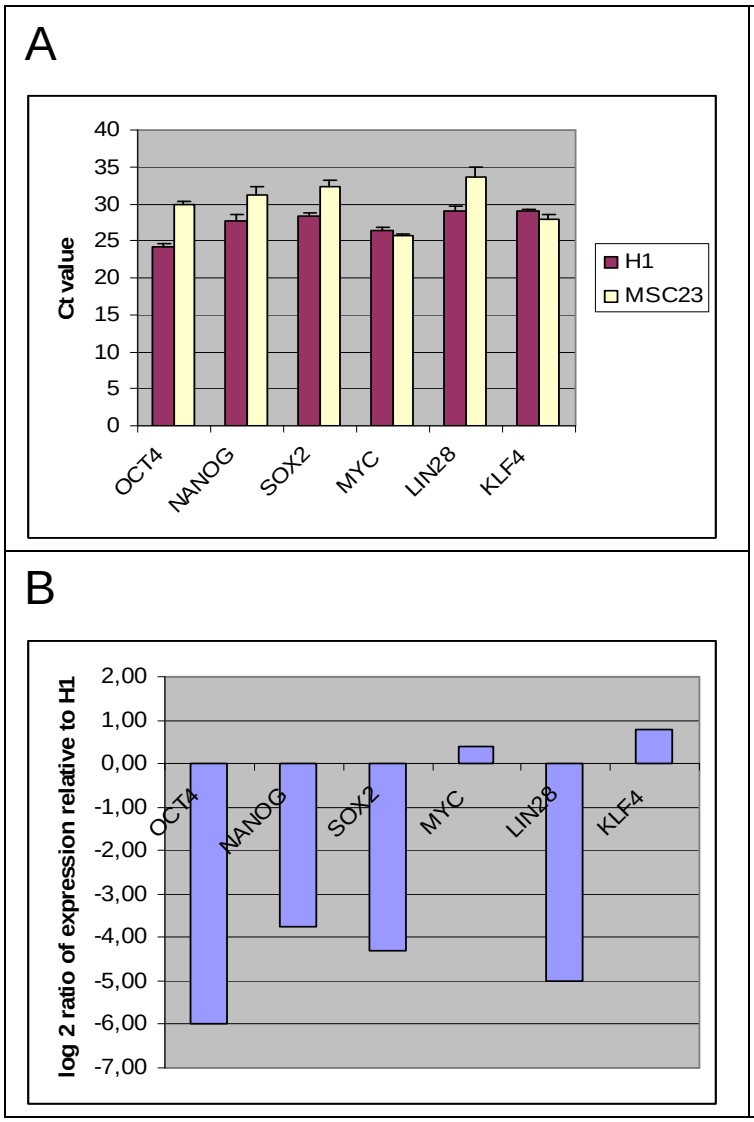


Figure 13 RNA expression of genes associated with pluripotency in hMSCs. A: C_t values of real time PCR conducted with RNA from MSC23 cells and from the embryonic stem cell line H1. The diagram shows the average C_t value of a measured triplicate. The standard deviation is depicted by the error bars. B: Log2 ratio of the fold change of the expression of pluripotency associated genes comparing the expression in MSC23 and in the embryonic stem cell line H1. The values were calculated and normalised as described in the methods section.

The increased expression of *KLF4* and *c-Myc* in MSC23 measured by real time PCR could be confirmed on the protein level by immunoflourescence staining of MSC74 cells. (Figure 14 A) Furthermore, *OCT4*, *NANOG* and *SOX2* were not expressed, which is consistent with the results for the RNA expression. (Figure 13 B) Immunofluorescence staining of *OCT4*, *NANOG*, *SOX2*, *KLF4* and *c-Myc* was carried out on the embryonic stem cell line H1 as a positive control. All proteins could be detected in H1 confirming that the used staining protocol is sufficient to visualise the analysed proteins in the determined MSC74 cells. (Figure 14 B)

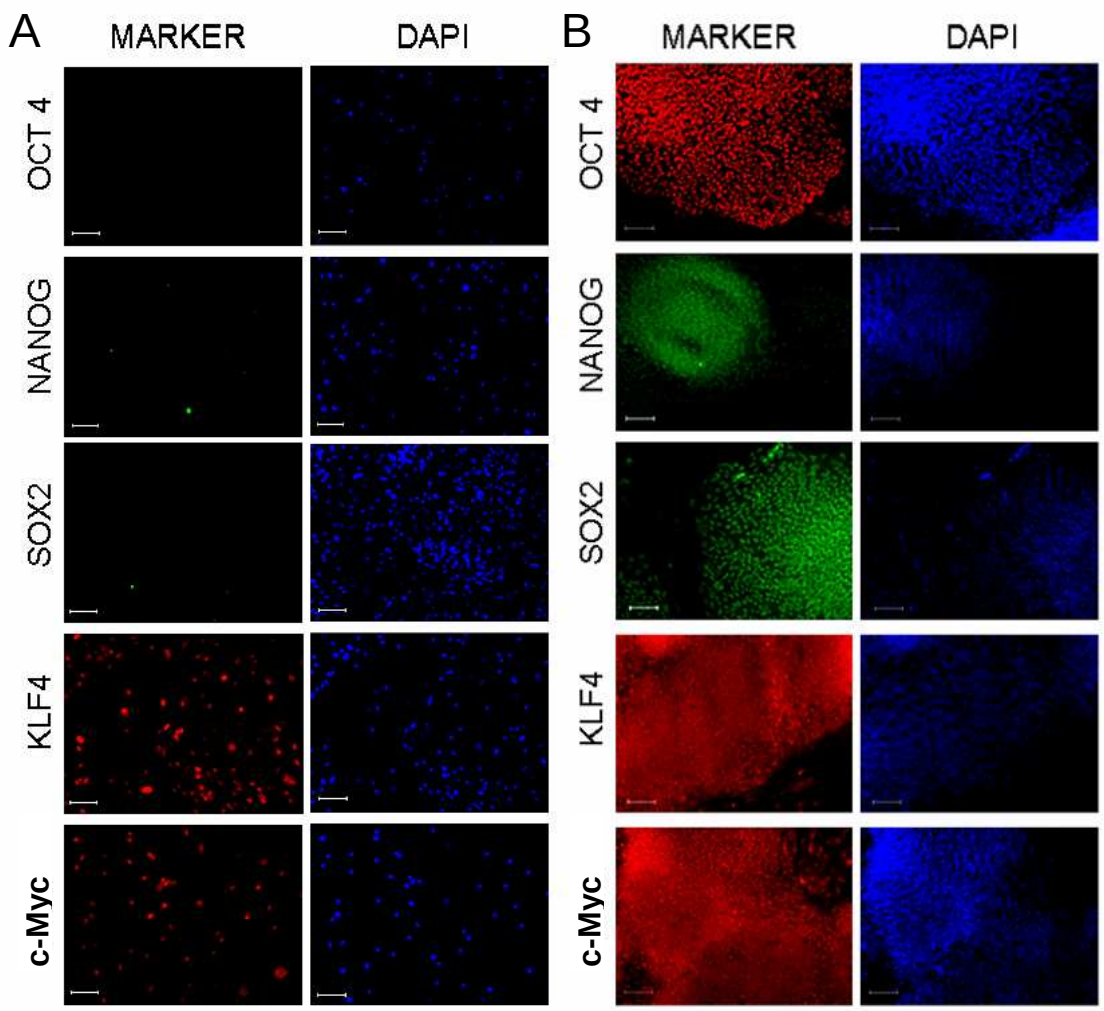


Figure 14 Protein expression of pluripotency associated genes in MSC74 (A) compared to the embryonic stem cell line H1 (B). Fluorescence microscopy, 5-fold magnification, scale bar: 100 µm. Alexa Fluor 488 (green), Alexa Fluor 594 (red).

4.2 Reprogramming of hMSCs using retroviruses

The microarray-based global gene expression analysis revealed that the gene expression patterns of MSC17 and MSC74 have the highest correlation. This could not be observed concerning the gene expression patterns of MSC17 cells and MSC23 cells as well as MSC74 cells and MSC23 cells. In order to better analyse a possible effect the donor age could have on hMSCs, the cell lines with the biggest age difference were chosen for reprogramming: MSC17 and MSC74.

Retroviruses carrying *OCT4*, *SOX2*, *KLF4* or *c-Myc* were generated as described in the materials and methods section. The produced retroviruses were tested on MSC17 in order to find out whether they could be applied in the reprogramming experiments.

MSC17 were infected with retroviruses carrying the genes either *OCT4*, *SOX2*, *KLF4* or *c-Myc* using the same amount of retrovirus that would be applied in the reprogramming experiments. Only one infection was performed per $2x10^5$ cells with one retrovirus for each gene. The genes could be successfully transduced into MSC17 as the factors OCT4, SOX2, KLF4 and c-Myc could be detected using immunofluorescence staining two days after the retroviral infection. (Figure 15) These results confirmed that the produced retroviruses work and can be applied in reprogramming experiments.

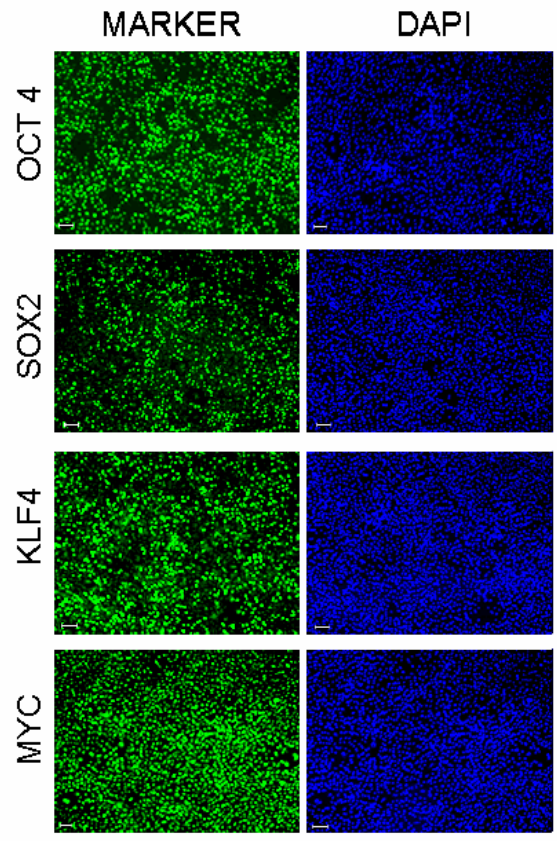


Figure 15 Successful infection of hMSCs by the produced retroviruses used in the reprogramming experiments of MSC17 and MSC74. Protein expression of *OCT4*, *SOX2*, *KLF4* and *c-Myc* two days after the retroviral transduction in MSC17. Fluorescence microscopy, 5-fold magnification, scale bar: 100 µm, Alexa Fluor 488 (green).

4.2.1 Generation of v-iPSCs

To monitor whether the retroviral infection of MSC17 and MSC74 was successful, $2x10^5$ cells of both cell lines were infected with retroviruses carrying the reporter gene *GFP*. An estimated 70 % of MSC17 and an estimated 20 % of MSC74 were found to be GFP positive. (Figure 16)

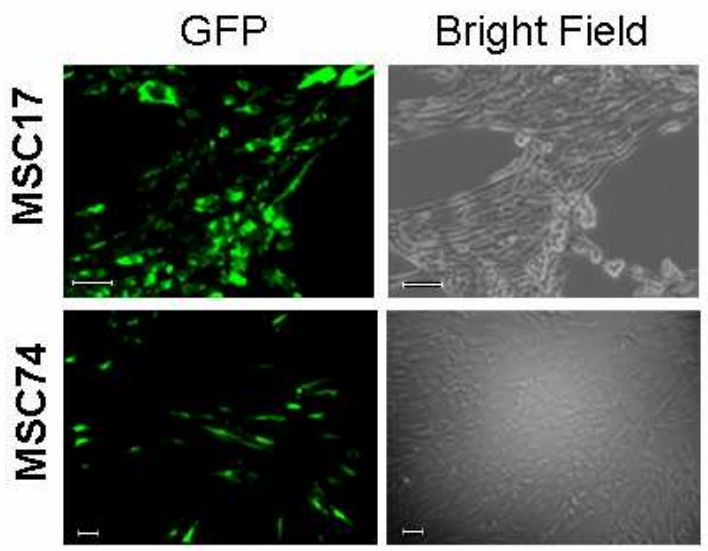


Figure 16 Protein expression of reporter gene *GFP* three days after retroviral transduction of the construct in MSC17 and MSC74. The infection served as positive control to monitor the transduction efficiency of the cells used in the viral reprogramming experiments. Bright field and fluorescence microscopy. MSC17: 10-fold magnification. scale bar: 100 µm.
MSC74: 5-fold magnification. scale bar: 100 µm.

Subsequently, the retroviral reprogramming experiments were carried out with MSC17 cells and MSC74 cells. In these experiments it was additionally tested, whether it is possible to enhance the efficiency of the iPSC derivation from hMSCs by adding inhibitors as described in Materials and Methods.
The results of the retroviral experiments are depicted in Table 7. MSC17 could not be reprogrammed to iPSCs by retroviral transduction of *OCT4*, *SOX2*, *KLF4* and *c-Myc*. The iPSC generation from MSC17 could not be enhanced by the inhibitors PD325901, SB431542 or Pifithrin α or their combined application. It was tested in this reprogramming experiment whether it has an effect when more cells are infected by retroviruses (experimental condition 4) as in the other conditions (control, 2i, p53 and 2i+p53). The increase of cell number had no effect towards the generation of iPSCs when used for MSC17 cells.
However, the combination of 2i and p53 inhibitor Pifithrin α resulted in the reprogramming of MSC74 to one colony of iPSCs 40 days after the factors *OCT4*, *SOX2*, *KLF4* and *c-Myc* were transduced into MSC74 cells. These cells were named v-iPSCs. The other experimental conditions did not lead to iPSC generation from MSC74. Considering that $2x10^5$ cells were infected with the retrovirus and one colony has formed out of one reprogrammed MSC74 cell the reprogramming efficiency is 0,0004 %.

Table 7 Results of viral reprogramming experiments conducted with MSC17 and MSC74. The mesenchymal stem cells were infected with equal amounts of retroviral constructs harbouring the open reading frame of either *OCT4*, *SOX2*, *KLF4* or *c-Myc* (4 factors), p53: culture in the presence of Pifithrin α, 2i: culture in the presence of SB431542 and PD325901, 2i+p53: combination of both. -: the experimental condition was not tested.

cell line	*cells plated*	*colonies with 4 factors*	*colonies with 4 factors +p53*	*colonies with 4 factors +2i*	*colonies with 4 factors +2i+p53 (days)*
MSC17	$2x10^5$	0	0	0	0
MSC17	$4x10^5$	0	-	-	-
MSC74	$2x10^5$	0	0	0	1 (40)

4.2.2 Characterisation of hMSCs after retroviral reprogramming

Retroviral reprogramming of MSC74 with an addition of PD325901, SB431542 and Pifithrin α to the culture media resulted in one iPSC colony. A part of this colony had a similar morphology to embryonic stem cells. The other part of the colony had shown features of cardiomyocytes. Both parts were separated. The part of the colony containing iPSC like features was cultured further as described in materials and methods and characterised as shown in this section.

4.2.2.1 v-iPSCs have a morphology similar to embryonic stem cells

iPCs derived from MSC74 (v-iPSCs) have a similar morphology compared to cells of the embryonic stem cell line H1 as depicted in Figure 17. v-iPSCs retain the same features like H1 cells when cultured in the same conditions such as growing on feeder cells and hESC culture media with addition of *FGF2*. v-iPSCs have the same compact cell shape like embryonic stem cell. They grow in colonies and differentiate when not kept in culture conditions for the maintenance of the undifferentiated state. The nuclei of v-iPSCs like the nuclei of ESCs fill out most of the entire cytoplasm of the cells.

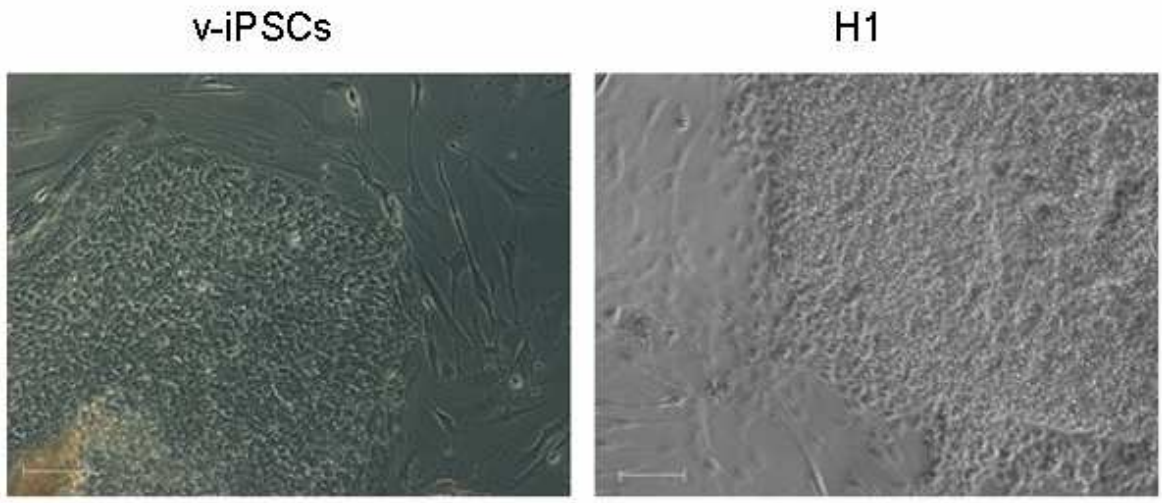


Figure 17 Morphology of iPSCs derived from MSC74 using retroviral reprogramming (v-iPCS) compared to the human embryonic stem cell line H1. Culture of both cell types was conducted on feeder cells. The pictures were taken when v-iPSCs were in passage 4 after isolation and H1 was in passage 71. Bright field microscopy, 10-fold magnification, scale bar: 100 µm.

4.2.2.2 v-iPSCs express alkaline phosphatase

The similar morphology of v-iPSCs to ESCs lead to the question whether these cells expressed alkaline phosphatase, a feature of hESCs often used to identify iPSCs.
Using cells of the embryonic stem cell line H1 as positive control (Figure 18 C) a staining procedure visualising alkaline phosphatase activity as purple was performed with v-iPSCs. The results of the staining revealed that v-iPSCs express alkaline phosphatase at a similar level like the H1 cells of the positive control. (Figure 18 B)

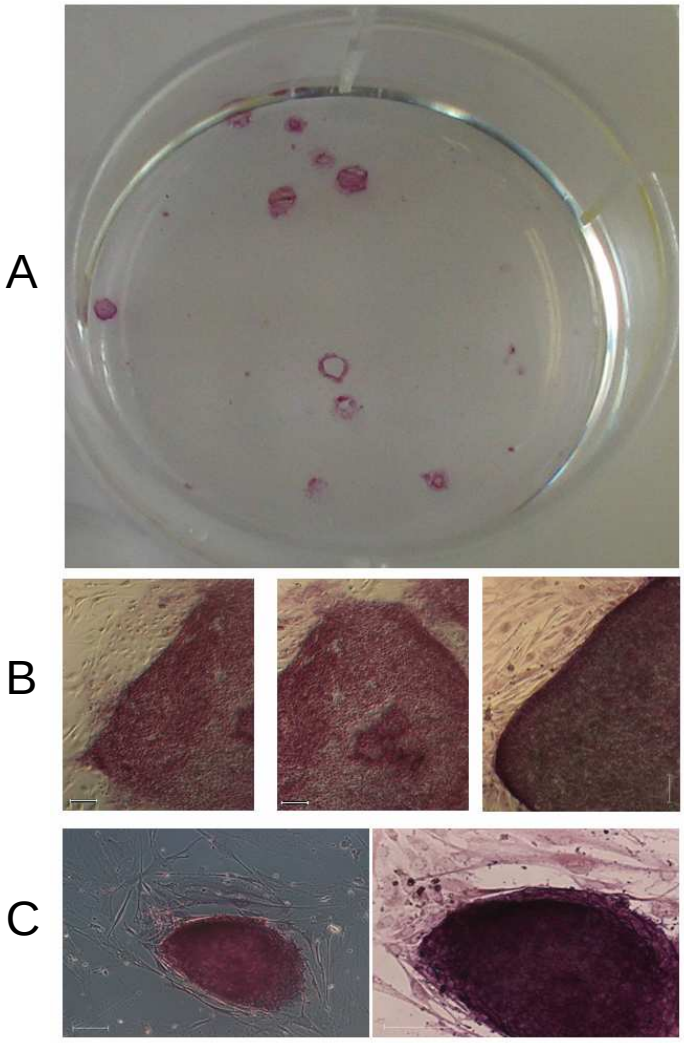


Figure 18 Similar activity of alkaline phosphatase in v-iPCS compared to the human embryonic stem cell line H1. A: alkaline phosphatase positive colonies of v-iPSCs in well of a 6-well cell culture dish. B: Example of a v-iPSC colony stained purple by alkaline phosphatase staining. 10-fld magnification, scale bar: 100 µm. All three pictures show the same colony. C: Colony of embryonic cell line H1 used as positive control. left: 10-fold magnification, right: 20-fold magnification, scale bar: 100 µm. v-iPSCs: passage 5, H1: passage 71.

4.2.2.3 v-iPSCs express NANOG

The confirmation of alkaline phosphatase expression alone is not enough to proof that v-iPSCs are indeed induced pluripotent stem cells. Therefore immunofluorescence staining of the pluripotency associated factor *NANOG* was carried out. H1 was used as positive control. The results clearly show that v-iPSCs express *NANOG* at a similar level like embryonic stem cells of the cell line H1. Only a very faint signal could be detected in the secondary antibody control underlining that the applied staining protocol for *NANOG* is not falsified by unspecific binding of the used secondary antibody. (Figure 19)

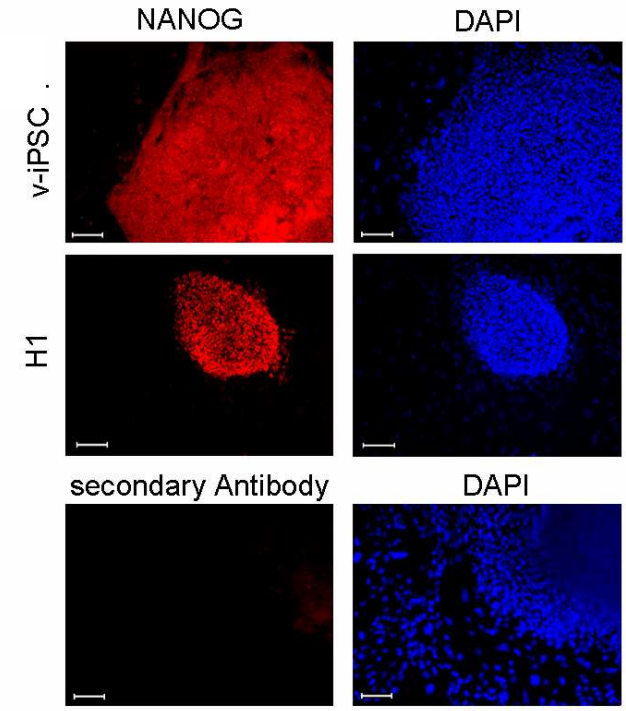


Figure 19 Similar protein expression of the pluripotency marker gene *NANOG* comparing v-iPSC to the embryonic stem cell line H1. v-iPSCs were used as second antibody control. v-iPSCs: passage 5, H1: passage 71. Fluorescence microscopy, 10-fold magnification, scale bar: 100 µm. Alexa Fluor 594 (red)

4.2.2.4 Spontaneous differentiation of hMSCs after retroviral reprogramming

Spontaneous differentiation of MSC17 derived potential iPSC-colonies into muscle cells

Cell accumulations of infected MSC17 appeared during retroviral reprogramming and were isolated at day 40 of the viral reprogramming experiment. Five of these colonies were isolated per experimental condition (control, p53, 2i and 2i+p53). When the cell clusters were cultured further under conditions for maintenance of hESCs without addition of inhibitors, only a very slow growth of the cells could be observed. The cells started to change their morphology one week after being isolated. The infected MSC17 cells became elongated and arranged themselves in bundles as shown in the first bright field picture of Figure 20. According to this observation it was postulated that the MSC17 cell accumulations differentiated into myocytes. To test the hypothesis the cells were stained by immunofluorecence staining specific for the protein alpha-smooth muscle actin. A positive fluorescence signal for alpha-smooth muscle actin could be found in all cell isolated cell clonies independent from the experimental condition during retroviral reprogramming. (Figure 20)

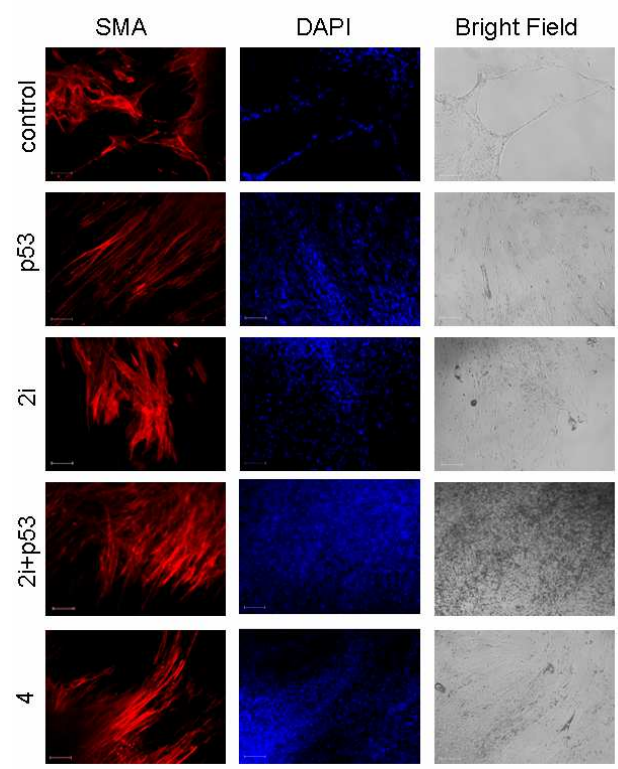


Figure 20 Possible differentiation of isolated cell colonies from viral reprogramming experiment carried out with MSC17 towards the myogenic lineage independent from the experimental condition. MSC17 cells could be stained positive for alpha-smooth muscle actin (left, red). Bright field and fluorescence microscopy, 10-fold magnification, scale bar: 100 µm, Alexa Fluor 594 (red). 2: no treatment p53: culture in the presence of Pifithrin α, 2i: culture in the presence of SB431542 and PD325901, 2i+p53: combination of both. 4: no treatment, $4x10^5$ cells were used for the retroviral infection instead of $2x10^5$ as for the other conditions.

Spontaneous differentiation of reprogrammed MSC74 cells into cardiomyocytes

The cellular reprogramming MSC74 by means of retroviruses resulted in one iPSC colony in the experimental condition 2i+p53 40 days after the retroviral infection. However, the isolated colony was not homogenous regarding the cells contained within. Differentiated cells could be observed growing next to the undifferentiated iPSC like cells in the colony. The iPSCs in the colony were mechanically isolated from the differentiated cells. Both parts of the colony were cultured further under conditions for the maintenance of hESCs. The cells of the already differentiated part of the original colony started to contract similar to cardiomyocytes. The contraction could be first observed four days after the differentiated part of the colony was servered from the part containing v-iPSCs.
To further determine whether the contracting cells are cardiomyocytes, immunofluorescence staining for the muscle specific marker alpha-smooth muscle actin and for the cardiomyocyte specific marker sarcomeric α actinin was performed. A positive fluorescence signal could be detected for both marker proteins. (Figure 21)

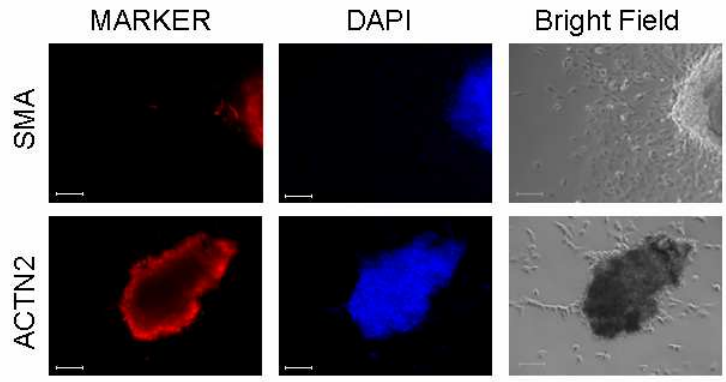


Figure 21 Spontaneous differentiation of a part of the isolated v-iPSC colony (experimental condition see Table 7) towards the cardiomyocyte lineage. Two parts of a contracting cell accumulation transferred to a cell culture dish coated with feeder cells and stained for alpha-smooth muscle actin (SMA) and cardiomyocyte specific marker sarcomeric α actinin (ACTN2) two days after the transfer. Bright field and fluorescence microscopy, 10-fold magnification, scale bar: 100 µm, Alexa Fluor 594 (red).

4.3 Reprogramming of hMSCs using non-integrating episomal plasmids

As only retroviral reprogramming of MSC74 cells and not of MSC17 cells resulted in iPSCs, the non viral reprogramming experiments were carried out with MSC74 cells.
Non viral reprogramming resulted in partially reprogrammed MSC74 cells. These cells are called p-iPSCs in this work and were more fully characterised than v-iPSCs. The reasons for this are that iPSCs produced in a non viral approach will be more secure when clinically applied than v-iPSCs that very likely contain random insertions of retroviruses throughout the genome. Furthermore, it has been published that hMSCs were reprogrammed with retroviruses carrying the factors *OCT4*, *SOX2*, *KLF4*, *c-Myc*, *SV40LT* and *hTERT* (Park et al., 2008). However, the efficiency of this method is very low. Non viral reprogramming of hMSCs using episomal plasmids has not been described until today.
In order to determine whether the hMSCs can be nucleofected with the episomal plasmids and if the genes on the episomal plasmids are expressed, MSC74 cells were nucleofected with the plasmid pEP4 E02S ET2K. ET2K contains the coding sequences of the genes *OCT4*, *SOX2* and *KLF4*. The protein products of theses genes could be detected by immunofluorescence staining. These findings indicate that episomal plasmids can be nucleofected into the nucleus of MSC74 and that the genes on ET2K are expressed in MSC74 cells even though the plasmid is not integrated into the genome of the cell. (Figure 22)

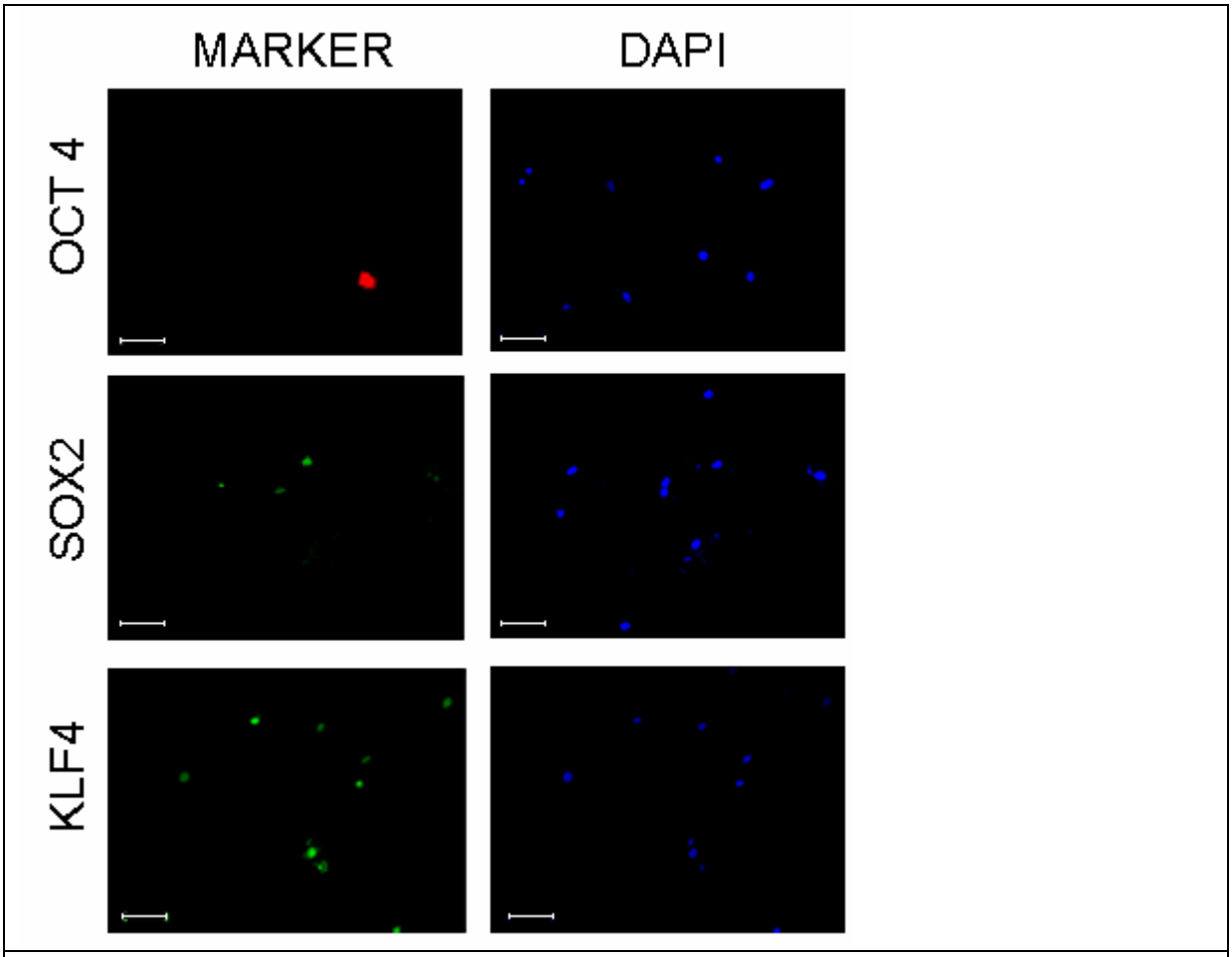


Figure 22 Protein expression of transgenes on the episomal plasmid pEP4 E02S ET2K (*OCT4*, *SOX2*, *KLF4*) after nucleofection into MSC74. The pictures were taken three days after nucleofection. Fluorescence microscopy, 10-fold magnification, scale bar: 100 µm, Alexa Flour 594 (red), Alexa Fluor 488 (green).

4.3.1 Generation of p-iPSCs

The Human MSC (Mesenchymal Stem Cell) Nucleofector® Kit (Lonza) was used for the nucleofection of the episomal plasmids into MSC74 cells. The plasmid pmaxGFP which was supplied with the kit was nucleofected into MSC74 as a positive control. One day after the nucleofection, *GFP* positive cells could be detected by fluorescence microscopy. An estimated 20 % of the nucelofected cells were detected as *GFP* positive indicating the nucleofection efficiency for single episomal plasmids in MSC74 in the plasmid based reprogramming experiments. (Figure 23)

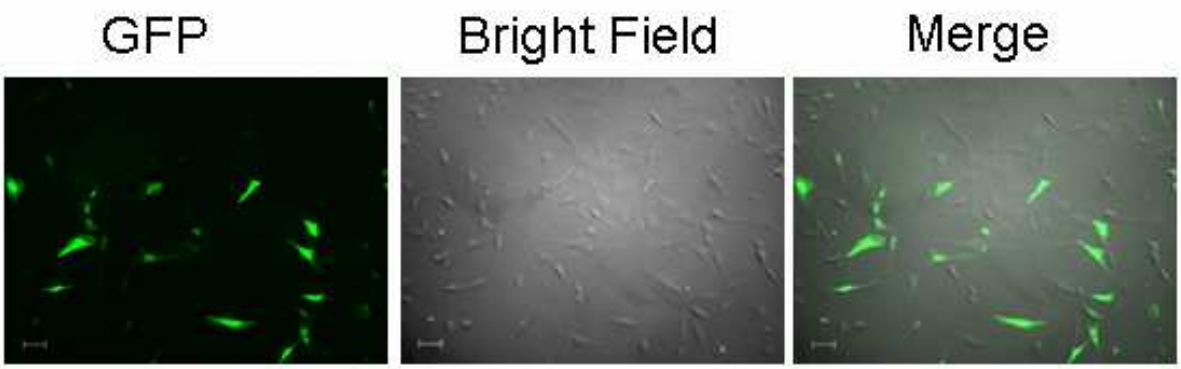


Figure 23 Protein expression of the *GFP* reporter gene on day one after nucleofection of plasmid pmaxGFP into MSC74. Bright field and fluorescence microscopy, 5-fold magnification, scale bar: 100 µm.

To achieve the aim of reprogramming hMSCs to induced pluripotent stem cell free of viral sequences episomal plasmids pEP4 E02S ET2K (ET2K) and pEP4 E02S EM2K (EM2K) were nucleofected in MSC74 cells.

Table 8 shows the results of the reprogramming experiments with episomal vectors using MSC74 cells. The fastest development of iPSC like cells could be observed in MSC74 cells nucleofected with ET2K and EM2K (condition 1). The plasmid combination resulted with additional treatment of 2i in three iPSC like colonies 20 days after the nucleofection. With an input of $1x10^6$ cells the efficiency of the iPSC generation in this condition was 0,0003 %. On day 27 after the nucleofection the iPSC like colonies were isolated as described in the methods section and further cultured. No iPSC lines could be established from the picked colonies of this experimental condition as the cells did not grow after their isolation.

Moreover, MSC74 cells were nucleofected with EM2K alone and cultured without 2i on Matrigel® (condition 4). This condition did not result in reprogrammed MSC74 cells.

Furthermore, MSC74 cells nucleofected with ET2K were seeded on Matrigel®, on feeder cells or on an uncoated cell culture surface and transferred after seven days on feeder cells (conditions 2, 3, 5, 6, 7, 8). In these three experimental conditions nucleofected MSC74 cells were cultured with and without 2i. Only the combination nucleofection of ET2K with subsequent seeding on an uncoated surface followed by reseeding on feeder cells and treatment with 2i (condition 8) resulted in four iPSC like colonies 20 days after the nucleofection. According to this number the efficiency of iPSC generation was 0,0004 %.

However, after further 30 days (day 50 after the nucleofection) of culture of the nucleofected MSC74 cells, 21 colonies of different sizes could be observed which changes the reprogramming efficiency to 0,0021 %. On day 27 after the nucleofection all four colonies that appeared from day 20 were isolated and cultured further. On day 60 14 iPSC like colonies were isolated from the same cell culture dish. The cell lines p-iPSC 1 and p-iPSC 2 were established from the colonies isolated on day 27. The cell lines p-iPSC 3 and p-iPSC 4 were established from the colonies that were isolated on day 60.

Table 8 General results of reprogramming experiments with episomal plasmids carried out with MSC74. Experimental conditions that resulted in colonies of potential induced pluripotent stem cells are marked bold. Experimental conditions 1 to 8 are described in the Methods section.

experimental condition	IPSC-like colonies obtained (day after nucleofection)	Picked colonies (day after nucleofection)	p-iPSC lines established
1	**3 (20)**	**3 (25)**	**0**
2	0	-	-
3	0	-	-
4	0	-	-
5	0	-	-
6	0	-	-
7	0	-	-
8	**4 (20)** **21 (50)**	**4 (27)** **14 (60)**	**2 (27)** **2 (60)**

4.3.2 Characterisation of p-iPSCs

4.3.2.1 No morphological similarity between p-iPSCs and hESCs

p-iPSCs have a different morphology compared to cells of the embryonic stem cell line H1. (Figure 24 A) p-iPSCs are bigger than ESCs and the cell shape is not round like H1 cells but more elongated with several edges. The nucleoli of p-iPSCs are not prominent compared to ESCs. Furthermore, p-iPSCs in one colony have different sizes whereas ESCs of the same colony have a similar size.

Figure 24 B shows that p-iPSCs grow in colonies like ESCs except that the borders of p-iPSC colonies are not defined as in hESC colonies. The area in the middle of a p-iPSC colony is elevated because the cells grow on top of each other, a feature which can not be found in ESCs.

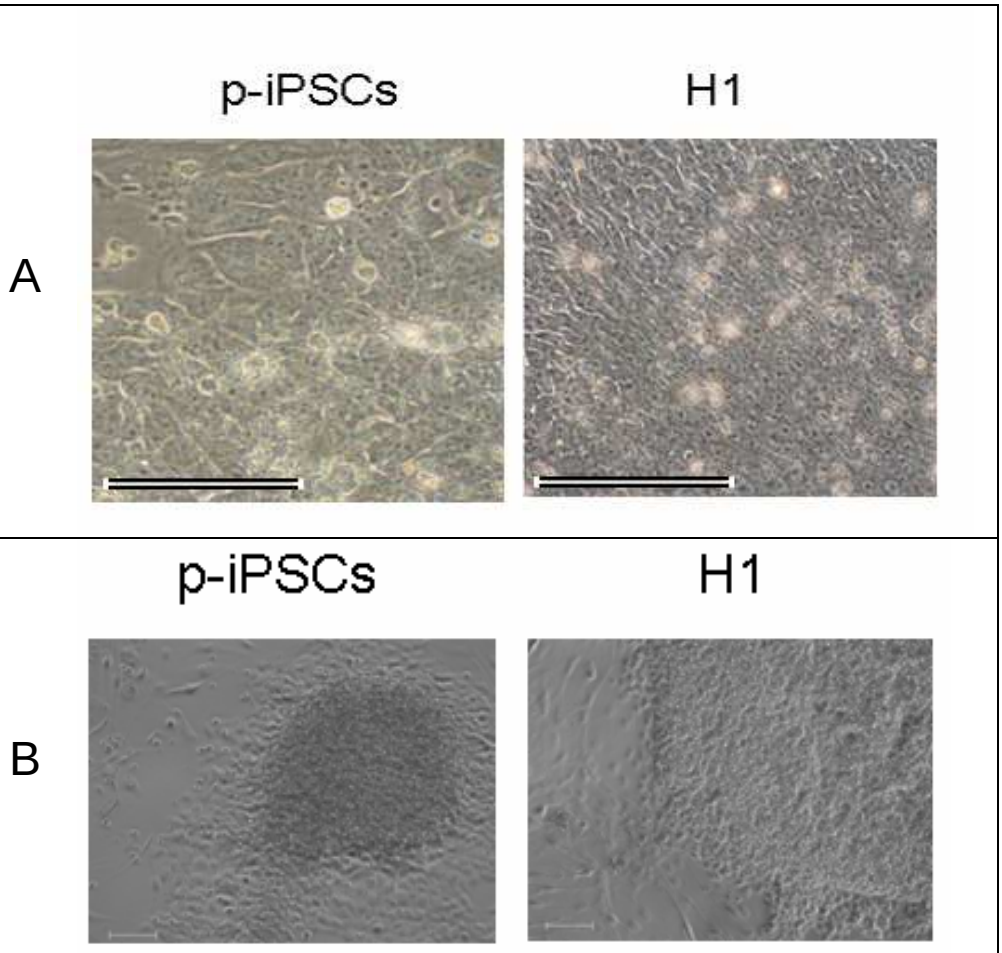


Figure 24 Morphology of p-iPSCs compared to the human embryonic stem cell line H1. The depicted cells were cultured on feeder cells. A: left: Example of the morphology of p-iPSCs (p-iPSC-1, passage 3 after isolation). right: morphology embryonic stem cell line H1 (passage 68). Bright field microscopy, pictures were taken with a digital camera (Canon; model Power shot A650IS) through the ocular of an inverted microscope (OLYMPUS; model CK2), magnification of 40-fold. Scale bar: 50 µm. B: Morphology of p-iPSC (p-iPSC-1, passage 7 after the isolation) and H1 (passage 72). Bright field microscopy, 10-fold magnification, scale bar: 100 µm. (microscope: Zeiss, LSM 510 Meta)

4.3.2.2 No expression of Alkaline phosphatase in p-iPSCs

The different morphology of p-iPSCs compared to ESCs lead to the question if p-iPSCs might express Alkaline phosphatase, a feature present in ESCs and iPSCs.
Therefore Alkaline phosphatase staining was performed on remaining p-iPSC colonies in the cell culture dish all p-iPSC lines were derived from. None of the remaining p-iPSC colonies were stained purple. This indicates that Alkaline phosphatase is not expressed in the cells of these colonies. (Figure 25 A right) Furthermore, none of the isolated p-iPSC lines were found to be Alkaline phosphatase positive as none of the cells were purple after the staining. (Figure 25 A left). The embryonic stem cell line H1 was used as positive control for the Alkaline phosphatase staining. H1 could be stained purple whereas p-iPSC 1 remained without staining using the same protocol for the Alkaline phosphatase activity assay as used for the staining of the p-iPSC lines. (Figure 25B)

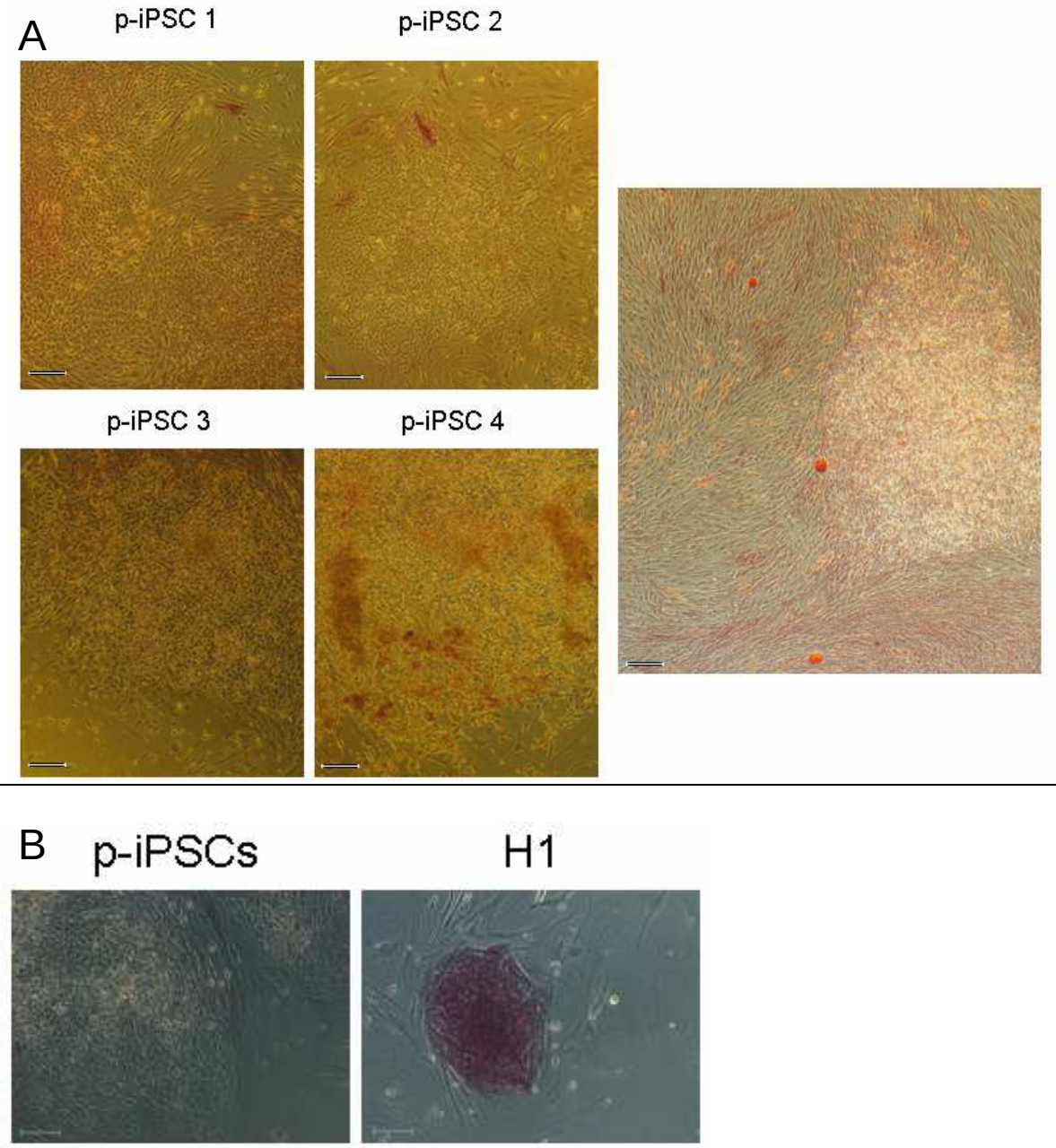


Figure 25 No alkaline phosphatase activity in p-iPSCs derived from MSC74. A: left: The four isolated p-iPSC lines after staining for alkaline phosphatase activity in passage 4. Right: example for a potential iPSC colony on the original plate after alkaline phosphatase staining and Oil Red O staining. Pictures were taken with a digital camera (Canon; model Power shot A650IS) through the ocular of an inverted microscope (OLYMPUS; model CK2), magnification 4-fold, scale bar: 50 µm.
B: p-iPSC 1 as example for the appearance of p-iPSCs after staining for alkaline phosphatase activity compared to alkaline phosphatase positivity in the embryonic stem cell line H1. Bright field microscopy, 10-fold magnification, scale bar: 100 µm. (microscope: Zeiss, LSM 510 Meta)

4.3.2.3 p-iPSCs are derived from MSC74 and not due to a contamination with H1, H9 or HFF1

The DNA fingerprint of all p-iPSC lines was compared to the fingerprint of MSC74, the embryonic stem cell lines H1, H9 and the human fibroblast line HFF1 to proof that p-iPSCs were not derived due to contamination of the cell culture with H1, H9 or HFF1.
The fingerprinting analysis revealed that all p-iPSC lines have the same DNA fingerprint as MSC74 and a fingerprint that is different from H1, H9 and HFF1. These results underline that all p-iPSCs are indeed derived from MSC74 and that the cell culture of the reprogramming experiments was not contaminated with H1, H9 or HFF1. (Figure 26)

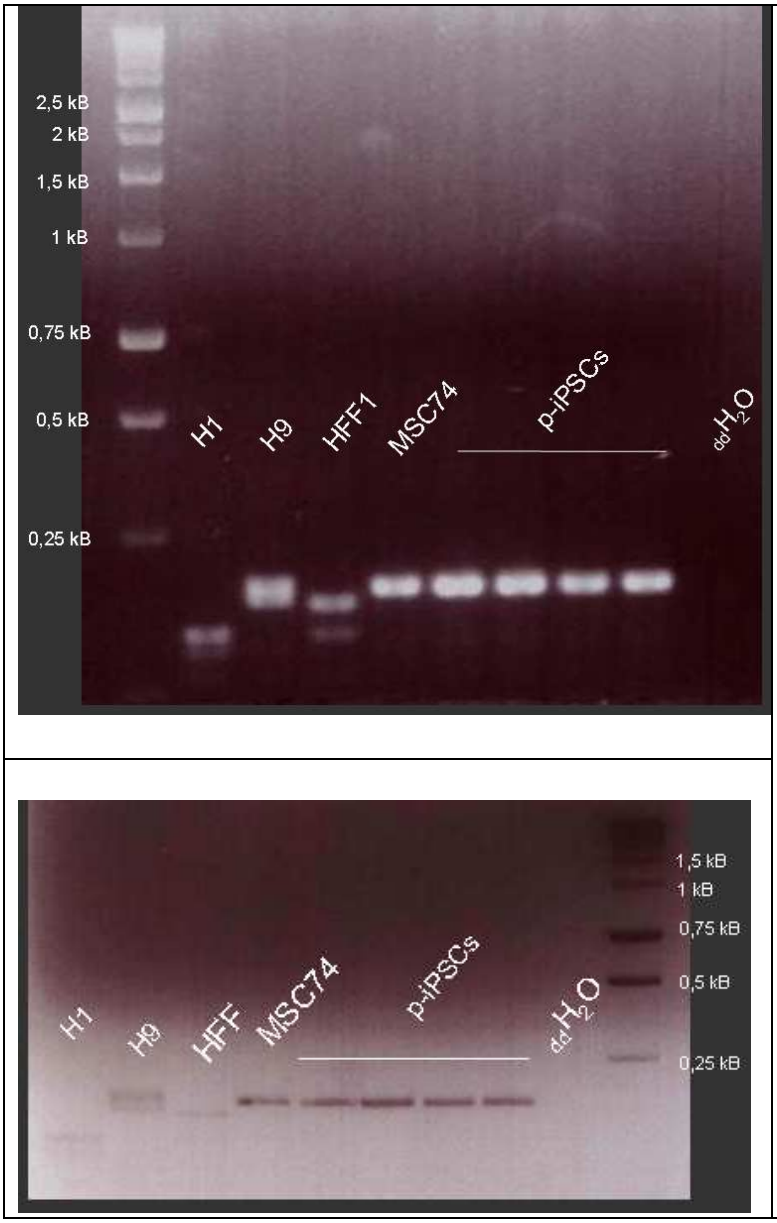


Figure 26 p-iPSCs have the same DNA fingerprint as MSC74 cells but a different DNA fingerprint compared to H1, H9 and HFF1. Left: Visualised fingerprinting PCR products, 2 % agarose gel. Right: Repetition of the gel electrophoresis using a 3 % agarose gel to enhance the resolution of sample H9. Samples: embryonic stem cell lines H1 and H9, Human foreskin fibroblasts (HFF1), human Mesenchymal stem cells from a 74 year old patient (MSC74), p-iPSC lines (from left to right p-iPSC 1, p-iPSC 2, p-iPSC 3, p-iPSC 4) and water control without template ($_{dd}H_2O$).

4.3.2.4 The oriP sequence of episomal plasmid pEP4 E02S ET2K is present in p-iPSCs

As episomal plasmids do not integrate into the genome and are gradually lost at 5 % per cell generation (Yu et al, 2009) it was postulated that the episomal plasmid ET2K which was used

to derive p-iPSCs from MSC74 is not present anymore in the cells of p-iPSC cell lines. To test whether the postulate was true a PCR was performed using primers specific for the oriP sequence on the episomal plasmid ET2K. Samples of the genomic DNA of MSC17 and MSC74 which were nucleofected with ET2K were used as positive controls. As shown in Figure 27 the oriP sequence was not detected in the genomic DNA of MSC74 but in the genomic DNA of the positive controls and in all p-iPSC lines as in these samples a PCR product was visible.

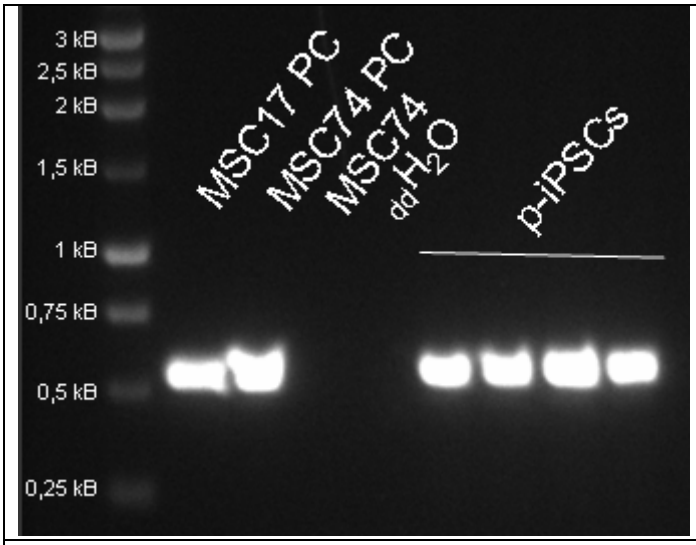


Figure 27 Genomic DNA of all four p-iPSC lines contain a sequence resulting in PCR products for primers specific for the oriP of the plasmid pEP4 E02S ET2K. Samples: positive control (MSC17 PC, MSC74 PC), MSC74, water control without template ($_{dd}H_2O$), p-iPSC lines (from left to right p-iPSC 1, p-iPSC 2, p-iPSC 3, p-iPSC 4). PCR products were separated in an 1,5 % agarose gel.

4.3.2.5 Protein expression of pluripotency associated genes in p-iPSCs

The protein expression of pluripotency associates marker genes *OCT4*, *NANOG*, *SOX2*, *KLF4* and *c-Myc* as well as the expression of the surface markers *SSEA-4*, *SSEA-1*, *TRA-1-60* and *TRA-1-81* was analysed in p-iPSC 1 and p-iPSC 4 compared to H1 as positive control by immunofluorescence staining.

OCT4, SOX2 and KLF4 were detected as clearly only expressed in the nucleus. The same nuclear expression could be detected in H1. (Figure 28 and Figure 29) The staining specific for NANOG and c-Myc resulted in a positive but not clearly nuclear fluorescence signal. As NANOG and c-Myc are nuclear proteins as also shown in H1 (Figure 29), it is not clear whether NANOG and c-Myc are expressed in p-iPSCs. Moreover, the surface marker SSEA-1 could not be detected in both p-iPSC lines and H1. This result was expected as SSEA-1 is not expressed on the surface of hESCs. A faint signal of the surface marker SSEA-4 could be detected for p-iPSC 1 and 4. However the signal was much higher when the same staining was applied to H1 cells. (Figure 28 and Figure 29)

Fluorescence signals for the surface marker TRA-1-60 could be detected for p-iPSC 1 and 4 after immunofluorescence staining. However, the detected fluorescence signal was higher in p-iPSC 4 and higher than both p-iPSC lines in the positive control H1. Furthermore, a very

faint signal for TRA-1-81 was detected for both p-iPSC lines which was much higher in H1. (Figure 28 and Figure 29)

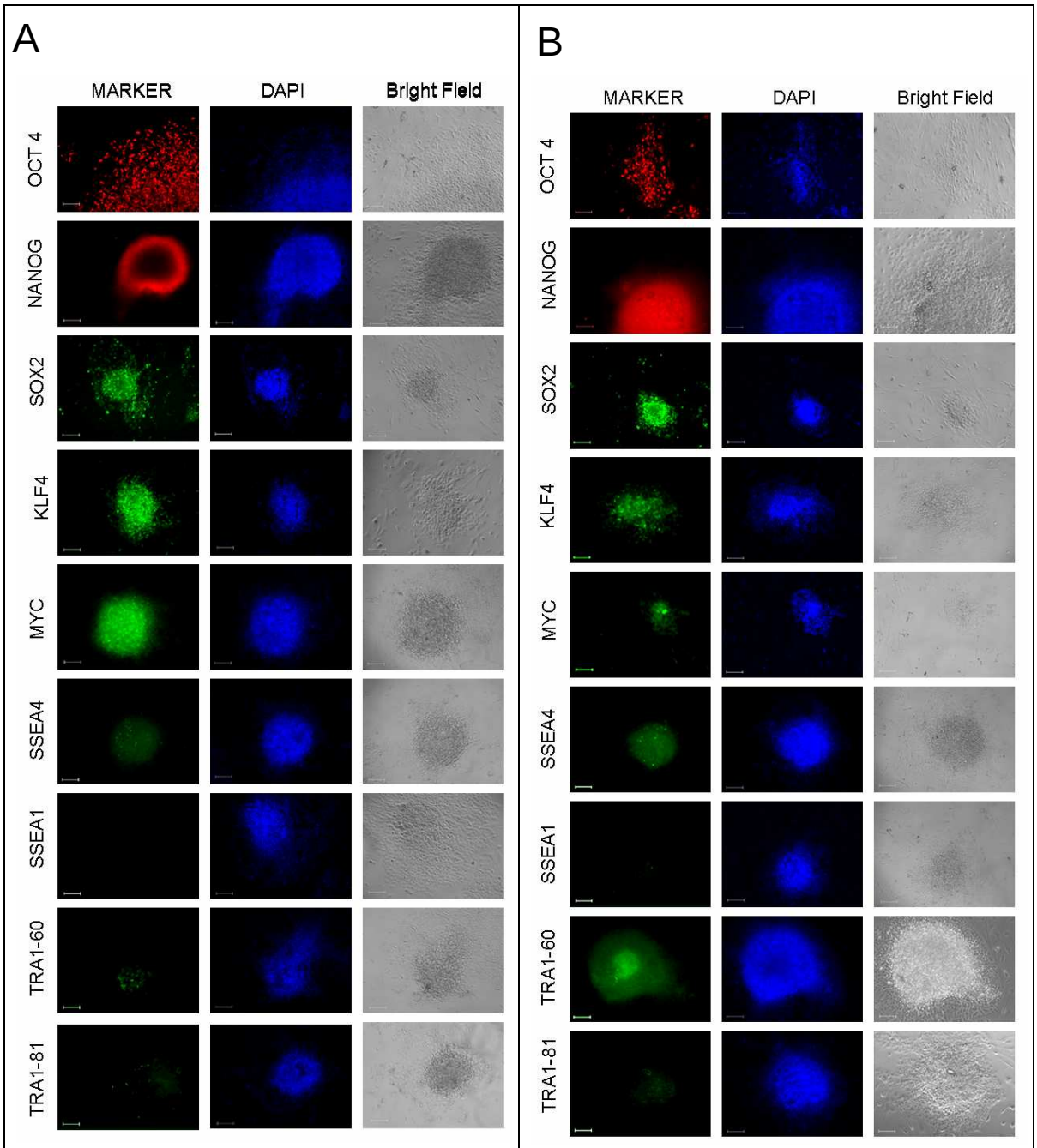


Figure 28 Protein expression of the pluripotency associated marker genes *OCT4 (OCT 4)*, *NANOG*, *SOX2*, *KLF4*, *c-Myc* (MYC) and the cell surface markers *SSEA-4 (SSEA4)*, *SSEA-1 (SSEA1)*, *TRA-1-60 (TRA1-60)* and *TRA-1-81 (TRA1-81)* in p-iPSC 1 (A) and p-iPSC 4 (B). Both cell lines were in passage 12 after their isolation. Culture on feeder cells. Bright field and fluorescence microscopy 10-fold magnification, scale bar: 100 µm, Alexa Flour 594 (red), Alexa Fluor 488 (green).

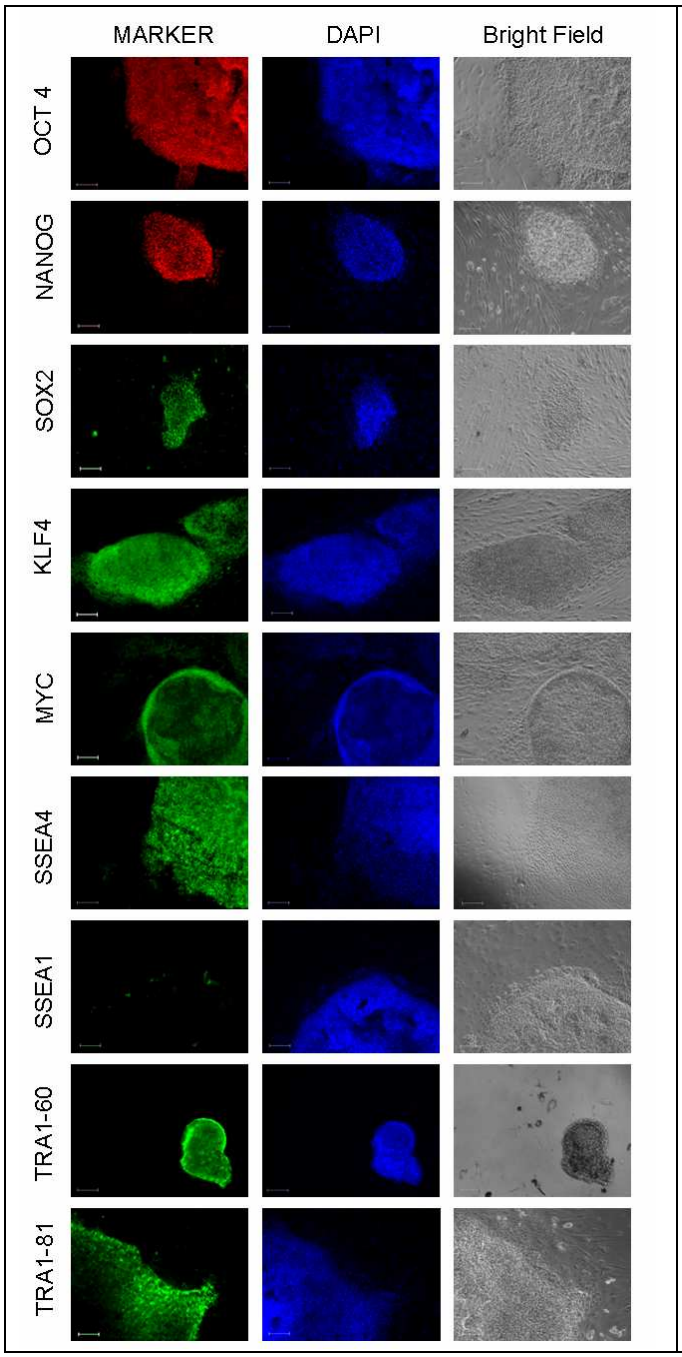


Figure 29 Protein expression of the pluripotency associated marker genes *OCT4 (OCT 4)*, *NANOG*, *SOX2*, *KLF4*, *c-Myc (MYC)* and the cell surface markers *SSEA-4 (SSEA4)*, *SSEA-1 (SSEA1)*, *TRA-1-60 (TRA1-60)* and *TRA-1-81 (TRA1-81)* in the human embryonic stem cell line H1. The cells were cultured on feeder cells. Positive control for the staining of the same factors in p-iPSC 1 and p-iPSC 4. Bright field and fluorescence microscopy, 10-fold magnification. Scale bar: 100 µm Alexa Flour 594 (red), Alexa Fluor 488 (green).

4.3.2.6 Extended differentiation potential of p-iPSCs

p-iPSCs form embryoid bodies that can be differentiated to cells of all three germ layers

Four iPSC like cell lines could be established by non viral reprogramming of MSC74. To analyse whether the cells of the p-iPSC cell lines are pluripotent, an embryoid body based differentiation into all three germ layers was carried out with cells of all p-iPSC lines as described in the Material and Methods section.
The content of three wells of a 6-well culture dish containing the respective p-iPSC line was cultured in low attachment cell culture dishes for seven days. All p-iPSC lines developed embryoid bodies as depicted in Figure 30 A.

The embryoid bodies were plated on gelatine coated cell culture dishes after seven days. 14 days after the embryoid bodies were plated, the outgrowing cells of the embryoid bodies of all four p-iPSC lines were stained by immunofluorescence staining specific for markers of the three germ layers. The result of the staining is depicted in Figure 30 B.
A positive staining signal for *SOX17*, a marker of endodermal differentaion was detected in all four p-iPSC lines. *SOX17* was only detected in the nucleus of the cells as expected.
A strong flourescence signal was detected in all p-iPSC lines that were stained for alpha-smooth muscle actin. However, the most cells stained positive for alpha-smooth muscle actin were found among p-iPSC4 cells. Furthermore, a positive fluorescence signal could be detected in all p-iPSC lines after being stained for *TUJ1*, a protein expressed in neurons and a marker of endodermal differentiation. However, the morphology of the stained cells did not display a network of elongated cells but cells with a fibroblast like morphology in the case of p-iPSC1, 2 and 4. The morphology of *TUJ1* positive p-iPSC 3 cells differed from the other cell lines. The cells were smaller and grew in clusters. In addition to that, fluorescence signals for *NESTIN*, a marker of ecdodermal differentiation which is expressed in neural progenitor cells could be detected in p-iPSC 1, 2 and 3. *NESTIN* positive fluorescence signals were detected in the cytoplasm of the cells as expected. *NESTIN* positive p-iPSCs had a smaller cell size compared to *TUJ1* positive p-iPSCs.

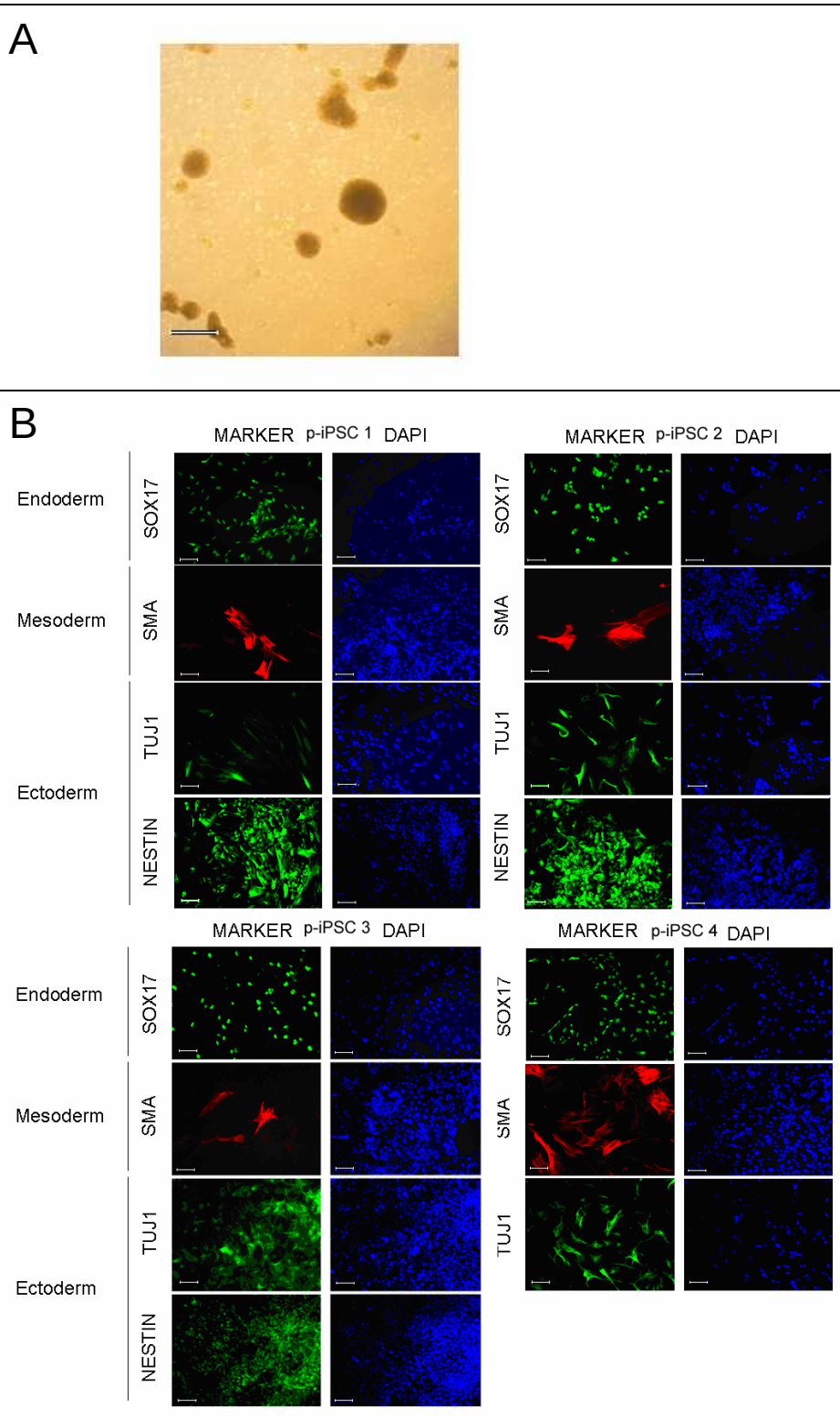


Figure 30 Embryoid body based differentiation of four isolated p-iPSC lines p-iPSC 1, 2, 3 and 4 in lineages of endoderm, ectoderm and mesoderm. A: left: example of morphology of embryoid bodies derived from p-iPSC 1 at day seven in suspension. Bright field microscopy, pictures were taken with a digital camera (Canon; model Power shot A650IS) through the ocular of an inverted microscope (OLYMPUS; model CK2), magnification of 4-fold, scale bar: 100 µm. B: Markers of endoderm (*SOX17*), mesoderm (*SMA*) and ectorderm (*NESTIN*, *TUJ1*) expressed in differentiated p-iPSCs after embryoid body formation and subsequent seeding on gelatine. Fluorescence microscopy, 10-fold magnification, scale bar: 100 µm, Alexa Flour 594 (red), Alexa Fluor 488 (green).

p-iPSCs retain a capability to differentiate into adipocytes and osteoblasts that is different to MSC74

The fact that p-iPSCs can be differentiated into cells that express all three germ layers lead in the same time to the question whether the cells retained the potential to differentiate into adipocytes and osteoblasts. To answer the question p-iPSC 1 cells were trypsinised and used in an *in vitro* adipocyte differentiation and osteoblast differentiation experiment. The differentiation protocol that was used for the *in vitro* differentiation of MSC74 was applied for p-iPSC 1. Only few and small (~1 % of the treated cells) adipocytes, stained positive for Oil red O, could be detected after 21 days in adipocyte differentiation media compared to culture in the control media. However, an estimated 90 % of p-iPSC 1 could be stained red by Alizarin Red S staining after 24 day of culture in osteoblast differentiation media compared to p-iPSC 1 cells cultured in control media for the same time. (Figure 31)

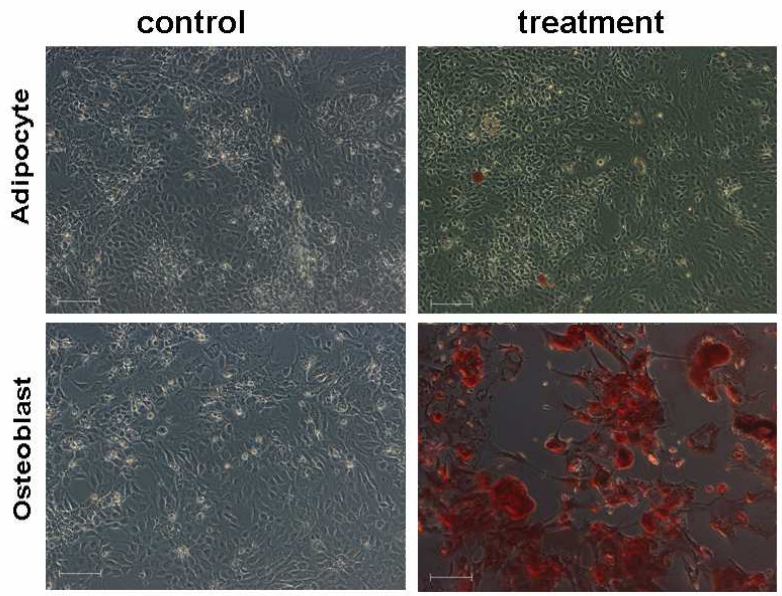


Figure 31 *In vitro* adipocyte differentiation (upper panel, Adipocyte) and osteoblast differentiation (lower panel, Osteoblast) of p-iPSC 1 in passage 7. Trypsinised p-iPSCs were treated according to the differentiation protocols for hMSC described in methods. Adipocyte: Oil red O stained cells after 21 days of culture in adipocyte differentiation media (treatment) or control media (control). Osteoblast: Cells stained with Alizarin Red S after 21 days of culture in osteoblast differentiation media (treatment) or control media (control). Bright field microscopy, 10-fold magnification, scale bar: 100 µm.

5 Discussion

The focus of this study was to comparatively reprogram human bone marrow derived mesenchymal stem cells obtained from patients of different ages to induced pluripotent stem cells with viral and non viral methods.

hMSCs derived from a 74 year old patient (MSC74) could be reprogrammed with retroviruses carrying the genes *OCT4*, *SOX2*, *KLF4* and *c-Myc* and additional inhibition of MEK, ALK4/5 and 7 as well as p53. A high similarity between hESCs and the derived v-iPSCs could be confirmed. hMSCs from a 17 year old patient (MSC17) could not be reprogrammed to pluripotency under the same conditions implying that age-related differences in the gene expression might have had an adverse effect on iPSC generation. A preliminarily conducted microarray-based gene expression analysis highlighted the down-regulation of cell cycle and p53 signaling associated genes in MSC74. It has been described that activation of p53 related pathways due to oxidative stress and DNA damage in cellular senescence, decreases the efficiency of iPSC generation (Banito et al., 2009; Prigione et al., 2010).Therefore a contribution of this age-related effectto reprogrammming of hMSCs is possible.

To derive iPSCs without virus integrations MSC74 cells were reprogrammed with episomal plasmids carrying the genes *OCT4*, *SOX2*, *KLF4* and *SV40LT* (ET2K) or *OCT4*, *SOX2*, *KLF4* and *c-Myc* (EM2K). The combination ET2K/EM2K and ET2K alone yielded partially reprogrammed p-iPSC colonies when MEK and ALK 4/5 and 7 were inhibited using the small molecule inhibitors PD325901 and SB431542. The efficiency of this method was with 0,0004 % equal to the efficiency of retroviral reprogramming of hMSCs of the same donor but lower than the previously reported efficiency of 0,003 % when only retroviruses and six instead of four factors are used (Park et al., 2008).

Four p-iPSC cell lines were established from MSC74 reprogrammed with ET2K.

p-iPSCs were distinct from hESCs and v-iPSCs in terms of morphology and expression of pluripotency markers. They do not express Alkaline phosphatase and NANOG but OCT4. Comparative global transcription analysis using embryonic stem cells as reference has not been carried out but planned.

However, the cells formed embryoid bodies and spontaneously differentiate into cells expressing markers of endoderm ectoderm and mesoderm. Yet, the morphology partly did not reflect the expressed marker due to non specific staining.

In addition, p-iPSCs could be differentiated into adipocytes with an estimated 20-fold lower and osteoblasts with an estimated two-fold higher efficiency compared to MSC74 suggesting a shift in cell commitment.

v-iPSCs could be used in the development of patient specific iPS cell based disease models.

However, the use of viruses is associated with several problems like random integrations in the genome, which might affect the differentiation potential and cell behaviour. Moreover, viruses can reactivate at a later time. Therefore, iPSCs that were generated without viruses are safer for the application in medicine.

In contrast to that, p-iPSCs could be a novel intermediate of iPSC generation. As they do not fulfil all the criteria of fully reprogrammed iPS cells they might not be pluripotent. However they retain hMSC differentiation and acquired additional differentiation properties as it was shown by the embryoid body based differentiation. p-iPSCs might not form teratomas in immune compromised mice as iPS cells due to their apparent partial reprogrammed state. When

this can be confirmed p-iPSCs could represent a safer source for regenerative medicine based applications.
In addition, p-iPSCs acquired an apparent unlimited propagation compared to their parental cells. Therefore, the shown protocol for p-iPSC generation could help to circumvent senescence related limitations for the *in vitro* expansion of hMSCs.

5.1 Age-related differences have an impact on reprogramming of hMSCs

It is still a matter of debate whether the donor age of hMSCs has an influence on the cells. There is evidence that the *in vitro* lifespan of hMSCs from older donors is about one fourth shorter than from younger individuals before the cells go into senescence (Shibata et al., 2007). Therefore, a higher number of senescent cells may have been present in the whole cell population of MSC74 cells compared to MSC17 cells at the time of retroviral infection. Furthermore, it is described that the four reprogramming factors *OCT4*, *SOX2*, *KLF4* and *c-Myc* trigger senescence by up-regulating p53 and other factors (Banito et al., 2009).
According to this the already present senescence of MSC74 cells was very likely multiplied by the transduction of *OCT4*, *SOX2*, *KLF4* and *c-Myc*. As senescence is described to block iPSC generation (Banito et al., 2009) it was expected that it is harderto reprogramm MSC74 than MSC17 due to elevated levels of senescence related p53 and associated genes. In contrast to that, the fact that MSC74 and not MSC17 could be reprogrammed confirms that hMSCs of older donors are easierto reprogramm to iPSCs.
A possible reason for this could be that p53 and cell cycle associated genes are down-regulated in MSC74 compared to MSC17.
One of these cell cycle associated genes is *CCNA2*. A down-regulation of CCNA2 through inhibition of OCT4 in human multipotent stem cells has been described (Seo et al., 2009). Therefore the higher level of CCNA2 in MSC17 might have impaired OCT4-regulated cell cycle function during retroviral reprogramming.
A further gene that could have blocked reprogramming of MSC17 is *PKMYT1*. This gene encodes the kinase MYT1. MYT1 negatively regulates the G2-phase Mitosis transition in the cell cycle (Fattaey and Booher, 1997). It might be possible that this MYT1 dependent regulation was impaired in MSC74 cells and that iPSC generation from MSC74 was therefore promoted
Additional genes that may play a role in the promotion of cellular reprogramming of MSC74 are *TTK*, which regulates the mitotic checkpoint and interacts with p53 (Huang et al., 2009), *PTTG1*, which represses p21 and p53 (Tong and Eigler, 2009) and BUB1, a gene that regulates the sensitivity of the mitotic spindle checkpoint (Tang et al., 2004).
A role of these genes in blocking iPSC generation from hMSCs is possible and has to be investigated in the future.
Among the down-regulated genes in MSC74 there were two genes associated with both p53 signaling and cell cycle regulation, namely CCNB2 and CHEK1.
CCNB2 encodes cyclin B2, and is mainly expressed during the G2-phase of the cell cycle (Brandeis et al., 1998). The transcription of CCNB2 is controlled by p53 (Müller and Engeland, 2010). The inhibition of p53 might have reduced the cyclin B2 levels in MSC74 during viral reprogramming thereby allowed iPSC generation.
In contrast to that, CHEK1 is involved in the negative regulation of cell proliferation and control of cell cycle progression upon DNA damage (Sanchez et al., 1997) and might have

blocked with this cellular function iPSC generation in MSC17. Moreover, the gene *GTSE1* may have blocked iPSC generation in hMSCs by its function in the G2 mitosis transition of the cell cycle (Sanchez et al., 1997).
The discussed cell cycle and p53 signaling related genes could have prevented iPSC generation from MSC17 in a concerted manner or a single gene or group of the genes might have caused the observed age dependent block of iPSC generation from hMSCs. Therefore, monitoring of the expression of the discussed genes at different time points by real time PCR during the reprogramming process may reveal their role in the reprogramming process of hMSCs.
Similar results were observed for fibroblasts as the efficiency of iPSC generation from these cells could be enhanced by modulating p53 and its downstream targets (Wang and Adjaye, unpublished data).

5.2 Comparison of viral and non viral reprogramming and effect of the application of inhibitors on reprogramming of hMSCs

hMSCs are multipotent cells with self-renewal abilities and have therefore features rendering them more similar to embryonic stem cells and induced pluripotent stem cells than differentiated fibroblasts, the cell type which has been used most in reprogramming experiments of human cells (Kiskinis and Eggan, 2010). Taking this higher similarity into account, it is more likely that hMSCs can be reprogrammed with a higher efficiency in terms of velocity and number of appearing colonies than human fibroblasts.
The results of the viral and non viral reprogramming experiments could not confirm this conclusion because only MSC74 could be fully reprogrammed with the help of a combination of three different inhibitors. The efficiency of iPSC generation was lower compared to human fibroblasts. This observation has been described for the retroviral reprogramming of hMSCs with six factors (Park et al., 2008).
Human fibroblasts were reprogrammed to iPSCs with an efficiency of 0,02 % measured on day 30 after the retroviral infection (Takahashi et al., 2007). However, no iPSC colonies could be derived from MSC17 and MSC74 when the same protocol was applied.
Only the addition of the inhibitors PD325901, SB431542 (2i) and Pifithrin α to the culture media resulted in one iPSC colony from $2x10^5$ infected MSC74 cells on day 40 after retroviral infection which is a 50-fold lower reprogramming efficiency compared to fibroblasts.
In addition to that, the application of PD325901 and SB431542 during retroviral reprogramming of human fibroblasts was described to enhance the efficiency of iPSC generation over 100-fold (Lin et al., 2009). Taking this finding into account, the reprogramming efficiency of MSC74 would be an estimated 100-fold lower than 0,0004 %.
Furthermore, the use of 2i alone did not promote iPSC derivation from MSC17 and MSC74 in retroviral reprogramming experiments underlining that the inhibition of p53 allowed reprogramming of MSC74 to iPSCs. This result confirms previous studies stating that p53 and related genes play a role in blocking iPSC generation (Hong et al., 2009).
Moreover, the number of cells infected by all four retroviruses leading to the intracellular expression of all four factors is due to statistical reasons very small. Therefore, the low efficiency of the iPSC generation from MSC74 may reflect the amount of cells infected with the right number of transgene carrying retroviruses.

In addition, it has been described that hMSCs transduced with *OCT4*, *SOX2*, *KLF4*, *c-Myc* as well as *SV40LT* and *hTERT* yielded 0,003 % iPSCs (Park et al., 2008) which is higher than the reprogramming efficiency of 0,0004 % of MSC74 cells reprogrammed with retroviruses in this study. Therefore, the combined use of PD325901, SB431542 and Pifithrin α might not completely replaced the effects of *SV40LT* and *hTERT* in cellular reprogramming of hMSCs.
Moreover, the fact that MSC17 could not be reprogrammed by retroviruses with the same conditions as MSC74 cannot be due to the function of the produced retroviruses. The preliminary infection of MSC17 and subsequent detection of the contained factors, demonstrated that protein expression of the transgenes could be detected in about 90 % of the MSC17 cells.

Furthermore, bone marrow derived mesenchymal stem cells of other species were reprogrammed to induced pluripotent stem cells, too. iPSCs could be derived from MSCs of the rat with an efficiency of 0,024 % with a lentiviral vector system (Liao et al., 2009). However, different cells require different induction levels of the reprogramming factors to induce pluripotency (Wernig et al., 2008). Thus, human mesenchymal stem cells might require different induction levels of the transcription factors than rat derived MSCs.
In the non viral reprogramming experiments two of the analysed experimental conditions lead to a partly reprogrammed state of the reprogrammed MSC74 cells (p-iPSCs).
To reprogram MSC74, the cells were nucleofected with the episomal plasmids pEP4 E02S ET2K (ET2K) and pEP4 E02S EM2K (EM2K) as well as ET2K alone and EM2K alone. p-iPSCs could only be generated when nucleofected MSC74 cells were additionally treated with the inhibitor combination 2i.
The reprogramming of multipotent adult human adipose stem cells on Matrigel® resulted in a lower reprogramming efficiency compared to adipose stem cells that were reprogrammed on feeder cells (Sun et al., 2009). Consistent with this MSC74 cells seeded on Matrigel® after the nucleofection of either EM2K or ET2K did not give rise to p-iPSCs as the efficiency might have been low due to effects of Matrigel®. Moreover, the addition of 2i did not help reprogramming MSC74 cells using Matrigel® and episomal plasmids. Therefore it might be that the enhancing effect of 2i depends on the presence of feeder cells during reprogramming.
The combined nucleofection of ET2K and EM2K with addition of 2i resulted in the appearance of three p-iPSC colonies on day 20 after the nucleofection. Thus, p-iPSC colonies could be derived faster than retroviral iPSCs, which could only be observed from day 40 after the retroviral infection. The efficiency of the non viral method was with 0,0003 % slightly lower than the efficiency of the viral method.
Furthermore, the lower reprogramming efficiency might be due to the very likely possibility that only a low percentage of the MSC74 cells contained both ET2K and EM2K and that only these cells gave rise to iPSCs in this experimental condition.
The second non viral experimental condition that leadsto reprogrammmed MSC74 cells was nucleofection with only ET2K, subsequent seeding on an uncoated surface followed by reseeding on feeder cells after seven days and treatment with the inhibitor combination 2i. Four p-iPSC colonies could be observed from 20 days after the nucleofection. Thus, p-iPSC colonies developed with an efficiency of 0,0004 %, which is similar but slightly higher than the discussed combination EM2K and ET2K. This is probably due to the reseeding step in this condition. As in this case only plasmid ET2K was used, it might be that rather SV40LT on ET2K alone leads to an enhanced p-iPSC generation than in combination with c-Myc on plasmid EM2K.
Surprisingly, 21 more p-iPSC colonies could be observed from day 50 after the nucleofection in this experimental condition, which elevates the reprogramming efficiency to 0,0021 %. A

possible explanation for the stepwise appearance of p-iPSCs is that the colonies which appeared first were infected with several ET2K plasmids resulting in higher levels of transgene expression in the respective cells.
Secondly, hMSCs derived from bone marrow aspirates consist of several subsets of cells with different features (Roobrouck et al., 2008). Therefore it is possible, that the different time points of the iPSC development is due to their belonging to different subpopulations within the MSC74 cells.
So far only human fibroblasts have been reprogrammed to iPSCs by means of episomal plasmids (Yu et al., 2009). However, successfully reprogrammed fibroblasts were nucleofected in addition to ET2K with episomal plasmids carrying further transgenes like *NANOG* or *LIN28* resulting in reprogramming efficiencies of 0,003-0,006 % of fully reprogrammed iPSCs compared to 0,0021 % of partially reprogrammed p-iPSCs. Therefore, the additional use of *NANOG* or *LIN28* might be useful for non viral reprogramming of hMSCs. It has not been shown, whether it is possible to derive iPSCs from fibroblasts using ET2K alone or in combination with EM2K.
Distinct morphological changes but no alkaline phosphatase positive iPSC colonies could be observed when ET2K was usedto reprogramm human fibroblasts together with a plasmid carrying *c-Myc* and *LIN28* (Yu et al., 2009). This could explain that p-iPSCs derived using ET2K were alkaline phosphatase negative but showed morphological changes. However, as hMSCs are different from fibroblasts in many aspects other factor combinations might be necessary to achieve similar results as in fibroblasts.
Moreover, there are other non viral methods for iPSC. One non viral method uses recombinant proteins for iPSC generation from fibroblasts. The reprogramming efficiency was higher than the non viral methods used for hMSCs. However, iPSCs appeared only after eight weeks (Kim et al., 2009). The time scale of this method shows that the slower reprogramming kinetics of MSC74 cells compared to fibroblasts might be normal for hMSCs. In contrast to that, MSC74 could be reprogrammed to p-iPSC colonies in 20 days with the episomal plasmids ET2K and the combination of ET2K and EM2K both with additional treatment with 2i. This time frame is similar to the retroviral reprogramming of human dermal fibroblasts which was shown to take around 25 days (Takahashi et al., 2007).
Moreover, the probably partially reprogrammed state of p-iPSCs might also be a result of the effects of the protein EBNA-1 in nucleofected hMSCs. It has been described that EBNA-1 proteins bind to cellular promoters in B cell lines and epithelial cell lines (Canaan et al., 2009). According to this, EBNA-1 might also bind to promoters in the genome of hMSCs which could have an impact on the reprogramming process.
Furthermore, p-iPSCs should lack integrated transgenes from the episomal plasmids as episomal plasmids are gradually lost at 5 % per cell generation due to defects in plasmid synthesis (Nanbo et al., 2007). However, the presence of the oriP of the episomal plasmid ET2K was demonstrated by PCR. This unexpected result could either suggest, that the episomal plasmid integrated into the genome or that it is still present in the episomal fraction which has been described for iPSC clones derived from fibroblasts (Yu et al., 2009). Real time PCR with transgene specific primers and southern blot analysis of the genomic DNA from p-iPSC lines will reveal whether the plasmids are integrated into the genome.

5.3 Possible effects of culture conditions on hMSCs during the reprogramming experiments

A different aspect of the reprogramming experiments is the influence of FGF2; cell density and the used inhibitors on hMSCs.
hMSCs are usually cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10 % fetal bovine serum. During the viral and non viral reprogramming experiments the hMSCs were cultured for up to 50 days in the presence of FGF2. It has been described that FGF2 allows self-renewal of human embryonic stem cells (Greber et al., 2007) and promotes multipotency and cell growth of hMSCs (Tsutsumi et al., 2001). Therefore it is likely that the proliferation of MSC17 and MSC74 was enhanced by FGF2 in the culture media during viral and non viral reprogramming. This might have contributed to the generation of v-iPSCs or p-iPSCs or impaired viral iPSC derivation from MSC17 and non viral iPSC generation from MSC74.
Moreover, hMSCs were cultured without being trypsinised beyond the time point when they reached confluence. It has been described that hMSCs show elevated levels of apoptosis when cultured at high densities (Song et al., 2009). This density induced increase of apoptotic cells might have lowered the reprogramming efficiencies of MSC74 in viral and non viral reprogramming experiments.
Pifithrin α, the p53 inhibitor used in retroviral reprogramming experiments, reversibly inhibits p53 mediated apoptosis and p53 dependent gene expression (Komarov et al., 1999). Furthermore, v- iPSCs that are closer to hESCs in terms of morphology, expression of alkaline phosphatase and *NANOG* could be generated only in the presence of Pifithrin α. This result shows that the use of Pifithrin α together with inhibitors of MEK and ALK 4/5 and 7 allowed to overcome the up-regulation of p53 that is triggered by the expression of *OCT4*, *SOX2*, *KLF4* and *c-Myc* in somatic cells and represents an obstacle to iPSC generation (Banito et al., 2009). In which way the parallel inhibition of p53, MEK and ALK 4/5 and 7 promoted the iPSC generation from MSC74 remains to be determined.

5.4 Comparison of v-iPSCs and p-iPSCs to hMSCs and hESCs

Before viral and non viral reprogramming of MSC17 and MSC74 cells it had to be confirmed that these cells display features of hMSCs. Therefore it was determined whether they show the typical pattern of marker gene expression and whether they are able to differentiate into the typical cell types such as adipocytes and osteoblasts.
Although it is still a matter of debate, hMSCs are commonly defined by the expression of the marker genes *NT5E* (*CD73*), *THY1*(*CD90*), *ENG*(*CD105*), *ALCAM* (*CD166*), *CD44* and the absence of the expression of *CD34*, *CD45* and *CD14* (Satija et al., 2007).
No expression of CD34 and CD45 could be found in MSC17 and MSC74, whereas all marker genes were found to be expressed. The data confirmed that the cells used for retroviral and non viral reprogramming in this work were mesenchymal stem cells. However, CD14 was found to be expressed. CD14 is mainly expressed in monocytes of the hematopoetic system (Mathai et al., 2010). Therefore the presence of these cells in the hMSC populations used is possible.

Moreover, mesenchymal stem cells are defined as multipotent progenitor cells present in the bone marrow that have the potential to differentiate into osteoblasts, adipocytes, chondrocytes and fibroblasts (Valtieri and Sorrentino, 2008).
In *in vitro* differentiation experiments it could be confirmed that MSC17 and MSC74 possess the ability to differentiate into adipocytes and osteoblasts.
The osteogenic and adipogenic differentiation potential of MSC17 was lower than the differentiation potential of MSC74. This difference may have been caused by a higher self-renewal capacity of MSC17 cells compared to MSC74 cells which probably has a higher differentiation potential or tendency to differentiate as the number of self renewing cells decreases with donor age in hMSCs (Stolzing et al., 2008).
Assuming that only self renewing MSCs within the hMSC population are easier to be reprogrammed to iPSCs, more efficient iPSC generation would be expected from MSC17. As MSC17 could not be reprogrammed, other parameters than the self-renewal capacity of the mesenchymal stem cells are likely to hamper viral reprogramming of hMSCs compared to fibroblasts.
As the cell line p-iPSC 1 can be differentiated into adipocytes and osteoblasts, it is very likely that p-iPSCs are in an undifferentiated state. Whether this state is closer to the undifferentiated state of iPSCs or the undifferentiated state if hMSCs has to be addressed in future studies of p-iPSCs. Furthermore, p-iPSCs might have retained the multilineage potential of MSC74 but also obtained new molecular features that shifted their commitment in the direction of osteoblast differentiation.
An important feature to determine the similarity between the reprogrammed hMSCs, iPSCs and hESCs is the morphology of the cells as human embryonic stem cells have distinct typical features that enable their recognition. The cells grow in flat colonies have prominent nucleoli and a high nucleus to cytoplasm ratio (Thomson et al., 1998). Induced pluripotent stem cells are described to display the same morphological features like hESCs (Takahashi et al., 2007).
v-iPSCs, obtained by viral reprogramming with additional inhibitors, show the same morphological features described for hESCs and iPS cells as prominent nucleoli, high nucleus to cytoplasm rate and they grow in flat colonies. However, p-iPSCs that were obtained with episomal plasmids show a morphology which is different from ESCs. p-iPSCs grow in colonies which are not round as iPSC or ES cell colonies, they are bigger than hESCs and have no prominent nucleoli. Therefore, v-iPSCs are very likely completely reprogrammed induced pluripotent stem cells whereas p-iPSCs might be partially reprogrammed cells which were observed before as non ES-like colonies during retroviral reprogramming of fibroblasts (Takahashi et al., 2007). The fact that no colonies with the morphologies similar to p-iPSCs were observed during the retroviral reprogramming of hMSCs suggests that the morphology of p-iPSCs may be caused by effects that are related to the different combination of transgenes which resulted in the generation of p-iPSCs compared to v-iPSCs.
A further result that supports the notion that v-iPSCs are fully reprogrammed in contrast to likely partially reprogrammed p-iPSCs is the different level of alkaline phosphatase activity in the two cell types. The absence of alkaline phosphatase activity in p-iPSCs could also be due to a not sufficiently high level of transgene expression in MSC74 that were nucleofected with ET2K or due to the reprogramming technique itself. The reason for this conclusion is that most of the episomal vector combinations that were tested for fibroblasts did not lead to colonies that displayed alkaline phosphatase activity but to morphological changes (Yu et al., 2009).

The analysis of protein expression of pluripotency-related genes revealed that v-iPSC clearly expressed NANOG, whereas NANOG could not be clearly detected in the nuclei of p-iPSCs. As NANOG is essential for the development of pluripotency in the inner call mass of the blastocyst and allows the pluripotent ground state of ESCs (Silva et al., 2009), it is a very reliable marker of pluripotency. Because NANOG expression was detected in v-iPSCs it can be concluded that v-iPSCs are pluripotent.
However, it remains to be clarified whether other pluripotency associated genes like *OCT4*, *SOX2*, *KLF4* and *c-Myc* are expressed in v-iPSCs and whether the pluripotency associated surface markers *SSEA-4*, *TRA-1-60* and *TRA-1-81* are expressed.
Moreover, the cell lines p-iPSC 1 and 4 were shown to express OCT4, SOX2 and KLF4 in a similar manner as H1 embryonic stem cells. However, the c-Myc protein could not be clearly detected in the nuclei of p-iPSCs. Furthermore, the analysed p-iPSCs did not express SSEA-1 and TRA-1-81 but possibly a low level of SSEA-4 and TRA-1-60 compared to embryonic stem cells.
As MSC74 cells that are cultured without additional treatment express KLF4, the expression of these factors could be retained in p-iPSCs.
In addition, the expression of SOX2 could be detected in clonal populations of bone marrow derived MSCs (Mareddy et al., 2010). Considering these publications, also the detected SOX2 and expression could be due to retained expression of these factors in p-iPSCs and not due to a pluripotent state of the cells. However, the expression of OCT4 and SOX2 as well as KLF4 is similar to the expression detected in H1 underlining that p-iPSCs may be reprogrammed to a pluripotent state.
In contrast to that, the detected expression of OCT4, SOX2 and KLF4 might be due to the presence of the episomal plasmid ET2K in the analysed p-iPSC cells. This conclusion is underlined by the detected presence of the oriP sequence of the episomal plasmid in all four p-iPSC lines.
According to the expression of only some of the analysed pluripotency associated factors, p-iPSCs may very likely be in a state between hMSCs and iPSCs and therefore partially reprogrammed cells. Taking this into account, microarray based gene expression analysis of transcriptional differences between hMSCs, iPSCs and p-iPSCs might yield useful results for the optimisation of non viral reprogramming of hMSCs.
Furthermore, the self-assembly to three-dimensional cell clusters called embryoid bodies is a hallmark of embryonic stem cells. As p-iPSCs are able to form embryoid bodies *in vitro*, it can be concluded that p-iPSCs are, despite displaying features of partially reprogrammed cells in a pluripotent state. However, analysis of teratoma formation *in vivo* is needed to confirm this.
This conclusion could be partly confirmed through embryoid body based differentiation of p-iPSCs into cell lineages of all three germ layers. However, in some p-iPSC clones the presence of lineage specific markers could be confirmed but the cells did not display the expected morphology. This might be due to unspecific staining of the secondary antibody and has to be determined by repeating the embryoid body based differentiation and including a secondary antibody control in the staining procedure.
In this context, it is noteworthy that it has been described that hMSCs can differentiate into ectodermal nerve cells (Kopen et al., 1999) and endodermal liver cells (Stock et al., 2010) in addition to mesodermal lineages. Therefore, the differentiated p-iPSCs expressing endodermal and ectodermal markers could be a result of the normal differentiation potential of

hMSCs. Thus, the differentiation potential might not have been broadened upon non viral reprogramming of hMSCs.
In addition, unrestricted somatic stem cells (USSC) from human umbilical cord blood are today the only somatic stem cells described to express SOX17. They are similar to hMSCs in many aspects but are still a different cell type (Simon et al., 2010). Therefore p-iPSCs may have gained additional feature to differentiate into SOX17 expressing cells of the endoderm and therefore it might be possible to derive terminally differentiated endodermal cells from p-iPSCs.
The expression of *ACTN2* which encodes alpha-smooth muscle actin could be detected in the cells that grew out of the embryoid bodies of all p-iPSC lines. As the expression of this gene is a marker for the differentiation into mesodermal smooth muscle cells (Skalli et al., 1989), it can be concluded that p-iPSCs retain the capacity to differentiate into mesodermal lineages.
The expression of NESTIN and TUJ1 has been demonstrated in bone marrow derived MSCs of the rat (Rooney et al., 2009). Therefore the expression of the ectodermal markers *TUJ1* and NESTIN in p-iPSCs could confirm that these factors were also present in MSC74 and that their expression is retained in p-iPSCs. However, this needs to be analysed by staining of MSC74 for these factors.
A second less likely explanation for the presence of endodermal and ectodermal markers in spontaneously differentiated p-iPSCs may be that the culture condition during cellular reprogramming favoured the proliferation of so called multipotent adult progenitor cells which are described to be able to differentiate into all three germ layers and to be present in purified hMSC populations (Jiang et al., 2002).
Ultimately, the pluripotency of p-iPSCs has to be tested by injection into mice in order to determination whether these cells form teratomas.

5.5 Spontaneous differentiations of hMSCs reprogrammed with retroviruses

Interestingly, colonies of MSC17 that were infected with retroviruses for iPSC generation were isolated and cultured under conditions for ES cell maintenance. The presence of alpha-smooth muscle actin in these cells confirmed, that the cells of the isolated colonies of MSC17 very likely differentiated into smooth muscle cells. A potential reason for this could be that proviruses integrated into genomic sites that control the differentiation of smooth muscle cells. However, as the inhibitor treatment had no influence on the differentiation into smooth muscle, it is most likely that the overexpression of *OCT4*, *SOX2*, *KLF4* or *c-Myc* triggered the smooth muscle cell differentiation. This mechanism could have impaired iPSC generation from MSC17, too.
Moreover, it has been described that bone marrow derived mesenchymal stem cells can differentiate into cardiomyocytes (Wang et al., 2006) and that cardiomyocytes can be derived from iPSCs (Freund et al., 2010). Therefore, the observed cardiomyocytes within the generated v-iPSC colony could have evolved either from non reprogrammed MSC74 cells or from v-iPSCs. In addition to that, hMSCs reprogrammed with *OCT4*, *SOX2*, *KLF4*, *c-Myc*, *hTERT* and *SV40LT* differentiated into beating cardiomyocytes during embryoid body formation (Park et al., 2008).

5.6 Implications for the use of v-iPSCs and p-iPSCs

hMSCs are currently the most popular cell type used in tissue engineering and cell therapeutic applications such as bone and cartilage regeneration (Chanda et al., 2010). The application of patient derived hMSC furthermore circumvents the problem of allograft rejection in cell therapies (Stolzing et al., 2008).
However, hMSCs go into senescence before or at 40 population doublings *in vitro* (Bruder et al., 1997), which limits the expansion in culture and therefore their application potential. The derivation of p-iPSCs by the non viral method demonstrated in this work could be a means of obtaining a source of mesenchymal cells with a longer life span and possibly enhanced differentiation capability. This possibility to circumvent the problem of cellular senescence in culture may allow cell therapies that would need a number of hMSCs beyond the actual expansion limit. However, it remains to be determined whether p-iPSCs develop teratoma *in vivo*. As somatic stem cells such as hMSCs do not form teratomas it might apply to p-iPSCs as they are partially reprogrammed and still possess features of hMSCs. In this case p-iPSCs would be a more secure cell source for regenerative therapies than iPSCs that form teratomas.
v-iPSCs that were derived from mesenchymal stem cells from a 74 year old patient in this work have to be further analysed toward their differentiation potential. After further confirmation that these cells are induced pluripotent stem cells the reprogramming protocol that allowed iPSC generation from MSC74 may be used to generate iPSCs from hMSCs of older patients to generate for instance disease models. In addition to that it may be easier to derive mesodermal cell lineages from these hMSCs derived iPSCs as it has been shown that human iPSCs retain in part the gene expression of their donor cells (Marchetto et al., 2009).

6 Summary

The *in vitro* expansion and application possibilities of primary human mesenchymal stem cells (hMSCs) are limited by their short life span in culture and restricted differentiation potential. These two features have been altered in human fibroblasts and other somatic cells by reprogramming them to induced pluripotent stem cells (iPSCs) that are similar to embryonic stem cells in terms of self-renewal properties and pluripotency. Therefore iPSC generation from hMSCs will broaden their application potential.
Increased donor age was described to be accompanied with elevated senescence of hMSCs *in vitro*. Accordingly, the inhibition of p53 may help increasing the efficiency of iPSC generation from hMSCs as senescence related elevated p53 expression has been described to impair iPSC generation.
Currently, most reprogramming techniques to generate iPSCs from somatic cells are using integrative retroviruses. This approach results in a low reprogramming efficiency which can be increased using the chemicals SB431542 (inhibitor of ALK4/5 and 7) and PD325901 (inhibitor of MEK). Moreover, hMSCs have been reprogrammed to iPSCs by retroviral expression of the six factors *OCT4*, *SOX2*, *KLF4*, *c-Myc*, *hTERT* and *SV40LT*. Yet, the yield of the method is low and multiple inserted proviruses decrease genomic stability. These problems could be circumvented if oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-based episomal vectors are used to generate iPSCs from fibroblasts. Therefore, the use of this method could help to derive virus free iPSCs from hMSCs.
It was the aim of this studyto reprogramm hMSCs comparing therefore the practicability of retroviral and episomal plasmid based non viral methods and the possibility of additionally enhancing the reprogramming efficiency by inhibition of TGFβ, MEK and/or p53 signaling. In addition, the putative effect of donor age-related transcriptional differences in hMSCs was analysed, as well.
hMSCs derived from a 74 year old patient (MSC74) could be reprogrammed with retroviruses carrying the genes *OCT4*, *SOX2*, *KLF4* and *c-Myc* and additional inhibition of MEK, ALK4/5 and 7 as well as p53. A high similarity between hESCs and the derived iPSCs (v-iPSCs) could be confirmed.
However, hMSCs from a 17 year old patient (MSC17) could not be reprogrammed under the same conditions implying an adverse effect of age-related differences in the gene expression on iPSC generation. A preliminarily conducted microarray-based gene expression analysis highlighted the down-regulation of cell cycle and p53 signaling associated genes in MSC74. Due to reported negative effects of p53 and senescence on iPSC generation these genes may have blocked the reprogramming of MSC17.
To derive iPSCs without virus integrations MSC74 cells were reprogrammed with episomal plasmids carrying the genes *OCT4*, *SOX2*, *KLF4* and *SV40LT* (ET2K) or *OCT4*, *SOX2*, *KLF4* and *c-Myc* (EM2K). The combination ET2K/EM2K and ET2K alone yielded partially reprogrammed iPSC colonies (p-iPSCs) when MEK and ALK 4/5 and 7 were inhibited. The efficiency was lower compared to which was reported for the use of this method for fibroblasts. Moreover, the efficiency was lower compared to viral methods.
Four p-iPSC cell lines were established from MSC74 reprogrammed with ET2K. p-iPSCs were distinct from ESCs and v-iPSCs in terms of morphology and expression of pluripotency markers. However, the cells formed embryoid bodies and spontaneously differentiated into

lineages expressing markers of endoderm, ectoderm and mesoderm. These results suggest that p-iPSCs are partially reprogrammed cells.
In addition p-iPSCs could be differentiated into adipocytes with lower and osteoblasts with higher efficiency compared to MSC74, thus, suggesting a shift in cell commitment.
In conclusion, this work demonstrates that hMSCs from older donors can be reprogrammed easier compared to hMSCs from younger individuals using retroviral expression of *OCT4*, *SOX2*, *KLF4* and *c-Myc* when MEK, ALK4/5 and 7 as well as p53 are inhibited. Moreover, non-integrating episomal vectors can be usedto reprogramm hMSCs to a partially reprogrammed state when MEK, ALK4/5 and 7 are inhibited. p-iPSCs could be a useful, novel intermediate of iPSC generation, which does not cause cancer, when pluripotency of these cells can be further confirmed and teratoma formation *in vivo* can be excluded.

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8 Appendix

8.1 Vector maps

Retroviral Vectors

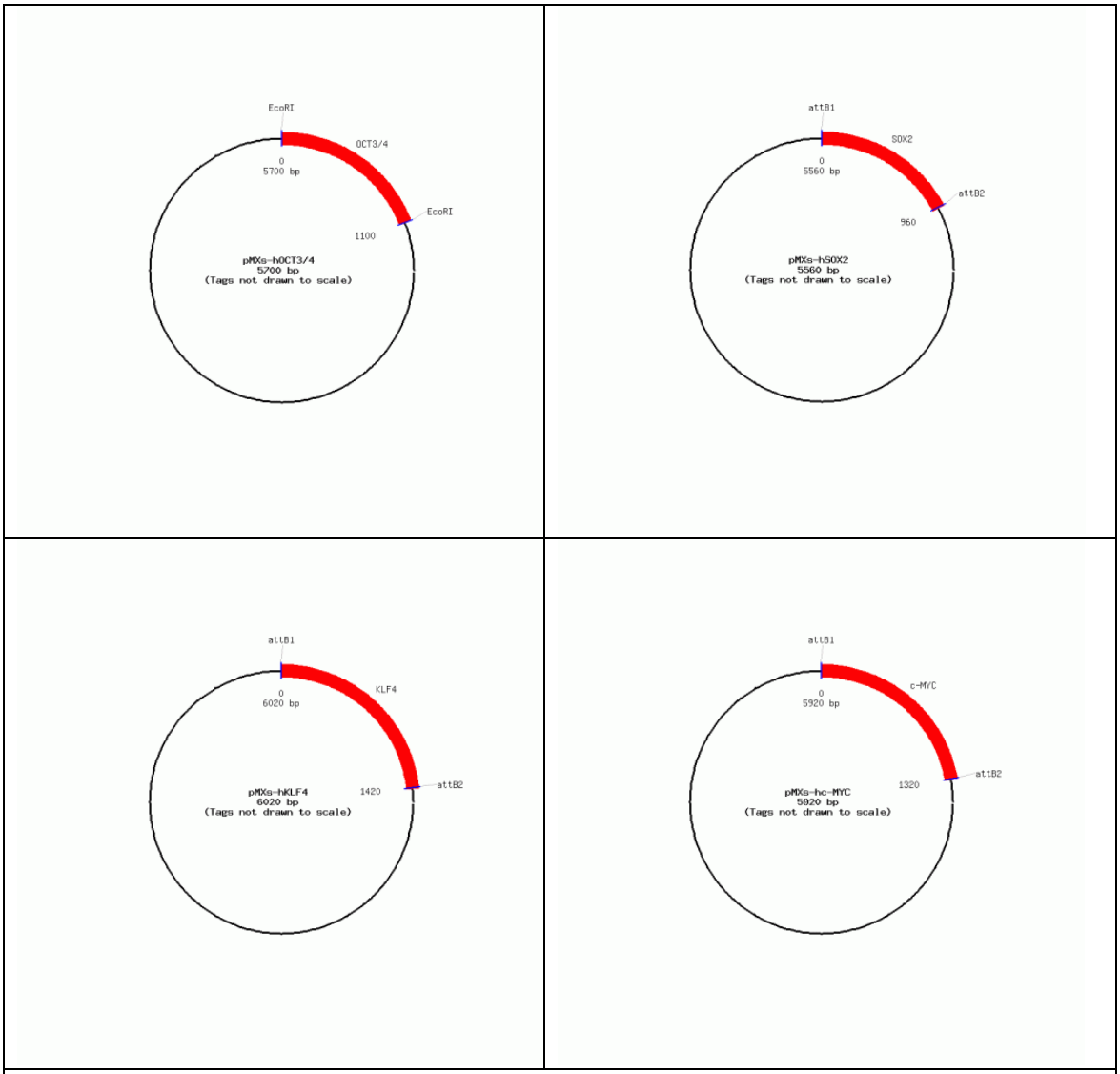
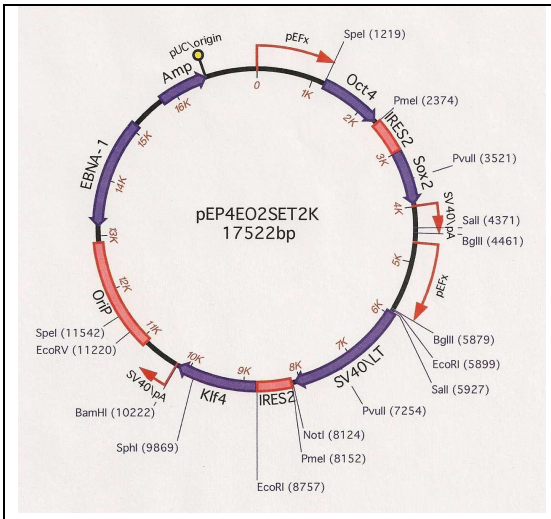


Figure 32 Maps of the vectors used for retroviral reprogramming of MSC17 and MSC74: pMXs-hOCT3/4, pMXs-hSOX2, pMXs-hKLF4 and pMXs-hc-Myc. (world wide web, http://www.addgene.org, 20.07. 2010)

oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-based episomal vectors



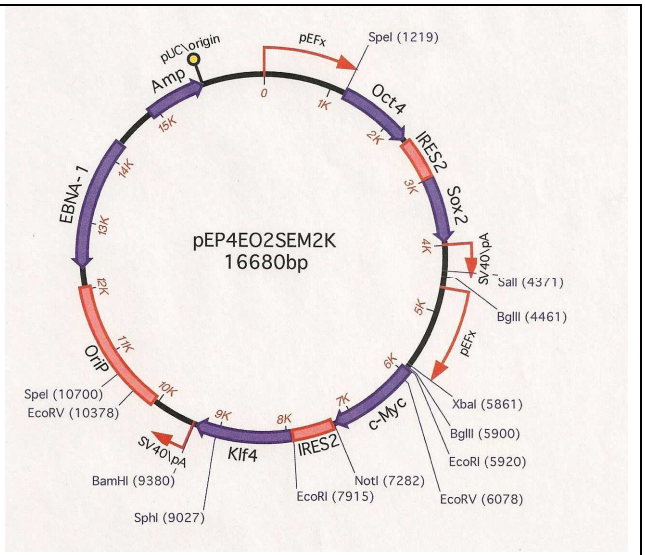


Figure 33 oriP/EBNA1-based episomal vectors that were used for reprogramming of MSC74. Amp: Ampicillin resistance gene, pEFx: eukaryotic elongation factor 1 alpha promoter, IRES2: internal ribosome entry site 2, oriP: origin of replication controlled by EBNA-1 (Epstein-Barr nuclear antigen-1), SV40\pA: SV40 PA terminator. (world wide web, http://www.addgene.org, 20.07. 2010)

9 Zusammenfassung

Die *in vitro*-Expansion und das Anwendungspotential von primären humanen mesenchymalen Stammzellen aus dem Knochenmark (hMSCs) sind durch die kurze Lebenspanne der Zellen in Kultur und ihr eingeschränktes Differenzierungspotential limitiert. Diese beiden Merkmale konnten in humanen Fibroblasten und anderen somatische Zellen verbessert werden, indem sie zu induziert pluripotenten Stammzellen (iPS-Zellen) reprogrammiert wurden. iPS-Zellen ähneln embryonalen Stammzellen bezüglich ihrer Fähigkeit zur Selbsterneuerung und ihrer Pluripotenz. Aus diesen Gründen kann die Herstellung von iPS-Zellen aus hMSCs das Verwendungspotential der hMSCs bedeutend erweitern.

Es ist bekannt, dass der Anteil an seneszenten Zellen in hMSCs, welche aus dem Knochenmark älterer Spender stammen, höher ist. Eine Verbesserung der Effizienz bei der Herstellung von iPS-Zellen aus hMSCs ist deshalb besonders durch Inhibition von p53 zu erwarten, da herausgefunden wurde, dass die durch Seneszenz induzierte Erhöhung der p53-Expression die Herstellung von iPS-Zellen verhindert.

Derzeitig verwendete Techniken der Reprogrammierung von somatischen Zellen zu iPS-Zellen schließen die Verwendung von Retroviren, die in das Genom integriert werden, mit ein. Dieser Ansatz führt zu einer niedrigen Effizienz bei der Herstellung der iPS-Zellen, die, wie kürzlich bekannt wurde, durch Verwendung der Substanzen SB431542 (Inhibitor von ALK4/5 und 7) und PD325901 (Inhibitor von MEK) erhöht werden kann.

iPS-Zellen können aus primären humanen mesenchymalen Stammzellen durch retrovirale Überexpression der sechs Gene *OCT4*, *SOX2*, *KLF4*, *c-Myc*, *hTERT* und *SV40LT* hergestellt werden. Jedoch ist die Ausbeute an iPS-Zellen sehr niedrig und die Stabilität des Genoms der iPS-Zellen ist aufgrund der Proviren, die in das Wirtsgenom integrierte sind, nicht gewährleistet. Diese Nachteile konnten bei der Reprogrammierung von Fibroblasten überwunden werden, indem man oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-basierte episomale Plasmide zur Herstellung von iPS-Zellen verwendete. Deswegen kann diese Methode zur Generierung von Virus-freien iPS-Zellen aus hMSCs sehr hilfreich sein.

Vor diesem Hintergund war es das Ziel dieser Arbeit, iPS-Zellen aus hMSCs herzustellen, wobei die Anwendbarkeit der retroviralen und der auf episomalen Plasmiden basierenden Methode verglichen werden sollte. Dabei wurde ebenfalls untersucht, ob es möglich ist, die Effizienz bei der Herstellung von iPS-Zellen aus hMSCs durch Inhibition der TGFβ, MEK und/oder p53-Signaltransduktionswege zu erhöhen.

Zusätzlich wurde der vermeintliche Effekt von Genexpressionsunterschieden, die durch das Spenderalter bedingt sind, analysiert.

hMSCs von einem 74-jährigen Spender (MSC74) konnten erfolgreich zu iPS-Zellen reprogrammiert werden, wobei Retroviren mit den Genen *OCT4*, *SOX2*, *KLF4* und *c-Myc* sowie die zusätzliche Inhibition von MEK, ALK4/5 und 7 und von p53 angewandt wurden.

Die hergestellten iPS-Zellen (v-iPSCs) waren embryonalen Stammzellen morphologisch sehr ähnlich. hMSCs von einem 17-jährigen Spender (MSC17) konnten unter den gleichen Bedingungen nicht zu iPS-Zellen reprogrammiert werden, was einen nachteiligen Effekt der vom Spenderalter abhängigen Genexpressionsunterschiede bei der iPS-Zell-Generierung impliziert. Eine einleitend durchgeführte, Mircoarray-basierte Genexpressionsanalyse zeigte die Herunterregulierung von Genen des Zellzykluses und des p53-Signalweges in MSC74-Zellen verglichen mit MSC17-Zellen auf.

Wegen des bekannten negativen Effektes von Seneszenz und der damit verbundenen Erhöhung der p53-Expression wäre es möglich, dass die Herunterregulierung dieser Gene an der Blockierung der Reprogrammierung von MSC17 zu IPS-Zellen beteiligt war. Zur Herstellung von Virus-freien iPS-Zellen wurden MSC74 Zellen mit episomalen Plasmiden, welche die Gene *OCT4*, *SOX2*, *KLF4* und *SV40LT* (ET2K) oder *OCT4*, *SOX2*, *KLF4* und *c-Myc* (EM2K) tragen, durch Nukleofektion transfiziert. Die Kombination ET2K/EM2K und ET2K allein führten zur Entstehung von Kolonien, die aus partiell reprogrammierten Zellen (p-iPSCs) bestehen, wenn MEK und ALK 4/5 und 7 inhibiert waren. Die Ausbeuten dieser Methode für hMSCs und Fibroblasten gleichen sich. Im Vergleich zu viralen Methoden zur Herstellung von iPS-Zellen war die Ausbeute jedoch niedriger. Vier Zelllinien wurden aus unterschiedlichen p-iPSC Kolonien, die mit dem episomalen Plasmid ET2K hergestellt wurden, generiert.
p-iPSCs unterscheiden sich von embryonalen Stammzellen und von v-iPSCs durch ihre Morphologie und die geringere Expression von Pluripotenz-Schlüsselgenen.
Dennoch bildeten alle p-iPSC-Zelllinien in Suspensionskultur kugelartige Zellaggregate, so genannte Embryoid Bodies. Sie differenzierten zudem in Zellarten, die jeweils Marker des Endoderms, Mesoderms oder Ectoderms exprimieren. Diese Ergebnisse legen nahe, dass es sich bei p-iPSCs um partiell reprogrammierte Zellen handelt.
Zusätzlich konnten p-iPSCs zu Adipozyten mit einer niedrigeren und zu Osteoblasten mit einer höheren Effizienz als MSC74 differenziert werden, was eine Verschiebung der Differenzierungsfähigkeit erkennen lässt.
Zusammenfassend lässt sich sagen, dass hMSCs von älteren Personen leichter zu iPS-Zellen reprogrammiert werden konnten als solche von jüngeren Spendern, wenn retrovirale Expression von *OCT4*, *SOX2*, *KLF4* und *c-Myc* sowie gleichzeitige Inhibition von MEK, ALK4/5/7 und p53 verwendet wurde. Des Weiteren konnten die benutzten episomalen Vektoren in hMSCs einen partiell reprogrammierten Zustand herbeiführen, wenn gleichzeitig MEK sowie ALK4/5 und 7 inhibiert wurden. p-iPSCs könnten ein nützliches, nicht zu Krebs führendes Zwischenprodukt bei der Herstellung von iPS-Zellen aus hMSCs sein, wenn die Pluripotenz dieser Zellen weiter belegt werden kann und wenn sie *in vivo* keine Teratome bilden.