THE ESTABLISHMENT AND CHARACTERIZATION OF GENETICALLY ENGINEERED IPS CELL LINES DERIVED FROM HUMAN SKIN FIBROBLASTS

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TIIVISTELMÄ

Ihmisen pluripotentit kantasolut pystyvät erilaistumaan kaikiksi elimistön soluiksi. Geneettisesti muokatut pluripotentit kantasolut olisivat oiva työkalu kantasolujen ominaisuuksien tutkimiseen, mutta niiden geneettinen muokkaaminen on osoittautunut teknisesti haastavaksi, toisin kuin erilaistuneiden solujen. iPS-soluteknologian avulla erilaistunut solu voidaan uudelleen ohjelmoida takaisin kantasoluksi. Tämän tutkimuksen tavoitteena oli tuottaa fluoresoivia ihmisen iPS-solulinjoja muokkaamalla ensin erilaistuneet solut geneettisesti ja uudelleen ohjelmoimalla ne iPS-soluiksi. Geneettisesti muokatut iPS-solut ja niistä erilaistetut sydänlihassolut visualisoitaisiin fluoresoivien merkkiaineiden avulla.

Erilaistuneiden solujen geneettiseen muokkaamiseen käytettiin SIN-lentivirus-vektoreita ja ihmisen iPS-solujen uudelleenohjelmointiin pMX-retrovirusvektoreita. Solulinjat karakterisoitiin immunosytokemiallisilla menetelmillä sekä käänteis-transkriptio-PCR:n avulla. Ihmisen iPS-solut erilaistettiin sydänlihassoluiksi yhteisviljelmällä END-2 solujen kanssa. Ihmisen iPS-solujen erilaistumiskyky *in vitro* selvitettiin erilaistamalla solut soluaggregaateiksi, joista analysoitiin eri alkiokerrosmerkkigeenien ilmentymistä.

Useita fluoresoivia ihmisen iPS-solulinjoja muodostettiin onnistuneesti, mutta yhden solulinjan kohdalla fluoresenssi katosi. Ihmisen iPS-solut ilmensivät kantasoluspesifisiä proteiineja ja endogeenisiä pluripotenttiusgeenejä. Retrovirus-peräiset eksogeeniset transgeenit eivät olleet hiljentyneet. Näin ollen, ihmisen iPS-solulinjat olivat osittain uudelleenohjelmoituneita. Tästä huolimatta erilaistaminen sydänlihassoluiksi onnistui, mutta ne eivät fluoresoineet. Erilaistetut sydänlihassolut ilmensivät sydänlihassolu-spesifisiä proteiineja. Erilaistetut soluaggregaatit ilmensivät markkerigeenejä kaikista kolmesta alkiokerroksesta osoittaen ihmisen iPS-solujen pluripotenttiuden.

Fluoresoivia ihmisen iPS-solulinjoja onnistuttiin muodostamaan, mutta solut olivat osittain uudelleenohjelmoituneita. Ihmisen iPS-soluissa havaittiin fluoresenssi-reportterigeenin hiljentymisilmiö, joka oletettavasti aiheutui SIN-lentivirusvektorien käytöstä. Tavoite tuottaa fluoresoivia sydänlihassoluja ei onnistunut. Tästä huolimatta, tuloksista saatiin hyödyllistä tietoa jatkotutkimuksiin.

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ABSTRACT

The human pluripotent stem cells are able to differentiate into all cell types of the three primary germ layers. Genetically modified human pluripotent stem cells represent a powerful tool to study stem cell characteristics such as differentiation. However, the genetic modification of human pluripotent stem cells has been technically challenging unlike with differentiated cells. With the emergence of iPS cell technology, differentiated cells can now be reprogrammed into stem cell-like state. The aim of this study was to establish genetically modified human iPS cell lines by first genetically modifying human skin fibroblasts before reprogramming to iPS cells. Genetically modified iPS cells and their derived cardiomyocytes would be visualized by fluorescent reporters.

SIN-lentiviral vectors were used to genetically modify human skin fibroblasts. Retroviral pMX-vectors were used for human iPS cell reprogramming. Cell lines were characterized by immunocytochemistry and reverse-transcriptase PCR. Human iPS cells were differentiated into cardiomyocytes by co-culturing with END-2 cells. Human iPS cell pluripotency was assessed *in vitro* by embryoid body formation and analyzing germ layer markers by reverse-transcriptase PCR.

Several fluorescent human iPS cell lines were successfully established. However, the fluorescence was extinguished in one cell line. Human iPS cell lines showed positive immunocytochemical staining for stem cell specific proteins. The endogenous pluripotency genes were expressed. The retroviral exogenous transgenes were not silenced. Thus, the human iPS cell lines were partially reprogrammed. Despite partial reprogramming, the differentiation to cardiomyocytes was successful, but no fluorescent cardiomyocytes were obtained. The cardiomyocytes expressed cardiac specific proteins. The embryoid bodies expressed markers from all the three germ layers indicating pluripotency of the iPS cells.

Fluorescent human iPS cell lines, albeit partially reprogrammed were successfully established. However, as in hESCs, the transgene silencing phenomenon was also observed in iPS cells probably due to SIN-lentiviral based genetic modification. The aim to generate fluorescent cardiomyocytes was not successful. Despite of this, valuable information was acquired for future studies.

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1. INTRODUCTION

Stem cells have two important features; they are self-renewing and capable of differentiating into different cell types. During fertilization totipotent cell is formed, which upon several cycles of cell division are able to produce extraembryonic tissues and specialize into pluripotent stem cells. These pluripotent stem cells in turn are able to differentiate into all cell types of the three germ layers but unlike totipotent cells, they are not capable for generating entire organism. Multipotent stem cells have more restricted differentiation potential than pluripotent cells, as they are capable of differentiating into few specific cell types. Unipotent stem cells or progenitor cells have lost their self-renewal capability and upon cell division only differentiated cells are generated. Toti- and pluripotent cells are found in embryonic tissues, where they contribute to the development of whole organism and tissues. Multi- and unipotent cells are primarily found in adult tissues, where they contribute to renewal of tissues.

Embryonic stem cells (ESCs) have advantage over adult stem cells as they are considered to be more potent in differentiation capability. In contrast, their disadvantages involve ethical, legal and practical issues as ESCs are derived from embryos, which are destroyed in the process. The availability of embryos is restricted and in some countries the establishment of new ESC-lines is prohibited.

Reprogramming somatic cells by defined factors can generate induced pluripotent stem cells, iPS cells (Takahashi & Yamanaka, 2006), which may provide an alternative source for ESCs. The iPS cells advantage over ESCs is that during iPS cell generation no embryos are destroyed and thus ethical conflicts are avoided. Moreover iPS cells have a remarkable potential in regenerative medicine and in biomedical research as patient-specific stem cells can be generated for several applications, such as disease modeling, toxicology and drug screening (Yamanaka, 2009b). Indeed, iPS cells have revolutionized stem cell research and the progress in the field has been tremendous in the past years.

Genetic modification of stem cells is a relatively new technique but it represents a powerful tool to study stem cell characteristics. For instance, it is challenging to develop efficient differentiation methods toward lineage-specific cells without the tools to monitor the appearance of desired, specific cell types (Giudice & Trounson, 2008). Thus, efficient,

rapid and easy detection system is needed for optimizing differentiation protocols. Fluorescent reporter genes may be useful, for instance in the context of differentiation. Unfortunately, the genetic modification of human embryonic stem cells (hESCs) has been traditionally technically challenging and to date only a handful of human knockin or knockout cell lines exist (Buecker et al., 2010). The genetic modification of somatic cells is technically easier than stem cells. Therefore, the objective of this study is the establishment of fluorescent and antibiotic selectable iPS-cell lines derived from human skin fibroblasts by first genetically modifying fibroblasts followed by reprogramming to iPS cells.

2. REVIEW OF THE LITERATURE

2.1 THE ORIGIN OF INDUCED PLURIPOTENT STEM CELLS

Previous attempts to establish artificial stem cells by somatic cell nuclear transfer or cell fusion have been hindered by technical and ethical problems (Geoghegan & Byrnes, 2008). All that changed in 2006 when Takahashi & Yamanaka (2006) published a groundbreaking report on stem cell research. They were able to reprogram mouse embryonic and adult fibroblasts into stem cell-like state by four transcription factors. The cells were pluripotent by nature and they were designated as "induced pluripotent stem cells", iPS cells. These iPS cells had an enormous impact on stem cell biology and they may revolutionize regenerative medicine and biomedical research. The knowledge on how to reprogram somatic cells into stem cell-like state has given whole new opportunities on generating patient-specific stem cells for studying different diseases, drug testing and toxicology screening on differentiated cells and ultimately, perhaps in the near-future, iPS cells can be used in regenerative medicine to repair tissues damaged through disease or injury (Yamanaka, 2009b).

2.2 HUMAN INDUCED PLURIPOTENT STEM CELLS

Human induced pluripotent stem cells (hiPS cells) were reported in 2007 by two groups (Takahashi et al., 2007), (Yu et al., 2007). Takahashi et al. (2007) were able to generate hiPS cells by the same defined factors as in mouse iPS (miPS) cell generation. Yu et al. (2007) approached the subject from a different point of view; they used their own transcription factor set which included Oct4, Sox2, Nanog and Lin-28.

Takahashi et al. (2007) generated pluripotent stem cells from human adult dermal fibroblasts (HDFs). They used ecotropic mouse retroviruses for the transduction of the transcription factors into the fibroblasts. Ecotropic mouse retroviruses infect efficiently only rodent cells, therefore optimization for the transduction efficiency was made by introducing mouse ecotropic retrovirus receptor (Slc7a1) to the HDFs with lentivirus. The transduction efficiency of mouse retroviruses reached to 60 %, which was sufficient to generate hiPS cells.

Yu et al. (2007) screened combination of genes responsible for reprogramming and identified their own set of transcription factors: Oct4, Sox2, Nanog and Lin-28. With these transcription factors they reprogrammed human fetal primary fibroblasts and human foreskin fibroblasts (hFFs) into hiPS cells by lentiviral transduction.

The resulting hiPS cells were investigated on their surface antigen and cell marker gene expression, differentiation capabilities and the epigenetic state of the pluripotency-related genes (Takahashi et al., 2007), (Yu et al., 2007). It was observed in both studies that hiPS cells expressed hES cell-specific surface antigens and cell marker genes, such as SSEA-3, SSEA-4, Oct4 and Nanog. The ES cell specific marker genes, such as Oct4 and Nanog were expressed at equivalent levels in hiPS cells as in hESCs. In addition, the protein levels of several pluripotency-related gene products, such as Oct4 and hTERT were similar in hES and hiPS cells (Takahashi et al., 2007).

In both studies, the gene expression profile of hiPS cells was compared to hESCs. The data showed that expression profile was similar to hESC but not identical. DNA microarray analyses showed that among 32 266 genes analyzed, 1 267 genes showed >5 fold difference between the hiPS cells and hESCs. In contrast, 5 107 genes showed >5 fold difference between HDFs and hiPS cells, whereas 6 083 genes showed >5 fold difference between hESCs and HDFs showing, that gene expression profile is highly dissimilar between HDFs and pluripotent stem cells. (Takahashi et al., 2007.)

Differentiation capability was determined by the formation of embryoid bodies (EBs), teratoma formation and differentiation into neural and cardiac cells. After culturing hiPS cells in suspension, the cells formed aggregates, EBs (Itskovitz-Eldor et al., 2000). These EBs comprise of the three embryonic germ layers; ecto-, endo- and mesoderm. EB immunocytochemistry detected cells positive of all three germ layers, therefore indicating that hiPS cells can be differentiated into all three germ layers *in vitro* (Takahashi et al., 2007), (Yu et al., 2007). Pluripotency was assessed *in vivo* by injecting hiPS cells into severe combined immunodeficient mouse (Takahashi et al., 2007) and monitoring teratoma formation. Nine weeks after injection, tumors were observed. Histological examinations confirmed that the tumors showed tissues from all the three germ layers. Finally, hiPS cells were successfully differentiated into neural and cardiac cells by methods reported for

hESCs. The expression levels of Oct4 and Nanog decreased markedly in both of the differentiations. (Takahashi et al., 2007.)

The epigenetic state of the pluripotency-related genes was assessed with bisulfite genomic sequencing, which evaluates the methylation status of the promoters. The promoters of pluripotency-related genes, such as Oct4, Rex-1 and Nanog were highly unmethylated in hiPS cells, whereas in HDFs the genes were highly methylated. This indicates that these pluripotency-related genes promoters are active in hiPS cells. (Takahashi et al., 2007.)

Finally, it was proven in both studies that hiPS cells are derived from HDFs (Takahashi et al., 2007) and from both human fetal and foreskin fibroblasts (Yu et al., 2007). Takahashi et al. (2007) showed that each iPS clone contains more than 20 retroviral transgene integration sites. Yu et al. (2007) used well-characterized DNA fingerprinted human fetal and foreskin fibroblasts for reprogramming, from which it was easy to identify that hiPS cells were derived from parental fibroblasts.

Since then, a variety of patient- and disease-specific cell lines have been generated from diseases such as amyotrophic lateral sclerosis (Dimos et al., 2008), Parkinson's disease (Soldner et al., 2009) and from a variety of genetic diseases, such as Huntington's disease and Down's syndrome (Park et al., 2008) among others. Ethical concerns regarding the use of embryonic stem cells are bypassed with iPS cell technology because there's no need of embryos in generating iPS cells. Also the generation of iPS cells requires only standard laboratory equipment and techniques (Yamanaka, 2009b).

The potential use of hiPS cells is enormous, ranging from regenerative medicine to studying the development of human tissues and diseases. However, there are several hurdles ahead regarding the clinical use of hiPS cells. It needs to be assessed whether hiPS cells can truly replace hESCs, for example in transplantation therapies. Also safety issue will be a big concern. In studies conducted by Takahashi et al. (2007) and Yu et al. (2007), both groups used retro- or lentiviruses to transduce the transcription factor genes into somatic cells. As retroviruses integrate into the genome, they may cause insertional mutagenesis, proto-oncogene activation and eventually tumorigenesis in cells. Therefore different induction methods need to be developed in order to get safer hiPS cells for clinical setting.

2.3 STRATEGIES FOR THE GENERATION OF IPS CELLS

The strategies for the generation of iPS cells can be divided into transgene genome integrating and non-integrating methods. Methods where the transgenes integrate into the genome include retroviral, lentiviral systems, Cre/LoxP lentiviral vector excision system and PiggyBac transposon/transposaase system. With the latter two systems it is possible to excise the integrated transgenes from host genome after the induction of iPS cells. Non-integrating methods include the use of adenoviruses, plasmid expression vectors, episomal vectors oriP/EBNA1, recombinant proteins and mRNA. Non-integrating methods are free from genetic modifications. (O'Malley et al., 2009.)

Takahashi & Yamanaka (2006) produced miPS cells by using a retroviral system to introduce the transgenes into the cells. Later on this system, with slight optimization, was employed on producing hiPS cells as well (Takahashi et al., 2007). The downside of retroviruses is that they integrate into the genome by random pattern. Thus, this may induce mutagenesis at the site of integration or alter the function of surrounding genes that may result in altered cell function or tumorigenesis (Robbins et al., 2010). From miPS cell studies (Okita et al., 2007) it was revealed that 20 % of the mice derived from miPS cells developed tumors. It was experimentally proven that at least in part, tumor formation occurred by reactivation of retroviral transgene c-Myc (Okita et al., 2007). It has been suggested that retroviral systems used for the generation of iPS cells might have an effect on cell function, if the exogenous reprogramming factors at least in part, are expressed. Therefore ideal iPS cells, suitable for clinical need, would need to be generated with minimal or total absence of genetic modifications (O'Malley et al., 2009).

Cre/loxP lentiviral vector excision system was used to generate Parkinson's disease patient-derived hiPS cells (Soldner et al., 2009). With Cre/loxP-system exogenous reprogrammable factors can be excised from integration sites in hiPS cells. A loxP site was placed in the 3'LTR region of lentiviral vector, which upon proviral replication is duplicated into the 5'LTR region. In between the 5'- and 3' LTR loxP sites individual reprogrammable factor with dox-inducible promoter CMV is located. Lentiviral vectors carrying the reprogramming factors were used to generate the patient-specific hiPS cells, in which each cell line contained 3–10 virus integration sites. After the generation of hiPS cells, Cre-recombinase was transiently expressed in the cells, which catalyses the site-

specific recombination at loxP sites. When Cre is introduced to the hiPS cells, the DNA sequence between loxP sites is deleted and thus reprogrammable factors are excised from the genome. However, not all vector DNA is excised. As the DNA fragment between loxP sites is excised, the system leaves a vector trace in the genome in the form of one loxP site and vector DNA external to loxP sites (O'Malley et al., 2009), (Yu et al., 2009). Interestingly, however, hiPS cells generated by Cre/loxP-system showed more closely related global gene expression profile to hESCs than to retrovirally generated hiPS cells. The effect of residual transgene expression, for instance to *in vitro* differentiation, has not been assessed. (Soldner et al., 2009.)

Another excisable system is called PiggyBac (PB) transposon/transposaase, which has an advantage over Cre/LoxP. With PB-system it is possible to excise the whole integrated vector DNA from the host genome by transient expression of transposaase (Woltjen et al., 2009). In the process somatic cells are transfected with PB expression plasmid vectors carrying the transgenes needed for reprogramming. After transfection, follows the identification of iPS cells. Firstly it is needed to identify cells with minimal-copy number plasmid insertions, and then map the insertion sites and excise transgenes with transposaase enzyme and finally validate the factor-free clones. All of these are time consuming and labor intensive processes, but needed for efficient transgene-free derivation of iPS cells. (O'Malley et al., 2009.)

Stadtfeld et al. (2008) successfully reprogrammed mouse hepatocytes into miPS cells by using transiently expressing adenoviruses. Adenoviruses do not integrate into the genome, but they allow high-level expression of exogenous genes required for reprogramming. Mouse hepatocytes were chosen because they are a cell type especially prone to adenoviral infection. Although the reprogramming was successful, the efficiency was many magnitudes lower than in retroviral method (Stadtfeld et al., 2008b). Zhou & Freed (2009) showed that adenoviral gene delivery can also reprogram human embryonic fibroblasts into pluripotent state, although at extremely low efficiency rate. The reprogrammed cells expressed hESC surface markers and they were able to form teratomas when injected into SCID mouse indicating their pluripotency. Albeit the virus-integration free hiPS cells were derived, the extremely low reprogramming efficiency rate possibly makes this technique impractical.

Okita et al. (2008) used another type of strategy for generating iPS cells. They developed two plasmid expression vectors containing the reprogramming factors for the transfection of MEFs. Since the reprogramming of mouse somatic cells takes 8–12 days, they had to transfect mouse embryonic fibroblasts repetitively. In this way they were able to generate miPS cells, which expressed ES cell markers at a comparable level to ES cells. The possible integration of plasmids into the genome was assessed by genomic PCR analysis. In 60 % of the experiments they were able to obtain iPS cell clones without plasmid integration. (Okita et al., 2008.) As was the case with adenoviruses, the reprogramming efficiency remained extremely low when compared to retroviral method. One important note is that no hiPS cells have been generated by plasmid expression vectors. A reason for this may be within the difference in reprogramming. Human cells require more time to be reprogrammed and therefore the level or the duration of transgene expression may not be enough with adenoviral or plasmid transfection systems (O'Malley et al., 2009).

Episomal plasmid vectors, oriP/Epstein-Barr virus nuclear antigen 1 (oriP/EBNA1), were used by Yu et al. (2009) to generate hiPS cells. The oriP/EBNA1 vector replicates only once in the cell cycle and with drug selection it can be maintained as low-copy number DNA episomes in 1 % of the transfected cells. Removing selection pressure results in the clearing of plasmids from cells. In order to generate hiPS cells, Yu and co-workers had to add three more factors (Nanog, Lin-28 and SV40LT) in addition to the Oct4, Sox2, c-Myc and Klf4. Still, the reprogramming efficiency remained extremely low, 3–6 hiPS cell colonies per 10^6 cells (Yu et al., 2009). General concern has risen that it cannot be completely ruled out that DNA used for the reprogramming of cells doesn't integrate into the genome (O'Malley et al., 2009). Therefore non-DNA reprogramming strategies have been studied.

First hiPS cells generated without genomic modifications were reported by Kim et al. (2009). They used recombinant proteins (Oct4, Sox2, c-Myc and Klf4) for the reprogramming of human newborn fibroblasts into hiPS cells. Fusing the factors with cell penetrating peptides allowed the recombinant proteins enter the cells when added in culture medium. Because of the low half-life of the proteins, the cells needed to be treated in cycles. The downside of this method is that it took 8 weeks to reprogram cells into hiPS

cells as in retroviral system it takes about 4 weeks (Takahashi et al., 2007). In addition, the reprogramming efficiency with recombinant proteins was significantly lower than in retroviral method (Kim et al., 2009).

Recently another type of reprogramming method without genetic modification was reported (Yakubov et al., 2010). Instead of proteins, they used mRNA of the transcription factors Oct4, Sox2, Nanog and Lin-28 to reprogram human foreskin fibroblasts. Human fibroblasts were transfected with mRNA containing plasmids 5 times in consecutive manner. The time required for the reprogramming was similar to the retroviral method as hiPS cell colonies were picked up around day 25. However, as proper characterization for the established lines was not reported, it is cautious to say that full reprogramming of somatic cells into pluripotent state had taken place. The authors showed only positive immunocytochemistry results on alkaline phosphatase and Nanog. More detailed characterizations would be needed to prove the successful reprogramming of fibroblasts into hiPS cells.

The iPS cell research has lead to new ways to generate iPS cells with the focus in mind to make the process more efficient and the cells safer for clinical use. However, iPS cells considered to be safe for clinical use do not exist yet, but new technologies are emerging. For instance, the use of synthetic antigene RNAs to activate specific endogenous genes and the development of synthetic regulatory proteins as for artificial transcription factors are being studied. (Selvaraj et al., 2010).

Nevertheless, the generation of human iPS cells, whether safe or not for clinical use, has allowed the valuable establishment of patient- and disease-specific cell lines. For instance, Park et al. (2008) established disease-specific hiPS cell lines by retroviral transgene transduction for a variety of genetic diseases. The differentiation of these iPS cells into target cells and studying them may result, for instance in better understanding of the disease mechanism and/or drug screening may yield suitable molecules for alleviating the disease.

2.4 THE FOUR DEFINED FACTORS: OCT4, SOX2, C-MYC AND KLF4

2.4.1 Oct4

Oct4 is a transcription factor, which belongs to the POU-protein family. It is specifically expressed in ES cells, early embryos and germ cells. Oct4 expression is required for the development of inner cell mass, embryonic stem cells and the maintenance of pluripotent state. (Nichols et al., 1998.)

Oct4 expression level has a critical role in the maintenance of pluripotent state and also in cellular differentiation. The precise expression levels of Oct4 govern the three distinct cell fates. First, if Oct4 expression is increased by less than twofold, it triggers the cell's differentiation into primitive endoderm and mesoderm. On the other hand, if expression is decreased by twofold, it triggers differentiation into trophectoderm and induces the loss of pluripotency. Thus, a critical amount of Oct4 is required for the maintenance of stem cell pluripotent state and self-renewal. (Niwa et al., 2000.) In reprogramming Oct4 has been shown to be the core factor as without it the generation of iPS cells won't succeed (Amabile & Meissner, 2009).

Recently Oct4 interaction network was examined (Pardo et al., 2010). Studying the interactome of Oct4 may provide information on how the pluripotent state is established and regulated. In the study Oct4-binding proteins were identified in mouse ESCs (mESCs). The set and variation of Oct4-binding proteins were quite considerable; in total 92 proteins were identified associated with Oct4 including regulators of gene expression, replication and chromatin remodeling. Although the experiment was done on mESCs, the authors note that because of the Oct4-binding proteins were found to be highly conserved between mice and human, it implies that these findings could be applied to hESCs. These results may have implications on human disease as some Oct4-interacting proteins were associated with genetic diseases, such as colorectal cancer. More detailed investigation on the specific interactions between Oct4 and different proteins in human cells may provide information not only on stem cell pluripotency but also on differentiation, reprogramming and possibly human diseases such as cancer. (Pardo et al., 2010.)

Oct4, Sox2 and Nanog share common binding sites in hESCs. These target genes frequently encode transcription factors, many of which are developmentally important.

The three factors form a regulatory circuitry, which consists of autoregulatory and feed forward loops. Oct4, Sox2 and Nanog have been shown to activate their own genes and repressing genes that are vital to developmental processes such as differentiation. Also the factors form feed forward transcriptional regulatory circuitry in which the factors act as activating regulators of specific hESC genes. These specific hESC genes encode for hES cell transcription factors, genes encoding components of chromatin remodeling and histone-modifying complexes and signaling pathways such as TGF-β signaling. Thus in hESCs, the three factors seem to be master regulators of pluripotency. (Boyer et al., 2005.)

2.4.2 Sox2

Sox2 belongs to a Sox (SRY-related HMG-box) transcription factor family. It is specifically expressed in ES cells and is required for the maintenance of pluripotency and self-renewal. Sox2 expression is restricted to cells with stem cell-like characteristics. Sox2 is down-regulated on cellular differentiation and thus it is not expressed on differentiated cells. (Avilion et al., 2003.)

Forced down-regulation of Sox2 in mESCs by RNAi promotes differentiation into trophectoderm and epiblast-derived lineages illustrating the importance of Sox2 expression for the maintenance of pluripotent state (Ivanova et al., 2006), (Masui et al., 2007). Masui et al. (2007) demonstrated the differentiation of mESCs into trophectoderm lineage was primarily due to inactivation of Oct4. As Oct4 expression levels need to be maintained at critical level for the maintenance of pluripotency, the repression of Oct4 also induces differentiation into trophectoderm. If Oct4 expression is artificially maintained after Sox2 reduction, the stem cell self-renewal-state can be restored. This indicates that the Sox2 has a function in the maintenance of pluripotency by transcriptional activation of Oct4. (Masui et al., 2007.)

2.4.3 c-Myc

C-Myc is a basic helix-loop-helix transcription factor, which is involved in several cellular functions including cellular growth, cell-cycle regulation and cellular proliferation (Schmidt, 1999). C-Myc has been identified as a potent oncogene and it plays a significant role in most human cancers (Geoghegan & Byrnes, 2008). Okita et al. (2007) generated

miPS cells from mouse embryonic fibroblasts (MEFs) by retroviral-mediated transgene transfer. The ability of miPS cells to produce adult chimaeras was assessed and it was found that 20 % of the offspring developed tumors. Detailed investigation of transgenes expressing the four transcription factors in the tumors revealed that retroviral transgene c-Myc was reactivated but not the other transgenes. Therefore, the data indicated that reactivation of the retroviral transgene c-Myc was attributable to tumor formation.

Two different signaling pathways activate c-Myc expression in stem cells, LIF/STAT3 in murine ES cells (Cartwright et al., 2005) and Wnt signaling pathway in murine and human ES cells (Sato et al., 2004). Both pathways control ES cell self-renewal and pluripotency independently but they possibly converge on the same targets. C-Myc has been identified as one of these downstream targets. In the LIF/STAT3 pathway STAT3 directly regulates the expression of c-Myc transcription factor. Normal c-Myc expression maintains the pluripotent state whereas over-expression promotes differentiation of mESCs. (Cartwright et al., 2005.) Thus c-Myc seems to be an important factor in maintaining pluripotency. Also Wnt signaling promotes the maintenance of pluripotency in hESCs and mESCs. Wnt signaling is thought to inhibit GSK3β, which in turn phosphorylates c-Myc leading to ubiqitin-mediated degradation. (Sato et al., 2004.)

The role of c-Myc in the reprogramming process is not fully understood. It is dispensable for iPS cell generation but removing it from the reprogramming mix significantly reduces the reprogramming efficiency (Amabile & Meissner, 2009).

2.4.4 Klf4

Klf4 (Krüppel-like factor 4) is a zinc finger transcription factor, which is highly expressed in epithelium of the skin, gastrointestinal tract and mESCs (Segre et al., 1999), (Zhang et al., 2000), (Yamanaka, 2008). Klf4 can activate and repress genes that are responsible for cell-cycle regulation and differentiation. Among the cell-cycle genes are up-regulated those that inhibit proliferation and down-regulated those that promote proliferation. Thus Klf4 may inhibit indirectly cellular proliferation by regulating cell-cycle gene expression and this has raised a possibility that Klf4 may have tumor-suppressor functions. Indeed, the expression of Klf4 has been diminished in various human cancer types. Contrariwise, elevated Klf4 expression levels are observed in mammary carcinomas.

Taken together, it seems that both the loss of Klf4 and over-expression of Klf4 may contribute to tumor formation. Thus, Klf4 has been proposed as a tumor-suppressor and oncogene. In the present light it seems that Klf4 inhibits the guardian of the genome, p53. It acts as a tumor-suppressor by inhibiting p53 and promoting p21 expression, which suppresses cellular proliferation. Conversely Klf4's role as an oncogene occurs when p21 is absent or inhibited by the presence of RAS/Cyclin-D1 signaling. (Rowland et al., 2005.)

For mESCs it seems that Klf4 over-expression maintains the mESC ability of self-renewal (Li et al., 2005). However, in miPS cells Klf4 seems to be dispensable for the reprogramming process as other Krüppel-like factors, such as Klf2 can replace it (Welstead et al., 2008). On the other hand, in a recent study the interactions of Klf4, Oct4 and Sox2 in miPS cells were investigated illustrating the critical importance of Klf4 (Wei et al., 2009). In the study it was shown that Klf4 interacts directly with Oct4 and Sox2. The complex containing Klf4, Oct4 and Sox2 was able to activate downstream gene targets, such as pluripotency gene Nanog. Interestingly, Klf4 alone can contribute to the activation of Nanog, but Oct4 and Sox2 alone cannot. (Wei et al., 2009.) Although not necessarily needed as exogenous reprogramming factor, Klf4's role for reprogramming in intrinsic gene regulatory network seems to be crucial.

2.5 IPS CELL REPROGRAMMING MECHANISM

Cell reprogramming is a complex process, which still is not thoroughly known. Partially reprogrammed iPS cells from an experiment conducted by Takahashi & Yamanaka (2006), have been a great use in deciphering the reprogramming mechanism (Hochedlinger & Plath, 2009). Partially reprogrammed cells are caught in the intermediate stage of full reprogramming. Thus, studying and comparing them to fully programmed cells can be informative in many ways regarding reprogramming mechanism. From the studies involving reprogramming mechanism, it has been found out that only temporal expression of the viral transgenes coding for transcription factors is needed for the activation of cell's own epigenetic network. This leads to the activation and deactivation of endogenous pluripotency genes and lineage-restricted gene expression, respectively. Endogenous pluripotency genes have their own network of activation circuitry, which upon fully activated leads to the gaining of pluripotent state and stem cell characteristics, such as

activation of pluripotency markers, for instance Oct4, Nanog and SSEA-4. Finally, in the fully reprogrammed iPS cells the exogenous transgenes, if integrated into the genome, are silenced (Figure 1). Thus differentiation into different cell types is possible without exogenous gene expression interfering. Here reprogramming mechanism is discussed from the perspective of retroviral induction.

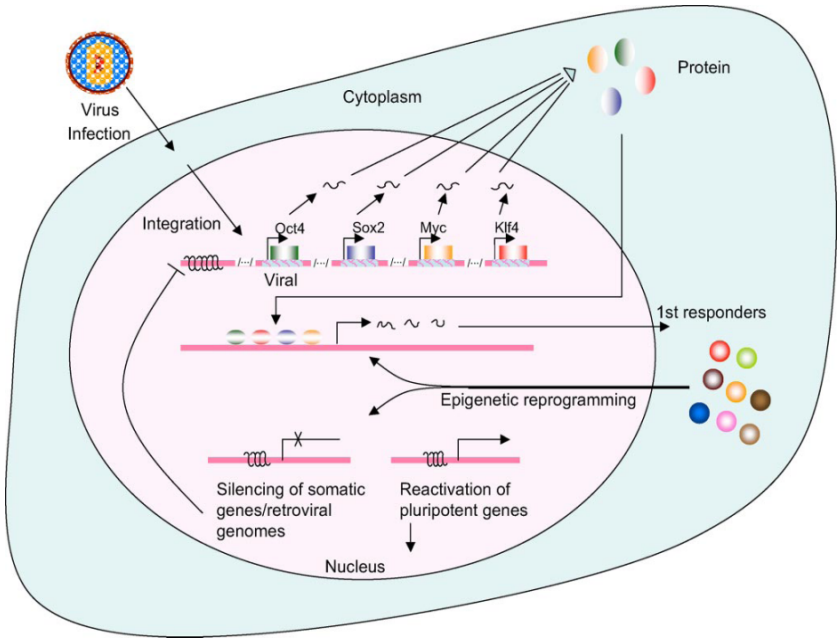

Figure 1. Mechanical steps involved in iPS cell reprogramming (Pei 2009).

2.5.1 Retroviral vectors and their integration into the genome

Retroviral ectopic expression of the defined factors reprograms the somatic cells into pluripotent state. As retroviral integration into the genome seem to occur at random pattern by non-homologous end-joining (Sakurai et al., 2010), a concern has risen that reprogramming requires additional activation of unknown factors. As retroviruses integrate into the genome causing insertional mutagenesis, they may activate or down-regulate genes important for reprogramming. As the procedure has been replicated around the world successfully albeit low reprogramming efficiency rates (Amabile & Meissner, 2009), it has been reasonable to argue that additional, unknown factors besides the four factors might be involved.

Retroviral and lentiviral integration sites have been studied in mouse and human models (Varas et al., 2009), (Winkler et al., 2010). Varas et al. (2008) infected mouse

fibroblasts with retroviruses carrying the defined reprogramming factors. The retroviral insertion sites were studied by ligation-mediated PCR in six iPS cell clones. No common insertion sites were detected among the clones. Furthermore, bioinformatics assays were conducted on genes targeted by retroviral insertions. No enrichment of specific genes or pathways was found, indicating that low reprogramming efficiency might have alternative explanations other than retroviral insertional activation of specific genes. Winkler et al. (2010) studied lentiviral integration sites in eight human iPS cell lines derived from human fetal fibroblasts or foreskin fibroblasts. The number of integration sites varied from 5 to 15 per clone but no common integration sites were detected between the clones. Remarkably, in six of the eight clones some of the integrations sites were found in pairs integrated into the same chromosomal allele within six base pairs of each other. According to their knowledge, this phenomenon has not been reported in any integration site assays. In order to generate iPS cells, the cells need to express all the transgenes responsible for reprogramming and also probably in appropriate stoichiometrial ratio as for instance, over-expression of Oct4 in ES cells results in differentiation (Niwa et al., 2000). Winkler et al. (2010) suggested that perhaps stoichiometrial requirement is better achieved when proviruses are integrated close each other.

Despite the differences in the viral vectors used in reprogramming, both studies came to the conclusion that no common integration sites exist between iPS cells and that efficient reprogramming is not dependent on insertional activation or deactivation of specific genes. How then the low efficiency in iPS cell generation can be explained? Yamanaka (2009) has considered three models explaining the low efficiency of iPS cell generation, which will be discussed next.

2.5.2 Reprogramming models for the generation of iPS cells

Yamanaka has considered two models: elite and stochastic for the generation of iPS cells (Figure 2). The elite model predicts that reprogramming takes place only in a subset of cells whereas stochastic model predicts that most of the cells, if not all, are capable for reprogramming. Current knowledge and evidence supports the stochastic model (Yamanaka, 2009a).

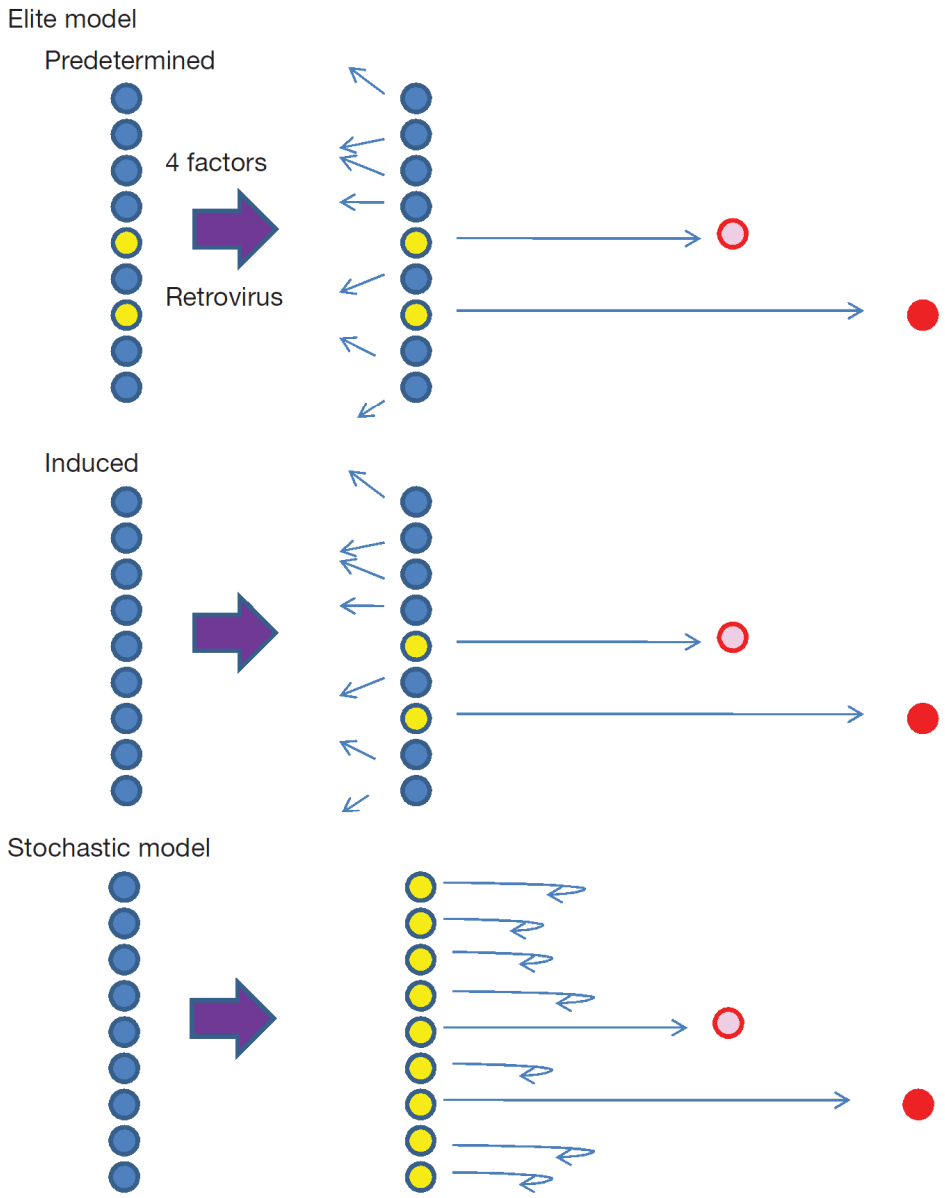

Figure 2. The elite and stochastic explaining the low efficiency of iPS cell generation. Yellow, cell competent for reprogramming; pink, partially reprogrammed iPS cell; red, fully reprogrammed iPS cell (Yamanaka, 2009a).

The elite model can be divided into two: predetermined and induced models. In the predetermined elite model small numbers of cells, 'elite' cells, are competent enough for reprogramming, such as tissue stem cells and other undifferentiated cells. Therefore the low reprogramming efficiency would be explained by the presence of these elite cells and their competency for reprogramming. In skin, stem cells comprise of~ 0,067 % of the cell population, which is similar to firstly reported reprogramming efficiency (Takahashi &

Yamanaka, 2006). However the efficiency of iPS cell generation in mouse is now higher than initially reported. It has been reported that MEF reprogramming efficiency can reach as high as 10 %. It is unlikely for tissue stem cells to exist in this magnitude in fibroblast cultures. Another line of evidence against predetermined model is that iPS cells have been generated from other tissues than skin as well, such as liver and pancreas and that genetic lineage tracing analyses showed that most iPS cells were originated from cells that expressed albumin or insulin. These findings indicate that lineage-committed cells can be reprogrammed into iPS cells. (Yamanaka, 2009a.)

In the induced elite model, factors other than the four factors must be activated in order to generate iPS cells. With viral induction methods this would mean insertional activation or deactivation of specific genes. However, as previously discussed, retro- or lentiviral integration sites seem to play no role in the activation of specific genes (Varas et al., 2009), (Winkler et al., 2010). Furthermore iPS cells have been generated without viral integration into the genome, such as adenoviral or recombinant protein based reprogramming. Therefore the current data does not support the induced elite model. (Yamanaka, 2009a.)

Although specific genes are not activated by viral vector integration, it does not exclude the significance of insertional mutagenesis. It may not be required for reprogramming but it might enhance the process. Yamanaka (2009) suggests that the amount, balance and silencing of the transgenes can be greatly influenced by the positions of retroviral integrations and it may explain why only a portion of cells complete the reprogramming process. Indeed, as Winkler et al. (2010) demonstrated, in most of the iPS cells viral integration sites were very close to each other in the same chromosomal allele. Therefore the position effect may play a different role other than specific gene activation or deactivation.

In the current light the stochastic model is favored over the other models discussed above. In this model most, if not all the cells are capable for reprogramming. According to Yamanaka (2009), there are at least two requirements for complete reprogramming. Firstly, the precise expression pattern for the four factors must be met in order to generate iPS cells. With current technology it is not possible to precisely control the expression pattern; therefore this requirement is met stochastically. Secondly, the cells need to maintain their

pluripotent state after the exogenous transgenes are silenced. In order to do this, cells own endogenous pluripotency network need to be reactivated, which involves in epigenetic changes, such as DNA methylation and histone modifications. The epigenetic state of somatic and stem cell is strikingly different, in somatic cells the pluripotency promoters are highly methylated whereas in stem cells they are hypomethylated. In complete reprogramming pluripotency promoters must be demethylated. The four factors don't have intrinsic DNA demethylation activity, yet pluripotency promoters are demethylated in complete reprogramming. (Yamanaka, 2009a.) The four factors may contribute to DNA demethylation either passively or actively. In passive demethylation the stochastic binding of reprogramming factors to target sites may hinder the activity of DNA demethylase Dnmt1. In active demethylation the reprogramming factors may recruit demethylases. However, the existence of demethylating enzymes in mammals has been highly controversial. (Hochedlinger & Plath, 2009.) At the moment it is not known how exactly pluripotency genes become demethylated. Moreover in favor of stochastic model is that the transcriptional status of genetically identical cells can be very different (Meissner et al., 2007). As genetically identical cells' transcriptional pattern is different, so is their epigenetic state. If the cells epigenetic state can differ, it probably means that binding sites for the reprogramming factors, due to histone and DNA modifications, can differ too. Therefore stochastic binding of reprogramming factors to target sites and possibly the hindering of Dmnt1 by stochastic manner may overall contribute stochastically to the iPS cell generation. (Hochedlinger & Plath, 2009.)

2.5.3 Epigenetic reprogramming and sequential activation of pluripotency markers

After the retroviral vectors have integrated into the genome and transgene expression driven by viral promoters is initiated, the transcription factors act co-operatively to reactivate pluripotency genes. This epigenetic reprogramming involves in remodeling the chromatin structure by DNA methylation and histone modification systems.

Pluripotent stem cells contain specific chromatin structures, termed 'bivalent domains'. These domains contain enriched regions of activating histones H3K4me3 and repressing histones H3K27me3 or H3K9me2. Pluripotent stem cells contain a large number

of bivalent domains (~2500) whereas progenitor cells such as neural progenitor cells contain much less (~200). (Amabile & Meissner, 2009.)

Maherali et al. (2007) and Mikkelsen et al. (2008) have investigated the presence of H3K4me3 and H3K27me3 in miPS cells. In order to complete reprogramming, the lineage-committed genes must be repressed and in contrast, pluripotency genes activated. Both studies came to the conclusion that complete reprogramming can epigenetically reset the somatic cell genome. It involves the establishment of bivalent domains, genome-wide H3K4me3 enrichment in pluripotency-associated genes and H3K27me3 enrichment in developmental and tissue-specific genes (Figure 3).

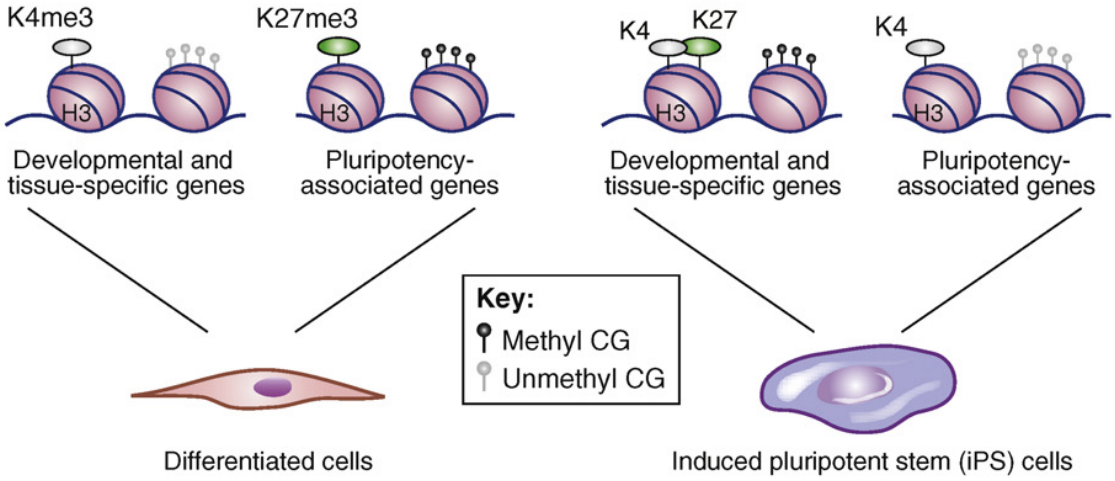

Figure 3. The epigenetic difference between differentiated cells and iPS cells (Adapted from Amabile & Meissner 2009).

The re-activation of endogenous pluripotency genes occurs in sequential manner (Figure 4). Brambrink et al. (2008) and Stadtfeld et al. (2008) studied the kinetics of pluripotency marker appearance in miPS cells. According to both studies, the exogenous transgene expression is required for at least 12–16 days in MEFs in order to generate miPS cells. Brambrink et al. (2008) observed more miPS cell colonies rising, if the exogenous transgene expression is continued beyond that time point.

What molecular events then happen in miPS cells during reprogramming? First pluripotency associated marker to activate is alkaline phosphatase (Brambrink et al., 2008).

At the same time surface antigen Thy1, which is highly expressed on fibroblasts and other differentiated cell types, was shown to be downregulated after 3 days of transgene expression. The downregulation of Thy1 occurs gradually in the majority of cells. (Stadtfeld et al., 2008a.) Recent study has shown that c-Myc promotes transcriptional repression of fibroblast-specific genes, including Thy1 (Sridharan et al., 2009). As the downregulation of Thy1 proceeds, simultaneously the next embryonic marker to activate is stage-specific embryonic antigen 1 (SSEA-1). However, SSEA-1 is not activated in all the cells, only in a fraction of cells. SSEA-1 positive cells then activate next endogenous genes Oct4, Sox2 and Nanog as the reprogramming process proceeds. The activation of these pluripotency markers represent late events of reprogramming, firstly observed around day 16 (Brambrink et al., 2008), (Stadtfeld et al., 2008a). In addition to the activation of pluripotency markers telomerase and X chromosome in female cells are also activated (Stadtfeld et al., 2008a). However, the activation of endogenous pluripotency genes doesn't necessarily represent full reprogramming of iPS cells. The retroviral transgenes must be silenced and only by then iPS cells complete their reprogramming.

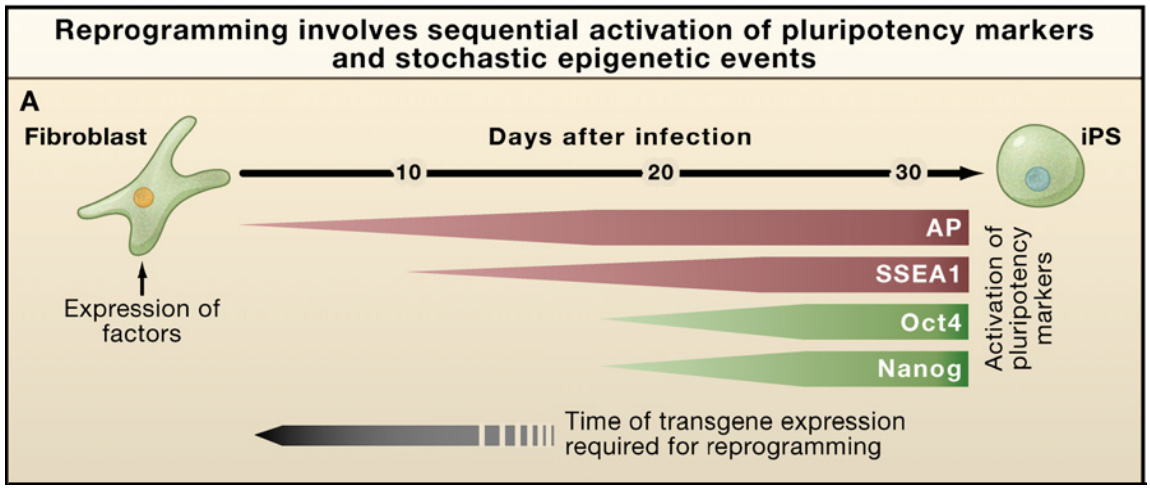

Figure 4. The sequential activation of pluripotency markers in miPS cells during reprogramming (Adapted from Jaenisch & Young 2008).

The reprogramming process involves intermediate steps, which result in sequential activation of pluripotency markers, changes in histone and DNA methylation statuses of pluripotency promoters and the changes of molecular circuitry between viral exogenous genes and endogenous genes (Figure 5).

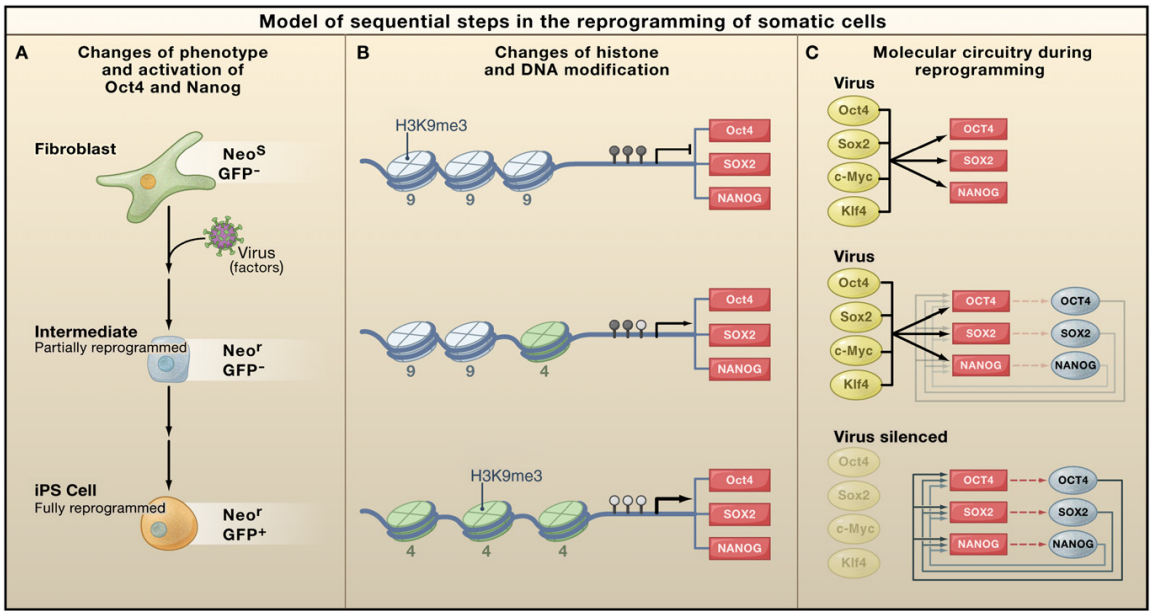

Figure 5. The model of sequential activation in the reprogramming process (Jaenisch & Young, 2008).

Fibroblasts do not express Oct4 or Nanog. Using genetic modification, MEFs were generated that carried neomycin-resistance marker inserted either endogenous Oct4 or Nanog locus. In addition, GFP-marker was inserted either under the control of promoters endogenous Oct4 or Nanog. (Maherali et al., 2007), (Okita et al., 2007), (Wernig et al., 2007.) Fibroblasts were then infected with viruses carrying the four factors. The endogenous Oct4 and Nanog became expressed at low levels sufficient for drug selection. However, the low level of expression was not enough for detection of GFP (Figure 5, A). The low level of expression may be explained by changes of histone and DNA methylation. The promoters of pluripotency markers Oct4 and Nanog may be partially activated by histone H3K4me3 (Figure 5, B). As the reprogramming proceeds the changes of histone and DNA methylation finally result in fully activated promoters and the detection of GFP in fully reprogrammed iPS cells. The transcription factor circuitry underlying the reprogramming process may be depicted as in Figure 5 C. At first exogenous transgene expression is required for the initiation of reprogramming process. Transcription factors bind also on their own promoters in addition to others. Thus, activated autoregulatory loop leads to endogenous gene activation and finally, as the exogenous genes are silenced, the

autoregulatory loop continues its function and pluripotency is sustained. (Jaenisch & Young, 2008.)

2.5.3 The silencing of retroviral transgenes

The retroviral vectors (pMXs) used for the generation of iPS cells were derived from Moloney murine leukemia virus (MoMLV). MoMLV has been extensively studied for retroviral silencing in pluripotent stem cells since 1977. (Hotta & Ellis, 2008.) Indeed, well-established evidence now exists for MoMLV silencing in embryonic cells (Jaenisch & Young, 2008). In the pMX vectors the transcription of transgenes is initiated at the 5' LTR promoter. The silencing elements have been primarily mapped to 5'LTR promoter. A number of transcriptional silencer molecules, such as TRIM28, have been identified that bind to these silencing elements. These silencers play an important role in the shutdown of transgene expression in ES cells. It is now evident that retroviral vector silencing occurs also in iPS cells and that marks an epigenetic beacon that distinct pluripotent states. In partially reprogrammed cells the transgene expression is not silenced, whereas in fully reprogrammed cells the transgenes are silenced. (Hotta & Ellis, 2008.)

It has been observed that when retroviral vectors are expressed in ES cells, their expression levels are significantly lower than in fibroblasts. This suggests that there are more repressor factors or less activator factors to transgene expression in ES cells. Also retroviral or lentiviral infected ES cells can be variegated, where some of the cells either express the transgene or are silent. This suggests that position effects of retroviral integration into the genome exist and that may influence on transgene expression. Studies on retroviral silencing have shown that epigenetic pathways are also involved. These pathways include histone deacetylation and methylation and DNA methylation. (Hotta & Ellis, 2008.)

Histone modifications, as previously discussed, play an important role in regulating the activity of pluripotency promoters. They may also play an important role in shutting down the exogenous transgene expression. Histone acetylation has been characterized as the best mark of transcriptional activation. In contrast, deacetylation of histones leads to silencing. In addition to histone deacetylation, also histone methylation plays a part in regulating transcriptional activity. As previously discussed, methylation of histone tails

H3K27 or H3K9 is recognized as repressive mark, whereas methylation of H3K4 is recognized as an active mark. (Hotta & Ellis, 2008.)

Cytosine methylation of DNA has been implicated in retroviral silencing. In miPS cells the expression levels of the retrovirally transduced transgenes were shown to decrease at the same time as the expression level of de novo methyltransferase Dmnt3a2 increased (Okita et al., 2007). In ES cells, it has been observed that silent retrovirus is heavily methylated at CpG sites. Although DNA methylation contributes to silencing, emerging evidence has shown that it is not required to establish silencing. DNA methylation seems to play a role in the maintenance of silent state. (Hotta & Ellis, 2008.)

Although several epigenetic pathways have been identified involving retroviral silencing, it is not known which factor initiates the silencing mechanism. Ultimately, full-scale genome screening may be required for the identification of the factors that initiate silencing in ES and iPS cells. (Hotta & Ellis, 2008.)

It has been observed that the same retroviral vector can be silent or variegated in ES cells (Yao et al., 2004). As retroviruses integrate into the genome in a random pattern, position effects on genome integration sites may contribute to gene expression and silencing (Hotta & Ellis, 2008). It has been shown that retro- and lentiviral vectors prefer to integrate into euchromatin region and disfavor the heterochromatin (Wang et al., 2007). During reprogramming, extensive chromatin remodeling occurs and some cells may acquire more advantageous stoichiometry of the four factors and destined to reprogram into iPS cells. As the epigenetic state of pluripotent and somatic cell is different, the chromatin restructuring may lead to decrease of expression levels of the viral vector or even transcriptional shutdown of the viral vector. However, ES cells appear to have more open chromatin state than in differentiated cells (Azuara et al., 2006) and therefore chromatin remodeling alone may not fully explain the silencing mechanism. Hotta & Ellis (2008) have proposed that complete silencing may be dependent on currently unknown factors that actively recognize integrated viruses in stem cells. These factors may be enriched in stem cells, as the retroviral expression in stem cells is significantly lower than in somatic cells.

Retroviral silencing is certainly required in the reprogramming of somatic cell to an pluripotent state as otherwise the ectopic expression of the four factors would presumably prevent differentiation. Although recognized as a critical point in generating quality iPS

cell lines, the actual silencing mechanism remains elusive. Partially reprogrammed iPS cells may provide a platform to study silencing mechanism. It has been proven that the treating partially reprogrammed mouse iPS cells with inhibitors of DNA methylation can reprogram them into fully-induced iPS cells, in which retroviral vectors are silenced (Mikkelsen et al., 2008). Thus unraveling the silencing mechanism may provide novel insights for viral vector designing, for instance to be used for the genetic manipulation of stem cells.

2.6 DIRECT LINEAGE REPROGRAMMING

Switching cell fate by reprogramming has offered new insights in generating lineage restricted cells. In lineage reprogramming the fate of differentiated cell can be switched to another without the intermediate pluripotent state. In fact, endocrine β cells have been generated from pancreatic endocrine progenitor cells (Zhou et al., 2008), neurons from dermal fibroblasts (Vierbuchen et al., 2008) and cardiomyocytes from fibroblasts (Ieda et al., 2010). In all of these experiments the certain combination of defined factors induced the reprogramming and the switching of cell fate.

Ieda et al. (2010) screened and identified three developmental transcription factors that were able to reprogram rapidly and efficiently mouse fibroblasts into cardiomyocytes. Induced cardiomyocytes (iCM) reprogramming takes place a much rapid pace than iPS cell reprogramming, as first iCMs appeared on day 3 after transduction of the defined factors. Authors note that the reprogramming process appears to continue for several weeks with progressive changes in gene expression, contractibility and in electrophysiology. Induced CMs express cardiac-specific markers and have a global gene expression profile resembling postnatal cardiomyocytes. The efficiency of cardiomyocyte induction was higher than in iPS cell induction. Up to 20 % of the initial fibroblast cell population was efficiently reprogrammed into cardiomyocytes. (Ieda et al., 2010.)

Although promising, the experimental procedure needs to be executed with human cells. If human cells can be reprogrammed efficiently into cardiomyocytes, that will give new insights into therapeutic applications. The avoidance of pluripotent cells may reduce the risk of tumorigenicity. As the starting material is fibroblast, it can be grown in high amounts and induced into cardiomyocytes with defined factors. These cells could then be

utilized in several applications, drug screening, toxicology, cell therapy and studying patient-specific diseases. Nevertheless, the reprogramming has given new insights on controlling cell fates and utilizing them into regenerative medicine and biomedical research.

2.7 GENETICALLY MODIFIED STEM CELLS

Pluripotent stem cells are able to differentiate into all cells originating from germ layers; ecto-, meso- and endoderm. The transcriptional networks that control the self-renewal and differentiation of stem cells are complex. Therefore genetic modification of stem cells is needed to study these networks. Genetic modification strategies include gain and loss of function approaches, generation of specific reporter-based cell lines, gene expression alteration, gene silencing via RNAi and ectopic expression of desired transgene (Giudice & Trounson, 2008), (Nieminen et al. 2010). Fluorescence reporter genes under the control of cell-specific promoters provide effective means to identify and isolate genetically modified stem cells and their derivatives. Also antibiotic resistance genes under the control of cell-specific promoters are used for selection to purify desired cell populations from mixed population of hESC-derived cells. (Giudice & Trounson, 2008.)

Genetic manipulation of stem cells is a relatively new technique (Giudice & Trounson, 2008). As different strategies for gene transfer have been investigated, it has been observed that human pluripotent stem cells are highly resistant to non-viral genetic manipulation (Buecker et al., 2010). The most effective transgene delivery method for stem cells seems to be viral-mediated. Viral infection of hESCs usually yields heterogeneous cell population. If not sorted, the transgene expression level starts to decrease as cells are passaged. Unsorted cells lose progressively their fluorescence by the 7^{th} passage. The problem for hESCs is that they are difficult to expand clonally. After viral infection clonal selection is needed to identify and isolate the desired cells. However, hESCs inability to grow from single cells greatly impedes clonal selection. (Kita-Matsuo et al., 2009.)

Viral-mediated transgene delivery seems to be the most effective method for the genetic modification of human stem cells, but it has at least two drawbacks. First, not all the viral vectors are suitable for genetic modification. For example, adenovirus vectors are poor at gene transfer to hESCs, they do not integrate into the genome and they only express

transiently in cells (Kane et al., 2008). On the other hand, retroviral vectors do integrate into the genome and they do express transgenes stably. However, problems may rise from gene silencing. Thus, the second major drawback is transgene silencing as was observed with pMXs vectors (Takahashi et al., 2007). However, pMXs vectors suits well for iPS cells as pluripotency inducing transgenes are silenced and thus differentiation is possible without the ectopic expression of the transgenes. If stable gene expression in pluripotent cells is required, self-inactivating (SIN) lentiviral vectors may prove useful. SIN lentiviral vectors are less subject to gene silencing, although they still contain unknown silencing elements (Ellis & Yao, 2005). Therefore viral vector design must be taken into account when applying them for the genetic modification of stem cells.

2.7.1 SIN lentiviral vectors for the genetic modifications of stem cells

SIN vectors represent the latest 3rd generation of lentiviral vectors (Dull et al., 1998), (Zufferey et al., 1998), (Miyoshi et al., 1998). SIN lentiviral vectors are designed so that the transcription of gene of interest is driven by internal promoter. Internal promoter can be active in specific cells, e.g. Oct4 in stem cells. Thus, the gene of interest will be expressed under Oct4 promoter only in stem cells.

SIN vectors have been modified to enhance their biosafety by deleting enhancer and promoters in the U3 region of the 3'LTR RNA. Once reverse transcribed in cells, the ΔU3 region deletion is transferred to 5'LTR of the proviral DNA. Thus, the deletion of ΔU3 region will inactivate any transcriptional activity in the 5'LTR region of the integrated vector – hence, the name self-inactivating vector (Figure 6). As 5'LTR promoter activity is inactivated, the gene of interest will only be expressed under the internal promoter. The system has several advantages. First, the self-inactivation of lentiviral vector minimizes the risk of emerging replication competent retroviruses, thus greatly improving their biosafety. Second, SIN vectors reduce the possibility of oncogene activation by insertional mutagenesis as 5'LTR promoter activity is diminished. Third, gene of interest can only be driven by internal promoter, thus facilitating cell-specific expression of the transgene. Moreover any transcriptional interference between internal promoter and 5'LTR are prevented. (Miyoshi et al., 1998), (Zufferey et al., 1998), (Julius et al., 2000), (Kittler & Moss 2006).

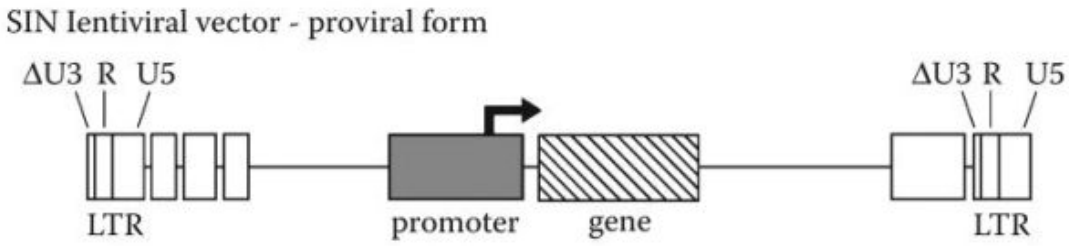

Figure 6. Schematic representation of the SIN lentiviral vector (Adapted from Kittler & Moss 2006).

SIN lentiviral vectors have been used for generating stable hESC lines for fluorescent tracking and drug resistance selection of cardiomyocytes (Kita-Matsuo et al., 2009). However, as noted before, clonal selection and expansion of genetically engineered hESCs is difficult and success depends on optimized cell culture conditions. The same vectors (Figure 7) will be used in this study to establish fluorescent and antibiotic selectable iPS-cell lines derived from human skin fibroblasts.

Figure 7. Lentiviral constructs used in the generation of genetically modified hESC lines.
A) Oct4 promoter drives the expression of eGFP in hiPS cells.
B) α myosin heavy chain (αMHC) is cardiac-specific promoter driving the expression of mCherry in cardiomyocytes. Rex-1 promoter drives the expression of antibiotic resistance gene blasticidine in hiPS cells.
C) Phosphoglycerate kinase (PGK) promoter is ubiquitously expressing and driving the expression of H2B-mCherry fusion protein, where H2B directs the proteins back to nucleus (Adapted from Kita-Matsuo et al., 2009).

3. AIMS OF THE RESEARCH

The aim of the study is the establishment of fluorescent and antibiotic selectable hiPS cell lines derived from human skin fibroblasts. Before reprogramming, fibroblasts will be genetically modified by SIN lentiviruses carrying cell type specific promoters driving the expression of fluorescent markers and antibiotic resistance gene blasticidine. The aim is to establish at the same time several different genetically engineered hiPS cell lines. If the establishment of genetically engineered hiPS cell lines is successful, the hiPS cells will be characterized by immunocytochemistry, RT-PCR and differentiation potential will be assessed by embryoid body formation and direct differentiation into cardiomyocytes. Differentiated cardiomyocytes will be characterized by immunocytochemistry.

Ultimately, the aim is to establish genetically engineered hiPS cell lines more effectively and more conveniently than genetically modifying hESCs. Genetically engineered hiPS cell lines can be used to study for example cardiac differentiation. The appearance of cardiomyocytes can be monitored by fluorescent reporter expression and these cardiomyocytes could be used for cell tracking *in vitro* or *in vivo*, evaluating differentiation protocols and for various quantification analyses.

4. METHODS

4.1 PLASMID DNA PURIFICATION

Plasmid DNAs, which contained the expression constructs to be later used in the production of lentiviral vectors, were purchased from Addgene. Bacterial cultures were grown in LB (Luria-Bertani)-medium supplemented with 50 µg/ml of ampicillin at +37 ºC overnight. The plasmids were purified from bacterial cultures by NucleoBond Xtra Midi kit (Macherey-Nagel) according to manufacturer's instructions. Oct4-eGFP plasmid had already been purified, thus only αMHC-mCherry-Rex-Bla and PGK-H2B-mCherry plasmids were purified. After isolation and purification, the DNA concentration was measured by UV-visible spectrophotometer (Ultrospec 3000 Pro). In addition, agarose gel electrophoresis was used to analyze the purity of the DNA.

4.2 CELL CULTURE CONDITIONS

Human foreskin fibroblasts (hFFs; ATCC) were cultured in knock-out Dulbecco's modified Eagle's medium (KO-DMEM; Invitrogen) supplemented with 10 % fetal bovine serum (FBS; PAA Laboratories GmbH), 2 mM L-glutamine (GlutaMAX; Invitrogen), 1 % Pen/Strep (10 U/ml penicillin, 10 U/ml streptomycin; Lonza) and 1 % non-essential amino acids (NEAA; Sigma).

The culture medium for 293FT cells consisted of DMEM (Lonza) supplemented with 10 % FBS (PAA Laboratories GmbH), 2 mM L-glutamine (Invitrogen) and 1 % NEAA (Sigma). Cell culture dishes (Sarstedt, USA) were coated with 0.1 % gelatin 10 minutes at +37 ºC before plating the cells.

The PLAT-E cells were cultured on DMEM (Lonza) containing 10 % FBS (PAA Laboratories GmbH). For PLAT-E cells the cell culture dishes were coated with 0.1 % gelatin 10 minutes at +37 ºC as well.

Mouse embryonic fibroblasts (MEFs; pMEF-p3, Millipore) were cultured in KO-DMEM (Invitrogen) supplemented with 10 % FBS (PAA Laboratories GmbH) and 2 mM L-glutamine (GlutaMAX; Invitrogen). The culture medium was sterile filtered using 0.45

μm filter (Acrodisc®; Pall Corporation) before use. Cell culture dishes and plates were coated with 0.1 % gelatin 1 hour at room temperature before plating the cells.

END-2 cells were cultured in DMEM/F-12 (Invitrogen) containing 7.5 % FBS (PAA Laboratories GmbH), 1 % NEAA (Lonza), 2 mM L-glutamine (GlutaMAX; Invitrogen) and 0.5 % Pen/Strep (10 U/ml penicillin, 10 U/ml streptomycin, Lonza). Cell culture plates were coated with 0.1 % gelatin 1 hour at room temperature before plating the cells.

Cardiomyocyte differentiation protocol required two different medium compositions. The first medium (0 % KO-SR hES) consisted of KO-DMEM (Invitrogen), 1 % NEAA (Lonza), 2 mM L-glutamine (GlutaMAX; Invitrogen), 0.5 % Pen/Strep (10 U/ml penicillin, 10 U/ml streptomycin; Lonza) and 0.1 mM β-mercaptoethanol (Invitrogen). The second medium (10 % KO-SR hES) was the same as previously described, except the addition of 10 % knock-out serum replacement (KO-SR; Invitrogen).

The embryoid body differentiation medium consisted of KO-DMEM (Invitrogen), 10 % FBS (PAA Laboratories GmbH), 1 % NEAA (Lonza), 2 mM L-glutamine (GlutaMAX; Invitrogen), 0.5 % Pen/Strep (10 U/ml penicillin, 10 U/ml streptomycin, Lonza).

Human iPS cells were cultured in KO-DMEM (Invitrogen) supplemented with 20 % KO-SR (Invitrogen), 1 % NEAA (Lonza), 2 mM L-glutamine (GlutaMAX; Invitrogen), 0.5 % Pen/Strep (10 U/ml penicillin, 10 U/ml streptomycin, Lonza), 0.1 mM β-mercaptoethanol (Invitrogen) and 4 ng/ml FGF (R&D systems).

Human iPS cells were grown on 6-well-plates (Corning Inc., Corning NY, USA) feeder layer consisting of MEFs (pMEF-p3; Millipore), which were plated at the density of 250’000 cells/well. The hiPS cells were passaged once a week by first removing the feeder layer, then adding 1 mg/ml collagenase IV (Gibco; Invitrogen) and incubating 5 minutes at +37 ºC. Cells were rinsed once with KO-DMEM (Invitrogen) before adding hiPS cell culture medium. The colonies were mechanically dispersed into smaller cell clusters, which were replated on new MEF feeder layer in 6-well-plate.

4.3 GENERATION OF HUMAN IPS CELLS

4.3.1 Lentivirus production

293FT cells were thawed and plated on 10 cm^2 cell culture dish and cultured in 293FT medium without antibiotics one day before transfection. 293FT cells were transfected with the instructions of Virapower™ Lentiviral expression system (Invitrogen). 9 µg Virapower™ Packaging Mix (Invitrogen) and 3 µg of expression plasmid DNA were diluted in 1 ml of OPTI-MEM® (Invitrogen). Next, 60 µl Lipofectamine™ 2000 (Invitrogen) was diluted in 2 ml of OPTI-MEM®. The DNA and Lipofectamine™ 2000 containing solutions were combined and incubated for 20 minutes at room temperature. 293FT culture medium was changed and DNA/Lipofectamine™ 2000 solution was added drop-wise to the cells and incubated overnight at +37 ºC, 5 % CO_2.

The next day hFFs were plated on 10cm^2 cell culture dishes (Falcon, Becton Dickinson) at the density of 800'000 cells and the culture medium for 293FT cells was changed.

On the fourth day hFFs (passage 6) were infected with lentiviruses. First 293FT cell supernatant was collected and sterile filtered using 0.45 µm filter (Whatman® Puradisc™ FP 30/0.45) and 4 µg/ml of polybrene was added into the supernatant. The hFFs were rinsed once with PBS (Lonza) before adding virus containing supernatant. The next day virus supernatant was replaced with fresh hFF culture medium, after rinsing the cells once with PBS.

In the first week hFFs were infected with lentiviruses that carried purified plasmid DNA expression constructs and the next week they were infected with lentiviruses that carried the mouse ecotropic retrovirus receptor gene Slc7a1. Fresh hFF culture medium was changed 1–2 times in between lentiviral infections.

4.3.2 Retrovirus production

PLAT-E cells were thawed and plated on 10 cm^2 cell culture dish (Sarstedt, USA) and cultured in PLAT-E medium without antibiotics one day before transfection. PLAT-E cells were transfected with retroviral vectors carrying the reprogramming factors. 27 µl of Fugene® 6 (Roche Applied Science) transfection reagent was diluted in 873 µl of OPTI-

MEM (Invitrogen) and incubated for 5 minutes at room temperature. Then, 9 µg of pMXs retroviral vector was added into diluted Fugene® 6 solution and incubated for 20 minutes at room temperature. Next, PLAT-E media was changed and DNA/Fugene® 6 solution was added drop-wise onto the cells and incubated overnight at +37 ºC, 5 % CO_2. One retroviral plasmid DNA involved one dish of PLAT-E cells.

The next day PLAT-E culture medium was changed and the day after that, 800'000 hFFs were infected with retroviruses carrying reprogramming factors. First viral supernatants were collected from PLAT-E dishes and sterile filtered using 0.45 µm filter (Whatman® Puradisc™ FP 30/0.45). The supernatants were combined in equal amounts and 4 µg/ml of polybrene was added. The hFFs were rinsed once with PBS and 10 ml of viral supernatant was added into each dish. After 24 hour infection the viral supernatant was replaced with fresh hFF medium.

1 week after retroviral infection, the hFFs were trypsinized and replated on 10 cm^2 cell culture dishes (Sarstedt, USA) on MEF feeder layers at the densities of 25'000 and 50'000. The next day culture medium was changed to hiPS cell culture medium supplemented with 10 mM Rhokinase-inhibitor (ROCK-I; Cal Biochem, Merck Biosciences). The hiPS cell medium was changed every other day.

The hiPS colonies were picked up 28 days after the retroviral infection. The hiPS colonies were rinsed once with PBS and 5 ml of PBS was added into each dish. Individual colonies were picked from the culture dish under Nikon SMZ800 Stereomicroscope with attached Thermoplate (Tokai Hit). Colonies were first transferred into 96-well plate containing 20 µl of hiPS cell medium, then 180 µl of hiPS cell medium was added and the colonies were dispersed mechanically into smaller cell clusters, which were plated on 24-well plate (Nunc) on MEF feeder layers. Beforehand, 300 µl of hiPS cell medium was added onto 24-well MEF-plate. The cells were incubated at +37 ºC, 5 % CO_2 (Galaxy R incubator, RS Biotech), for one week before passaging. The fluorescence of the hiPS cell colonies was monitored with Olympus IX 51 fluorescence microscope and photographed using DP30BW camera (Olympus).

4.4 CELL LINE CHARACTERIZATIONS

4.4.1 Immunocytochemistry

Human iPS cells were characterized by immunocytochemical staining for Oct4, Nanog and SSEA-4. Differentiated cardiomyocytes were immunocytochemically stained for αMHC and Troponin T. Cells were washed twice with PBS (Lonza) and fixed with 4 % paraformaldehyde for 20 minutes in room temperature. After fixing, cells were gently washed twice with PBS for 5 minutes. Non-specific binding of the antibody was prevented by blocking solution, which contained 10 % normal donkey serum (NDS), 0.1 % TritonX-100 (Sigma) and 1 % BSA (Sigma) in PBS. Cells were blocked for 45 minutes in room temperature. Primary antibody solutions were made as follows: antibody Oct4 (R&D Systems, goat IgG) was diluted 1:400, Nanog (R&D Systems, goat IgG) 1:200 and SSEA-4 (Santa Cruz Biotechnology, mouse monoclonal IgG) 1:200, αMHC (Chemicon, mouse IgG) 1:100, Troponin T (Abcam, goat IgG) 1:1500 in 1 % NDS, 0.1 % TritonX-100 and 1 % BSA in PBS. Cells were washed once with dilution solution for antibodies. Primary antibodies were incubated on cells at +4 °C overnight.

The next day cells were first washed three times with 1 % BSA in PBS for 5 minutes. Secondary antibodies for Oct4 and Nanog (Alexa Fluor 568 nm donkey anti-goat IgG, Invitrogen), SSEA-4 (Alexa Fluor 568 nm donkey anti-mouse IgG Invitrogen), αMHC (Alexa Fluor 488 nm donkey anti-mouse IgG, Invitrogen) and Troponin T (Alexa Fluor 568 nm donkey anti-goat IgG, Invitrogen) were diluted in 1:800 in 1 % BSA in PBS. Secondary antibodies were incubated on cells for 1 hour protected from light in room temperature. After incubation, cells were washed twice with PBS for 5 minutes and twice with phosphate buffer for 5 minutes. Washed cells were mounted with Vectashield (Vector Laboratories Inc., Burlingame CA) containing 4',6-diamino-2-phenylindole (DAPI) and covered with cover slip. The imaging was done using an Olympus IX 51 fluorescence microscope and DP30BW camera (Olympus).

4.4.2 RNA extraction and cDNA-synthesis

Total RNA from cells was isolated according to NucleoSpin RNA II (Macherey-Nagal) kit instructions. The purity and concentration of RNA samples were measured on

NanoDrop (NanoDrop Spectrophotometer ND-1000). The RNA samples were stored at -80 °C (Vip Series -86 °C; Sanyo)

Reverse transcription to cDNA was performed by the instructions of High-Capacity cDNA reverse transcriptase-kit (Applied Biosystems). The total RNA of the samples to be used in reverse transcription reaction was 100 and 200 ng. Two negative controls were used in the reaction: 1) H_2O control and 2) -RT control, which contained 200 ng of RNA but no reverse transcriptase enzyme. RNAs were reverse transcribed using MasterCycler Personal (Eppendorf). Reaction condition contained four steps: 1) 25 °C, 10 minutes 2) 37 °C, 120 minutes 3) 85 °C, 5 seconds and 4) 4 °C, hold. The cDNA samples were stored at -20 °C.

4.4.3 RT-PCR and agarose gel electrophoresis

The synthesized cDNAs were used as templates to probe the expression of specific mRNAs from endogenous genes Oct4, Sox2, c-Myc, Nanog, Rex-1, β-actin and exogenous transgenes Oct4, Sox2, c-Myc and Klf4. The β-actin acts as a housekeeping gene and thus was used as a control. The primers (biomers.net) were diluted from stock to 5 µM concentrations. For exogenous transgenes Oct4, c-Myc and Klf4 retroviral plasmid backbone pMXs-L3205-AS was used as antisense primer whereas for exogenous Sox2 transgene pMXs-Tg-AS was used as antisense primer.

Embryoid body cDNAs were used as templates to probe the expression of the germ layer markers. Endogenous markers were α-fetoprotein (AFP) and Sox17, mesodermal α-cardiactin and kinase insert domain receptor (KDR), ectodermal markers Sox1, paired box gene 6 (PAX6), Nestin and Musashi 1. The 5 µm primer concentrations were used.

PCR was performed using Dynazyme II (Finnzymes; Espoo, Finland) and by the manufacturer's instructions. PCR master mix for each reaction (24 µl/reaction) contained 2,5 µl of 10x Dynazyme buffer, 0,5 µl of dNTPmix (Fermentas) 2,5 µl of sense and antisense primers, 0,25 µl of Dynazyme II and 15,75 µl of H_2O. In addition, optimized concentration of 0.5 mM $MgCl_2$ (Fermentas) and 5 % dimethyl sulfoxide (DMSO) were used for endogenous genes. 1 µl of the template cDNA was used in the reaction. For each exogenous transgene specific positive control was used in the reaction. In addition, negative H_2O control was used both in exogenous and endogenous reactions.

PCR was performed on MasterCycler epgradient (Eppendorf) and the reaction condition for endogenous genes Oct4, Sox2, c-Myc, Rex-1 and β-actin was as follows: denaturation step at 94 °C for 2 minutes, followed by 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 1 minute and finally after last cycle extension at 72 °C for 5 minutes. Reaction conditions for exogenous genes and Nanog were the same as above except annealing temperature was 55 °C on exogenous genes and 45 °C on Nanog.

PCR reaction conditions for embryoid body markers was as follows: denaturation step at 95 °C for 3 minutes, followed by 40 cycles of denaturing at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 minute and finally after last cycle extension at 72 °C for 5 minutes.

PCR results were analyzed by agarose gel electrophoresis (Amersham Biosciences Electrophoresis). 2 % agarose gels were made containing 0.25 μg/ml of ethidium bromide (Sigma). 50 bp DNA ladder (Generuler; Fermentas) was used for DNA sizing. Gels were photographed under UV-light using Bio-Rad Universal Hood II and ChemiDoc XRS scanner.

4.5 BLASTICIDINE SELECTION FOR HUMAN IPS CELLS

Blasticidine (Sigma) was weighed and dissolved into H_2O and aliquated for -20 °C storage. For blasticidine selection 6-well-plates (Corning Inc., Corning NY, USA) were first coated with Matrigel™ (BD Biosciences) followed by plating of MEFs. Matrigel was thawed on ice and diluted into DMEM/F-12 (Invitrogen), which was used to coat 6-well-plates for 1 hour at room temperature. After 1 hour coating, 250’000 MEFs were plated on each well of 6-well-plate. The next day hiPS cells were passaged on Matrigel-coated MEFs. Cultured hiPS cells were allowed to grow for 6 days before administering 5 μg/ml of blasticidine.

4.6 HUMAN IPS CELL DIFFERENTIATION

4.6.1 END-2-differentiation

Human iPS cells were directly differentiated into cardiomyocytes by coculturing them with endoderm-like cells (END-2 cells), which have been derived from mouse embryonal carcinoma cells (Passier et al., 2005). END-2 cells were first treated with 5 µl/ml of Mitomycin C (Sigma) for 3 hours. Cells were trypsinized (Lonza) and counted with hemocytometer (Neubauer). END-2 cells were plated on 12-well-plate (Nunc) at a density of 175'000 cells/well.

The next day END2-medium was changed to 0 % KO-SR hES medium supplemented with 2.92 mg/ml of ascorbic acid 1 hour before plating hiPS cells. MEF feeders were removed from cultured hiPS cells and colonies were cell scraped as whole into the culture medium. Colonies were transferred on END-2 cell layer with minimum amount of medium as possible. Approximately 30 colony pieces were transferred on each well. After plating, cells were cultured at +37 °C (Galaxy R incubator, RS Biotech).

The 0 % KO-SR hES medium was changed at days 5, 8 and 12. At day 14 the culture medium was changed to 10 % KO-SR hES medium and subsequently medium was changed every other day except weekends. The beating areas were dissociated into individual cardiomyocytes, which were immunocytochemically stained. The emerging cardiomyocytes were monitored by Olympus IX51 Fluorescence microscope and photographed using DP30BW camera (Olympus).

4.6.2 EB-differentiation

Embryoid bodies, which contain differentiated cells from all germ layers, were formed from hiPS cells by culturing them in suspension. First MEF feeders were removed from hiPS cells and culture medium was changed to EB-medium. Colonies were then scraped as a whole into the culture medium, which were transferred to low-cell binding 6-well-plate (Nalge Nunc) and cultured at +37 °C, 5 % CO_2 (Galaxy R incubator, RS Biotech) for 37 days. The culture medium was changed every other day except weekends. After cultivation, EBs were lysed and RNA extracted, which was used for RT-PCR.

5. RESULTS

5.1 HUMAN IPS CELL GENERATION

Human iPS colonies were picked up on 28 days after retroviral infection. Fluorescent and non-fluorescent ES-like colonies could be seen in the cell culture plates and both types of colonies were picked up. In total, 17 hiPS colonies were picked up from which 11 were fluorescent. Overall, 9 hiPS cell lines with 5 of them fluorescent, were successfully established from these 17 hiPS colonies. After passaging only 3 hiPS cell lines retained fluorescence. These 3 hiPS cell lines had been genetically modified by Oct-eGFP (h3/01, figure 8 A,B), PGK-H2B-mCherry (h3/03, figure 8 C,D) and by Oct-eGFP + αMHC-mCherry-Rex-Bla (h3/06, figure 8 E,F).

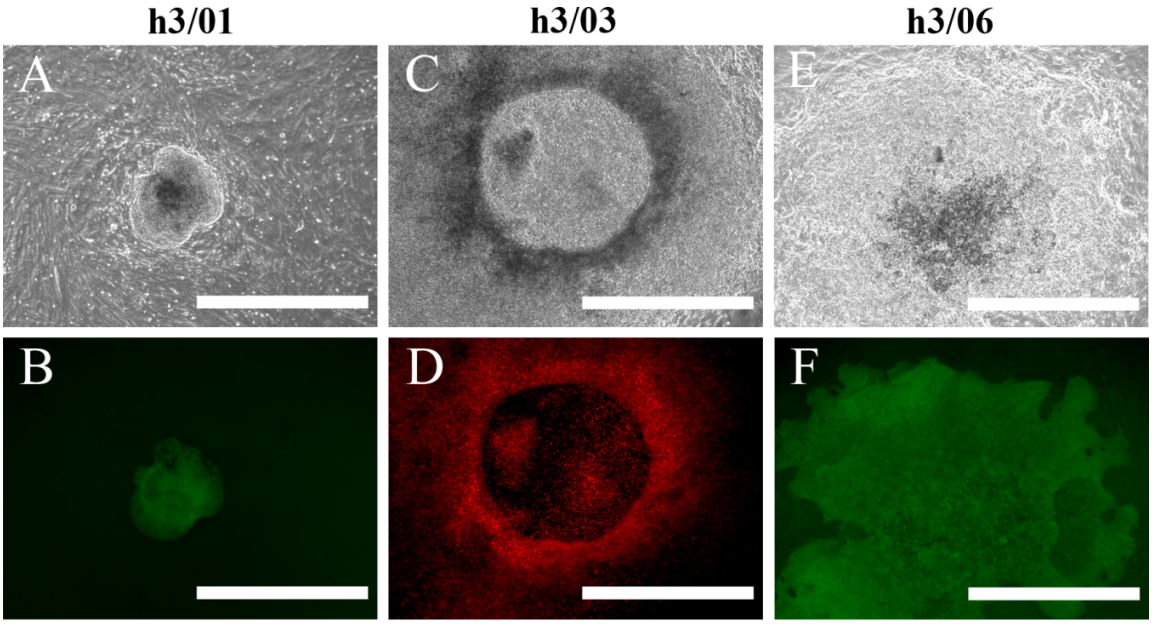

Figure 8. Phase contrast and fluorescence images of colonies to be picked up. The cells had been genetically modified by different vectors: A, B) Oct-eGFP. C, D) PGK-H2B-mCherry. E, F) Oct-eGFP + αMHC-mCherry-Rex-Bla. Scale bar 1.0 mm.

Overall, the main criteria for colony picking were the fluorescence and morphology of the colonies. However, most of the picked fluorescent colonies did not exhibit clear morphology of ES-like colonies such as smooth boundaries and single layered cells. On the other hand, clear ES-like colonies were observed but they were not fluorescent. Therefore,

mostly fluorescent colonies that necessarily did not exhibit clear ES-like colony morphology features were chosen (figure 8 C, E). However, one fluorescent colony had also ES-like morphology and from which a hiPS cell line, named h3/01, was established (figure 8 A, B).

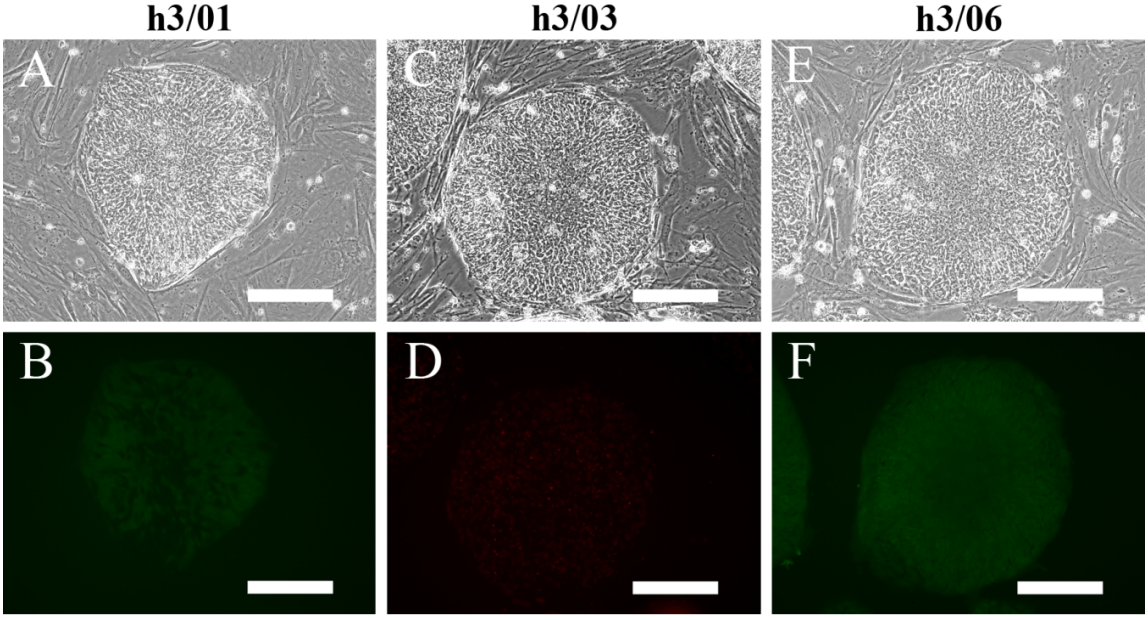

Figure 9. Phase contrast and fluorescence images of individual colonies from hiPS cell lines at passage 5. The individual colonies from different cell lines showed morphology similar to hESCs. Between cell lines, there were differences in fluorescence and pattern. A, B) In the h3/01 not all the iPS cells in a colony were fluorescent. C, D) In the h3/03 only a few colonies were fluorescent and the intensity was weaker compared to other hiPS cell lines. E, F) The h3/06 showed the most strongest and uniform fluorescence. Scale bar 200 µm.

Only 3 hiPS cell lines retained fluorescence after passage 2 from the established 5 fluorescent hiPS cell lines. Interestingly, the difference in fluorescence intensity was observed between cell lines h3/01 and h3/06, which both expressed the same vector Oct-eGFP. The cell line h3/06 seemed to fluorescent more intensively than h3/01. At passage 2 fluorescent and non-fluorescent hiPS colonies were observed in all of the fluorescent cell lines: h3/01, h3/03 and h3/06. Only the fluorescent colonies were selected for passaging. As a result only fluorescent colonies were observed at passage 3 in cell lines h3/01 and h3/06. However, the selection of fluorescent colonies did not result in establishment of stable, fluorescent cell line in the case of h3/03 despite constant selection of fluorescent colonies

throughout the passages. Although a few colonies showed fluorescence (figure 9 D), the rest of the colonies were non-fluorescent. Thus, cell line h3/03 was excluded from cell line characterizations.

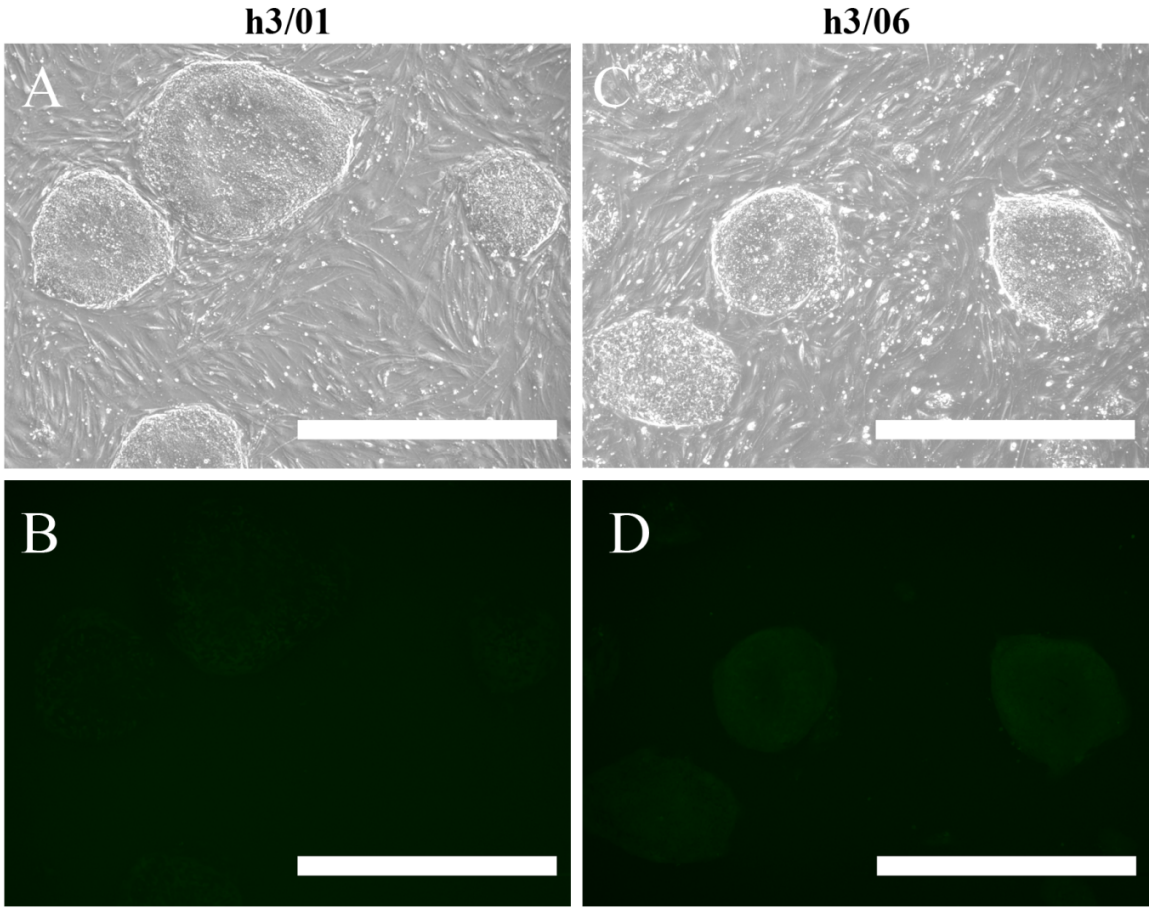

Figure 10. The general view of the hiPS colonies in cell lines h3/01 and h3/06 at passage 5. A, B) The fluorescence of the individual colonies is not uniform; some cells seem to have lost fluorescence within the colony. The fluorescence was weaker in h3/01 than in h3/06. C, D) The fluorescence is uniform in h3/06 and stronger compared to h3/01. Scale bar 1.0 mm.

In cell line h3/01 at passage 5, colonies began to emerge that were not fully fluorescent. Some cells in the colonies seemed to have lost fluorescence (figure 9 B, figure 10 B). In contrast, the fluorescence of the colonies in the h3/06 line had been strong and constant throughout the passages (figure 10 D, figure 11 D). The selection of only the fully fluorescent colonies for passaging did not help the situation in h3/01 line. The fluorescence was lost almost completely by passage 7 and only a few cells were very faintly fluorescent

at passage 8. On the other hand, the fluorescence of the colonies in h3/06 line at passage 8 was stable (figure 11 B). In the latest passage 19, the cells have still retained their fluorescence in h3/06 line (figure 11 D). Overall, from picked up total 17 colonies only 1 stable and fluorescent hiPS cell line was established.

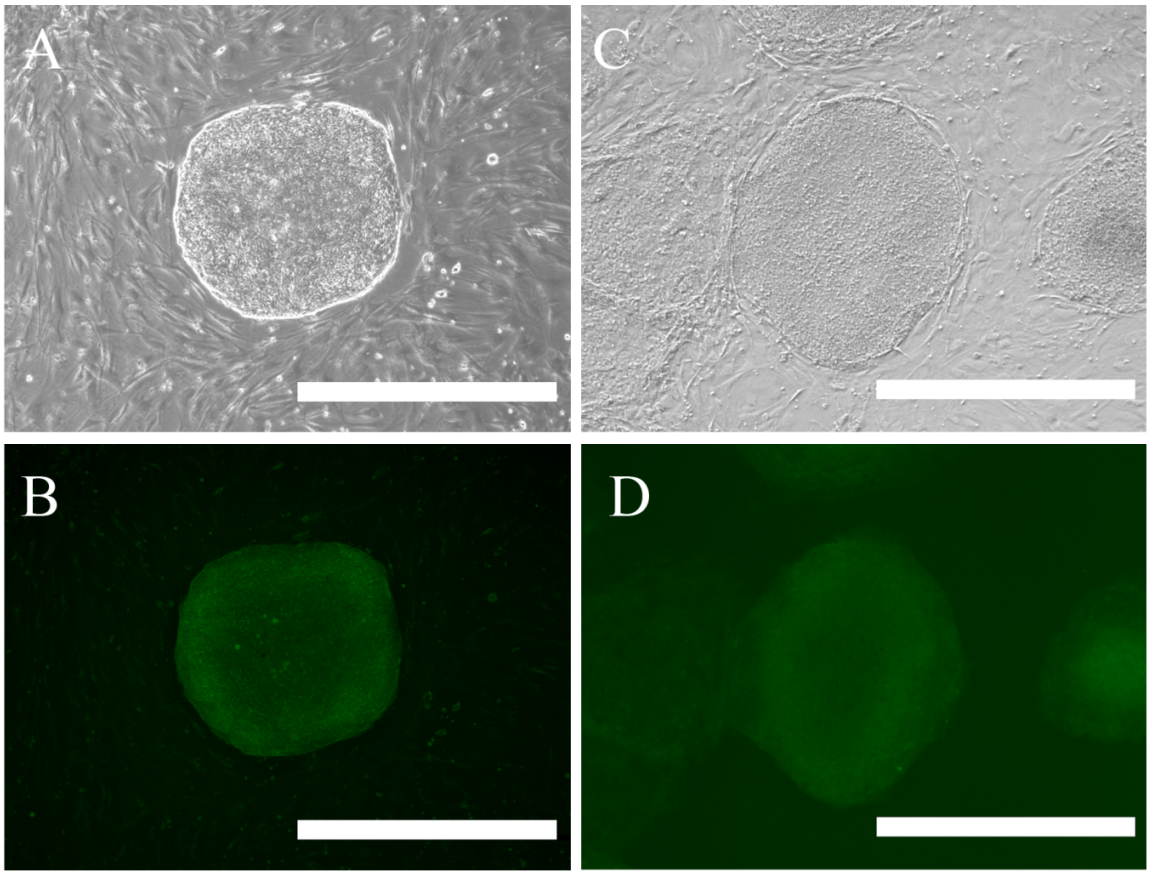

Figure 11. The hiPS colony morphology and fluorescence in h3/06 cell line at passages 8 and 19. A, B) In h3/01 the fluorescence was lost by passage 8 but in the corresponding passage, the cells in h3/06 continued to have a strong fluorescence. C, D) Cells in h3/06 have continued to fluorescent until passage 19.

5.2 HUMAN IPS CELL CHARACTERIZATIONS

5.2.1 Immunocytochemistry

Cell lines h3/01 and h3/06 were immunocytochemically stained at passage 6 for proteins Oct4, Nanog and SSEA-4. Both cell lines expressed Oct4, Nanog and SSEA-4 (figure 12).

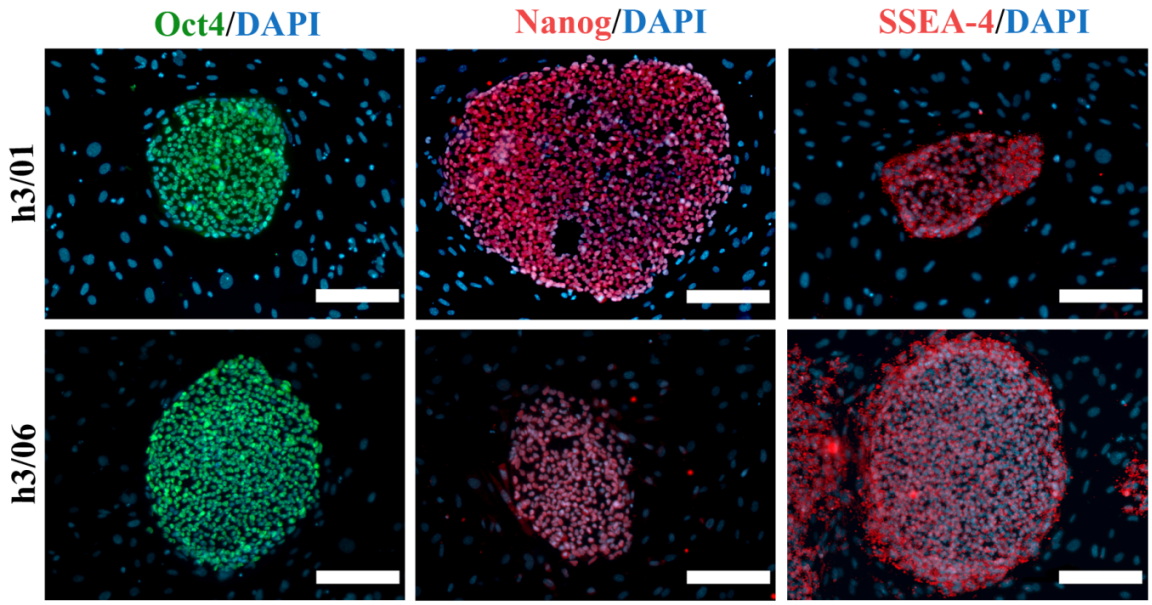

Figure 12. Immunocytochemistry for h3/01 and h3/06 cell lines at passage 6. In both cell lines, cells expressed Oct4, Nanog and SSEA-4. DAPI stained the nuclei. Scale bar 200 µm.

5.2.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of viral exogenous transgenes Oct4, Sox2, Klf4 and c-Myc was investigated at passages 8 and 12 in the cell lines h3/01 and h3/06. In addition, the expression of several endogenous pluripotency genes, Oct4, Sox2, c-Myc, Nanog and Rex-1 was analyzed in the same lines.

At passage 8 the expression of all the endogenous genes was detected (figure 13). However, at the same passage the exogenous transgenes were also expressed (figure 14). As most of the retroviral transgenes were expressed at passage 8, another exogenous transgene expression analysis was made at passage 12 (figure 15). The results showed that retroviral transgenes were still expressed. However, some divergence was observed in exogenous transgene expression at both passages. For instance, in cell line h3/06 exogenous Sox2 expression was not detected at passage 8 but later, at passage 12 the transgene expression was detected. This led to repeat the PCR experiment for exogenous Sox2 and c-Myc at passage 8 in cell lines h3/06 and h3/01, respectively and exogenous Oct4 at passage 12 in cell line h3/01 (figure 16). Taken together, the results indicate that all the exogenous transgenes were expressed already from passage 8 and have continued to express until

passage 12 in both of the cell lines. In conclusion, the RT-PCR results indicated that hiPS cell reprogramming had successfully activated endogenous pluripotency genes but failed to shut down the expression of the viral exogenous genes.

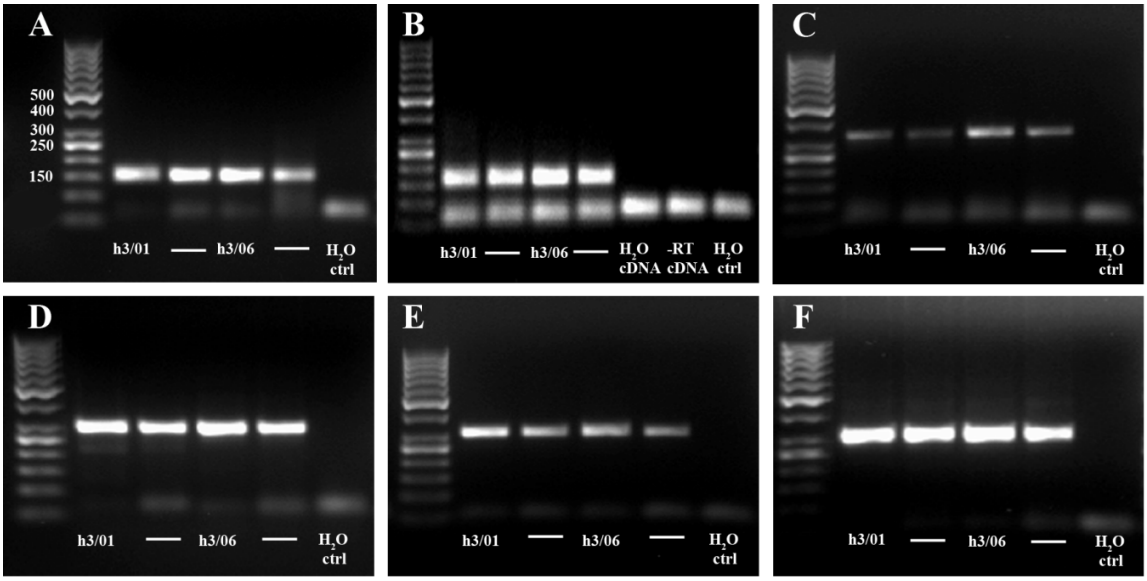

Figure 13. The expression of endogenous pluripotency markers in h3/01 and h3/06 at passage 8. Both cell lines expressed all the studied genes: A) Oct4, B) Sox2, C) c-Myc, D) Nanog, E) Rex-1 and F) β-actin, which was used as a positive control.

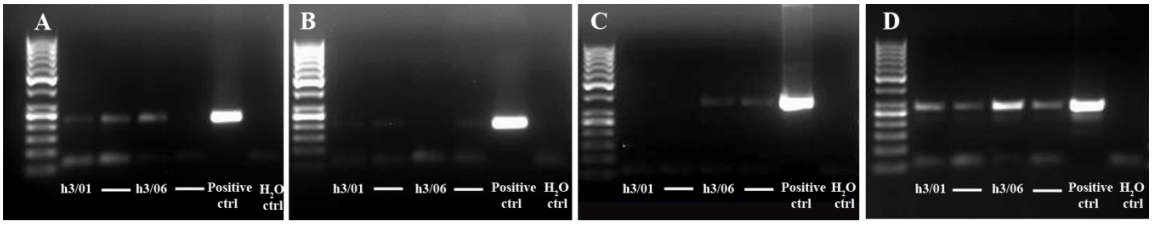

Figure 14. The expression of retroviral exogenous transgenes in h3/01 and h3/06 at passage 8. A) Exogenous Oct4, B) exogenous Sox2, C) exogenous c-Myc, D) exogenous Klf4. The retroviral plasmid vectors were used as positive controls (bright bands, lanes on the right).

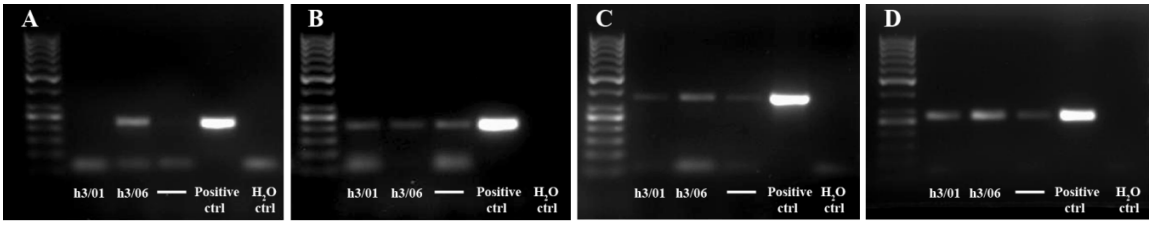

Figure 15. The expression of retroviral exogenous transgenes in h3/01 and h3/06 at passage 12. A) Exogenous Oct4, B) exogenous Sox2, C) exogenous c-Myc, D) exogenous Klf4. The retroviral plasmid vectors were used as positive controls.

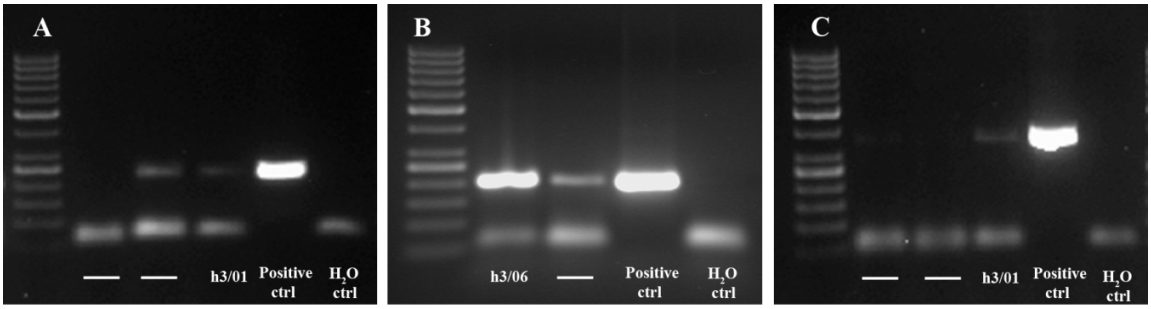

Figure 16. The expression of retroviral exogenous transgenes Oct4, Sox2 and c-Myc in h3/01 and h3/06 at passages 8 and 12. The reanalysis revealed the expression of above-mentioned transgenes in both cell lines. A) Exogenous Oct4 at passage 12 from h3/01 cell line B) exogenous Sox2 at passage 8 from h3/06 cell line C) exogenous C-Myc at passage 8 from h3/01 cell line.

5.2.3 Blasticidine administration for h3/06 cell line

Blasticidine was administered on h3/06 cell line cultured on Matrigel™ at passage 8 one day before passaging (figure 11 A, B). Cells were examined 21 hours after drug administration. As a result blasticidine had killed all the hiPS cells even though Rex-1 expression was confirmed by RT-PCR. Thus, the vector αMHC-mCherry-Rex-Bla was absent from the h3/06 cell line.

5.3 HUMAN IPS CELL DIFFERENTIATIONS AND CHARACTERIZATIONS

Human iPS cells from the cell line h3/06 were differentiated into cardiomyocytes using END-2-differentiation. The emerging beating cells were immunocytochemically stained for cardiac markers. The pluripotency of h3/01 was assessed *in vitro* by differentiating cells by EB-formation and investigating the appearance of germ layer

markers by RT-PCR. END-2 differentiation was initiated at passage 9 and EB-differentiation at passage 13. EBs were grown in suspension for 37 days.

5.3.1 END-2-differentiated cardiomyocytes and immunocytochemistry

The cells from h3/06 cell line were plated on END-2 cells to induce cardiomyocyte differentiation. The fluorescence of Oct-eGFP was lost by day 9 after the initiation of the differentiation but the fluorescence of mCherry was not detected at all during differentiation. Eventually, two beating areas emerged by day 30. The first beating area emerged by day 17 and the second by day 30 (figure 17 A). Both of the beating areas were enzymatically dissociated into individual cardiomyocytes (figure 17 B), which were immunocytochemically stained for ventricular αMHC and troponin T (figure 18).

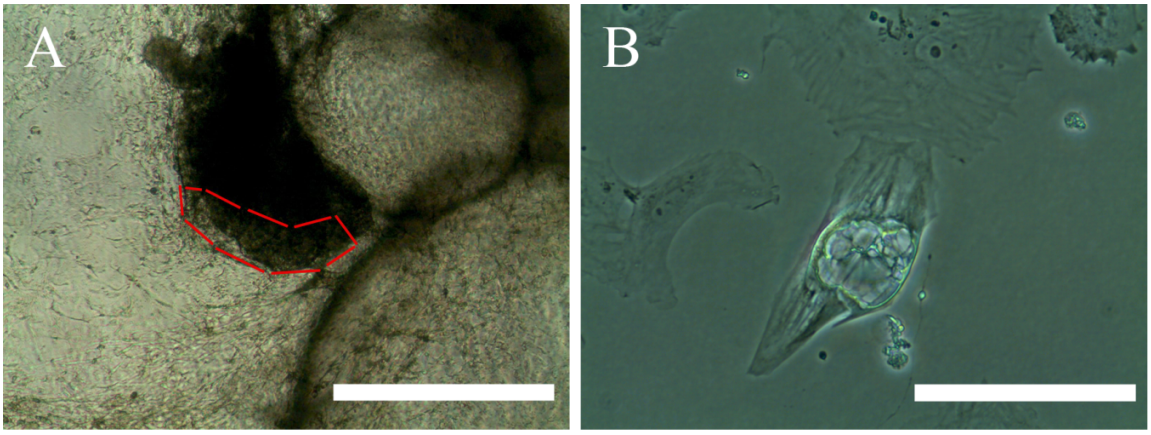

Figure 17. END-2-differentiated cardiomyocytes from h3/06. A) The beating area of the differentiated cardiomyocytes at day 30. Scale bar 1.0 mm. B) Individual beating cardiomyocyte 7 days after dissociation. Scale bar 200 µm.

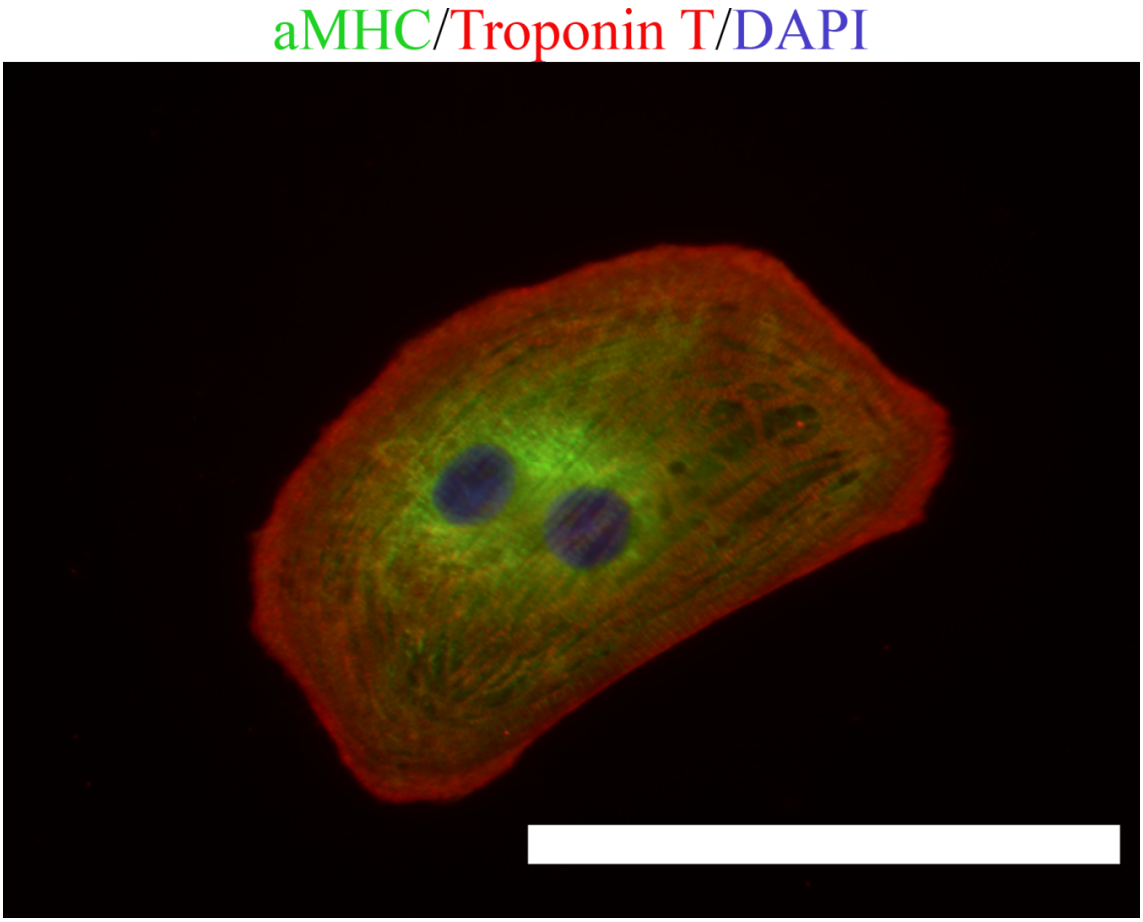

Figure 18. Immunocytochemistry for cardiomyocytes. Differentiated cardiomyocytes expressed ventricular αMHC and troponin T. DAPI stained the nuclei. Scale bar 100 µm.

5.3.2 EB-differentiation and RT-PCR

The expression of several germ layer markers was analyzed from differentiated EBs, which were grown in suspension for 37 days. In addition, the expression of exogenous transgenes Oct4, Sox2, c-Myc and Klf4 was also analyzed.

Figure 19 shows the expression of several germ layer markers; AFP and Sox17, which are endodermal markers, KDR which is a mesodermal marker and Musashi 1, which is ectodermal marker. In summary, the h3/06 cell line seems to be pluripotent *in vitro*.

As the exogenous transgenes were expressed in h3/06 cell line before EB-differentiation, their expression was investigated after the EB-differentiation. The expression of exogenous transgenes Oct4, Klf4 and to a lesser extent, Sox2, was detected (figure 20). On the other hand, c-Myc expression was completely absent.

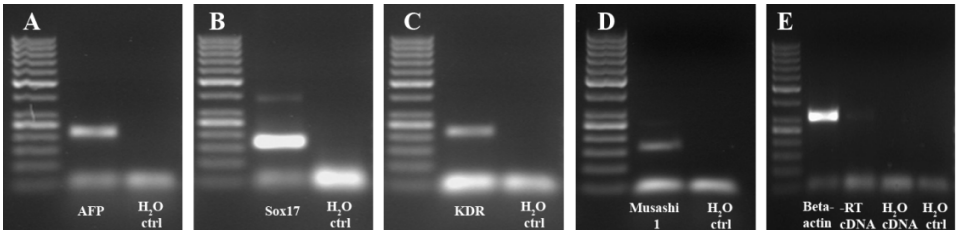

Figure 19. The expression of several germ layer markers from differentiated EBs. A,B) Endodermal markers AFP and Sox17, respectively. C) Mesodermal marker KDR, D) Ectodermal marker Musashi 1 E) β-actin, which was used as a positive control.

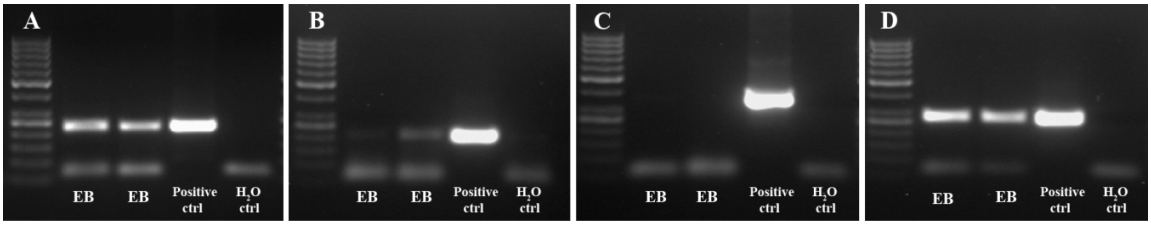

Figure 20. The expression of exogenous transgenes in differentiated EBs. Parallel samples of EBs were used. A) Exogenous Oct4, B) exogenous Sox2, C) exogenous c-Myc, D) exogenous Klf4.

6. DISCUSSION

The aim of the study was to generate genetically engineered hiPS cells and characterize them by immunocytochemistry and RT-PCR. The differentiation potential was assessed by EB-formation and by differentiation to cardiomyocytes. The aim was to establish several different genetically engineered hiPS cell lines for various research purposes. Fluorescent proteins allow visualization and tracking of hiPS cells and differentiated cardiomyocytes. During differentiation, the extinguishment of stem cell-labeled fluorescence and the emergence of cardiomyocyte-labeled fluorescence could be monitored. In addition, after differentiation the possible presence of undifferentiated hiPS cells could be easily monitored. Fluorescent reporters could also be used to evaluate cell migration and differentiation and for various quantitative analyses (Kita-Matsuo et al., 2009). Antibiotic selection provides the derivation of pure populations of cells. If fluorescent tracking is combined with antibiotic-selection in cardiomyocytes, then it could be possible to generate pure, fluorescent traceable cardiomyocytes for basic developmental research and for high-throughput screening applications, such as microelectrode array (MEA) studies and patch clamp.

6.1 THE GENERATION OF GENETICALLY MODIFIED HIPS CELLS

In total 17 human iPS colonies were picked from which 11 were fluorescent. Overall, out of the total 11 fluorescent colonies only one stable fluorescent human iPS cell line was established that has been shown to be fluorescent at least for 19 passages. Thus, the efficiency of generating genetically engineered human iPS cell lines by methods used in this study was relatively low. In fact, this was the third and successful try to generate genetically engineered hiPS cells. In the first attempt fluorescent hiPS colonies were picked up and hiPS cell lines were established but the fluorescence was lost immediately after the first passage. The same phenomenon occurred also in this experiment. As total of 5 fluorescent hiPS cell lines were established, immediately after first passaging the fluorescence was lost in two hiPS cell lines. Although the fluorescence was lost, the cells continued to grow and showed ESC-like properties such as proliferation and morphology. However, these cell lines were not included in hiPS cell line characterizations. Rather, the focus was on the fluorescent hiPS cell lines.

After the first passage three hiPS cell lines retained fluorescence, which were h3/01, h3/03 and h3/06. During the first passages the colony morphologies continued to mature more into ESC-like state. Interestingly, at passage 2 all the three fluorescent hiPS cell lines showed also non-fluorescent colonies. The reason for this may be in colony picking. It may be possible that some additional non-fluorescent cells had been transferred along with the colony resulting in the formation of non-fluorescent colonies. Thus, selection system should be implemented in order to keep the cell population homogenous or on the other hand, generate clonal lines from genetically modified stem cells. However, clonal selection in hESCs has been technically challenging as clonal growth efficiency is extremely low when plated as single cells. The appearance of non-fluorescent colonies may also be explained by the reprogramming process. During the first passages the colony morphology continued to mature more into ESC-like state indicating changes in cellular level. The reprogramming process is gradual and involves changes in gene expression, the epigenetic state of the pluripotency promoters and the activation of endogenous molecular circuitry (Jaenisch & Young, 2008). Although the cells may be genetically identical, their transcriptional status can be very different (Meissner et al., 2007). As cellular reprogramming occurs gradually, it may be expected that not all the cells act in the same way to the effect of ectopic expression of the exogenous factors at the epigenetic level. This may result in different epigenetic states among the cells which in turn may have influence on the lentiviral vector construct resulting in silencing of the vector in some of the cells and thus, in part creates heterogeneity within the cell line. However, as selection for fluorescent colonies was made at early passages, the following passages of h3/01 and h3/06 cell lines showed only homogenous fluorescent colonies. Curiously, the selection of fluorescent colonies in h3/03 cell line did not result in the establishment of a stable, fluorescent state in the following passages. Although some individual colonies showed fluorescence, the majority of the colonies were non-fluorescent. Due to this heterogeneity in terms of fluorescence, the cell line was not characterized.

Although hiPS cell lines h3/01 and h3/06 were genetically modified by the same vector Oct-eGFP, notable differences were seen in fluorescence levels between the cells. Generally, the fluorescence of h3/01 cell line seemed weaker than in h3/06 cell line. Although this was not investigated any further, it is reasonable to think that the issue may

relate to the lentiviral integration sites, position effects and proviral copy number (Hotta & Ellis, 2008). Lentiviral integration sites are randomly located in the genome of the infected cell. Thus, enhancers or repressors of neighboring genes may affect the expression of the integrated transgene. Although not assessed, also the proviral copy number of SIN-lentiviruses in different cell lines might have been different. Proviral copy number determines the number of integrated viruses and thus, more integrated viruses in a cell population, the better the transgene expression. Also the reprogramming process might have an effect on transgene expression. The gene expression profile and epigenetic state of somatic cell and hiPS cell is highly dissimilar (Takahashi et al., 2007), (Yu et al., 2007). It has been shown that major changes in gene expression and in epigenetic state of the cells occur during reprogramming (Maherali et al., 2007), (Brambrink et al., 2008), (Mikkelsen et al., 2008), (Stadtfeld et al., 2008). Thus, the expression of the transgene may be affected by position effects originating from the remodeling of the epigenetic state.

Interestingly and unexpectedly cells in h3/01 began to lose their fluorescence around passage 5 resulting in partially fluorescent colonies. The disappearance of fluorescence was quite rapid, by passage 7 the fluorescence was basically lost and in passage 8 only a few cells were very faintly fluorescent. Although not verified experimentally, presumably the transgene was silenced in cells, which over time took over the cell culture. Bisulphate sequencing of the transgene promoter would have revealed the methylation state of the promoter indicating the activity of the promoter. Another possibility for verifying silencing would have been to utilize RT-PCR and check whether the transgene is effectively transcribed. However, as it is not exactly known in what stage silencing occurs it would be also reasonable to analyze the expression of the transgene on protein level. However, the key question is why and how silencing occurs in particular set of cells? The loss of fluorescence in some of the cells could indicate the existence of heterogeneity among the cells. Therefore, some cells may be more vulnerable to silencing whereas the rest are not. This may explain the sudden appearance of non-fluorescent colonies immediately after the second passage. Another concern is that does silencing occur in all of the cells that result in the complete loss of fluorescence or do transgene-silenced cells create selection pressure, which will eventually lead to the enrichment of non-fluorescent cells in the cell culture. If the latter would be the explanation, then the selection of the most fluorescent colonies for

passaging would presumably keep the cell line stable. However, the selection for the most fluorescent colonies was made in h3/01 cell line at passage 5 but that did not result in the establishment of stable, fluorescent cell line. One possibility is that the selection was made too late or it may be so that the silencing occurs in all of the cells within certain time frame. In order to generate new genetically engineered hiPS cell lines, the silencing issue must be addressed. Although evidence exists that retroviral silencing occurs in embryonic cells, the actual mechanism stays elusive. SIN-lentiviral vectors are shown to be less subject to silencing (Ellis & Yao, 2005) but still they contain unknown silencing elements. As the SIN-lentiviral vectors used in the genetic modification of hFFs were the same, some differences between the cell lines h3/01 and h3/06 must have existed that resulted in the silencing of transgene in h3/01. In comparison to h3/01, the cells in h3/06 were highly fluorescent in passage 8. Overall, when comparing fluorescence levels between passages it seems that the fluorescence level was decreasing as cells were passaged further. However, quantifying fluorescence intensity was not possible and therefore it is difficult to draw conclusions. The fact is that h3/06 cell line has been shown to be fluorescent for 19 passages with no signs of silencing.

The generation of genetically engineered hiPS cell lines seems to be a delicate process. In total 11 fluorescent colonies were picked up from which only 5 continued growths. After the second passage the fluorescence was lost in 2 hiPS cell lines. From the 3 remaining fluorescent hiPS cell lines eventually only one retained fluorescence and has been shown to be stable until now.

6.2 THE CHARACTERIZATION OF GENETICALLY MODIFIED HIPS CELLS

The cell lines h3/01 and h3/06 were characterized by immunocytochemistry and RT-PCR for the expression of pluripotency associated proteins and genes. Indeed, at passage 6 Oct4, Nanog and SSEA-4 proteins and at passage 8 the endogenous genes Oct4, Sox2, c-Myc, Nanog and Rex-1 were expressed. Oct4 and Nanog have been shown to be master regulators of pluripotency in hESCs (Boyer et al., 2005) and SSEA-4 is a specific marker that is expressed on the surface of human embryonal carcinoma cells, human embryonic germ cells, hESCs and hiPS cells. Oct4, Sox2 and c-Myc are the same factors which were used in iPS cell reprogramming (Takahashi & Yamanaka, 2006). Rex-1 was first

characterized in mouse embryonal carcinoma cells and in hESCs Rex-1 expression is a marker of pluripotency (Kita-Matsuo et al., 2009). The activation of endogenous pluripotency gene network is crucial for full reprogramming. The results indicated that in both of the cell lines the reprogramming had been successful on the part of activating endogenous genes.

Analysis of exogenous transgene expressions showed that exogenous transgenes were not silenced in either h3/01 or h3/06, at least until passage 12. However, the extinguishment of fluorescence in h3/01 cell line was firstly observed around passage 5 indicating that the SIN-lentiviral vector had possibly been silenced. Why pMX-vectors were not silenced in cell lines, remains to be determined. The fact that retroviral exogenous transgenes are actively expressed in both cell lines indicates that they are partially reprogrammed (Hotta & Ellis, 2008). However, the partially reprogrammed cells fail to activate endogenous pluripotency genes and thus, pluripotency is only driven by ectopic expression of the reprogramming factors. In this case, the reprogramming seems to have activated the master regulators of pluripotency. It would be interesting to compare these cell lines to the fully reprogrammed lines and see whether differences lie between different reprogramming states. What effects partial reprogramming has on hiPS cells is elusive. The ectopic expression of exogenous transgenes may have an effect on differentiation potential.

At passage 8 blasticidine was administered on Matrigel™ coated h3/06 cell line. After 21 hours of exposure the cells were examined and it turned out that blasticidine had killed all the cells. The aim was to establish genetically engineered hiPS cell line, which would have had two vectors: Oct-eGFP and αMHC-mCherry-Rex-Bla. If integrated into the genome, the antibiotic resistance gene Bla would have been expressed in the cells under Rex-1 promoter. As RT-PCR results showed, Rex-1 was expressed in h3/06 cell line at passage 8. However, blasticidine selection was unsuccessful. The possible integration of the vector αMHC-mCherry-Rex-Bla was not verified directly by PCR from genomic DNA. Therefore, it is uncertain whether the vector had integrated into the genome or had it been silenced. If the vector had integrated into the genome, it may have been silenced during reprogramming.

6.3 THE DIFFERENTIATION OF GENETICALLY ENGINEERED HIPS CELLS

The cell line h3/06 was used for differentiation of cardiomyocytes using END-2-differentiation (Passier et al., 2005). The differentiation was initiated at passage 9. The fluorescence was extinguished by day 9 indicating the Oct4 promoter expression down-regulation. Even though all the exogenous transgenes were actively expressed at passage 8 and 12, surprisingly beating areas were formed. However, the beating areas appeared late, the first at day 17 and the second at day 30. Usually beating areas appear from day 7 to day 12 (Passier et al., 2005). The late appearance of beating areas may indicate that the time period required for differentiation somehow prolonged and this might be due to expression of exogenous transgenes.

The dissociation of the beating areas revealed individual beating cardiomyocytes. These cardiomyocytes were immunocytochemically stained for cardiomyocyte markers αMHC and Troponin T. αMHC expression on protein level was confirmed, but no fluorescent cardiomyocytes were observed. Thus, the vector aMHC-mCherry-Rex-Bla was either silenced or was not integrated into the genome of h3/06 cell line.

During reprogramming the core regulatory circuitry of pluripotency is jumpstarted with the introduction of the reprogramming factors into human cells (Takahashi et al., 2007), (Yu et al, 2007). The ectopic expression of these factors forces the reprogramming of somatic cell into stem cell-state. If the expression of exogenous transgenes remains active, it would be likely that differentiation would not be possible. Here the result, that differentiation into cardiomyocytes is possible even though retroviral exogenous genes are actively expressed was unexpected. This raises some issues. First, from this study it cannot be said for sure that specifically hiPS cell expressing retroviral transgenes differentiate into cardiomyocyte. Second, hiPS cells having possibly lower or down-regulated retroviral transgene expression, which have the ability to differentiate into cardiomyocytes might have been existed. It would be very interesting to see, how the expression of exogenous transgenes changes during differentiation and if silenced, what kind of changes would induce silencing. Lastly, the functionality of the differentiated cardiomyocytes was not assessed by electrophysiological studies. It may be possible that the expression of exogenous transgenes might have an effect on the functionality of the differentiated cells.

The h3/06 cell line was also differentiated by EB-method. The differentiated EBs expressed endogenous markers AFP and Sox17, mesodermal marker KDR and ectodermal Musashi 1. The results indicate that h3/06 cell line is pluripotent *in vitro* despite partial reprogramming. However, not all the investigated germ layer markers were expressed. The expression of mesodermal marker α-cardiactin and ectodermal markers PAX6, Sox1 and Nestin was not detected. In the cardiomyocyte differentiation the beating areas appeared late, possibly due to effects of partial reprogramming. Therefore, the time required for all the germ layer markers to be activated in EBs may also take more time to develop. In EBs it is thought that the endoderm layer is formed first (Freund & Mummery, 2009). Here, the endoderm markers AFP and Sox17 were both expressed, which is in line with the current model. However, according to the results here it might be possible that h3/06 cell line is not capable to differentiate purely into various cell types of mesodermal or ectodermal origin.

The exogenous transgenes Oct4, Klf4 and to a lesser extent, Sox2 were expressed in differentiated EBs. On the other hand, the expression of exogenous c-Myc was not detected. The result is kind of curious in a way that these four factors are required for switching cell fate from somatic state to pluripotent state, yet upon differentiation to different germ layer cells, some of the factors remain expressed. Simultaneously, as the exogenous transgenes remain expressed in EBs, it is also curious how the expression of exogenous c-Myc has been extinguished in the process. This may involve chromatin remodeling during differentiation and as somatic cells have more restricted chromatin state than in ES cells (Azuara et al., 2006), it may be possible that these changes affect to the expression of transgenes. However, the bands for exogenous Oct4 and Klf4 observed in differentiated EBs are exceptionally strong compared to the expression of the corresponding transgenes in hiPS cells. In these cases, the chromatin remodeling seemed to have no decreasing effect on transgene expression; instead it seems that the expression had strengthened. This may raise the possibility that undifferentiated hiPS cells may have existed in EBs that contribute to the expression of the retroviral transgenes.

6.4 FUTURE PERSPECTIVES

The results from this study show that several issues need to be addressed in future attempts to establish genetically engineered hiPS cell lines. First, fully reprogrammed hiPS

cells need to be obtained. The fluorescence intensity of the vector constructs needs to be strengthened. If using multiple vector constructs, the appropriate cells carrying these vector constructs need to be selected before or during reprogramming process. Lastly, alternative gene transfer methods would be needed to avoid transgene silencing.

The utilization of Oct4-driven GFP in colony picking did not result in establishing cell lines that had been fully reprogrammed. In order to establish fully reprogrammed hiPS cell lines, the retroviral transgenes need to be turned off. The problem is that retroviral transgene silencing is not well known phenomenon and thus it is difficult to have an effect on. Cre/loxP-system in hiPS cells reported by Soldner et al. (2009) could be used to excise the integrated retroviral transgenes. However, as retroviral transgene expression indicates partial reprogramming, simple excision of the transgenes may not be enough. The total effect of partial reprogramming on hiPS cell characteristics is not known and thus, is to be avoided. Well-established evidence exists that pMX-vectors are silenced in fully reprogrammed hiPS cells. Thus, pMX-driven fluorescent marker would reveal the silencing state of pMX-vectors in cell culture. In order to generate genetically modified fully reprogrammed hiPS cell lines, pMX-driven fluorescent markers along with the stem cell-specific promoters that mark pluripotent stem cells could be used to identify hiPS colonies that have been fully reprogrammed. In order to intensify fluorescence, stronger promoters than Oct4 could be used instead. Also the use of different cell-type specific enhancers may be used to intensify fluorescence.

The current view is that stochastic events are involved in hiPS reprogramming (Yamanaka, 2009a). With the current technological understanding it is not possible to control the generation of hiPS cell from somatic cell. Thus, in the genetic engineering of hiPS cells, it will be important to have homogeneity among cells in terms of integrated vectors before reprogramming them to pluripotent state. Thus, selection system for the integrated vectors needs to be developed. The easiest way is to include antibiotic resistance genes under specific promoters to select those cells carrying the vector. However, usually the selection needs to be maintained in the hiPS cell culture. If using multiple antibiotics, this may prove problematic in technical aspect.

Retro- and lentiviral vectors are frequently silenced in stem cells. In order to avoid this, alternative gene transfer methods would be needed. One of the possibilities could be

homologous recombination, which could prove useful in integrating the transgene into a defined locus. However, the reported efficiency for gene targeting in mammalian somatic cells is very low (Porteus, 2007) and only a handful of reports exists for the genetic modification of hES and hiPS cells (Tenzen et al., 2009). Despite the current technical challenges, the advantage of homologous recombination is that the transgene is integrated into a defined locus and is most likely to avoid gene silencing.

Novel genetic modification strategies for human pluripotent stem cells may come from the realization that stem cells may exist in distinct pluripotent states. Recent publication demonstrates that different culture conditions can be used to derive hiPS cells with mESC-like characteristics (Buecker et al., 2010). In general, mouse and human ESCs differ in terms of colony morphology, proliferation rate, cell culture requirements, and cell-surface marker expression (Kerr & Cheng, 2010). In addition, genetic modification in mESCs is easier and more effective than in hESCs. Buecker et al. (2010) generated metastable hiPS cells using mESC culture conditions. The metastable hiPS cells, designated as hLR5, resembled mESCs in terms of colony morphology, culture dynamics and certain molecular properties. The hLR5 also demonstrated much higher clonal growth efficiency when plated as single cells, which facilitates gene targeting. The hLR5 were shown to allow efficient homologous recombination gene targeting via standard protocols established for mESCs. Although hLR5 were derived and maintained under mESC culture conditions, it was shown that hLR5 can convert into stable, conventional hiPS cells when hESC culture conditions were applied. Thus, in genetic engineering the utilization of these metastable human pluripotent stem cells may prove useful.

Direct reprogramming may also prove useful in the context of genetic engineering. As the pluripotency intermediate state and differentiation is avoided, it may be reasonable that gene silencing phenomenon may also be evaded. Direct reprogramming from dermal fibroblast to cardiomyocyte has been proven in mouse (Ieda et al., 2010). As Oct4, Sox2 and Nanog are the master regulators of pluripotency in human stem cells (Boyer et al., 2005), the core transcription factors in early heart development in mouse seems to be Tbx5, Gata4 and Mef2c (Ieda et al., 2010). However, direct reprogramming to cardiomyocytes has not been yet reported in humans. If successful in humans, fibroblasts could be first infected or transfected with vectors carrying cardiac specific promoters that would drive the

expression of fluorescent markers or antibiotic resistance genes. As cardiac differentiation is a multi-phased process, it may be possible to isolate progenitor cells or cells with different differentiation state using different cell-type specific promoters. Also the gene silencing phenomenon observed in stem cells may be evaded by direct reprogramming. Although promising, several issues remain. First, the direct reprogramming of cardiomyocytes has not been yet proven in human cells. Secondly, the identification of the master regulators is important as if more down-stream regulators are used it may be possible that the induced cardiomyocyte may not be truly equivalent to cardiomyocyte that has been differentiated from stem cell. Thorough characterization and refinement is needed in order to generate high quality cardiomyocytes. As reprogramming of cells into different states is relatively new concept, there is much to learn from.

7. CONCLUSIONS

Genetically modified and seemingly pluripotent hiPS cell lines, albeit partially reprogrammed, were successfully established. However, as in hESCs fluorescent reporter extinguishment was observed during early passages. Although SIN-lentiviral system is employed in the genetic modification of somatic cells followed by reprogramming to hiPS cells, the gene silencing phenomenon remains an issue for establishing stable, genetically modified hiPS cell lines.

The aim of the study was to establish genetically modified hiPS cell lines that had been fluorescent-labeled for hiPS cells and differentiated cardiomyocytes. Although fluorescent hiPS cells were generated and differentiation to cardiomyocytes was successful, no fluorescent cardiomyocytes were obtained. Thus, the cells carrying the multiple, desired vectors need to be selected before reprogramming them into pluripotent state.

The difference between fluorescence intensity was observed between established hiPS cell lines. In order to enhance fluorescent protein expression, more powerful promoters and enhancers could be used.

In order to establish new genetically modified hiPS cell lines, the issues of gene silencing, full reprogramming, stable and highlighted transgene expression and selection of cells carrying the desired vectors for reprogramming need to be solved.

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