Evaluation of the pluripotency of human induced pluripotent stem cells (hiPSCs) reprogrammed with integrative and non-integrative protocols and their differentiation into cardiomyocytes

Tanja Elo
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University of Tampere
Institute of Biosciences and Medical Technology (BioMediTech)
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Tekijä: ELO, TANJA HELENA
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TIIVISTELMÄ

Tutkielman tausta ja tavoitteet Tutkimuksen tarkoitus oli arvioida integroituvalla retroviruksella ja ei-integroituvalla Sendai-viruksella uudelleenohjelmoitujen ihmisen indusoitujen pluripotenttien kantasolujen (hiPSCs) pluripotenttisuutta ja sydänlihassoluiksi erilaistamista. Eksogeenisten pluripotenttimarkkereiden geeni-ilmentymisen analyysi oli tärkeä osa tutkimusta. Eksogeenisten markkerigeenien on arveltu voivan uudelleenaktivoitua pitkään kasvatetuissa integroituvilla menetelmillä valmistetuissa hiPS-soluissa, mikä saattaa aiheuttaa matalan erilaistustehokkuuden.
Tutkimusmenetelmät hiPS-soluja viljeltiin yhteiskasvatuksessa MEF-solujen kanssa. hiPS-solujen pluripotenttisuuden määrityksiin sisältyi eksogeenisten ja endogeenisten pluripotenttimarkkereiden geeniaktiivisuuden tutkiminen RT-PCR-menetelmällä, pluripotenttimarkkereiden immunosytokemia sekä embryoid body (EB) -aggregaattien muodostuminen ja alkiokerrosmarkkereiden RT-PCR. Karakterisoidut hiPS-solut erilaistettiin sydänlihassoluiksi viljelemällä niitä END-2 solujen kanssa yhteiskasvatuksessa. Eksogeenisten pluripotenttimarkkereiden geeniaktiivisuus tutkittiin RT-PCR-menetelmällä myös sydänerilaistuksen jälkeen. Sydänlihassolujen karakterisointiin kuului myös sydänmarkkereiden immunosytokemia. Sendai- ja retroviraalisesti ohjelmoitujen hiPSC-linjojen sydänerilaistusta arvioitiin ja verrattiin keskenään.
Tutkimustulokset Kaikki hiPS-solulinjat todettiin pluripotenteiksi. Toisessa retroviraalisesti ohjelmoidussa linjassa (R-00208) eksogeenisen pluripotenttigeenin Oct-3/4 havaittiin olevan aktiivinen. Sydänerilaistuksessa havaittiin eroja sykkivien alueiden morfologiassa, koossa ja lukumäärässä eri linjojen välillä. Retroviruksella ohjelmoidut linjat erilaistuivat tehokkaammin kuin Sendai-viruksella johdetut linjat. Eksogeeninen Oct-3/4 pysyi aktiivisena sydänerilaistuksen jälkeenkin linjassa R-00208. Kyseinen linja erilaistui sydänlihassoluiksi kaikkein tehokkaimmin. Sydänerilaistus ei siten hiljentänyt eksogeenista Oct-3/4 aktiivisuutta.
Johtopäätökset Eksogeenisen pluripotenttimarkkeriaktiivisuuden ajateltiin heikentävän sydänerilaistustehokkuutta, mutta tämän tutkimuksen perusteella eksogeenisen Oct-3/4 -markkerin ilmentyminen saattaa jopa parantaa sydänerilaistusta. Uusia erilaistuskertoja useammilla iPSC-linjoilla eri kasvatusnumeroilla kuitenkin tarvitaan transgeenien vaikutuksen arvioimiseksi erilaistuksessa. Myös Oct-3/4 -markkerin kaksijakoinen rooli pluripotenttisuuden ylläpitämisessä ja erilaistussuunnan määrityksessä vaatii lisätutkimuksia.

Avainsanat ihmisen indusoidut pluripotentit kantasolut (hiPSC), Sendai-viruksella indusointi, retroviruksella indusointi, transgeenien jäännösaktiivisuus, sydänlihassolu, END-2 sydänerilaistus, erilaistustehokkuus

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ABSTRACT

Research background and aims The purpose of this research was to evaluate the pluripotency of integrative retro and non-integrative Sendai virally generated human induced pluripotent stem cells (hiPSCs) and their cardiac differentiation efficiency, giving value on gene activity analysis of exogenous pluripotency factors. These exogenes have been hypothesized to be reactivated in higher passages of iPSCs generated with integrative methods, resulting in low differentiation efficiency.
Materials and methods hiPSCs reprogrammed with Sendai and retro virus were cultivated by co-culture of MEF cells and characterized for their pluripotency by RT-PCR analysis for exogenous and endogenous pluripotency markers, immunocytochemistry for pluripotency markers and embryoid body (EB) formation with sequential RT-PCR analysis for germ layer markers. These characterized hiPSCs were differentiated into cardiomyocytes by co-culturing with END-2 cells. Exogenous pluripotency gene activity was studied by RT-PCR analysis after cardiac differentiation as well. Cardiomyocytes were characterized also by immunocytochemistry for cardiac markers. The cardiac differentiation efficiency was evaluated between Sendai and retro virally induced hiPSC lines.
Results The pluripotency of all hiPSC lines was confirmed, except one retro virally induced line (R-00208) was expressing exogenous pluripotency gene Oct-3/4. Cardiac differentiation showed variable morphology, size and number of the beating areas between cell lines. Retro virally induced lines differentiated more efficiently into cardiomyocytes than Sendai virally induced lines. Exogene Oct-3/4 stayed active in R-00208 even after cardiac differentiation. This line was the most efficient to differentiate into cardiomyocytes. Thus differentiation didn't switch off exogenous Oct-3/4 expression.
Conclusions Traces of exogenous pluripotency activity was hypothesized to impair the cardiac differentiation but this study suggests that exogenous Oct-3/4 might actually enhance cardiac differentiation. Several differentiation experiments with different iPSC lines in many passages are required in order to determine the effect of transgene activity in differentiation. The mechanism behind the dualistic role of Oct-3/4 in pluripotency maintenance and lineage commitment is another problem to be solved.

Key words human induced pluripotent stem cells (hiPSC), Sendai viral induction, retro viral induction, residual transgene activity, cardiomyocyte, END-2 cardiac differentiation, differentiation efficiency

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ABBREVIATIONS

AA	Ascorbic Acid
ASC	Adult Stem Cell
AFP	α-fetoprotein
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CM	Cardiomyocyte
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle medium
EB	Embryoid Body
ESC	Embryonic Stem Cell
END-2	Mouse Visceral Endoderm-like Cells
ESC	Embryonic Stem Cell
FBS	Fetal Bovine Serum
hESC	Human Embryonic Stem Cell
hiPSC	Human Induced Pluripotent Stem Cell
iPSC	Induced Pluripotent Stem Cell
KLF	Krüppel-like factor
KO	Knock-Out
KSR	Knock-Out Serum
MEA	Microelectrode Array
MEF	Mouse Embryonic Fibroblast
miPSC	Mouse Induced Pluripotent Stem Cell
NEAA	Non-essential Amino Acid
NDS	Normal Donkey Serum
OCT	Octamer-binding transcription factor
PAX	Paired box gene
PBS	Phosphate-Buffered Saline
PSC	Pluripotent Stem Cell
REX	Reduced Expression
ROCK	Rho-associated Kinase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SOX	Sex Determining Region Y-box / SRY-related High-Mobility-Group Box / SRY-related HMG Box
SR	Serum Replacement
SSEA	Stage-specific Embryonic Antigen
TGF	Transforming Growth Factor
TRA	Tumor-related Antigen
VEGFR/KDR	Vascular Endothelial Growth Factor Receptor 2/Kinase Insert Domain Receptor

1. INTRODUCTION

The discovery of induced pluripotent stem cells (iPSCs) in 2006 by Takahashi and Yamanaka started a new era in regenerative medicine and biomedical research. iPS cells have the potential to differentiate into most type of cells (if not all) and therefore they have the potential to be used for repairing any damaged tissue. Previous regenerative studies have utilized embryonic stem cells (ESCs) for regenerative medicine but iPS cells are more promising for therapy than ES cells as iPSCs can be derived from patient's own cells. iPS technology can be used instead of ES technology to obtain human pluripotent stem cells (hPSCs) and it has two advantages over ES technology: it solves the ethical concerns with ESCs and eliminates the risk of immune rejection. ESCs are derived from *in vitro* fertilized embryos which raises ethical concerns. The limited source of embryos also hinders broad studies (Bayart and Cohen-Haguenauer, 2013). Moreover, immunocompatibility of tissues generated from iPSCs with the patient's immune system is an advantage. Because iPS cells are easy to generate in principle and there are much less ethical concerns about their use, many laboratories can conduct studies with them (Bayart and Cohen-Haguenauer, 2013). Since the discovery of iPS cells, various methods of reprogramming, combinations of transcription factors and small chemicals, donor cells and species have been studied. Furthermore, iPS cells have been demonstrated to be equivalent to ES cells in many aspects.

iPS cells have been successfully differentiated into various types of tissues such as neural cells, cardiac cells, hepatocytes, pancreatic cells, smooth muscle cells, endothelial cells (Wang et al., 2013) and retinal pigmented epithelial cells (Cox and Rizzino, 2010). Only cardiac differentiation is considered within this thesis. The ultimate goal of iPSC technology in cardiology is the repair of the heart by cardiomyocyte replacement. By far cardiomyogenesis *in vitro* has been challenging but methods are improving (Rajala et al., 2011). iPSC-derived cardiomyocytes are structurally and functionally immature when comparing to adult cardiomyocytes. Problems to be solved in the future include higher cardiomyocyte maturation *in vitro*, guiding the differentiation into a desired cardiac subtype and to isolate the cardiomyocytes from the heterogeneous population of differentiated cells (Rajala et al., 2011).

2. REVIEW OF THE LITERATURE

2.1 Stem cells

Stem cells are undifferentiated cells characterized by their ability to self-renew and differentiate into different cell types. Different stem cell types have different differentiation potency. The most primitive totipotent stem cell is the zygote, which results from fusion of the two germ cells oocyte and sperm in fertilization (Ratajczak et al., 2008). Totipotent stem cells give rise to embryo and placenta. As zygote undergoes its first division, blastomers are formed and they are still totipotent. When the blastomers have divided into 32-cell stage, the embryo is called morula. The cells of morula are pluripotent stem cells which can give rise to all three germ layers of embryo (mesoderm, ectoderm and endoderm) but not to trophoblast that gives rise to placenta. During early embryogenesis, the morula grows into blastocyst and develops a central cavity. Blastocyst consists of precursor cells for extra-embryonic tissues and cells of the inner cell mass (ICM). The cells of the ICM are still pluripotent and they give rise to the three germ layers during gastrulation. The cells within one germ layer are multipotent stem cells and are committed into one germ layer. They give rise to tissue-specific stem cells (monopotent) that are committed to a certain tissue and give rise to cells of one lineage. There are for example tissue-specific or monopotent hematopoietic, epidermal, intestinal, neural, liver and muscle stem cells (Ratajczak et al., 2008). The different tissues or organs that develop from different germ layers are presented in **Table 1**.

Table 1. Different tissues or organs derived from the three germ layer (Derived from Ratajczak et al., 2008).

Ectoderm	Brain, sympathetic ganglions, peripheral nerves, eye, epidermis, skin appendices, pigment cells
Mesoderm	Hemato/lymphopoietic cells, endothelium, skeletal muscles, heart, adipocytes, connective tissues (bone, tendon, cartilage), smooth muscles, tubule cells of the kidney
Endoderm	Liver, pancreas, lung, stomach and intestines, thyroid gland

Human stem cells are classified into subgroups according to their origin and differentiation potential: embryonic stem cells (ESCs), adult stem cells (ASCs) and induced pluripotent stem cells (iPSCs). ES

cells are pluripotent stem cells and can be isolated from the ICM of the blastocyst. They can be maintained in cell culture without differentiation (Bayart and Cohen-Haguenauer, 2013). AS cells are multipotent cells being able to differentiate into cell types specific to the tissue in which they reside. They can be obtained for example from bone marrow. iPS cells are pluripotent stem cells generated from somatic cells to an embryonic-like state and are able to differentiate into the three germ layers (Bayart and Cohen-Haguenauer, 2013).

2.2 Induced pluripotent stem cells

Takahashi and Yamanaka (2006) discovered that forced expression of pluripotency factors can convert somatic cells to embryonic-like stem cells, called induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006). They reprogrammed mouse fibroblasts by ectopic expression of four transcription factors (out of 24 studied): Oct-3/4, Sox-2, c-Myc and Klf-4. These factors were later named Yamanaka factors. The following year also human iPS cells (hiPSCs) were generated by the same group (Takahashi et al., 2007) and by another group (Yu et al., 2007). For hiPSC generation, Yu et al. (2007) studied 14 transcription factor genes, out of which Oct-3/4, Sox-2, Nanog and LIN-28 were found to be sufficient to convert both human fetal and adult fibroblasts into hiPSCs. These factors are nowadays known as Thomson factors.

Currently iPSC technology shows promise in drug screening, toxicology testing and disease modeling (**Figure 1**). hiPS cells can be generated from patients with any disease and differentiated into the desired cell type (Wang et al., 2013). These patient-derived hiPSCs can be used to study pathogenesis of the disease. The role of genetic variants can be studied when comparing disease-specific iPS cells to healthy iPS cells. Disease-specific iPS cells have included for instance Alzheimer's disease, Parkinson's disease, Down syndrome, schizophrenia (Wang et al., 2013), type I diabetes (Cox and Rizzino, 2010) and cardiovascular diseases such as long QT syndromes and cardiomyopathy (Bellin et al., 2012). Disease-specific hiPS cells would be more relevant for toxicology testing compared to current animal models or cell models derived from cancer cells (Wang et al., 2013). Personalized cell therapy is possible with iPS cells, as gene defects in patient-specific iPS cells could be corrected, iPSCs differentiated into desired cells and returned to the patient (Bellin et al., 2012; Wang et al., 2013).

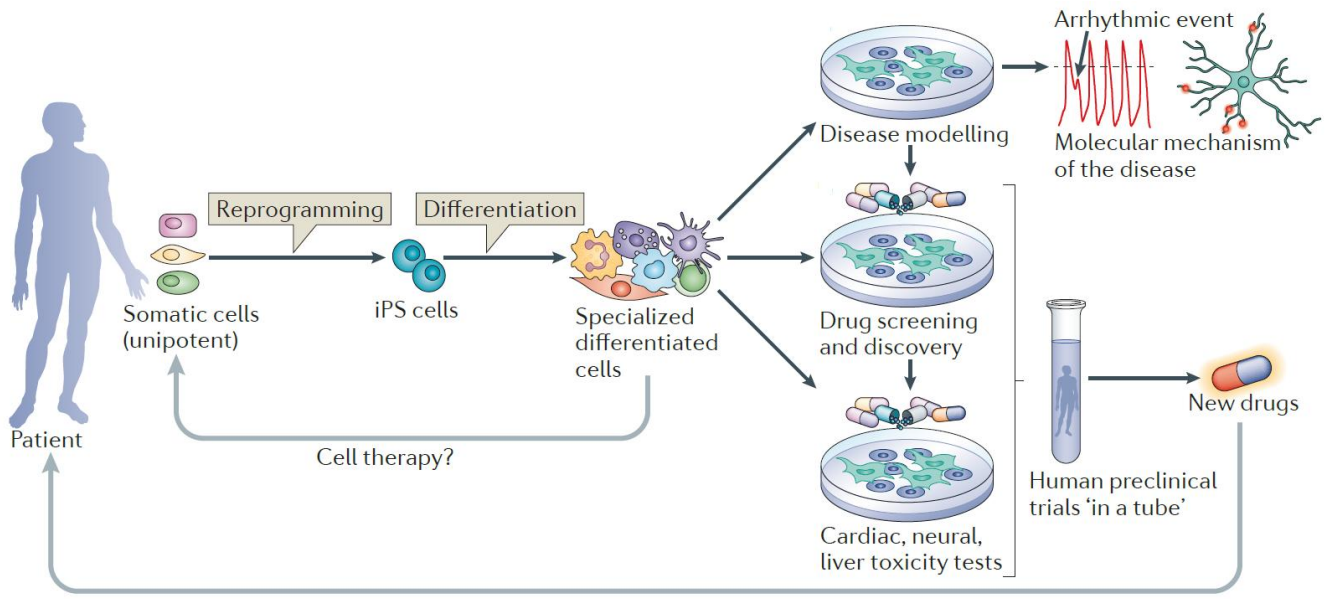


Figure 1. Derivation, differentiation and applications of hiPSCs. Patient-derived somatic cells can be converted into iPS cells and differentiated into the desired cell type. These cells have several applications in disease modeling, drug screening and toxicity testing. In addition, hiPSCs have potential for cell therapy. (Modified from Bellin et al., 2012)

2.2.1 Characterization of induced pluripotent stem cells

Somatic cells have only limited proliferation ability and active G1 cell cycle checkpoint (Cox and Rizzino, 2010). They show tissue-specific cell morphology and express somatic cell-specific markers. Pluripotency genes are inactive. X-chromosome is inactivated in female cells. Fully reprogrammed iPS cells on the other hand can proliferate nearly unlimitedly and have lost the G1 cell cycle checkpoint. High telomerase activity has a role in their self-renewal as telomerases are involved in maintaining telomere length, which is known to be crucial for replicative life span in ES cells. iPS cells are morphologically similar to ES cells. iPS cells express ES-specific markers. Endogenous genes essential for pluripotency and self-renewal are reactivated and exogenous pluripotency genes used in reprogramming are silenced. Extensive epigenetic remodeling is required for gene expression changes. X-chromosome is reactivated in female mouse iPSCs (miPSCs). Different human iPSC lines have been examined to have either one or two active X-chromosomes (Cox and Rizzino, 2010).

iPSCs are showing very similar with ESCs in expression of pluripotency markers, ability to differentiate into germ layers, proliferation, morphology and teratoma formation. Gene expression and DNA methylation pattern is however different in many ways (Takahashi and Yamanaka 2006;

Takahashi et al., 2007; Yu et al., 2007). iPSCs have been found to show subtle, but non-random genetic and epigenetic variability, that occurs at certain genes or loci (Wang et al., 2013). Interestingly, late-passage (p. 35 and above) iPS cells have more similar gene expression profile with ES cells than early-passage (below p. 12) iPS cells have (Chin et al., 2009). Therefore extended culturing brings iPS cells and ES cells closer. This maturation might affect the differentiation potential of the iPSCs.

Takahashi et al. (2007) and Yu et al. (2007) conducted wide characterization analysis on the generated hiPSCs. They had a typical ESC morphology and showed high telomerase activity as well. DNA fingerprinting analyses confirmed that the hiPS cells were derived from the parental fibroblasts. The hiPSCs showed generation of cell surface markers and expression of genes specific for human ES at equivalent levels: They were stained positive in immunocytochemistry for hES cell-specific surface antigens stage-specific embryonic antigens (SSEA-3 and SSEA-4); tumor-related antigens (TRA-1-60 and TRA-1-81) (Takahashi et al., 2007; Yu et al., 2007) and alkaline phosphatase (AP) (Takahashi et al., 2007). RT-PCR showed expression of many undifferentiated ES cell markers such as Oct-3/4, Nanog, (Takahashi et al., 2007; Yu et al., 2007), Sox-2, reduced expression 1 (Rex-1), growth and differentiation factor 3 (GDF3), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated (DPPA2 and DPPA4), and telomerase reverse transcriptase (hTERT) (Takahashi et al., 2007). hESCs have been demonstrated to be able to spontaneously develop into three dimensional cell aggregates called embryoid bodies (EB) when cultured in suspension (Itskovitz-Eldor et al., 2000). These EBs consist of derivatives of the three germ layers. The differentiation ability of the hiPSCs was analyzed *in vitro* by floating cultivation to form embryoid bodies (Takahashi et al., 2007; Yu et al., 2007). Pluripotency *in vivo* was evaluated by teratoma formation in immunodeficient mice. Teratomas are benign tumors that compose of tissues of more than one embryonic germ layer. Histological analysis confirmed tumors from three germ layers (Takahashi et al., 2007; Yu et al., 2007). Another method for evaluation pluripotency *in vivo* would be generating chimeric mice (Hotta and Ellis, 2008). In addition, Takahashi et al. (2007) initiated differentiation into functional cardiac and neural cells.

Epigenetic status of the hiPS cells was studied by bisulfite genomic sequencing and chromatin immunoprecipitation (Takahashi et al., 2007). Bisulfite genomic sequencing analyses were conducted to study the methylation status of promoters of pluripotent associated gene such as Oct-3/4, Rex-1 and Nanog. The cytosine guanide dinucleotides (CpG) of the promoters were highly unmethylated, on

contrary to CpG regions on parental cell promoters that were highly methylated. Therefore the pluripotency genes were active in iPSCs and inactive in the parental fibroblasts. Histone modification status was analyzed by chromatin immunoprecipitation. It showed many histone modifications characteristic of hESCs: histone H3 lysine 4 was methylated and H3 lysine 27 was demethylated in the promoter regions of Oct-3/4, Sox-2, and Nanog. Development-associated genes, such as Gata6, Msx2, Pax6, and Hand1 had bivalent histone methylation pattern typical for ESCs. Yu et al. (2007) found that demethylation pattern of their hiPSCs was similar to ES cells but Oct-3/4 promoter showed different methylation pattern compared to ES cells. Exogenous transgene Nanog was not expressed in hiPSCs indicating silencing, but Oct-3/4 was expressed in variable levels (Yu et al., 2007).

2.3 Methods of reprogramming somatic cells to a pluripotent state

Presence of pluripotent stem cells is transient in normal development. During normal development these pluripotent cells commit to certain lineage and their differentiation potential becomes more restricted (Wang et al., 2013). It was believed for a long time that this differentiation process is irreversible. Nevertheless, it was demonstrated already in 1958 by Gurdon et al., that somatic cells in a frog can be to some extent reprogrammed to pluripotency by nuclear reprogramming (Wang et al., 2013). Wilmut et al. (1997) was the first to show that in mammals terminally differentiated cells can return to their pluripotent state and therefore the developmental process is reversible. They demonstrated somatic cell nuclear transfer (SCNT) and reprogrammed adult somatic cell nucleus with unfertilized enucleated oocyte. Moreover, cell fusion was later introduced as another method of reprogramming by Tada et al. (2001). They fused adult somatic cells with embryonic stem cells or embryonic germ cells to restore pluripotency of the somatic cells. Takahashi and Yamanaka (2006), Yamanaka et al. (2007) and Yu et al. (2007) showed that ectopic expression of pluripotency factors can convert somatic cells to embryonic-like stem cells, iPS cells. Reprogramming with transcription factors is inefficient, slow and epigenetic variation is high compared to SCNT (Wang et al., 2013). Ethical issues are also of concern with SCNT since lots of oocytes are needed in this process. Somatic cell conversion into pluripotent stem cells has been successful without introduction of exogenous transcription factors as well. Different reprogramming methods are discussed in the next two chapters.

2.3.1 iPSC reprogramming by transcription factors

The reprogramming of somatic cells into iPSCs is traditionally performed by forced expression of four transcription factors Oct-3/4, Sox-2, Klf-4 and c-Myc, so-called OSKM cocktail. There are integrative and non-integrative methods of reprogramming iPS cells with transcription factors. Integrative methods include viral (retro and lenti viruses) and non-viral (plasmid, excisable Cre/LoxP and excisable transposons). Non-integrative methods are viral (adeno and Sendai viruses) and non-viral (episomal vector, mRNA and protein). Mostly used method for iPSC generation has been gene delivery by viral vectors (Bayart and Cohen-Haguenauer, 2013). Integrative methods based on retro virus or transposons are most efficient reprogramming methods (0,1-1 %) but improvements in non-integrative methods have increased their efficiency. Non-integrative Sendai virus has been shown to have quite a high reprogramming efficiency as well (up to 1 %) (Bayart and Cohen-Haguenauer, 2013). Different delivery methods of reprogramming factors are presented in **Figure 2**.

In integrative reprogramming systems, the delivered transgenes are randomly integrated into the host genome, and might become reactivated. Reactivation of transgene c-Myc has resulted in tumor formation (Okita et al., 2007). The reactivation or persistence of transgenes has been found to interfere with the differentiation of the iPSCs and suggested to result in poor differentiation efficiency (Toivonen et al., 2013). Non-integrative reprogramming methods result in transient expression of transgenes. Transgenes do not integrate into the host genome but stay as episomes.

2.3.1.1 Integrative reprogramming methods

Different integrative reprogramming methods include viral and non-viral based systems. Retro and lenti viruses are viral integrative vectors, whereas plasmids, excisable Cre/LoxP and excisable transposons are non-viral integrative vectors. Integrative methods are more efficient than non-integrative methods. However, integration has a risk of undesired genetic modification of the host genome, which is a disadvantage when considering clinical usage of iPS cells (Bayart and Cohen-Haguenauer, 2013).

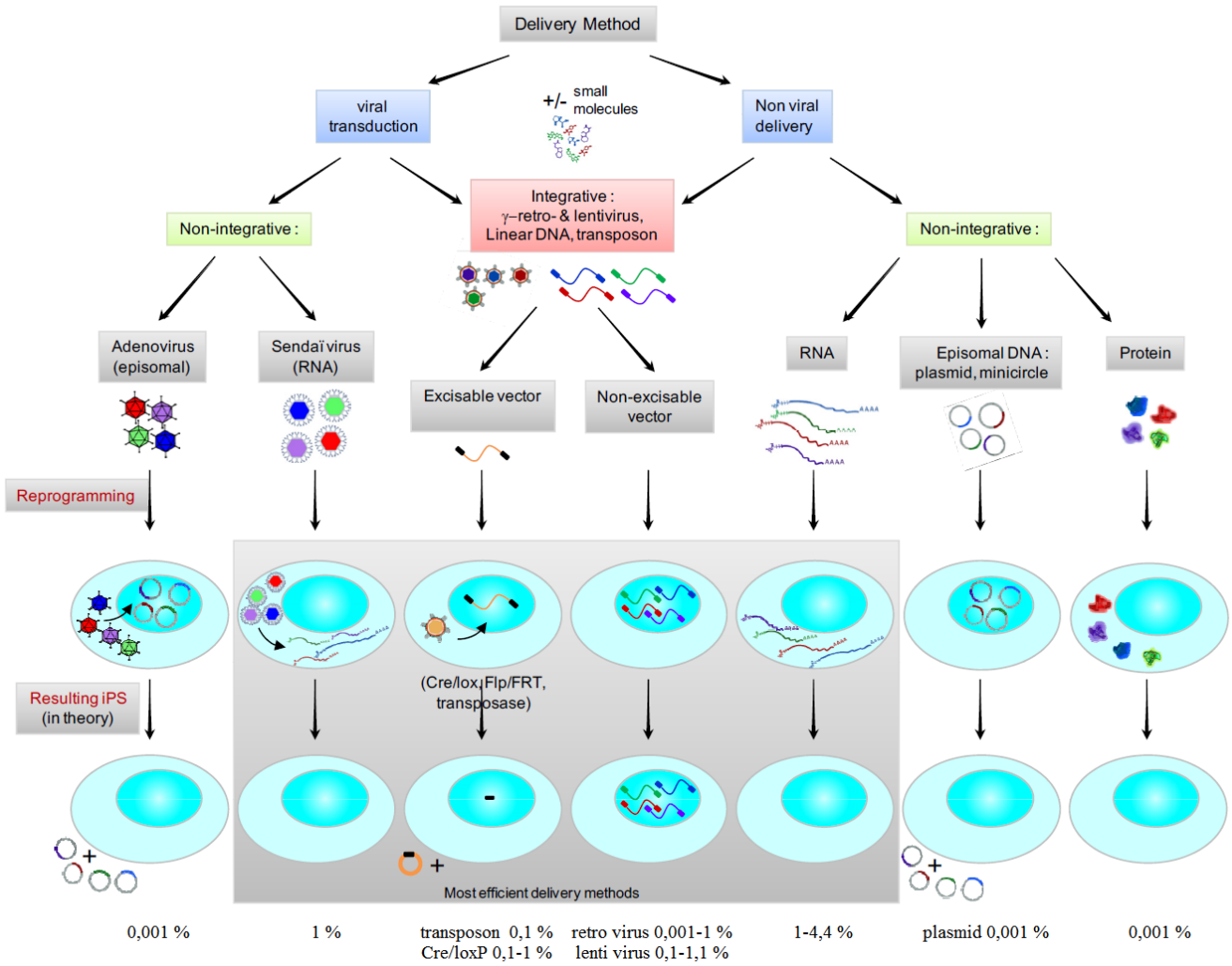


Figure 2. Transcription factor based reprogramming methods of iPSCs divided into viral and non-viral delivery methods. The efficiencies are given for each method (Robinton and Daley, 2012; Wang et al., 2013). (Figure modified from Bayart and Cohen-Haguenauer, 2013)

Takahashi and Yamanaka (2006) and Takahashi et al. (2007) delivered the transcription factors via gammaretrovirus Mo-MLV (Moloney murine Leukemia Virus) based vector pMXs or pMSCV. Regions encoding replication and packaging proteins are deleted from viral genomes and therefore the virus vectors are not capable of replicating in the host cell. Transgene delivery is efficient in actively dividing cells (efficiency up to 90 %) but slowly or non-diving cells are resistant to transfection (Bayart and Cohen-Haguenauer, 2013). Major disadvantage of the retro viral reprogramming method is that vectors integrate randomly in the host genome. This may result in insertional mutagenesis, proto-oncogene activation and tumorigenesis (Bayart and Cohen-Haguenauer, 2013). In addition, reactivation of transgenes has been demonstrated to cause problems as well. Okita et al. (2007) studied mouse iPSC

(miPSC) derived mice and tumors were developed in 20 % of the offspring. This tumor formation was suggested to result from reactivation of retro viral transgene c-Myc. It was found that reprogramming factors are required only for the induction stage and not for the maintenance of pluripotency of the iPSCs. Therefore reprogramming methods with transient expression of transgenes were suggested (Okita et al., 2007). Transgenes have been studied to be silenced during reprogramming but persistence or reactivation is however possible (Hotta and Ellis, 2008). Transgene reactivation could result in deregulation of neighboring genes and affect the cell cycle (Bayart and Cohen-Haguenauer, 2013). The reprogramming efficiency of retro viral based methods is 0,001-1 % (Robinton and Daley, 2012).

Yu et al. (2007) utilized lenti viral vectors to generate hiPSCs. Lenti virus based vectors are derived from HIV (human), SIV (simian) or EAIV (equine) viruses. They are able to transduce both dividing and non-dividing cells. Their cloning capacity is broader and transduction efficiency is higher compared to gammaretroviruses (Bayart and Cohen-Haguenauer, 2013). Transgenes are silenced during reprogramming but to a lower extent than retro viral transgenes, possibly preventing full reprogramming (Hotta and Ellis, 2008). The reprogramming efficiency has been reported to be 0,1-1,1 % (Robinton and Daley, 2012).

In addition to viral integrative methods, there are also non-viral integrative protocols to generate iPS cells with transcription factors, as plasmid DNA may be transfected also via liposomes or electroporation. Transduction efficiency is however extremely low compared to viral vectors (Bayart and Cohen-Haguenauer, 2013). These non-viral integrative methods include excisable vectors. Excisable vectors have been designed to generate transgene-free iPSCs. In excisable vector systems, the integrated transgenes are excised from the integration sites after the induction of iPS cells (Bayart and Cohen-Haguenauer, 2013). Excisable integrative vectors include Cre/LoxP and transposons.

Soldner et al. (2009) used excisable Cre/LoxP lenti virus vectors for their studies. They included both LoxP site in the 3'LTR (Long terminal repeat) and inducible promoter to drive transgene expression. During virus replication LoxP site was duplicated in the 5'LTR and the integrated transgene was located between the 3' and 5'LTR sites. After hiPSC generation, reprogramming factors were excised with transiently expressed Cre-recombinase as the sequence between LoxP sites was deleted by recombination (Soldner et al., 2009). A problem with the vectors was that reprogramming factors were

primarily integrated at different independent sites resulting in multiple excisions, which could lead to genomic rearrangements (Bayart and Cohen-Haguenauer, 2013). The reprogramming efficiency of Cre/LoxP based methods is 0,1-1 % (Robinton and Daley, 2012). There are also Cre/LoxP based retro virus vector systems for reprogramming.

Another excisable integrative vector system is based on transposons. Transposons are DNA sequences that can move from one position to another within the genome by excision and insertion. Woltjen et al. (2009) used a PiggyBack (PB) transposon system to generate hiPSCs. The PB system was composed of transposon plasmid and transiently expressed transposase that catalyses insertion and excision. The reprogramming transgenes were flanked by the 5' and 3' ITRs (inverted terminal repeats) and excised after iPSC generation by targeted recombination (Woltjen et al., 2009). An advantage over Cre/LoxP is that the whole integrated vector DNA can be excised from the host genome. However, the transposition reaction is not always precise and genomic instability of the host cells might be a problem (Bayart and Cohen-Haguenauer, 2013). The reprogramming efficiency is 0,1 % (Robinton and Daley, 2012).

2.3.1.2 Non-integrative reprogramming methods

Insertional mutagenesis can be prevented by non-integrative vectors that are transiently expressed. They dilute with time and cell division (Bayart and Cohen-Haguenauer, 2013). Different non-integrative vectors include viral adeno and Sendai vectors, episomal vectors, RNA and (recombinant) proteins. Integration deficient retro virus vectors and lenti virus vectors have also been studied but no iPSC generation with them has been demonstrated by far (Bayart and Cohen-Haguenauer, 2013).

Stadtfeld et al. (2008b) used adeno virus vectors to generate integration free miPS cells. Adeno virus vectors are replication deficient. In principle, they do not integrate in most cell types but remain as episomes in the host cell (Bayart and Cohen-Haguenauer, 2013). Generation of iPSCs with adeno viral transgene expression was however low in efficiency and was successful only in genetically engineered cells with inducible Oct-3/4 expression cassette (Stadtfeld et al., 2008b). hiPSCs were generated in Zhou and Freed (2009) studies by adeno virus vectors, however the efficiency was low (0,001 %) and reprogramming required repeated infection cycles.

Fusion gene F-deficient Sendai virus (SeV) vectors are another non-integrative vectors used in iPSC generation (Bayart and Cohen-Haguenauer, 2013). These modified Sendai viruses are incapable of producing infectious particles and are also commercially available nowadays. Fusaki et al. (2009) generated hiPSCs efficiently (1 %) with Sendai virus and after reprogramming the virus vectors were diluted during cell proliferation. Sendai viruses can infect a wide range of cells. Viruses replicate constitutively and therefore their elimination from host cells may be both important and difficult (Bayart and Cohen-Haguenauer, 2013). However, it has been demonstrated that Sendai vectors can be eliminated from the host cells simply by proliferation and usually after 10 passages they can not be detected in the host cells by normal methods such as PCR (Griesenbach et al., 2005).

Transient episomal vectors are other non-integrative vectors for iPSC reprogramming. One of the advantages of this method is ease to perform and no virus particles are required to be produced (Bayart and Cohen-Haguenauer, 2013). Yu et al. (2009) used oriP/Epstein-Barr nuclear antigen-1-based episomal vectors (oriP/EBNA1) to generate hiPSCs. These vectors replicate autonomously as episomal elements and can be maintained as low copy number episomes with drug selection (Yu et al., 2009). However, it is not completely proven that these vectors are not integrated into host genome (Bayart and Cohen-Haguenauer, 2013).

There are also transgene-free delivery methods to generate iPSCs, based on delivery of transcription factors in the form of RNA or protein. They aim to avoid introducing exogenous material into host cells (Bayart and Cohen-Haguenauer, 2013).

Successful reprogramming of hiPSCs with *in vitro* transcribed capped mRNAs of transcription factors was demonstrated by Yakubov et al. (2010). However this method required repeated transfection cycles and RNA vector production is expensive (Bayart and Cohen-Haguenauer, 2013). Kim et al. (2009) generated hiPS cells by recombinant reprogramming factor proteins fused with poly-arginine tract to facilitate transfection to host cells. The process was however slow and the efficiency rather low as they could isolate only few colonies. Another disadvantage is that recombinant proteins are needed in rather high concentrations (Bayart and Cohen-Haguenauer, 2013).

2.3.2 iPSC reprogramming without transcription factors

Reprogramming methods without introducing transcription factors have been developed as well. These methods include chemicals and microRNAs (miRNAs).

Small molecule –mediated methods have been developed to replace reprogramming with transcription factors. Since these methods do not cause any genetic modification, they can decrease the risk of tumorigenesis. These chemical methods are also assumed to be non-immunogenic. They target different cell signaling pathways such as TGF-β and nuclear epigenetic factors (Wang et al., 2013). miPS cells have been generated by using chemical compounds only, termed chemically induced iPS cells (CiPSCs) (Hou et al., 2013). Their 0,2 % reprogramming efficiency is comparable to standard reprogramming methods (Wang et al., 2013).

Miyoshi et al. (2011) were able to generate iPS cells with miRNAs, without exogenous transcription factors. miRNAs are small non-coding RNAs that induce mRNA degradation or translational silencing. The reprogramming efficiency was 0,1 % (Robinton and Daley, 2012).

In addition, somatic cell conversion into pluripotent stem cells has been successful with exposure to strong external stress such as low pH (Obokata et al., 2014). These STAP-cells (stimulus-triggered acquisition of pluripotency) were able to differentiate into placental cells, indicating that they would be more potent than ESCs or iPSCs. However, questions of whether the results were falsified were raised as other researchers could not replicate the study.

2.4 Reprogramming factors

Transcription factors define the cell fate during development by activating or repressing target gene expression (Adachi and Schöler, 2008). The exogenous transcription factors that were first used in iPSC generation are Oct-3/4, Sox-2, c-Myc and Klf-4. In addition, there are other transcription factors for somatic cell reprogramming. Nanog together with LIN-28 can be used in reprogramming to replace c-Myc and Klf-4 (Yu et al., 2007).

Oct-3/4 is a transcription factor (Oct3, Oct4 or POU5F1) belonging to the family of POU transcription factors (Zhao et al., 2012). It is critical for establishing pluripotency in the embryo (Nichols et al., 1998). It has a key role in maintenance of self-renewal in ES cells and it inhibits the expression of genes related to differentiation. Oct-3/4 is expressed in the pluripotent inner cell mass of the blastocyst and epiblast lineage. Oct-3/4 regulates several genes during early embryogenesis, including Sox-2, Nanog and Rex-1 (Zhao et al., 2012). The regulatory elements of the target genes often are located close to Sox-2 binding sites (Lewitzky et al., 2007). Oct-3/4 is the only exogenous transcription known to be required for somatic cell conversion into pluripotent state (Wang et al., 2013). Niwa et al. (2000) established the role of Oct-3/4 as the master regulator of pluripotency. They studied expression and repression of transgenous Oct-3/4 in ES cells. Oct-3/4 was demonstrated to control lineage commitment and its expression level was crucial for maintenance of stem cell pluripotent state and self-renewal, as maintenance and self-renewal require Oct-3/4 expression in a critical amount. For instance, twofold increase of Oct-3/4 resulted in differentiation into primitive endoderm and mesoderm. On the other hand, repression of Oct-3/4 induced a loss of pluripotency and resulted in dedifferentiation into trophectoderm (Niwa et al., 2000).

Sox-2 is a transcription factor belonging to the Sox (Sry-related High-mobility Group (HMG) Box-containing) family of HMG box transcription factors (Zhao et al., 2012). Sox-2 has a critical role in embryonal development and maintaining pluripotency of ES cells. Sox-2 and Oct-3/4 co-regulate many pluripotency-associated genes in ES cells. Sox-2 has been hypothesized to co-operate with other transcription factors such as Nanog to activate transcription of pluripotency markers (Lewitzky et al., 2007). However, other members of the Sox-2 family play a role in induction as well and therefore Sox-2 can be replaced in reprogramming by other Sox-2 family members (Zhao et al., 2012).

Klf-4 is a transcription factor of the Klf family (Krüppel-like factor) of zinc finger transcription factors (Zhao et al., 2012). Klf family members regulate cell proliferation, differentiation, development and apoptosis. Klf-4 interacts with the pluripotency network proteins, including Oct-3/4 and Sox-2 (Zhao et al., 2012). It inhibits cell death, as it has been studied to directly repress the guardian of the genome, p53 (Rowland et al., 2005). In addition, Klf-4 has been found to act both as an oncogene and a tumor suppressor (Lewitzky et al., 2007). It is a target of STAT3 in LIF pathway. The overexpression of Klf-4 inhibits differentiation of ES cells (Lewitzky et al., 2007).

c-Myc is a helix-loop-helix transcription factor (Lewitzky et al., 2007). It has a role in many cellular functions such as cell growth, proliferation and cell-cycle regulation. The many downstream targets of c-Myc enhance proliferation and transformation. c-Myc is a substrate for GSK3β in Wnt signaling. In addition, it is a downstream effector of STAT3 in the LIF signaling pathway (Lewitzky et al., 2007). c-Myc is a potent oncogene and reactivation of transgenous c-Myc has resulted in tumor formation in miPSC derived mice (Okita et al., 2007). c-Myc is not essential for reprogramming, but it increases the efficiency of iPSC colony formation (Wang et al., 2013).

Nanog is a transcription factor having a key role in the maintenance of pluripotency and self-renewal of ES cells (Zhao et al., 2012). However, exogenous Nanog is not required for reprogramming as it is activated by Oct-3/4 and other transcription factors such as Sox-2 (Wang et al., 2013). p53 has been found to be a negative regulator of Nanog (Lin et al., 2005).

Yu et al. (2007) generated hiPSCs by introduction of Oct-3/4, Sox-2, Nanog and LIN-28 into somatic cells. Nanog and LIN-28 interact and bind cooperatively with Oct-3/4 and Sox-2. LIN-28 is a target of c-Myc (Adachi and Schöler, 2012). Few iPSC clones were generated even in the absence of LIN-28 integration, thus it was suggested that LIN-28 is not required for reprogramming (Yu et al., 2007).

Oct-3/4 upregulates Sox-2 and Nanog (Wang et al., 2013). Boyer et al. (2005) have identified Oct-3/4, Sox-2 and Nanog as master regulators of pluripotency. These transcription factors co-occupy promoters of large gene population. Many of the target genes are important in early development. Oct-3/4, Sox-2 and Nanog co-operate to form a regulatory network of autoregulatory and feed forward loops. Autoregulatory indicates that Oct-3/4, Sox-2 and Nanog bind and activate the promoters of their own genes. The feed forward loops activate gene expression of hES cell transcription factors and of various components of chromatin remodeling and histone modification complexes and of signaling pathways such as TGF-β. This network activation results in self-renewal and repression of differentiation in ES cells (Boyer et al., 2005).

2.5 Mechanisms of iPSC reprogramming

iPSC reprogramming from somatic cells is a complex process that involves genome wide changes and epigenetic modifications. Endogenous pluripotency genes that were silent in the differentiated state are

activated, whereas genes that were specific for the somatic state are silenced during the reprogramming process. The exogenous pluripotency factors bind to specific genes across the genome and interact with other factors to modify the genetic and epigenetic profile of the cells. The result is establishment of the endogenous pluripotency network. Only temporal expression of the exogenous transcription factors is required for the activation of endogenous pluripotency genes. The transgenes are silenced epigenetically in integrative reprogramming methods or diluted with proliferation in non-integrative reprogramming methods (Bayart and Cohen-Haguenauer, 2013). Silencing of exogenous pluripotency genes is considered to be required for further differentiation of hiPS cells, since persistent expression of exogenous pluripotency genes might interfere with the natural differentiation signaling (Hotta and Ellis, 2008). Non-integrative methods result in transient expression of transgenes: The transgenes do not integrate into the host genome but stay as episomes which dilute during proliferation.

2.5.1 Epigenetic modifications during reprogramming

Variety of epigenetic modifications is required for conversion of somatic cells into pluripotent by exogenous transcription factors: 1) bivalent and altered histone and DNA methylation pattern is established, 2) the endogenous pluripotency genes are activated and endogenous pluripotency network is established, 3) the exogenous pluripotency genes are silenced and 4) chromatin structure is reorganized.

Histone modifications and DNA methylation are important in the epigenetic regulation of gene activity (Hotta and Ellis, 2008). Histone modifications include histone acetylation and methylation. Acetylation of histone tails is a sign of transcriptional activity and results in open euchromatin, whereas histone deacetylation results in condensed heterochromatin and transcriptional repression (Hotta and Ellis, 2008). Open chromatin (euchromatin) is a mark of active transcription, whereas closed chromatin (heterochromatin) is an inactive sign. Methylation of histone tails H3 lysine 4 (mono-, di- or trimethylation: H3K4me1/me2/me3) is an active mark (Cox and Rizzino, 2010). Trimethylation of histone H3 lysine 27 (H3K27me3) or dimethylation of lysine 9 (H3K9me2) is a repressing mark (Hotta and Ellis, 2008). DNA methylation takes place primarily at cytosine on CpG sites of promoters in mammals but up to 25 % of DNA methylation can occur on non-CpG sites in ES cells (Wang et al., 2013). DNA methylation represses gene activity.

Somatic genes are silenced and pluripotency genes are activated during reprogramming. The core pluripotency genes are activated to establish the endogenous pluripotency network. Transcription factors co-operate to repress somatic genes directly or indirectly (Adachi and Schöler, 2012). Some somatic genes with non-CpG island promoters are downregulated by removal of their activating H3K4me2 mark. Co-operation between the transcription factors is required for activation of pluripotency genes that are not silenced by DNA methylation. When non-methylated promoters of pluripotency genes and developmental regulators are activated, they gain histone H3 lysine 4 mono or dimethylation (H3K4me1/me2). These genes are enriched for H3K4me2 in ES cells as well. Methylated pluripotency genes are activated only later in reprogramming by unknown mechanisms (Adachi and Schöler, 2012). The promoters of the core endogenous pluripotency genes Oct-3/4, Nanog and Sox-2 are repressed in somatic cells by DNA methylation at CpG sites and histone H3 lysine 9 trimethylation (H3K9me3) (Wang et al., 2013). During reprogramming these CpG sites become demethylated and H3K9me3 mark is replaced by activating H3K4me3 mark. Promoters of many pluripotency genes are enriched for H3K4me3 in ES cells (Adachi and Schöler, 2012). Pluripotent stem cells (PSCs) have many bivalent chromatin sites. These bivalent sites contain regions that are enriched of both activating H3K4me3 mark and repressing H3K27me3 or H3K9me2 marks. These bivalent domains are established in iPS cells during reprogramming (Hotta and Ellis, 2008). Bivalent chromatin domains might not be required in the induction of reprogramming but on the other hand required for the differentiation ability of iPS cells (Djuric and Ellis, 2010).

De novo methylation of somatic genes has been demonstrated to have a minor role in reprogramming (Wang et al., 2013). De novo methylation is catalyzed by methyltransferases DNMT3a and 3b and maintained by DNMT1. On the contrary, demethylation of pluripotency genes is important for cell conversion. As mentioned in the previous paragraph, activation of endogenous Oct-3/4, Nanog and other pluripotent genes is associated with demethylation of their promoter CpG regions. Demethylation of pluripotency genes is hypothesized to occur either by DNA replication-independent active DNA demethylation or DNA replication-dependent passive demethylation. DNA replication-independent active DNA demethylation might involve the reprogramming factors or their targets that antagonize the methylation maintaining DNMT1. DNA replication-dependent passive demethylation would be catalyzed by deaminase Aid (or Aicda) (Wang et al., 2013).

Epigenetic changes are involved in silencing of retro viral and lenti viral exogenes (Hotta and Ellis, 2008). Histone modifications including acetylation and methylation have been suggested essential for silencing the exogenes, in addition to cytosine methylation of DNA. Deacetylation of histone H3 or H4 is found in silent retro and lenti viruses. Silent retro viruses have been demonstrated to undergo histone methylation as well. In addition, silent retro viruses and lenti viruses have been demonstrated to be heavily methylated on their CpG sites. However, DNA methylation might not be required to establish the silencing of transgenes, but rather to maintain the silent state (Hotta and Ellis, 2008).

Chromatin reorganization is required for somatic cell conversion into iPSC (Wang et al., 2013). Complete chromatin reorganization allows the activation of endogenous pluripotency network. Open, active chromatin structure is a mark of PSCs (Cox and Rizzino, 2010). Open chromatin allows the transcription factors to activate a variety of genes needed for self-renewal or to prepare bivalent genes for rapid activation in differentiation. At the time of differentiation, not-needed chromatin regions are closed (condensed) and genes in these regions are inactivated. Different histone markers such as activating histone 3 lysine 4 di- or tri-methylation (H3K4me2/me3) marks are associated with open chromatin in ES cells. Chromatin remodeling proteins associate with the markers and maintain the chromatin open (Cox and Rizzino, 2010).

Different iPS cell lines have been found to be variable in their epigenetic, transcriptional and differentiation potential (Bayart and Cohen-Haguenauer, 2013; Wang et al., 2013). The genetic and epigenetic variability is not random but occurs at hotspots across the genome (Wang et al., 2013). Epigenetic alterations occur in histone modification and methylation. Reprogramming requires genome wide genetic alteration and therefore different mutations may arise during reprogramming process (Bayart and Cohen-Haguenauer, 2013).

2.5.2 Steps of reprogramming

Somatic cell conversion into pluripotent state follows sequential steps driven by reprogramming factors (Adachi and Schöler, 2012). Many molecular and cellular events take place during reprogramming. Details of the first steps of reprogramming have been found, but later steps are not well known. This might partly be due to low efficiency of reprogramming (Adachi and Schöler, 2012). **Figure 3** presents the sequence of events in reprogramming.

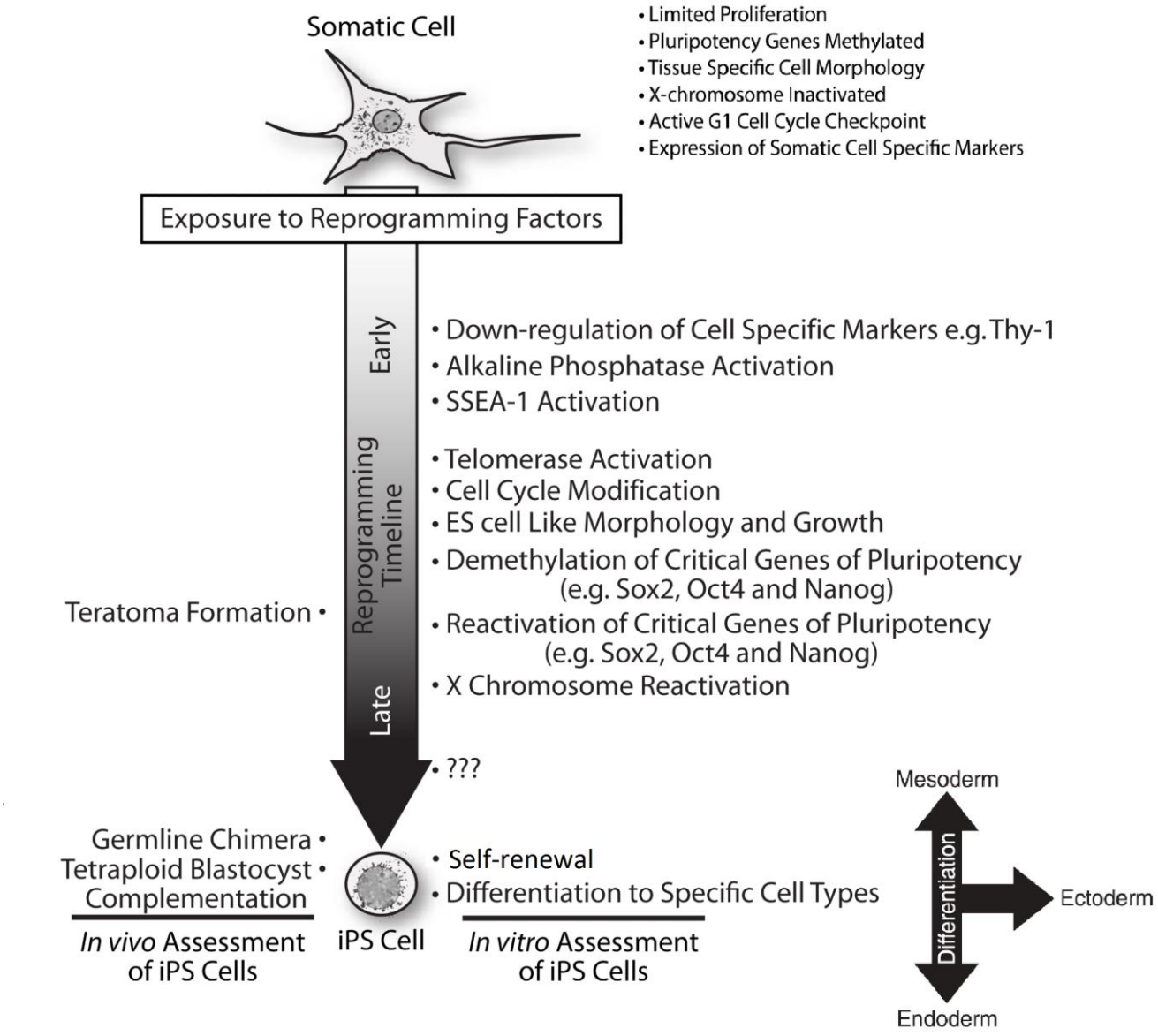


Figure 3. The sequence of events in reprogramming somatic cells into iPS cells. The latter steps are not well known. (Modified from Cox and Rizzino, 2010; Greenow and Clarke, 2012)

The first step of reprogramming is identified by an increase in proliferation rate of a small percentage of cells (Adachi and Schöler, 2012). The size of the cells decrease and cell cycle promoters are upregulated. The first pluripotency associated marker that is activated is alkaline phosphatase (AP) (Stadtfeld et al., 2008a). Cell-specific markers such as Thymocyte differentiation antigen 1 (Thy-1) are downregulated. Next pluripotency marker to be activated is stage-specific embryonic antigen 1 (SSEA-1). Not all cells however express AP and SSEA-1 (Stadtfeld et al., 2008a). The second step includes suppression of somatic cell programs and gaining of some properties of pluripotent cell type, such as

formation of compact colonies (Adachi and Schöler, 2012). The endogenous pluripotency network is established and the exogenous pluripotency genes are silenced in the last steps of reprograming. The exogenous pluripotency genes are considered to be silenced only after endogenous pluripotency genes are activated (Hotta and Ellis, 2008). SSEA-1 positive cells reactivate the core endogenous pluripotency genes Oct-3/4, Sox-2 and Nanog (Stadtfeld et al., 2008a). The reactivation of endogenous pluripotency genes is associated with expression of embryonic antigens SSEA-3, TRA-1-60, TRA-1-81, DNA methyltransferase 3β (DNMT3β) and Rex-1 (Robinton and Daley, 2012). In addition, chromatin remodeling takes place during reprogramming but it is not known when and how it is established. Chromatin remodeling appears to take place over an extended time period (Cox and Rizzino, 2010). Reactivation of inactive X-chromosome in female cells (at least in mice iPSCs) and upregulated telomerase activity occur before exogenous pluripotency gene expression is silenced (Stadtfeld et al., 2008a). Late reprogramming includes remodeling of the cell cycle structure as well, resulting in loss of G1 checkpoint but mechanisms are not known (Cox and Rizzino, 2010). In addition, reprogramming involves changes in microRNA expression (Wang et al., 2013). miRNAs have a role in the regulation of self-renewal and differentiation potential of PSCs. Some miRNAs are required for efficient reprogramming and others for reprogramming inhibitors (Wang et al., 2013).

Each of the four reprogramming factors may have their role in every step of reprogramming (Adachi and Schöler, 2012). The reprogramming factors are primarily thought to bind their putative binding sites, then alter the target gene expression and change cell fate. Co-operation between transcription factors is required for activation. Oct-3/4 might act as a pioneering factor by binding to enhancer regions in order to prepare chromatin sites more accessible to other factors such as Sox-2 and Klf-4 (Adachi and Schöler, 2012). Oct-3/4, Sox-2 and Nanog have been shown to regulate X-chromosome inactivation (Cox and Rizzino, 2010). Nanog has been studied to initiate the reactivation of inactive X-chromosome by initiating silencing of Xist, one of the genes that regulate X-chromosome inactivation. On the other hand, Sox-2 and Oct-3/4 repress Xist in the absence of Nanog (Cox and Rizzino, 2010). c-Myc is likely to be the primary regulator in the change of proliferation rate as it is important in cell proliferation and transformation (Adachi and Schöler, 2012). c-Myc effects mainly during the first steps of reprogramming where it upregulates cell cycle and represses the expression of somatic genes. It activates the expression of housekeeping genes that are involved in cell proliferation and metabolism

c-Myc recruits many histone acetyltransferase complexes and chromatin remodeling factors. c-Myc also promotes nucleosome disassembly (Adachi and Schöler, 2012).

2.5.3 Partial reprogramming

Genome wide epigenetic modifications are required for successful reprogramming. Variation in epigenetic modifications, the extent of methylation, the persistence of expression of exogenous pluripotency genes and other factors affect the success of reprogramming (Robinton and Daley, 2012). Indeed, reprogramming is considered as an inefficient process and it can result in partially reprogrammed cells: They express some pluripotency markers but the core pluripotency markers Oct-3/4, Sox-2 and Nanog are inactive (Cox and Rizzino, 2010). Their promoters are still methylated and histone modifications have been established only partially. Exogenous pluripotency genes are not silenced and pluripotency maintenance is dependent on their expression (Cox and Rizzino, 2010). iPS cells are considered to be fully reprogrammed only when endogenous pluripotency genes are up-regulated and transgenes downregulated (Hotta and Ellis, 2008). Fully reprogrammed iPS cells are thus independent of exogenous pluripotency genes in maintaining their pluripotency. ES cells lack G1 checkpoint and therefore it has been suggested that partially reprogrammed iPS cells have insufficiently remodeled their cell cycle control (Cox and Rizzino, 2010). Constant expression level of exogenous Oct-3/4 is required in intermediate stages of reprogramming to maintain pluripotency. If exogenes are silenced too early in the reprogramming process, the endogenous pluripotency network might not be activated, resulting in partial reprogramming (Hotta and Ellis, 2008).

Two barriers for reprogramming have been suggested (Boué et al., 2010). The first barrier is the stress generated by the overexpression of transcription factors. The stress results in stimulation of apoptosis and reduction of cell viability. The cells that overcome this first barrier are partially reprogrammed: self-renewing and pluripotent but their pluripotency is maintained by transgenous pluripotency genes that are not yet silenced. The second barrier is the genome wide epigenetic remodeling that allows the activation of the endogenous pluripotency network, resulting in silencing of transgenes (Boué et al., 2010). This epigenetic remodeling is the rate-limiting step of reprogramming and also known as epigenetic barrier of reprogramming (Bayart and Cohen-Haguenauer, 2013). Different compounds

affecting for example DNA methylation or histone acetylation have been studied to overcome this epigenetic barrier.

Although full reprogramming should silence all somatic marks, studies have revealed that iPS cells have epigenetic memory, meaning that they have epigenetic characteristics of the tissue that they originate from (Kim et al., 2010). This epigenetic memory might favor differentiation into lineages that are related to the original tissue. iPS cells have been found to express somatic genes especially in low passages (Bayart and Cohen-Haguenauer, 2013). This phenomenon is defined as transcriptional memory. It is considered to be a result from incomplete silencing of tissue-specific genes and possibly incomplete reactivation of ES-specific genes during reprogramming. Incomplete DNA methylation of the promoters is thought to result in this incomplete silencing and reactivation of ES-specific genes (Ohi et al., 2011).

2.5.4 Elite, stochastic and deterministic model of reprogramming

Reprogramming is considered to be stochastic at the early stages but deterministic during the late stages (Wang et al., 2013). Two models for reprogramming were first suggested: elite and stochastic. In elite model only few somatic cells would be able to convert into iPSCs: The donor cell population would consist of stem cells and differentiated cells, out of which only the stem cells would generate iPS cells. However, improvements of reprogramming methods have generated iPS cells in efficiencies of 10-20 % and it is unlikely for stem cells to be present in such a high level in tissues. In addition, lineage tracing has proven that iPSC colonies are derived from terminally differentiated cells instead of stem cells. Therefore elite model has been ruled out. Stochastic model assumes that almost all differentiated cells are capable of reprogramming. The cells just need to overcome the epigenetic barrier for successful reprogramming. However, the stochastic model cannot predict whether a given cell would be converted to iPS cell and when conversion would take place. Deterministic model states that once crucial pluripotency network is established, reprogrammed cells would be generated with a fixed timescale (Wang et al., 2013).

2.5.5 Reprogramming efficiency

Direct reprogramming is in principle a simple process and involves ectopic expression of defined factors that induce cell conversion. It is however a slow and inefficient process, as already discussed in earlier chapters. The parameters affecting reprogramming efficiency and quality of the established iPS cells include reprogramming method, reprogramming factors and donor cell type. Different reprogramming methods were already discussed in chapter 2.3.

Different factor combinations are more efficient with some reprogramming methods than other methods (Bayart and Cohen-Haguenauer, 2013). The Yamanaka cocktail OSKM (Oct-3/4, Sox-2, Klf-4 and c-Myc) is efficient with many integrative and also non-integrative vectors such as Sendai virus and mRNA but not efficient with adeno virus, episomal plasmid and proteins. Moreover, other cocktails including more or less than the common set of four transcription factors have been demonstrated to generate iPS cells. However, the efficiencies have been lower than with the OSKM cocktail. For example, OSK, OSN and OKM which contain three of the four elements of Yamanaka factors have been proved to be successful for iPSC reprogramming. In addition, OS or OK when combined with some other factors such as cell cycle regulators, chromatin modelers, short hairpin RNAs (shRNAs) or microRNAs (miRNAs) can be used for iPSC generation (Bayart and Cohen-Haguenauer, 2013).

Relative expression level of the four reprogramming factors has been found to affect reprogramming efficiency and quality of the resulting iPS cells (Wang et al., 2013). Higher expression of Oct-3/4 than Sox-2, Klf-4 and c-Myc generates more iPSC colonies. On the other hand, reprogramming efficiency decreases when expression of Oct-3/4 is lower than expression of the other factors. The order of OSKM polycistronic expression cassette compared to OKSM has been found to enhance the reprogramming efficiency and iPSC quality as well. Stoichiometry of the reprogramming factors has been suggested to be important in the early stages of reprogramming, where Oct-3/4 and Klf-4 may have higher expression than Sox-2 and c-Myc. However, once pluripotency is established, reduced Oct-3/4 expression enhances self-renewal and delays differentiation (Wang et al., 2013).

A wide variety of cell types have been studied for reprogramming: for instance embryonic and adult fibroblasts, keratinocytes, neural progenitor cells, hepatocytes, stomach and intestinal epithelial cells and pancreatic β cells (Cox and Rizzino, 2010). The most common donor cells are fibroblasts (80%)

and keratinocytes (Bayart and Cohen-Haguenauer, 2013). Usually iPS cells are derived from fibroblasts as they are easy to extract from skin biopsies. Embryonic tissues are more efficient in reprogramming than adult tissues. However, embryonic tissues are not as easily accessible as adult tissues for iPSC generation. In addition, progenitor cells are more prone for conversion compared to terminally differentiated cells (Bayart and Cohen-Haguenauer, 2013). The requirements for the exogenous transcription factors depend on the donor cell type. It has been demonstrated that if donor cells show endogenously high expression level of one or more pluripotency factors, reprogramming with fewer factors is possible. Kim et al. (2008) applied only two exogenous transcription factors Oct-3/4 and Klf-4 for iPSC reprogramming as the host cells were already expressing Sox-2 and c-Myc in higher level than ES cells.

Reprogramming efficiency has been proven to depend on cell proliferation of the initial cells. In fact, the higher the proliferation rate is the more efficient the reprogramming would be (Ruiz et al., 2011). Positive regulators of cell proliferation have been found to facilitate reprogramming. p53 has been identified as a guardian against reprogramming (Menendez et al., 2010) and inhibiting p53 has been hypothesized to enhance reprogramming. Short hairpin RNAs (shRNAs) against p53 have been demonstrated to enhance iPSC generation. Kawamura et al. (2009) was able to generate iPSCs with shRNAs against p53, without exogenous c-Myc and Klf-4. Moreover, reprogramming efficiency has been shown to increase when applying certain microRNAs (miRNAs) that are involved in cell cycle (Bayart and Cohen-Haguenauer, 2013).

2.6 Cardiac differentiation

2.6.1 Cardiogenesis

Formation of the heart, cardiogenesis is the earliest event of organogenesis during embryonic development (Rajala et al., 2011). Heart tissue is composed of three cell lineages derived from mesoderm: the cardiac myocyte, the vascular smooth muscle and the endothelial cell lineages. The three main subtypes of cardiomyocytes are ventricular, atrial and nodal (pacemaker) cells. Knowledge on different stages and molecular mechanisms in cardiogenesis has been gained from animal models but many aspects still remain unclear (Rajala et al., 2011).

The three embryonic germ layers endoderm, mesoderm and ectoderm are formed soon after gastrulation. Many tissues derive from the primitive streak of the primitive endoderm but posterior primitive streak is the origin of cardiac progenitor cells (Rajala et al., 2011). Posterior primitive streak gives rise to mesodermal cells that migrate to anterior region (Mummery et al, 2012). Signaling from anterior endodermal cells promote the induction of cardiac mesoderm. Cardiac mesodermal cells give rise to heart field progenitor cells that eventually form the ventricles and atriums of the heart. The committed cardiac progenitors maturate into functional cardiomyocytes (Mummery et al, 2011).

Cardiac differentiation *in vivo* and *in vitro* includes a complex signaling network (Rajala et al., 2011). Many signaling pathways and growth factors have been identified in cardiac differentiation but mechanisms are still in many cases unclear. The most studied signaling pathways involved in cardiac differentiation are Wnt/Nodal, BMPs and FGFs. In addition, microRNAs (miRNAs) have been studied to contribute to differentiation process. The first step is well characterized but the signaling processes in latter three steps are poorly understood. Wnts, BMPs and TFG- β family member Nodal have been confirmed to induce mesoderm (Rajala et al., 2011).

2.6.2 Differentiation of hPSCs into cardiomyocytes

Cardiomyocytes can be generated *in vitro* from pluripotent stem cells (PSCs) in four steps that mimic the stages of embryonic cardiac development: 1) mesoderm induction, 2) the patterning of mesoderm toward anterior mesoderm or cardiogenic mesoderm, 3) formation of cardiac mesoderm and 4) maturation of early cardiomyocytes. These steps can be characterized by the expression of typical transcription factors, such as T/Brachyury of primitive streak mesoderm, Mesp-1 of cardiogenic mesoderm, Nkx2.5 of cardiac mesoderm, and α-actinin and cardiac Troponin T (cTnT) of maturing cardiomyocytes (Rajala et al., 2012). **Figure 4** presents the steps of *in vitro* cardiac differentiation.

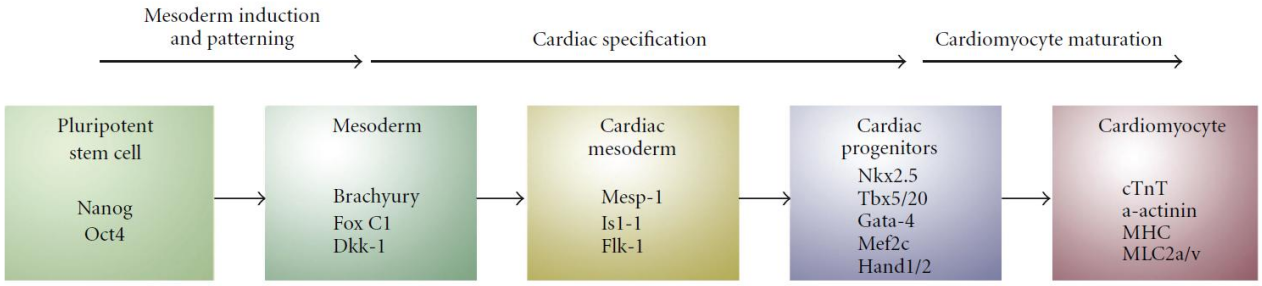


Figure 4. The steps of cardiac differentiation *in vitro* and their typical markers. (Rajala et al., 2011)

Different human tissues could be used as a source of stem cells but cardiac progenitor cells have been found to be the only multipotent adult stem cells that are capable of differentiating into beating cardiomyocytes (Rajala et al., 2011). However, spontaneously contracting cardiomyocyte-like cells have been efficiently differentiated only from pluripotent stem cells. Different methods have been developed to differentiate human ES cells into functional cardiomyocytes. hiPS cells are promising as an additional source for cardiomyocyte differentiation as the iPSC-derived cardiomyocytes are similar to hESC-derived cardiomyocytes (Takahashi et al., 2007; Rajala et al., 2011).

Current cardiomyocyte differentiation methods from pluripotent stem cells are 1) spontaneous embryoid body differentiation in suspension, 2) co-culture with cardioinductive mouse endoderm-like cells (END-2) and 3) guidance with defined growth factors in monolayer or suspension. Many of the protocols have first been used in differentiating hESCs. After discovery of iPSCs, many of the methods have been successful in hiPSC differentiation as well. The disadvantages of the current protocols include generation of heterogeneous cardiomyocyte populations and low differentiation efficiency (Rajala et al., 2011). Moreover, direct reprogramming of somatic cells into cardiomyocytes has been demonstrated using only transcription factors. Fibroblasts can be directly reprogrammed into cardiomyocytes with a combination of three developmental transcription factors (i.e., Gata4, Mef2c, and Tbx5) (Ieda et al., 2010). Different cardiac differentiation methods are presented in **Figure 5**.

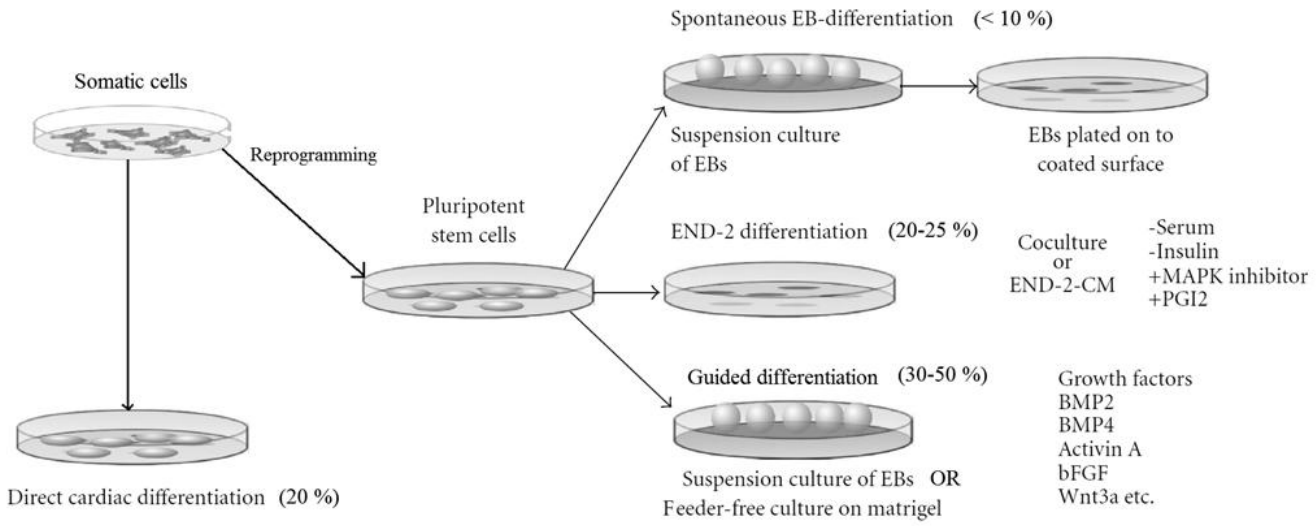


Figure 5. Cardiac differentiation methods *in vitro*. Pluripotent stem cells can be differentiated into cardiomyocytes with spontaneous embryoid body (EB) differentiation, in co-culture with END-2 cells or with guided differentiation. Newer protocols have suggested that somatic cells can be directly differentiated into cardiomyocytes by defined factors. Efficiencies of each differentiation method are given in brackets (Rajala et al., 2011 except direct cardiac differentiation Ieda et al., 2010). (Figure modified from Rajala et al., 2011)

2.6.2.1 Embryoid body differentiation

Pluripotent stem cells are able to spontaneously differentiate into three dimensional cell aggregates called embryoid bodies (EB) by floating cultivation technique. It was first demonstrated by Itskovitz-Eldor et al. (2000). Kehat et al. (2001) was the first to differentiate hES cells into spontaneously contracting cardiomyocyte-like cells using EB method. Zhang et al., (2009) was the first to demonstrate hiPSC-derived cardiomyocytes by EB method. These embryoid bodies are usually plated on matrix-coated culture plates to enhance the differentiation. The spontaneous differentiation efficiency of hPSCs into cardiomyocytes with EB method is quite low (below 10 %) and it is highly dependent on cell line (Kehat et al., 2001). Embryoid bodies often differ in size and morphology. Hanging drop or forced aggregation methods have been studied in order to increase the reproducibility of the results (Rajala et al., 2011). Manipulation of the differentiation process by directing it with growth factors, morphogens or transgenes has been successful as well. The EB differentiation method is simple and inexpensive and thus a widely used cardiac differentiation method (Rajala et al., 2011).

2.6.2.2 Co-culture with END-2 cells

After the discovery that anterior endoderm plays a role in cardiac induction from the adjacent mesoderm in cardiac development, a co-culture method with visceral endoderm-like cells (END-2) was developed for *in vitro* cardiac differentiation of hPSCs (Mummery et al., 2003). The END-2 cells were derived from mouse P19 embryonal carcinoma (EC) cells. In this method, END-2 cells are treated with Mitomycin C to prevent their proliferation. Beating cardiomyocyte aggregates (beating areas) usually begin to emerge in 12-20 days in co-culture. The differentiation results in heterogeneous population of cells (Mummery et al., 2003). Direct cell to cell interaction with END-2 cells and/or stimulating factors secreted by END-2 cells are considered to induce cardiac differentiation but the exact mechanism is not yet clear (Rajala et al., 2011). The END-2 method has been improved by removing serum from the culture and applying ascorbic acid to the medium. It has been demonstrated that serum inhibits (Passier et al., 2005) and ascorbic acid enhances (Takahashi et al., 2003) the cardiac differentiation of hESCs. Furthermore, END-2 conditioned medium (END-2-CM) can be used. The cardiac differentiation with END-2 method is nevertheless rather inefficient. However, the method has been demonstrated to be rather robust in iPS cell differentiation (Rajala et al., 2011).

2.6.2.3 Guidance with defined factors

Growth factors of endodermal origin such as fibroblast growth factors (FGF), bone morphogenic proteins (BMP) and repressors of Wnt signaling pathway have been suggested to mediate the cardiac induction (Mummery et al., 2003). BMP4, Wnt3a and Activin A have been demonstrated to induce gastrulation-like events and the meso/endoderm development of PSCs (Xu et al., 2006). Cardiac differentiation induced with growth factors such as FGFs, BMPs, and Wnts is called guided cardiac differentiation. This method involves culturing cells as a high-density monolayer in feeder cell-free systems or in EB suspensions (Rajala et al., 2011). Laflamme et al. (2007) were able to enhance the cardiac differentiation efficiency of hESCs into 30 % with sequential supplementation of BMP4 and Activin A.

2.6.3 Characterization of hPSC-derived cardiomyocytes

Basic characteristics of pluripotent stem cell derived cardiomyocytes have been widely studied. The characteristics include expression of specific molecular markers, structure and functionality (Rajala et al., 2011). The markers include transcription factors, structural proteins, ion channels, tight junction

proteins and hormones. Functional studies include pharmacological and electrophysiological studies (Rajala et al., 2011). Characterization methods of PSC-derived cardiomyocytes include flow cytometry, RT-PCR and immunofluorescence for cardiac-specific markers, in addition to functional analyses (Mummery et al., 2012). These functional analyses of PSC-derived cardiomyocytes are for instance electrophysiological measurements of action potential, electrical activity or Ca^{2+} transients. Electrophysiological measurements can be conducted by microelectrode array (MEA) or patch clamp.

hiPSC-derived cardiomyocytes are comparable to hESC-derived cardiomyocytes in cardiac phenotype (Takahashi et al., 2007). PSC-derived cardiomyocytes mature over time *in vitro* but do not achieve structure and functionality equal to adult cardiomyocyte stages (Rajala et al., 2011). Therefore the result of differentiation *in vitro* is a mix of non-cardiac cells and cardiomyocytes with different subtypes and maturation stages.

Potentials for *in vitro* differentiated human cardiomyocytes include models of early human cardiogenesis, disease models, drug discovery and safety toxicology (Rajala et al., 2011). Many disease models and pharmaceutical testing utilize animal models but because of the basic physiology differences between different species, human models would give more applicable results. Derivation from patients, possibility of genetic manipulation and thus targeted treatment are advantages of hiPSCs in cardiac disease models (Rajala et al., 2011).

3. AIMS OF THE RESEARCH

Patient derived hiPS cells that have been reprogrammed with two different protocols (integrative retro virus and non-integrative Sendai virus) were studied in this study. It has been hypothesized that the exogenous pluripotency genes used in integrative reprogramming methods might reactivate in higher passages, which may affect the efficiency and direction of differentiation (Takahashi and Yamanaka, 2006; Toivonen et al., 2013). The purpose of this study was to examine the reactivation of transgenes in hiPSCs generated by integrative retro virus method and evaluate its effect on cardiomyocyte differentiation. The results were compared with iPSCs generated by non-integrative Sendai virus method.

The hiPS cells were cultivated and their pluripotency was characterized. These characterized hiPS cells were differentiated into cardiomyocytes by END-2 protocol and the cardiac differentiation efficiency was studied. Exogene activity was studied both before and after cardiac differentiation. The original plan was also to study hepatocyte differentiation differences of these hiPS cells but setting up the differentiation protocol by another researcher was not as fast and effortless as first was considered and therefore only cardiac differentiation is included in this thesis.

The questions to be answered during this study are 1) whether the hiPSCs generated by integrative retro viral reprogramming method at high passage (p. 40-60) show signs of reactivated transgenes in undifferentiated state and 2) whether there is any evidence of reactivation of transgenes in these retro virally reprogrammed hiPSCs after cardiac differentiation compared to Sendai virally reprogrammed (non-integrative) hiPSCs at low passage. hiPSCs reprogrammed with non-integrative Sendai virus method should show no sign of transgene reactivation and differentiate efficiently.

4. MATERIALS AND METHODS

Three hiPS cell lines reprogrammed by non-integrative Sendai virus and two cell lines reprogrammed by integrative retro virus were selected. After culturing, they were first characterized for pluripotency and then were differentiated into cardiomyocytes.

4.1 hiPSC lines and reprogramming

This study includes five patient derived hiPSC lines: UTA.10101.EURCAs, UTA.11304.EURCCs, UTA.10311.EURCSs, UTA.05105.HcMM and UTA.00208.LQT1. The first three lines are new lines that have not been studied before. The latter two lines are older lines that have been cultivated in the Heart group for a longer period of time. The abbreviations after line number are disease-specific and will be omitted in the latter sections of this thesis, as patient background is not relevant in this study. In addition, the Sendai virally induced lines will be referred as Sendai lines S-10101, S-11304 and S-10311 whereas retro virally induced lines as retro lines R-05105 and R-00208 later in this thesis.

All the five hiPS cell lines were previously reprogrammed by the laboratory technician from patient derived skin biopsies and isolated dermal fibroblasts. Three of them (S-10101, S-11304 and S-10311) were established by CytoTune™-iPS Reprogramming Kit (Invitrogen). The kit contains four CytoTune™ reprogramming Sendai virus vectors from F-gene deficient Sendai virus, each vector capable of expressing one of the four Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc). The two other cell lines (R-05105 and R-00208) were established by lenti virus infection followed by infection of pMX retro viruses each encoding either Oct3/4, Sox-2, c-Myc or Klf-4 (Takahashi et al., 2007).

4.2 Maintenance and characterization of hiPSCs

4.2.1 Cell culture conditions of hiPSCs

The hiPS cells were grown in ESC culture conditions in co-culture with inactivated mouse embryonic fibroblasts (MEF; CF1 MEF P3, MitomycinC treated; Applied StemCell, Inc) at 37 °C with 5 % CO_2 (Kojair). MEF cells are feeder cells that support the growth of iPSCs. MEF cells were cultured in

KnockOut™ Dulbecco's Modified Eagle Medium (KO-DMEM; Gibco by Life Technologies), supplemented with 10 % Fetal Bovine Serum (FBS Gold, PAA Laboratories GmbH) and 2 mM % L-glutamine (GlutaMAX™, Life Technologies). The medium was sterile filtered before usage with 0.8/0.2 µm filter (Acrodisc® PSF Syringe Filters 32 mm, Pall Corporation). 6-well plates (Nunc, ThermoFisher Scientific) were coated with 0,1 % gelatin (Type A from porcine skin, Sigma) for one hour at room temperature before plating MEFs in density of 100 000 cells/ml. MEFs were cultured overnight at 37 °C and 5 % CO_2 (Heraeus Hera cell 150; Thermo Scientific) before submitting the iPS cells on top of them.

The hiPS cells were cultured in KO-KSR (serum replaced Knock Out DMEM) containing KO-DMEM (KnockOut™ DMEM; Gibco by Life Technologies), 20 % KO-SR (KnockOut™ Serum Replacement; Gibco by Life Technologies), 1 % L-glutamine (Sigma Aldrich), 1 % NEAA (100 x NEAA MEM; non-essential amino acids; Sigma Aldrich or Lonza BioWhittaker), 1 % Pen-Strep (Penicillin Streptomycin; Sigma Aldrich), 0.1 mM β-mercaptoethanol (Sigma Aldrich) and 4 ng/ml bFGF (basic fibroblast growth factor; Peprotech). The medium was changed three times per week and the undifferentiated state of the colonies was confirmed as well. The hiPSCs were passaged once a week: First the MEF feeder cell layer was removed with a pipet tip. Then the hiPSCs were incubated 3-5 min with 1 mg/ml collagenase IV (Gibco by Life Technologies) at +37 °C thermoplate. They were finally detached from the well bottom and transferred on top of MEF cells with KSR medium. Stocks of hiPSCs were prepared by freezing the cells with freezing medium that contained 10 % DMSO (Sigma Aldrich) and 90 % FBS (Gold, PAA Laboratories GmbH), in isopropanol chamber at -80 °C overnight. The stocks were stored in liquid nitrogen.

4.2.2 Characterization of hiPSCs for pluripotency

Characterization methods consisted of RNA extraction, RT-PCR and PCR analysis, gel electrophoresis, immunocytochemistry and formation of embryoid bodies. The characterization of the three Sendai lines S-10101, S-11304 and S-10311 was initiated at passage 10, as Sendai virally induced hiPSCs are Sendai virus free at that passage at the latest (Griesenbach et al., 2005). The two retro lines R-00208 and R-05105 were characterized at passage 40 or 60, respectively. The retro lines had already been characterized once at passage 10 by the same methods as in this study. Samples have been reported negative for mycoplasma infection performed by Quality Controllers.

4.2.2.1 RNA extraction, RT-PCR, PCR and gel electrophoresis

The collected hiPSC colonies were lysed with lysis buffer (β-mercaptoethanol added as a denaturing agent) and the RNA was extracted with NucleoSpin® RNA II (Macherey Nagel) according to the manufacturer's instructions. DNA was digested from the samples, following washing steps and eluation of the pure RNA. Eluation was enhanced with incubation of the eluation, prewarming the eluation water and centrifuging in two steps. The concentration and purity of isolated RNA was measured with spectrophotometer (NanoDrop Spectrophotometer ND-1000). RNA was stored at -80 °C.

Extracted RNA was reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. The master mixes contained 10x RT buffer, 25x dNTP mix, 10x primers and reverse transcriptase (all Applied Biosystems). Also RNase inhibitor (Riboblock RNase Inhibitor, Thermo Scientific) and RNAse free H_2O was added into the reactions. Total amount of 1000 ng RNA was synthesized. Two negative controls were used: 1) sample and the previous master mix without reverse transcriptase (-RT control) and 2) the previous master mix and H_2O instead of sample (-H_2O control). Reaction conditions were 1) 25 °C 10 min, 2) 37 °C 120 min, 3) 85 °C 5 min and 4) 4 °C hold. PRC reactions were run with Thermal Cycler Mastercycler (Eppendorf). cDNA was stored at -20 °C.

Expression of different pluripotency genes at mRNA level was studied with PCR analysis using the synthesized cDNA as a template. The expression of exogenous pluripotency genes Oct-3/4. Klf-4, c-Myc and Sox-2 and the expression of endogenous pluripotency genes Nanog, Rex-1, Oct-3/4, Sox-2 and c-Myc was studied. Housekeeping genes GAPDH and/or β-actin were used as a positive control. In addition, there were positive controls for each exogene: retro exogene controls were derived from the plasmids that were used in retro viral transfection. Sendai exogene controls were derived from patient fibroblasts that had been infected with the Sendai delivered exogenes. Negative controls were the same as in RT-PCR: -RT control and H_2O control.

Master mixes of PCR reactions contained 10x Dynazyme buffer and Dynazyme II polymerase (Thermo Scientific), forward and reverse primers designed for each gene and diluted into 5 μM (biomers.net), 10mM dNTP mix Fermentas), 25 mM MgCl (Fermentas) and RNAse free H_2O. DMSO was added in all reactions except for GAPDH and endogenes Oct-3/4, c-Myc and Rex-1. Reaction conditions of

exogenous PCR reactions were 1) 94 °C 2 min, 2) hybridization 94 °C 30 s, 3) annealing 55 °C 30 s, 4) elongation 72 °C 1 min and 5) last elongation 72 °C 5 min. Steps 2-4 were repeated for 40 cycles. Reaction conditions of endogenous PCR reactions were 1) 94 °C 2 min, 2) hybridization 94 °C 30 s, 3) annealing 45-60 °C 30 s, 4) elongation 72 °C 30 s and 5) last elongation 72 °C 5 min . The annealing temperatures were 45 °C for Nanog, 55 °C for Rex-1 and 60 °C for Oct-3/4, Sox-2 and c-Myc. PRC reactions were run with Thermal Cycler Mastercycler (Eppendorf).

The PCR products were identified with agarose gel electrophoresis. The PCR products were loaded with 6x DNA Loading Dye (Fermentas). They were run with 2 % agarose gel (peqGOLD Universal Agarose; PeqLab) containing 0,25 μg/ml ethidium bromide (10 mg/ml; Sigma) for 1 hour at 80 V (BioRad and Amersham Biosciences). 50 bp DNA Ladder (Generuler; Fermentas) was used as a molecular weight marker. Agarose gels were imaged with UV gel documentation system (UVidoc; UVitec).

Two of the Sendai lines S-11304 and S-10311 did not give high enough yield of RNA for PCR products to be visualized on agarose gel at passage 10. Therefore new RNA samples were collected at passage 14 and protocol was continued as above.

4.2.2.2 Immunocytochemistry

After removing the feeder layer the culture plates were fixed with 4 % paraformaldehyde (PFA; Sigma Aldrich) for 20 minutes at room temperature. At this point the plates were either stored at + 4 °C or continued for immunocytochemistry.

Unspecific binding was blocked with blocking solution containing 10 % NDS (Normal Donkey Serum; Millipore), 0,1 % TritonX-100 (Sigma Aldrich) and 1 % BSA (Bovine Serum Albumin; Sigma Aldrich) in PBS for 45 minutes at room temperature. Samples were washed with washing solution containing 1 % NDS, 0,1 % TritonX-100 and 1 % BSA in PBS. Primary antibodies diluted in 1 % NDS, 0,1 % TritonX-100 and 1 % BSA in PBS were incubated overnight at +4 °C. The primary antibodies included pluripotency markers Nanog (Anti-human Nanog Antibody goat IgG; R&D Systems, Inc) diluted 1:100, Oct-3/4 (Anti-human Oct-3/4 antibody goat IgG; R&D Systems, Inc.) 1:400, SOX-2 (SOX-2 goat IgG; Santa Cruz) 1:200, SSEA-4 (SSEA-4 mouse IgG; Santa Cruz) 1:50 or

1:100, TRA-1-60 (Anti-TRA-1-60 mouse IgM; Millipore) 1:100 or 1:200 and TRA-1-81 (Anti-TRA-1-81 mouse IgM; Millipore) 1:100 or 1:200.

Secondary antibodies diluted in 1:800 in 1 % BSA in PBS were incubated for 1 hour at room temperature and light protected. Secondary antibodies included Alexa Fluor 568 nm donkey anti-goat IgG (Invitrogen) for Nanog, Oct-3/4 and Sox-2; Alexa Fluor 568 nm goat anti-mouse IgG H&L (Invitrogen) for SSEA-4; and Alexa Fluor 568 nm goat anti-mouse IgM M chain (Invitrogen) for TRA-1-60 and TRA-181. Samples were mounted with Vectashield Mounting Medium fluorescence with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories) and covered with 19 mm cover slips (Menzel Gläser). Images were captured with fluorescence microscope (Olympus IX51) connected to a camera (Olympus DP30BW). Plates were stored at + 4 °C protected from light.

4.2.2.3 Formation of embryoid bodies

Floating cultivation of hiPS cells was used for embryoid body (EB) differentiation (Itskovitz-Eldor et al., 2000). MEF feeder layer was first removed and hiPS cells were scraped into EB medium containing KO-DMEM (Gibco by Life Technologies), supplemented with 20 % FBS (Gold, PAA Laboratories GmbH), 1 % NEAA (MEM 100 x; Fisher Scientific), 2 mM % L-glutamine (GlutaMAX™, Life Technologies) and 0,5 % Pen-Strep (Fisher Scientific). hiPSCs were seeded on non-attachment 12-well plates (MPC treatment; Thermo Fisher Scientific) and cultured for 5 weeks at 36,5 °C and 5 % CO_2 (HERAcell 150i; Thermo Scientific). Medium was changed once a week under microscope.

Expression of the three germ layer marker was studied by RT-PCR analysis. EBs were collected, RNA was extracted and cDNA was synthesized as presented in chapter 4.2.2.1. Master mixes and reaction conditions were the same as in exogenous PCR reactions (see chapter 4.2.2.1), except that DMSO was added in all reaction. The primers (biomers.net) were: Sox-17 and AFP (α-fetoprotein) as endoderm markers, α-cardiac actinin and VEGFR2/KDR (vascular endothelial growth factor receptor 2/Kinase insert domain receptor) as mesoderm markers and Sox-1 and Pax-6 (Paired box gene 6) as ectoderm markers. Housekeeping genes GAPDH and/or β-actin were used as a positive control and -RT control and H_2O control as negative controls.

In the first EB differentiation experiment, two of the Sendai lines (S-11304 and S-10311) did not form enough EBs for PCR reactions. Therefore at passage 17, the cells were seeded again in higher density for EB differentiation and cultured for 5 weeks. Second experiment was not essentially more efficient, and therefore three different mediums were tested in the third differentiation experiment at passage 26: 1) KO-KSR without bFGF, 2) EB medium with 5 mM rock inhibitor (Rho-associated protein kinase inhibitor; R&D Systems) 3) normal EB medium. Cells were plated as above in high density. Rock free EB medium was changed the following day in the case of the second medium. RNA was extracted after 5 weeks. 500 ng of RNA was used for cDNA synthesis. PCR for germ layer markers was run without DMSO and annealing temperature was decreased by 1 °C from 55 °C to 54 °C. Otherwise the reaction conditions were the same as above. cDNA samples were amplified in one extra PCR reaction for endoderm markers, before running on agarose gel.

4.3 Differentiation of hiPSCs into cardiomyocytes

Characterized hiPSCs were co-cultured with END-2 cells to initiate cardiac differentiation (Mummery et al., 2003). The formed beating areas were counted and cardiac differentiation efficiency was evaluated at the end of differentiation. Exogene activity was studied by RT-PCR. One cell line (R-05105) was enzymatically dissociated and immunocytochemically stained for cardiac markers.

4.3.1 Cell culture conditions

Mouse visceral endoderm-like cells (END-2; a friendly gift from professor Mummery, Humbrecht Institute, Utrecht, The Netherlands) were cultured in END-2 medium containing DMEM F-12 (Gibco by Life Technologies) supplemented with 7,5 % FBS (Gold, PAA Laboratories GmbH), 1 % NEAA (MEM 100 x; Fisher Scientific), 2 mM % L-glutamine (GlutaMAX™, Life Technologies) and 0,5 % Pen-Strep (Fisher Scientific). Cells were cultured at + 36,5 °C and 5 % CO_2 (HERAcell 150i; Thermo Scientific) and passaged twice a week with trypsin (Lonza).

Co-cultures were first cultured in 0% KO-SR hES medium containing KO-DMEM (Gibco by Life Technologies) supplemented with 2,92 mg/ml ascorbic acid (Takahashi et al., 2003; Sigma Aldrich), 1 % NEAA (MEM 100 x; Fisher Scientific), 2 mM % L-glutamine (GlutaMAX™, Life Technologies) and 0,5 % Pen-Strep (Fisher Scientific) and 0.1 mM β-mercaptoethanol (Sigma Aldrich). 0% KO-SR

hES medium was changed at days 6, 8 and 13. At day 15 the medium was changed into 10 % KO-SR hES medium containing same supplements as 0 % KO-SR hES medium except with 10 % KO-SR (Gibco by Life Technologies) and without ascorbic acid. 10 % KO-SR hES medium was changed three times per week. Cells were cultured at + 36,5 °C and 5 % CO_2 (HERAcell 150i; Thermo Scientific).

4.3.2 Differentiation of hiPSCs with END-2 protocol

Before the start of differentiation END-2 cells were treated with 5µl/ml Mitomycin C (R&D Systems) for 3 hours at + 37 °C. They were dissociated with trypsin (Lonza) and counted with hemocytometer (Neubauer). The cells were plated with END-2 medium in density of 175 000 cells/well on 0,1 % gelatin (Type A from porcine skin, Sigma) treated 12-well plates (Nunc, ThermoFisher Scientific).

On the following day the END-2 medium on END-2 cells was changed into 0% KO-SR hES medium. MEF feeder layer was removed from hiPSCs and hiPS cells were scraped into medium. Approximately 30 colony pieces of hiPSCs were transferred onto one well of END-2 cells in as little amount of KO.KSR medium as possible. The cells were co-cultured for 31 days. The appearance of beating areas was monitored under microscope twice a week.

The first cardiac differentiation experiment with line S-10101 initiated at passage 12 did not succeed as expected. Thus another differentiation was initiated at higher passage (23). The retro lines R-050105 and R-00208 were known to differentiate well from previous experiments so they were used as positive controls for the Sendai lines: S-11304 was initiated for differentiation at passage 17 alongside with R-05105 at passage 69. When first beating areas appeared in R-05105 to indicate no researcher bias, S-10101 and S-10311 were differentiated at passage 23 and 21 respectively, with R-00208 at passage 46.

4.3.3 Characterization of hiPSC-derived cardiomyocytes

Cardiomyocytes were characterized for their morphology and number, and differentiation efficiency was evaluated. Immunocytochemistry for cardiac markers was performed on one line. Expression of exogenous pluripotency genes was analyzed by RT-PCR.

4.3.3.1 Evaluation of cardiac differentiation and efficiency

The number of beating areas per each well and per each plate was counted on day 31 from the start of the differentiation. The morphology and size of the beating areas was evaluated by photographing them. The differentiation efficiency was evaluated by dividing the total number of beating areas with the total number of plated colony pieces.

4.3.3.2 Dissociation of beating areas

10 beating areas of the line R-05105 were dissociated into single cardiomyocytes by Collagenase A treatment (Mummery et al., 2003). First the 10 beating areas were removed from the culture plates by cutting with a microscalpel (Feather, no 15) under microscope. The beating areas were then collected and washed in Low-Ca buffer for 30 minutes at room temperature. After washing they were incubated in enzyme medium containing collagenase A (Roche Diagnostics) for 45 minutes at +37 °C and in KB medium containing high K^+ for 1 hour at room temperature. Lastly the beating areas were transferred into EB medium, resuspended into single cells and seeded on 0,1 % gelatin (Type A from porcine skin, Sigma) coated plate. The dissociated cells were cultured for 5 days at + 36,5 °C and 5 % CO_2 (HERAcell 150i; Thermo Scientific).

4.3.3.3 Immunocytochemistry of dissociated cardiomyocytes

The dissociated cardiomyocytes were double stained immunocytochemically for two cardiac markers: Troponin T and cardiac α-actinin. The ICC protocol was carried out as in chapter 4.2.2.2. Primary antibodies included Troponin T (goat cardiac Troponin T IgG; Abcam) diluted in 1:2000 and cardiac α-actinin (monoclonal sarcomeric anti α-actinin mouse IgG; Sigma) diluted in 1:1500. Secondary antibodies were Alexa Fluor 568 nm donkey anti-goat IgG (Invitrogen) for Troponin T and Alexa Fluor 488 nm donkey anti-mouse IgG (Invitrogen). DAPI was used as a counterstain.

4.3.3.4 Exogenous pluripotency gene expression in hiPSC-derived cardiomyocytes

The cells were collected for RNA extraction and cDNA synthesis to study the activity of exogenous pluripotency markers Oct-3/4, Klf-4, c-Myc and Sox-2 by PCR analysis. The protocol and reaction conditions were as presented in chapter 4.2.2.1 for exogenous PRC reactions, with negative and positive controls. Reaction products were identified by agarose gel electrophoresis.

5. RESULTS

5.1 Characterization of hiPSCs for pluripotency

hiPS cells were confirmed to be pluripotent by RT-PCR, immunocytochemistry and EB formation.

5.1.1 Morphology

hiPS cells grew in tightly packed clusters called colonies (**Figure 6**). The hiPS cells are small and have round morphology, and can therefore grow tightly packed. hiPSC colonies containing non-differentiated cells were flat (grown in one layer culture), even in color and density. The colonies had defined edges and were usually round in shape. Non-differentiated hiPS cells attach firmly to the plate.

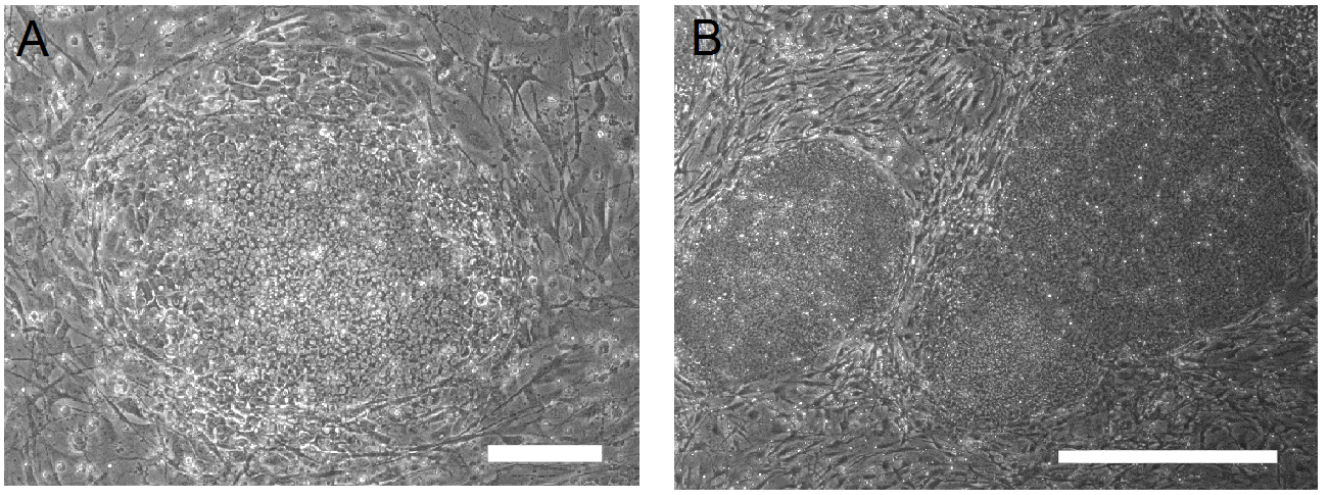


Figure 6. hiPSC colonies surrounded by MEF cells. **A**) Line S-10311, one colony. hiPSCs can be seen to push MEF cells aside at the edge of the colony. 10x magnification, scale bar 200 μm. **B**) Line S-10304, three colonies out of which two are grown together. 4x magnification, scale bar 1 mm.

5.1.2 RNA extraction, RT-PCR, PCR and gel electrophoresis

Only temporal exogenous pluripotency factor expression is required to activate the endogenous pluripotency network. In fully reprogrammed iPSCs the exogenous pluripotency genes should be silenced and endogenous pluripotency genes activated. In this study, exogenous and endogenous pluripotency gene expressions at mRNA level were studied by PCR analysis from the synthesized cDNA samples and visualized by gel electrophoresis. Exogenous pluripotency genes Oct-3/4, Klf-4, c-Myc and Sox-2 were not expressed. These PCR analyses confirm that all the exogenous genes had

either turned off (integrative reprogramming method, retro virus) or diluted out (non-integrative reprogramming method, Sendai virus) in the reprogrammed cells during their proliferation. The only exception was cell line R-00208 that was expressing exogenous Oct-3/4. No exogene activity had been encountered when the line had once been characterized at passage 10. Since this gene had been already shown silenced at lower passage, it could be concluded that it was reactivated during the cell growth at high passage (p. 40). The endogenous pluripotency markers Nanog, Rex-1, Oct-3/4, Sox-2 and c-Myc were all expressed. Therefore reprogramming had successfully activated endogenous pluripotency genes for all cell lines. **Figure 7** presents the results of exogenous PCR analysis and **Figure 8** of endogenous PCR analysis of lines S-11304, S-10311 and R-00208. Rest of the PCR figures are given in **Appendix 1**.

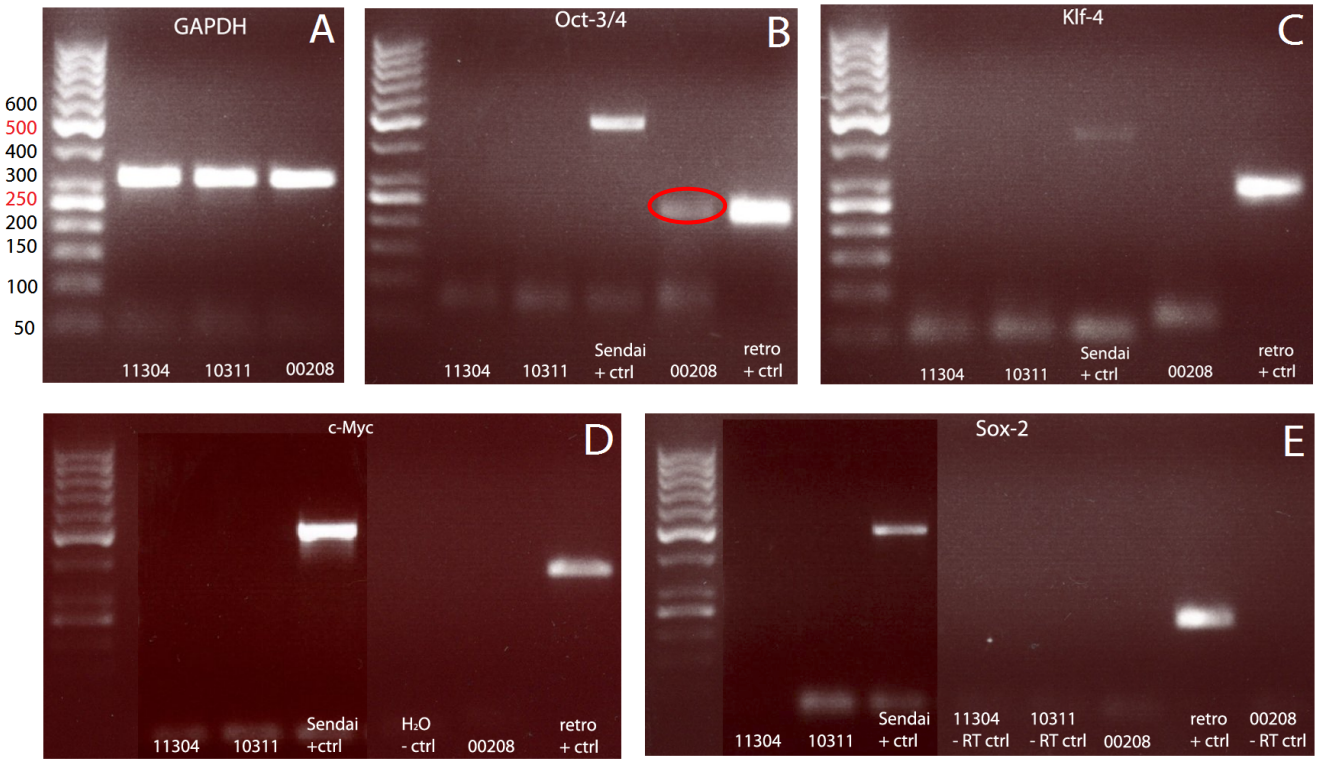


Figure 7. PCR analysis of four exogenous pluripotency genes in hiPS cell lines S-11304, S-10311 and R-00208. **A**) housekeeping gene GAPDH *(302 bp)* as a positive control, **B**) Oct-3/4 *(Sendai 483 bp and retro 200-250 bp)*, where red circle marks the residual Oct-3/4 expression in line R-00208, **C**) Klf-4 *(Sendai 410 bp and retro 250-300 bp)*, **D**) c-Myc *(Sendai 532 bp and retro <400 bp)* and **E**) Sox-2 *(Sendai 451 bp and retro >200 bp)*. Positive Sendai and retro transgene controls were used, negative controls were 1) cDNA sample without reverse transcriptase (-RT ctrl) and 2) H_2O as a template (-H_2O ctrl). The size of the molecular weight marker is given in base pairs (bp), red numbers represent the brightest bands.

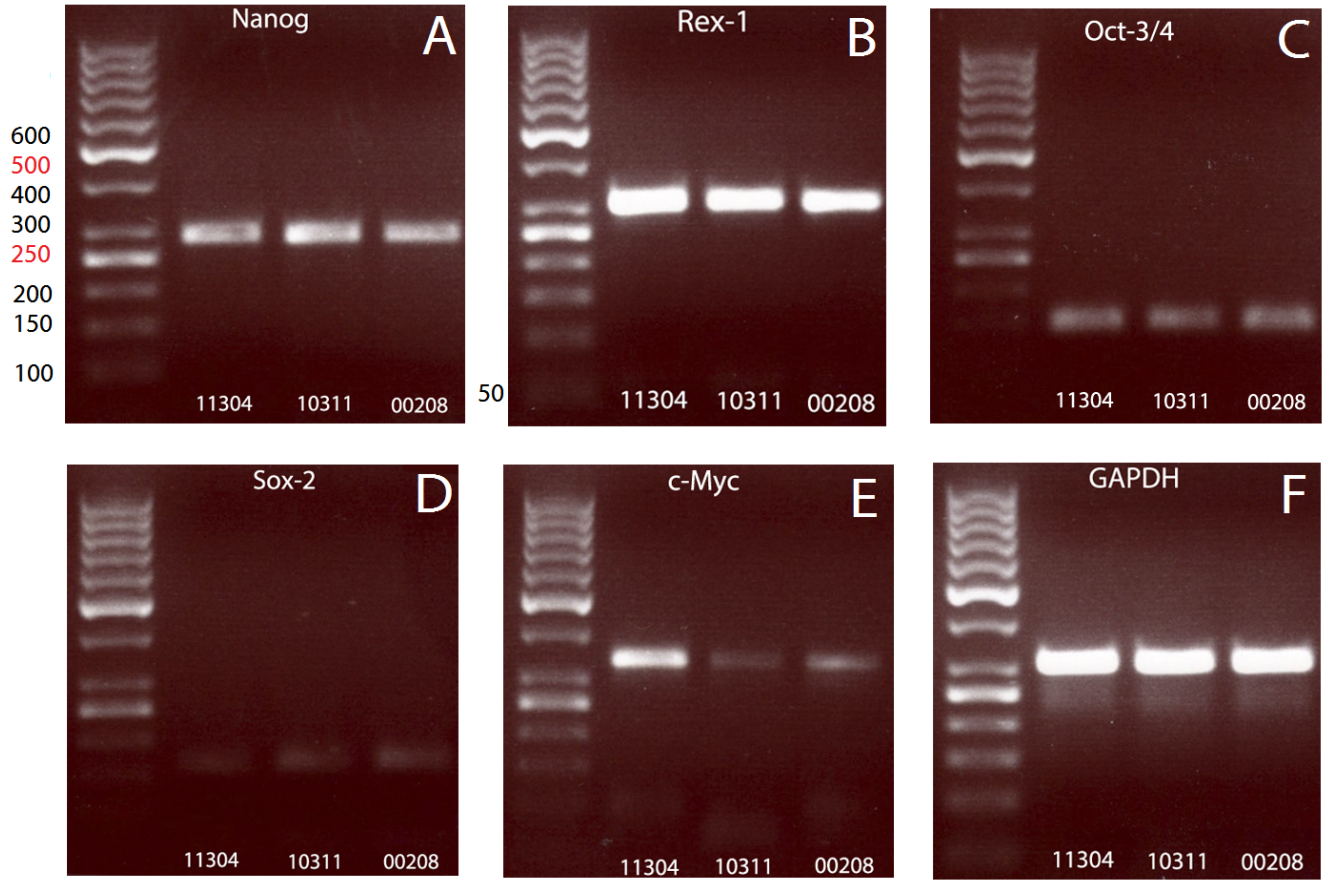


Figure 8. PCR analysis of five endogenous pluripotency genes in hiPS cell lines S-11034, S-10311 and R-00208. **A**) Nanog *(287 bp)*, **B**) Rex-1 *(306 bp)*, **C**) Oct-3/4 *(144 bp)*, **D**) Sox-2 *(151 bp)*, **E**) c-Myc *(328 bp)* and **F**) housekeeping gene GAPDH *(302 bp)* as a positive control. The positive bands represent the activity of the gene of interest.

5.1.3 Immunocytochemistry

All five hiPSC lines were immunocytochemically stained for the six pluripotency markers Nanog, Oct-3/4, Sox-2, SSEA-4, TRA-1-60 and TRA-1-81. Every marker stained positively. Different markers were stained st slightly variable degrees. Positive immunocytochemistry results indicate that these pluripotency marker proteins were generated in the cells. Oct-3/4 was most intensively stained in the retro virally induced lines but there was no considerable difference in other markers compared to the Sendai virally induced lines. The immunocytochemistry results are given in **Figure 9** for line R-05105. As there was no essential difference between lines in immunocytochemical staining, the results of immunocytochemistry for pluripotency of the other hiPSC lines are found in **Appendix 2**.

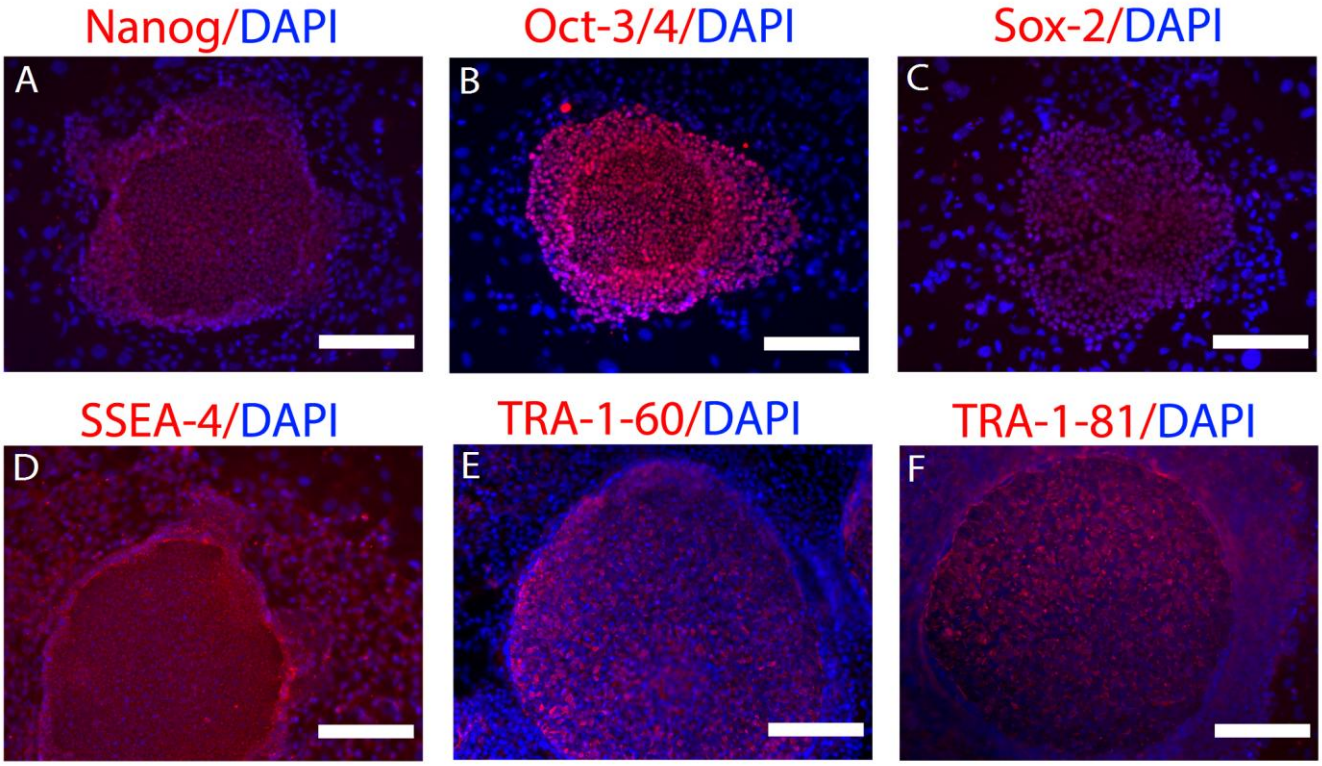


Figure 9. Immunocytochemistry of six pluripotency markers in hiPSC line R-05105. **A**) Nanog, **B**) Oct-3/4, **C**) Sox-2, **D**) SSEA-4, **E**) TRA-1-60 and **F**) TRA-1-81. DAPI was used as a counterstain to stain the nucleus. The cells around the hiPSC colonies staining only with DAPI are MEF cells. 10x magnification, scale bars 200 µm.

5.1.4 Formation of embryoid bodies

All five cell lines were evaluated for their ability to form embryoid bodies (EB) by floating cultivation technique (**Figure 10**). All cell lines were able to form embryoid bodies although some cell lines showed more ability for this (R-05105, R-00208 and S-10101). Embryoid bodies consisted of different cell types and for example beating areas and pigmented cells were seen. Cells with pigment were seen especially in Sendai lines. For the lines S-11304 and S-10311, KO-KSR without bFGF produced the highest amount of EBs and the other two medias (EB medium with rock inhibitor in the beginning and normal EB medium) resulted in high apoptosis of cells.

Presence of all three germ layers was studied at mRNA level by PCR analysis for germ layer markers. For each cell line at least one gene in each germ layer was expressed (**Figure 11** for R-05105 and S-10101, rest lines in **Appendix 1**). The results of EB formation conclude the *in vitro* pluripotency of the cell lines.

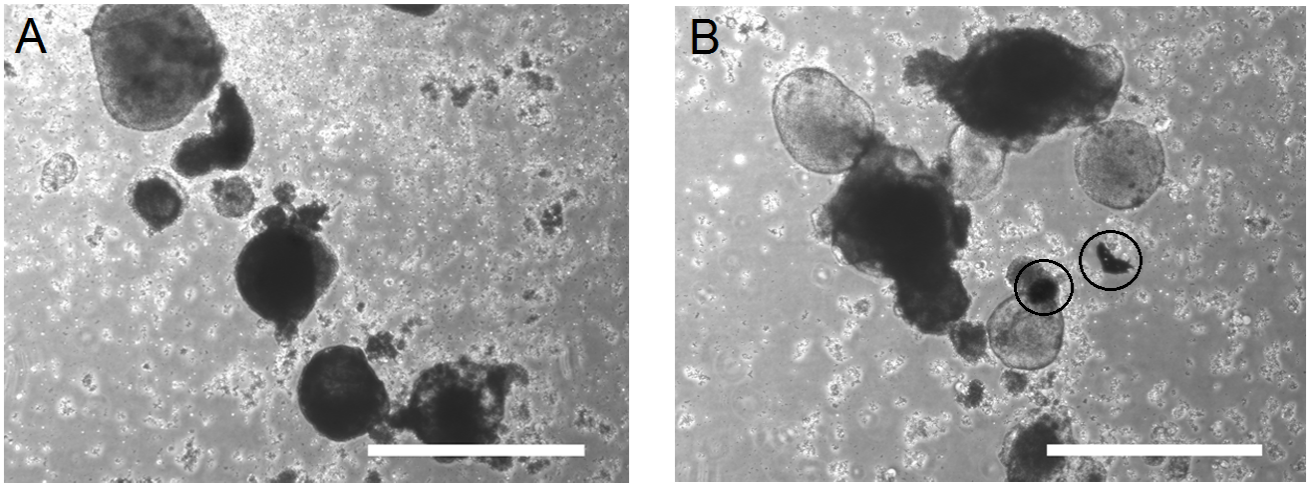


Figure 10. Images of embryoid bodies generated in the hiPSC line S-11304 at p.26. **A**) EB differentiation in KO-KSR medium and **B**) pigmented areas in EBs, circled. 4x magnification, scale bars 1 mm.

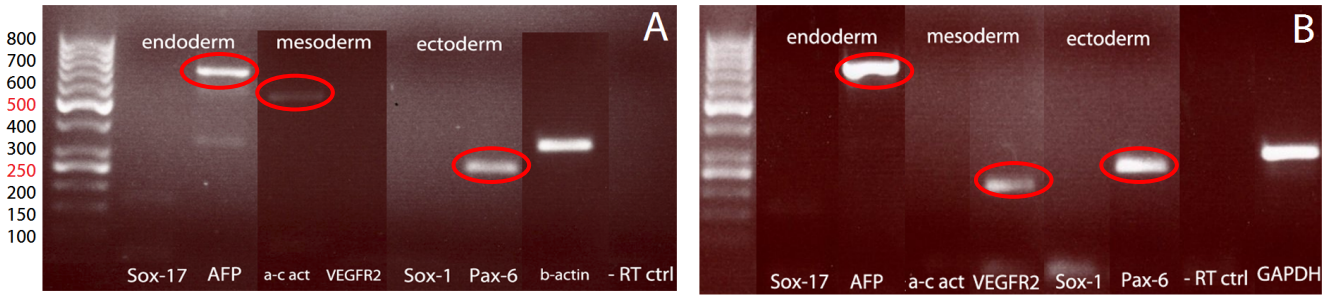


Figure 11. PCR analysis of germ layer markers in hiPSC lines R-05105 (**A**) and S-10101 (**B**). One gene of each germ layer was expressed: endoderm Sox-17 *(120 bp)* or AFP *(672 bp)*; mesoderm α-cardiac actinin (a-c act) *(486 bp)* or VEGFR2 *(218 bp)*; and ectoderm Sox-1 *(171 bp)* or Pax-6 *(274 bp)*. The positive bands represent the activity of the gene of interest, marked with a red circle. Housekeeping gene GAPDH *(302 bp)* or β-actin *(302 bp)* was used as positive control. Negative control was cDNA sample without reverse transcriptase (-RT ctrl).

5.2 Characterization of hiPSC-derived cardiomyocytes

All five hiPS cell lines were initiated for cardiac differentiation by co-culturing them with END-2 cells. Every line differentiated into spontaneously beating cell aggregates of cardiomyocytes, called beating areas. The number, size and morphology of the beating area varied between different lines, in addition to the time of the appearance of the first beating area.

5.2.1 Evaluation of cardiac differentiation

The first beating areas appeared on day 8 at earliest (line R-05105) and between days 24 and 29 at latest (line S-10101, first experiment). Retro virally induced hiPSCs formed more beating areas (57 and 130 for lines R-05105 and R-00208, respectively) than the Sendai virally induced hiPSCs (14, 18 and 2 for lines S-10101, S-11304 and S-10311 respectively). Furthermore, retro lines had higher number of beating areas per one well and total number of wells with beating areas than Sendai lines.

Different kinds of beating areas are presented in **Figure 12**. The morphology of the beating areas varied from almost round (**Figure 12B**) to oval and highly irregular (**Figure 12D, E**) The size was also variable ranging from ~30 μm single beating cells into enormous ~2700 μm areas. Retro lines differentiated into larger beating areas in general compared to Sendai lines. Thickness of the beating areas also varied from layer-like to more compact three dimensional. Appearance of cystic beating areas was more usual for Sendai lines compared to the retro lines that formed more compact beating areas consisting of single or multiple cell layers. Cystic non-beating structures filled with growth medium appeared more in Sendai lines as well.

5.2.2 Evaluation of cardiac differentiation efficiency

Cardiac differentiation efficiency was evaluated by dividing the total number of beating areas with the total number of plated colony pieces. Total number of plated colony pieces was 360 pieces since 30 pieces were plated per well in a 12-well plate. The first experiment of S-10101 was conducted on three 12-well plates, thus total number of plated colony pieces is 1080. The differentiation efficiencies are given in **Figure 13**. The beating area evaluations are summarized in **Table 2**.

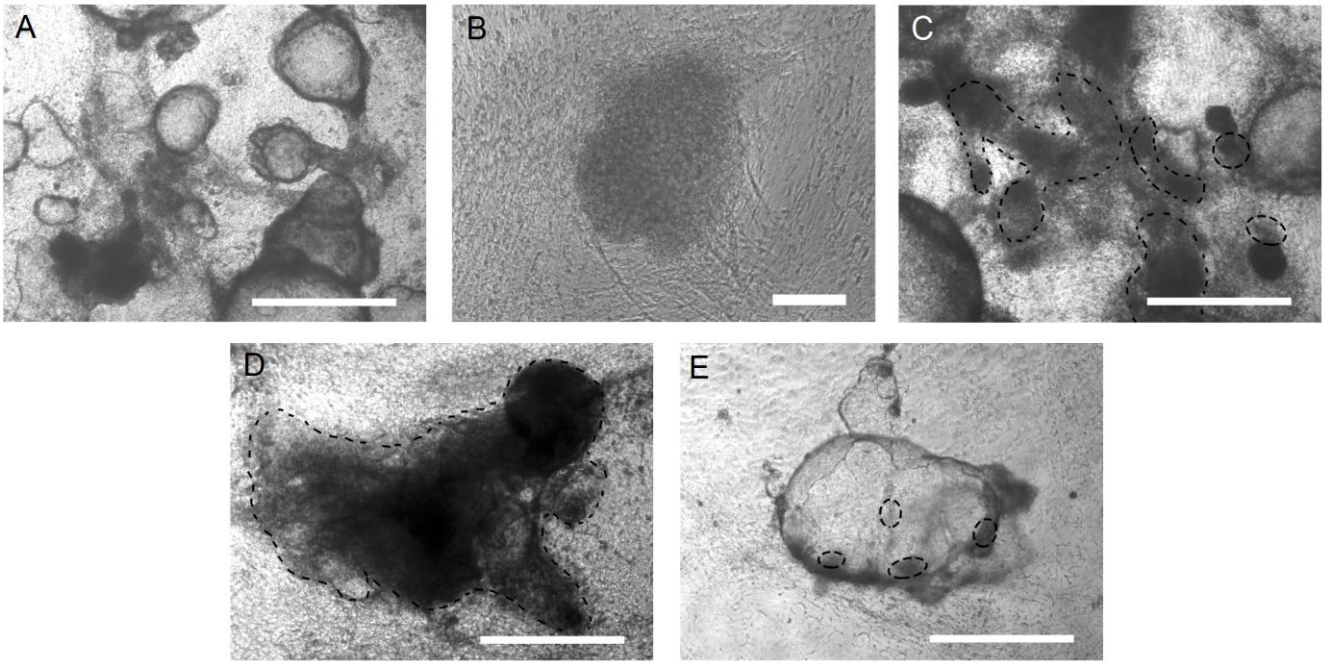


Figure 12. Images of the beating areas from hiPSC-derived cardiomyocytes. **A**) Line S-11304 on day 30. No beating areas but cysts. **B**) Line R-05105 on day 31. Single round beating area. **C**) Line R-00208 on day 30. Five beating areas of different shape and size marked with dashed lines. **D**) Line R-05105 on day 31. Single large beating area marked with a dashed line. **E**) Line S-10311 on day 30. Cyst with four pigmented areas marked with dashed lines. 4x magnification, scale bar 1 mm for (**A**), (**C**), (**D**) and (**E**). 10x magnification, scale bar 200 µm for (**B**).

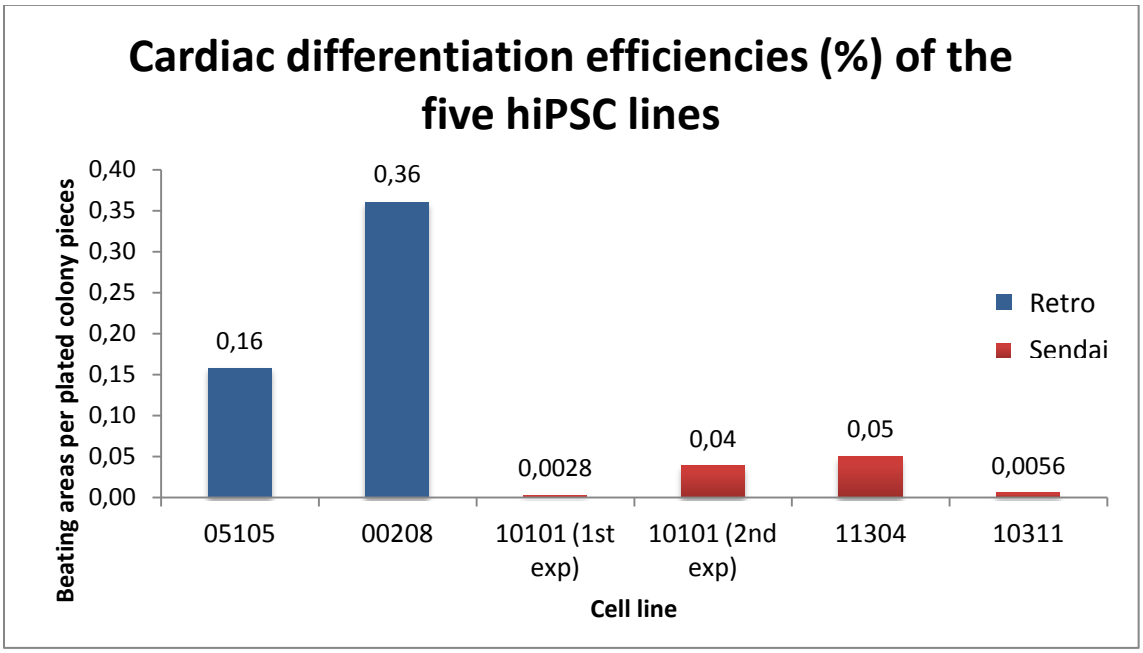


Figure 13. The cardiac differentiation efficiencies of the five hiPSC lines. The efficiencies were counted as a percentage by dividing the total number of the beating areas by the total number of plated colony pieces.

Table 2. Evaluation of the beating areas in END-2 differentiation of the five hiPS cell lines and cardiac efficiency.
** Total number of wells was 12, except 36 in 1st experiment of line S-10101*
*** Counted as total number of beating areas per total number of plated colony pieces. Plated colony pieces is 12*30=360, except 3*360=1080 in 1st experiment of line S-10101*

	Cell line	Passage	1st beating area, on day	Total no. of beating areas	No. of beating areas/well	Mean of beating areas/well	Total no. of wells with beating areas *	Differen-tiation efficiency **	Thickness (single layer = S, multiple layer = M)	Morpho-logy	Size
Retro vir. induced	**05105**	69	day 8	57	0-10	4,75	11	0,16	S + M	generally round	small, medium, large (90-2700 μm); 200-300 μm on average
	00208	46	day 17 at latest	130	1-28	10,8	12	0,36	S	round and irregular	small, medium, large (80-1900 μm); 300-400 μm on average
Sendai vir. induced	**10101 (1st exp)**	12	between days 24-29	3	1	0,083	3	0,0028	S + M	generally round	small, medium (300-400 μm); 350 μm on average
	10101 (2nd exp)	23	day 17 at latest	14	1-8	1,17	5	0,04	S + M	generally round	small, medium (50-500 μm); 200-300 μm on average
	11304	17	day 13	18	0-6	1,33	6	0,05	S	generally irregular	small, medium, large (100-1100 μm); 200-300 μm on average
	10311	21	day 17 at latest	2	0-1	0,17	2	0,0056	S	round	medium (450-900 μm)

5.2.3 Immunocytochemistry of dissociated cardiomyocytes

The dissociated cardiomyocytes of line R-05105 were immunocytochemically stained for two cardiac markers positively: Troponin T and cardiac α-actinin. Positive results indicate that these structural proteins were generated in the hiPSC-derived cardiomyocytes. **Figure 14** presents dissociated cardiomyocytes without and with immunocytochemical staining of the two cardiac markers.

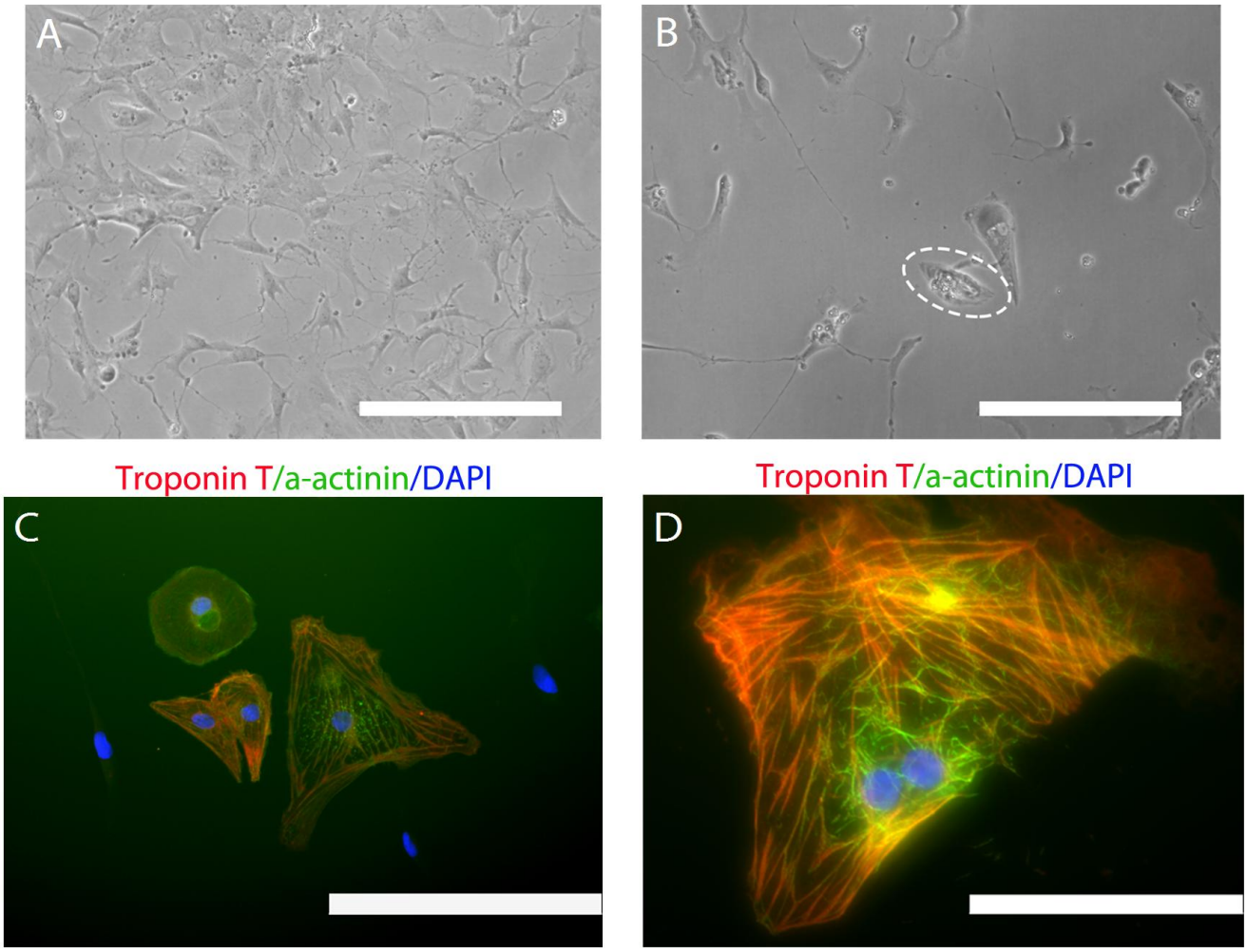


Figure 14. Images of the dissociated hiPSC-derived cardiomyocytes of line R-05105 without (**A** and **B**) and with immunocytochemical double staining (**C** and **D**) for two cardiac markers: Troponin T and cardiac α-actinin. **A**) Partially dissociated cardiomyocyte aggregates. **B**) Single dissociated cardiomyocytes. Single beating cardiomyocyte marked with a dashed circle. **C**) Cardiomyocytes of different morphology. **D**) Binucleated cardiomyocyte. 20x magnification, scale bar 200 µm for (**A**), (**B**) and (**C**), 40x magnification, scale bar 100 µm for (**D**).

5.2.4 Exogenous pluripotency gene expression in hiPSC-derived cardiomyocytes

The expression of exogenous pluripotency genes was evaluated by PCR analysis in all cell lines after cardiac differentiation. None of the exogenous pluripotency genes Oct-3/4, Klf-4, c-Myc and Sox-2 were expressed, indicating their silencing. The exception was expression of Oct-3/4 in line R-00208, indicating that cardiac differentiation did not silence the gene. **Figure 15** presents the results of exogenous PCR analysis after cardiac differentiation of all lines.

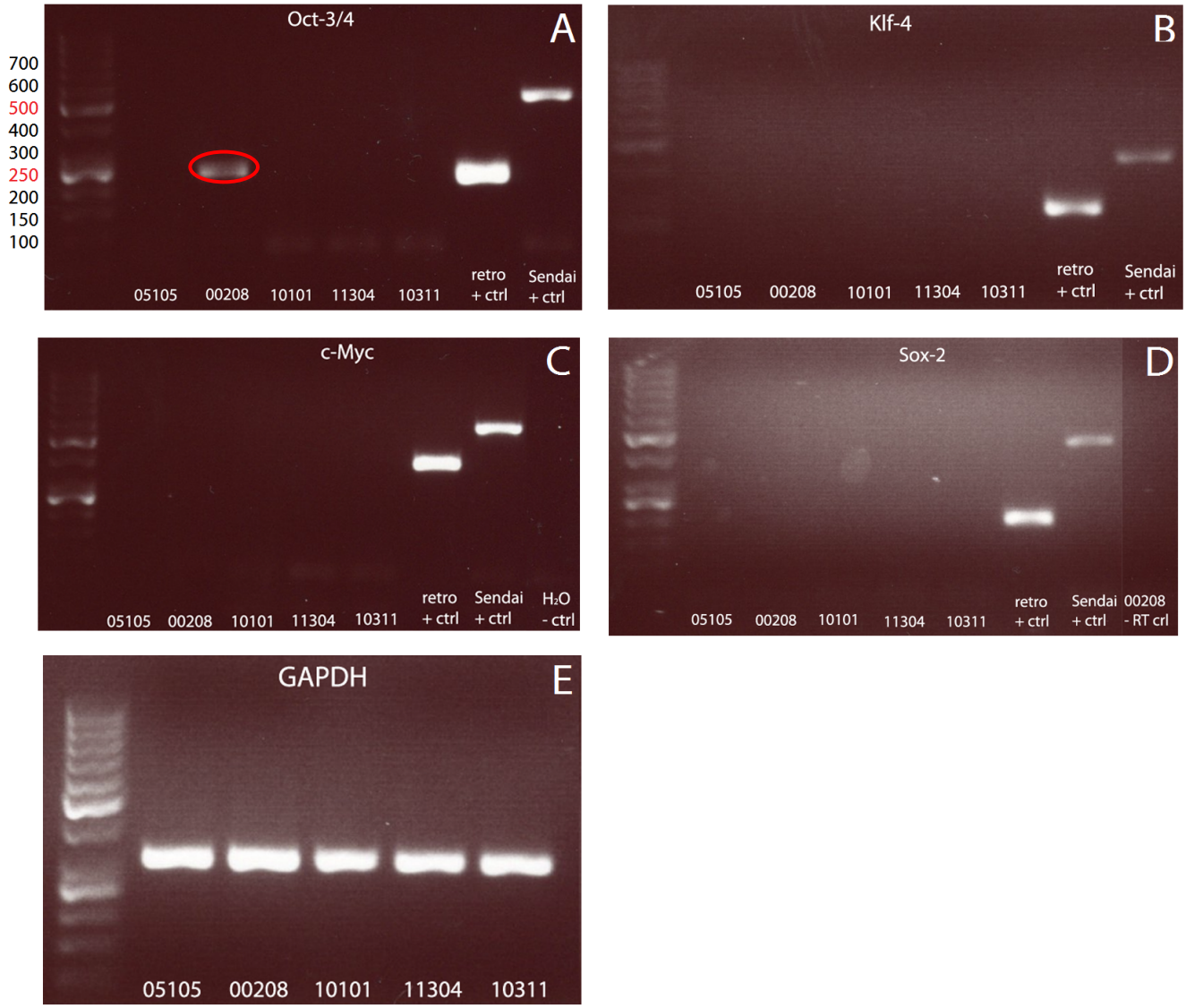


Figure 15. PCR analysis of 4 exogenous pluripotency genes in each five hiPSC-derived cardiomyocyte samples. **A**) housekeeping gene GAPDH *(302 bp)* as a positive control, **B**) Oct-3/4 *(Sendai 483 bp and retro 200-250 bp)*, where red circle marks the residual Oct-3/4 expression in line R-00208, **C**) Klf-4 *(Sendai 410 bp and retro 250-300 bp)*, **D**) c-Myc *(Sendai 532 bp and retro <400 bp)* and **E**) Sox-2 *(Sendai 451 bp and retro >200 bp)*. Positive Sendai and retro transgene controls were used, negative controls were 1) cDNA without reverse transcriptase (-RT ctrl) and 2) H_2O as a template (-H_2O ctrl).

6. DISCUSSION

Somatic cells can be converted into iPSCs by ectopic expression of transcription factors, delivered by either integrative or non-integrative vectors. Pluripotency genes are inactive in somatic cells (Cox & Rizzino, 2010). These endogenous pluripotency genes are activated during reprogramming into iPSCs. iPS cells are considered to be fully reprogrammed only when endogenous pluripotency genes are up-regulated and exogenous transgenes downregulated (Hotta and Ellis, 2008). Fully reprogrammed iPS cells are thus independent of exogenous pluripotency genes in maintaining their pluripotency. Exogenous transgenes are hypothesized to become reactivated in higher passages of hiPSCs derived by integrative methods (Toivonen et al., 2013). Residual exogenous pluripotency gene expression may affect differentiation efficiency and direction (Yamanaka and Takahashi, 2006; Toivonen et al., 2013).

The aim of this study was to evaluate the pluripotency of integrative retro virally derived hiPSCs and non-integrative Sendai virally derived hiPSCs and their cardiac differentiation efficiency. The hiPS cells were first characterized for their pluripotency by PCR for exogenous and endogenous pluripotency markers, by immunocytochemistry and embryoid body (EB) formation followed by PCR for markers of the three germ layers. Characterized hiPS cells were differentiated into cardiomyocytes, some of which were characterized by immunocytochemistry. Because of the possible reactivation of exogenous pluripotency factors, this study included analysis of the activity of exogenous pluripotency factors after cardiac differentiation as well. In addition, differentiation efficiency was evaluated between retro virally and Sendai virally derived hiPSCs.

6.1 Growth and pluripotency of hiPSCs

Three new Sendai virally derived hiPSC lines were successfully generated from patient derived skin biopsies. All the hiPSCs were derived from dermal fibroblasts. During reprogramming the fibroblasts gradually lost their typical elongated, bi- or multipolar (single cells) appearance and became small, round and grew in tightly packed colonies with defined edges. These cells are hiPSCs which showed typical ESC morphology. In general, the hiPS cells grew well in culture and were passaged once a week. Some colonies showed some sign of differentiation to some extent. Differentiating colonies varied in their density, which was seen as paler or darker areas in the middle of the colony. They also lost the defined edges and could detach from the plate, which was seen as multi-layered 3D-structures. Cells with increased passage number (p. 20 or higher) could grow faster and differentiate

spontaneously more easily. Moreover, overconfluency of colonies could increase the number of differentiating colonies.

This study showed the pluripotency of the hiPSCs by PCR and immunocytochemistry for the expression of pluripotency associated genes and generation of pluripotency associated proteins. All hiPSC lines showed activity of endogenous pluripotency genes Oct-3/4, Sox-2, Nanog, c-Myc and Rex-1. Oct-3/4, Sox-2 and Nanog have been identified as master regulators of pluripotency (Boyer et al., 2005). Rex-1 (Reduced Expression 1) encodes for a zinc finger family transcription family member, which is highly expressed in mouse and human ES cells and is a marker of pluripotency (Zhao et al., 2012). These results indicate that reprogramming had successfully activated the endogenous core pluripotency genes. In addition, all six pluripotency markers Nanog, Oct-3/4, SSEA-4, Sox-2, TRA-1-60 and TRA-1-81 were immunocytochemically demonstrated to be generated in each of the hiPS cell line. SSEA-4, TRA-1-60 and TRA-1-81 are hES cell-specific surface antigens and Nanog, Oct-3/4 and Sox-2 are hES cell-specific proteins (Takahashi et al., 2007).

The results of PCR analysis of exogenous pluripotency genes proved that reprogramming process had successfully silenced the exogenous pluripotency genes Oct-3/4, Klf-4, c-Myc and Sox-2, except exogenous Oct-3/4 was expressed in line R-00208. Transgenes are demonstrated to be silenced in late steps of reprogramming (Stadtfeld et al., 2008a). Expression of retro viral transgene Oct-3/4 might indicate that the line R-00208 is only partially reprogrammed. However, partially reprogrammed iPS cells fail to fully activate the expression of endogenous pluripotency genes and pluripotency is maintained by exogene expression (Hotta and Ellis, 2008). Yet, all the core endogenous pluripotency genes were studied to be activated in line R-00208. Expression of exogenous Oct-3/4 was found at passage 40 in this line. However, no exogenous Oct-3/4 activity had been encountered when the line was once characterized at passage 10. As this gene had been already shown silenced at lower passage, it could be concluded that Oct-3/4 was reactivated during culturing into high passage. This finding of the study is in line with the hypothesis of exogene activation during extended culturing by Toivonen et al. (2013). The mechanism of transgene reactivation is not understood yet. Analysis of the epigenetic status of the pluripotency promoters by bisulfite genomic sequencing and/or chromatin immunoprecipitation (Takahashi et al., 2007) could be beneficial. It might explain whether incomplete silencing of Oct-3/4 would be due to incomplete histone modification and DNA methylation.

All the lines were confirmed to be pluripotent *in vitro* by successful EB formation. Although the expression of germ layer markers was quite low in some cases, at least one marker of each germ layer was expressed in each cell line. Expression of markers of each germ layer proves that these cells are capable of differentiating towards all three germ layers: mesoderm, endoderm and ectoderm. In addition, pluripotency could be evaluated *in vivo* by teratoma formation in immunodeficient mice or generation of chimeric mice (Hotta and Ellis, 2008). However, performing teratoma test could not fit in with the timescale of this study but might be performed later.

6.2 Differentiation of hiPSCs into cardiomyocytes and characterization

Each of the hiPSC lines were successfully differentiated into spontaneously beating cell aggregates called beating areas when co-cultured with END-2 cells. Cardiomyocytes are of mesodermal origin (Rajala et al., 2011).

First beating area usually appears between days 8 to 14, most areas before day 21 but new beating areas may appear until day 30. In this study the first beating area of line R-05105 appeared therefore rather early (day 8). First beating areas for the other lines appeared in usual time period (between days 13-17). The first experiment of line S-10101 showed however prolonged differentiation (first beating area between days 24-29). Changing cell fate from iPSC back to a somatic cell requires switching off the pluripotency gene pattern and switching on the tissue-specific somatic cell gene pattern. This requires epigenetic remodeling and control. Successful cardiac differentiation *in vitro* requires a complex signaling network (Rajala et al., 2011). Prolonged cardiac differentiation might be due to impeded signaling at a certain point of the process. Expression analysis of cardiac specific markers during differentiation might shed a light of the differentiation process and the steps in which it is impeded. Indeed, it has been demonstrated that individual hiPSC lines might require different times for cardiac differentiation (Ohno et al., 2013). Expression of mesodermal and cardiac-specific markers is decreased and delayed in those lines showing delayed cardiac differentiation.

The amount, size and morphology of the formed beating areas varied between different hiPSC lines. Retro lines produced much more beating areas than Sendai lines. There was no notifiable difference in the morphology of the beating areas between Sendai lines and retro lines. Beating areas were round, elliptic and irregular. Retro lines formed the largest beating areas (~2700 μm and ~1900 μm), but also S-11304 differentiated into a large beating area (~1100 μm). Indeed, END-2 protocol has been

demonstrated to generate beating areas of different morphology and size (Mummery et al., 2003), which was the case in this study as well. Nevertheless, on average the sizes were quite equal between lines. Every hiPSC line formed fluid filled cysts on END-2 cells. These kinds of structures are presumed to be epithelial cells derived from extraembryonic visceral endoderm as they stain positively for α-fetoprotein (AFP) (Mummery et al., 2003). The differentiation of Sendai lines resulted in more of these cystic structures that contained only few cells. However, beating areas could be found under these structures. The function of these structures is not well known.

END-2 differentiation is more based on spontaneous differentiation and results in heterogeneous differentiation (Mummery et al., 2003). This method has been shown to induce not only cardiomyocytes but also cell types of other germ layers. Whole-genome microarray analysis of hESCs during END-2 differentiation showed that co-cultures differentiate predominantly toward mesodermal and endodermal tissues, with some ectodermal differentiation taking place (Beqqali et al., 2006). The temporal gene expression was highest for heart-specific genes (62 %; mesoderm) but many other tissue-specific genes were expressed as well: liver (58 %; endoderm), kidney (47 %; mesoderm), uterus (32 %; mesoderm), lung (30 %; endoderm), vascular (30 %; mesoderm), pancreatic islet (27 %; endoderm), colon (26 %; endoderm), testis (14 %; mesoderm), stomach (13 %; mesoderm), brain (12 %; ectoderm) and skin (11 %; ectoderm). In this study, lines S-11304 and S-10311 differentiated rather poorly into cardiomyocytes but more numerously into cells with pigment compared to retro lines. These pigmented cells were hypothesized to be retinal pigment epithelium cells (RPE) because of the pigment, but they were not studied in more detail. Low-passage (below 12) iPSCs have been demonstrated to show residual DNA methylation patterns that are characteristic to their somatic tissue of origin (epigenetic memory; Kim et al., 2010). This would favor their differentiation along the lineages related to the donor cell and restrict other lineages. However, in this study the hiPSCs were derived from patient skin fibroblasts. Dermal fibroblasts are derived from mesenchymal stem cells, which are of mesodermal origin (Ratajczak et al., 2008). RPE cells derive from ectoderm (Toivonen et al., 2013) and therefore epigenetic memory would not explain the direction of differentiation in this study. Surprisingly many pigmented cells appeared in EB differentiation of these lines as well.

The beating areas of R-05105 were dissociated into individual cardiomyocytes, many of which maintained their beating ability after dissociation. The immunocytochemical staining of typical cardiac markers Troponin T and α-cardiac actinin was positive. When studying the germ layer gene expression

from the EBs of this line, α-cardiac actinin was also the marker expressed in mesoderm. Troponin T and α-actinin are both important structural proteins of cardiomyocytes (Rajala et al., 2011). Troponin T is a part of Troponin complex that regulates the contraction of heart muscle. α-actinin is a protein of the sarcomer, the contracting unit of a cardiomyocyte (Rajala et al., 2011). As many dissociated cardiomyocytes maintained their beating ability, it already indicated positive results in the immunocytochemical staining. The immunocytochemistry results proved that hiPSC-derived cardiomyocytes had typical structure required for their function.

6.2.1 Cardiac differentiation efficiency

In this study retro virally derived hiPS cells had higher cardiac differentiation efficiency (R-05105 0,16 % and R-00208 0,36 %) compared to Sendai virally derived hiPSCs (S-10101 0,04 %; S-11304 0,05 %; S-10311 0,0056 %). iPSCs have been shown to resemble ESCs gradually more when maturing *in vitro* (Chin et al., 2009). Thus their differentiation potential might improve during extended culture. Maturing might explain partially why retro lines (late-passage 46 or 69) differentiated more efficiently than Sendai lines (middle-passage 17, 21 or 23). The first experiment of S-10101 had low efficiency (total of three beating areas in three plates; 0,0028 %) and it was hypothesized that it might be due to the early passage of the line (p. 12) when differentiation was initiated. However, second experiment (p. 23) was not essentially more efficient. On the other hand, S-10101 was at middle passage and extended culturing into late passage might improve the differentiation efficiency. In fact, the first differentiation experiment of S-10101 showed prolonged cardiac differentiation but the second was in normal range. Therefore some enhancement in differentiation signaling could have been taken place in the cells. Enhanced cell signaling could be due to epigenetic or genetic changes during the hiPSC proliferation between different differentiation experiments.

END-2 differentiation method has been found to be the most robust, but not the most efficient for hiPSCs (Rajala et al., 2011). Therefore other differentiation methods could be initiated for these studied hiPSC lines, as the same method might not be the most suitable and efficient to every line. Some lines may be more prone to differentiate in certain direction (Toivonen et al., 2013) as might be the case with the Sendai lines in this study. As END-2 differentiation is based more on spontaneous differentiation, the genetic profile of the cells has more effect on differentiation compared to more directed differentiation methods. Therefore more targeted differentiation method such as guided differentiation with defined factors might enhance the efficiency. It has been found that there is natural difference

between different hiPS cell lines in genetic and epigenetic profile (Bayart and Cohen-Haguenauer, 2013; Toivonen et al., 2013), that possibly affects the behavior and differentiation to different lineages. Indeed, it has been demonstrated that different iPSC lines show different cardiac differentiation efficiencies (Ohno et al., 2013; Toivonen et al., 2013). Moreover, different differentiation experiments from the same hiPSC line have demonstrated differences in differentiation efficiency (Toivonen et al., 2013), which was the situation in this study as well.

Cardiac differentiation efficiency was calculated as a percentage: the total number of beating areas divided with the total number of plated hiPSC colonies. Another method for the evaluating the cardiac differentiation efficiency is quantitative immunocytochemical analysis of cytospin samples for Troponin T positivity. In this analysis the percentage (or number) of Troponin T positive cells is determined versus the total cell number (Toivonen et al., 2013). Troponin T is a cardiac marker. In future studies with these cells it would be interesting to count the Troponin T positive cells versus the total number of cells, since many colonies of the Sendai lines never attached and also many detached during the differentiation process. In addition, EB differentiation of Sendai lines resulted in lots of dead cells. Detaching was not a serious problem for the retro lines, but some detaching was encountered with them as well. However, the commonly used immunocytochemical counterstain DAPI stains both live and dead cells. Therefore another counterstain should be used. Another option would be to determine Troponin T positive cells versus END-2 cells. END-2 cells would be stained for some END-2 specific marker. This kind of analysis might show higher efficiency for the Sendai lines compared to counting the beating areas, as END-2 cells were dominating in many wells of Sendai line co-cultures.

6.2.2 Exogenous pluripotency gene expression

It would have been expected that exogenous pluripotency gene activity interferes with differentiation resulting in poor efficiency (Toivonen et al., 2013). However, in this study line R-00208 with exogene Oct-3/4 being expressed showed the highest cardiac differentiation efficiency out of the five lines studied. It might be possible that hiPSC population was heterogeneous in the line R-00208 and it was consisting of cells with higher, lower and no exogenous Oct-3/4 activity. The cells with lower or no Oct-3/4 activity might have been the ones to differentiate. The mechanisms of transgene reactivation are not well known.

Ohno et al. (2013) suggested that high quality iPSCs show higher cardiac differentiation efficiency than low quality iPSCs, which hardly differentiate. High quality iPSCs would succeed in silencing the transgenes and low quality cells would still express transgenes resulting in lower cardiac differentiation efficiency. However, it was not clear in the study by Ohno et al. (2013) whether the residual transgene expression affected directly to cardiac differentiation or whether residual transgene expression was a marker of low-quality iPSCs that showed low differentiation efficiency. The different iPSC lines that they studied expressed different exogenes but the ones expressing Klf-4 showed lowest differentiation efficiency. Therefore residual expression of Klf-4 might have a negative effect on cardiac differentiation (Ohno et al., 2013). However, in this study Sendai lines showed low cardiac differentiation efficiency even though all exogenes were successfully silenced and endogenes activated, which would indicate their full reprogramming state. Moreover, in this study the most efficient line to differentiate into cardiomyocytes was the only one (R-00208) with residual exogene expression (Oct-3/4). This finding would suggest that residual transgene expression of Oct-3/4 in fact could enhance the cardiac differentiation efficiency.

Critical amount of Oct-3/4 is needed for ESC pluripotency maintenance. In addition, Oct-3/4 controls lineage commitment as twofold increase in expression initiates differentiation into primitive endoderm and mesoderm and repression induces dedifferentiation to trophectoderm (Niwa et al., 2000). Some studies have reported that Oct-3/4 suppresses *in vitro* differentiation into neuroectoderm and definitive endoderm but Radzisheuskaya et al. (2013) disagree with this suppressing role of Oct-3/4 in differentiation, as they found constitutive expression of Oct-3/4 at ESC level contribute to differentiation into all germ layers. This dualistic role of Oct-3/4 in pluripotency maintenance and lineage commitment might explain partially the high cardiac differentiation efficiency of R-00208. The expression of endogenous Oct-3/4 was quite equal in all hiPSC lines, and the high Oct-3/4 expression in line R-00208 was due to the persistence of exogenous Oct-3/4 expression.

It was shown in this study that cardiac differentiation process did not switch off the transgene expression of Oct-3/4 in line R-00208. Endogenous Oct-3/4 expression has been found to progressively decrease during EB differentiation (Yamanaka and Takahashi, 2006) and END-2 differentiation (Ojala et al., 2012). However, reactivation of exogenous Oct-3/4 expression has been detected in RPE differentiation (Toivonen et al., 2013). It would be interesting to study whether there was any change in the level of expression of Oct-3/4 during cardiac differentiation of the line R-00208.

6.3 Future perspectives

As only five hiPSC lines altogether were studied, no general conclusions about behavior of hiPSCs and their differentiation can be made. To gain statistically relevant information on differentiation of these hiPSC lines, more differentiation experiments would need to be carried out. Parallel differentiation from the same hiPSC line in different passages (low, intermediate, high) would be required to understand the effect of iPSC maturing. This would require same passages for retro and Sendai virally derived cells. In this study it was relevant to study high-passage retro virally derived iPSCs due to the transgene activation during culturing, but also lower retro and higher Sendai virally derived lines would be reasonable to study. As different iPSC lines have been demonstrated to be variable in their epigenetic and genetic profile, each iPSC line may need a differentiation protocol of their own, as suggested already by Rajala et al. (2011).

Various reprogramming methods have been demonstrated during the eight years since the discovery of iPSC technology. In order to evaluate the effect of reprogramming method on differentiation, it would be interesting to study hiPS cell lines induced with different protocols and derived from same patients. Integrative reprogramming methods (retro virus as the most important of them) have been more efficient in cell conversion than non-integrative reprogramming methods but Sendai virus has been a promising method with rather a high efficiency (Bayart and Cohen-Haguenauer, 2013). Therefore it may be difficult to synchronize the establishment of hiPSC lines with Sendai and retro virus for parallel differentiation experiments at same passages. The reprogramming method might not affect directly in differentiation but indirectly by efficiently generating fully reprogrammed hiPSC lines. The natural variability between different cell lines in their epigenetic and genetic profile might be the most important factor affecting the differentiation efficiency and direction.

The original purpose of this study was to differentiate these hiPSCs also into hepatocytes that derive from endoderm (Ratajczak et al., 2008). Cardiomyocytes derive from mesoderm. Hepatocyte differentiation would have given information on whether these hiPSCs are more prone to differentiate into certain lineage. Differentiation into cells derived from ectoderm would be interesting as well. For example retinal and neural cells are derived from ectoderm, and two of the Sendai lines S-11304 and S-10311 were differentiating into pigmented cells surprisingly well with both END-2 and EB differentiation protocols. These cells were suggested to be retinal pigmented epithelial, RPE cells that derive from ectoderm. Furthermore, differentiating the cells into lineages originating from each germ

layer would show whether transgene activity of the line R-00208 would affect the efficiency and direction of differentiation.

As majority (76 %) of the current iPSC lines are retro virally derived lines (Bellin et al., 2012), exogenous pluripotency gene activity might be a hidden problem interfering differentiation of these iPSC lines, especially if the pluripotency of the lines is not regularly confirmed. This hidden exogene activity was shown in this study. Reactivation of exogenes encountered in this study shows the unpredictability of the iPSCs. The integration free methods have shown no transgene activity. For disease modeling and therapeutic screens, absence of genetic modifications is not an absolute requirement but genetically stable iPS cells are required in clinical cell therapy use (Bayart and Cohen-Haguenauer, 2013). Whole-genome sequencing and epigenome screening might be needed to validate safe iPS cells for their transcriptional and epigenetic status, genomic integrity and stability, differentiation ability and tumorigenesis potential.

It would be interesting to study hiPSC-derived cardiomyocytes by patch clamp or micro electrode array measurements to see whether transgene activity affects the functionality of the cardiomyocytes. However, cell lines with same characteristics should be selected for this purpose. The lines in this study were derived from patients with different diseases and therefore with different mutations. Different mutations might affect the electrophysiological properties of the hiPSC-derived cardiomyocytes were different.

7. CONCLUSIONS

The aim of this study was to evaluate the pluripotency of retro and Sendai virally reprogrammed hiPSCs and to evaluate the cardiac differentiation efficiency of these hiPS cells in consideration of exogenous pluripotency activity. Residual exogene activity was hypothesized to interfere with cardiac differentiation resulting in lower differentiation efficiency.

In this study three new Sendai virally induced hiPSC lines were successfully generated. All Sendai and retro virally induced hiPSC lines showed pluripotency at gene expression and protein level, in addition to the ability to produce EBs. However, it was shown that exogenous pluripotency marker Oct-3/4 had been activated in one retro virally derived hiPSC line R-00208 during culturing to high passage (p. 40). Sendai virally induced hiPSCs showed no transgene activity as expected.

All five hiPS cell lines could successfully differentiate into cardiomyocytes with END-2 protocol. However, the efficiency was dramatically different between individual cell lines. Retro virally reprogrammed cell lines showed better cardiac differentiation efficiency than Sendai virally reprogrammed lines. Even though exogenous Oct-3/4 was found reactivated in one retro virally induced line R-00208, it did not negatively affect its cardiac differentiation. In fact, this line showed the highest efficiency. This suggests that activity of exogenous Oct-3/4 might actually enhance the cardiac differentiation. In addition, the PCR analyses showed that exogenous Oct-3/4 did not turn off during cardiac differentiation.

In order to study the effect of residual transgene activity to differentiation, more differentiation experiments of different lines in many passages would need to be conducted. Relative gene expression studies by qPCR would give more detailed information on exogene activity level and whether differentiation might up- or downregulate exogene activity. The mechanisms behind the reprogramming process are not well known and especially late reprogramming events including transgene silencing remain to be determined. The role of residual exogene activity in differentiation process is another interesting but poorly understood field of study. Transgene free iPSCs generated by non-integrative reprogramming methods should however be preferred. The most viable option would be to identify the methods of how to avoid transgene reactivation. The mechanisms behind the dualistic role of Oct-3/4 in self-renewal and lineage commitment need to be discovered as well.

8. REFERENCES

Bellin M, Marchetto MC, Gage FH, Mummery CL. 2012. Induced pluripotent stem cells: The new patient? Nature Reviews Molecular Cell Biology 13(11):713-26.

Beqqali A, Kloots J, Ward-van Oostwaard D, Mummery C, Passier R. 2006. Genome-Wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells 24(8):1956-67.

Boué S, Paramonov I, Barrero MJ, Belmonte JCI. 2010. Analysis of human and mouse reprogramming of somatic cells to induced pluripotent stem cells. what is in the plate? PLoS One 5(9):e12664.

Boyer LA, Lee TI, Cole MF et al. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122(6):947-56.

Chang C, Lai Y, Pawlik KM, et al. 2009. Polycistronic lentiviral vector for "hit and run" reprogramming of adult skin fibroblasts to induced pluripotent stem cells. Stem Cells 27(5):1042-9.

Chin MH, Mason MJ, Xie W et al. 2009. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell 5(1):111-23.

Djuric U and Ellis J. 2010. Epigenetics of induced pluripotency, the seven-headed dragon. Stem Cell Res Ther 1(1):3.

Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. 2009. Efficient induction of transgene-free human pluripotent stem cells using a vector based on sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 85(8):348-62.

Greenow K and Clarke AR. 2012. Controlling the stem cell compartment and regeneration in vivo: The role of pluripotency pathways. Physiol Rev 92(1):75.

Griesenbach U, Inoue M, Hasegawa M, Alton EW. 2005. Sendai virus for gene therapy and vaccination. Curr Opin Mol Ther 7(4):346-52.

Hotta A and Ellis J. 2008. Retroviral vector silencing during iPS cell induction: An epigenetic beacon that signals distinct pluripotent states. J Cell Biochem 105(4):940-8.

Hou P, Li Y, Zhang X et al. 2013. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341(6146):651-4.

Ieda M, Fu J, Delgado-Olguin P et al. 2010. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 142(3):375-86.

Itskovitz-Eldor J, Schuldiner M, Karsenti D et al. 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6(2):88-95.

Kawamura T, Suzuki J, Wang YV et al. 2009. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 460(7259):1140-4.

Kehat I, Kenyagin-Karsenti D, Snir M et al. 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108(3):407-14.

Kim JB, Zaehres H, Wu G, Gentile et al. 2008. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454(7204):646-50.

Kim K, Doi A, Wen B et al. 2010. Epigenetic memory in induced pluripotent stem cells. Nature 467(7313):285-90.

Kim D, Kim CH, Moon JI et al. 2009. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4(6):472-6.

Laflamme MA, Chen KY, Naumova AV et al. 2007. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25(9):1015-24.

Lewitzky M and Yamanaka S. 2007. Reprogramming somatic cells towards pluripotency by defined factors. Curr Opin Biotechnol 18(5):467-73.

Lin T, Chao C, Saito S et al. 2005. p53 induces differentiation of mouse embryonic stem cells by suppressing nanog expression. Nat Cell Biol 7(2):165-71.

Menendez S, Camus S, Izpisua Belmonte JC. 2010. p53: guardian of reprogramming. Cell Cycle 9(19):3887-91.

Mikkelsen TS, Hanna J, Zhang X et al. 2008. Dissecting direct reprogramming through integrative genomic analysis. Nature 454(7200):49-55.

Miyoshi N, Ishii H, Nagano H et al. 2011. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8(6):633-8.

Mummery CL, Zhang J, Ng ES, Elliott DA, Elefanty AG, Kamp TJ. 2012. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: A methods overview. Circ Res 111(3):344-58.

Obokata H, Wakayama T, Sasai Y et al. 2014. Stimulus-triggered fate conversion of somatic cells into pluripotency. Nature 505(7485):641-7.

Ohi Y, Qin H, Hong C et al. 2011. Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. Nat Cell Biol 13(5):541-9.

Ohno Y, Yuasa S, Egashira T et al. 2013. Distinct iPS cells show different cardiac differentiation efficiency. Stem Cells International 2013.

Ojala M, Rajala K, Pekkanen-Mattila M, Miettinen M, Huhtala H, Aalto-Setälä K. 2012. Culture conditions affect cardiac differentiation potential of human pluripotent stem cells. PloS One 7(10):e48659.

Okita K, Ichisaka T, Yamanaka S. 2007. Generation of germline-competent induced pluripotent stem cells. Nature 448(7151):313-7.

Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. 2008. Generation of mouse induced pluripotent stem cells without viral vectors. Science 322(5903):949-53.

Passier R, Oostwaard DW, Snapper J et al. 2005. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 23(6):772-80.

Radzisheuskaya A, Chia GLB, dos Santos RL et al. 2013. A defined Oct4 level governs cell state transitions of pluripotency entry and differentiation into all embryonic lineages. Nat Cell Biol 15(6):579-90.

Rajala K, Pekkanen-Mattila M, Aalto-Setala K. 2011. Cardiac differentiation of pluripotent stem cells. Stem Cells Int 2011:383709.

Ratajczak MZ, Zuba-Surma EK, Wysoczynski M et al. 2008. Hunt for pluripotent stem cell–regenerative medicine search for almighty cell. J Autoimmun 30(3):151-62.

Robinton DA and Daley GQ. 2012. The promise of induced pluripotent stem cells in research and therapy. Nature 481(7381):295-305.

Rowland BD, Bernards R, Peeper DS. 2005. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat Cell Biol 7(11):1074-82.

Ruiz S, Panopoulos AD, Herrerías A et al. 2011. A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity. Current Biology 21(1):45-52.

Soldner F, Hockemeyer D, Beard C et al. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell 136(5):964-77.

Stadtfeld M, Maherali N, Breault DT, Hochedlinger K. 2008a. Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell 2(3):230-40.

Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. 2008b. Induced pluripotent stem cells generated without viral integration. Science 322(5903):945-9.

Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T. 2001. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Current Biology 11(19):1553-8.

Takahashi K and Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663-76.

Takahashi K, Tanabe K, Ohnuki M et al. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861-72.

Takahashi T, Lord B, Schulze PC et al. 2003. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 107(14):1912-6.

Toivonen S, Ojala M, Hyysalo A et al. 2013. Comparative analysis of targeted differentiation of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells reveals variability associated with incomplete transgene silencing in retrovirally derived hiPSC lines. Stem Cells Transl Med 2(2):83-93.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810-3.

Woltjen K, Michael IP, Mohseni P et al. 2009. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458(7239):766-70.

Xu C, Police S, Hassanipour M, Gold JD. 2006. Cardiac bodies: A novel culture method for enrichment of cardiomyocytes derived from human embryonic stem cells. Stem Cells and Development 15(5):631-9.

Yakubov E, Rechavi G, Rozenblatt S, Givol D. 2010. Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. Biochem Biophys Res Commun 394(1):189-93.

Yu J, Hu K, Smuga-Otto K et al. 2009. Human induced pluripotent stem cells free of vector and transgene sequences. Science 324(5928):797-801.

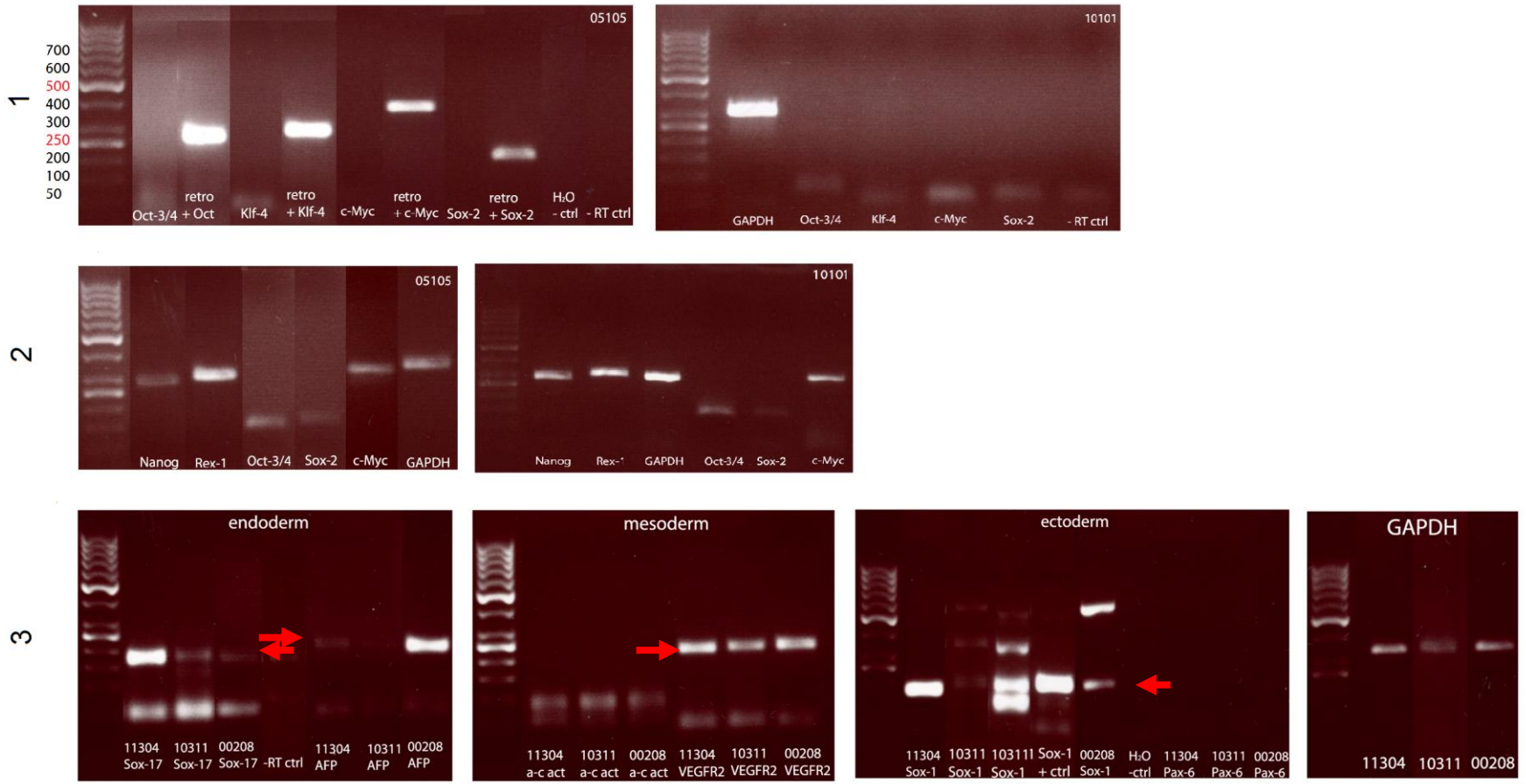
Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858):1917-20.

Zhang J, Wilson GF, Soerens AG et al. 2009. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 104(4):e30-41.

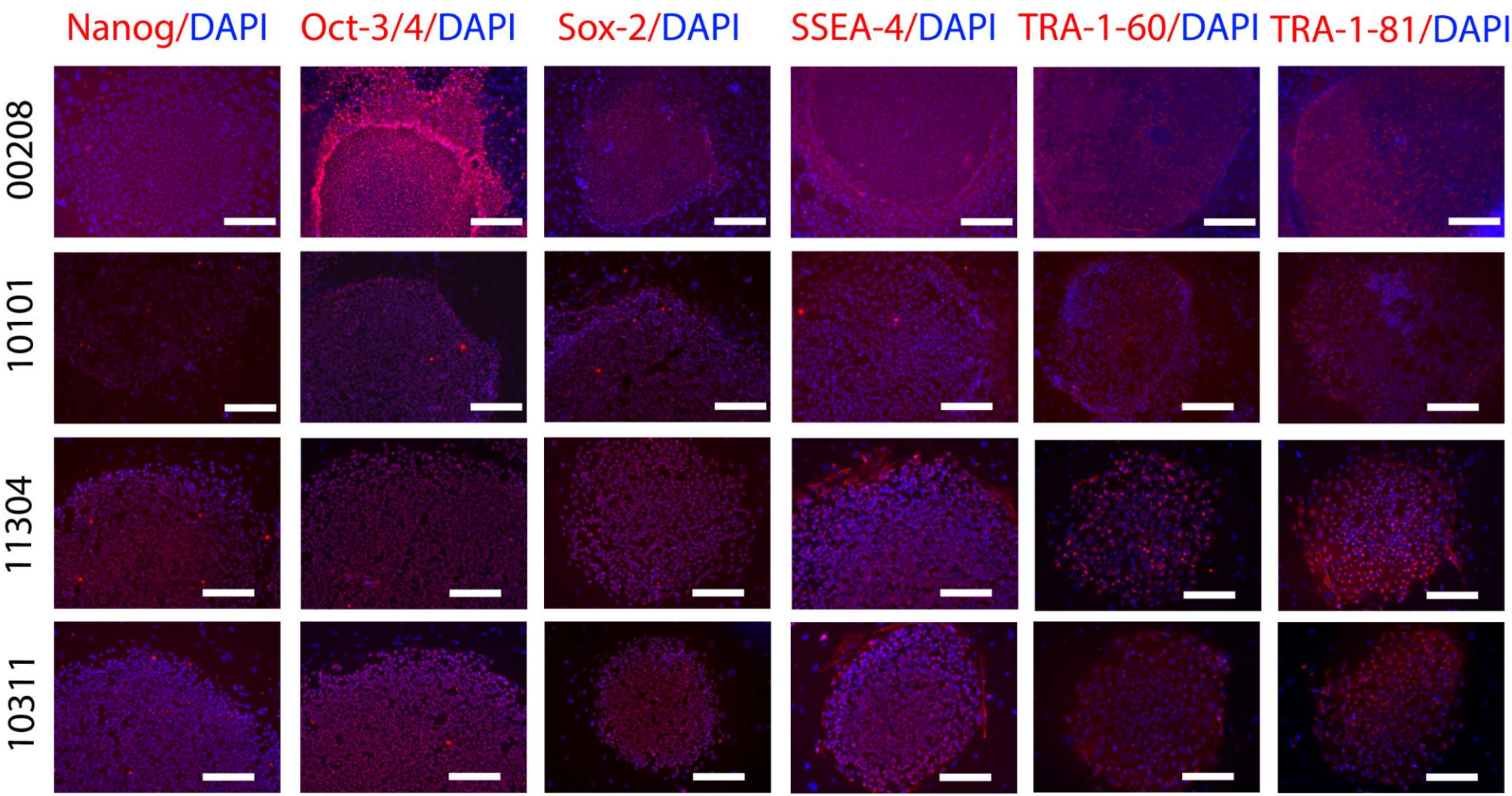
Zhao W, Ji X, Zhang F, Li L, Ma L. 2012. Embryonic stem cell markers. Molecules 17(6):6196-236.

Zhou W and Freed CR. 2009. Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27(11):2667-74.

APPENDICES



Appendix 1. PCR analysis of **1**) exogenous and **2**) endogenous pluripotency genes in hiPSC lines R-05105 and S-10101; and **3**) germ layer markers in hiPSC lines S-11304, S-10311 and R-00208. The positive bands represent the activity of the gene of interest in **2**) and **3**) and red arrow indicates the correct size in **3**). The genes are GAPDH *(302 bp)* and **1**) exogenous markers Oct-3/4 *(Sendai 483 bp and retro 200-250 bp)*, Klf-4 *(Sendai 410 bp and retro 250-300 bp)*, c-Myc *(Sendai 532 bp and retro <400 bp)* and Sox-2 *(Sendai 451 bp and retro >200 bp)*; **2**) endogenous markers Nanog *(287 bp)*, Rex-1 *(306 bp)*, Oct-3/4 *(144 bp)*, Sox-2 *(151 bp)*, and c-Myc *(328 bp)* and **3**) germ layer markers endoderm Sox-17 *(120 bp)* and AFP *(209 bp*); mesoderm α-cardiac actinin *(a-c act, 486 bp)* and VEGFR2 *(218 bp*); and ectoderm Sox-1 *(171 bp)* and Pax-6 *(274 bp*).



Appendix 2. Immunocytochemistry for pluripotency markers (Nanog, Oct-3/4, Sox-2, SSEA-4, TRA-1-60 and TRA-1-81) in hiPSC lines R-00208, S-10101, S-11304 and S-10311. 10x magnification, scale bars 200 µm.