Derivation of factor-free human induced pluripotent stem cells by Cre protein transduction and their differentiation towards cardiogenic fates

Thesis

Submitted for a Doctoral Degree in Natural Sciences

(Dr. rer. nat.)

Faculty of Mathematics and Natural Sciences

Rheinische Friedrich Wilhelms Universität Bonn

by

Asifiqbal Kadari

from Dholka, India

Bonn 2014

Prepared with the consent from the Faculty of Mathematics and Natural Sciences Rheinische Friedrich Wilhelms Universität, Bonn.

This thesis is available online on the Hochschulschriftenserver of the ULB Bonn http://hss.ulb.uni-bonn.de/diss_online.

1. Reviewer: Prof. Dr. Frank Edenhofer
2. Reviewer: Prof. Dr. Hubert Schorle

Date of examination: 23.01.2015

Publication year: 2015

INDEX OF ABBREVIATIONS

%	Percentage
°C	Degree celsius
AP	Action potential
bp	Base pair
BSA	Bovine serum albumin
BMP	Bone morphogenic protein
cDNA	Complementary DNA
CMV	Cytomegalovirus
CMs	Cardiomyocytes
CPCs	Cardiac precursors
CVPCs	Cardiovascular precursors
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetate
EF1alpha	Elongation factor 1-alpha
ES cell	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FLK1	Fetal liver kinase 1

GSK3	Glycogen synthase kinase 3
HIV	Human immunodeficiency virus
HES	Human embryonic stem cells
iPSC	Induced pluripotent stem cell
ICM	Inner cell mass
ISL	Insulin gene enhancer protein
kb	Kilo base
L	Litre
LB	Luria Bertani medium
LIF	Leukemia inhibitory factor
loxP	Locus of crossing over in P1
LTR	Long terminal repeat
min	Minute
µl	Microliter
µm	Micrometer
µM	Micro molar
MDP	Maximum diastolic potential
MEF	Mouse embryonic fibroblast
NEAA	Non-essential amino acids
MOI	Multiplicity of infection
ng	Nanogram
nm	Nanometer
NLS	Nuclear localizing sequence

PBS   Phosphate buffered saline

PCR   Polymerase chain reaction

PFA   Paraformaldehyde

PTD   Protein transduction domain

rpm   Rounds per minute

RPMI   Roswell Park Memorial Institute

RNA   Ribonucleic acid

SSEA   Stage-specific embryonic antigen

SMA   Smooth muscle actin

Stat3   Signal transducer and activator of transcription 3

TAT   Transactivator protein derived from HIV

TTFs   Tail tip fibroblasts

TGFβ   Transforming growth factor beta

WPRE   WHP posttranscriptional regulatory element

INDEX

1 INTRODUCTION

1.1   Stem cells

Stem cells are characterized by their unique ability to self-renew and differentiation capabilities. These two properties enable them to repair the damaged tissue (reviewed by George Q. Daley. 2010). Depending upon the differentiation potential, stem cells can be divided in three groups namely totipotent, pluripotent and multipotent stem cells. A totipotent cell can give rise to whole organism e.g. zygote, a pluripotent stem cell can give rise to all cells except extra embryonic tissue e.g. cells from inner cell mass. Other stem cells can be oligopotent, bipotent or unipotent depending on their ability to develop into few, two or one other cell type(s) (Winslow. 2001). Stem cells have attracted attention of scientific community because of their regenerative capacity. First idea about therapeutic potential of stem cells came during early experiments with bone marrow transplants when Canadian scientists Ernest A McCulloch and James E Till described the self renewing properties of mouse bone marrow stem cells in 1963 (Becker *et al.*, 1963), which initiated adult stem cell research. Further momentum in the stem cell research came with the discovery of pluripotent ES cells, when Martin *et al* showed successful isolation of mouse ES cells and their pluripotent potential (Martin *et al*., 1981). Major breakthrough in this field came in 1998 when James Thomson, a scientist at the University of Wisconsin-Madison, isolated cells from human embryo and grew them in culture (Thomson *et al*., 1998), these human ES cells had a normal karyotype and also showed the potential to develop into all three germ layers. Since then extensive research has been carried out in isolation, characterization and clinical application of human ES cells. However, it has provoked serious ethical debates with respect to the source of these cells, which is eight days old human embryo. This controversy had great impact on the flow and direction of stem cell research. Scientists started looking for alternative sources of stem cells. This lead to the emergence of reprogramming field, where somatic cells are dedifferentiated to primitive pluripotent stem cells with *in vitro* manipulation (Marc Lewitzky and Shinya Yamanaka. 2007). Earlier attempts in this direction were carried out by exposing somatic cells to the extracts from pluripotent stem cells or by fusing it with enucleated embryo thus forcing it to revert back to pluripotent state (Marc Lewitzky and Shinya Yamanaka. 2007). However those approaches were extremely inefficient and practically

complicated. Nevertheless, these attempts shed light on the signaling pathways as well as transcription factors involved in pluripotency.

1.2 Reprogramming somatic cells towards pluripotency by defined factors

Advancement in the field of developmental biology and increased understanding about cellular signaling brought the idea of using defined factors for the direct reprogramming of somatic cells.

1.2.1 Discovery of reprogramming factors and generation of induced pluripotent stem (iPS) cells

Takahashi and Yamanaka did first successful reprogramming of mouse somatic cell in 2007 (Takahashi *et al.,* 2006).
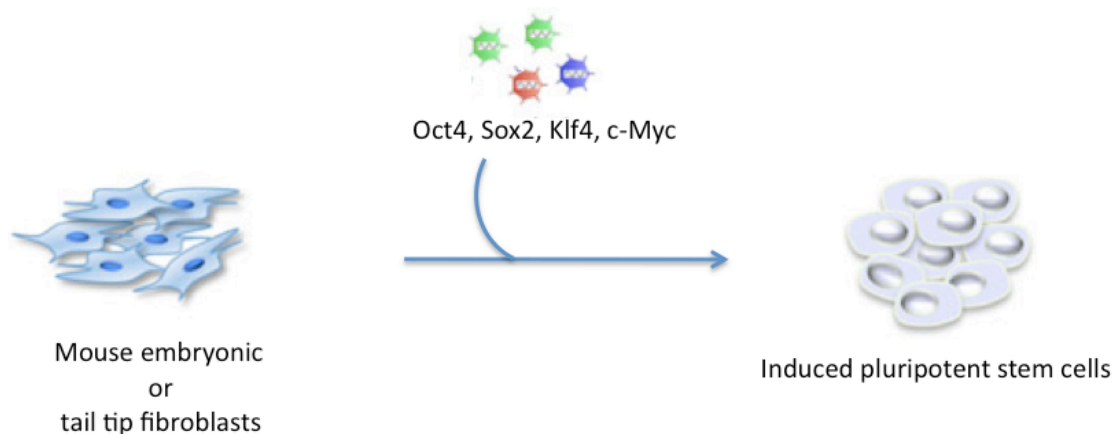
Figure 1: Induction of pluripotency with defined factors. Generation of induced pluripotent stem (iPS) cells from differentiated fibroblasts using retroviral transduction of defined transcription factors namely Oct4, Sox2, Klf4, and c-Myc (modified from Takahashi *et al.,* 2006).

During the study their first objective was to find the combination of factors capable of reprogramming. For this purpose they used mouse embryonic fibroblasts (MEFs) and tail-tip fibroblasts (TTFs) of mice homozygous for a knock-in of a neomycin-reporter cassette into promoter region of Fbx15 gene. Fbx15 expression is restricted to early embryonic development and ES cells but it is dispensable for mouse development and maintenance of pluripotency (Tokuzawa Y *et al.*, 2003). Initially, 24 potential reprogramming factors were retrovirally transduced into Fbx15-reporter-fibroblasts. The rationale behind the experiment was: upon becoming pluripotent,

cells would express the neomycin resistance gene under control of the Fbx15 promoter and thus become resistant to G418 selection. Indeed the G418 selected cells formed the ES-like colonies and termed induced pluripotent stem (iPS) cells (Figure 1). The experiment was repeated with stepwise reduction of each of the 24 factors. Ultimately it was reduced to four factors namely Oct4, Sox2, Klf4 and c-Myc as described in figure 2. These results were also reproduced in the case of human by generating human iPS cells using similar reprogramming approach (Yu *et al.,* 2007). iPS cells obtained were similar to ES cells with respect to pluripotency and differentiation potential with some minor differences in the gene expression patterns (Takahashi *et al.,* 2006, Yu *et al.,* 2007). Therefore, iPS cells offers suitable alternative to embryonic stem cells in terms of their applicability in medical research.

1.2.2 Biomedical application of human iPS cells

Human iPS cells are immensely valuable in the field of biomedical research in terms of making relevant cellular platform from disease modeling, drug screening and toxicity studies (Figure 2). Patient specific iPS cells have been successfully utilized to assess disease phenotype in case of cardiac as well as neurological
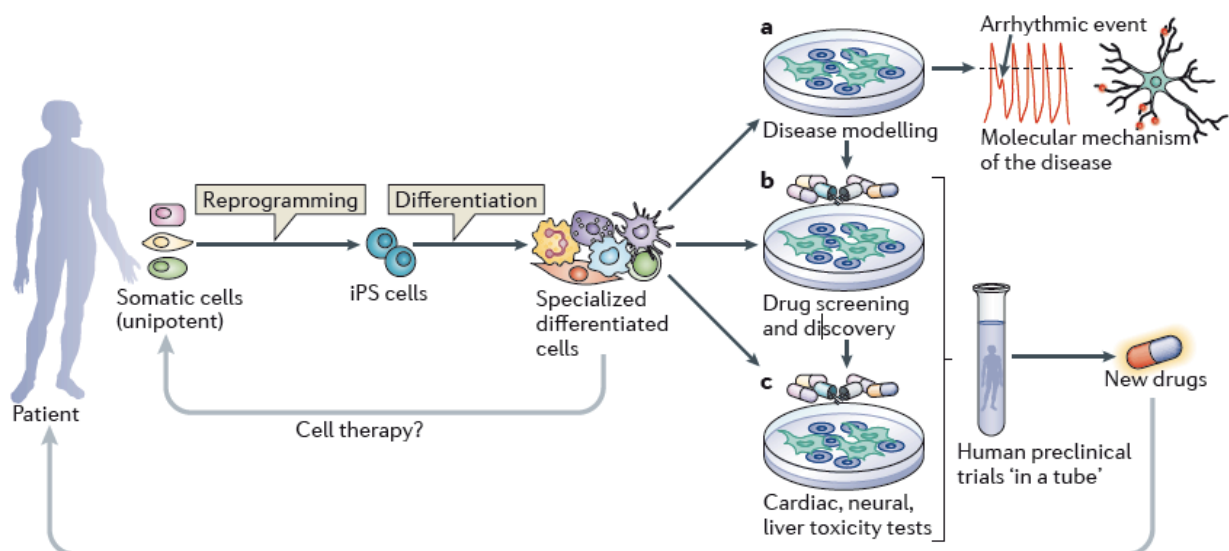
Figure 2: Biomedical application of human iPS cells. Human iPS cells can be used in several ways. a) Patients specific iPS cells can be differentiated into relevant cell types in order to establish cellular models for studying the disease mechanism. b) Cells derived from human iPS cells can be used for drug screening to evaluate its potency on targeted cell population. c) Toxic side effects of drugs can be evaluated on iPS cell derived cells before its clinical application (Bellin *et al.,* 2012).

disorders such as Long QT, Catecholaminergic Polymorphic Ventricular Tachycardia, Alzheime's disease, Parkinson's disease, Huntington's disease etc

(Itzhaki *et al*., 2011; Itzhaki *et al*., 2012; Egashira *et al*., 2012, Israel *et al*., 2012, Soldner *et a*l., 2009, Park *et al.,* 2008). Apart from disease modeling, iPS derived cells hold great potential for pharmaceutical industry by providing cellular platform for drug screening and identifying novel targets for therapy (Bellin *et al.,* 2012). Moreover, toxic side effects of drugs can be evaluated directly on targeted cell types derived from iPS cells. Thus enabling focused development of drugs without toxic side effects. This is crucial as many drugs are removed from the market due to its unexpected toxicity on vital organs in humans. Such late phase removal of drug causes immense capital loss to the company (Braam *et al.,* 2009). Therefore, pre-testing of drug toxicity on human iPS derived cells could provide vital screening step during drug development.

1.3 Derivation of transgene-free human iPS cells

Initial reports for generating iPS cells involved retroviruses as a mode of transgene delivery (Takahashi *et al*., 2006). Although retroviruses are capable of reprogramming somatic cells to iPS cells, the clinical applicability of such iPS cells
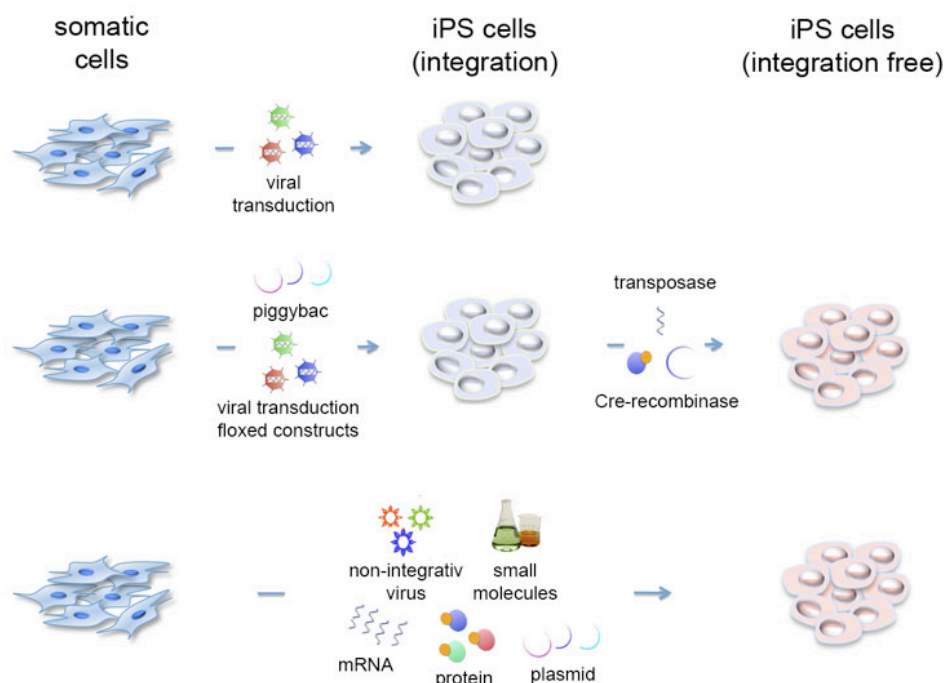
Figure 3: Summary of different methods for iPS cell induction. Overall methods are divided in three groups. Methods utilizing retroviruses result in iPS cells with integrated transgenes. Second group belongs to excisable vectors such as transposons and Cre excisable viral vectors. Third category includes the methods overcoming genomic modification by using proteins, chemicals, mRNA, non-integrating viruses and episomal plasmids (Wörsdorfer P, Thier M and Kadari A. 2013).

is limited due to the integrated transgenes carrying the risk of insertional mutagenesis (Mikkers H. & A. Berns, 2003) and tumor formation (Okita *et al*., 2007). Moreover, continuous expression of transgenes in iPS cells negatively affects the pluripotency (Kopp *et al*., 2008) and limits their differentiation potential. This has been shown by inability to yield live chimeric mice and diminished endodermal differentiation of iPS cells carrying transgenes (Sommer *et al*., 2009). Alternative approaches were explored to obtain iPS cells with higher efficiency with minimal genetic modifications of the cells. Various protocols circumventing viral vectors have been published, including the use of transposons (Kaji K *et al.,* 2009) episomal plasmids (Chou *et al*., 2011, Valamehr *et al.,* 2014), synthetic mRNA (Warren *et al*., 2010), micro RNAs (Anokye-Danso *et al*., 2011), synthetic self-replicating RNAs (Yoshioka et al., 2013), sendai viruses (Fusaki *et al*., 2009, Ye *et al.,* 2013) as well as protein transduction (Kim *et al*., 2009; Zhou *et al*., 2009) as summarized in figure 3. Human iPS cells generated via above methods contain minimal or no genetic modifications and are generally more suitable for clinical applications than virus-based protocols. However, still there is no gold standard of an iPS cell reprogramming strategy since these non-integrating approaches exhibit limitations such as low reprogramming efficiencies, slow reprogramming kinetics, narrow range of cell specificity, and poor reproducibility (Gonzalez *et al*., 2011; Wörsdörfer *et al*., 2011, Lee *et al.,* 2013). Thus, in terms of robustness and efficacy lentiviral system still represents the method of choice for iPS cell derivation.

1.3.1 Polycistronic and Cre-excisable lentiviral vector systems

As mentioned above, integrated transgenes harbor a risk of tumor formation due to insertional mutagenesis as well as reactivation of silenced oncogenic transcription factors. A first improvement in iPS cell generation was the usage of polycistronic vectors in order to reduce the number of integration sites. The core element of those vectors is a cassette coding for all four transcription factors, which are linked together via self cleaving peptide sequences such as the 2A peptide (Szymczak *et al*., 2004, Carey *et al*., 2009). This strategy allows translation of four separate polypeptides from a single mRNA strand. Thus, instead of different viruses, one construct is sufficient to induce epigenetic reprogramming, which decreases the number of inserted transgenes and therefore minimizes the risk of tumor formation (Okita *et al*., 2007). Subsequently, the polycistronic concept was combined with the

Cre/loxP-system (Sauer *et al*., 1989) to generate a lentiviral vector that integrates a polycistronic reprogramming cassette flanked by loxP sites. Hence, transgenes could be excised from iPS cells via transient expression of the DNA recombinase Cre (Soldner *et al*., 2009). Recently, a new loxP-modified polycistronic lentiviral vector system called stemcca was reported that allows efficient iPS induction of about 0.1-1% reprogramming efficiency (Sommer at al., 2009). The cassette consists of the coding regions of Oct4 and Klf4, separated by a 2A peptide sequence, followed by an internal ribosomal entry site (IRES) (Pelletier *et al*., 1988) and the coding regions of Sox2 and c-Myc, also separated by a 2A sequence. The expression of the transgene is driven either by the human EF1α promoter or a Tet inducible minimal CMV promoter (Sommer *et al*., 2009, Somers *et al*., 2010). This structure leads to a strong transgene expression and increases the probability to obtain an appropriate stochiometry of ectopic transcription factors, which turned out to be important for efficient reprogramming (Papapetrou *et al*., 2009). In fact, this polycistronic reprogramming system proved to be functional even in peripheral blood cells that are usually quite resistant towards reprogramming (Staerk *et al*., 2010)**.** Moreover, stemcca was also used recently in order to obtain transgene-free human iPS cells with putative clinical grade status (Awe *et al.,* 2013). Similar approaches were published utilizing polycistronic construct in combination with application of lentiviral Cre vector in order to obtain transgene-free human iPS cells (Papapetrou *et al*., 2011).

1.4 Direct delivery of Cre protein

Use of Cre recombinase in a protein form for transgene deletion promises clean way to remove transgenes, as it involves no genetic modifications of target cells. In order to directly deliver the Cre protein to the cells, it has been modified with the inclusion of protein transduction domain (PTD) and nucleus localization signals and resulting fusion protein is called TAT-Cre (Peiz et al., 2002). PTD confers the ability to pass through cell membrane and NLS allows the targeting of Cre protein to the nucleus (reviewed by Patch and Edenhofer. 2007). TAT-Cre has been used successfully for Cre mediated recombination in human ES cells (Nolden et al., 2006). Therefore TAT-Cre offers robust alternative for Cre mediated transgene excision in case of human iPS cells.

1.4.1 Alternative ways of Cre mediated reprogramming construct excision

The usage of polycistronic vectors harboring loxP sites allows transgene excision from iPS cells via transient expression of the Cre recombinase (Soldner *et al*., 2009). However, the reprogramming efficiency using these vectors was reported to be 0.01% only (Soldner *et al*., 2009). In 2009 Sommer et al reported the lentiviral vector to overcome this limitation by yielding a reprogramming efficiency of 0.1 to 1.5% (Sommer *et al*., 2009). However, deletion of the loxP-modified transgene cassette requires introduction of Cre recombinase activity. This has been accomplished by either transfection of iPS cells with a Cre-encoding plasmid (Soldner *et al.,* 2009; Somers et al., 2010) or using an adenoviral Cre construct (Awe *et al.,* 2013; Sommer *et al.,* 2010) and subsequent genetic identification of successfully recombined clones (figure 4). However the efficiency of transgene excision using those approaches were very low e.g Soldner et al showed successful deletion of 16 out of 180 analyzed clones (Soldner *et al.,* 2009).
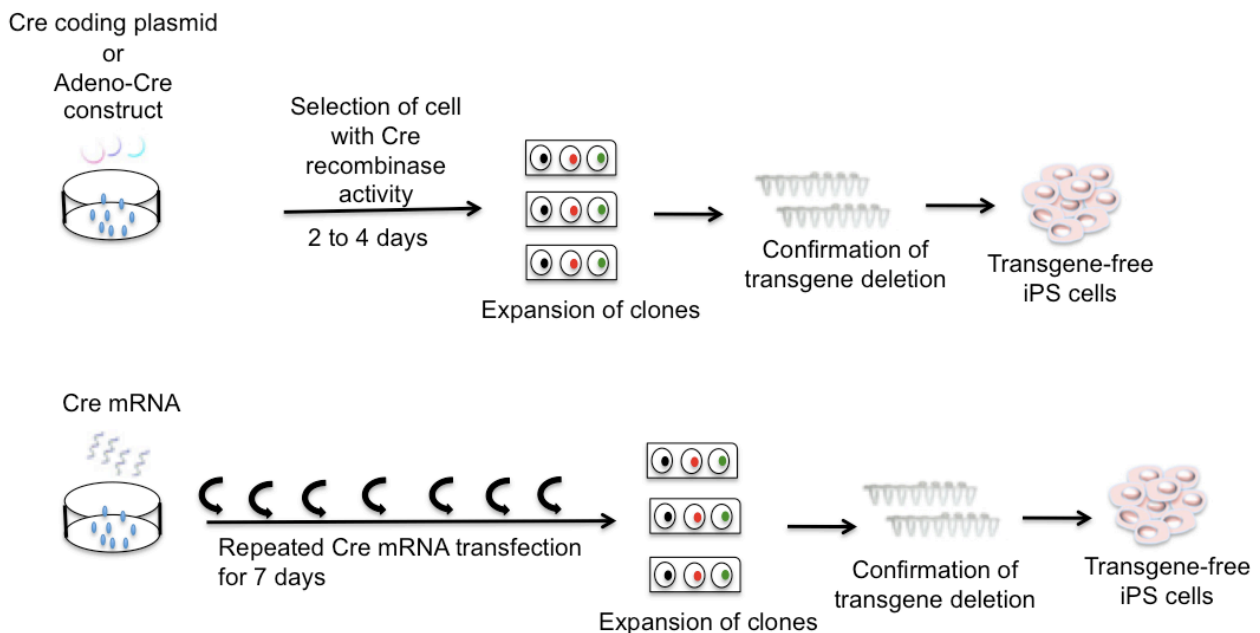
Figure 4: Summary of different approaches for Cre mediated transgene deletion. Earlier attempts to excise reprogramming factors using Cre-coding plasmids, adenovirus and mRNA.

More recently, transgene-free iPS cells were obtained by excising the transgene cassette by delivery of Cre mRNA (Loh *et al*., 2012) as described in figure 4. However, this protocol involves daily transfection of mRNA for a week to perform excision. Such repeated transfection could be stressful to the cells. This rather inefficient and laborious transfection and selection procedures make Cre/loxP-based

iPS cell derivation less appealing for obtaining transgene-free iPS cells. In fact, efficient and reliable induction of Cre recombinase activity in loxP-modified iPS cells and subsequent selection of cleaned clones represents a roadblock for the widespread use of Cre-deletable iPS cell systems.

1.5 Advantages of transgene-free iPS cells

Apart from earlier discussed advantages with respect to insertional mutagenesis and tumorogenesis, removal of transgenes enables iPS cells with putative clinical grade status (Awe et al., 2013). Moreover, there are several studies showing the improved differentiation potential as well as enhancement in the quality of iPS cells after the transgene removal (Soldner *et al.,* 2009, Sommer *et al.,* 2010). Sommer *et al* systematically showed the improvements of transgene-free iPS cell towards endodermal lineage as well as they could obtain live chimeras only with iPS cells devoid of transgenes thus showing enhancement their *in vivo* developmental potential (Sommer *et al.,* 2010). There has been recent study showing successful transplantation of neuronal cells derived from transgene-free human iPS cells with improvement of disease phenotype without any signs of tumor formation in rodent model (Mohamad *et al.,* 2014). Thus in order to make iPS based treatments routine practices in clinic, it would be of high importance to devise approaches to derive iPS cells devoid of integrated transgenes.

1.6 Cardiomyocyte differentiation

Due to the immense potential of human iPS derived cardiomyocytes, it would be of great of value to devise robust approaches to obtain reprogramming-factor free iPS cells and their subsequent differentiation to cardiomyocytes.

1.6.1 *In vivo* Cardiac development and regeneration

Heart is the first organ to form during the mammalian development. Evidence gathered in rodent developmental models suggest that cardiovascular system is derived from mesoderm, which originates during gastrulation from the primitive streak population (Buckingham *et al*., 2005). Complex interplay amongst different developmental signals further specifies the cells from primitive steak into cardiac precursors states which forms first and second heart field. More than 70% of the heart is formed from second heart field and rest from first heart field population (Buckingham *et al*., 2005). Unlike in most of amphibians and fish, human myocardium is unable to repair itself after injuries. Studies utilizing genetic fate mapping and C^{14} incorporation assays have revealed that human myocardium renewal rate is approximately 1% during the early age (20 years), 0.4% during old age (75 years) (Laflamme and Murry *et al*. 2011). Such low regeneration capacity is not sufficient in the case cardiac injuries such as infarction where 25% of the cells are lost (Laflamme and Murry *et al*. 2011).

1.6.2 Different stem cell sources for cardiac regeneration

In spite of recent advances in medicine, cardiovascular disorders remain a major cause of mortality in the world (Lopez *et, al* 2006). Supply with human cardiomyocytes is generally limited due to lack of donors as well as restricted proliferation rate of adult cardiomyocytes. Thus, with respect to use of human cardiomyocytes for regenerative therapies and drug toxicity studies as well as disease modeling, alternative sources are highly desired. There were many attempts in this direction using adult stem cells such as bone marrow derived stem cells (BMSCs) (Orlic *et al*., 2001), mesenchymal stem cell (MSCs) (Calpan *et al.,* 2006), c-kit and ISL1 positive cardiac stem cells (CSCs) (Zaruba *et al*., 2010, Laugwitz *et al*., 2005). However, there is little evidence that BMSCs and MSCs differentiate into cardioymocytes after transplantation, since positive effects observed using those

cells are mainly due to angiogenesis and paracrine effects (Choi *et al.*, 2012). Although it has been shown that CSCs can be differentiated into all cardiovascular lineages in an animal model (Cai *et al.*, 2003), there have been few studies in the case of human due to lack of donors, limited *in vitro* amplifications as well as complicated isolation procedures (Choi *et al.*, 2012, Hou *et al.*, 2013).

1.6.3 Cardiac differentiation of human pluripotent stem cells

Discovery of ES cells has pave new hopes in the field of regenerative medicine (Thomson *et al.*, 1998). It holds great potential for providing unlimited source of cardiac cells. However, ethical concerns associated with use of human embryo have made it difficult to bring the technology further with respect to clinical research (Burridge *et al.*, 2012). Recent iPS technology offers generation pluripotent stem cells from somatic cells.

Evidence obtained from the studies involving mouse and human pluripotent stem cells suggest involvement of BMP, WNT and TGF-β signals to play critical role by blocking the ectodermal differentiation and promoting primitive steak-like population (Keller *et al.,* 2005).

1.6.3.1 Stages of cardiac differentiation of pluripotent stem cells
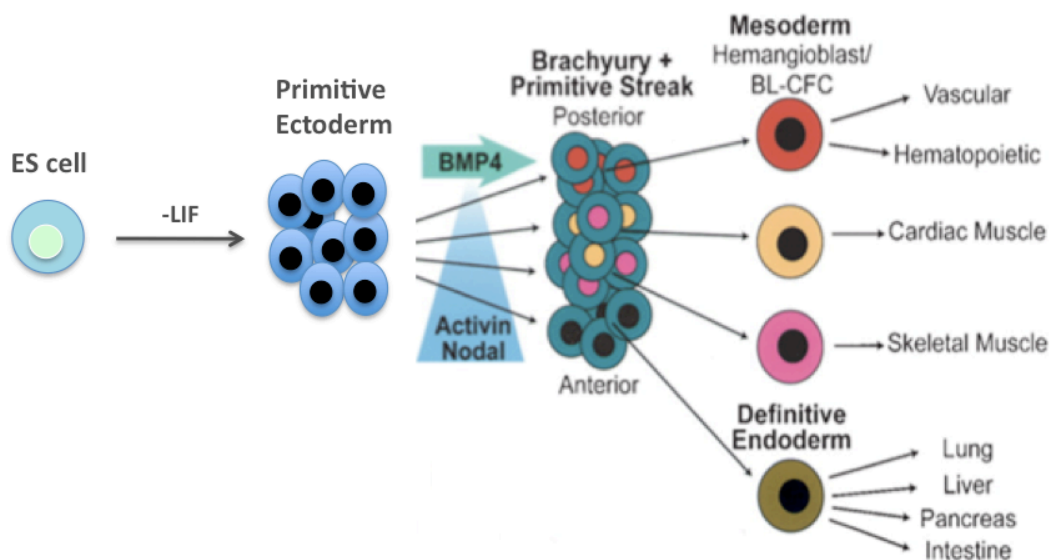

Figure 5: Differentiation of ES cells towards mesendodermal lineages. Overview of important cell signaling pathways specifying ES towards different lineages (modified from Keller *et al.*, 2005).

As described in figure 5, T (Brachyury) positive cells characterize first phase of cardiac differentiation, which comprises of mesendodermal precursor cells (Keller *et*

al., 2005). These early precursors are then further specified into respective cell type depending upon critical interplay of Nodal and WNT signals.
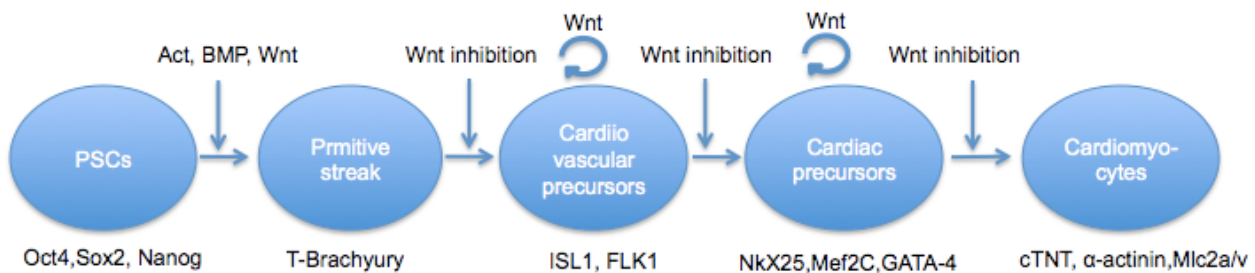
Figure 6: Cardiac differentiation of pluripotent stem cells towards cardiomyocytes. Overview of different precursor populations arising during cardiac specification of pluripotent stem cells (modified from Gessert and Kühl. 2013).

During cardiac specification, cells pass through a series of precursor states as described in the figure 6. First precursor cells in this series includes cardiovascular precursor cells (CVPCs), these cells expresses markers such as ISL1 and FLK1. CVPCs hold the potential to differentiate into the cardiac as well as vascular lineages (Zaruba *et al*., 2010, Laugwitz et al., 2005). Next step in specification leads to the cardiac precursors (CPCs) characterized by the expression of Nkx2.5, GATA-4 and Mef2c. These cells could give rise to first and second heart field derivatives (Gessert and Kühl. 2013). Evidence from several studies indicates that CVPCs and CPCs require WNT/β-catenin signal for their proliferation (Gessert and Kühl. 2013).

1.6.3.2 Current state of cardiac differentiation of pluripotent stem cells

Until recently human cardiomyocytes can only be isolated from heart biopsies, which is limited with respect to the availability of tissue. Moreover, obtained cardiomyocytes remain viable not more than a day. Hence obtaining functional cells in large quantities remains a major obstacle in making physiologically relevant cellular models (Rajala *et al*., 2011). Pluripotent stem cells offer an attractive option to obtain cardiomyocytes as they provide unlimited source of undifferentiated cells which has potential to differentiate into any cell type provided suitable media conditions are provided. Figure 7 describes recent systematic approaches for derivation of cardiomyocytes from pluripotent stem cells (Burridge *et al*., 2012, Mummery *et al*., 2013). Following the heart development *in vivo*, there are three different approaches to direct the pluripotent stem cells towards cardiac lineages (see figure 7). The first approach involves formation of embryoid bodies (EBs) in the presence of growth factors known to be involved in cardiac development. Spontaneous differentiation of

these EBs in medium containing fetal bovine serum results in contracting areas having cardiomyocytes. However the efficiency of this approach remains very low (5 to 20%) (Kehat *et al*., 2001). There have been improvements in efficiency by using a precise cell number for EB formation in a V-Shaped 96-well plate (Burridge *et al*., 2007). Moreover, application of bone morphogenic protein 4 (BMP4) and Activin A further increases the efficiency of this protocol (Ng *et al*., 2005; Filipczyk *et al*., 2007). The second approach uses the monolayer culture of pluripotent stem cells in the presence of cardiac-specific growth factors without going through EB formation. Several attempts have been published utilizing monolayer culture of cells in a serum-free condition having growth factors such as BMP4, Activin A, FGF2, VEGF and small molecules in order to increase the efficiency while reducing the heterogeneity arising during EB based differentiation (Laflamme *et al*., 2007; Zhang *et al*., 2011; Uosaki *et al.,* 2011, Hudson *et al*., 2011, Carpenter *et al*., 2013, Lian *et al*., 2013, Minami *et al.,* 2013, Dambrot *et al.,* 2014). However, optimum concentration of extrinsic factors varies among different iPS cell lines requiring the optimization of protocol for each cell line, which makes it laborious (Kattman *et al*., 2011, Buriddge *et al*., 2012). The third approach utilizes co-culture of END2 (visceral endoderm-like cells), where they instruct the pluripotent stem cells to differentiate towards cardiomyocytes by secreting cardio-inductive growth factors (Mummery *et al*., 2003).
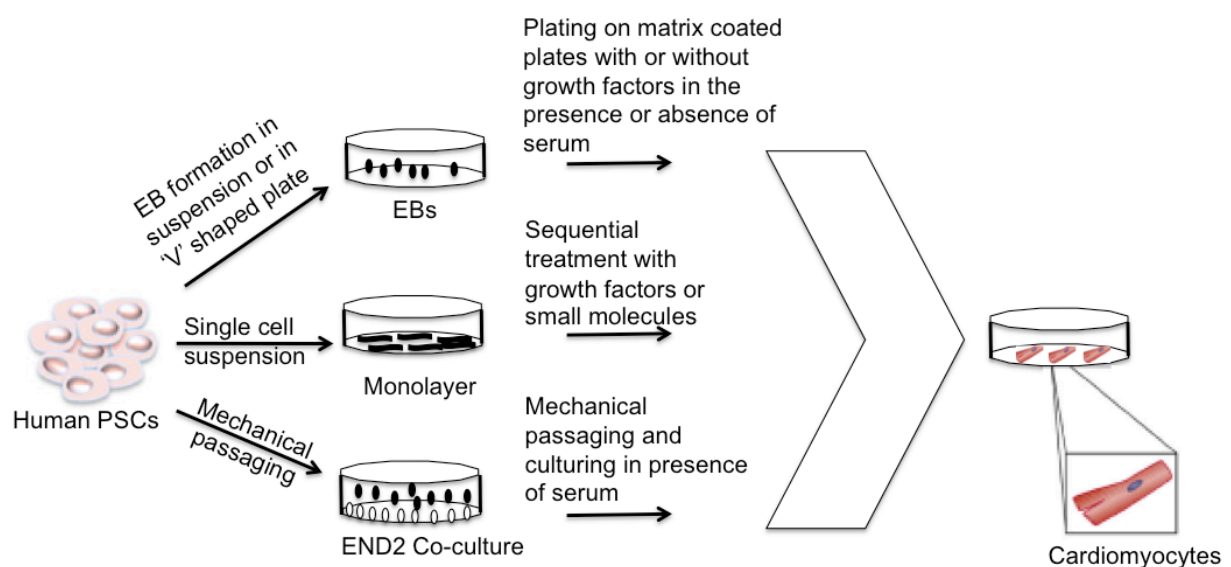

Figure 7: Overview of different methods for cardiac differentiation of pluripotent stem cells. Summary of different approaches for cardiac differentiation of human pluripotent stem cells using EBs, END-2 co-culture and monolayer based methods (Modified from Mummery *et al*., 2013)

Recent advances in cell signaling studies have shed light on detailed signaling pathways involved during cardiac differentiation. It has been shown that WNT signaling plays very critical role during cardiogenesis (Marvin *et al.*, 2001). It has been suggested that WNT signaling during the early differentiation is required for mesodermal induction, however later on during differentiation cardiac specification is hampered by WNT signaling and inhibition of WNT signal is absolutely essential for the formation of cardiomyocytes (Ueno *et al.*, 2007). Recent studies have shown the use of WNT inhibiting small molecules for increasing the cardiomyocyte yield in the case of EB (Ren *et al.*, 2011) as well as monolayer based protocols (Lian *et al.*, 2013, Minami *et al.*, 2013). Two recent studies by Cao *et al.* have shown importance of ascorbic acid in increasing the efficiency by affecting MEK/ERK pathway (Cao *et al.*, 2011). Moreover, by combining the application BMP4, Chir99021 and ascorbic acid they were successful in isolation of cardiovascular precursors cells from human pluripotent stem cells (Cao *et al.*, 2013). However the heterogeneity among the different lines still remains a major issue even in the case small molecules based protocols. Hence it is very important to devise simple and highly efficient new protocols combining the previous findings.

Apart from differentiation of pluripotent stem cells, the new branch of trans differentiation is emerging where fibroblasts are directly converted into desired cell types via over expression of lineage-specific transcription factors. Recently, Ieda *et al.* have shown direct conversion of mouse fibroblasts to cardiomyocytes using defined transcription factors (Gata4, Mef2c, and Tbx5) (Ieda *et al.*, 2010). Another group obtained cardiomyocytes by partially reprogramming somatic cells and then diverting them towards cardiac lineage by providing cardio-specific media conditions (Efe *et al.*, 2011). However such a transdifferentiation approach has not yet been shown in the case of human cells. Hence it would be of high interest to devise direct transdifferentiation protocols to obtain human cardiomyocytes.

1.7 Enrichment of human iPS derived cardiomyocytes

iPS and ES cells provide unlimited source of cardiomyocytes, however, major applications of obtained cardiomyocytes will strictly require pure population of cardiomyocytes. There have been several attempts in order to enrich the cardiac population. Earlier attempts in this direction include genetic methods where the ES

cells having antibiotic resistance or fluorescent protein under the control of cardiac-specific transcription factors were used for differentiation. Enrichment of cardiac populations can be achieved via antibiotic selection or by FACS analysis (Fijnvandraat *et al*., 2003; Gassanov *et al*., 2004; Hidaka *et al*., 2003). Major disadvantage of above methods include use of genetically modified cell lines, which limits its application. Hattori *et al* showed the successful isolation of more than 90% pure cardiac population using a non-genetic method utilizing mitochondrial specific dye. Since cardiomyocytes have higher number of mitochondria, they will absorb most of the dye and can be sorted out using FACS (Hattori *et al*., 2010). This approach is limited due to its purity as well as toxicity of dye and the requirement of FACS sorting. There have been several studies utilizing the cardiac-specific antibodies in order to isolate cardiac cells from mixed population of cells. Use of specific antibodies improved the purity of cardiomyocytes up to 99% but the wide application of such protocols is limited as they require FACS analysis (Dubois *et al*., 2011; Uosaki *et al*., 2011). Recently Tohyama *et al* showed the successful purification of cardiomyocytes utilizing different metabolic requirements of cardiomyocytes. Their protocol uses the medium devoid of glucose and contains lactate as a main energy source. Since only fetal cardiomyocytes could utilize lactate as an energy source, unwanted cells die out due to lack of energy source (Tohyama *et al*., 2013).

1.8 Potential applications of human iPS derived cardiomyocytes

1.8.1 Cellular models of disease pathophysiology and drug toxicity studies

Until recently animals have been used as a model to uncover mechanism of disease progression, drug efficacy as well as toxicity studies (Goldsmith *et al*., 1975). Although animal models are helpful in many regards, there are fundamental differences compared to human physiology. In order to faithfully replicate the human cellular models, it is of great potential to use the cellular platform of human origin. Emergence of iPS technology has given immense momentum to the field of disease modeling. In spite of structural and functional immaturity there have been many studies showing the successful cellular models of cardiac diseases such as long QT (Moretti *et al*., 2010; Itzhaki *et al*., 2011; Itzhaki *et al*., 2012; Egashira *et al*., 2012), and Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) (Itzhaki *et al*.,

2012; Jung *et al*., 2012; Kujala *et al*., 2012). These studies were extremely informative in terms of disease phenotype and its potential treatment. For example Itzhaki *et al* obtained the cardiomyocytes from LQTS-2 patients and showed the disease phenotype and using drug they achieved reversal of disease phenotype thus establishing reliable platform for drug efficacy screening (Itzhaki *et al*., 2011). Jung *et al* obtained similar results in case of disease modeling of CPVT, where they could rescue the disease phenotype with the use of drug called dantrolene (Jung *et al*., 2012).
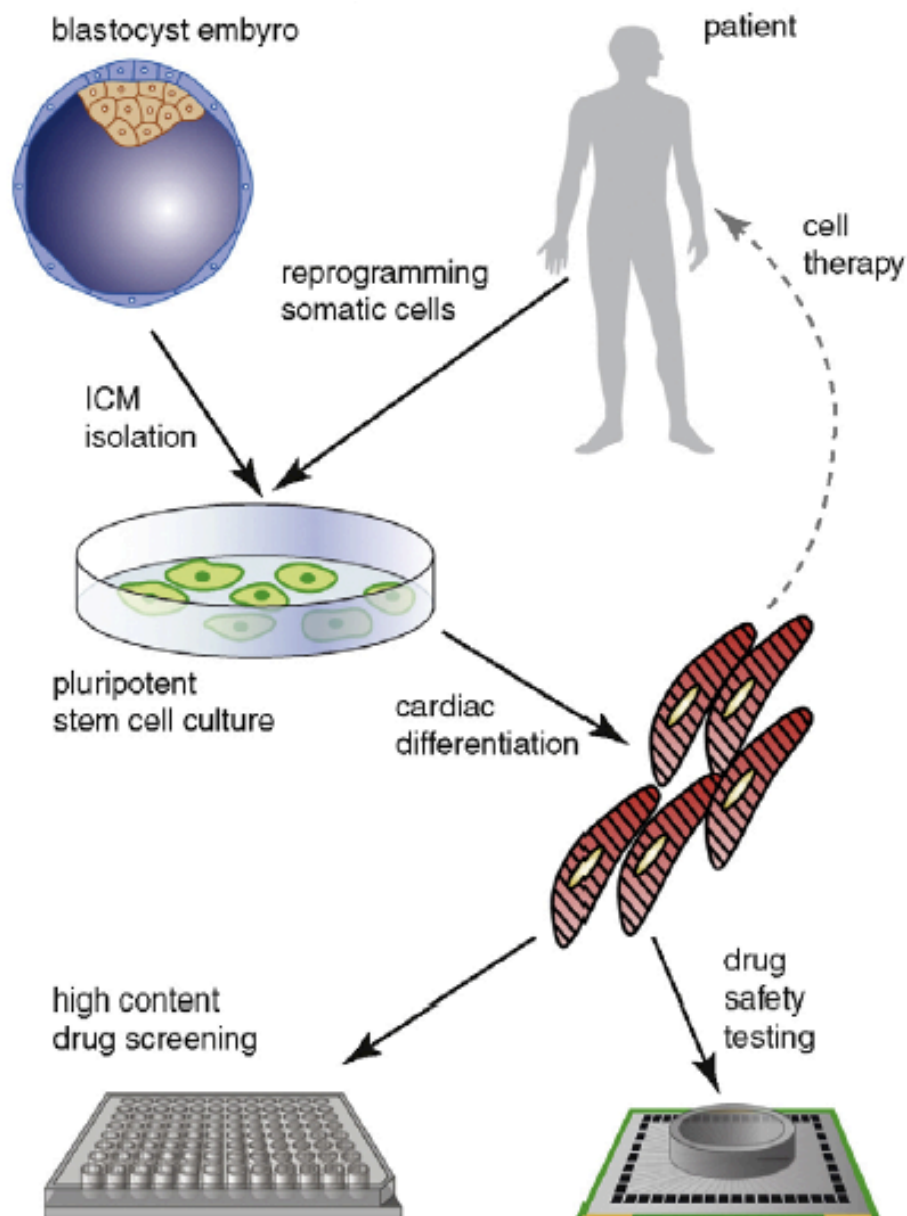

Figure 8: Potential application of pluripotent stem cell derived cardiomyocytes. Overview of applications of human cardiomyocytes for pharmaceutical research and regenerative medicine (Braam *et al.,* 2009)

Apart from disease modeling, iPS CMs offer unlimited source of cardiac cells for drug toxicity studies (Figure 8). Many drugs are removed from market due to their side effects causing fatal cardiac conditions (Lasser *et al*., 2002; Mandenius *et al* 2010). Conventional methods for toxicity screening include cell lines from animal models as well as heterologous cell systems where cardiac channels are expressed in HEK (human Embryonic Kidney) or CHO (Chinese Hamster Ovary) cell lines (Lu *et al*., 2008). These methods are helpful however there are obvious limitations of such non-human cellular platforms. Recent studies have achieved the successful establishment of cardiac tissue from human ES and iPS cells derived cardiomyocytes, which could validate the toxic effects of drugs (Schaaf et al., 2012; Moya et al., 2013). Therefore iPS CMs hold the great potential as a platform for studying disease mechanism, pharmacology and drug screening studies provided the field evolves further with respect to differentiation efficiency and maturity of iPS CMs.

1.8.2 Cell therapy and tissue engineering

Ultimate aim of stem cell field is to readily obtain the cells for regenerative therapy in order to replace dead or damaged tissue. iPS CMs are the most suitable candidates for cell replacement approaches for cardiac repair. However it is absolutely essential to provide the cardiomyocytes in a proper transplantable format as to enhance their functional integration into the host tissue (Braam *et al*., 2009). It is quite a challenging task as recent transplantation studies have shown the poor integration of transplanted cardiomyocytes can lead to fetal arrhythmia (Liao et al., 2011; Zhang et al., 2002). Hence it is important to merge the cardiac regeneration field with tissue engineering techniques, which deals with construction of 2D or 3D tissues *in vitro*. There are several recent attempts in this direction by constructing 3D tissue models using cardiomyocytes derived from pluripotent stem cells (Schaaf *et al*., 2012; Moya et al., 2013). Such 3D models resemble tissue in native form and have been successfully used for drug toxicity screens (Schaaf et al., 2012; Moya et al., 2013) as well as cell therapy in case of rodent models of myocardial infarction (Eschegen et al., 2002). In spite of recent success, the field remains in infancy as cell replacement studies were carried out in rodent models hence it is required to carry out similar studies in larger animal models. More improvements are needed in terms of

strategies to enhance the functional integration of cells as well as follow up methods for detection of functional improvement of heart after transplantation. Moreover it is of high interest to design more inert scaffolds assisting successful transplantation and minimizing adverse immune reaction.

1.9 Functional properties of human iPS derived cardiomyocytes

1.9.1 Structural features of human iPS derived cardiomyocytes

Cardiac differentiation of human iPS cells is marked by spontaneous beating (Zhang J *et al*., 2009). Indeed human iPS derived cardiomyocytes have all the skeletal apparatus required for beating. Morphologically they are round or polygonal in shape and subtype wise are of mixed populations of ventricular, atrial and pacemaker like cells with 38 to 70% of ventricular like cells (Lee *et al*., 2011, Ma *et al*., 2011). There are some recent reports of obtaining subtype specific cardiomyocytes with inclusion of growth factors and small molecules (Karakikes *et al*., 2014, Zhanng *et al*., 2010). Ultra structure analysis using electron microscopy reveals the presence of sarcomeric structures. Although it is not as mature as found in their adult counterparts (Mummery *et al*., 2012). Characteristics proteins present in cardiomyocytes includes α-MHC, c-TNT, α-actinin which make up the contractile apparatus (Gupta *et al*., 2010). Moreover they have important proteins such as connexin 43, 40 and 45 for intracellular gap junction formation (Gupta *et al*., 2010, Saric T *et al*., 2014).

1.9.2 Electrophysiological properties of cardiomyocytes

Action potential

Human iPS derived cardiomyocytes (CMs) exhibit action potential (AP), which is indicative of ventricular, atrial and nodal-like cells (Ma *et al*., 2011). AP recordings from iPS CMs are different from the adult CMs with respect to certain parameters such as maximum diastolic potential (MDP), Vmax and depolarization which is suggestive of immature fetal cardiomyocytes like phenotype. Generally MDP of human iPS CMs is positive, Vmax is lower and depolarization slightly delayed compared to adult CMS (Saric T *et al.,* 2014).

Ion channels

As indicated in figure 9 human iPS CMs exhibits all major currents such as I_{Na}, $I_{Ca,L}$, I_{to}, I_{K1}, I_{Kr}, I_{Ks}, I_F which differ in their properties compare to adult CMs resulting in immature fetal-like AP (Honda et la., 2011, Saric T *et al.,* 2014). Frequencies of occurrence of certain ion channels varies due to differences in the culture conditions used to obtain CMs for example I_{Ks} and I_{K1} have been shown to present in some studies while completely absent in other reports (Lieu *et al*., 2013, Cordeiro *et al*., 2013, Ma *et al*., 2011).
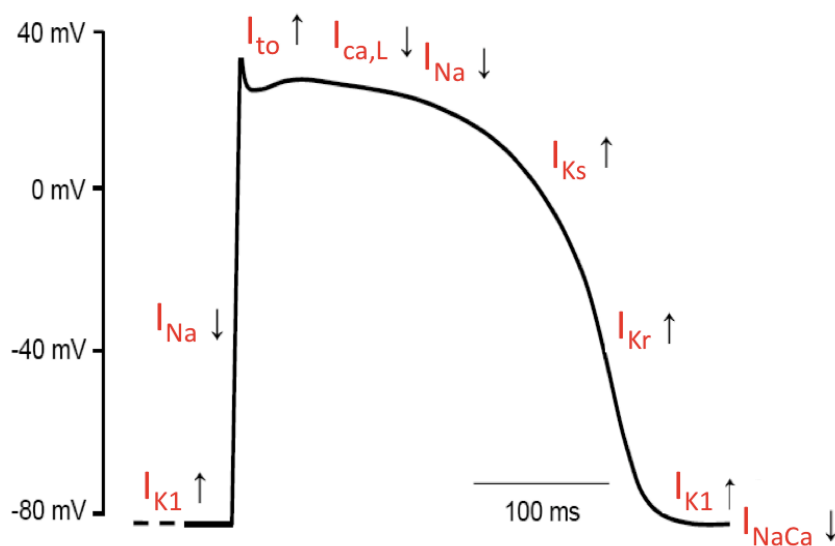
Figure 9: Overview of different ionic currents contributing to the ventricular-like action potential in human iPS derived cardiomyocyte. Contribution of individual ion currents for the formation of different phases of action potential. Direction of arrow indicates the direction of ionic flux where upward represent outwards and downwards represents inwards ionic current flow (modified from Saric *et al*., 2014).

Such variations observed in current profile of iPS CMs represent the major roadblock towards making reliable disease models involving disorder associated with those ionic channels. Hence further improvements are required in order to obtain more mature iPS CMs capable of providing robust platform for drug toxicity and disease modeling.

Calcium handling

Readily detectable beating in human iPS CMs implies functional excitation-contraction coupling. Indeed there are recent studies indicating presence of all important calcium handling proteins such as Rynodine receptor protein (RyRs), SERCA, L-type calcium channel, inositol-1,4,5-trisphosphate receptors (IP3Rs), etc.

in human iPS CMs (Itzhaki *et al*., 2011, Li *et al*., 2013). Therefore iPS CMs are of great potential as a reliable model for calcium signaling and the disorders associated with calcium signaling.

1.10 AIM OF THE STUDY

Aim of the present study was to obtain suitable human iPS cells free of reprogramming factors utilizing Cre excisable viral vectors. Further we checked enhancement of the quality of transgene excised iPS cells in terms of pluripotency as well as differentiation potential. The second objective of the study was the establishment of robust protocol to obtain cardiomyocytes from multiple human iPS lines. It was achieved with precise modulation of key signaling pathways as well as enrichment of cardiomyocytes based on their metabolic requirement to enhance the purity of obtained cells. Our next goal was to perform detailed characterization of obtained cardiomyocytes in order to validate their functional properties. Overall we intend to provide the robust basis for scale up production of human cardiomyocytes suitable for drug toxicity, disease modeling and cellular therapy.

2 MATERIAL AND METHODS

2.1 Materials

2.1.1 Instruments and technical equipments

Autoclave (D-150, Systec)

Block heater (Thermomixer compact, Eppendorf)

Centrifuges and rotors (Megafuge 1.0R, Rotor #2704, Rotor BS4402/A, Heraeus)

Counting chamber (Fuchs-Rosenthal, Faust)

Fluorescence microscope (Axiovert 40 CFL, Carl Zeiss)

Freezer -80°C (Hera freeze, Heraeus)

Gel electrophoresis chamber (Agagel, Biometra)

Gel electrophoresis documentation (GelDoc, BIO-RAD)

Horizontal Hood (Hera guard, Hereaus)

Incubator (HERAcell 150, Heraeus)

Incubator Shaker (Innova 44, New Brunswick Scientific)

Inverse light microscope (Axiovert 40C, Carl Zeiss)

Liquid nitrogen store (MVE 611, Chart Industries)

Microwave (Microwave 800, Severin)

Microscope (Axiovert 200M Carl Zeiss)

pH-Meter (HI 9321, HANNA Instruments)

Power supply electrophoresis (Standard Power Pack P25, Biometra)

Sterile laminar flow hood (HERAsafe, Heraeus)

Table centrifuges (Centrifuge 5415R, BIOFUGEpico, Galaxy Mini, Eppendorf, Heraeus, VWR)

Thermocycler (T3 Thermocycler, Biometra)

Transmission electron microscope (TEM) (LEO AB 912, Zeiss NTC)

Ultracentrifuge (Discovery 90SE, Sorval)

2.1.2 Chemicals and biochemicals

Activin A (Life technologies)

Agar (Roth)

Agarose (PEQLAB)

Ampicillin (Sigma-Aldrich)

Ascorbic acid (Sigma-Aldrich)

bFGF (Life tehnologies)

BMP4 (Life technologies)

BSA (Sigma-Aldrich)

B-27 supplement (Life technologies)

B-27 supplement minus insulin (Life technologies)

Chir99021 (Sigma-Aldrich)

DMEM high glucose (Sigma-Aldrich)

DMSO (Sigma-Aldrich)

DNA ladder (1kb) (New England Biolabs)

DNA-loading buffer (10x) (New England Biolabs)

2-mercaptoethanol (Life technologies)

dNTPs (PEQLAB)

EDTA (Sigma-Aldrich)

Ethanol (Roth)

Ethidium bromide (Sigma-Aldrich)

EPC 10 amplifier (HEKA Electronics)

FCS (PAN Biotech)

Gelatin (Life technologies)

Isopropanol (Roth)

IWR1 (Sigma-Aldrich)

Knockout-DMEM (Life technologies)

L-Glutamine (Life technologies)

mTESR1 (Stem cell technologies)

Matrigel (Stem cell technologies)

Non-essential amino acids (Life technologies)

PBS (Life technologies)

Polybrine (Merck Millipore)

Poly-D-Lysine (Sigma-Aldrich)

Serum replacement (Life technologies)

Sodium pyruvate (Life technologies)

Sodium-L-lactate (Sigma-Aldrich)

Rock inhibitor Y27632 (Sigma-Aldrich)

RPMI1640 (Life technologies)

XAV939 (Sigma-Aldrich)

2.1.3 Buffers
Gel loading Buffer

H_2O	50%(V/V)
Glycerol	49%(V/V)
Bromphenol blue	0.5%(V/V)
Xylene Cyanol	0.5%(V/V)

Cell lysis Buffer

Tris	0.1 M
EDTA	5 mM
NaCl	0.2 M
10%SDS	0.2%(V/V)
Adjust PH to 8.5.	

2.1.4 Enzymes

goTaq - Polymerase (Promega)

Phusion™ Hot Start High-Fidelity DNA Polymerase (Finnzymes)

T4 DNA Ligase (New England Biolabs)

Collagenase (Sigma-Aldrich)

Alphazyme (PAA)

Accutase (PAA)

DNase (Fermentas)

2.1.5 Antibodies

Oct4 (1:100; Santacruz)

SSEA-4 (1:200; Millipore)

ISL1 (1:200,Biorbyt)

cTNT (1:100, abcam)

alpha-actinin (1:400, Sigma-Aldrich)

SMA (1:200, Santacruz)

Alexa 555 and Alexa 488-cojuagted anti-mouse IgG (1:1000; Life technologies)

2.1.6 Kits

Plasmid Maxi Prep (Life technologies)

Plasmid Mini prep (Life technologies)

PCR Purification / Gel Extraction (Promega)

mRNA isolation kit (Macherey Nagel)

Lenti-X-Concentration kit (Clontech)

Letnti-X-GoSticks (Clontech)

Reverse Transcriptase (BIO-RAD)

2.1.7 Media for bacterial cultures

LB agar	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	10 g
H_2O was added to 1L and the mixture was autoclaved and stored at 4°C	

LB medium	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

SOC medium	Amount
Tryptone	20 g
Yeast extract	5 g
$MgSO4 \cdot 7 H_2O$	5 g
NaCl	0.5 g
KCl	0.19 g
Glucose	20 mM
H_2O was added to 1L and the mixture was autoclaved and stored at -20°C	

2.1.8 Cell culture reagents

Cell culture media

Freezing medium for fibroblasts and cardiomyocytes	
10%	DMSO
90%	FCS
Freezing medium for human iPS cells	
10%	DMSO
90%	Knockout serum replacement

Standard MEF medium	Volume
DMEM-high-glucose	500 ml
Non-essential amino acids	5 ml
Sodium pyruvate (100 mM)	5 ml
L-Glutamine (200 mM)	5 ml

Standard human ES cell medium	Volume
DMEM/F12	400 ml
Non-essential amino acid (100X)	5 ml
β-Mercaptoethanol (500X)	1 ml
L-Glutamine (200mM)	2.4 ml
Ascorbic acid	50 µg/ml
Knock-Out serum replacement	100 ml
bFGF	10 ng/ml

Cardiac differentiation basal medium	Volume
RPMI1640 with Glutamine	500 ml
B-27 supplement (50X) with and without insulin	10 ml
Penicillin/Streptomycin (100X)	5 ml

Cell culture solutions

1X Trypsin EDTA	Conc.
10x Trypsin EDTA	10%(V/V)
PBS	90%(V/V)

2X HBS Buffer PH 7	Conc.
10x Trypsin EDTA	10%(V/V)
PBS	90%(V/V)

Coatings

Gelatin	Conc.
Gelatin	0.1%(V/V)
in dd H_2O	
Incubation 20 minutes, 37°C	

Matrigel	Conc.
Matrigel	According to manual
in 24 ml DMEM-F12	
Incubation overnight at 4°C	

2.1.9 PCR Primers

Purpose	Primer	Sequence
Confirmation of excision of stemcca construct	WPRE-Fw	5-ATCATGCTATTGCTTCCCGTATGGC
	WPRE-Rev	5- GGAGATCCGACTCGTCTGAGG
	β-actin-Fw	5-GGCTACAGCTTCACCACCAC
	β-actin-Rev	5- CCACCTTCCAGCAGATGTGG
Cardiac differentiation follow up (Yang et al., 2008)	RT Oct4 Fw	5-AACCTGGAGTTTGTGCCAGGGTTT
	RT Oct4 Rev	5-TGAACTTCACCTTCCCTCCAACCA
	RT T Fw	5-TGTCCCAGGTGGCTTACAGATGAA
	RT T Rev	5-GGTGTGCCAAAGTTGCCAATACAC
	RT ISL1 Fw	5-CACAAGCGTCTCGGGATTGTGTTT
	RT ISL1 Rev	5-AGTGGCAAGTCTTCCGACAA
	RT Nkx2.5 Fw	5-GCGATTATGCAGCGTGCAATGAGT
	RT Nkx2.5 Rev	5-AACATAAATACGGGTGGGTGCGTG
	RT cTNT Fw	5-TTCACCAAAGATCTGCTCCTCGCT
	RT cTNT Rev	5-TTATTACTGGTGTGGAGTGGGTGTGG
	RT β-actin Fw	5-TTTGAATGATGAGCCTTCGTCCCC
	RT β-actin Rev	5-GGTCTCAAGTCAGTGTACAGGTAAGC

Table 1 Primer used during present study

2.1.10 Plasmids

stemcca lentiviral construct

It is a lentiviral vector used for reprogramming purpose during the thesis work. It has Cre excisable polycistronic coding frame containing four stem cell factors (Oct4, Sox2, Klf4, c-Myc) linked with 2A peptides (Sommer *et al*., 2009).

pMD2.G

pMD2.G codes for a CMV-promoter driven VSV-G envelope protein used for lentivirus production (Addgene plasmid 12259 – D. Trono).

psPAX2

Packaging plasmid coding for HIV-GAG, used for lentivirus production (Addgene plasmid 12260 – D. Trono).

2.1.11 Cell lines

293 T cells

293 T cells are derived from human embryonic kidney cells grown in tissue culture (ATCC – Manassas, USA). They grow rapidly and can be very efficiently transfected by the calcium phosphate transfection.

Human dermal fibroblasts (AR1034ZIMA)

The human fibroblasts used in this study were obtained from a skin punch biopsy of a 24 year old male after getting informed consent and ethical clearance by the ethics committee of the University of Würzburg, Germany (ethical report no: 96/11 dated 10.06.2011).

Human cardiac fibroblasts

Human cardiac fibroblasts were obtained from a myocardial biopsy with an informed consent and ethical clearance by the ethics committee of the University of Würzburg, Germany (ethical report no: 182/10 dated 04.04.2011).

Feeder cells

These are mitotically inactivated MEFs. It supports the growth of human iPS cells by secreting growth factor to the medium.

Human iPS cell lines

del-AR1034ZIMA 001 and fl-AR1034ZIMA 001 are lentiviral reprogramming derived iPS sister clones from human dermal fibroblasts (del: transgene excised, fl: transgene floxed. Human iPS cell line (k-hiPS) (Linta *et al*., 2012) is a lentivirally derived iPS cell line from human keratinocytes kindly gifted by Dr. Stefan Liebau from University of Tübingen, Germany. Human iPS cell line iLB-C-50-s9 is a Sendai

virus derived iPS cell line from human cord blood cells. Human iPS cell line iLB-C1-30 m-r12 is a retroviral reprogramming derived iPS cell line from human dermal fibroblasts cells (Koch *et al.,* 2011).

AR1034ZIMA Cre reporter iPS cell line

It is human iPS cell line (del-AR1034ZIMA 001) having a Cre reporter construct (modified from original construct (Russ *et al*., 2008) by replacing CMV promoter by EF1α promoter). Cre-mediated recombination induces the expression of GFP, by deleting the loxP-flanking RFP gene. This reporter line was used to check the efficiency of TAT-Cre mediated recombination.

WNT reporter cell line

It is the neural stem cell line (I3 lt-NES) carrying 7TGP WNT reporter construct developed by Fuerer et al., 2009. It was kindly gifted by Laura Stappert and Katharina Doll from University of Bonn, Bonn, Germany. It was used to screen for the small molecules for WNT modulation.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Maintenance and passaging of cells

Pluripotent stem cells

Human iPS and ES colonies were maintained on 6 well plates having mitotically inactivated feeder cells and 2 ml of standard HES medium having 10 ng/µl of basic FGF in each well. Cells were given fresh medium every day, every two days IPS colonies achieve the appropriate size and are ready for splitting. For this purpose medium was removed and colonies were treated with enzyme collagenase (1 mg/ml) for 1 hour at 37°C. Once colonies have detached from the bottom, 1 ml of medium was added to stop the collagenase action further. Now colonies from one well were taken into 15 ml falcon and centrifuged at 800 rpm for 3 minutes at 4°C. After centrifugation medium with collagenase was removed carefully without disturbing the pellet. Pellet was then resuspended in 1 ml of standard HES medium very carefully,

because it is very important that during resuspension colonies were shattered into appropriate size (not too big or not too small). After shattering the colonies, 2 ml of standard HES medium was added and this 3 ml of suspension was equally distributed over 6 wells of new 6 well plate.

In order to have feeder free cultivation of pluripotent cells, cells were maintained in matrigel coated plates having m-TESR medium. Passaging of cells was achieved by obtaining single cell suspension using alphazyme or accutase (1 ml/well of 6-well plate) for 7 to 10 min at 37°C. Cell were then spllited (1:3) on new plates having m-TESR medium with 10 µM Rock inhibitor, Y27632 to prevent the cell apoptosis.

Somatic cells

Human fibroblasts and 293T cells were maintained in MEF medium. Passaging of cells was achieved using trypsin (1ml/ well of 6-well plate) for 5 min at 37°C. Once cells have detached from the bottom, 1 ml of medium was added to stop the trypsin action. Cells were then centrifuged and distributed to new plates. Human cardiomyocytes were maintained in cardiac differentiation basal medium. Passaging was achieved using accutase (1ml/ well of 6-well plate) for 7 to 10 minutes at 37°C. Cell were then spllited (1:2) on new plates having cardiac differentiation basal medium.

2.2.1.2 Freezing and thawing of cells

Human pluripotent stem cells were cryopreserved by freezing approximately 2 million cells in 2 ml cryovials in a freezing medium (see material part). Vials were kept for one day in -80°C and next day transferred in liquid N2 tank. In order to thaw the cells, vials were kept in one to two mins in water bath at 37°C and resuspended in appropriate medium in 15 ml falcon tube. Cell were then centrifuged and redistributed to appropriate plates as described earlier. Freezing and thawing of somatic cells was achieved in a similar manner with only different freezing medium composition (see material part).

2.2.1.3 Virus production

For lentivirus production 3×10^6 293T cells were grown on poly-D-lysine coated 10 cm culture dishes having 12 ml of MEF medium. Cells were seeded 24 hours before

transfection during which they reached the confluency of 80 to 90%. 1 to 2 hours before transfection medium was changed to 2% Advanced MEF medium. The cells were co-transfected with a lentiviral constructs together with the envelope and packaging plasmids pMD2.G and psPAX2 (see materials part) using calcium phosphate precipitation protocol. According to this protocol 61.5µl of $CaCl_2$, 37 µg lentivirus plasmid, 18.5 µg helper plasmid (psPAX2), 18.5 µg envelope plasmid (pMD2.G) were mixed in cryovials and final volume was made to 600µl with ultra pure H_2O and then 600µl of 2x HBS buffer (pH 7) was added and transfection mix was shaken vigorously and incubated for 15 minutes. During this time 25µM chloroquine was added to 293T cells. Transfection mix was then added drop wise to 293T cells and was dispensed evenly by gentle shaking. Cells were then incubated at 37°C and 5% CO_2 for 16-20 hours. Then medium was replaced with 12 ml of 5% advanced MEF medium and cells were incubated at 37°C and 5% CO_2 for 24 hours. The virus containing supernatant was harvested 42 hours (first harvest) and optionally 66 hours (second harvest) after transfection and filtered through a 0.45 µm polysulfone filter into a 15 ml Falcon tube. Viral titer was measured using Lenti-X-GoStix kit. 20 µl of viral harvest was applied to stripes provided in the kit according to manual instructions. After few minutes (5 to 10 minutes) of incubation band appeared with particular intensity which correlates to the viral titer. Viral titer could be estimated by comparing the intensity of band with that of control. In order to concentrate virus, Lenti-X concentration kit was used. Briefly filtered medium was mixed with Lenti-X-concentrator with 1 part Lenti-X-concentrator and 3 parts filtered medium. After gentle mixing, it was kept at 4°C for 30 minutes (overnight optional). Mixture was then centrifuged for 45 minutes at 2000 rpm in order to obtain pellet containing virus concentrate. Pellet was resuspended in an appropriate volume of medium as to obtain 10X concentration.

2.2.1.4 Infection of fibroblasts with lentivirus

100,000 to 200,000 cells were seeded in 6-well plates one day before virus infection. For infection, culture medium was removed and for each virus, 100 to 500 µl of 10X virus concentrate was diluted in fresh medium into final volume of 1ml containing 6 µg/ml final concentration of polybrene. Incubation was performed over night. Next day, medium containing the virus was removed and fresh culture medium was added.

2.2.1.5 Picking of colonies

Putative iPS colonies were picked in a tissue culture hood with outward pressure by gently scratching them from primary plate. After all desired colonies have been picked, it was transferred into 48-well plate coated with matrigel having mTESR medium with 10 µM Rock inhibitor. Once they have grown enough, they were transferred into 12-well plate and eventually to 6-well plate for further expansions.

2.2.1.6 Reprogramming of human fibroblasts and maintenance of human iPS cells

Human fibroblasts were infected with the Human stemcca Cre-excisable constitutive polycistronic (OKSM) lentiviral vector. Cells were kept for first two days in MEF medium and afterwards in reprogramming medium. Infected fibroblasts were allowed to grow up to 6 days (* infected cells can also be frozen down up to one year at this time point and reprogramming can be resumed at later time points). Afterwards cells were seeded on irradiated MEFs in a reprogramming medium consisting DMEM/F12 with 20% KnockOut Serum Replacement, 1 mM non-animal L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, 50 µg/ml ascorbic acid and 10 ng/ml of FGF2. After three to four weeks iPS-like colonies were picked, expanded on matrigel-coated dishes and characterized for pluripotency markers Oct4 and SSEA-4 antibodies. Human iPS cells were maintained on matrigel-coated dishes in mTeSR1 medium.

2.2.1.7 TAT-Cre treatment of human iPS cells

Human iPS colonies were treated with alphazyme or accutase (1 ml/well in 6-well plate) for 5 to 10 minutes to obtain single cells. 100,000 to 200,000 cells were seeded in each well of a 6-well plate having mTeSR1 medium with 10 µM Rock inhibitor, Y27632 to prevent the cell apoptosis. 24 hours later medium was changed to mTeSR1 containing TAT-Cre with different concentrations (0.5 µM, 1 µM and 2 µM). Cells were incubated with TAT-Cre recombinant protein for 1 to 5 hours. Cells were grown for one week and colonies were expanded either monoclonally or polyclonaly and PCR was performed to assess transgene deletion. Transgene deleted clones were expanded and characterized further by immunostaining and differentiation.

2.2.1.8 Generation of Cre reporter human iPS cell line

Human iPS cell line del-AR1034ZIMA 001 were treated with alfazyme to obtain single cell suspension. 200,000 cells were seeded on one well of a 6-well plate in mTESR1 medium with 10µM Rock inhibitor. Next day cells were infected with lentivirus containing EF1α-Cre reporter-puromycin construct. 48 hours later medium was changed to mTESR1 with puromycin (1 µg/ml) for 5 days to obtain colonies with stably integrated Cre reporter construct.

2.2.1.9 Cardiac differentiation of human iPS cells

Human iPS cells were maintained on matrigel-coated plates in mTESR1 medium until they reached 80 to 90% confluency. Cardiac differentiation was induced by BMP4 (25 ng/ml) and Chir99021 (5 µM) in RPMI1640 medium containing B-27 and 2mM glutamine and 50 µg/ml Ascorbic acid as a basal medium for 24 hours. Following 24 hours, cell were kept in same basal medium with only Chir (5 µM). Cells were then kept in RPMI basal medium with B-27 without insulin for next 24 hours and then medium was replaced with similar basal medium having WNT inhibitor either 10 µM of XAV939 or IWR1 for 96 hours. Once WNT inhibition is complete, cells were kept for 96 hours in basal medium (B-27 + insulin) and then medium was replaced with cardiac enrichment medium (RPMI 1640 without glucose + 4 mM Lactate). Cells were kept in enrichment medium for 4 to 5 days. In case cells were too dense, they were spillted (1:2) before starting with enrichment.

2.2.2 Other methods

2.2.2.1 Immunostaining

For iPS cell characterization, immunostaining was performed using Oct4 and SSEA-4 antibodies. Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) for 15 minutes and permeabilized in PBS containing 0.1% Triton X-100 and 5% FCS for 30 minutes. Cells were then incubated overnight with the Oct4 and SSEA-4 antibodies. Next day, secondary antibodies Alexa 488 and Alexa 555 were used to detect and visualize the primary antibodies. All antibodies were diluted in blocking solution. Micrographs were taken with an Axiovert 200M microscope. The above immunostaining protocol was also performed to characterize cardiomyocytes using cTNT and alpha-actinin as a primary antibodies and Alexa 488-conjugated anti

mouse IgG as a secondary antibody. Similar protocol was also used for antibodies of ISL1 and SMA.

2.2.2.2 PCR to confirm the excision of reprogramming cassette

To confirm the transgene deletion, genomic DNA from TAT-Cre treated subclones were isolated and PCR was performed with following conditions; 95°C for 2 minutes; followed by 33 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds; followed by a single cycle of 72°C for 5 minutes using the primers described in Table 2.

2.2.2.3 RT-PCR to follow up cardiomyocyte differentiation

Total RNA was prepared with the NucleoSpin RNA kit and treated with DNase. RNA (1 μg) was reverse ranscribed into cDNA via Oligo (dT) with SuperScript III Reverse Transcriptase. PCR was performed using Go Taq polymerase kit with following conditions; 95°C for 2 minutes, followed by 34 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds, followed by a single cycle of 72°C for 5 minutes using the primers mentioned in table 2.

2.2.2.4 Gene expression analysis

RNA was isolated using the RNeasy-Kit. Expression analysis had been performed following the Illumina (Illumina Inc., San Diego, CA, USA) Whole-Genome Gene Expression Direct Hybridization Assay analysis pipeline. mRNA transcription levels were evaluated using the Human HT-12 (version 4 revision 2) array which consists of 47323 probes and described mRNA features. All samples were processed in at least duplicates to reduce signals arising from processing artifacts. Data processing was performed using the GenomeStudio suite version 2011.1 and the Gene expression module version 1.9.4 (both Illumina Inc., San Diego, CA, USA). Gene expression data analysis was carried out with R and Bioconductor packages and their intensities were quintile normalized. The differentially expressed genes were determined applying the empirical bayes test statistics and Benjamini-Hochberg false discovery method was used for multiple testing corrections. Genes with fold change greater than 2 and p-value less than or equal to 0.5 were considered differentially expressed and used for subsequent analysis.

2.2.2.5 Flow Cytometry

1×10^6 cells were trypsinized and fixed with 4% PFA for 10 minutes. Cells were then washed with phosphate buffered saline (PBS), permeabilized in PBS containing 0.1% Triton X-100 and 5% FCS for 30 minutes and incubated for 2 hours with cTNT antibody. No antibody was taken as a negative control. Cells were then washed once with PBS containing 0.1% Tween-20 and resuspended in PBS containing 0.1% Triton-X 100 and 5% FCS and secondary antibody Alexa 488 anti-mouse IgG for 1 hour in dark. Finally, cells were washed again with PBS containing 0.1% Tween-20 and measured for FACS analysis. Analysis was performed by Flow Jo program.

3 Results

3.1 Derivation of human iPS cells free of reprogramming factors

3.1.1 Generation of human iPS cells

There are several ways of obtaining human iPS cells as described by earlier studies using retro- or lenti- or sendai-viruses, episomal plasmids, transposons, protein, mRNA etc (Takahashi *et al*., 2006, Soldner *et al.,* 2009, Fusaki *et al*., 2009, Chou *et al*., 2011, Kim *et al*., 2009; Zhou *et al*., 2009, Warren *et al*., 2010). In order to generate human iPS cells for subsequent experiments, we decided to use the polycistronic
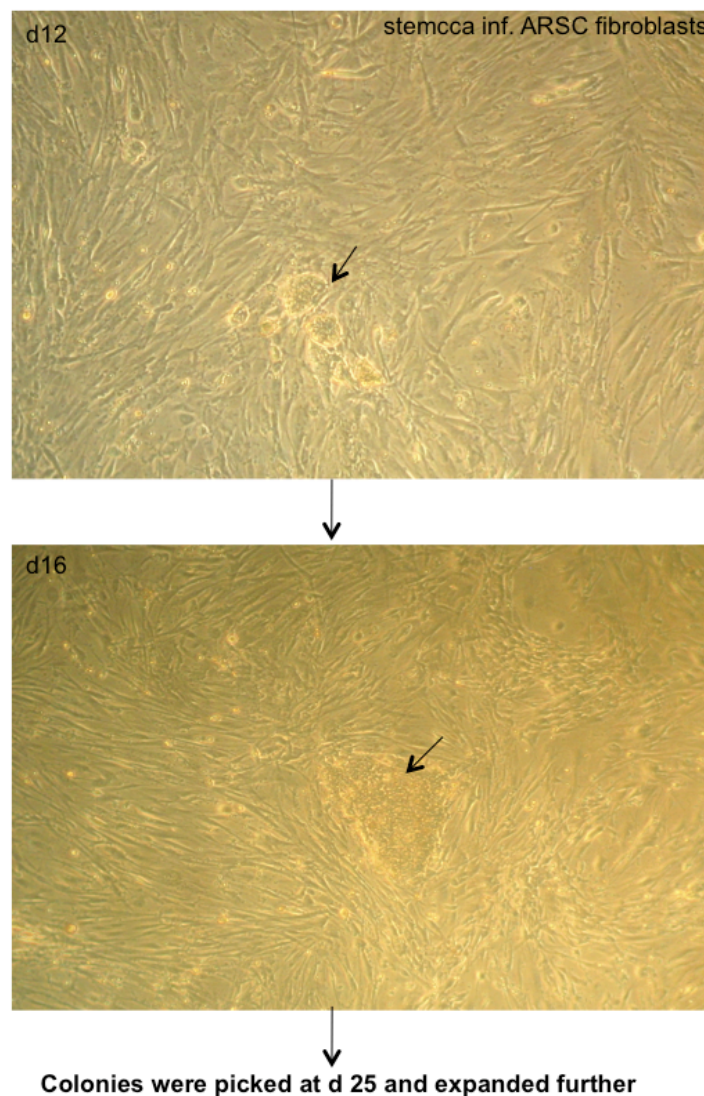

Colonies were picked at d 25 and expanded further

Figure 10: **Generation of human iPS cell line fl-AR1034ZIMA 001**. Appearance of iPS-like colonies two weeks after the initiation of stemcca induced reprogramming of AR1034ZIMA fibroblasts.

lentiviral vector stemcca due to its practical robustness (Sommer *et al.,* 2010). Initial attempts of reprogramming failed either due to high titer of virus which appeared stressful to the cells or too less virus which resulted in less efficient infection. Therefore, we first optimized the viral preparation and infection paradigm by concentrating the virus using lentiviral concentration kit as described in method part. Moreover, we also included lentiviral titer check stripes, which gives approximation of viral titer (see the material part). Along with stemcca virus, we also included lentivirus called pSico expressing green fluorescent protein (Ventura *et al.*, 2004) as a control virus in order to follow the infection process. After optimization of viral preparation, human fibroblasts obtained from the skin biopsy (see material part) were infected with stemcca vector and putative reprogrammed colonies approximately 30 to 40 appeared around day 25. 12 colonies were carefully picked and expanded as described in methods part (Figure 10). Pluripotency of 5 obtained clones were confirmed by staining the cells with pluripotency marker Oct4 and SSEA-4 (Figure 11) as well as microarray analysis (described later in the result part). During our transgene deletion studies, we used three lines fl-AR1034ZIMAiPS 001 (fl-ARiPS cl1 hereafter), del-ARiPS cl1.2 and del-ARiPS cl1.4 ('fl' means AR1034ZIMA iPS 001 containing loxP flanked reprogramming cassette 'del' means daughter clones after reprogramming cassette removal).
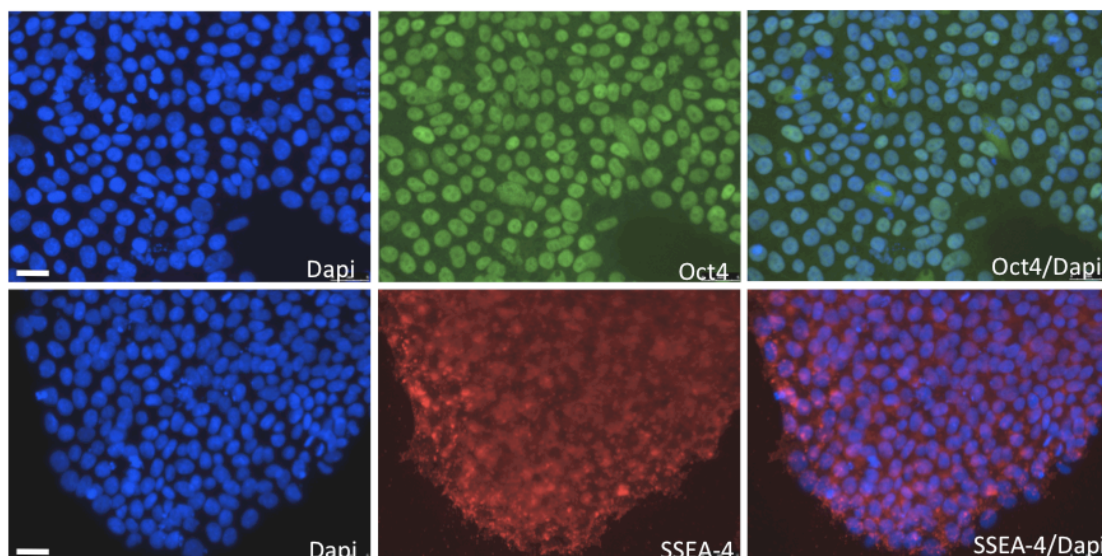

Figure 11: **Generation of human iPS cell line fl-AR1034ZIMA 001**. Pluripotency analysis of fl-ARiPS clone1. Cells stained positive for pluripotency markers Oct4, SSEA-4. Scale bar: 40 μm.

3.1.2 Removal of transgene cassette from human iPS cells

After obtaining iPS cells, we aimed for excising the reprogramming cassette in order to obtain transgene-free iPS cells. It has been shown in earlier studies that transgene-free human iPS cells resemble more to human ES cells with respect to gene expression and exhibit better differentiation potential than iPS cells still harboring the integrated reprogramming factors (Sommer *et al*., 2009). Previous studies have shown successful derivation of transgene-free iPS cells by excising a loxP-flanked transgene cassette with Cre plasmid (Soldner *et al*., 2009, Somers *et al*., 2010), Adeno-Cre (Sommer *et al*., 2010, Awe et al 2013) or Cre mRNA (Loh *et al.,* 2013). However these approaches have limitations such as low excision efficiency and thus laborious selection procedure. Moreover use of antibiotics appears stressful to the cells thus limiting its application to narrow range of the cells,
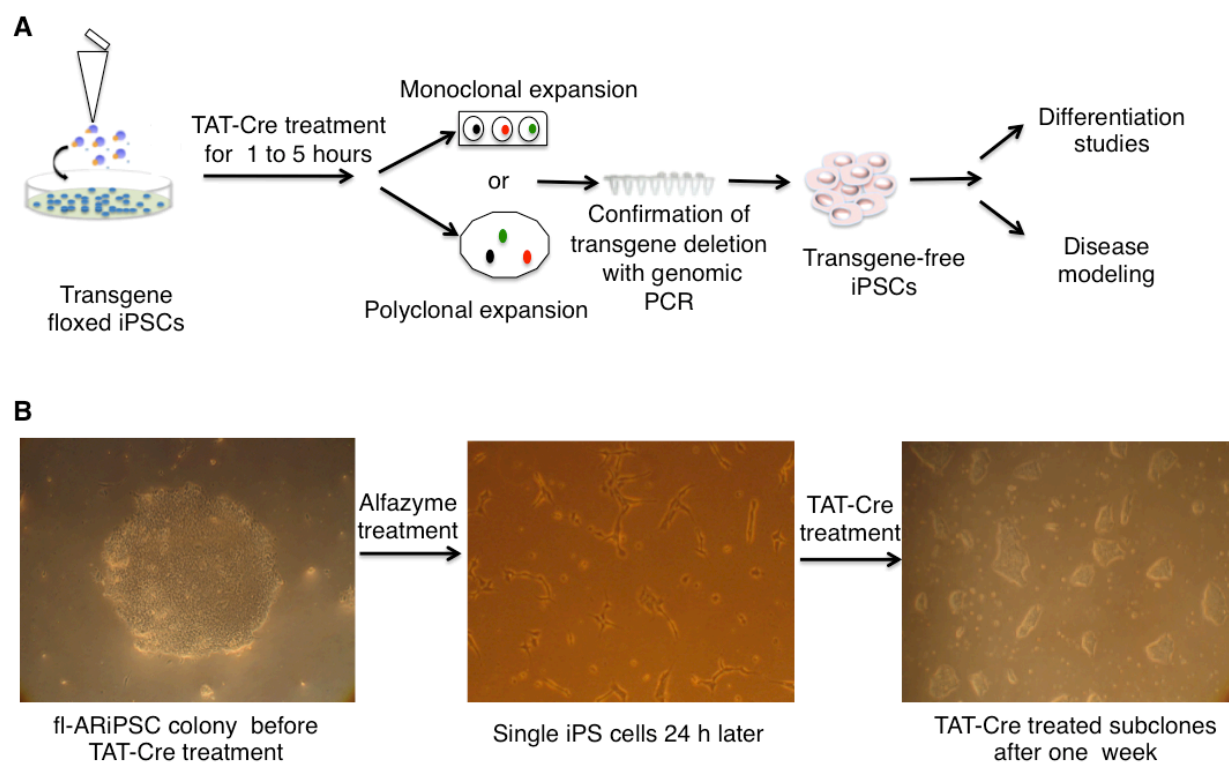

Figure 12: TAT-Cre protein transduction to obtain transgene-free iPS cells. A) Schematic representation of TAT-Cre treatment to obtain transgene-free iPS cells. B) TAT-Cre treatment of fl-ARiPS cells. iPS colonies were treated with alfazyme to obtain single cells. Cells were treated with different concentrations of TAT-Cre prorein 24 hours later. TAT-Cre treated colonies were obtained one week later.

which could be manipulated (Moran *et al*., 2009). Moreover, there is the possibility that Cre-encoding plasmids or viral constructs integrate into the genome (Glover *et al*., 2005). Therefore we aimed at improving this approach by employing Cre protein

transduction to enhance the efficiency as well as to accelerate the process of obtaining transgene-free iPS cells. This protocol does not require repeated transfections or viral preparations (See schematic overview in Figure 12A). As described in the scheme, we treated a single cell suspension of fl-ARiPS with a single shot application of TAT-Cre recombinant protein for 1 to 5 hours. TAT-Cre treated cells were expanded either monoclonally or polyclonally and analyzed further for transgene excision (Figure 12B).

3.1.3 Optimization of TAT-Cre mediated transgene deletion efficiency

First, we used Cre protein transduction conditions that was elaborated for Cre-mediated excision in human ES cells cultivated on mouse feeder cells (Nolden *et al*., 2006). Moreover, in order to make it practically more feasible we adopted our iPS cell lines to feeder-free conditions, which we assume to result in higher transgene deletion efficiency with a lower concentration of TAT-Cre. In particular, we prepared single cell suspension of fl-ARiPS cells by treating them with alfazyme and seeded them on matrigel-coated plates. 24 hours later cells were treated with different concentrations (0.5 to 6 μM) of TAT-Cre for 1 to 5 hours. TAT-Cre treated fl-ARiPS monoclones were expanded and analyzed for transgene deletion. For the confirmation of transgene excision, PCR against the viral WPRE element was used (Figure 13A). All three monoclones treated with 2 μM TAT-Cre showed excision of the transgenes, while in the case of 0.5 and 1 μM TAT-Cre treatment, we observed 1 and 2 deleted clones, respectively (Figure 13B). In order to explore the possibility of deletion and subsequent polyclonal cell expansion, we treated the fl-ARiPS cells with 0.5, 1 and 2 μM TAT-Cre for 5 hours and expanded them polyclonally. Genomic PCR analysis revealed a faint band in the case 0.5 and 1 μM and no band was observed after treatment with 2 μM TAT-Cre, indicating a high excision efficiency which was consistent with the monoclonal analysis (Figure 13C) and previously reported results employing human ES cells (Nolden *et al*., 2006). In order to validate the PCR results, we mixed genomic DNA from fl-ARiPS and human ES clones I3 in a standardized manner representing deletion efficiencies of 0 to 99%. Where, 0% and 100% means only DNA from fl-ARiPS or HES I3 cells respectively. PCR analysis of this dilution series yielded a faint band even in the case of 99% mixture while no band was observed with 100% HES I3 DNA (Figure 13D). Protein transduction was repeated with 7 monoclones treated with 1.5, 3 and 6

μM TAT-Cre in each case. As listed in figure 13E all clones analyzed showed the deletion of transgenes. Increasing concentrations of TAT-Cre beyond 3 μM resulted in significant cell death and affected the recovery of iPS colonies after the treatment (data not shown). Upon using higher concentrations of TAT-Cre, excessive cell death was prevented by shortening the time duration of TAT-Cre treatment (5 μM TAT-Cre for 1 hour) without compromising the recombination efficiency. We obtained 7 transgene-free clones out of 12 clones tested (Figure 13E).
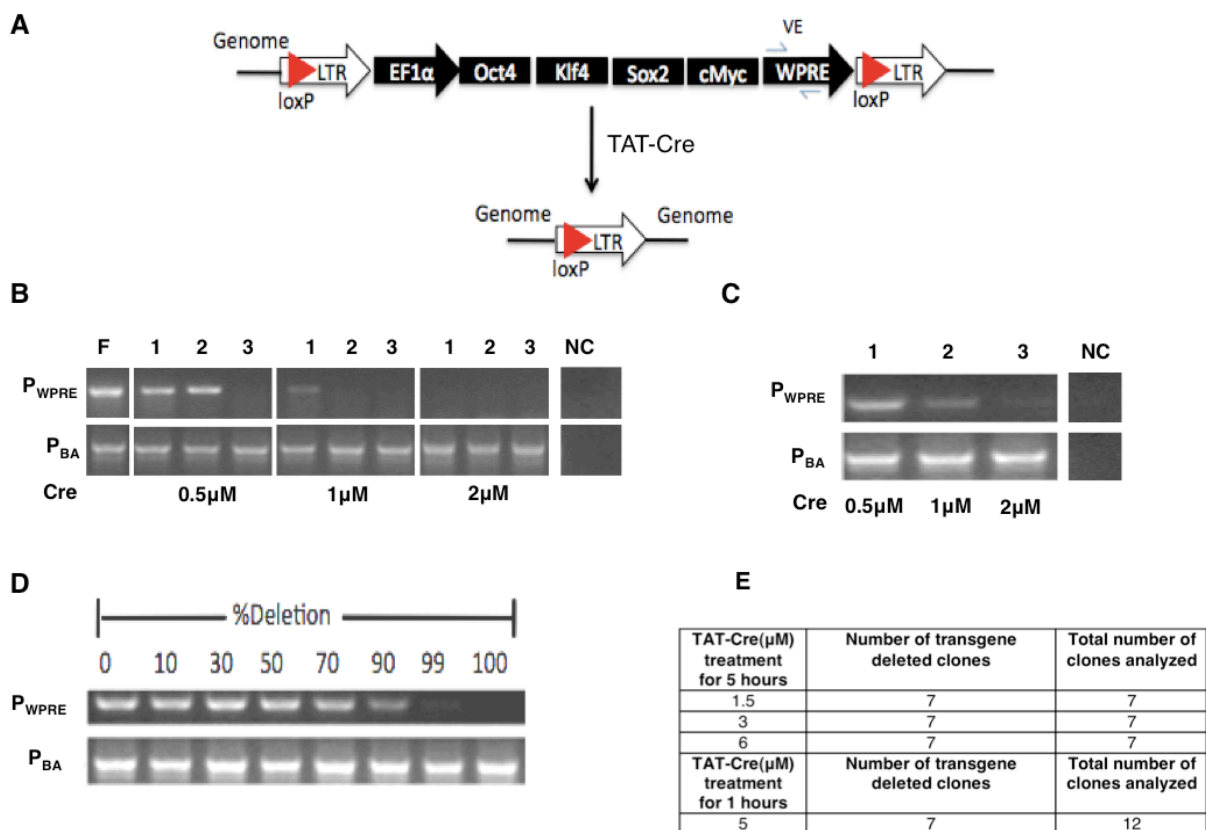

Figure 13: Optimization of TAT-Cre mediated transgene deletion efficiency. A) PCR strategy to screen for transgene-free iPS cells. B) Genomic PCR for the confirmation of transgene deletion. Individual clones were treated 0.5, 1 or 2 μM of TAT-Cre for 5 hours and PCR was performed using primers against WPRE and β-actin. fl: Floxed ARiPS cells clone 1, NC: water control, WPRE: Viral element, BA: β-actin. 1, 2 and 3 represents the fl-ARiPS cells subclones treated with 0.5, 1 and 2 μM of TAT-Cre. C) Genomic PCR for the confirmation of transgene deletion. fl-ARiPS cells were treated with 0.5, 1 or 2 μM of TAT-Cre for 5 hours. Cells were expanded polyclonally in each condition and PCR was performed using primers against WPRE and β-actin. D) Validation of genomic PCR. Genomic DNA from floxed and HES I3 cells were mixed to create defined solutions representing the deletion efficiency from 0 to 100%. E) Quantification of transgene deleted clones using different concentrations and time duration of TAT-Cre.

To further confirm the efficiency of TAT-Cre, we monitored the recombination event by integrating a double fluorescence Cre reporter cassette through lentiviral transduction of fl-ARiPS cells. Where TAT-Cre mediated excision will delete the RFP

and put the EGFP construct under the control of EF1α promoter resulting in red to green switch. We observed more than 95% of cells showing GFP expression with 2µM TAT-Cre for 5 hours (Figure 14) confirming the high recombination efficiency determined by PCR analyses.
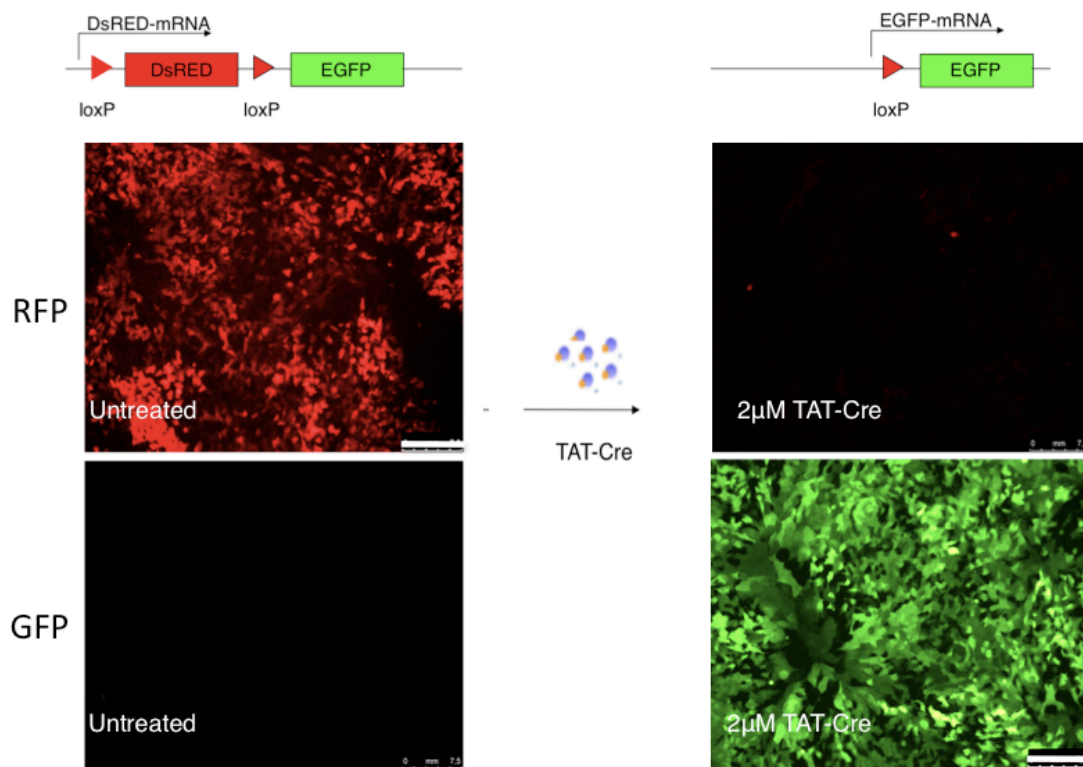

Figure 14: **Assessment of TAT-Cre mediated transgene deletion efficiency using Cre reporter line.** ARiPS-Cre reporter cell line was treated with 2µM of Cre protein for 5 hours to validate recombination efficiency. Cre-mediated recombination induced the expression of GFP, by deleting the loxP-flanking RFP gene. Scale bar: 40µM

3.1.4 Characterization of transgene-free iPS cells

In order to assess the pluripotency status, transgene excised iPS clones del-AR1034 ZIMA 001 (del-ARiPS cl1.4) were expanded until passage 15 and stained with pluripotency-associated markers Oct4 and SSEA-4. Cells stained positive for both, nuclear Oct4 and SSEA-4 at the cell surface (Figure 15).
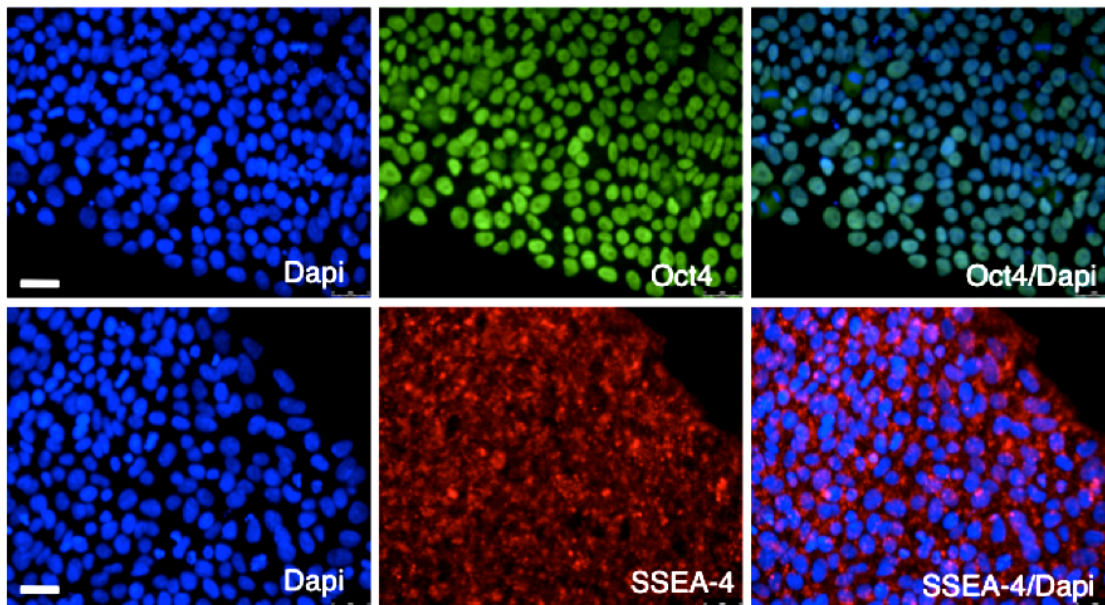
Figure 15: Pluripotency analysis of transgene excised clones. Cells stained positive for pluripotency markers Oct4, SSEA-4 in the case of del-ARiPS cl1.4 cells. Scale bar: 40μM.

Furthermore, we did detailed pluripotency analysis by performing genome-wide gene expression profiling on del-ARiPS cl1.2 and cl1.4 and fl-ARiPS cells. The gene expression datasets
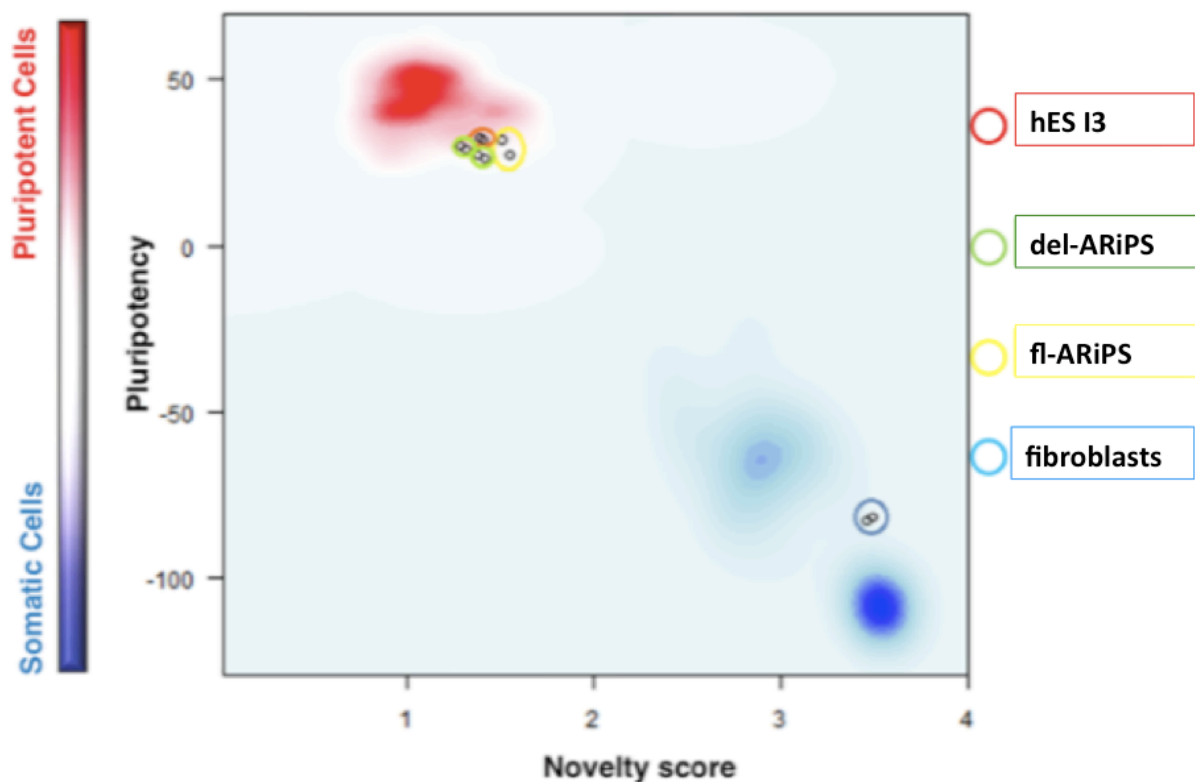

Figure 16: PluriTest analysis of the human ES cells I3, fl-ARiPS cells, del-ARiPS cl1.2 and cl1.4 cells and fibroblasts to assess pluripotency. Cells are distributed based on pluripotency and novelty scores as indicated by color density background. Red color indicates pluripotency and blue indicates non-pluripotency.

50

were subjected to the recently published bioinformatics assay, PluriTest (Muller *et al*., 2011) to assess the pluripotency status of reprogrammed cells. It is an easy substitute for time-consuming teratoma analysis and requires just input of a gene expression dataset of experimental samples, which are distributed according to their pluripotency. According to this analysis both, fl-ARiPS and del-ARiPS clones cluster with human ES cell line I3 in the red colored background indicating pluripotency, while fibroblasts are located in the blue region confirming their non-pluripotent nature (Figure 16). Notably, the del-ARiPS clones appear a bit more shifted to the human ES I3 cells as compared to fl-ARiPS. In fact, there were 63 differentially expressed genes
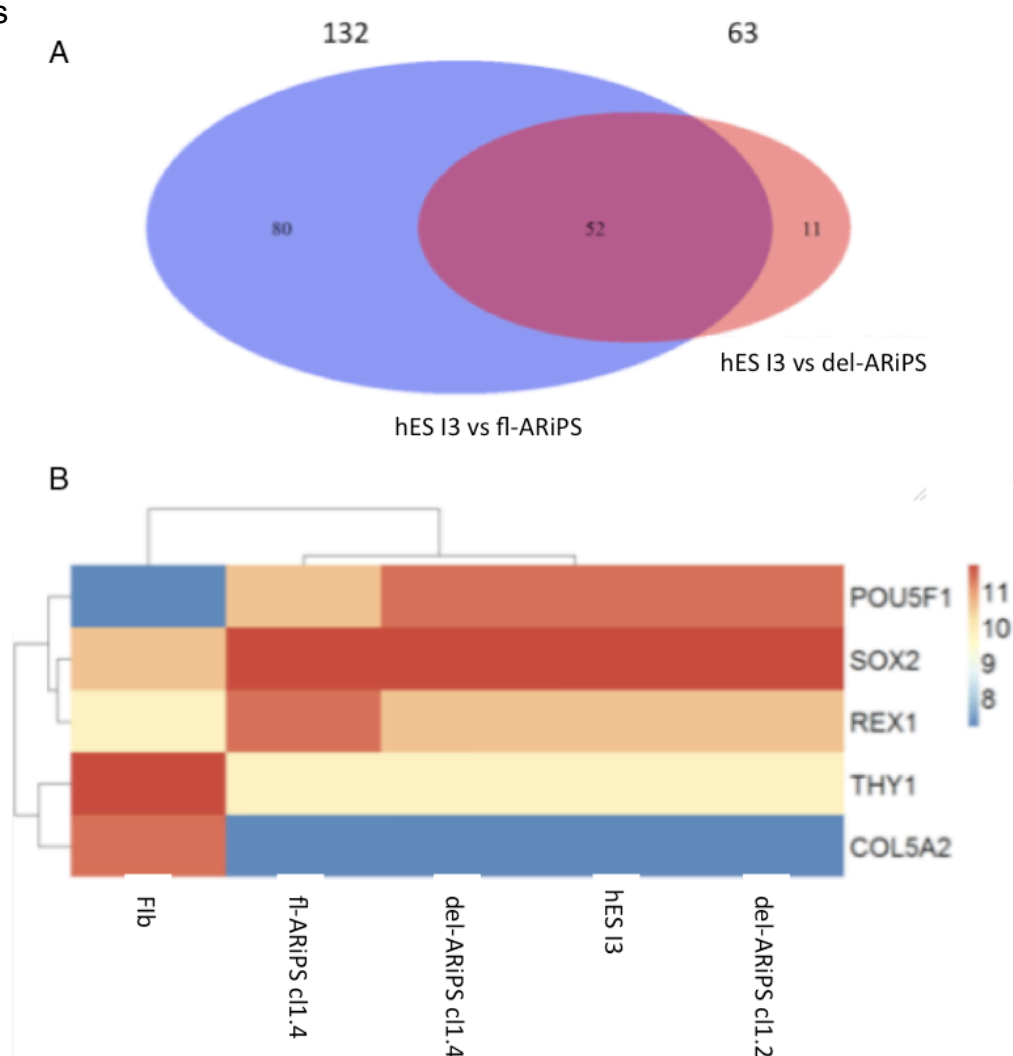

Figure 17: Gene expression analysis of transgene containing as well as transgene excised clones. A) Venn diagram showing differentially expressed genes. Comparison of differentially expressed genes amongst human ES cell I3, fl-ARiPS cells and del-ARIPS cells. B) Heatmap representation of pluripotency- and fibroblast-specific markers. Expression of pluripotency markers Oct4, Sox2, Rex1 and fibroblast markers Thy1 and Col5a2 in iPS cells and human ES cell used for this study.

51

between human ES cell I3 and del-ARiPS cl1.4 cells as compared to more than 130 genes in the case of the parental fl-ARiPS clone (Figure 17A). Expression profiling of pluripotency-associated genes Oct4, Sox2, Rex1, as well as fibroblast genes Thy1 and Col5a2 showed similar expression pattern across the iPS cells and human ES cells. Again del-ARiPS clones appears more similar to human ES I3 than fl-ARiPS (Figure 17B)

3.1.5 Improved differentiation potential of transgene-free iPS cells

In order to explore whether the deletion of transgenes results not only in genome-wide transcriptional differences but also has functional consequences, we analyzed the differentiation potential by differentiation into the cardiac lineage.
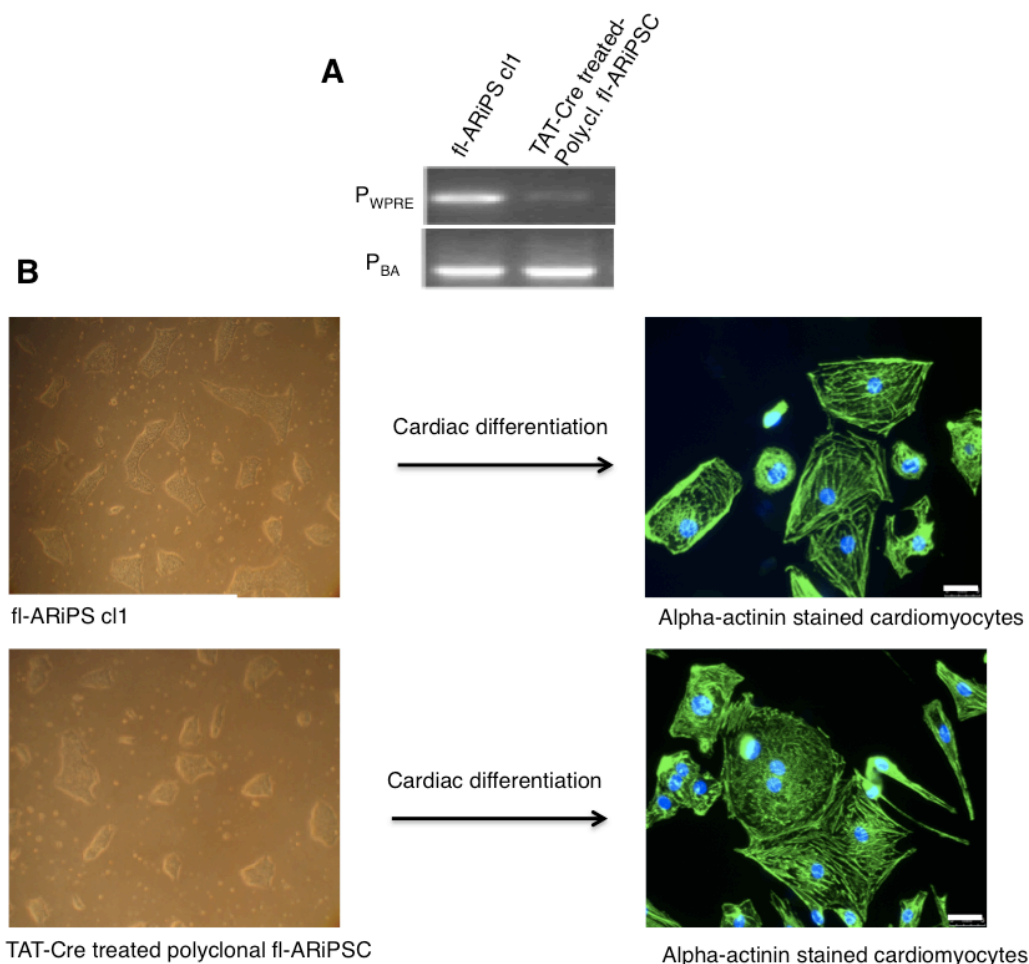

Figure 18: Cardiac differentiation of human iPS cells. A) Genomic PCR for the confirmation of transgene deletion. fl-ARiPS was treated with 2 µM of TAT-Cre for 5 hours and PCR was performed using primers against WPRE and β-actin. B) Cardiomyocyte differentiation of iPS cell lines. fl-ARiPS and its TAT-Cre treated polyclonal cell population were differentiated to cardiomyocytes using cardio-inductive medium. Cells were stained with α-actinin antibody at day 15. Scale bar: 40µM.

We employed our protocol described in methods part for the cardiac differentiation of fl-ARiPS and a polyclonal TAT-Cre treated daughter cell population (2 µM TAT-Cre

for 5 hours) (Figure 18A and 18B). We decided to do polyclonal expansion in order to reduce the time duration of the entire procedure and check whether it is possible to see the overall enhancement in differentiation capacity of a polyclonal TAT-Cre treated population. Indeed, flow cytometry analysis using cardiac troponin T (cTNT) as a cardiomyocyte-specific marker indicates a strongly increased differentiation capability of polyclonal TAT-Cre treated daughter cell population. More than 55% of differentiated are cTNT-positive in the case of TAT-Cre treated cell population, whereas only 37% of the parental fl-ARiPS cells stained positive for cTNT (Figure 19).
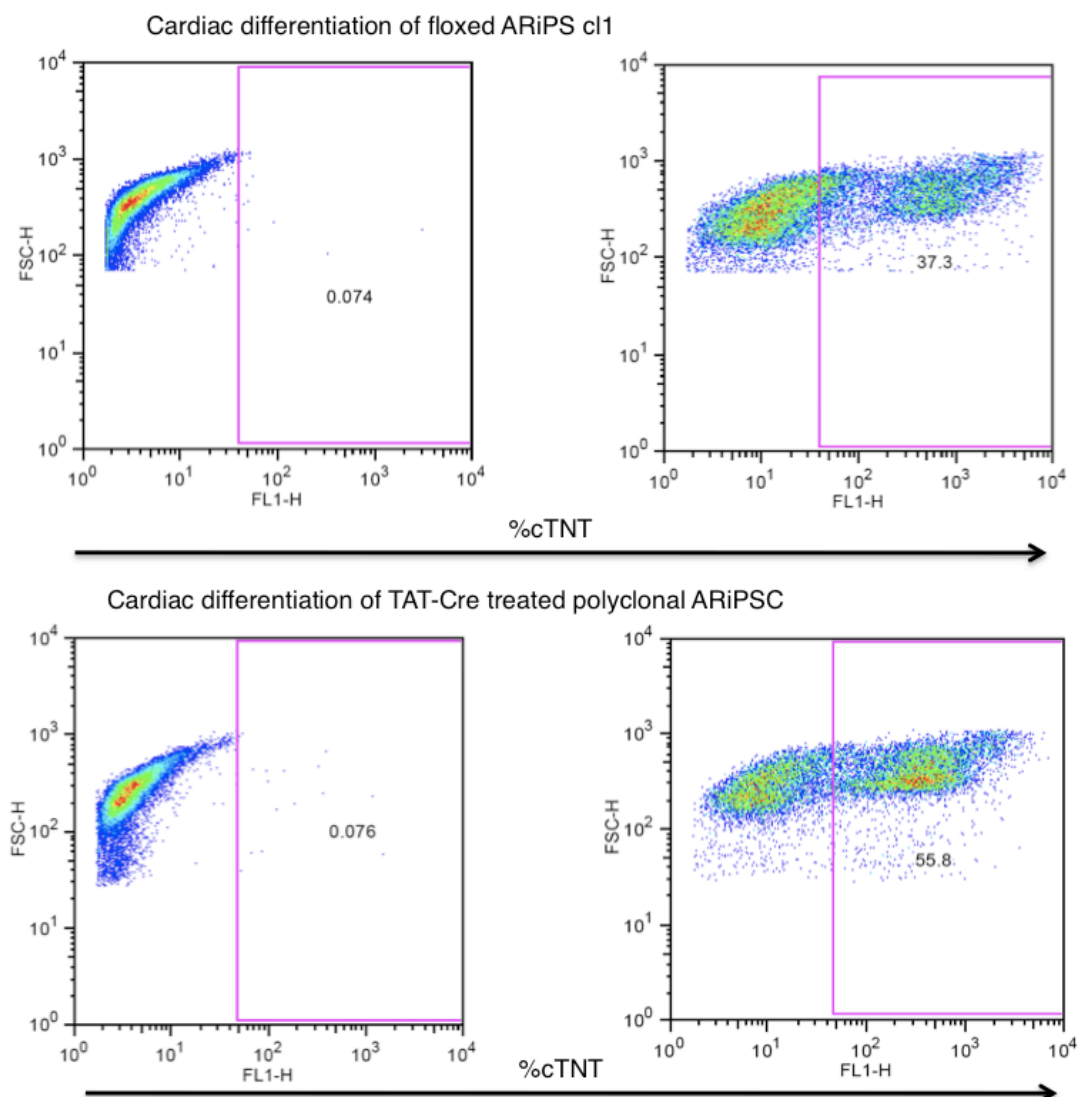
Figure 19: Flow cytometry analysis of cardiac-specific troponin T staining at day 15 of cardiac differentiation. Cardiac differentiation showed an increase from 37 to 55% cTNT positive cardiomyocytes in the case of TAT-Cre treated cell populations. The experiment was repeated twice with similar results. Note: Sharp boundaries in above flow cytometry results are due to set threshold value of FL1-H channel and gating of the cell population.

3.2 Cardiomyocytes differentiation of human iPS cells

3.2.1 Optimization of cardiomyocytes differentiation

In order to obtain cardiomyocytes for our subsequent experiments, we referred to the earlier studies showing successful cardiac differentiation of human pluripotent stem cells using EB based as well as monolayer approaches (Yang *et al.,* 2008, Hudson *et al.*, 2011,Carpenter *et al.,* 2011). First we did the selection of basal medium by thoroughly comparing the medium described in earlier studies. After our literature findings, we decided for three mediums namely DMEM F/12, RPMI1640 and Stempro as a basis of our initial experiments in parallel. Moreover we always kept B-27 supplement in order to replace serum. Ascorbic acid was also included in our formulation due to its positive effect on cardiomyocytes differentiation (Cao *et al.*, 2011). The first approach we tried involves formation of embryoid bodies (EBs) in the presence of growth factors known to be involved in cardiac development such as ActivinA, BMP4, FGF-2 and VEGF as well as DKK-1, which is a WNT inhibitor that directs the EB differentiation into cardiomyocytes lineage (Yang *et al.*2008). In spite of repeated attempts, we failed to obtain beating clusters (Figure 20).
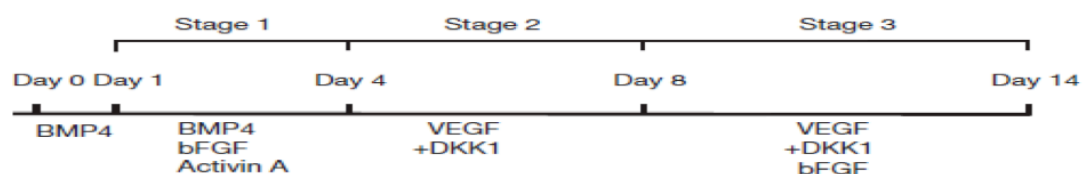
Figure 20: Cardiomyocytes differentiation of iLB-C-50-s9 cl1 using EB based approach. A) Scheme of cardiac differentiation of human iPS cells using EB formation B) Cardiac differentiation in the presence of growth factors resulted in non-beating cell clusters after plating of the EBs.

Since EBs have complex microenvironments, we reasoned that it would be easy to manipulate the key signals in the monolayer based approach and hence we switched

to differentiation protocol utilizing monolayer of human iPS cells. During our first attempts in this direction includes the protocol described by Hudson *et al* (Hudson *et al.*, 2011). We treated human iPS cells with BMP4 and Activin A in combination with WNT inhibitors IWR1 as described in the protocol (Figure 21A). In spite of several repetitions, we did not observe beating in the case of DMEM F/12 and Stempro medium but we got few beating patches in the case of RPMI1640 medium. Hence we could narrow down our selection to only RPMI1640 as a basal medium for subsequent attempts. In order to follow the differentiation, we did the gene expression analysis during different time points and stained the cells at the end of differentiation. During our analysis we observed early cardiovascular precursor marker ISL1 appeared from day 4 till day 10 of differentiation, while the cardiac specific marker Nkx2.5 was not expressed throughout differentiation (Figure 21B).

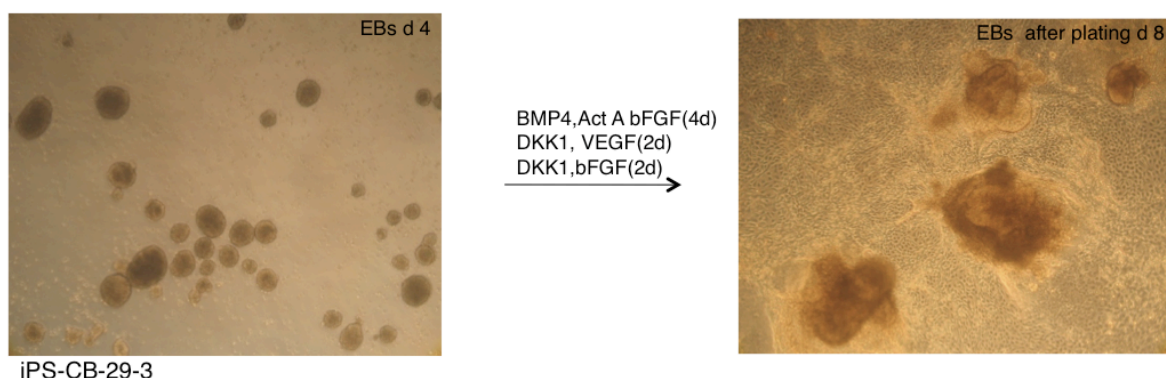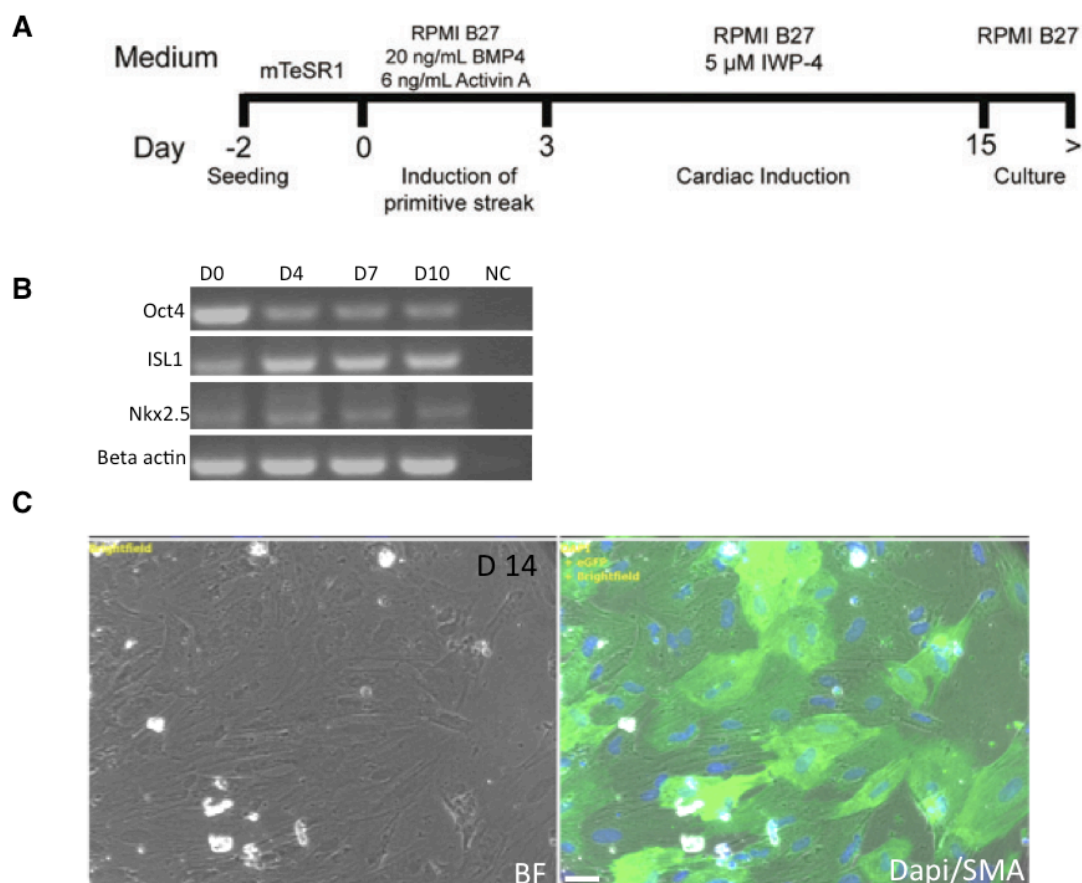**Figure 21: Analysis of lineage specific marker expression during cardiomyocytes differentiation of iLB-C-50-s9 using monolayer based approach**. A) Scheme of cardiac differentiation of human iPS cells using monolayer approach .B) Gene expression analysis at different time points during cardiac differentiation. C) Staining of SMA at day 14 of differentiation indicated majority of positively stained cells. Scale bar: 40 µM

This observation led us to conclude that we could differentiate the cells till the early cardiovascular fate but they were not further specified in to cardiomyocytes instead

differentiated mostly into smooth muscle cells as indicated by smooth muscle actin (SMA) staining (Figure 21C). Based upon our initial attempts, we realized that cardiac specific growth factors alone are not sufficient to efficiently drive the cells into cardiomyocytes fate. Hence to achieve efficient cardiac differentiation we also focused on WNT signaling. Since cardiomyocyte differentiation critically depends upon WNT signaling as described in earlier studies (Cohen *et al.,* 2008), our next step was to screen for the molecules with which one can tightly control WNT signaling. In order to assess the activity of WNT signaling, we used WNT reporter cell line where expression of GFP correlates to WNT activity (described in the materials and method part). We used previously described WNT modulators Chir99021 (designated as Chir hereafter), BIO, XAV939, KY02111 and WNT-C59 as a basis for our screening. During our analysis we found out that 5 µM Chir strongly activates WNT signaling as judged by GFP positive cells during FACS analysis and 10µM XAV939 showed strongest WNT inhibition without causing excessive cell death, while KY0211 failed to suppress WNT activation (Figure 22) and WNT-C59 and BIO appeared toxic to the cells. Hence we did the selection of Chir as WNT activator and XAV939 as WNT inhibitor during the subsequent experiments.
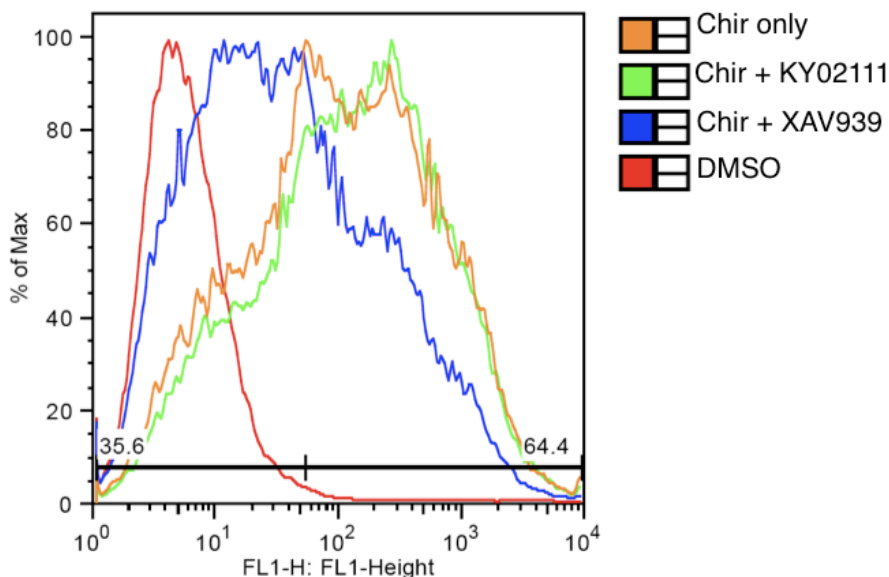
Figure 22: Flow cytometry analysis of GFP expression after the treatment of WNT reporter line with WNT modulators. Cells showing GFP expression after treatment of WNT reporter lines with Chir, XAV939 and KY02111 respectively.

After the selection of WNT modulators, we decided to formulate different combination of the previously tried out growth factors and small molecules in order to enhance cardiac differentiation. As a quick read out for cardiovascular induction we

decided to check the expression of T (Brachyury) and ISL1 at day two and five of differentiation respectively. After trying out different combinations and concentrations of small molecules and growth factors (Table 2 and 3), we found out that combination of BMP4 and Chir resulted in synchronously beating culture at the end of differentiation. In particular BMP4 (25 ng/ml) and Chir (5 µM) strongly enhanced expression of T (Brachyury) (Figure 23A) and cardiovascular marker ISL1 (Figure 23B) and eventually beating of culture. Apart from concentration, time duration of treatment also appeared very critical e.g only 48 hours treatment of Chir was most effective. Similar was true for BMP4, only 24 hours treatment resulted in desired beating of culture. Rest all combinations either increasing or decreasing the time duration of treatment of Chir and BMP4 resulted in decrease of observed beating.

Application of Chir in combination with 24h BMP4 (25 ng/ml)		
Conc (µM). of Chir	Time in hours	Extent of cardiac differentiation
5	24	-
5	48	+++
5	72	+
10	24	-
10	48	-
10	72	-
15	24	-
15	48	-
15	72	-
Application of Chir in combination with 48h BMP4 (25 ng/ml)		
5	24	-
5	48	++
5	72	-
Application of Chir only		
5	24	-
5	48	+
5	72	-
10	24	+
10	48	-
10	72	-
15	24	-

Table 2: Optimization of cardiac differentiation of iLB-C-50-s9 by varying concentration of Chir in combination with 25 ng/ml of BMP4. Here (-): no beating, (+): few beating patches, (+++): synchronous beating throughout well. All above combinations were tried in combination with WNT inhibition phase from Day 3 to 8 of cardiac differentiation.

Application of BMP4 in combination with 48h Chir (5 µM)		
Conc (ng/ml). of BMP4	Time in hours	Extent of cardiac differentiation
12.5	24	+
25	24	+++
50	24	+

Table 3: Optimization of cardiac differentiation of iLB-C-50-s9 by varying concentration of BMP4 in combination with 5 µM of Chir. Here (-): no beating, (+): few beating patches, (+++): synchronous beating throughout well. All above combinations were tried in combination with WNT inhibition phase from Day 3 to 8 of cardiac differentiation.

In order to achieve proper cardiac specification of early cardiac mesoderm induced by BMP4 and Chir treatment, we used WNT inhibitor XAV939. During WNT reporter assay 10µM XAV939 was most effective, hence we used it in same concentration and applied it to different time points during our cardiac differentiation. In our analysis we found out that early time point application (Day 1 or 2 of cardiac differentiation) of WNT inhibition completely block the cardiac differentiation. While WNT inhibition from day 3 onwards resulted in appearance of beating, with day 3 to 7 was most of effective time window for achieving efficient cardiac specification (see Table 4). We also used another WNT inhibitor IWR1 and obtained similar results as in case of XAV939. After several rounds of repetition, we could only get reproducible results in a basal medium devoid of insulin. Hence cardiac specification was always done in absence of insulin. Detailed reasons for insulin effect are explained in discussion part.

Optimization of Wnt inhibitor application	
Time window of application of 10 µM XAV939 or 10 µM IWR1	Extent of cardiac differentiation
Day 1 to 3	-
Day 2 to 5	-
Day 3 to 6	++
Day 3 to 7	+++

Table 4: Optimization of cardiac differentiation of iLB-C-50-s9 by varying time window of WNT inhibition. Here (-): no beating, (+): few beating patches, (+++): synchronous beating throughout well. All above combinations were tried in combination with cardiac induction phase using 24hours BMP4 and 48 hours Chir treatment during Day 1 to 3 of cardiac differentiation.

Upon combining efficient cardiovascular induction with WNT inhibition (see Table 4) during late phase of differentiation using XAV939 or IWR1 (10µM), we achieved

synchronously beating cardiomyocytes up to 92% of purity at day 15 of differentiation as judged by flow cytometry analysis using cTNT specific staining (Figure 23). Removal of any compounds from the cocktail resulted in decrease of T (Brachyury), ISL1 and eventually cTNT positive cells (Figure 23 and 24).
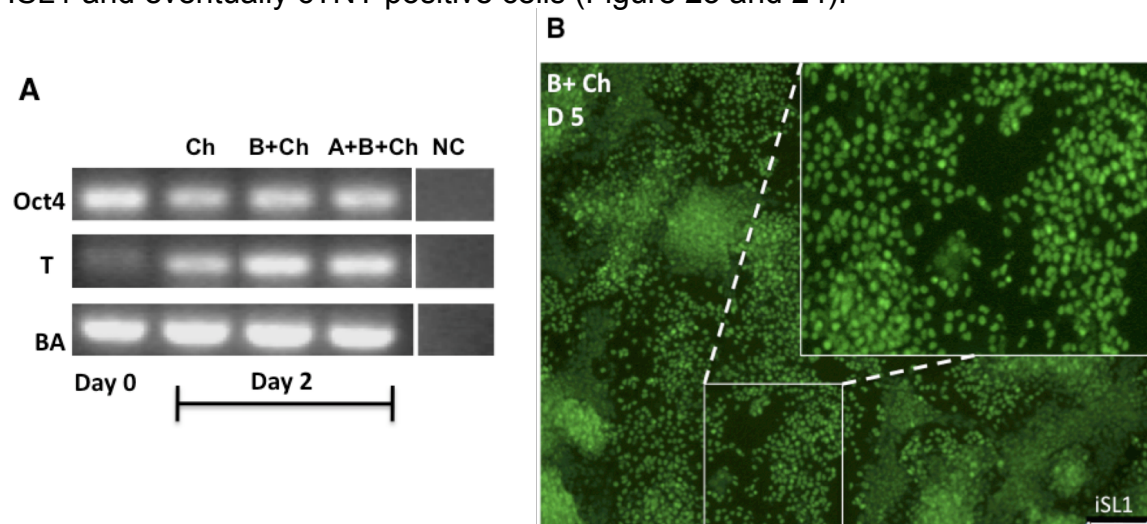

Figure 23: Optimization of cardiac differentiation of iLB-C-50-s9 A) RT PCR analysis to assess the expression of T (Brachyury) expression at day 2 of cardiac induction using different conditions namely Ch, B+Ch and A+B+Ch B) Immunostaining using cardiac precursors maker ISL1 at day 5 of cardiac differentiation using small molecule combination (B+Ch). Scale bar: 80 µM. Abbreviations, GFP: green fluorescent protein, Ch: Chir99021, B: BMP4, A: Activin A.
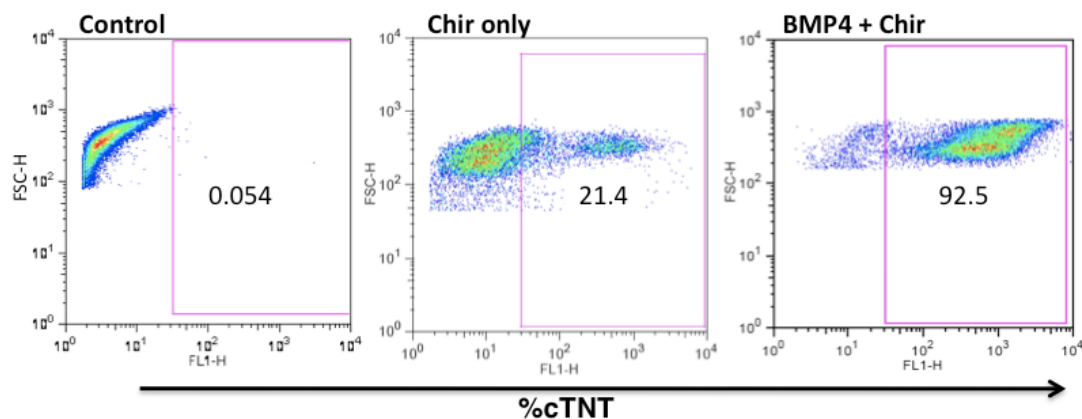

Figure 24: Optimization of cardiac differentiation of iLB-C-50-s9 Flow cytometry analysis of cardiac-specific troponin T staining at day 15 of cardiac differentiation showed 21.4% cTNT positive cardiomyocytes in the case of a WNT activator Chir and 92.5% cTNT positive cardiomyocytes with combined application of BMP4 and Chir.

We also performed RT-PCR analysis with cardiovascular and cardiac specific markers to follow the differentiation process. During the induction phase iPS cells were treated with our formulation (BMP4 and Chir) in a basal medium, which resulted in strong up regulation mesendodermal marker T (Brachyury) (Figure 25). Induction phase was followed by treatment with WNT inhibitors in a basal medium

devoid of insulin in order to achieve proper specification into cardiac mesoderm, which was confirmed by expression of early and late cardiac precursor markers ISL1 and Nkx2.5 respectively (Figure 25). Cells then further matured into beating cardiomyocytes expressing specific marker cTNT (Figure 25).
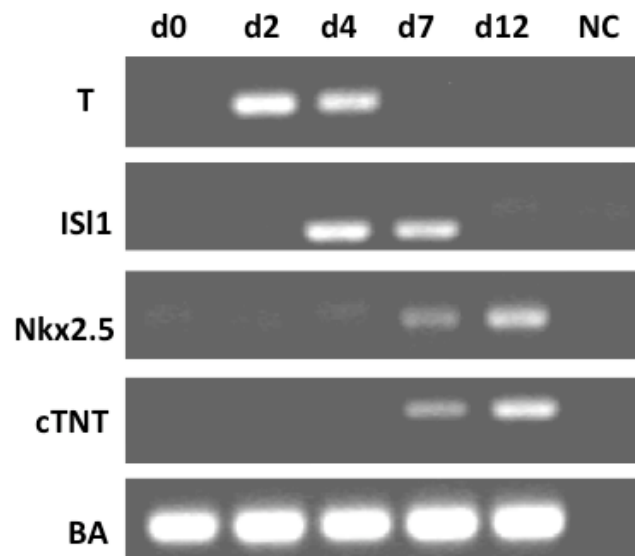

Figure 25: Gene expression analysis for cardiovascular and cardiac specific genes. mRNAs at different time points during the differentiation were isolated and RT-PCR analysis was performed showing the subsequent passage of cells though the mesendodermal, mesoderm and cardiac specific precursor state.

3.2.2 Cardiac differentiation of multiple human iPS cell lines and subsequent enrichment of cardiomyocytes

After optimization of cardiac differentiation of our standard iPS cell line (iLB-C-50-s9), we checked the effectiveness of our devised protocol on multiple iPS cell lines representing different origin of cells (fibroblasts, keratinocyte and cord blood cells) as well as methods of reprogramming (retrovirus, lentivirus and sendai virus). Although this optimized protocol gave rise to high purity population of beating cells using our standard iPS cell line, the outcome with several other iPS cell lines varied substantially. In particular, we obtained the yield of cTNT-positive cells from 33.8% and 92.5% (Figure 26A). In order to bring purity of cardiomyocytes from different iPS cell lines to the same level, it was necessary to devise step where cardiomyocytes can be separated out from non-cardiac cells. There are several strategies published in order to achieve the enrichment such as FACS sorting using cardiac specific marker or dye (as described in introduction). Since such additional steps are time

consuming and stressful to the cells, we decided to apply lactate based cardiac enrichment (Tohyama *et al*., 2013) in the late phase of our protocol. This approach utilizes clever trick considering the different metabolic requirement of cardiac and non-cardiac cell. In particular cardiomyocytes can metabolize the lactate as a source of energy unlike non-cardiac cells, hence providing the basal medium having only lactate as a source of energy will result in cardiac enrichment as non-cardiac cells will die out due to lack of energy source. In order to achieve this, we switched the medium at day 12 of cardiac differentiation to basal medium without glucose but supplemented with 4mM lactate. In fact when we combined lactate enrichment, we could obtain 95% pure cTNT positive cardiomyocytes from the iPS cell lines iLB-C-30-r12 which otherwise gave 62% positive cardiomyocytes. Similar enrichment was achieved in the case of fl-AR1034ZIMA 001 iPS cell line from 33 to 74% cTNT positive cells (Figure 26B).

A

Human iPS lines	% cTNT w/o enrichment	% cTNT with enrichment
iLB-C-50-s9 (Cord blood)	92.5	ND
del-AR1034ZIMA 001(Fibroblast)	82.4	ND
k-hiPS (Keratinocyte)	42.4	ND
iLB-C1-30m-r12 (Fibroblast)	62.8	95.5
fl-AR1034ZIMA 001 (Fibroblast)	33.8	74.3

B
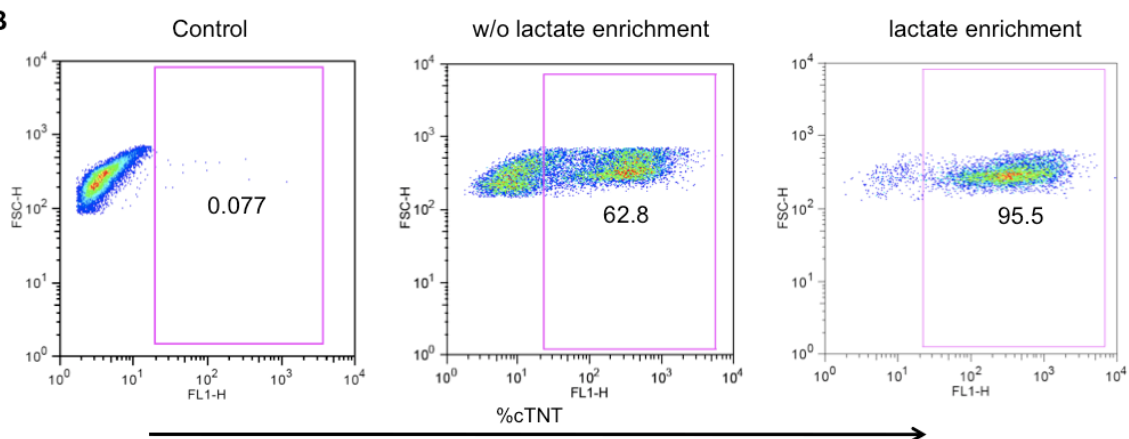
Figure 26: Enrichment of cardiomyocytes with sodium-L-lactate. A) Summary of cardiac differentiation of different human iPS cell lines using efficient cardiac differentiation followed by cardiac enrichment. B) Flow cytometry analysis of cardiac-specific troponin T staining at day 16 of cardiac differentiation showed about 62.8% cTNT positive cardiomyocytes without lactate enrichment and 95.5% cTNT positive cells after lactate enrichment. Note: Sharp boundaries in above flow cytometry results are due to set threshold value of FL1-H channel and gating of the cell population.

In conclusion, our optimized protocol consists of three phases namely cardiovascular induction, cardiac specification and cardiomyocyte enrichment as described in figure 27 in order to achieve robust cardiac differentiation of human iPS cells.
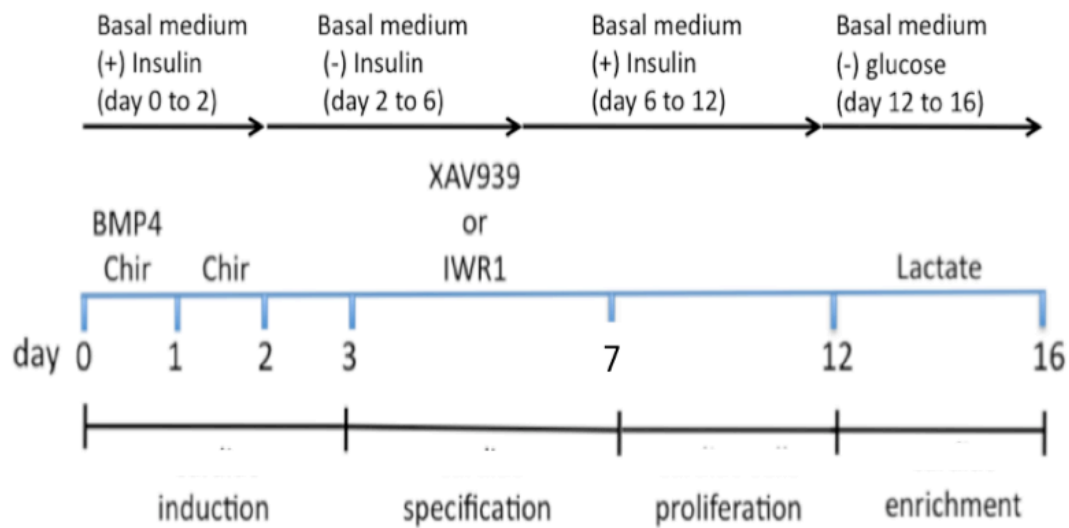

Figure 27: Optimized protocol for cardiac differentiation of human iPS cells. Scheme of efficient cardiac differentiation of human iPS cells with combination of strong cardiac induction in early phase and cardiac enrichment in late phase

3.3 Characterization of human iPS derived cardiomyocytes

3.3.1 Structural features of human iPS-CM

After obtaining cardiomyocytes using optimized protocol, we performed standard immunohistochemical stainings as well as electron microscopy to study structural characteristics. Obtained cardiomyocytes showed strong cardiac specific alpha-actinin staining with typical striation pattern as well as cTNT staining (Figure 28).
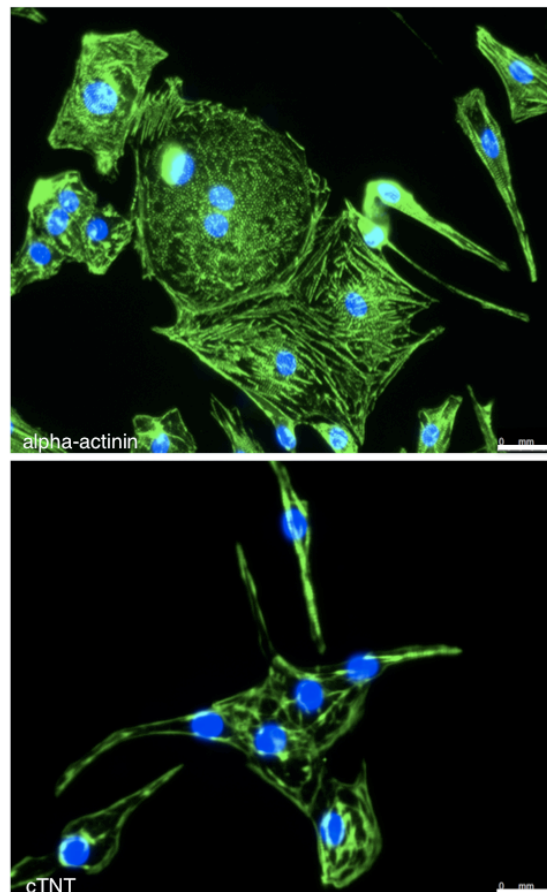
Figure 28: Characterization of human iPS-CMs by immunostainings of cytoskeleton proteins. Immunohistochemical characterization of human iPS (del-AR1034ZIMA 001) derived cardiomyocytes using antibody against alpha-actinin (top) and cardiac troponin T (bottom). Scale bar: 40 µm

In order to study the maturation state of iPS-derived cardiomyocytes, we performed ultra-structural analysis employing TEM of 21 day old cardiomyocytes. Many cells show nascent parallel arrays of myofilament bundles anchored at Z-band like electron dense structures. They show different spatial orientation within the same cell as well as branching. Moreover, we observed sarcomer-like organization of

Figure 29: Ultrastructural analysis of 21-day old human iPS-CMs (del-AR1034ZIMA 001). A) Two cells in close contact displaying sarcomer-like organization of contractile fila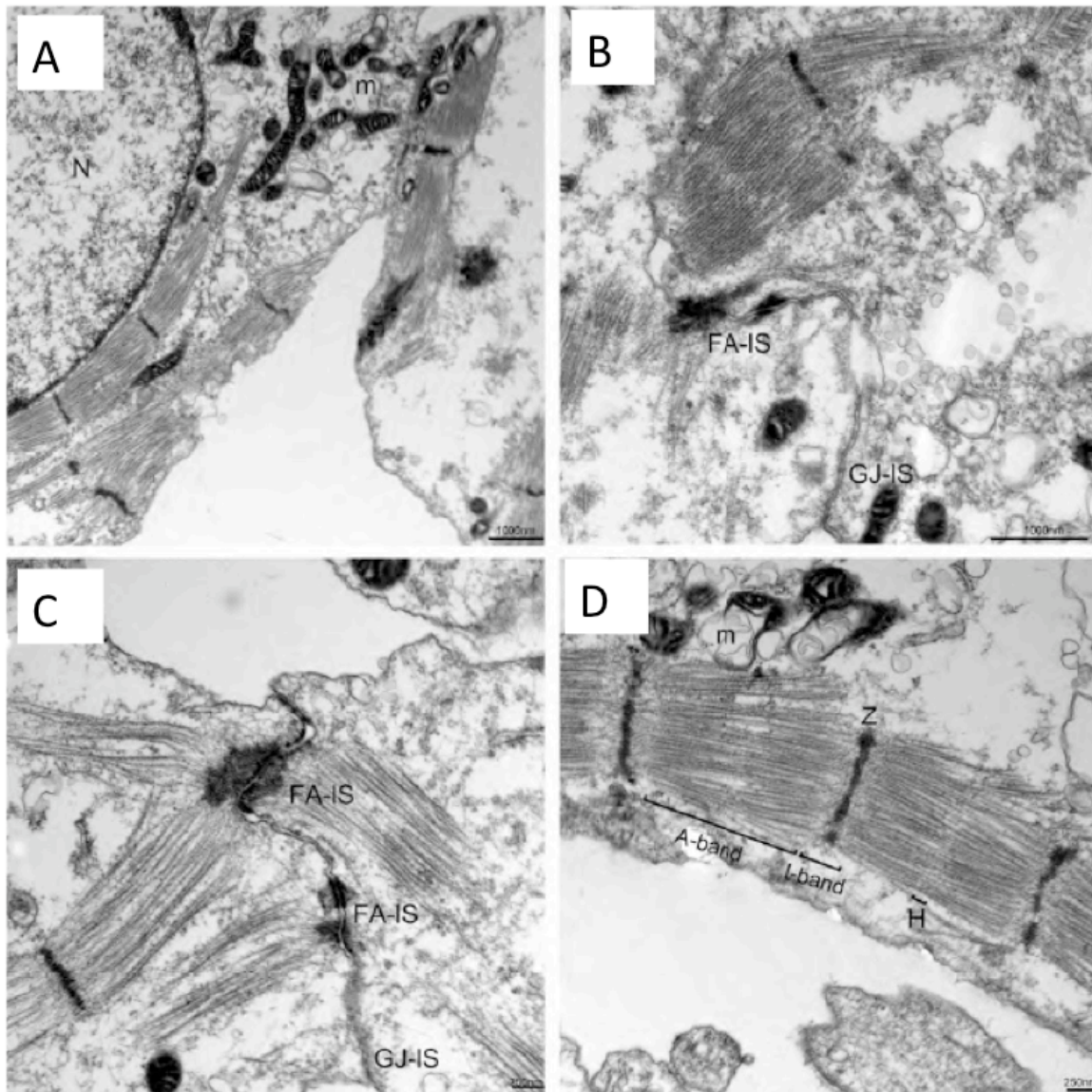ments. Scale bar: 1000nm B-C) Higher magnification showing the presence of fascia adherens-like and gap-junctions like cellular contacts and initial sarcomeric organisation of actin and myosin filaments. Scale bar: (B) 1000nm, (C) 250nm. D) iPS cell-derived cardiomyocyte-like cells show sarcomer organization of contractile filaments with already identifiable A-, I-, Z- and H-bands. Abbrevations: m: mitochondria; N: Nucleus; FA-IS: Fascia adherens-like structure; GJ-IS: Gap junctions-like structure; Z: Z-band; H: H-band. Scale Bar: 250nm. **(* These results were obtained with help of Subbarao Mekkala, Nicole Wagner and Süleyman Ergün from University of Würzburg)**

contractile filaments (Figure 29A). Additionally, fascia adherens-like and gap-junctions-like cellular contacts as well as initial sarcomeric organisation of actin and myosin filaments were detected. The sarcomeric structures of contractile filaments exhibited already identifiable A-, I-, Z- and H-bands (Figure 29B-D).

3.3.2 Electrophysiological properties of human iPS-CMs

It is essential to obtain cardiomyocytes exhibiting characteristics electrical activities for their future applications. Therefore we carried out detailed electrophysiological analysis using standard patch-clamp methods. Action potential recordings on single beating cardiomyocytes showed typical spontaneous action potentials (Figure 30A), with atrial (n=2) or ventricular (n=4)-like morphology as well as characteristic voltage dependent inward and outward currents using voltage ramps (Figure 30B).
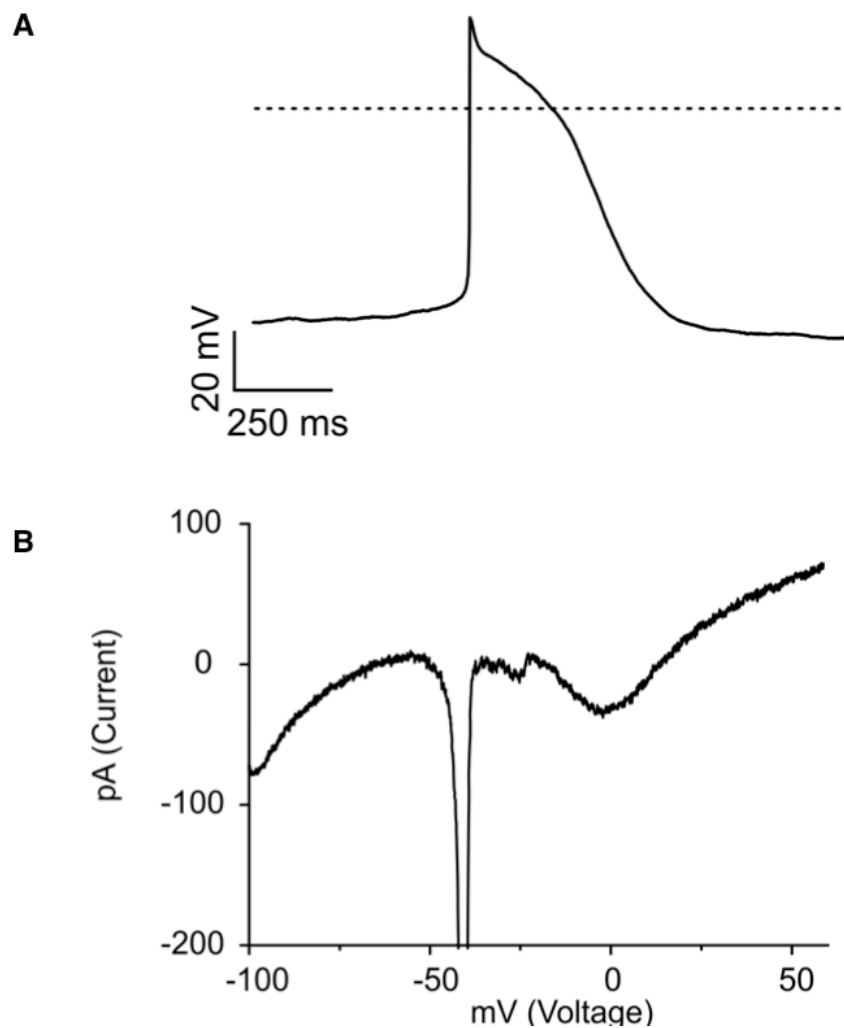
Figure 30: Electrophysiological characterization of human iPS-CMs (del-AR1034ZIMA 001). A) Action potential recorded from a ventricular like cardiomyocyte. B) Typical activation of voltage dependent inward and outward currents following a ramp protocol in voltage clamp (-100 to +60 in 250ms). **(* These results were obtained with help of Daniela Malan and Philipp Sasse from University of Bonn)**

In order to check for calcium current activity, we performed whole cell calcium currents recording. L-type Ca^{2+} currents carried by cardiac $Ca_V1.2$ (Figure 31A) showed a half-maximum activation at -13.69±0.97 mV, reached a maximum current

density of -11.55±1.6 pA/pF at 0 mV and inactivated nearly completely during 150 ms of depolarization (n=4) (Figure 31B).
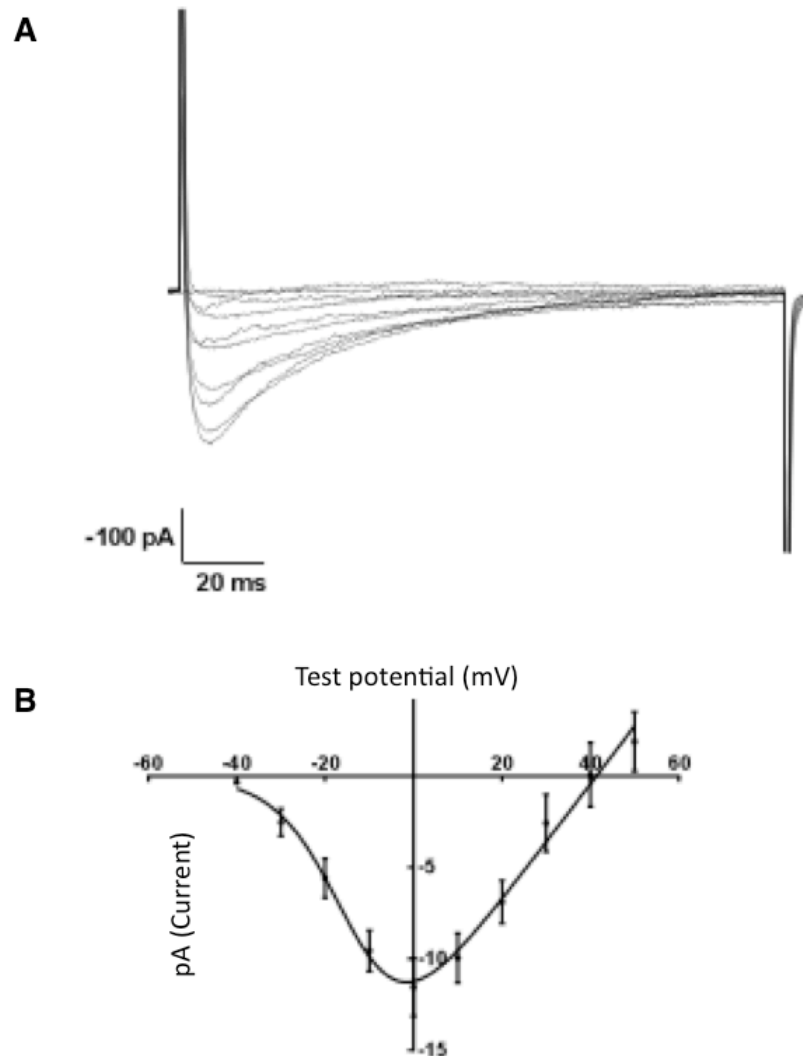
Figure 31: Electrophysiological characterization of human iPS-CMs (del-AR1034ZIMA 001). A) Representative whole cell calcium current recording (2 mM extracellular Ca^{2+}). Cells were depolarized from a holding potential of -80 mV to -40 mV for 45 ms in order to inactivate sodium channels. This prepulse was followed by test voltages ranging from -40 to +50 mV in 10 mV steps (pulse duration 150 ms). B) Whole cell calcium current density-voltage relationship (n=4). **(* These results were obtained with help of Jessica Köth and Jan Matthes from University of Cologne)**

4 Discussion

Our aim during the present study was to establish a robust strategy to obtain transgene-free human iPS cells. Excision of reprogramming cassette was achieved by optimizing direct delivery of TAT-Cre protein. We also performed the detailed pluripotency and differentiation analysis to assess the quality of iPS cells after removal of reprogramming factors. Second part of the study describes in detail optimization of cardiomyocytes differentiation with modulation of WNT and BMP signaling as well as devising enrichment step based on energy supply. During the last phase of study, we did in depth characterization of cardiomyocytes by performing immunostainings, ultra structural analysis and electrophysiological analysis for the validation of their functionality. By doing this, we tried to develop a methodology providing an unlimited source of cardiomyocytes for various biomedical applications.

4.1 Generation of reprogramming factor-free human iPS cells

4.1.1 Reprogramming of human fibroblasts

First step towards our aim was to reprogram human fibroblasts to obtain suitable iPS cells for further differentiation study. It was a critical step to decide which methodology to adopt as there were several reprogramming approaches available employing retro- or lenti- or sendai-viruses, episomes, synthetic mRNA, microRNA, transposons, recombinant proteins (Takahashi *et al*., 2006; Soldner *et al*., 2009; Fusaki *et al*., 2009; Chou *et al*., 2011; Kim *et al*., 2009; Zhou *et al*., 2009; Warren *et al.*, 2010). Each protocol has its advantages and disadvantages as described in the introduction chapter. We decided to use Cre-excisable lentiviral construct stemcca for our study due to its efficiency and possibility of removing reprogramming cassette using Cre recombination (Sommer *et al*., 2009). Initial attempts of reprogramming were unsuccessful due to non-optimized viral preparation. During our analysis we found that the viral titer plays a critical role in success of reprogramming. We observed that too much of virus appears toxic to the cells and too less results in less efficient infection. Once we were successful in controlling the number of virus particle for the infection that it became relatively easy to reproducibly reprogram human fibroblasts used for the study. We always kept the MOI (multiplicity of infection)

between 0.1 and 1. Virus concentration out of the stated limits results in less efficient reprogramming either due to less virus generating few colonies or too much virus, which is toxic to the cells. Moreover, it is also very important to apply regulated number of viral particle in order to control the number of viral integration. Study by Somers *et al* showed that viral titer with MOI of 0.1 to 1 results in only single integration of stemcca construct with 94% of probability (Somers *et al*., 2010). Overall we were successful in generating five human iPS lines using lentiviral stemcca as confirmed by standard pluripotency characterizations in the result part.

4.1.2 Transgene deletion using TAT-Cre

After performing systemic optimization of TAT-Cre mediated transgene deletion using genomic PCR and Cre-reporter cell line, we found out that 2 µM of TAT-Cre for 5 hours is the optimal combination to achieve efficient transgene deletion. In contrast to earlier study by Nolden *et al* where they used 6 µM of TAT-Cre for 24 hours, we achieved the same transgene excision efficiency up to 90% using less concentration of TAT-Cre. The reason of efficient transgene deletion using less concentration of TAT-Cre presumably is feeder-free cultivation of iPS cells. Due to high transgene deletion efficiency, it is also possible to expand transgene-excised clones polyclonally. Decreasing or increasing TAT-Cre concentration and time window of its application resulted in less efficient transgene excision. We used the genomic PCR and Cre-reporter iPS lines for assessing transgene deletion instead of southern blot analysis. We did the optimization of genomic PCR by mixing genomic DNA from human ES and transgene containing iPS cells to ensure that appearance of no band correlates to complete excision. Taking into consideration the results from genomic PCR together with Cre reporter iPS lines, one can reasonably assume our transgene deletion assessment to be equivalent to that of southern blot analysis. As described in the introduction part, transgene-free iPS cells are of better quality in terms of pluripotency and differentiation potential. Due to their better differentiation propensity, we argue that they are better pluripotent stem cells candidate as a source of obtaining desired cells, in our case cardiomyocytes. Earlier studies have reported the deletion of transgenes by delivering Cre as a plasmid, Adeno-Cre, or by mRNA transfection (Soldner *et al*., 2009, Somers *et al*., 2010; Sommer *et al*., 2009, Awe *et al*., 2013, Loh *et al*., 2012). The protocol elaborated by Soldner *et al* yielded only 16 transgene-deleted clones out of 180 analyzed after transfecting iPS cells

with a Cre-encoding plasmid and subsequent selection either with GFP fluorescence or puromycin (Soldner *et al*., 2009). The relatively low efficiency might be due to the transient transfection of Cre, which is limiting intracellular DNA recombinase activity. Sommer *et al* and Somers *et al* used Adeno-Cre and Cre-IRES-Puro constructs respectively to achieve higher excision efficiencies (Somers *et al*., 2010; Sommer *et al*., 2009). A more recent study by Awe et al demonstrate transgene excision in iPS clones with putative clinical grade status using Adeno-Cre mediated transgene deletion. During their transgene excision analysis they obtained only one transgene-excised subclone out of six Adeno-Cre treated clones (Awe *et al*., 2013). Furthermore, deletion approaches using Cre plasmids or Adeno-Cre constructs require transfection and subsequent selection of cells with Cre recombinase activity either by flow cytometry sorting or antibiotic selection. Such relatively complicated steps are undesirable as it might be stressful to the cells (Moran *et al*., 2009) and time-consuming thus costly. Moreover, there is possibility that Cre-encoding plasmids or viral constructs integrate into the genome (Glover *et al*., 2005). More recently, the group of George Daley has reported transgene excision by transfecting loxP-modified iPS cells with Cre-encoding mRNA (Loh *et al*., 2012). Notably, this procedure involves daily transfections of Cre mRNA for 4 hours up to 7 days, which again represents a complicated and stressful procedure for the cells. Overall protocol developed in our study, in contrast to previous Cre recombinase based approaches requires just a single shot application of TAT-Cre recombinant protein for 5 hours due to its high recombination efficiency. By this, the use of TAT-Cre accelerates the process of obtaining transgene-free iPS cells with minimal technical complexity. Other reprogramming methods utilizing non-integrating approach such m-RNAs, Sendai viruses, episomes and proteins are more suitable compared Cre excisable methods for generating clinically safe iPS cells. However these methods are limited with respect to practical robustness and reproducibility (Gonzalez *et al*., 2011; Wörsdörfer *et al*., 2011, Lee *et al.,* 2013). Moreover not all biomedical applications strictly require iPS cells completely devoid of transgenes such as disease modeling and differentiation studies. Hence for such purpose our protocol provides easier way to efficiently obtained transgene-free iPS cells. Although Cre mediated excision removes the transgenes cassette, one loxP site together with LTR remains integrated. Hence detailed sequence analysis is necessary in order to map the precise integration to ensure safety before clinical applications.

4.1.3 Effect of transgene excision on pluripotency and differentiation potential of human iPS cells

After achieving successful deletion of transgenes from human iPS cells, it was important to check for negative as well as positive effect of TAT-Cre mediated excision on pluripotency characteristics of resulting iPS cells. During our analysis transgene-free iPS cells remain pluripotent and did not show any alteration with respect to pluripotency, which is in consistent with earlier studies showing transgene excision without causing any abnormalities to the iPS cells (Soldner *et al*., 2009, Somers *et al.,* 2010; Sommer *et al*., 2009, Awe *et al.,* 2013, Loh *et al*., 2012). In contrast, microarray analysis indicates enhancement in the quality of iPS cells, as transgene-excised iPS cells appeared more similar to human ES cells with respect to gene expression profiling confirming previous studies (Soldner *et al*., 2009). In fact, it has been reported that transgene-free iPS cells exhibit an improved differentiation potential by showing enhanced endodermal differentiation of transgene excised iPS cells (Sommer *et al*., 2010). In our study, we show enhanced cardiac differentiation of TAT-Cre treated polyclonal iPS cells. By this we show an improvement in differentiation capacity of a polyclonal cell population after the removal of transgenes by Cre-mediated recombination. This makes TAT-Cre protein an attractive tool to obtain transgene-free iPS cells even in a polyclonal manner as recently suggested (Willmann *et al*., 2013) circumventing the laborious selection procedure of transgene-excised clones. In conclusion, our study provides a simple, rapid and robust protocol for the generation of superior transgene-free iPS cells suitable for disease modeling and drug toxicity screening.

4.2 Cardiomyocyte differentiation of human iPS cells

4.2.1 Optimization of cardiomyocyte differentiation paradigm using gene expression analysis of cardiac specific markers

Our study reports a novel strategy to obtain cardiomyocytes from diverse human iPS cell lines using an optimal combination of well-orchestrated extrinsic stimuli such as BMP4 and Chir followed by WNT inhibition using XAV939 or IWR1 and enrichment of cardiomyocytes by supplying lactate as an energy source. In order to establish it, we started with protocols published in earlier reports employing EBs as well as a monolayer based approach (Yang *et al.,* 2008, Hudson *et al*., 2011). We realized the practical issues, which need to be addressed in order to achieve efficient differentiation. During our study we were not successful in differentiating human iPS cell lines into cardiomyocytes using the EB based approach. One explanation for this could be the complex microenvironments within EBs, which makes hard to modulate key signals (Mummery *et al*., 2013). Hence we switched to monolayer culture of differentiation to make them more accessible to extrinsic signals applied during the differentiation. Although initial attempts were unsuccessful but it gave us clues about critical signaling pathways. In particular analysis of markers such as T (Brachyury), ISL1 as well as Nkx2.5 was an essential part of the optimization process. T (Brachyury) is a marker for mesendodermal population, which is expected to appear during the cardiac differentiation of human ES and iPS cells around day 3 (Osafune *et al.*, 2008). Therefore we used it as a quick read out to check the efficacy of our growth factors formulations. We used different combinations of growth factors as well as small molecules for the modulation of Nodal, FGF and WNT signaling pathways. There are several studies reporting the importance of each of these pathways for efficient cardiogenesis as described in introduction chapter. According to our analysis T (Brachyury) was strongly induced using combination of BMP4 and Chir. Hence we used this combination as rapid induction for early mesoderm. In order to follow the further specification of early mesoderm towards cardiac fate, we checked the expression of ISL1. Expression of ISL1 marks the entry of differentiating cells towards cardiac fate (Laugwitz *et al*., 2005, Cai *et al*., 2003). It was also used as basis of cardiac precursor isolation and their subsequent differentiation into cardiovascular linkages (Zaruba *et al*., 2010, Laugwitz *et al.,* 2005). During our initial

experiments we observed a strong induction of ISL1 from day 4 of differentiation that remained constantly upregulated through the differentiation till day 10. This observation was critical in many respects. First of all it indicated that we were able to differentiate the cells towards cardiovascular fate and second constant upregulation of ISL1 indicated improper specification towards cardiac fate. Earlier studies describing efficient cardiac differentiation of human iPS cells showed that ISL1 expression peaked at day 4 and slowly decreased around day 7 of differentiation (Lian *et al*., 2013). Decrease of ISL1 expression was accompanied with up regulation of cardiac marker such as Nkx2.5, hence indicating proper cardiac specification (Lian *et al*., 2013). Taking into consideration the fact that we obtained SMA positive cells led us to conclude that we were differentiating the cells towards vascular fate due the lack of signals which could further specify cardiovascular cells into cardiac fate. Thus we also included Nkx2.5 marker analysis during differentiation. It marks the entry of cardiac precursors into the cardiomyocyte lineage (Lyons *et al*., 1995, Terami *et al*., 2004). It was also used as selection marker for cardiac cells in earlier studies (Elliott *et al.,* 2011). Absence of Nkx2.5 marker during our earlier attempts gave us an idea about the problem of lack of specification towards cardiomyocytes lineage.

4.2.2 Optimization of cardiac specification with WNT modulation

Repeated failure of differentiation led us to include molecules for modulation of WNT signaling. It has been reported that WNT signal plays crucial role in cardiogenesis *in vivo* as well as during cardiac differentiation of ES cells. It plays biphasic role during cardiac differentiation with early WNT activation and late inhibition seems essential for proper cardiac induction and specification (Cohen *et al*., 2008). We used previously described molecules as basis of our screening for WNT modulators and performed the WNT reporter analysis as described in result part. After performing WNT reporter assay, we decided for Chir, XAV939 and IWR-1 as WNT modulators. In order to confirm the finding from WNT reporter assay, we performed systematic comparative cardiac differentiation using all small molecules in combination with growth factors as described in result part (Table 2, 3 and 4). The outcome was indeed diverse, we only obtained synchroneously beating cultures using Chir and XAV939 as WNT modulators. We obtained similar result in terms cardiomyocytes yield by replacing XAV939 with IWR1 as WNT inhibitor. All other known molecules we tried failed to support cardiac differentiation. Thus our comprehensive analysis

revealed that efficient cardiac differentiation of human iPS cell requires combination growth factors with WNT modulators. In particular, we achieved potent formulation of BMP4 and Chir in combination with XAV939 or IWR1 generating robust cardiomyocytes up to 90% as described in the result part. We observed that cardiac differentiation to be very sensitive to the proper concentration as well as time window of growth factor BMP4 and small molecules Chir, XAV939 and IWR1.

4.2.3 Significance of insulin for cardiac differentiation

During our earlier attempts, we faced the problem of reproducibility using similar conditions with the same human iPS cell line (iLB-C-50-s9). Our first step toward solving this problem was to use fresh medium components and avoid repeated freezing and thawing of growth factors and small molecules. However, it did not solve the problem, hence we did literature research in order to find out the reason for non-reproducibility. It was reported in several studies that insulin plays critical role during cardiac differentiation. In case of mouse pluripotent stem cells it supports (Naito *et al*., 2005) while in human pluripotent stem cells it negatively influences cardiomyocytes formation (Xu *et al*., 2008). Therefore, recent studies have used insulin-free medium during cardiac differentiation (Lian *et al*., 2012). Since we also had insulin as one of the component of B-27 supplement that we were using, we switched to B-27 supplement without insulin. However using basal medium without insulin appeared stressful to the cells and many cells died in the beginning of differentiation. In order to avoid cell death, we kept basal medium with insulin for first two days and then switched to the insulin free medium during specification phase. In fact we found recent study to support our insulin switch. It has been described systematically by Lian *et al* that insulin does not inhibit the formation T (Brachyury) positive cells but it interferes with the specification of early mesendodermal population towards cardiac fate (Lian *et al*., 2013). Hence it was a reasonable to keep the insulin for initial two days during which cells are converted into mesendodermal precursors and remove it during the specification phase. Indeed by doing so we now achieved reproducible results in terms of robust cardiac differentiation using the cardiac differentiation scheme shown in figure 27 (see result part).

Although we found out effective formulation in order to efficiently obtain cardiomyocytes from iPS lines, we still could not effectively differentiate all the iPS lines that we used for our studies. Very important trend that we observed was reprogramming factor-free iPS lines showed high propensity of cardiac differentiation up to 90% than iPS lines still having reprogramming factors (see figure 26). Thus it further strengthens our claim for using transgene-free human iPS cells for differentiation study. However we still do not exclude the possibility that our formulation would not work robustly on all iPS cell line as different iPS lines might respond to different concentration of Chir and BMP4. Hence it is advisable to optimize the concentration of BMP4 and Chir in case given the iPS line does not respond to the concentration that we used in our studies. Nevertheless, the overall approach that we have devised in our study would help the scientific community to further enhance the protocols for iPS based cardiomyocytes derivation. Moreover, systematic analysis of cardiac precursor markers during the cardiac differentiation offers the possibility of devising a protocol for isolating cardiac precursors. Such precursors cell would be of high interest as they can be expanded and differentiated to cardiovascular cell types when needed.

4.3 Enrichment of cardiomyocytes

We observed different cardiac differentiation efficiencies from 33 to 92% in multiple iPS cell lines presumably due to the complexity of the signals, which is in accordance with recent studies (Kaichi *et al*., 2010, Ohno *et al*., 2013). We therefore elaborated further purification steps to improve the yield of cardiomyocytes with reduced line-to-line variability. Various studies have shown successful purification of cardiomyocytes from heterogeneous cell populations using antibodies or a dyes specific to cardiomyocytes (Hattori *et la.,* 2010, Dubois *et al*., 2011). We decided to assess the potential of lactate enrichment of cardiomyocytes, since it has bee shown that cardiomyocyte culture can be enriched by supplying only lactate as a source of energy (Tohyama *et al*., 2013). Indeed, we show a substantial cardiac enrichment of our two iPS cell lines (iLB-C-30-r12 and fl-AR1034ZIMA 001) that exhibited relatively poor cardiomyocytes yield using our optimized chemical cocktail only. We were successful in enriching cardiac population from 62 till 95% in case of iLB-C-30-r12 and from 33 till 74% in case of fl-AR1034ZIMA 001 PS line. By that we demonstrate

that the combination of extrinsically induced differentiation stimuli together with metabolic enrichment is an efficient means to overcome line-to-line variability of cardiomyocytes differentiation. We observed the cell density affects the enrichment, if the cells are too dense (more than 90% confluent) than it is difficult to achieve proper enrichment. Therefore before enrichment it is advisable to split the cells with a ratio of 1:2 into new plates and then begin with the enrichment. Apart from that it is also helpful to change medium everyday during enrichment process to remove dead cells.

4.4 Characterization of human iPS derived cardiomyocytes

4.4.1 Structural characteristics

Differentiated cells showed synchronous spontaneous beating and remain beating till day 150 to 200. Obtained cardiomyocytes had limited proliferation rate with maximum cell division observed till two passages. In order to study detailed structural features, we performed immunostainings as well as TEM. Cells stained positive for cTNT and alpha-actinin with typical striation pattern as described in earlier studies (Mummery *et al*., 2013). Ultra structure analysis using TEM showed well-organized sarcomeric structures in 21 day old cardiomyocytes with distinct Z-, H-, I- and A-bands. Different degrees of myofibril organizations were seen in different areas of cells, which is indicative of fetal cardiomyocytes-like phenotype (Minami *et al*., 2012, Mummery *et al*., 2012). Moreover cells appear to be coupled with each other by gap junction complexes as well as facia adherence-like structures. Overall obtained results are indicative of functional cardiomyocytes with typical fetal-like features.

4.4.2 Electrophysiological characteristics

Obtained cardiomyocytes were beating spontaneously thus indicating the functional excitation-contraction coupling with functional calcium handling as seen in the case of cardiomyocytes obtained from pluripotent stem cells (Itzhaki *et al*., 2011, Li *et al*., 2013). In depth analysis was carried out using patch-camp methods measuring action potential and ionic currents. Spontaneous action potentials of differentiated cells showed typical cardiomyocyte pattern and we identified both ventricular and atrial-like shapes. Out of 6 analyzed cells 4 showed ventricular- and 2 showed atrial-

like phenotypes. Hence we obtained heterogeneous cell populations with respect to cardiac subtype. Voltage ramps identified fast sodium, calcium as well as potassium currents. Characteristics of Ca^{2+} currents obtained from iPS-derived cardiomyocytes were similar to those recently obtained from murine ventricular cardiomyocytes (Beetz *et al*., 2009). I-V relationship furthermore perfectly correlate with data from HEK293 cells expressing recombinant human L-type Ca^{2+}-channels suggesting that indeed iPS-derived cells express cardiac-like channel complexes consisting of a pore-forming and auxiliary subunits (Jangsangthong *et al*., 2010).

4.4.3 Outlook

Invention of iPS cell technology has pave new hopes in the field of regenerative therapies. It offers the possibility of autologous transplantation without any ethical constraints. Considering new developments in the field, the ultimate goal to effectively treat disorders with cell replacement therapy appears in the reach in near future. Many countries have already started with iPS cell banking in order to supply unlimited cell sources for regenerative therapies. It is an exciting time not only for the scientists but also for the general public with possibility of having better treatments. However still there are several critical refinements in terms of efficiency, safety as well as cost of iPS cell derivation need to be addressed in order to make it routine practices in clinics. Various differentiation protocols are available for generation of cardiomyocytes from human iPS cell lines. However, varied differentiation propensities of multiple pluripotent stem cell lines to a particular protocol require more alternative approaches. Hence, still it is highly desired to devise simple and efficient new protocols to achieve high robustness and efficacy. Moreover, it is of great importance to obtain homogenous cell population of cardiomyocyte subtypes in terms of establishing reliable platforms for disease modeling and differentiation studies. Latest research showing the modulation of key signals using chemicals will provide better control over differentiation process. Hence the iPS generation and their differentiation research hold the great potential for drug discovery and regenerative medicine.

5 Summary

Reprogramming of somatic cells to iPS cells represents promising way towards regenerative therapy. However, several refinements in iPS technology are needed in terms of efficiency and clinical safety. Classical protocols of iPS cells generation harness infection by retro- or lenti-viruses. Although such integrating viruses represent robust tools for reprogramming, the presence of viral transgenes in iPS cells is deleterious as it holds the risk of insertional mutagenesis leading to malignant transformation. Moreover, remaining reprogramming transgenes have been shown to affect the differentiation potential of iPS cells. Alternative protocols have been explored to derive transgene-free iPS cells. However, the utility of such protocols remains limited due to low efficiency and narrow range of cell specificity. During first part of our study, we set out towards efficient derivation of factor-free human iPS cell line using a lentiviral polycistronic vector and TAT–Cre protein transduction. We have shown enhancement in the quality of transgene-free iPS using microarray analysis and cardiac differentiation. Moreover, we show polyclonal expansion of transgene-deleted clones, which circumvents laborious selection procedures, and time-consuming analysis of subclones.

Second part of the study deals with the systematic optimization of cardiac differentiation of human iPS cells by extrinsic stimuli in monolayer culture. Protocol developed herein divides the whole differentiation process into three phases, namely cardiovascular induction, cardiac specification and cardiomyocyte enrichment. Our analysis revealed that efficient cardiac induction requires precise concentrations of extrinsic instructors such as BMP4, WNT modulators Chir and XAV939 as well as determination of effective time window for application of each growth factor or small molecule. We observed different cardiac differentiation efficiencies in multiple human iPS lines and elaborated further purification steps to improve the purity up to 90% of cardiomyocytes. We demonstrate that the combination of extrinsically induced differentiation stimuli together with metabolic enrichment is an efficient means to overcome line-to-line variability of cardiomyocyte differentiation. Furthermore, we did detailed functional validation of cardiomyocytes using immunostaining, electrophysiology as well as ultra-structural analysis. We conclude that

cardiomyocytes obtained using our protocol have the potential for drug toxicity as well as disease modeling studies.

6 Affirmation

I hereby declare that the work in this thesis is original and has been carried out by myself at the Stem Cell Engineering Group, Institute for Reconstructive Neurobiology, LIFE & BRAIN center, University of Bonn, Bonn, Germany. This thesis was prepared under the supervision of Prof. Dr. Frank Edenhofer in fulfillment of the requirements of the doctoral degree in natural sciences at the University of Bonn. I further declare that I have independently written this thesis with careful consideration from the best of my scientific knowledge, used no other than the indicated sources and means, and indicated any citations from literature and personal communications.

Place: Bonn, Germany

Date:

Asifiqbal Kadari

7 ACKNOWLEDGEMENTS

Finally, it is now that page of the thesis, which most people find interesting to take a first look. But I must confess, it is the most challenging when it comes to writing. This book certainly would not have been possible without the direct or indirect contribution of many people. I wish to extend my deep gratitude to all of you but if I forget someone, please forgive me.

*First of all, I am extremely grateful to **Prof. Dr. Frank Edenhofer** for providing me an opportunity to do PhD thesis in his lab and placing trust in me with respect to this new research topic carried out in the group and for the realization of this thesis. Frank has always been open to my suggestions or ideas, and that helped me to think about my research projects more critically. I am deeply indebted to him for his consistent support, encouragement, understanding and help during the PhD and for his willingness to discuss and correct this thesis thoroughly in a short time. Thank you very much for providing excellent lab facilities and for your valuable supervision.*

*I am also thankful to **Prof. Dr. Oliver Brüstle** for giving me an opportunity to pursue my PhD research in this institute. It was an excellent experience to be a part of this institute. I admire his scientific and administrative expertise to ensure that the department with teams from different disciplines functions smoothly and for creating an environment that builds a healthy relationship among team members. I am very grateful for the regular progress and journal clubs and the following discussions which were useful during the whole project.*

*I would also like to thank **Prof. Dr. Hubert Schorle** for being my examiner and providing me valuable suggestions for writing my thesis and **Prof. Dr. Oliver Brüstle** and **Prof. Jörg Höhfeld** for being additional reviewers of my thesis.*

*I would also thank **Dr. Rajkumar P. Thummer** for the supervision of the practical work and his stimulating suggestions helped me all the time during my research work. I would also thank **Kathrin Vogt, Nicole Russ** and **Martina Gebhardt** for providing the best technical support during the practical work. My sincere thanks to*

8 Publications

Kadari Asifiqbal, Mekkala Subbarao, Wagner Nicole, Malan Daniela, Köth Jessica, Sasse Phillip, Matthes Jan, Doll Katharina, Stappert Laura, Eckert Daniela, Peitz Michael, Brüstle Oliver, Herzig Stefan, and Edenhofer Frank. Efficient generation of cardiomyocytes from human iPSCs requires precise modulation of Bmp and Wnt signaling (2014). Stem cell Reviews and Reports

Kadari Asifiqbal, Min Lu, Ming Li, Thileepan Sekaran, Thummer Rajkumar, Naomi Guyette, Vi Chu, and Edenhofer Frank (2014). Excision of viral reprogramming cassettes by Cre protein transduction enables rapid, robust and efficient derivation of transgene-free human iPS cells. Stem Cell Research and Therapy

Wörsdörfer Phillip*, Thier Marc*, **Kadari Asifiqbal*** & Edenhofer Frank (2013). Roadmap to cellular reprogramming – Manipulating transcriptional networks with DNA, RNA, proteins and small molecules. Current Molecular Medicine (*these authors contributed equally to this work)

* Author contributed equally.

9 References

Anokye-Danso F, Trivedi CM, Juhr D, et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell. 2011;8:376-388.

Avilion AA, Nicolis SK, Pevny LH, et al. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 2003;17:126-140.

Awe JP, Lee PC, Ramathal C, et al. Generation and characterization of transgene-free human induced pluripotent stem cells and conversion to putative clinical-grade status. Stem Cell Res Ther. 2013;4:87.

Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 1963;197:452-454.

Beetz N, Hein L, Meszaros J, et al. Transgenic simulation of human heart failure-like L-type Ca2+-channels: implications for fibrosis and heart rate in mice. Cardiovasc Res. 2009;84:396-406.

Ben-Porath I, Thomson MW, Carey VJ, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet. 2008;40:499-507.

Braam SR, Passier R, Mummery CL. Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery. Trends Pharmacol Sci. 2009;30:536-545.

Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet. 2005;6:826-835.

Burridge PW, Keller G, Gold JD, et al. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. Cell Stem Cell. 2012;10:16-28.

Cai CL, Liang X, Shi Y, et al. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell. 2003;5:877-889.

Cao N, Liang H, Huang J, et al. Highly efficient induction and long-term maintenance of multipotent cardiovascular progenitors from human pluripotent stem cells under defined conditions. Cell Res. 2013;23:1119-1132.

Cao N, Liu Z, Chen Z, et al. Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells through promoting the proliferation of cardiac progenitor cells. Cell Res. 2011;22:219-236.

Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006;98:1076-1084.

Carey BW, Markoulaki S, Hanna J, et al. Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc Natl Acad Sci U S A. 2009;106:157-162.

Carpenter L, Carr C, Yang CT, et al. Efficient differentiation of human induced pluripotent stem cells generates cardiac cells that provide protection following myocardial infarction in the rat. Stem Cells Dev. 2012;21:977-986.

Cartwright P, McLean C, Sheppard A, et al. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development. 2005;132:885-896.

Chew JL, Loh YH, Zhang W, et al. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol Cell Biol. 2005;25:6031-6046.

Choi SH, Jung SY, Kwon SM, et al. Perspectives on stem cell therapy for cardiac regeneration. Advances and challenges. Circ J. 2012;76:1307-1312.

Chou BK, Mali P, Huang X, et al. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res. 2011;21:518-529.

Cohen ED, Tian Y, Morrisey EE. WNT signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. Development. 2008;135:789-798.

Cordeiro JM, Nesterenko VV, Sicouri S, et al. Identification and characterization of a transient outward K+ current in human induced pluripotent stem cell-derived cardiomyocytes. J Mol Cell Cardiol. 2013;60:36-46.

Daley GQ. Stem cells: roadmap to the clinic. J Clin Invest. 2010;120:8-10.

Dalla-Favera R, Bregni M, Erikson J, et al. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A. 1982;79:7824-7827.

Dambrot C, Buermans HP, Varga E, et al. Strategies for rapidly mapping proviral integration sites and assessing cardiogenic potential of nascent human induced pluripotent stem cell clones. Exp Cell Res. 2014.

Egashira T, Yuasa S, Suzuki T, et al. Disease characterization using LQTS-specific induced pluripotent stem cells. Cardiovasc Res. 2012;95:419-429.

Elliott DA, Braam SR, Koutsis K, et al. NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. Nat Methods. 2011;8:1037-1040.

Eschenhagen T, Didie M, Munzel F, et al. 3D engineered heart tissue for replacement therapy. Basic Res Cardiol. 2002;97 Suppl 1:I146-152.

Fijnvandraat AC, van Ginneken AC, Schumacher CA, et al. Cardiomyocytes purified from differentiated embryonic stem cells exhibit characteristics of early chamber

myocardium. J Mol Cell Cardiol. 2003;35:1461-1472.

Fuerer C, Nusse R. Lentiviral vectors to probe and manipulate the WNT signaling pathway. PLoS One. 2010;5:e9370.

Fusaki N, Ban H, Nishiyama A, et al. 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. 2009;85:348-362.

Gassanov N, Er F, Zagidullin N, et al. Endothelin induces differentiation of ANP-EGFP expressing embryonic stem cells towards a pacemaker phenotype. FASEB J. 2004;18:1710-1712.

Gessert S, Kuhl M. The multiple phases and faces of WNT signaling during cardiac differentiation and development. Circ Res. 2010;107:186-199.

Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet. 2005;6:299-310.

Gordon K. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes & Dev. 2005. 19:1129-1155.

Goldsmith MA, Slavik M, Carter SK. Quantitative prediction of drug toxicity in humans from toxicology in small and large animals. Cancer Res. 1975;35:1354-1364.

Gonzalez F, Boue S, Izpisua Belmonte JC. Methods for making induced pluripotent stem cells: reprogramming a la carte. Nat Rev Genet. 2011;12:231-242.

Gupta MK, Illich DJ, Gaarz A, et al. Global transcriptional profiles of beating clusters derived from human induced pluripotent stem cells and embryonic stem cells are highly similar. BMC Dev Biol. 2010;10:98.

Haupt S, Edenhofer F, Peitz M, et al. Stage-specific conditional mutagenesis in mouse embryonic stem cell-derived neural cells and postmitotic neurons by direct delivery of biologically active Cre recombinase. Stem Cells. 2007;25:181-188.

Hidaka K, Lee JK, Kim HS, et al. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. FASEB J. 2003;17:740-742.

Honda M, Kiyokawa J, Tabo M, et al. Electrophysiological characterization of cardiomyocytes derived from human induced pluripotent stem cells. J Pharmacol Sci. 2011;117:149-159.

Hou J, Wang L, Jiang J, et al. Cardiac stem cells and their roles in myocardial infarction. Stem Cell Rev. 2013;9:326-338.

Hudson J, Titmarsh D, Hidalgo A, et al. Primitive cardiac cells from human embryonic stem cells. Stem Cells Dev. 2011;21:1513-1523.

Humphrey RK, Beattie GM, Lopez AD, et al. Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. Stem Cells. 2004;22:522-530.

Israel MA, Yuan SH, Bardy C, et al. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature. 2012;482:216-220.

Itzhaki I, Maizels L, Huber I, et al. Modeling of catecholaminergic polymorphic ventricular tachycardia with patient-specific human-induced pluripotent stem cells. J Am Coll Cardiol. 2012;60:990-1000.

Itzhaki I, Maizels L, Huber I, et al. Modelling the long QT syndrome with induced pluripotent stem cells. Nature. 2011;471:225-229.

Itzhaki I, Rapoport S, Huber I, et al. Calcium handling in human induced pluripotent stem cell derived cardiomyocytes. PLoS One. 2011;6:e18037.

Ivanova N, Dobrin R, Lu R, et al. Dissecting self-renewal in stem cells with RNA interference. Nature. 2006;442:533-538.

Jangsangthong W, Kuzmenkina E, Khan IF, et al. Inactivation of L-type calcium channels is determined by the length of the N terminus of mutant beta(1) subunits. Pflugers Arch. 2010;459:399-411.

Jung CB, Moretti A, Mederos y Schnitzler M, et al. Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. EMBO Mol Med. 2012;4:180-191.

Kaichi S, Hasegawa K, Takaya T, et al. Cell line-dependent differentiation of induced pluripotent stem cells into cardiomyocytes in mice. Cardiovasc Res. 2010;88:314-323.

Kaji K, Norrby K, Paca A, et al. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature. 2009;458:771-775.

Karakikes I, Senyei GD, Hansen J, et al. Small molecule-mediated directed differentiation of human embryonic stem cells toward ventricular cardiomyocytes. Stem Cells Transl Med. 2013;3:18-31.

Kattman SJ, Witty AD, Gagliardi M, et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell. 2011;8:228-240.

Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest. 2001;108:407-414.

Kehat I, Khimovich L, Caspi O, et al. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nat Biotechnol.

2004;22:1282-1289.

Kim D, Kim CH, Moon JI, et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell. 2009;4:472-476.

Koch, P., Breuer, P., Peitz, M., et al. (2011). Excitation-induced ataxin-3 aggregation in neurons from patients with Machado- Joseph disease. Nature, 480, 543–546.

Kopp JL, Ormsbee BD, Desler M, et al. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells. 2008;26:903-911.

Kujala K, Paavola J, Lahti A, et al. Cell model of catecholaminergic polymorphic ventricular tachycardia reveals early and delayed afterdepolarizations. PLoS One. 2012;7:e44660.

Laflamme MA, Chen KY, Naumova AV, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol. 2007;25:1015-1024.

Laflamme MA, Murry CE. Heart regeneration. Nature. 2011;473:326-335.

Lasser KE, Allen PD, Woolhandler SJ, et al. Timing of new black box warnings and withdrawals for prescription medications. JAMA. 2002;287:2215-2220.

Laugwitz KL, Moretti A, Lam J, et al. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature. 2005;433:647-653.

Lee YK, Ng KM, Lai WH, et al. Calcium homeostasis in human induced pluripotent stem cell-derived cardiomyocytes. Stem Cell Rev. 2011;7:976-986.

Lewitzky M, Yamanaka S. Reprogramming somatic cells towards pluripotency by defined factors. Curr Opin Biotechnol. 2007;18:467-473.

Li Y, McClintick J, Zhong L, et al. Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. Blood.

2005;105:635-637.

Lian X, Hsiao C, Wilson G, et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical WNT signaling. Proc Natl Acad Sci U S A. 2012;109:E1848-1857.

Lian X, Zhang J, Zhu K, et al. Insulin inhibits cardiac mesoderm, not mesendoderm, formation during cardiac differentiation of human pluripotent stem cells and modulation of canonical WNT signaling can rescue this inhibition. Stem Cells. 2013;31:447-457.

Liao SY, Liu Y, Siu CW, et al. Proarrhythmic risk of embryonic stem cell-derived cardiomyocyte transplantation in infarcted myocardium. Heart Rhythm. 2010;7:1852-1859.

Lieu DK, Fu JD, Chiamvimonvat N, et al. Mechanism-based facilitated maturation of human pluripotent stem cell-derived cardiomyocytes. Circ Arrhythm Electrophysiol. 2013;6:191-201.

Linta, L., Stockmann, M., Kleinhans, K. N., et al. (2012). Rat embryonic fibroblasts improve reprogramming of human keratinocytes into induced pluripotent stem cells. Stem Cells and Development, 21, 965–976.

Loh YH, Yang JC, De Los Angeles A, et al. Excision of a viral reprogramming cassette by delivery of synthetic Cre mRNA. Curr Protoc Stem Cell Biol. 2012;Chapter 4:Unit4A 5.

Lopez AD, Mathers CD, Ezzati M, et al. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet. 2006;367:1747-1757.

Lu HR, Marien R, Saels A, et al. Species plays an important role in drug-induced prolongation of action potential duration and early afterdepolarizations in isolated Purkinje fibers. J Cardiovasc Electrophysiol. 2001;12:93-102.

Lu HR, Vlaminckx E, Hermans AN, et al. Predicting drug-induced changes in QT interval and arrhythmias: QT-shortening drugs point to gaps in the ICHS7B Guidelines. Br J Pharmacol. 2008;154:1427-1438.

Lyons I, Parsons LM, Hartley L, et al. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev. 1995;9:1654-1666.

Ma J, Guo L, Fiene SJ, et al. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. Am J Physiol Heart Circ Physiol. 2011;301:H2006-2017.

Malan D, Friedrichs S, Fleischmann BK, et al. Cardiomyocytes obtained from induced pluripotent stem cells with long-QT syndrome 3 recapitulate typical disease-specific features in vitro. Circ Res. 2011;109:841-847.

Mandenius CF, Steel D, Noor F, et al. Cardiotoxicity testing using pluripotent stem cell-derived human cardiomyocytes and state-of-the-art bioanalytics: a review. J Appl Toxicol. 2011;31:191-205.

Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981;78:7634-7638.

Marvin MJ, Di Rocco G, Gardiner A, et al. Inhibition of WNT activity induces heart formation from posterior mesoderm. Genes Dev. 2001;15:316-327.

Mikkers H, Berns A. Retroviral insertional mutagenesis: tagging cancer pathways. Adv Cancer Res. 2003;88:53-99.

Minami I, Yamada K, Otsuji TG, et al. A small molecule that promotes cardiac differentiation of human pluripotent stem cells under defined, cytokine- and xeno-free conditions. Cell Rep. 2012;2:1448-1460.

Mohamad O, Drury-Stewart D, Song M, et al. Vector-free and transgene-free human iPS cells differentiate into functional neurons and enhance functional recovery after ischemic stroke in mice. PLoS One. 2013;8:e64160.

Moran DM, Shen H, Maki CG. Puromycin-based vectors promote a ROS-dependent recruitment of PML to nuclear inclusions enriched with HSP70 and Proteasomes. BMC Cell Biol. 2009;10:32.

Moretti A, Bellin M, Welling A, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N Engl J Med. 2010;363:1397-1409.

Moya M, Tran D, George SC. An integrated in vitro model of perfused tumor and cardiac tissue. Stem Cell Res Ther. 2013;4 Suppl 1:S15.

Mummery CL, Zhang J, Ng ES, et al. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. Circ Res. 2012;111:344-358.

Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell. 2008;132:661-680.

Naito AT, Akazawa H, Takano H, et al. Phosphatidylinositol 3-kinase-Akt pathway plays a critical role in early cardiomyogenesis by regulating canonical WNT signaling. Circ Res. 2005;97:144-151.

Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell. 1998;95:379-391.

Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet. 2000;24:372-376.

Nolden L, Edenhofer F, Haupt S, et al. Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase. Nat Methods. 2006;3:461-467.

Ohno Y, Yuasa S, Egashira T, et al. Distinct iPS Cells Show Different Cardiac Differentiation Efficiency. Stem Cells Int. 2013;2013:659739.

Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007;448:313-317.

Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A. 2001;98:10344-10349.

Osafune K, Caron L, Borowiak M, et al. Marked differences in differentiation propensity among human embryonic stem cell lines. Nat Biotechnol. 2008;26:313-315.

Papapetrou EP, Sadelain M. Generation of transgene-free human induced pluripotent stem cells with an excisable single polycistronic vector. Nat Protoc. 2011;6:1251-1273.

Park IH, Arora N, Huo H, et al. Disease-specific induced pluripotent stem cells. Cell. 2008;134:877-886.

Passier R, Oostwaard DW, Snapper J, et al. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells. 2005;23:772-780.

Peitz M, Jager R, Patsch C, et al. Enhanced purification of cell-permeant Cre and germline transmission after transduction into mouse embryonic stem cells. Genesis. 2007;45:508-517.

Peitz M, Pfannkuche K, Rajewsky K, et al. Ability of the hydrophobic FGF and basic

TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. Proc Natl Acad Sci U S A. 2002;99:4489-4494.

Ren Y, Lee MY, Schliffke S, et al. Small molecule WNT inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. J Mol Cell Cardiol. 2011;51:280-287.

Rowland BD, Bernards R, Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat Cell Biol. 2005;7:1074-1082.

Russ HA, Bar Y, Ravassard P, Efrat S: In vitro proliferation of cells derived from adult human beta cells revealel by cell lineage tracing. *Diabetes* 2008, **57**:1575-1583

Sa S, McCloskey KE. Stage-specific cardiomyocyte differentiation method for H7 and H9 human embryonic stem cells. Stem Cell Rev. 2012;8:1120-1128.

Saric T, Halbach M, Khalil M, et al. Induced pluripotent stem cells as cardiac arrhythmic in vitro models and the impact for drug discovery. Expert Opin Drug Discov. 2014;9:55-76.

Schaaf S, Shibamiya A, Mewe M, et al. Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. PLoS One. 2011;6:e26397.

Schuh R, Aicher W, Gaul U, et al. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene. Cell. 1986;47:1025-1032.

Segre JA, Bauer C, Fuchs E. Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet. 1999;22:356-360.

Shiba Y, Fernandes S, Zhu WZ, et al. Human ES-cell-derived cardiomyocytes

electrically couple and suppress arrhythmias in injured hearts. Nature. 2012;489:322-325.

Soldner F, Hockemeyer D, Beard C, et al. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell. 2009;136:964-977.

Somers A, Jean JC, Sommer CA, et al. Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette. Stem Cells. 2010;28:1728-1740.

Sommer CA, Sommer AG, Longmire TA, et al. Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector. Stem Cells. 2010;28:64-74.

Sommer CA, Stadtfeld M, Murphy GJ, et al. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. Stem Cells. 2009;27:543-549.

Staerk J, Dawlaty MM, Gao Q, et al. Reprogramming of human peripheral blood cells to induced pluripotent stem cells. Cell Stem Cell. 2010;7:20-24.

Straube WL, Tanaka EM. Reversibility of the differentiated state: regeneration in amphibians. Artif Organs. 2006;30:743-755.

Szymczak AL, Workman CJ, Wang Y, et al. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol. 2004;22:589-594.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663-676.

Takei S, Ichikawa H, Johkura K, et al. Bone morphogenetic protein-4 promotes induction of cardiomyocytes from human embryonic stem cells in serum-based embryoid body development. Am J Physiol Heart Circ Physiol. 2009;296:H1793-

1803.

Terami H, Hidaka K, Katsumata T, et al. WNT11 facilitates embryonic stem cell differentiation to Nkx2.5-positive cardiomyocytes. Biochem Biophys Res Commun. 2004;325:968-975.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-1147.

Tohyama S, Hattori F, Sano M, et al. Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. Cell Stem Cell. 2013;12:127-137.

Tokuzawa Y, Kaiho E, Maruyama M, et al. Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Mol Cell Biol. 2003;23:2699-2708.

Ueno S, Weidinger G, Osugi T, et al. Biphasic role for WNT/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. Proc Natl Acad Sci U S A. 2007;104:9685-9690.

Uosaki H, Fukushima H, Takeuchi A, et al. Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. PLoS One. 2011;6:e23657.

Valamehr B, Robinson M, Abujarour R, et al. Platform for Induction and Maintenance of Transgene-free hiPSCs Resembling Ground State Pluripotent Stem Cells. Stem Cell Reports. 2014;2:366-381.

van Laake LW, Passier R, Doevendans PA, et al. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. Circ Res. 2008;102:1008-1010.

Ventura A, Meissner A, Dillon CP, et al. Cre-lox-regulated conditional RNA

interference from transgenes. Proc Natl Acad Sci U S A. 2004;101:10380-10385.

Warren L, Manos PD, Ahfeldt T, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell. 2010;7:618-630.

Willmann CA, Hemeda H, Pieper LA, et al. To clone or not to clone? Induced pluripotent stem cells can be generated in bulk culture. PLoS One. 2013;8:e65324.

Worsdorfer P, Thier M, Kadari A, et al. Roadmap to cellular reprogramming - manipulating transcriptional networks with DNA, RNA, proteins and small molecules. Curr Mol Med. 2011;13:868-878.

Xu XQ, Graichen R, Soo SY, et al. Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. Differentiation. 2008;76:958-970.

Yang, L., Soonpaa, M. H., Adler, E. D., et al. (2008). Human cardiovascular progenitor cells develop from a KDR+embryonicstem-cell-derived population. Nature, 453, 524–528

Ye L, Muench MO, Fusaki N, et al. Blood cell-derived induced pluripotent stem cells free of reprogramming factors generated by Sendai viral vectors. Stem Cells Transl Med. 2013;2:558-566.

Yoshioka N, Gros E, Li HR, et al. Efficient generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell. 2013;13:246-254.

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

Yuan H, Corbi N, Basilico C, et al. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev. 1995;9:2635-2645.

Zaruba MM, Soonpaa M, Reuter S, et al. Cardiomyogenic potential of C-kit(+)-expressing cells derived from neonatal and adult mouse hearts. Circulation. 2010;121:1992-2000.

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

Zhang Q, Jiang J, Han P, et al. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. Cell Res. 2011;21:579-587.

Zhang YM, Hartzell C, Narlow M, et al. Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. Circulation. 2002;106:1294-1299.