Functional and molecular analysis of cardiomyocytes derived from reprogrammed pluripotent cells and embryonic stem cells

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List of Publications

Highly purified cardiomyocytes generated by drug selection from murine induced pluripotent stem cells and embryonic stem cells are functionally and transcriptionally indistinguishable

Azra Fatima, Filomain Nguemo, Tobias Hannes, Guoxing Xu, Markus Khalil, Alexey Kuzmenkin Andrea Gaarz, Sabine Classen, Eugen Kolossov, Michael Reppel, Joachim L. Schultze, Jürgen Hescheler, Tomo Šarić . ***(Manuscript prepared).***

In vitro modeling of ryanodine receptor 2 dysfunction using induced pluripotent stem cells

Azra Fatima, Guoxing Xu, Shao Kaifeng, Symeon Papadopoulos, Martin Lehmann, Juan-Jose Arnaiz Cot, Angelo O. Rosa, Filomain Nguemo, Sven Dittmann, Matthias Matzkies, Susannah L. Stone, Matthias Linke, Ulrich Zechner, Vera Beyer, Hans-Christian Hennies, Stephan Rosenkranz, Baerbel Klauke, Martin Farr, Abdul S. Parwani, Wilhelm Haverkamp, Lars Cleemann, Martin Morad, Hendrik Milting, Jürgen Hescheler and Tomo Šarić. ***(In press)***

Dual-color photoactivation localization microscopy of cardiomyopathy associated desmin mutants

Andreas Brodehl, Per Niklas Hedde, Mareike Dieding, **Azra Fatima**, Volker Walhorn, Susan Gayda, Tomo Šarić, Bärbel Klauke, Jan Gummert, Dario Anselmetti, Mike Heilemann, Gerd Ulrich Nienhaus, Hendrik Milting. ***(Under revision)***

1. **Comparison of contractile behaviour of native murine ventricular tissue and cardiomyocytes derived from embryonic or induced pluripotent stem cells.**
   Xi J, Khalil M, Shishechian N, Hannes T, Pfannkuche K, Liang H, **Fatima A**, Haustein M, Suhr F, Bloch W, Reppel M, Sarić T, Wernig M, Jaenisch R, Brockmeier K, Hescheler J, Pillekamp F. ***FASEB J****. 2010 Aug; 24(8):2739-51.*

2. **Initial colony morphology-based selection for iPS cells derived from adult fibroblasts is substantially improved by temporary UTF1-based selection.**
   Pfannkuche K, **Fatima A**, Gupta MK, Dieterich R, Hescheler *J. **PLoS One**. 2010 Mar 8; 5(3):e9580.*

3. **Functional characterization of cardiomyocytes derived from murine induced pluripotent stem cells in vitro.**
   Kuzmenkin A, Liang H, Xu G, Pfannkuche K, Eichhorn H, **Fatima A**, Luo H, Saric T, Wernig M, Jaenisch R, Hescheler J. ***FASEB J****. 2009 Dec; 23(12):4168-80.*

4. **Cardiac myocytes derived from murine reprogrammed fibroblasts: intact hormonal regulation, cardiac ion channel expression and development of contractility.**
Pfannkuche K, Liang H, Hannes T, Xi J, **Fatima A**, Nguemo F, Matzkies M, Wernig M, Jaenisch R, Pillekamp F, Halbach M, Schunkert H, Sarić T, Hescheler J, Reppel M.
Cell Physiol Biochem*. 2009; 24(1-2):73-86.*

5. **Proteomic Analysis Of The "Side Population" (SP) Cells From Murine Bone Marrow**
Ravipati Satyavani, **Azra Fatima**, Curam S. Sundaram, Charumathi Anabalagan, CV Saritha, G Srinivas, Aleem Ahmed Khan, CM Habibullah and Gopal Pande
Journal of Proteomics & Bioinformatics
http://scitechnol.com/journals/journal_fulltext.php?id=236&jid=34

Book Chapter

Embryonic Stem Cells, Cardiomyoplasty, and the Risk of Teratoma Formation
Tomo Šarić, Lukas P. Frenzel, **Azra Fatima**, Manoj K. Gupta and Jürgen Hescheler
Trends in Stem Cell Biology and Technology 2009, 229-260.

CONTENTS

ABBREVIATIONS

AP	Action potentials
BMP4	Bone morphogenetic protein 4
CCs	Cardiac clusters
CMs	Cardiomyocytes
cTnT	cardiac troponin T
EBs	Embryoid bodies
ES cells	Embryonic stem cells
FH	Fusion hybrid
GFP	Green fluorescent protein
HAT	Hypoxanthine, aminopterin and thymidines
ICM	Inner cell mass
IFNγ	Interferon gamma
iPS	Induced pluripotent stem
LIF	Leukemia inhibitory factor
MEFs	Mouse embryonic fibroblasts
MHC	Major histocompatibility complex
MLC	Myosin light chain
PEG	Polyethylene glycol
PI	Propidium iodide
SCNT	Somatic cell nuclear transfer
SNP	Single-nucleotide polymorphism
SSEA1	Stage Specific Embryonic Antigen 1
TTF	Tail tip fibroblasts
UTF	Undifferentiated transcription factor1
α-MHC	Alpha myosin heavy chain
α-PIG	Vector in which puromycin N-acetyltransferase (PAC) and enhanced green fluorescent protein (eGFP) linked with the internal ribosomal entry site (IRES) are expressed under the control of α-myosin heavy chain (MHC) promoter
β-MHC	β-myosin heavy chain

ABSTRACT

Pluripotent stem cells can be obtained from embryonic stage or generated by *in vitro* reprogramming of terminally differentiated somatic cells. Such reprogrammed cells possess similar developmental potential as embryonic stem (ES) cells and therefore are an indispensible source of diverse cell types, including cardiomyocytes (CMs), for basic research. CMs derived from ES cells and reprogrammed cells can provide possibilities for study of development, differentiation processes, stem cell malignancy and genetic diseases *in vitro*. Such studies can allow for development of novel compounds for drug discovery and toxicity testing which might help to develop more efficient and safer drugs

The present thesis is based on analyzing the impact of switching the cell fate on the differentiated state of reprogrammed cells using cardiomyocytes as a model. Detailed structural molecular and functional characterization of CMs derived from ES cells and reprogrammed cells like fusion hybrid (FH) cells and induced pluripotent stem (iPS) cells was performed.

Somatic cells (spleenocytes and bone marrow cells) were fused with ES cells to generate FH cells. The formed hybrid cells were morphologically similar to pluripotent ES cells and retained a tetraploid genome through many passages. Spontaneous differentiation led to formation of embryoid bodies (EBs) with appearance of beating areas representing differentiation to cardiac lineage. The EBs derived from FH cells also retained tetraploid genome and expressed major histocompatibility (MHC) class I molecules of both fusing partners. FH derived CM expressed typical cardiac structural proteins and intact β-adrenergic and muscarinic signaling receptors. Atrial, ventricular and pacemaker cardiac subtypes could be found in FH-CMs. Thus, CMs derived from tetraploid FH cells appear to be structurally and functionally intact.

Ongoing research proved iPS cell technology to be more robust reprogramming strategy. In order to obtain homogenous population of CMs for further studies an iPS cell line TiB7.4 was genetically manipulated to allow for antibiotic-based purification of cardiomyocytes after spontaneous differentiation. We generated highly purified CMs from transgenic murine iPS and ES cell lines expressing puromycin N-

acetyltransferase and EGFP under the control of α-myosin heavy chain promoter. iPS and ES cells differentiated into CMs at comparable efficiencies yielding highly purified CMs after drug selection. Purified iPS- and ES-CMs exhibited indistinguishable structural properties, similarly responded to pharmacological agents, expressed functional voltage-gated sodium, calcium and potassium channels and possessed comparable current densities. Global transcriptional profile and gene ontology signature of transgenic iPS-CMs were very similar to that of ES-CMs but clearly distinct from fibroblasts that were used to generate iPS cells and differentiated cells in iPS or ES cell-derived embryoid bodies. After puromycin selection, iPS-CMs did not contain any residual pluripotent cells nor formed teratoma in immunodeficient mice.

Therefore, cell fate switching brought about by reprogramming does not affect the structural, molecular and functional characteristics of cardiac derivatives of the reprogrammed cells.

ZUSAMMENFASSUNG

Pluripotente Stammzellen können aus Embryonen gewonnen oder durch Reprogrammierung vollständig differenzierter somatischer Zellen *in vitro* generiert werden. Diese reprogrammierten Zellen besitzen ein ähnliches Differenzierungspotential wie embryonale Stammzellen (ES) und sind somit eine vielversprechende Quelle unterschiedlicher Zelltypen, wie Kardiomyozyten (KM), für die Grundlagenforschung. Die aus ES Zellen und reprogrammierten Zellen differenzierte KM, ermöglichen Untersuchungen von Entwicklungsprozessen, Mechanismen genetischer Krankheiten des Herzens, Entwicklung neuer Medikamente und Bestimmung ihrer Toxizität.

Die hier vorliegende Arbeit basiert auf der Analyse des Einflusses von Zellschicksalsänderungen auf den differenzierten Zustand von reprogrammierten Zellen.

Dabei wurde eine detaillierte strukturelle, molekulare und funktionale Charakterisierung der KM durchgeführt, die aus ES Zellen und reprogrammierten Zellen, wie Fusionshybrodoma (FH) Zellen und induzierten pluripotenten (iPS) Zellen, gewonnen wurden.

Die FH Zellen wurden durch Fusion somatischer Zellen der Milz oder des Knochenmarks mit ES Zellen generiert. Die erhaltenen Hybridzellen waren morphologisch pluripotent, aber behielten ein tetraploides Genom über mehrere Passagen. Spontane Differenzierung dieser Zellen führte zur Formation von `embryoid bodies' (EB), welche schlagende Areale und somit die Differenzierung zur kardialen Linie aufwiesen. Die EB der FH Zellen behielten ebenfalls ein tetraploides Genom und exprimierten MHC Klasse I Moleküle beider Fusionspartner. Kardiomyozyten aus FH Zellen zeigten typische kardiale Strukturproteine auf und exprimierten intakte β-adrenerge und muskarine Signalwege. Obwohl die KM von tetraploiden FH Zellen generiert wurden, waren sie strukturell und funktionell normal.

Die gegenwärtige Forschung bewies, dass die iPS-Technologie die robustere Reprogrammierungsstrategie ist. Um KM besser zu analysieren, wurde eine transgene iPS Zelllinie generiert, welche Puromyzin-N-Acetyltransferase und EGFP unter der Kontrolle des Promoters für die schwere Kette des α-Myosin Gens exprimiert. Diese

Zelllinie ermöglichte die Aufreinigung einer homogenen Population der KM und somit die Analyse ihrer physiologischer und transkriptioneller Eigenschaften unter kontrollierten Bedingungen. Transgene iPS- und ES-Zellen differenzierten zu KM mit einer vergleichbaren Effizienz und lieferten nach Antibiotikaselektion hoch reine KM. Gereinigte iPS- und ES-KM zeigten ähnliche strukturelle Eigenschaften, expremierten funktionelle spannungskontrollierte Natrium-, Kalzium- und Kaliumkanäle und besaßen eine vergleichbare elektrische Stromdichte. Globale Transkriptionsprofile und Genontologiesignaturen transgener iPS- und von ES-KM waren auch vergleichbar und unterschieden sich deutlich von den Fibroblasten, welche zur Generierung der iPS Zellen verwendet wurden. Nach Puromyzinselektion enthielten die iPS-KM weder verbliebene pluripotente Stammzellen noch bildeten sie Teratome in immundefizienten Mäusen.

Somit beeinflusst die Veränderung des Zellschicksals mittels Reporgrammierung weder die strukturellen noch molekularen oder funktionellen Charkateristika der von ihnen abgeleiteten kardialen Zellen.

1 INTRODUCTION

Development of a new organism begins with fertilization of an oocyte by the sperm leading to the formation of single cell called zygote. The zygote undergoes several rounds of cleavage to form a ball of cells called morula. Each cell in morula is called blastomere. The characteristic of the zygote and blastomere is a state of totipotency which marks their ability to give rise to all the different cells of an adult organism including the extra-embryonic membranes. The cells of the morula undergo more divisions to form a blastocyst, a hollow ball of cells in which the outer cells become trophoblast and develop into placenta. Some cells trapped in the interior of the blastocyst, termed the inner cell mass (ICM); generate the epiblast and the hyphoblast. The epiblast and hyphoblast will form the embryo and the yolk sac, respectively. The cells of the ICM are termed pluripotent as they are able to form each of the three germ layers; the endoderm, the ectoderm and the mesoderm. These germ layers form distinct lineages of terminally differentiated post-mitotic cells, which contribute to specific organ function. As a fertilized egg develops into an adult organism, specialized cells are formed by a one-way process, and they become increasingly, and normally irreversibly, committed to their fate.

1.1 Pluripotent stem cells

Pluripotency is the potential of stem cells to give rise to any cell of the embryo proper. The study of pluripotent stem cells began with the study of teratocarcinomas (Evans and Kaufman 1981), which contain a haphazard array of almost any somatic cell type found in the developing embryo. The stem cells of these tumours are embryonal carcinoma (EC) cells, which express characteristics including a developmental potential similar to those of the ICM of the early embryo (Na *et al.* 2010). Mouse Embryonic stem (ES) cells were first pluripotent cells derived from the ICM of mouse blastocyst-stage embryos (Evans and Kaufman 1981). Human ES (hES) cells were isolated from the ICM of human blastocysts, generated by *in vitro* fertilization (IVF)-produced embryos that were no longer designated for clinical use, and donated by individuals after informed consent (Thomson *et al.* 1998).The importance of ES cells to modern biology and medicine derives from two unique distinguishing characteristics. First, they have the ability to be maintained in an

undifferentiated state (self renewal) indefinitely in culture. Second, they are pluripotent, possessing the capacity to generate every cell type in the body (**Fig.1**). The pluripotent nature of mouse ES cells was formally demonstrated by their ability to contribute to all tissues of adult mice, including the germ-line, following their injection into host blastocyst (Bradley *et al.* 1984). ES cells are of great interest for both basic science and medicine. Studies on ES cells can provide information about the complex events that occur during embryonic development by identifying how undifferentiated stem cells become the differentiated cells that form the tissues and organs. ES cells provide opportunities for basic research on developmental gene regulation through functional genomics, signaling, molecular mechanisms of disease, drug screening and toxicological testing. As gene modulation techniques, including gene targeting, are well established in ES cells, it is relatively easy to identify a role of a specific gene in the development of a cell lineage (Keller *et al.* 1993).

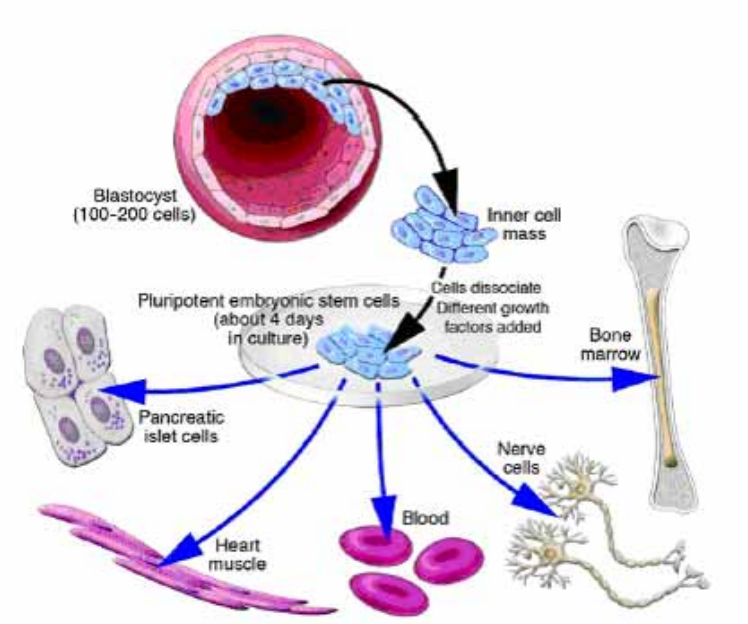


Figure 1. Pluripotent stem cells, isolated from the ICM in the blastocyst, have the ability to give rise to all types of cells in the human body, but not the placenta and other supporting tissues. www.innovitaresearch.org/news/res/04121501_01.jpg

1.2 Developmental potential of pluripotent stem cells

In addition to their developmental potential *in vivo*, ES cells display a remarkable capacity to form differentiated cell types *in vitro* (Keller 1995; Smith 2001).Studies during the past 20 years have led to the development of appropriate culture conditions and protocols for the generation of a broad spectrum of lineages. Because of their ability to give rise to multiple lineages, ES cells open exciting new opportunities to model embryonic development *in vitro* for studying the events regulating the earliest stages of lineage induction and specification. Comparable studies are difficult in the mouse embryo and impossible in the human embryo.

When injected into immuno-compromised animals, pluripotent cells form teratomas composed of multiple differentiated tissues in all three germ layers: ectoderm, mesoderm, and endoderm including the germ line of chimeric animals. Tetraploid complementation assay test is the most stringent test for the pluripotency of ES cells. In this test a tetraploid embryo, incapable of developing beyond blastocyst stage, is fused with diploid ES cells. The resulting embryo develops normally wherein the fetus is exclusively derived from ES cells and the extra embryonic tissues develop exclusively from tetraploid embryo. In addition to these the *in vivo* tests, ES cells are able to differentiate into multiple cell types *in vitro.* When removed from the factors that maintain them as stem cells, ES cells will differentiate and, under appropriate conditions, generate progeny consisting of derivatives of the three embryonic germ layers: mesoderm, endoderm, and ectoderm ES cells can be differentiated in the form of three-dimensional aggregates, known as embryoid bodies (EBs), containing various cell types like cardiac myocytes, neuronal cells, erythrocytes, melanocytes, and others (Doetschman *et al.* 1985; Yamane *et al.* 1999; Lee *et al.* 2000).

The mouse ES cell differentiation system can be regarded as an unlimited source of cells and tissues for a broad spectrum of research. To date, mouse ES cells have been successfully differentiated into hematopoietic precursors (Potocnik *et al.* 1997), CMs (Yamashita *et al.* 2000), neural precursors (Nakano *et al.* 1996; Brustle *et al.* 1997; Garrington *et al.* 2000), insulin-producing cells (Klug et al. 1996), and mast cells (Tsai *et al.* 2000) and have been transplanted successfully into recipient animals. *In*

vitro differentiation of ES cells has been used in basic science to study gene expression during development of specific cell types.

Studies of pluripotent mouse ES cells have led to *in vitro* models of cardiomyocyte differentiation. Much of what we know about the differentiation of pluripotent ES cells to CMs *in vitro* has been learned from studies with mouse ES cells. Morphological, electrophysiological, and molecular techniques indicate that the *in vitro* differentiation process recapitulates the developmental pattern of early cardiogenesis, and genetic studies have shown how signaling molecules, transcription factors, ECM components, and calcium-handling proteins affect this process. Genomic studies, coupled with *in vitro* differentiation, have also led to the identification of developmentally regulated genes (gene trapping) and to the identification of differentiation-responsive genes.

The potential use of ES cells for research is challenged by ethical considerations and more so due to imposition of strict regulations. Although ES cells are regarded as a 'gold standard' for pluripotency, there is a need for alternate sources of pluripotent cells which can be easily accessible and possess the same developmental potential as ES cells.

1.3 Nuclear reprogramming

Embryonic development is a unidirectional process characterized by constant loss of developmental potential with progress in time. Cells progress from totipotency to pluripotency, multipotency and then finally commit to differentiated cell fates. Genomic content of totipotent zygote is identical to that of its pluripotent progeny and terminally differentiated somatic cells. This content remains unchanged throughout development. Thus the difference in developmental potential of these cell types is imposed by a cell specific transcriptional state that is ultimately dependent on epigenetic changes of genome. The cell fate of a defined specialized cell type can be reversed, returning the cell to an embryonic state. *Reprogramming is a term used to describe the process that reverts nuclear gene expression of fully differentiated somatic cells to a pluripotent state.* This process is of interest for three reasons. Understanding of reprogramming process can help us to understand how cell differentiation and specialized gene expression are normally maintained. Nuclear

reprogramming enables the culture of lines of cells from diseased tissues, and hence allows us to analyze the nature of the disease and to screen for therapeutic drugs. It can open several new opportunities of *in vitro* disease models to help in understanding disease mechanisms thus leading to devising new strategies for treatment. Nuclear reprogramming could represent a first major step in cell-replacement therapy, providing replacement of heart, pancreas, or other types of cells from the skin of the same individual.

1.4 Strategies of nuclear reprogramming

1.4.1 Reprogramming by somatic cell nuclear transfer (SCNT)

Nuclear reprogramming is initiated when a nucleus from a differentiated somatic cell is transplanted into an enucleated oocyte leading to the generation of a live offspring, which is a genetically identical clone of the original somatic cell. Such nuclear-transfer experiments, also known as cloning, have shown definitively that all of the genes required to create an entire organism are present in the nucleus of the specialized cell and can be activated on exposure to reprogramming factors present in the oocyte. Briggs and King (Briggs and King 1952) first succeeded in producing normal swimming tadpoles of *Rana pipiens* by transplanting the nuclei of embryo (blastula) cells into enucleated eggs. Similar experiments were carried out with enucleated eggs of *Xenopus laevis (Gurdon and Uehlinger 1966)*, which were transplanted with the nuclei of intestinal epithelium of feeding tadpoles to obtain entirely normal and fertile male and female frogs. The major conclusion from these and subsequent findings was that development imposes reversible epigenetic rather than irreversible genetic changes on the genome during cellular differentiation. The first successful cloning of a higher animal using SCNT was demonstrated by the cloning of normal adult sheep (Dolly) by transplanting the nuclei of cultured mammary gland cells derived from an adult sheep to enucleated sheep eggs (Wilmut *et al.* 1997). Cloning experiments have been successfully performed with different kinds of terminally differentiated cells, such as T lymphocytes (Hochedlinger and Jaenisch 2002) post-mitotic olfactory neurons (Eggan *et al.* 2004), natural killer T cells (Inoue *et al.* 2005), peripheral blood granulocytes, and malignant melanoma (Hochedlinger, 2004). In addition to sheep and mice, a wide range of species have

now been successfully cloned using SCNT, ranging from domesticated animals such as dogs and goats, and their hybrids such as mules, to wild animals such as African wildcats and wolves (Gomez *et al.* 2004). Extinct animals could also be successfully cloned by transplantation of nuclei from frozen cells into enucleated oocytes a decade after tissue freezing (Wakayama *et al.* 2008). However, most cloned animals with apparently normal gross anatomy can have numerous abnormalities. Common abnormalities include aberrant gene expression in embryos, telomere elongation, obesity in adults, impaired immune systems and, often, increased cancer susceptibility and premature death (Gomez *et al.* 2004)

SCNT ES cells have been derived from an immunodeficient mouse and then used *in vitro* to correct the original genetic defect by homologous recombination. Subsequently, the repaired SCNT ES cells were differentiated into hematopoietic stem cells and transplanted back to the immunodeficient donor mouse to restore lymphopoiesis (Rideout *et al.* 2002). Although no human SCNT ES cell lines have been created yet, the successful recent generation of SCNT ES cell lines in primates (Byrne *et al.* 2007) suggests that therapeutic cloning in humans may be feasible in the future. **Fig. 2** shows the process of the derivation of ES cells from a blastocyst derived from oocyte which has received nucleus from somatic cell.

To increase the frequency of cloned animals various technical modifications have been tested in mice, including attempts at chemically activating oocytes to make them more responsive, changing the time of enucleation, inhibiting cytokinesis and using cell fusion instead of nuclear injection, but these alterations have led to only modest results of 1-3% increase (Thuan *et al.* 2010).

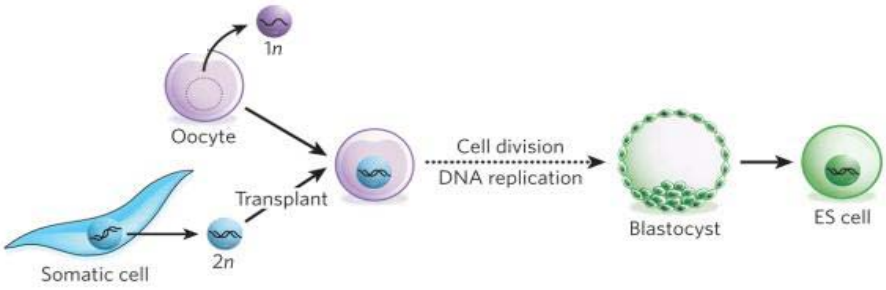


Figure 2. Schematic representation of the technique of somatic cell nuclear transfer (SCNT). Yamanaka and Blau; Nature 465, 704–712.

The need for a large number of donated human oocytes, ethical concerns and extremely low cloning efficiency by SCNT in mammals render this approach impractical. Nuclear transfers between different strains or subspecies are just as successful as those within a species; however, eggs produced by transfers between very different species such as human, mouse, cow, or pig generally die before the 32-cell stage (Tecirlioglu *et al.* 2006). So far, there is no confirmed evidence that proliferating ES cells can be obtained from such distant combinations, including human nuclei in monkey cytoplasm.

1.4.2 Reprogramming by fusion of somatic cells

Cell fusion involves fusing two or more cell types to form a single entity. The possibility that two different cell types can fuse, known as heterotypic fusion, and form a somatic cell hybrid was initially suggested back in 1965 (Harris and Watkins 1965). The hybrid cells were called 'heterokaryons'. Cell-cell fusion between pluripotent teratocarcinoma and differentiated thymocyte cells resulted in hybrid cells maintaining the potential for unlimited self renewal and differentiation into a variety of cell types. The authors had hypothesized that the teratocarcinoma might lose pluripotency by fusion with differentiated somatic cells, but instead, the hybrid cells obtained pluripotency and resembled EC cell morphology without expressing a tissue specific gene such as *Thy* (Miller and Ruddle 1976; Miller and Ruddle 1977). Following this initial study, many studies have shown that various somatic cells can be reprogrammed by fusing with pluripotent stem cells like ES, EG, or EC cells (Matveeva *et al.* 1998; Tada *et al.* 2001; Flasza *et al.* 2003; Cowan *et al.* 2005).Cell fusion also was suggested as a mechanism for somatic cell plasticity. Phenotype and potency of somatic cells (bone marrow cells and brain cell) were changed by spontaneous cell fusion with ES cells after co-culture with ES cells (Terada *et al.*, 2002, Ying *et al.*, 2002). The hybrid cells that showed over-diploid DNA content expressed pluripotency related genes and could differentiate into all three embryonic germ layer *in vivo* and *in vitro*.

EC cells are another source of pluripotent cells that can reprogram somatic cells by fusion. Since EC cells share many of the key pluripotent characteristics with ES therefore EC cells can provide a readily amenable alternative source for

reprogramming (Do *et al.*, 2009a; Flasza *et al.*, 2003; Mise *et al.*, 1996). Moreover, mouse EC cells can reprogram human somatic cells into pluripotent state, indicating that reprogramming factors can cross-act through another species (Flasza *et al.*, 2003).

The fusion hybrid cells present pluripotential characteristics, such as inactivation of tissue-specific genes, reactivation of pluripotency related genes, differentiation potential to all three germ layers, and a specific epigenetic state corresponding to the pluripotent cells (Do *et al*., 2006). The differentiated state of somatic cells could also be altered by fusion with another type of somatic cell, suggesting that cellular factors between the two different types of cells dynamically interact and might be responsible for the plasticity and re-establishment of new characteristics. However, the fusion hybrid cells are not identical to the pluripotent fusion partner cells.

When somatic cells acquire pluripotency through cell-cell fusion with pluripotent stem cells, the reprogrammed hybrid cells express pluripotency-related genes but did not express tissue-specific genes. The 'memory' of somatic cells is almost like a dogma considered to be erased by fusion with pluripotent cells during fusion-induced pluripotential reprogramming. Fusion-induced reprogramming was thought to be a unidirectional process resulting in an ES cell phenotype without other viable cell states (Silva *et al.*, 2006). But Do *et al* in 2009 demonstrated that pluripotent stem cells also could acquire some characteristics of differentiated cells indicating that the reprogramming direction in pluripotent hybrid cells is not solely unidirectional, and some genes could be reprogrammed opposite to that of the pluripotent fusion partner (Do *et al.* 2009).

Under differentiation-inducing conditions, fusion hybrid cells exhibit similar differentiation potential of their pluripotent fusion partner, and are not preferentially committed to the lineage of the somatic cells that had been fused with the pluripotent cells. Inability for neural differentiation of the F9- neural stem cells (NSC) fusion hybrid cells demonstrated that NSCs lose their memory and adopt the similar differentiation potential of F9 cells. Therefore, the differentiation potential of fusion hybrid cells is contingent on the type of pluripotent fusion partner cells, and the resulting hybrid cells have the same potential as the pluripotent fusion partner cells. Pluripotent cells reprogram somatic cells and may induce erasure of somatic cell memory by fusion.

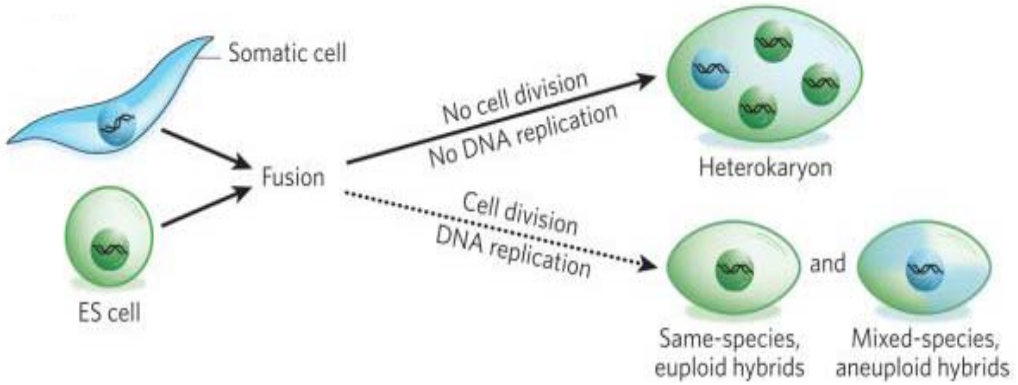


Figure 3. Schematic representation of reprogramming brought about fusion of somatic cells with ES cells leading to formation of fusion hybrids. Yamanaka and Blau; Nature 465,704-712.

Major disadvantage of this approach of reprogramming is that it leads to tetraploid cells or aneuploid cells **(Fig. 3)**. Variable chromosome number ranging from 40-85 was observed in hybrids between mouse spleenocytes and murine ES cells due to extensive loss or gain of individual chromosomes. This indicates that spontaneous segregation leading to diploid genome in the hybrid is not achieved (Matveeva et al. 1998).

Experiments into cell fusion mediated by PEG have provided important information about the molecules and pathways that have been implicated in the regulation of reprogramming. Specifically, it has been seen that over-expression of the embryonic factor Nanog in ES cells, or Sall4 in mouse embryonic fibroblasts (MEFs), can increase the number of reprogrammed somatic cells after fusion (Wong *et al.* 2008; Silva *et al.* 2009). On the other hand, the periodic activation of the Wnt/β-catenin signaling pathway in ES cells and the activation of Akt signaling remarkably enhance cell-fusion-mediated reprogramming (Lluis, 2008; Nakamura, 2008). Interestingly, in ES cells over-expression of Nanog, and at the same time activation of the Wnt/β-catenin pathway, can enhance the reprogramming efficiency of somatic cells even further after fusion (Lluis and Cosma 2009).The method of cell fusion might be regarded superior to SCNT, as it is technically less challenging and does not require the use of oocytes and pre-implantation embryos.

Fusion hybrid cells can be used to study gene expression, basics of cell division, and transformation of normal cells to malignant cells, obtain viral replication, chromosome or gene mapping, production of monoclonal antibodies and as a tool for investigating the mechanism of cellular plasticity.

1.4.3 Nuclear reprogramming by cell extracts

In this technique of reprogramming, the plasma membrane of an adult cell to be reprogrammed is reversibly permeabilized with the bacterial toxin Streptolysin O. Permeabilized cells are then incubated with a nuclear and cytoplasmic extract derived from another type of cell. After transient exposure to extract, cells are resealed and cultured **(Fig. 4)**. During incubation, factors in the cell extract required for reprogramming diffuse into permeabilized cells and activate genes that are typically expressed in cells from which the extract has been prepared. This principle has been used to reprogram kidney epithelial 293T cells to express T lymphocyte or neuronal markers by incubating them with an extract prepared from T cells or neuronal precursor cells, respectively (Hakelien *et al.* 2002). However, although reprogrammed fibroblasts expressed T cell specific surface markers, such as CD3, CD4, CD8, CD45 and T cell receptor (TCR) αβ chains, and a T cell specific function, the reprogramming of the 293T cells into T cells was not complete, as the reprogrammed cells did not express a pure T cell specific phenotype and the expression profile of many genes did not match that of T cells. In addition to human 293T cells, the cell extract based reprogramming approach was also applied to other mammalian cells such as human adipose tissue stem cells, which adopted cardiomyocyte properties following transient exposure to rat cardiomyocyte extract (Gaustad *et al.* 2004).

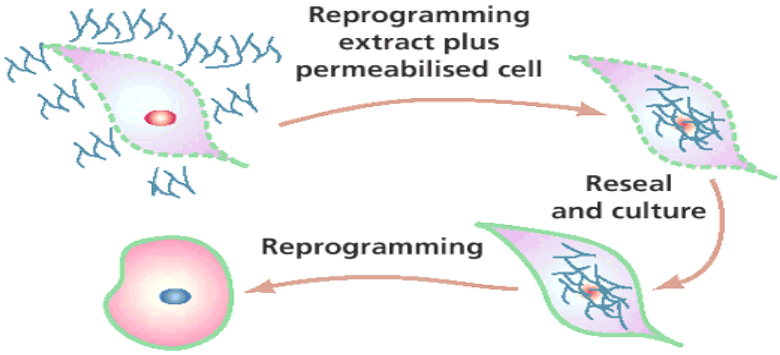


Figure 4: Reprogramming by cell extracts. Yamanaka and Blau; Nature 465. 704-712.

At present it is unclear if complete and stable reprogramming to a pluripotent state can be achieved with crude cell extracts and if reprogramming efficiencies would be different with different types of cells. However, this method is very attractive because cell extract derived factors are presumably not permanently active in target cells but turn over at kinetics corresponding to their half lives. This approach may prove useful for dissecting the molecular machinery involved in reprogramming using biochemical methods (Hansis *et al.* 2004).

1.4.4 Reprogramming by transcription-factor transduction

Successful reprogramming of somatic cells by fusion with ES cells indicates that ES cells have factors that induce pluripotency. It seemed likely that these pluripotency-inducing factors also play important roles in the maintenance of pluripotency. Based on this hypothesis, Yamanaka and Takahashi devised an elegant screen for factors within a pool of 24 pluripotency-associated candidate genes and tested for their ability to induce pluripotency and activate a dormant drug resistant allele integrated into the ES cell-specific *Fbx15* locus. The combination of 24 factors, when co-expressed from retroviral vectors in mouse fibroblasts, indeed activated *Fbx15* and induced the formation of drug-resistant colonies with characteristic ES cell morphology (Takahashi and Yamanaka, 2006). Successive rounds of elimination of individual factors then led to the identification of the minimally required core set of four genes, comprising *Klf4*, *Sox2*, *c-Myc*, and *Oct4*.

Retrovirus-mediated introduction of four transcription factors (*Oct-3/4, Sox2, c-Myc,* and *Klf4*) into mouse embryonic or adult fibroblasts and selection for the expression of *Fbx15*, a target of *Oct-3/4* and *Sox2*, resulted in the generation of induced pluripotent stem (iPS) cells, which are similar to ES cells in morphology, proliferation, and teratoma formation (Takahashi and Yamanaka 2006) **(Fig. 5)**. The selected iPS cells are, however, significantly different from ES cells in gene expression and DNA methylation patterns. When transplanted into blastocysts, these iPS cells only give rise to chimeric embryos, but not adult or germ line competent chimeras. These data indicate that reprogramming in Fbx15-selected iPS cells is incomplete. These “first-generation” iPS cells therefore appeared to be only partially reprogrammed.

Although both *Fbx15* and *Nanog* are targets of *Oct-3/4* and *Sox2*, the former is dispensable for pluripotency, while the latter plays crucial roles. By selecting for the reactivation of the essential pluripotency genes *Nanog* or *Oct4* instead of *Fbx15*, iPS cells were generated that molecularly and functionally more closely resembled ES cells (Maherali *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007). Nanog-selected iPS cells showed reactivation of a somatically silenced X chromosome and underwent random X-inactivation upon differentiation (Maherali *et al.* 2007). These data demonstrated that full reprogramming can be achieved by expression of the four factors and using an appropriate selection procedure.

iPS cells have also been derived from a number of different species-including humans (Takahashi *et al.* 2007; Yu *et al.* 2007; Park *et al.* 2008; Li *et al.* 2009), and rhesus monkeys (Liu *et al.* 2008) by expression of the four Yamanaka factors demonstrating that fundamental features of the transcriptional network governing pluripotency remain conserved during evolution. Similarly, iPS cells have been derived from other somatic cell populations, such as keratinocytes (Maherali *et al.* 2007; Aasen *et al.* 2008), neural progenitor cells (Eminli *et al.* 2008), stomach and liver cells (Aoi *et al.* 2008) and melanocytes (Utikal *et al.* 2009), as well as from genetically labeled pancreatic β cells (Stadtfeld *et al.* 2008) and terminally differentiated lymphocytes (Hanna *et al.* 2008; Eminli *et al.* 2009), further underscoring the universality of induced pluripotency.

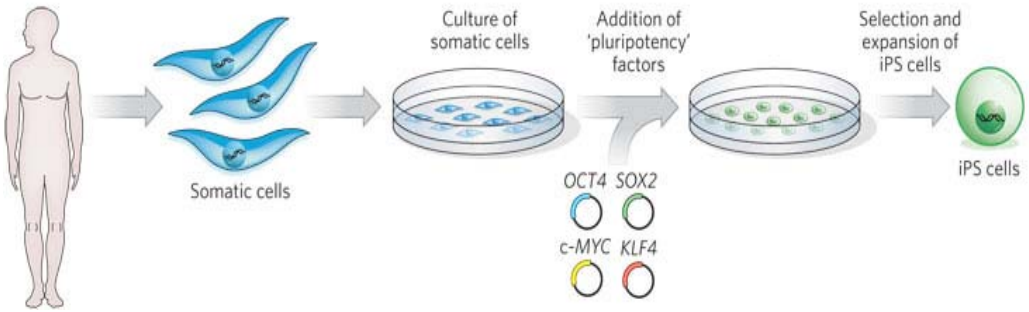


Figure 5. Induction of pluripotency in adult cells by retroviral transduction of transcription factors. Yamanaka and Blau; Nature 465, 704–712.

The unique properties of ES and iPS cells also provide for practical approaches in pharmaceutical toxicology and pharmacogenomics. In particular, hepato-toxicity and cardiotoxicity are two principal causes of drug failure during preclinical testing, while the variability in individual responses to potential therapeutic agents is also a major problem in effective drug development (Rubin 2008). The advantage of iPS cell technology is that it allows for the first time the generation of a library of cell lines that may to a substantial extent represent the genetic and potentially epigenetic variation of a broad spectrum of the population. The use of this tool in high-throughput screening assays could allow better prediction of the toxicology caused by and therapeutic responses induced by newly developed dugs and offer insight into the underlying mechanisms. The net result of this approach would substantially decrease the risk and cost associated with early-stage clinical trials and could lead toward a more personalized approach in drug administration.

iPS cell derivation is ethically and legally less problematic and technically more feasible than SCNT. In order to use iPS cells as efficient research tools suitable techniques of factor delivery and efficient identification of faithfully reprogrammed cells are crucial.

1.5 Advancement of methods for iPS cell generation

1.5.1 Factor delivery into target cells

A number of different approaches have been devised to shuttle reprogramming factors into somatic cells, which can affect the efficiency of reprogramming and the quality of resultant iPS cells. The first studies on iPS cells used constitutively active retroviral vectors that stably integrated into the host cell genome to introduce *c-Myc*, *Klf4*, *Oct4*, and *Sox2* (Takahashi and Yamanaka 2006; Wernig *et al.* 2007; Wilber *et al.* 2007). While retroviral transgenes are usually silenced toward the end of reprogramming (Stadtfeld *et al.* 2008), this process is often incomplete, resulting in partially reprogrammed cell lines that continue to depend on exogenous factor expression and fail to activate the corresponding endogenous genes. In addition, residual activity or reactivation of viral transgenes in iPS cell-derived somatic cells can interfere with their developmental potential and frequently leads to the formation of tumors in chimeric animals (Okita *et al.* 2007). When constitutively active lentiviral vectors are

used to produce iPS cells, which are even less efficiently silenced in pluripotent cells than retroviral vectors, it can lead to differentiation block (Brambrink *et al.* 2008; Sommer *et al.* 2010). The use of inducible lentiviral vectors, whose expression can be controlled by the inert drug doxycycline, diminishes the risk of continued transgene expression and allows for the selection of fully reprogrammed iPS cells, since cells that depend on exogenous factor expression readily stop proliferating upon doxycycline withdrawal (Brambrink *et al.* 2008; Stadtfeld *et al.* 2008). Lentiviral vectors are also more efficient than retroviral vectors at infecting different somatic cell types and can be used to express polycistronic cassettes encoding all four reprogramming factors, thus increasing reprogramming efficiency ((Carey *et al.* 2009; Sommer *et al.* 2009; Sommer *et al.* 2010). Inducible vector systems have been employed to generate so-called "secondary" reprogramming systems, which do not rely on direct factor delivery into target cells. These systems entail differentiating "primary" iPS cell clones, generated with doxycycline-inducible lentiviral vectors or transposons, into genetically homogeneous somatic cells using either *in vitro* differentiation (for human cells) (Hockemeyer *et al.* 2008) or blastocyst injection (for mice) (Wernig *et al.* 2007; Woltjen *et al.* 2009). These somatic cells are then cultured in doxycycline-containing media, thus triggering the formation of "secondary" iPS cells at efficiencies that depend on the specific cell type used but are generally several orders of magnitude higher than the efficiencies obtained after primary infection. Secondary systems therefore (1) allow for the reprogramming of large quantities of genetically homogeneous cells for biochemical studies and cells that are difficult to culture or transduce, and (2) facilitate the comparison of genetically matched iPS cells derived from different somatic cell types. The recent development of "reprogrammable" mouse strains, which contain a single inducible polycistronic transgene in a defined genomic position enables the breeding of animals into desired mutant backgrounds for mechanistic studies (Carey *et al.* 2009).

1.5.2 Integration-free iPS cells

Potentially harmful effects of leaky transgene expression and insertional mutagenesis can be overcome by producing transgene free iPS cells. Techniques to generate integration-free iPS cells can be subdivided into three categories **(Fig. 6)**:

(a) Non integrating viruses. Human fibroblasts have also been reprogrammed into iPS cells with adenoviral vectors (Zhou and Freed 2009) and Sendai virus (Fusaki *et al.* 2009), as well as with polycistronic minicircle vectors (Jia *et al.* 2010) and self-replicating selectable episomes (Yu *et al.* 2011), albeit the latter system required the simultaneous over-expression of additional factors, including another potent oncogene (*Oct4, Sox2, c-Myc, and Klf4*, together with *Nanog, Lin28*, and SV40 large T antigen). Reprogramming efficiencies with current non-integrating methods are several orders of magnitude lower (~0.001%) than those achieved with integrating vectors (0.1%–1%); most likely because factor expression is not maintained for a sufficient length of time to allow complete epigenetic remodeling.

(b) Integrating vectors that can be excised: Several laboratories have developed integration-dependent gene delivery vectors with incorporated loxP sites that can be subsequently excised from the host genome by transient expression of Cre recombinase (Kaji *et al.* 2009; Soldner *et al.* 2009). This approach enables the efficient generation of iPS cells from different cell types. The use of polycistronic vectors have especially shown to be very useful (Chang *et al.* 2009; Sommer *et al.* 2009; Sommer *et al.* 2010). Transgene-free iPS cells can also be generated with *piggyBac* transposons, mobile genetic elements that can be introduced into and removed from the host genome by transient expression of transposase (Woltjen *et al.* 2009; Yusa *et al.* 2009). It remains unclear if transposase expression can induce nonspecific genomic alterations in iPS cells (Stadtfeld and Hochedlinger 2009).

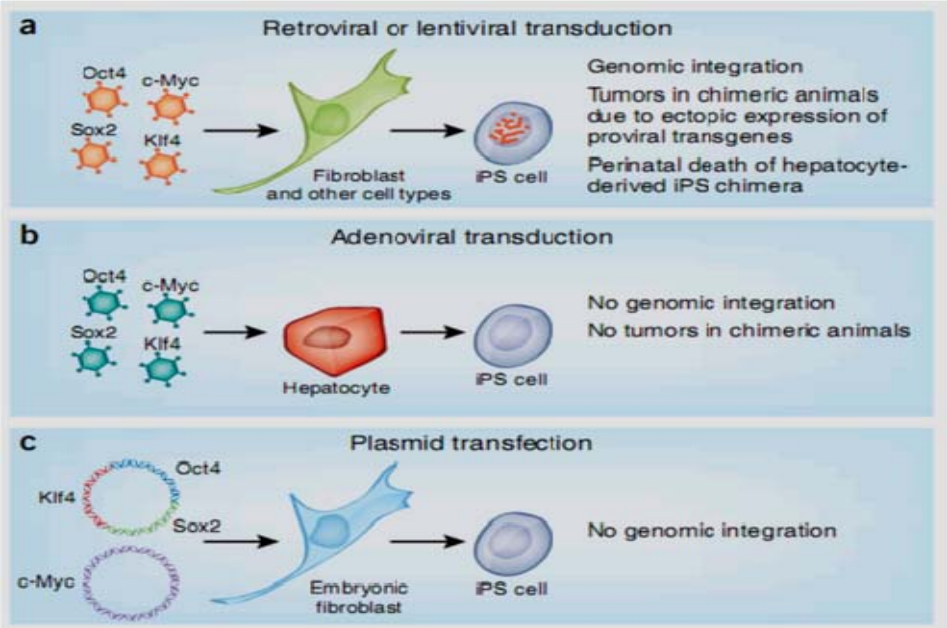


Figure 6. Different approaches to generate iPS cells. Modified from Lowry and Plath, Nature Biotechnology 26, 1246 - 1248 (2008).

(c) non nucleic acid reprogrammers: iPS cells have been derived from both mouse and human fibroblasts by delivering the reprogramming factors as purified recombinant proteins (Zhou and Freed 2009) or as whole-cell extracts isolated from either ES cells (Cho *et al.* 2010) or EK293 cells genetically engineered to stably express reprogramming factors fused with 9 arginine and myc tag (Kim *et al.* 2009). While the use of purified proteins represents an attractive approach for the generation of transgene-free iPS cells, its efficiency is extremely low and requires chemical compounds that promote reprogramming. Small molecules were utilized that could significantly increase reprogramming efficiencies in the context of Oct4, Klf4, Sox2, and c-Myc over expression ((Desponts and Ding 2010). Notably, some of these molecules can also replace individual reprogramming factors, raising the possibility of deriving iPS cells solely with chemicals. Chemical substitution of a reprogramming factor is, in most cases, associated with a significant decrease in the number of iPS cells clones generated, indicating that no single chemical compound is able to entirely replace the function of a transcription factor. Another potential caveat of chemical reprogramming approaches is the introduction of genetic or epigenetic abnormalities into resultant iPS cells, especially since many of the reported compounds are potent modulators of DNA and chromatin modifications.

A more efficient and safer way of producing integration-free iPS cells may be the introduction of modified RNA molecules encoding for the reprogramming factors into somatic cells, which has been validated recently (Warren *et al.* 2010).These authors have demonstrated a simple, non-integrating strategy for reprogramming cell fate based on administration of synthetic mRNA modified to overcome innate antiviral responses. The modified RNA induced pluripotent cells thus obtained were termed as RiPSC.

In earlier reprogramming strategies, microRNAs (miRNAs) were used to promote the transcription factor-mediated reprogramming process but recently two independent groups have derived human and mouse iPS cells by adding miRNAs, in the absence of any additional protein factors (Anokye-Danso *et al.* 2011) shows that expression of a single primary miRNA transcript, the miR-302/367 cluster, is in rapidly and efficiently reprograms mouse and human somatic cells to an iPS cell state without a requirement for exogenous transcription factors. The resulting iPS cells exhibit gene expression and functional properties characteristic of fully reprogrammed pluripotent cells. Approximately 10% of fibroblasts form iPS cell colonies, an improvement in efficiency of >100-fold compared with OSKM. Moreover, the appearance of iPS cell colonies and the activation of pluripotency markers occur sooner. Miyoshi et al also demonstrated the possibility of reprogramming human and murine fibroblasts by transfection of a combination of mature double-stranded microRNAs (miRNAs) mir-200c plus mir-302 s and mir-369 s family miRNAs(Miyoshi *et al.* 2011).

The unique properties of ES and iPS cells also provide for practical approaches in pharmaceutical toxicology and pharmacogenomics. In particular, hepatotoxicity and cardiotoxicity are two principal causes of drug failure during preclinical testing, while the variability in individual responses to potential therapeutic agents is also a major problem in effective drug development. The advantage of iPS cell technology is that it allows for the first time the generation of a library of cell lines that may, to a substantial extent, represent the genetic and potentially epigenetic variation of a broad spectrum of the population. The use of this tool in high-throughput screening assays could allow better prediction of the toxicology caused by and therapeutic responses induced by newly developed dugs and offer insight into the underlying mechanisms. The net result of this approach would substantially decrease the risk and cost

associated with early-stage clinical trials and could lead toward a more personalized approach in drug administration.

1.5.3 Identification of iPS cell colonies

A technical roadblock to efficient iPS cell derivation is the inability to distinguish successfully reprogrammed clones from partially reprogrammed or simply transformed colonies. The reactivation of endogenous pluripotency-associated genes such as *Fbx15* (Takahashi and Yamanaka 2006)), *Nanog* or *Oct4* (Maherali *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007), linked to drug selection cassettes has been successfully employed for this purpose. As mentioned above, *Fbx15* selection generates partially reprogrammed cells, likely because activation of this gene occurs early in the reprogramming process when the majority of cells are not yet fully reprogrammed (Stadtfeld *et al.* 2008). High quality iPS cells can be derived from unmodified somatic cells without drug selection or fluorescent reporters by simply using morphological criteria (Blelloch *et al.* 2007; Maherali *et al.* 2007; Meissner *et al.* 2007), although this approach requires careful characterization of the resultant cell lines. A more stringent approach to identify fully reprogrammed human iPS cells without the use of drug selection combines the detection of surface markers with that of "indicator retroviruses" expressing fluorescent proteins, which become silenced upon acquisition of pluripotency (Chan *et al.* 2009). For human iPS cells, expression of surface markers such as TRA-1-81 has been shown to enrich for reprogrammed cells (Lowry *et al.* 2008).

It has been demonstrated recently with human ES cells, that G418 resistance driven by the *undifferentiated transcription factor1 (UTF1)* promoter plus enhancer elements is very efficient to enrich the fraction of pluripotent cells within ES cell cultures (Tan *et al.* 2007). *UTF1* is expressed in EC cells, ES cells and cells of the germ line but absent in adult tissues (Okuda *et al.* 1998). The *UTF1* promoter consists of a short TATA less region and a downstream enhancer at the 3-prime end of the coding sequence (Okuda *et al.* 1998; Nishimoto *et al.* 1999; Nishimoto *et al.* 2001). Functional binding sites for Oct-3/4 and Sox2 were identified within this enhancer region. In addition, a genetic element called M1 could be found within the enhancer and carries an octamer sequence important for Nanog expression (Rodda *et al.* 2005;

Tan *et al.* 2007). Besides these elements, it is likely that additional, yet unknown factors contribute to the regulation of *UTF1*. Experiments that demonstrate a fast down regulation of *UTF1*, which responds even faster to spontaneous and induced differentiation than Oct4 or Nanog, clearly point to a more complex regulation of the *UTF1* expression (Nishimoto *et al.* 1999; Wei *et al.* 2005; Cai *et al.* 2006; Tan *et al.* 2007). The fast response of the *UTF1* expression upon induction of differentiation, which is probably supported by its low endogenous expression level, makes the *UTF1* promoter an interesting tool for the enrichment of high quality, homogenous pluripotent cell lines (Tan *et al.* 2007). In this context, it is important to note that reprogramming of human fibroblasts demonstrated that *UTF1* over-expression and a p53 knockdown act synergistically to enhance reprogramming efficiency of *Oct4, Klf4, Sox2* and *c-Myc* by 200-fold (Zhao *et al.* 2008). Knockdown of p53, which is believed to be suppressed by *Klf4* or over-expression of UTF1 alone, had reduced supporting effects on reprogramming (Zhao *et al.* 2008).

1.6 Direct reprogramming: Conversion of one somatic-cell type to another

The fate of a cell can be altered by forced expression of single tissue-specific transcription factors. Gehring and colleagues (Schneuwly *et al.* 1987) were the first to show that in *D. melanogaster* larvae, ectopic over expression of a homeotic gene, *Antennapedia*, under the control of a heat-shock gene promoter led to a change in body plan, with an additional set of legs being formed instead of antennae. Even more striking was the finding by Gehring (Gehring 1996) almost a decade later that ectopic expression of *eyeless* (known as *Pax6* in mice), a master controller of a cascade of 2,500 genes, led to the development of functional eyes on the legs, wings and antennae of *D. melanogaster*. In mice, the first tissue-specific master regulatory transcription factor was identified by Weintraub and colleagues (Davis *et al.* 1987) in 1987. They found that it was possible to induce a phenotypic conversion of fibroblasts to the myogenic lineage by expressing a single muscle helix–loop–helix protein MYOD. In addition, in 2004, the mouse C/EBP family of transcription factors was shown by Graf and colleagues to have a key role in the conversion of one blood cell type to another (from lymphocytes to macrophages) (Xie *et al.* 2004). In addition, when the gene encoding the transcription factor PAX5 was removed from B cells,

these cells reverted to less specialized progenitors (Cobaleda *et al.* 2007). It should be noted that, in mammals, altering the expression of single transcription factors generally results in the phenotype of somatic cells changing only to that of closely related cell types, so the effects of transcription factors are highly context dependent (Schafer *et al.* 1990; Farah *et al.* 2000).

Direct reprogramming of mature cells from one lineage to another has emerged recently as an alternative strategy for generating cell types of interest. Basing on the studies of Weintraub and colleagues and on the information derived from the robust methodologies of generating iPS cells, it has been possible to directly reprogram mouse embryonic fibroblasts to neurons, CMs and to blood cell progenitors without an intermediate pluripotent state.

Induced neurons (iNs) were formed by lentiviral transduction of a cocktail of 19 neuronal specific transcription factors leading to neural specific expression of Tau in mouse embryonic fibroblasts(Vierbuchen *et al.* 2011). Later the cocktail was reduced to a minimum of four transcription factors Ascl1, Brn2, and Mytl1 or Zic1 required to form iNs which were convincingly demonstrated to function as typical neurons *in vitro*. In a similar study a cocktail of cardiac specific 14 transcription factors, related to heart development, were used to induce CMs (iCMs) from mouse tail tip fibroblasts and cardiac fibroblasts isolated from mice expressing EGFP/puromycin under a cardiac-specific alpha myosin heavy chain (αMHC) reporter (Ieda *et al.* 2011). Out of the initial pool of 14 factors, Gata4, Mef2c, and Tbx5 (T-box transcription factor) were found to optimally induce the αMHC-GFP reporter (25% of cells) and cardiac Troponin T (8% of cells). The iCMs exhibited spontaneous contractions and electrical activity *in vitro*. Another group used the 'Yamanaka factors'-OCT4 (also known as POU5F1), SOX2, KLF4 and c-MYC — to initiate reprogramming, but they blocked signaling through the JAK–STAT pathway, which is required for pluripotency in the mouse, and added the cardiogenic factor BMP4 (Efe *et al.* 2011). These modifications yielded minimal generation of iPS cells and instead activated the cardiac progenitor program and, within 2 weeks, generated substantial numbers of beating colonies. By 18 days after induction, approximately 40% of the cells expressed cardiac troponin T.

Later human dermal fibroblasts were reprogrammed into $CD45^{+}/CD34^{+}$ hematopoietic progenitor colonies by over expression of single factor (Oct4), in

combination with exposure to the cytokines SCF (stem cell factor) and FLT3LG (FMS-like tyrosine kinase 3 ligand). The resulting cells possess the potential to form both erythroid and myeloid cells when exposed to the appropriate cytokines (Szabo *et al.* 2011). Zabierowski et al have shown that only one factor, the active intracellular form of Notch1, is sufficient to convert mature pigmented epidermal-derived melanocytes into functional multipotent neural crest stem-like cells (Zabierowski *et al.* 2011). These induced neural crest stem cells (iNCSCs) proliferate as spheres under stem cell media conditions, re-express neural crest-related genes and differentiate into multiple neural crest derived mesenchymal and neuronal lineages. Moreover, iNCSCs are highly migratory and functional *in vivo*.

Ambasudhan et al have reported that a combination of a microRNA (miR-124) and two transcription factors (MYT1L and BRN2) is sufficient to directly reprogram postnatal and adult human primary dermal fibroblasts (mesoderm) to functional neurons (ectoderm) under precisely defined conditions. These human induced neurons (hiNs) exhibit typical neuronal morphology and marker gene expression, fire action potentials, and produce functional synapses between each other. Success of direct reprogramming has opened new questions of efficiency, diversity, degree of developmental timing, resemblance of the characteristics of the reprogrammed cell to its target cell and the epigenetic of maintenance of the reprogrammed cell fate. Like the advent of the iPS cell field, it may soon be possible to induce reprogrammed cells from all the known cell types (Ambasudhan *et al.* 2011).

It is also important to consider the advantages and disadvantages of direct reprogramming in comparison to iPS cell derived cell types. iPS cells offer great flexibility, scalability and multiple cell type derivation. When starting with pluripotent cells, current protocols yield differentiated cells that appear to correspond to fetal stages of human development. This is a considerable challenge in the context of modeling late-onset human diseases such as Alzheimer's or Parkinson's diseases (Saha and Jaenisch 2009). It may be only a matter of time before directed reprogramming can match the cell-type diversity currently accessible only through a pluripotent intermediate. One weakness of iPS that can be overcome by direct reprogramming is the age of the target tissue. Another potential advantage of direct reprogramming is the overall speed and simplicity of the differentiation conditions. In contrast, disease

modeling using iPS cells requires time to generate, expand, characterize, and differentiate pluripotent cells.

1.7 Epigenetics of reprogrammed cells

DNA methylation, acetylation and methylation of histone H3 and H4 amino terminal tail are crucial epigenetic modifications involved in regulating gene activity (Lachner *et al.* 2003). Embryonic stem cells carry several epigenetic characteristics that distinguish them from differentiated cells and that are thought to contribute to stem cell identity. For example, key pluripotency genes such as Oct4 and Nanog are free of DNA methylation in ES cells and can be actively transcribed, while lineage commitment results in silencing of these genes by *de novo* DNA methylation (Feldman *et al.* 2006). In addition to DNA methylation, histone modification patterns also differ between ES cells and differentiated cells. For example, the repression of developmental genes in ES cells is regulated in a remarkable way. Paradoxically, both activating H3K4me3 and repressive H3K27me3 histone modifications are found at these genes (Azuara *et al.* 2006; Bernstein *et al.* 2006). The H3K4me3 marks of these 'bivalent' domains (chromatin regions bearing both activating and repressive histone modifications) allow transcription to be initiated at these genes, but transcription elongation is obstructed by proteins of the Polycomb group (PcG)—an evolutionarily conserved family of proteins that regulate expression of developmental genes through gene silencing that bind to the repressive H3K27me3 mark (Guenther *et al.* 2007). Intriguingly, most silenced developmental genes that are regulated by Oct4, Sox2 and Nanog in ES cells are also targeted by PcG proteins, indicating an important role for PcG proteins in ES cell pluripotency (Bernstein *et al.* 2006; Boyer *et al.* 2006). Bivalent domains are believed to be instrumental to the transcriptional flexibility of ES cells by allowing developmental genes to be stably repressed without irrevocably silencing them. Bivalent domains are virtually exclusive to ES cells and that they are an important characteristic of the pluripotent state.

During nuclear reprogramming it is expected that the final structure of chromatin, which is believed to function in establishing cell-type-specific gene expression pattern, should be significantly modified by two major events of epigenetics, histone modification and DNA methylation. Following hybridization of ES cells with

thymocytes, the somatic cells undergo chromatin remodeling which is induced by reprogramming factors residing in ES cells (Kimura *et al.* 2002). Therefore, the erasure of somatic cell-specific histone modifications is a crucial step in the induction of successful nuclear reprogramming. DNA methylation is also a crucial remodeler of chromatin structure and gene expression regulation, which control differentiation, cell cycle progression, and early embryonic development However, tissue-specific genes of somatic cells become methylated and pluripotency-related genes become demethylated after being reprogrammed to the pluripotent state by fusion induced reprogramming. For example, the *Oct4* promoter region of NSCs become demethylated during reprogramming; partially methylated patterns of the *Oct4* proximal enhancer (55.0%) and the promoter region (46.0%) in NSCs were completely demethylated just day 2 post-fusion with ES or EC cells (Do *et al.* 2007).The reprogramming of the imprinted genes of somatic cells is dependent on the pluripotent fusion partners. Previous studies suggest that the resulting fusion hybrid cells display an identical potential to their respective pluripotent fusion partners. For example, the methylation pattern of the *Igf2r* region of thymocytes was not changed after fusion with ES cells (methylated on the maternal allele as in thymocytes), but was changed after fusion with EG cells (not methylated on both alleles) (Tada *et al.* 2001).

A key event in induced reprogramming may be the restoration of repressive bivalent domains at loci containing lineage-associated genes. In this way, lineage-associated genes that have been activated during differentiation and have lost repressive H3K27me3 marks may be re-silenced when these bivalent domains are restored. In contrast, chromatin of pluripotency genes must undergo different modifications, since these genes must be reactivated rather than silenced. It is thought that repressive H3K9me3 marks that were deposited in a G9a histone methyltransferase-dependent manner on Oct4 (but not Nanog and Sox2) upon lineage commitment (Feldman *et al.* 2006), are removed from the Oct4 locus during reprogramming. Interestingly, it has been reported that the chromatin of fully reprogrammed iPS cells carries bivalent histone marks that are virtually identical to ES cells(Maherali *et al.* 2007; Takahashi *et al.* 2007; Wernig *et al.* 2007; Mikkelsen *et al.* 2008), indicating that the bivalent profile is indeed restored during reprogramming.

Induced reprogramming is associated with alterations in DNA methylation profiles as well. Pluripotency genes such as Oct4, Sox2 and Nanog are repressed by *de novo* DNA methylation upon differentiation, and these methylation marks are removed during the reprogramming process (Takahashi and Yamanaka 2006; Maherali *et al.* 2007). In fact, the degree of demethylation at these loci may indicate the progression and faithfulness of reprogramming, as incompletely reprogrammed Fbx15-selected iPS cells display only partial demethylation of the Oct4 promoter (Takahashi and Yamanaka 2006).

Recent reports indicate existence of epigenomic differences between ES cells and iPS cells and alterations in the differentiation potential of iPS cells compared to ES cells (Bourne *et al.* 2004; Mikkelsen *et al.* 2008). Lister et al demonstrated that although on a global scale ES cell and iPS cells methylomes are very similar, every iPS cell line shows significant reprogramming variability compared to both ES cells and other iPS cells, including both somatic 'memory' and iPS cell-specific differential DNA methylation (Lister *et al.* 2011). Further, all iPS cell lines share numerous non-randomly distributed megabase-scale regions that are aberrantly methylated in the non-CG context, associated with alterations in CG methylation, histone modifications and gene expression. They also show that differentially methylated regions in iPS cells are transmitted to differentiated cells at a high frequency.

1.8 Differences of iPS cells and ES cells

Pluripotent stem cells can be derived by various reprogramming strategies as described above. Although reprogrammed cells have been shown to be similar to ES cells, their artificial nature of pluripotency raises the question of whether iPS cells, fusion hybrid cells and blastocyst-derived ES cells are molecularly and functionally equivalent. The initial reports on iPS cell derivation supported the morphological similarity with ES cells including expression of key pluripotency markers and cell surface markers. Analysis of genome-wide expression patterns and global histone modifications have shown a high degree of similarity between ES cells and iPS cells (Maherali *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007; Mikkelsen *et al.* 2008). However, substantial differences between the two cell types have been reported as well. For example, a reduced and more variable neuronal potential has been described

for a number of human iPS cell lines, regardless of whether they carried reprogramming transgenes in their genome or not (Hu *et al.* 2010). Likewise, an increased propensity of iPS cell-derived neural cells to form tumors after transplantation into the brains of immuno-compromised mice has been observed (Miura *et al.* 2009). In addition, human iPS cells-derived early blood progenitor cells appear to undergo premature senescence (Feng *et al.* 2010). At the molecular level, gene-specific and global differences in DNA methylation (Deng *et al.* 2009; Doi *et al.* 2009; Pick *et al.* 2009) and in the expression of mRNAs and miRNAs (Chin *et al.* 2009; Wilson *et al.* 2009) have been reported between both mouse and human ES cells and iPS cells.

These findings indicate that subtle differences between ES and iPS cells exist. An additional study found gene expression differences indicative of a transcriptional memory in human iPS cells derived from fibroblasts, adipose tissue, and keratinocytes (Marchetto *et al.* 2009). While the cell lines analyzed in this study were derived in independent laboratories and with different technologies, which can confound gene expression analyses (Newman and Cooper 2010), another study confirmed and extended this finding by comparing genetically matched iPS cells derived from granulocytes, muscle progenitors, fibroblasts, and lymphocytes (Polo *et al.* 2010). iPS cells derived from these cell types exhibited discernible gene expression and DNA methylation patterns as well as differentiation biases into hematopoietic cells *in vitro*, some of which could be attributed to their cell type of origin. A parallel study corroborated these conclusions and further discovered that analysis of DNA methylation patterns in a given iPS cell clone could predict the somatic cell from which it was derived (Kim *et al.* 2011). Notably, continuous passaging of iPS cells (Polo *et al.* 2010) or the treatment of cells with chromatin-modifying drugs (Kim *et al.* 2009; Kim *et al.* 2011) attenuated these differences. Together, these results suggest that low-passage iPS cells retain a transient epigenetic memory of their cell type of origin that can influence their differentiation potential.

1.9 Cardiac differentiation of pluripotent stem cells

CMs are one of the first cell types induced from ES cells. Appearance of self-beating cells in EBs was first reported four years after the derivation of mouse ES cells

(Doetschman *et al.* 1985). It was shown that ES cell-derived $Flk1^+$ cells can give rise to vascular cells as well as CMs (Yamashita *et al.* 2005). When $Flk1^+$ cells were cultured on OP9 stroma cells, self-beating CMs appeared in 4 days. Flk1, Nkx2.5, and/or Isl1 were reported to mark multipotent cardiac progenitor population (Garry and Olson 2006). Though these markers mainly represent lateral plate mesoderm, primary heart field, and secondary heart field, respectively, these marker expressions overlap each other (Yan *et al.* 2009). The development of the cardiac lineage in ES cell differentiation cultures is easily detected by the appearance of areas of contracting cells that display characteristics of CMs. With continued differentiation, the number of spontaneously beating foci increases and all the EBs may contain localized beating cells. The rate of contraction within each beating area rapidly increases with differentiation, followed by a decrease in average beating rate with maturation. Depending on the number of cells in the initial aggregation step, the change in beating rate and the presence of spontaneous contractions continue from several days to >1 month. Fully differentiated CMs often stop contracting but can be maintained in culture for many weeks. Thus, developmental changes of CMs can be correlated with the length of time in culture and can be readily divided into 3 stages of differentiation: early (pacemaker-like or primary myocardial–like cells), intermediate, and terminal (atrial-, ventricular-, nodal-, His-, and Purkinje-like cells).

As observed with the hematopoietic and vascular systems, development of the cardiomyocyte lineage progresses through distinct stages that are similar to development of the lineage *in vivo*. An ordered pattern of expression of cardiac genes is observed in the differentiation cultures. CMs express cardiac gene products in a developmentally controlled manner. mRNAs encoding GATA-4 and Nkx2.5 transcription factors *GATANkx* appear first. Transcripts encoding *atrial natriuretic* factor (ANF), *myosin light chain* (*MLC*)*-2v*, α*-myosin heavy chain* (α*-MHC*), β*-myosin heavy chain* (β*-MHC*), Na^+-Ca^{2+} exchanger, and phospholamban appear next. Sarcomeric proteins of ES cell–derived CMs are also established developmentally in the following order: titin (Z disk), α-actinin, myomesin, titin (M band), MHC, α-actin, cardiac troponin T, and M protein. CMs with characteristics of fetal/neonatal rodent CMs express slow skeletal muscle troponin I isoforms and a greater proportion of β-MHC versus α-MHC, whereas CMs that more rapidly contract preferentially express cardiac troponin I and α-MHC. Thus, the appearance of cardiac-associated gene

products is a function of differentiation time, similar to that seen in normal myocardial development.

Maturation of the lineage in the cultures is associated with changes in cell size and shape, progressing from small, round cells to elongated cells with well-developed myofibrils and sarcomeres (Boheler *et al.* 2002). Electrophysiological measurements of cells from different times in culture suggest that the cardiomyocyte population undergoes a change from early-stage cells with pacemaker-like activity to more terminally differentiated atrial- and ventricular-like cells (Maltsev *et al.* 1993; Hescheler *et al.* 1997; Banach *et al.* 2003).

The identification and isolation of a cardiac precursor cell population is expected to provide a source of cells for tissue regeneration, while also providing valuable insight into cardiac development. Several recent studies focused on identifying these progenitor cells have reported that cardiac cells, including CMs, endothelial cells, and smooth muscle cells, may arise from cardiovascular progenitor populations with the expression of specific markers, such as Flk-1, c-kit, and Isl-1 (Yamashita *et al.* 2000; Yamashita *et al.* 2005; Kattman *et al.* 2006; Moretti *et al.* 2006; Yang *et al.* 2008; Bu *et al.* 2009).

1.9.1 Purification of cardiomyocytes from differentiating pluripotent stem cell cultures

While these studies clearly demonstrate the development of the cardiomyocyte lineage from differentiating ES cells, they are carried out in heterogeneous cultures in which these cells represent a minority of the entire population (Klug *et al.* 1996). A limitation for many studies is the identification of CMs, especially of the early stage. This appears particularly critical for molecular biological and biochemical studies, where a pure population of CMs is needed. This may help to address such critical questions as time point of differentiation into the cardiac lineage and regulation of gene expression. As there are relatively few antibodies available for the isolation of cardiac progenitors, investigators have genetically engineered ES cells to enable specific selection of cells representing different stages of development within the lineage. An ES cell line has be reported in which lacZ expression is under the control of the cardiac specific promoter human cardiac α-actin (Metzger *et al.* 1996). This

allows also a vital stain approach, in order to investigate early stage CMs functionally. This approach has been further improvised by the establishment of stably transfected ES-cell lines, where *in vivo* reporter genes are under control of very early, cardiac specific promoters. ES cells have been generated to express either drug-resistance or fluorescent genes under the control of promoters that drive expression at specific stages of cardiac development. In the first of these approaches, (Klug *et al.* 1996) expressed the neomycin-resistance gene under the control of the cardiac α-myosin heavy chain (α-MHC) promoter. With G418 selection at appropriate stages of development, populations highly enriched (>99%) for CMs were isolated. When applied to large-scale cultures, this strategy enabled the generation of large numbers of CMs (Zandstra *et al.* 2003). Other strategies involve expressing the green fluorescent protein (GFP) from cardiac specific promoters including Nkx2.5 (Hidaka *et al.* 2003), cardiac α-actin (Kolossov *et al.* 1998), and myosin light chain-2v (Muller *et al.* 2000). Expression from myosin light chain-2v was designed to specifically select for ventricular cells from the ES cell differentiation cultures. Cells selected on this basis displayed electrophysiological properties of ventricular CMs, indicating that the strategy was successful. Recently, a FACS-based isolation of cardiac progenitors from murine iPS cells expressing green fluorescent protein (GFP) under the control of Nkx2.5 promoter has been reported (van Laake *et al.* 2010). Similarly Rust et al have used CD166 (ALCAM) specifically expressed in cardiac tissue during the cardiac crescent and heart tube stage, to select a population of differentiated cells that are enriched for CMs in hES cell cultures. However, scalable purification of differentiated iPS-CMs and their characterization has not yet been achieved.

1.10 Potential application of research on cardiomyocytes derived from reprogrammed cells

1.10.1 A model system for study of embryonic development

The fully developed heart is composed of several diverse cell lineages including cardiomyocytes, endothelial cells, vascular smooth muscle and fibroblast cells that are derived from distinct subsets of mesoderm during the course of embryonic development. During cardiogenesis, the differentiation of these multiple heart lineages is under tight spatial and temporal control, resulting in the coordinated

formation of the distinct tissue components of the heart. Insight into the development of the heart can be acquired through the observation of differentiation of ES cells into CMs. Cardiac differentiation from ES cells closely mimics cardiac development in the embryo. In either case, the specification of the cardiovascular lineages involves a transition through a sequence of increasingly restricted progenitor cells, proceeding from a pluripotent state to mesoderm and then to cells committed to cardiovascular fates.

Precursor cells in vertebrate mesoderm express the T-box transcription factor Brachyury T. Brachyury is considered a marker of mesodermal progenitors which defines the earliest induction of the lineage. However, as these cells progress into the precardiac mesodermal stage of development they begin to express mesoderm posterior 1 and 2 (*Mesp1* and *Mesp2* respectively). Cells expressing *Mesp1* contribute to all four lineages in the heart. These factors are expressed transiently in the primitive streak and contribute to both primary and secondary heart fields, in addition to regulating the migration of cells to the anterior region of the embryo. Brachyury expression diminishes as newly formed mesodermal cells exit the primitive streak and migrate to varying sites in the developing embryo. Another gene associated with mesodermal differentiation is the fetal liver kinase-1 (*flk1*) gene encoding the vascular endothelial growth factors receptor 2 (VEGFR-2). Mesp1, induced via T brachyury, acts as a key regulator of the cardiovascular transcriptional network by inducing directly and/or indirectly the expression of the majority of key cardiovascular transcription factors including *Hand2*, Myocardin, *Nkx2-5*, *Gata4*, *Mef2c*, *Foxc1* and *Foxc2*. Primary heart field is derived from the anterior splanchnic mesoderm and from cardiac crescent. Primary heart field is positive for Nkx2.5, Tbx5, and Hand1, and gives rise to mainly the left ventricle. Secondary heart field originates from the pharyngeal mesoderm and is situated medially to the primary heart field. Secondary heart field is positive for Isl1, Tbx1, FGF8 and FGF10, and gives rise to mainly the right ventricle, outflow tract and inflow region. In addition to these two populations, proepicardial cells and neural crest cells also contribute to the heart structure(Gonzales and Pedrazzini 2009).

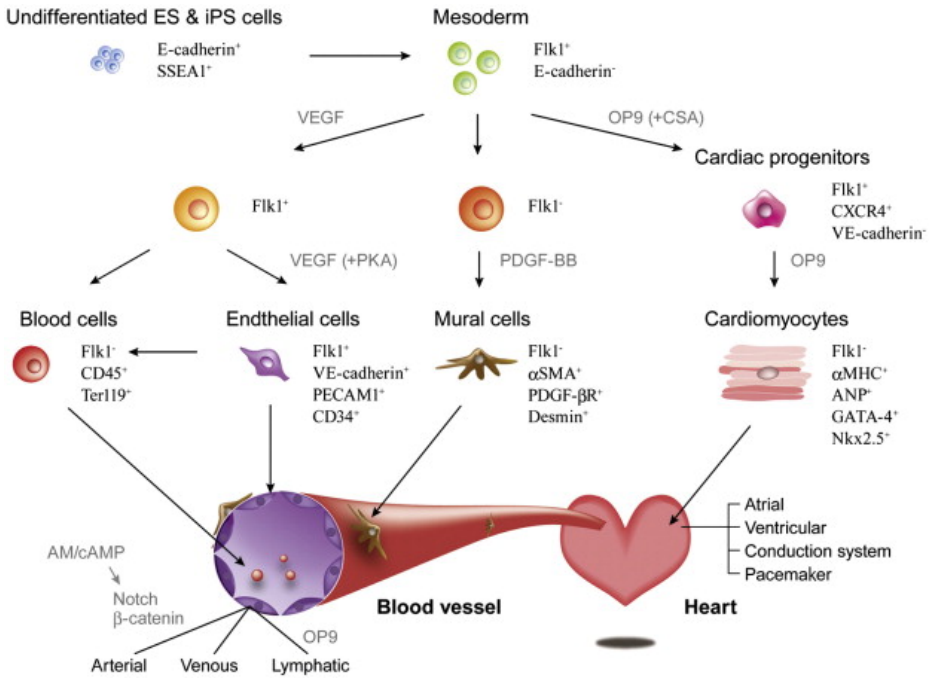


Figure 7: Cardiac cell types generated by *in vitro* differentiation of ES and iPS cells. Yamashita Experimental Cell Research, Volume 316, Issue 16, 1 Oct 2010, Pages 2555-2559.

The ability to access and manipulate populations representing early developmental stages in the ES cell differentiation cultures provides a new approach for addressing questions of embryonic development due to difficulty in isolating cells from the embryonic stages. Developmental studies of human CMs are now possible as a result of the recent availability of human ES/iPS cells. It is from these studies that we know today of the function of Cripto and Notch signaling in cardiac development of ES cells. Parisi and coworkers demonstrated that Cripto, known to be essential for cardiomyocyte development *in vivo*, plays a pivotal role in differentiation of ES cells to the cardiac lineage (Parisi *et al.* 2003). *Cripto*$^{-/-}$ES cells display a deficiency in generating CMs in culture that could be restored by the addition of soluble Cripto to the differentiation cultures. Notch signaling also plays a role in cardiac development from ES cells (Schroeder *et al.* 2003).

Cardiomyocyte induction from mouse iPS cells was first reported in 2008 (Mauritz *et al.* 2008; Narazaki *et al.* 2008; Schenke-Layland *et al.* 2008). CMs could be induced

from mouse iPS cells with similar methods as from mouse ES cells using EBs or stepwise methods. Various cardiovascular cells, such as CMs, arterial, venous, and lymphatic cells, and blood cells, were systematically induced from $Flk1^+$ progenitor cells (Narazaki *et al.* 2008). Comparable levels of cardiovascular cells could be induced from iPS cells and ES cells **(Fig. 7)**. As for human iPS cells, cardiomyocyte induction using EB methods was reported for the first time in 2009 (Zhang *et al.* 2009). Though functional analyses of induced CMs suggest that human cardiac cell models could be established from human iPS cells, induction efficiency and stability are still not sufficient. Further improvements for more robust differentiation methods are still required.

A critical area of investigation is the regulation of germ layer induction and tissue specification using the ES cell differentiation cultures. These issues are most easily addressed using lineage-specific markers that allow quantification of the response to the specific factor or sets of factors being tested. Given their role in the development of the early embryo, factors from the TGFβ, Wnt, and FGF families would be obvious candidates to be tested. The outcome of these experiments will establish a role for the different factors in these early developmental steps and ultimately lead to the establishment of defined conditions that will enable efficient and reproducible lineage-specific induction in both mouse and human ES cell differentiation cultures. It will be important to expand this approach to introduce different selectable markers into genes that define distinct developmental steps within a given lineage. These strategies will not only provide progenitors for cell-based therapy, they will also generate closely related populations that can be used for the identification of genes involved in the development and maturation of the lineage under investigation. Once genes have been identified, the ES cell system is an ideal model with which to study their role in lineage development.

Furthermore, this cell model is valuable for more detailed studies on commitment and differentiation of CMs, on the role of growth factors, extracellular matrix components and connexins, on cardiac myogenesis, as well as on pharmacological and toxicological effects on morphology, gene expression, cardiac-specific ionic currents and action potentials. It may further provide a unique model to analyze the quantitative expression of ion channels and the corresponding structural changes

during the cardiac development which may reveal new insights into inborn heart diseases (Robbins 1993). A first attempt for studying the role of the extracellular matrix (β1-integrins) has been made already in 1996 (Fassler *et al.* 1996) by finding that the lack of integrins significantly influence cardiac development, in particular the expression of ion channels as well as myofibrillar proteins. The absence of integrins leads to a retarded differentiation of CMs.

Simultaneous quantitative analysis of channel expression and ultra structure in ES cell-derived CMs as well as temporally and spatially controlled gene expression may offer for the first time the possibility to study pathophysiological phenomena. For example, an abnormal development of ion channels may lead to an electrical instability of the cardiomyocyte and consequently to arrhythmias.

1.10.2 Regenerative medicine

A variety of medical and surgical strategies have been developed for the treatment of heart failure. However, heart failure still remains a major cause of morbidity and mortality in developed countries. Therefore, a new strategy to improve the cardiac function and inhibit cardiac remodeling needs to be established. A number of strategies to regenerate heart tissue have been devised to resolve the shortage of available transplantation organs, including the transplantation of CMs or cardiomyogenic stem cells.

The foremost aim of use of cell therapy to repair the heart is to introduce new CMs into the heart to replace those that are damaged or dead, for example after an infarct. ES cell can serve as new source of differentiated cell types for cell replacement therapy. hES-cell-derived CMs have been used in xenogeneic transplantation as “biologic pacemakers” for the treatment of bradycardia. Studies have shown that hES cell-CM introduced into mouse or rat hearts will survive and mature. However, the cells are often separated from the animal heart by fibrotic tissue, and these hearts have only shown short-term functional improvements. Whereas most studies have only been performed in small animals, studies in pigs showed improved cardiac function after intra-myocardial injection of hES cell-CM compared with the controls, but any long-term follow-up was not described. Despite these transient improvements in cardiac function following hES cell-CM transplantation in animals, there are a

number of other obstacles which need to be overcome before transfer to humans may even be considered. *In vivo*, human ES cell-CM triggers an immune response. Therefore all of the studies thus far have involved immune suppression or used immuno-compromised animals. The discovery of iPS cell may help overcome this by creating immune-matched cells for transplantation, but a large cell bank would still be necessary to match most of the population since human iPS cell lines for individual patients, at present, would not be economically available. Not only would cheaper reagents be necessary for scaling up the cell production, but the whole procedure would also have to be carried out under conditions of GMP (good manufacturing practice). These practices are by themselves costly and labor intensive. A second obstacle is the removal of all undifferentiated cells before the introduction into the body since undifferentiated pluripotent stem cells can give rise to teratomas when injected into a living organism, animal or human. The most important consideration though in human patients is the potential risk of arrhythmias, which can be caused by the introduction of immature CMs with intrinsic pacemaker-like activity into the human heart, as well as anisotropy and other scar-implanted cell interactions.

iPS cells can be regarded as a renewable source of CMs. The major advantage of iPS derived CM over ES derived CM is that they can be derived from the same patient for whom disease treatment is being sought. Several elegant studies have already proven the potential of iPS cells to treat disorders. In a humanized sickle cell anemia model, mice have been rescued after transplantation of hematopoietic progenitors derived from autologous iPS cells with a genetically corrected β-globin locus (Hanna *et al.* 2007). In a further report, iPS cells were differentiated into neurons that functionally integrated into the host brain upon transplantation into fetal mouse brain. In addition, such cells were able to improve behavior in a rat model of Parkinson's disease (Wernig *et al.* 2007; Wernig *et al.* 2008). In another study, injection of undifferentiated iPS cells into diseased myocardium of mice led to multilineage repair (Nelson *et al.* 2009; Raya *et al.* 2009). Patient-specific iPS cells from Fanconi anemia patients have been generated that can, upon correction of the genetic defect, give rise to healthy hematopoietic progenitors (Raya *et al.* 2009). Furthermore, skin fibroblasts of a patient with homozygous β-thalassaemia have been reprogrammed into iPS cells, which could be differentiated into hematopoietic cells that synthesized hemoglobin

(Ye *et al.* 2009). There is no doubt that these studies demonstrate the enormous therapeutic potential of iPS cells, but there are still major hurdles to overcome.

The best cell type in terms of safety and efficiency for reprogramming still has to be determined. Risk of pluripotent stem cells to cause teratomas after transplantation has to be eliminated by excluding them from *in vitro*-differentiated cultures. Those pure populations of disease-relevant cells then would have to be transplanted into patients by safe and effective methods that still have to be established. Reprogramming efficiency must be increased and generation of iPS cells must be scaled up and standardized to allow high-throughput drug screens and toxicity tests, as well as fast availability for an increasing number of patients. Finally, comprehensive pre-clinical trials are needed to evaluate safety and efficacy of 'clinical grade' iPS cells.

1.10.3 Drug discovery

Cell types such as CMs and hepatocytes generated from hES cells could provide ideal populations for predictive toxicology. These human cells could reveal the toxicity of certain drugs that might not be detected using conventional assays that rely on animal models. Other advantages of such ES-cell-based assays is the requirement of significantly less test compound than *in vivo* assays, enabling earlier use in the drug discovery phase, resulting in earlier prioritization between drug leads.

ES cells offer many different strategies to develop drugs that can be used for regenerative medicine, that is, the therapeutic regrowth and/or repair of damaged cells. A unique strength of the ES cell system is the ability to engineer the ES cells to enable one to easily quantify the effect of drugs. This strategy could be used for drug discovery applications by introducing reporter molecules, such as GFP, into genes indicative of the development of specific progenitor populations or into genes associated with cell maturation and function. These customized assays could be used to identify those compounds that induce the growth and/or maturation of the cell types of interest. This capability offers a unique opportunity to develop clinically relevant commercial-grade pharmaceutical screening assays for certain human cell types that are not possible to produce by any other approach. hES cell lines with genotypes characteristic of various genetic diseases could not only provide novel insights into the mechanisms of the disease process, but also offer powerful screening systems for

developing drugs for treating those diseases. Such lines have already been established from embryos carrying genetic diseases, identified through pre-implantation diagnosis (Verlinsky *et al.* 2005). hiPS cell lines from patients suffering from a variety of different diseases can be generated (Park *et al.* 2008). The ability of human iPS cells to form various cardiac lineages, thus, makes them good candidates for some of the drug screening presently carried out using animals.

Several exciting demonstrations of the disease modeling capability of hiPS cell-CMs have recently been published. In all studies to date, the resulting patient-specific hiPS cell-CMs have been found to at least partially exhibit the phenotype of the diseases under investigation: long-QT syndrome, Timothy syndrome, and LEOPARD syndrome.

A seminal study evaluated the use of hiPS cells in modeling LEOPARD syndrome, an autosomal-dominant developmental disorder of multiple organ systems resulting from a missense mutation in the *PTPN11* gene (Vergara *et al.* 2011). Compared with control hES cell-CMs, diseased hiPS cell-CMs were noted to have a higher mean cell surface area as well as nuclear translocation of the NFATC4 transcription factor, perhaps representing *in vitro* molecular surrogates of the disease's cardiac hypertrophy phenotype. Subsequently, evaluation of type 1 long-QT syndrome revealed that CMs derived from iPS cells containing a missense mutation in the KCNQ1 gene exhibit ventricular and atrial myocyte action potentials with significantly longer QT intervals and slower repolarization velocity as compared with wild-type CMs. Single-cell electrophysiological analysis on the ventricular patient-specific hiPS-CMs revealed a reduction in current, confirming that the KCNQ1 mutant interferes with the function of the wild-type subunit. Immunocytochemical tests of both populations of CMs suggested that the phenotype is the result of a trafficking defect, in which the mutated KCNQ1 protein fails to achieve membrane targeting. Last, stimulation of the ventricular patient-specific hiPS-CMs using isoproterenol had little to no effect on the patient-specific hiPS-CM repolarization and currents, whereas a significant reduction of both was produced in the wild-type cells. Additionally, the patient-specific hiPS-CMs had reduced action potential duration: action potential interval ratios. These adrenergically stimulating tests cumulatively suggest that long-QT syndrome hiPS-CMs are predisposed to arrhythmic events.

Another study has extended the above-mentioned findings by modeling type 2 long-QT syndrome using similar methods (Itzhaki *et al.* 2011). These hiPS cells were derived from type 2 long-QT syndrome patients and contained a missense mutation in the KCNH2 gene. The resulting hiPS cell-CMs exhibited the electrophysiological hallmarks of the disease, including prolonged action potential duration and early after depolarizations in patch-clamping studies, as well as prolonged field potential duration in microelectrode array studies. Diseased hiPS cell-CMs displayed the expected defect in I_{Kr} (delayed-rectifier potassium current) as well as increased susceptibility to pharmacologically induced arrhythmogenesis. The authors also demonstrate an important proof-of-concept drug screening experiment to evaluate the effects of nifedipine, pinacidil, and ranolazine on the electrophysiological properties of the diseased hiPS cell-CMs.

Yazawa et al (Yazawa *et al.* 2011) derived hiPS cells from patients with Timothy syndrome, a disorder in which patients have long-QT syndrome, autism, immune deficiency, and syndactyly caused by a mutation in the *CACNA1C* gene encoding the $Ca_v1.2$ L-type channel. Beating hiPS cell-derived EBs displayed irregular contraction rates, whereas single hiPS cells-CMs displayed increased action potential duration as well as reduced voltage-dependent inactivation of the L-type calcium channel current. Interestingly, ventricular CMs but not atrial CMs displayed the prolonged action potential phenotype indicative of long-QT syndrome, in contrast to the results of Moretti et al. (Moretti *et al.* 2010).

1.10.4 Gene targeting to create reporter lines and disease models

The modification of ES cells by introduction of ectopic reporters or by targeting developmentally important loci is becoming a very useful tool in understanding the differentiation of CMs. It is of significant interest in the study of heart disease, since it represents an opportunity to introduce different mutations against a single genetic background if using just one hES cell line. In this respect, genetic modification of hES cell lines can compliment studies with human iPS cells since deriving lines from different individual patients automatically implies a different genetic background.

Genetic modifications can be introduced through non-homologous, homologous, site-specific or transpositional recombination. The efficiency of these processes varies

between species and cells types. The efficiency of homologous recombination has been found to be extremely low. Zinc-finger nucleases have been used in targeting a number of human genes including *VEGFA* (vascular endothelial growth factor A), *HoxB13* (homeobox B13) and *CFTR* (cystic fibrosis transmembrane conductance regulator). Site-specific recombination allows for more controlled introduction of exogenous DNA sequences. Another technique uses transposons to modify hES cell, employing three different systems, Sleeping Beauty (Wilber *et al.* 2007), PiggyBac (Lacoste *et al.* 2009) and Mu (Paatero *et al.* 2008).

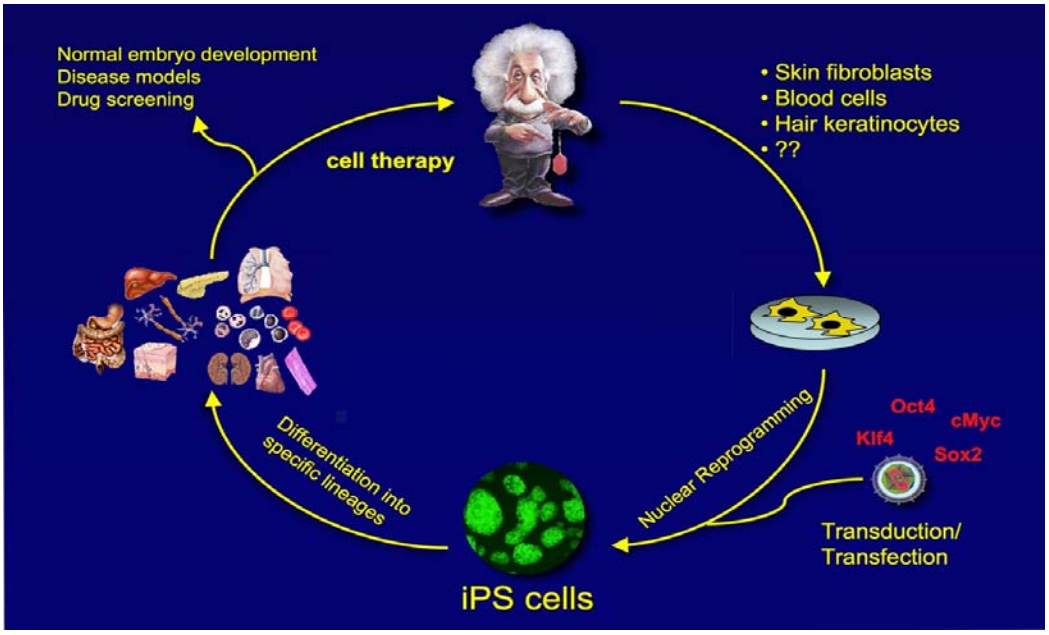


Figure 8: Potential applications of research on reprogrammed cells.

Thus research on cardiac derivatives of iPS cells will allow the development of novel, scalable screening platforms for compound discovery and toxicity testing which might help to develop more efficient and safer drugs **(Fig.8)**. Moreover, the study of developmental and differentiation processes as well as stem cell malignancy and genetic disorders *in vitro* will become feasible.

2 AIM

FH cells and iPS cells, which have been induced to pluripotency by reversing their biological clock, have undergone many epigenetic changes during the course of reprogramming and hence are more amenable to variable fates depending on the degree of epigenetic strain. When such cells undertake an *in vitro* differentiation process as that of ES cells, the following questions arise

1. Are the cells generated by different reprogramming methods similar to ES cells?
2. Will the cellular origin influence the *in vitro* differentiation potentials of reprogrammed cells?
3. Can the distinct transcriptional and epigenetic patterns of reprogrammed cells dictate the altered differentiation capacity leading to aberrant cell fates?

CMs can be readily generated by spontaneous differentiation of ES cells, FH cells, and iPS cells. Cardiac cells can be used as a model system to systematically decipher cardiogenesis, cardiac signaling processes, toxicological studies, and to understand disease mechanisms. Moreover many functional, molecular and physiological studies can be performed on CMs. In the present thesis work, I have chosen cardiomyocytes as a tool for understanding the effect of cell fate switching (brought about by reprogramming) on the developmental attributes of reprogrammed cells. CMs were derived by *in vitro* differentiation of ES cells and reprogrammed cells to asses the variability in their structural, molecular and functional characteristics.

Specific Aims:

a) To determine the pluripotent nature of reprogrammed cells.
b) To evaluate the influence of altered genome on cardiac differentiation potential of reprogrammed cells.
c) To obtain purified CMs from reprogrammed cells
d) To compare the molecular, structural and functional properties of reprogrammed cell derived CM with those of ES derived CM.
e) To analyze the transcriptional profile of ES and iPS cells at various developmental stages.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell culture reagents

Name	Manufacturer	Catalogue Number
ß-Mercaptoethanol	Invitrogen	31350-010
Dimethylsulfoxide DMSO	Applichem	2045215
Trypan Blue Stain,0.4%	Invitrogen	15250-061
PBS, Dulbecco's (1x) with Ca^{2+} and Mg^{2+}	Invitrogen	14040174
PBS, Dulbecco's (1x) without Ca^{2+} and Mg^{2+}	Invitrogen	14190169
Dulbecco's modified eagle medium (DMEM)	Invitrogen	41965039
RPMI-1640	Invitrogen	A1049-01
Fibronectin from Bovine Plasma	Sigma	F1141-1Mg
FBS heat inactivated Charge (Lot#)944855K	Invitrogen	10500064
Gelatin 2% Solution from Bovine Skin	Sigma	G1393-100ml
Iscove's Modified Dulbecco's Media(IMDM)	Invitrogen	31980022
L-glutamine 200mM	Invitrogen	25030024
Leukemia inhibitory factor (LIF-ESGRO)	Millipore	ESG1107
Geneticin (G418)	PAA	P11-019
MEM Non essential amino acids 100x	Invitrogen	11140035
Penicillin/Streptomycin 100x	Invitrogen	15140122
Puromycin	PAA	P15-019
Trypsin/EDTA(1x), 0.25%	Invitrogen	25300-054
Propidium iodide	Sigma	P4864
H.A.T. Supplement (50x)	Invitrogen	21060017
Polyethylene glycol 1500	Roche	10783641001

3.1.2 Consumables

Material	Specification	Manufacturer	Catalogue number
96 Well Cell Culture Plate, flat, sterile	Cell culture	Applichem	655180
Micro Amp 96 well plates	Real time PCR	Applied Biosystems	4346906
Bacteriological dishes	6 and 10cm	Cornig	430167,430166
Cell culture pipettes	5, 10, 25 and 50 ml	Greiner	606180
Cell strainers	40 µm	Becton Dickinson	352340
Falcon tubes	15 a and 50 ml	Becton Dickinson	358206,3582097
Cryotubes	1.8 µl	Nunc	375418
Electroporation cuvettes	ES cells-0.4 cm	Bio-Rad	165-2081
	Bacteria-0.1 cm		165-2082
FACS polystyrene round bottom tube	5 ml, 12 x 75 mm	Becton Dickinson	352052
GeneChip mouse expression array set	Mouse Genome 430 Version 2 array	Affymetrix	900495
Microcentrifuge tube	0.5, 1.5 and 2.0 ml	Eppendorf	0030 108.035
Multi-well tissue culture plates	6 wells and 96 wells	Greiner	665180, 650180
PCR tubes, thin-wall, sterile	0.2 ml	Eppendorf	0030 124.332
Pipette tips	With aerosol protection	Axygen	T-1000-B, T-200-C, TF-20
Acrodisc syringe filters	GxF/0.2 µm AutoPack Tubes	Pall Life sciences	AP-4798
Tissue culture dishes	3.5, 6 and 10 cm	Becton Dickinson	356517
Tissue culture Flask	T-25, T-75	Becton Dickinson	353810
µ-Dish 35mm low ibiTreat, sterile	60x 35mm µ-dishes	Ibidi	80136

3.1.3 Enzymes, Nucleotides and Markers

Name	Supplier	Catalogue Number
2x JumpStart™ Taq ReadyMix™	Sigma Aldrich	P0982
DNase I, Amplification Grade, 100U	Invitrogen	18068-015
SuperScript™ II Reverse Transcriptase,10,000U	Invitrogen	18064-014
Random primer, 1x	Invitrogen	48190-011
10 mM dNTP Mix	Invitrogen	18427013
100 bp DNA ladder	Fermentas	SM0242
GeneRuler™ 1kb DNA Ladder	Fermentas	SM0311
Agarsoe	Invitrogen	16500-500
Oligonucleotides / Primers *	Sigma Aldrich	
TRIzol	Invitrogen	15596-026

*See section 3.1.6 and 3.1.7

3.1.4 Kits

Kit	Supplier	Catalogue Number
Qiagen Plasmid Maxi Kit	Qiagen	12163
DNAeasy Blood and Tissue kit	Qiagen	69504
QIAquick Gel Extraction Kit (50)	Qiagen	28704
QIAprep Spin Miniprep Kit (50)	Qiagen	27104
QIAquick PCR purification Kit (50)	Qiagen	28104
Sybr Advantage qPCR Premix	Clontech	639676
DNeasy Blood & Tissue Kit (50)	Qiagen	69504
Mycoplasma Detection Kit for PCR	Venor GeM	11-1050

3.1.5 Laboratory apparatus

Apparatus	Specifications	Manufacturer
Cell culture hood	Clean Air Techniek	Thermo Scientific
Incubator	Direct Heat CO_2	Thermo Scientific
ApoTome	Flourescence microscope	Carl Zeiss
Electrophoresis chamber	Long horizontal	Starlab
FACScan	Calibur	Becton Dickinson
Microscope	Brightfield	Carl Zeiss
Mastercycler Personal	25x0.2ml PCR tubes	Eppendorf
Real time PCR machine	7500 Fast	Applied biosytems
Spectrophotometer	Nanodrop100	Peqlab
pH meter	Seven Easy	Mettler Toledo
Micro liter Pipettes	P10, p20,P200,P1000	Gilson
Pipetmann	Liquid handling with sterile filter	Thermoscientific
Thermomixer Comfort	24x0.5ml,24x2.0ml	Eppendorf

3.1.6 PCR primers used for SNP genotyping

Name	ref seq	Primer	Product size	Annealing temp., °C
SNPa1	rs3722007	**F:** GGCTTCTGGCTCTGTTTTTG **R:** GGAAACAGCCAATCTTCAGG	483	58
SNPb2	rs13476485	**F:** AGGCAAGGTGCTTGTGATCT **R:** TGGTATTCACATGCCACAGG	323	54
SNPa3	rs31512068	**F:** TCGGGCATAGTCTCTGGTTC **R:** GCCAGGGAACTACAACTCCA	445	58
SNPa4	rs13459075	**F:** TGAAGAGTCAGGCAGAAGCA **R**: CTAAGGAGCAGACCCAGCAC	330	58
SNPb5	rs3664494	**F:** GAGCTTGGAAGGGGAAGG **R:** AAATGAAGGGAGGCGACATA	330	54
SNPa6	rs13472545	**F:** CCAGCCCACAGTGAGTTGTA **R:** GGGAAACCACAAAGACAGGA	492	58

Name	ref seq	Primer	Product size	Annealing temp., °C
SNPb8	rs13479805	**F:** ATAACCAGCTGAGGGTGTGC **R:** ATGAGAGCCACATGGAGGAG	310	54
SNPa9	rs13480095	**F:** GACAGGCACAGCAAGGTACA **R:** CCTGTGGATCTCACCTGTCA	377	58
SNPa10	rs13480662	**F:** CCCTGCTGTCTTTCTCTGCT **R:** TGTGGGGGACATCTTCATCT	365	58
SNPb11	rs26891750	**F:** ATGAAGGCTGCAGGAAAAGA **R:** CCAAAGAAGGACCCTGTTCA	346	54
SNPa12	rs29196570	**F:** ACACAGAACGGTCAGGTGGT **R:** CGGAGAGGGGCATACATAAG	352	58
SNPa13	rs13481715	**F:** CCAGGAGTGTGTCTGCTCAA **R:** GCAGAGTTGCCTGAGAATCC	423	58
SNPa14	rs30707092	**F:** GTTGCCACTTCTTCCTCTGC **R:** CACATTTCGTGGGTCACAAG	412	58
SNPa15	rs13482486	**F:** ACTTAGTCATGGGCGGGTTT **R:** TTGCCCTGACACTTGACATC	431	58
SNPa16	rs4163196	**F:** ACGGAGGTGTGTTCTGGTGT **R:** GAGATGGGCAGAGGAGAGTG	377	58
SNPa17	rs3023450	**F:** CCATCCCTTTTATGCCTCCT **R:** CACCTCCTTCTTGCTCACCT	349	58
SNPa18	rs29882799	**F:** ACCGGGAAGAACTGGAAACT **R:** AGCCACACAGAGGAACAACA	300	58
SNPa19	rs4223757	**F:** TGCAAGACTGTCAGGAGGTG **R:** CTGCCCACACTGGTTACCTT	352	58
SNPbX	rs13483822	**F:** CCGACTGTTCCCAAAACACT **R:** GCATTTGCTACTGGGATGCT	382	54
SNPb14	rs6262654	**F:** CGAACCCACACAGCAGATAA **R:** CACATCTTCCCAGGCTTGAT	364	54

* temp.,-temperature

3.1.7 PCR primers used for gene expression analyses

Gene*	NCBI Accession	Sequence (5' to 3')	Product size (nt)	Annealing temp., °C
Oct3/4	NM_013633	**F:** AGCCGACAACAATGAGAACC **R:** TGATTGGCGATGTGAGTGAT	168	61
Sox2	NM_011443	**F:** TACCTCTTCCTCCCACTCCA **R:** TCTCCAGTTCGCAGTCCAG	212	61
Klf4	NM_010637	**F:** CCCACCAGGACTACCCCTAC **R:** GTGTGGGTGGCTGTTCTTTT	218	61
c-Myc	NM_010849	**F:** ACACGGAGGAAAACGACAAG **R:** CTTGTGCTCGTCTGCTTGAA	295	61
rv-Oct3/4	NM_013633	**F:** AGAAGGCGAAGTCTGAAGCC **R:** ATCCTCCCTTTATCCAGCCC	200	61
rv-Sox2	NM_011443	**F:** TACACCCTAAGCCTCCGCCT **R:** TCAGCTCCGTCTCCATCATG	220	61
rv-Klf4	NM_010637	**F:** TCTCTAGGCGCCGGAATTC **R:** CCATGTCAGACTCGCCAGGT	190	61
rv-c-Myc	NM_010849	**F:** CTTCTCTAGGCGCCGGAATT **R:**TGGTGAAGTTCACGTTGAGGG	160	61
Nkx2.5	NM_008700.2	**F:** CCACTCTCTGCTACCCACCT **R:** CCAGGTTCAGGATGTCTTTGA	107	60
MLC2v	NM_010861.2	**F:** AAAGAGGCTCCAGGTCCAAT **R:** TCAGCCTTCAGTGACCCTTT	140	60
Troponin T	NM_011618.1	**F:** GAGGAGGTGGTGGAGGAGTA **R:** GGCTTCTTCATCAGGACCAA	150	60
CD31	NM_008816.2	**F:** CAGGTGTGCGAAATGCTCT **R:** ATGGGTGCAGTTCCATTTTC	113	60
Troponin C	NM_009393.2	**F:** CAGCAAAGGGAAGTCTGAGG **R:** CGTAATGGTCTCACCTGTGG	124	60
Sox17	NM_011441	**F:** TAAAGGTGAAAGGCGAGGTG **R:** GCTTCTCTGCCAAGGTCAAC	219	60
AFP	NM_007423.3	**F:** CCTATGCCCCTCCCCCATTC **R:**CTCACACCAAAGCGTCAACACAT T	324	60
GAPDH	NM_008084.2	**F:** GGTGCTGAGTATGTCGTGGA **R:** CGGAGATGATGACCCTTTTG	97	60

*Abbreviations: rv – denotes the sequence expressed from the retroviral vector, MLC2v - ; AFP - ; GAPDH - Glycerinaldehyd-3-phosphat-Dehydrogenase; temp.,-temperature

3.1.8 Primary and secondary antibodies

Antibody	Supplier and Catalogue No.	Final concentration	Use in
Mouse monoclonal antimouse α-sarcomeric actinin	Sigma A7732	1.2 µg/ml	IHC
Mouse monoclonal Antimouse SSEA1	Santa Cruz sc-21702	8µg/ml 1µg/ml	FACS IHC
Troponin T monoclonal antibody	Thermoscientific ms-295	4 µg/ml	IHC
FITC conjugated anti-mouse H-2K^b	BD Pharmingen 553456	10 µg/ml	FACS
PE-conjugated anti-mouse H-2K^d	BD Pharmingen 562004	10 µg/ml	FACS
Normal mouse IgG2a-PE	Santa Cruz Sc-2867	8 µg/ml	FACS
Normal mouse IgG2a-FITC	Santa Cruz Sc-2010	8 µg/ml	FACS
Anti-mouse Oct3/4	Santa Cruz Sc-5279	1 µg/ml	IHC
Mouse monoclonal anti-mouse α-myosin heavy chain	Santa Cruz Sc-168676	1 µg/ml	IHC
IgG1 Isotype Control, purified, unlabelled	Molecular Probes A105538	10 µg/ml	IHC
Goat Anti-mouse IgG1, AlexaFluor 555 conjugate	Santa cruz Sc-3890	1 µg/ml	IHC
Goat anti-mouse IgM, FITC conjugate	Sigma-Aldrich F-9259	1 µg/ml	IHC
Hoechst 33324	Molecular Probes H-21492	1 µg/ml	IHC

*IHC-immunohistochemistry
*FACS-Florescence activated cell sorting

3.1.9 Buffers and Solutions

0.1% DEPC-treated water

Diethyl Pyrocarbonate (DEPC) was dissolved in tridistilled water (500 µl DEPC in 500 ml tdH_2O). Incubated overnight at 37°C with constant shaking and sterilized by autoclaving.

0.5 M EDTA, pH 8.0

93.05g Ethylenediaminetetraaceticacid (EDTA) disodium salt dihydrate (Sigma, catalogue no. E-5134) was dissolved in 500 ml of tdH_2O. It was heated and NaOH (Applichem, catalogue no. A1551.0500) was added to aid the salt in dissolving. pH was adjusted to 8.0 and the solution was sterilized by autoclaving.

10x TBE

108 g Tris-Base (0.89 M, Promega, catalogue No. H513a), 55 g Boric Acid (0.89 M, Sigma, catalogue no. B-6768) and 7.44 g EDTA-Na_2-salt (0.02 M) were dissolved in 800 ml tdH_2O and finally made to 1 liter.

10x TAE

48.46 g Tris-Base (0.4 M), 11.4 ml Glacial Acetic Acid (Sigma, catalogue no. A-6283) and 3.72 g EDTA-Na_2-salt (0.01 M) were dissolved in 800 ml tdH_2O and finally made to 1 liter.

4% Para-formaldehyde fixative

16% Para-formaldehyde (Roth, catalogue no. 335.2) was diluted to a final concentration of 4% before being used for fixing live cells for immunocytochemistry.

LB medium and agar plates

10 g tryptone (Scharlau, catalogue no. 07-119), 5 g yeast extract (Scharlau, catalogue no. 07-079) and 10 g sodium chloride (Roth, catalogue no. 3957.1) were dissolved in 500 ml distilled water, mixed well and made to 1 liter. The solution was sterilized by autoclaving. For preparing agar plates, 15 g agar (Roth, catalogue no. 5210.2) was added to 1 liter LB medium and autoclaved. LB agar was cooled down to 50°C and the

appropriate antibiotic was added and poured into 10 cm bacteriological dishes. Prepared plates were stored at 4℃ and used within two weeks.

TSS buffer (Transformation and Storage Solution)

20ml of 50% PEG4000 solution is added to 70 ml sterile LB medium and mixed thoroughly. Then 5ml of dimethyl sulfoxide (DMSO; Sigma, catalogue no. C6295) and 5ml of 1M $MgCl_2$ (pH6.5, sterile filtered) is then mixed with the LB medium 10% PEG solution to obtain TSS buffer which can be used for freezing bacterial competent cells.

Alkaline phosphatase staining solution

Solution A: Napthol AS-MX phosphate (Sigma, catalogue no. 4875) is dissolved to a concentration of 2mg/ml in 0.1M Tris/HCl (pH 9.2).Solution B: Fast Red TR salt TM (Sigma, catalogue no. F2768) is dissolved to a concentration of 1mg/ml in 0.1M Tris/ HCl pH 9.2.Solution A is then mixed with solution B to obtain a final concentration of Napthol at 200µg/ml (1:10 dilution). The resulting solution is mixed well and used immediately for staining methanol fixed and dried cells. The solution works only when used fresh.

Crystal violet staining solution

3% solution of crystal-violet (Fluka, catalogue no. 61135) in Methanol

3.1.10 Sterilization of Solutions and Equipments

All solutions (heat insensitive) and plastic ware was sterilized at 121°C, 105 Pa for 60 min in an autoclave. Heat sensitive solutions were sterile-filtered through a disposable sterile filter (0.2 to 0.45 µm pore size). Glassware were sterilized overnight in an oven at 220°C. The solutions for RNA preparations were handled with DEPC-treated water and the equipment for RNA analyses was cleansed using RNase Zap (Ambion, catalogue no. AM9780) to inactivate any RNase contamination.

3.2 Methods

3.2.1 Molecular Biology Methods

3.2.1.1 Preparation of *E. coli* competent cells

Competent cells were prepared according to the protocol described (Chung and Miller, 1988). 5 ml of LB medium containing ampicillin at final concentration of 100µg/ml was inoculated with a single bacterial colony (DH5α) and incubated overnight at 37°C with constant shaking. Next day, 1 ml of the overnight culture was added to 100 ml of LB medium and incubated at 37°C with vigorous shaking until the optical density at 600 nm reached 0.4 (between 0.40 and 0.45). The culture was quickly chilled on ice for 5 min and the cells were pelleted down by spinning at 3000g for 5-8 min at 4°C. The supernatant was discarded and the pellet was resuspended in ice cold TSS buffer using 1/10 volume of initial culture (e.g. 10 ml TSS for 100 ml culture). The suspension was aliquoted in desired amounts (typically in 100 µl/vial), snaps frozen in liquid nitrogen and stored at -80°C.

3.2.1.2 Transformation of competent bacterial cells

200 µl of competent *E. coli* cells (DH5α) were thawed on ice (10 min) mixed with 10 µl of plasmid (10-100 ng) and placed on ice for 30 min with occasional mixing. The transformation reaction mixture was heat shocked at 42°C for 90 sec and immediately placed on ice for 2 min. In order to accelerate the bacterial growth, 800 µl of LB medium was added to the reaction mixture and incubated at 37°C with shaking for 1h. Cells were collected by centrifugation in a table-top centrifuge (provide model and the manufacturer) at 8,000 rpm (6000x*g*) for 30 sec at RT and plated on LB-agar plate containing appropriate antibiotic. The plate was incubated overnight at 37°C in the upside-down position.

3.2.1.3 Plasmid DNA preparation

Transformed *E. coli* cells were cultivated for at least 12 hrs in LB medium containing appropriate antibiotics at 37°C. Plasmid DNA (10 µg) was isolated from 5 ml bacterial cultures using the Mini-prep kit (Qiagen) according to manufacturer's recommendations. For larger amounts up to 100 µg of plasmid DNA, the Midi-Prep Kit (Qiagen) was used.

3.2.1.4 RNA isolation

500µl of TRIzol reagent (Invitrogen) is added to the cell sample. The sample is homogenized by pipetting 4x up/down with 27G ¾ Nr. 20 needle. The homogenized samples are then incubated for 5 min at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes.100µl of chloroform is add to the sample and tubes are capped securely and vigorously shaken by hand for 15 sec. and later incubated at RT for 2-3 min. The samples are then centrifuged at 10,500 rpm for 15 min at 4°C. The resulting aqueous phase is then transferred to a fresh tube. RNA is then precipitated from the aqueous phase by mixing with 500µl isopropanol per tube. After 10 min incubation at room temperature, the tubes are centrifuged at 10,500 rpm for 10 min at 4°C. The supernatant is then discarded and the RNA pellet is washed once with 1ml of 75% ethanol per tube. The pellet is then mixed by vortexing and then the tube is centrifuged at 8,500 rpm for 5 min at 4°C.At the end of the procedure, the RNA pellet is briefly dried on air. RNA is then dissolved in sterile, RNase-free water.1µl of RNA is used to determine the concentration by Nanodrop. RNA samples are aliquoted 2x and stored until use at –80°C.

3.2.1.5 Quantification of nucleic acids

Nucleic acid concentrations were determined by spectrophotometric measurement of the amount of ultraviolet radiation absorbed by the bases. The readings were taken at 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An optical density (OD) of 1 corresponds to approximately 50 µg /ml for double-stranded DNA and 40 µg /ml for single-stranded DNA and RNA. The ratio between the readings at 260 nm and 280 nm (OD260/OD280) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 and 2.0, respectively. Alternatively the nucleic acids were quantified using Nanodrop ND-1000 spectrophotometer which uses a patented sample retention system that holds 1 µl of sample without any need for traditional containment devices like cuvettes and capillaries. Using fiber optic technology and surface tension, the sample is held between two optical surfaces that define the path length in vertical orientation.

3.2.1.6 Restriction digestion of DNA

The restriction analysis was performed with 1 µg DNA in a total volume of 20 µl. The DNA sample was digested with restriction enzyme in the prescribed buffer at the recommended temperature for 1-2 h for double digestion, in most cases Tango buffer was used according to the manufacturer's (Fermentas) instruction. At the end of incubation, the single and/or double digested DNA samples and undigested controls were analyzed by agarose gel electrophoresis (100 ng /lane) and purified if necessary.

3.2.1.7 Semi quantitative RT-PCR analysis

1 µg of total RNA was treated with DNAseI (amplification grade, Invitrogen) in the presence of 10xBuffer at 37°C for 15 min. DNAse treated RNA was used for reverse transcription. cDNA synthesis was performed with Superscript II Reverse transcriptase (Invitrogen) after priming using random hexamers. Generation of cDNA was performed in a total reaction volume of 30 µl. Finally the cDNA was dilute 1:4 (filled up to 120 µl) with nuclease free water. cDNA samples were frozen at –20°c until used for PCR amplifications of gene of interest.

3.2.1.8 PCR (Polymerase Chain Reaction) amplification

In order to amplify the double stranded genomic DNA or single stranded cDNA, polymerase chain reaction (PCR) was performed. For PCR amplification, cDNAs were amplified using JumpStart REDTaq ReadyMix and 0.25 µM of each primer. All amplifications were performed using Eppendorf PCR Cycler under optimized conditions for each target sequence. PCR reactions were performed in sterile 0.2 ml thin-wall tubes as described below.

PCR component	Volume	Final concentration
2x JumpStart *Taq* ReadyMix	10 µl	1x
Forward primer (5 µM)	1.2. µ l	0.25 µM
Reverse primer (5 µM)	1.2. µ l	0.25 µM
cDNA*	2.0 µ l	
H_2O	4.6 µ l	
Total volume	20 µl	

PCR conditions

1. Denaturation 2 min 94°C
2. Denaturation 35 sec 94°C
3. Annealing 45 sec 60°C
4. Elongation (1 min/1Kb fragment) 72°C
5. Repeat step 2- 4 for 29-35 cycles
6. Extension 10 min 72°C
7. Hold 4°C

After amplification the products were confirmed by electrophoresis on ethidium bromide stained (final concentration of (10µg/µl) agarose gels.

3.2.1.9 Quantitative real-time PCR

Total RNA isolation and cDNA synthesis were performed as described above. For PCR amplification, 2 µl of cDNAs (1:30 dilution of cDNA as in 3.2.1.7) were amplified using 1x QuantiTect SYBR Green PCR Master Mix (Clontech) and 0.3 µM of each primer pair in final volume of 20 µl/well. Amplification was performed in MicroAmp 96-well plates (Applied Biosystems) starting with an initial step for 2.0 min at 50 °C, 10 min template denaturation/hot start step at 95 °C, followed by 40 cycles (95°C for 15 sec, 58°C for 30 sec and 60°C for 45 sec). Quantitative PCR analysis for each sample was performed in triplicates. GAPDH was used as an internal control. Relative gene expression values were obtained by normalizing C_T (threshold cycle) values of the target genes in comparison with C_T values of the housekeeping gene (GAPDH) using the ΔC_T method.

3.2.1.10 DNA Electrophoresis in agarose gels

To analyze the PCR products, samples were electrophoretically separated according to their molecular size in (0.6%-2%). agarose gels The agarose was boiled in a microwave for 2-3 min in 0.5x TBE or 0.5x TAE buffer and after cooling down (~ 60°C) 2-3 µl of ethidium bromide solution (10 mg/ml) per 100 ml gel was added and poured in a gel caster. The amount of DNA loaded was as follows on a gel: 10µl of PCR reaction volume/well, for plasmid after purification 100 ng/well, for the restriction digest 100ng/well. Migration of the DNA in the gel can be judged by visually monitoring the migration of the tracking dyes, that is Bromophenol blue and Xylene cyanol present in

the DNA loading buffer. After adequate migration, DNA fragments are visualized on an ultraviolet transilluminator (due to the intercalation of the fluorescent dye ethidium bromide into the double strand of DNA, DNA becomes fluorescent) and photographed by a camera attached to a gel documentation system. In order to define the size of the DNA fragments, DNA size marker was also loaded on the gel.

3.2.1.11 Isolation of DNA fragments from agarose gels

For the isolation of DNA fragments from agarose gels, the appropriate DNA band was excised from the gel with a sterile scalpel, transferred into 500µl eppendorf tubes and extracted using QIAquick Gel Extraction Kit (Quiagen) according to manufacturer's recommendations. The isolated DNA was eluted/ redissolved in elution buffer and the purity and integrity of the isolated DNA fragment determined electrophoretically.

3.2.1.12 Microarray expression analysis

Three independent total RNA samples of each cell type were prepared using TRIzol. Samples used in this study are summarized in the Table 2. Biotin-labeled cRNAs were prepared using the Ambion® Illumina RNA amplification kit (Ambion Europe), 1.5 µg was hybridized to Sentrix® whole genome bead chips (Mouse WG-6 v2.0, Illumina) carrying 45281 probe sets and scanned on the Illumina® BeadStation 500x.

Raw data extraction of mRNA microarrays was performed with Beadstudio 3.1.1.0 software using the Beadstudio Gene Expression Analysis Module 3.1.8. All further analysis was performed in R (http://www.r-project.org, version 2.8.0) using Bioconductor packages. For further analysis we used quantile normalization implemented in the affy package. Variable genes were defined by a coefficient of variation (SD/mean) between 0.5 - 10. Determination of present calls was based on the detection p-value assessed by Beadstudio software; a gene was called present if the detection p-value was < 0.05. Otherwise the mRNA transcript was called absent. Differentially expressed genes were selected using a fold change/P value filter with the following criteria: Only p-values smaller than 0.05 and an expression change higher than 2 fold and a difference between mean intensity signals greater 100 were considered statistically significant for further analysis. The Benjamini-Hochberg method was used to adjust the raw p-values to control the false discovery rate. The fold-change was calculated by dividing the mean intensity of the genes in one group by that in the other

group. If this number was less than one, the negative reciprocal was used. Hierarchic cluster analysis was performed using the hcluster method in R. Before clustering, the data were log2 transformed. Distances of the samples were calculated using Pearson correlation and clusters were formed by taking the average of each cluster. PCA analysis was performed using the p-curve package in R. When visualizing PCA results, the first 3 principal components (coordinates) were z-transformed (mean= 0, standard deviation = 1) and subsequently plotted in 3D.

3.2.1.13 Selection of SNPs and design of primers for their detection

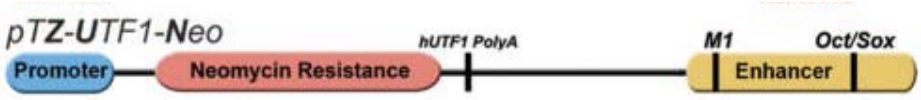
A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide in the genome differs between members of a biological species or paired chromosomes in an individual. We use SNP sequencing as stool to confirm the presence of variant alleles in fusion cell hybrids each originating from a genome of different mouse strain - 129S2 (source of HM-1 ES cells) and F1 generation of DBA/2J x C57BL/6 (source of bone marrow cells and spleenocytes). The SNPs were identified by a genome-wide screen of the SNP variation between these two strains of mice to identify single nucleotide variations on each of chromosome The database for Mouse Genome Informatics was used.
(http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snpQF). SNP sites located on each chromosome were randomly selected and primers were designed in order that 200 bases spanning 3' and 5' ends of the variant nucleotide could be amplified and recognize two sequences harboring one nucleotide variation on a single chromosome. In this way 21 primer pairs were designed to recognize a single sequence with one allelic variation for each of the 21 chromosomes of the mouse genome. Genomic DNA was isolated from HM-1 ES cells, spleenocytes and FH cells (clones1.2, 2.1 3.1 and 4.2) using DNeasy Blood and Tissue kit and PCR was performed with 100 ng of DNA to amplify the sequence encompassing the specific SNP using primers listed in chapter 3.1.6 at final concentration of 0.15µM. PCR products pooled from 4-5 PCR reactions were purified using QIAquick PCR Purification kit and then sequenced using the Big Dye Terminator kit and primers listed in chapter 3.1.6 to determine the allelic variation at one position of each chromosomes. The sequencing was performed in the sequencing facility of Institute for Biochemistry, Cologne, Germany. Sequences were analyzed using Chromas Pro1.5 software.

3.2.1.14 Preparation of vector constructs

The UTF-Neo vector was kindly provided by Prof. Peter Dröge and is described in the publication by Tan *et al*. 2007. Full-length *UTF1* was cloned into phagemid *pTZ-18R* yielding *pTZ-UTF1*. Its coding region was subsequently replaced with neomycin (*Neo*), yielding *pTZ-UTF1-Neo* **(Fig. 9a)**

The α-MHC–PAC–IRES–eGFP (αPIG) vector was generated previously by Eugen Kolossov (Kolossov *et al.* 1998). The pIRES2-EGFP vector (CLONTECH Laboratories, Inc.) was truncated via excision of the CMV-IE promoter by AseI-NheI and re-ligated. The α-MHC promoter (5.5-kb BamHI–Sal I fragment) of the α-MHC–pBK plasmid (provided by J. Robbins, University of Cincinnati, Cincinnati, OH) and the Puromycin^R cassette (HindIII-ClaI fragment) of the pCre-Pac vector were inserted into the multiple cloning site of the truncated pIRES2-EGFP vector. The resulting vector was named αPIG vector henceforth **(Fig. 9b).**

A.



B.

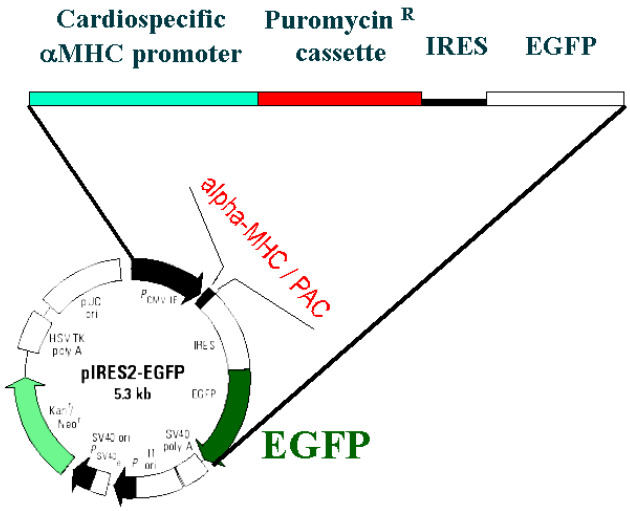


Figure 9. Schematic drawing of vectors. **αPIG vector. (A)** ***pTZ-UTF1-Neo*** vector in which the coding region of UTF1 is replaced with neomycin resistance gene. **(B)pIRES2-EGFP** vector truncated with puromycin resistance, IRES and eGFP under the control of cardiospecific α-MHC promoter.

3.2.1.15 Establishment of transgenic cell lines

The αPIG vector was verified by sequencing and 14 µg of the vector was digested using the enzyme SacI (1U per 10µg plasmid) at 37°C overnight. After overnight digestion, the enzyme was inactivated by heating the reaction mix at 65°C for 20 minutes and then stored at -20°C until used for transfection. An aliquot of the restriction digest was verified for the completeness of plasmid linearization by agarose gel electrophoresis. A confluent dish of murine iPS cells (TiB7.4, provided by Prof. Rudolf Jaenisch and Prof. Alexander Meissner, MIT, Cambridge, USA) was trypsinized and cell number was determined. Approximately $5x10^6$ cells were resuspended in 700 µl PBS (without Ca/Mg). 20 µg of the linearized plasmid vector was diluted in 100 µl of PBS and mixed with the cell suspension. The mixture was transferred into a GenePulser cuvette (0.4 cm electrode, gap 50). The cell and plasmid mix was incubated on ice for 20 minutes. Electroporation was then performed using GenePulser Electroporator (BioRad) at 260 V and 500 µF. Immediately after electroporation the cell suspension was transferred to 10 cm dish containing 10 ml of iPS medium with LIF and layered with irradiated MEFs prepared one day earlier. Selection of resistant colonies was started 48 hours later by addition of neomycin at a concentration of 500 µg/ml. Approximately 10 days later resistant colonies were obtained. 40 colonies were picked and sub cultured as separate clones. All clones were expanded and frozen in multiple vials. At least ten clones were chosen for the initial round of screening for cardiac differentiation. Three clones-clone 25, clone 15 and clone 11 were routinely used for cardiac differentiation. All data described in this thesis were generated with αPIG-iPS clone 25. The transgenic murine D3 ES cell line αPIG-ES (clone 44) was generated with the same αPIG vector previously and was used for comparative analyses.

To enrich pluripotent cells from the non-homogenous population of TiB7-4 cells, *UTF1*-Neo was used as selection vector. TiB7-4 cells were electroporated with circular *UTF1*-Neo plasmid and selected with G418 24 hours after the transfection, which ultimately resulted in several colonies. Three were isolated two weeks after electroporation and expanded as subclones UTF-1, UTF-2 and UTF-3. No further selection with G418 was applied. UTF-1, -2 and –3 were sub-cultured for 40 passages.

3.2.2 Cell Culture Techniques

3.2.2.1 Culture conditions for murine ES, iPS and fusion hybrid cells

The murine iPS cell line TiB7.4 was kindly provided by Rudolf Jaenisch and Alexander Meissner. The transgenic ES cell line αPIG44 derived from a parental ES cell line D3 was described previously (Kolossov *et al.* 2006). The ES cell line HM-1 was obtained from ATCC. Fusion hybrid cells were providedby Bernd Fleishcmann and Hans Schöler. The ES , iPS and FH cells were cultured in ES/iPS cell medium composed of Dulbecco's modified minimal essential medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 100 μM β-mercaptoethanol, 1x non-essential amino acids and 1000 U/ml leukemia inhibitory factor (LIF). The cells were grown on monolayer of mitotically inactivated murine embryonic fibroblasts (MEFs). Pluripotent ES and iPS cells grow in the form of compact colonies with clearly defined borders. Cells were passaged every 2-3 days by trypsinization and seeding onto irradiated MEFs.

3.2.2.2. Inactivation of MEFs and preparation of feeder plates

ES cells are usually grown on a layer of mitotically inactivated primary MEFs in the presence of LIF to promote growth and prevent differentiation. These cells stop dividing after a couple of passages, so embryonic fibroblasts need to be isolated freshly from time to time. This requires pregnant female mice at day 13 post coitum (p.c., assuming day one is the first day the plug is observed). F1 hybrid or outbred mice are favored because of increased litter size. Murine embryonic fibroblasts (MEFs) were isolated from 12.5 to 13.5 p.c. mouse embryos. The head is discarded and the body parts including the limbs are collected and dissociated by trypsinization to produce single-cell suspensions. After expansion, aliquots can be frozen and stored in liquid nitrogen indefinitely. Alternatively, MEFs suitable for ES cell culture may be obtained from commercial sources. MEFs must be inactivated prior to use as a feeder layer for mouse ES cells. Mitotic inactivation prevents the dilution of ES cell lines with dividing fibroblasts. MEFs can be inactivated using γ-irradiation, or mitomycin C treatment .We routinely used irradiated MEFs for ES cell maintenance.

A frozen vial MEFs is thawed and plated in a 75-cm^2 tissue culture flask or 100-mm plate in MEF medium (DMEM supplemented with 10% FBS and 1x non-essential

amino acids). The cells are allowed to grow until confluent (3 to 5 days). Medium is changed after the first day and every other day thereafter. The cells are then passaged by trypsinizing and resuspending the cell pellet in 10 to 50 ml MEF medium without penicillin/streptomycin, and plating at a 1:10 dilution. Medium is added to a final volume of 10 to 15 ml per 75-cm^2 flask or100-mm plate. The cells are allowed to grow until confluent (3 to 5 days), and passage at a 1:5 to 1:10 dilution, using twenty-five to fifty 150-cm^2 flasks. Medium from confluent flasks is removed and the cells are rinsed with 15 ml Ca^{2+} and Mg^{2+} free PBS, and trypsinized again. MEFs from ten to fifteen 150-cm^2 flasks can be processed together. The pellet is resuspended in 10 ml MEF medium without penicillin/streptomycin and transferred in suspension to a 50 ml Falcon tube. The cells are then exposed 2 times to 4000 rads of γ-radiation. The cells suspension is then immediately diluted to 50 ml with MEF medium without penicillin/streptomycin. The number of cells is counted and either used freshly for ES cell culture by plating ($0.8x10^6$ cell/6cm dish) or frozen ($10x10^6$ cells/ vial) for later use.

3.2.2.2 Passaging of ES, iPS cells and fusion hybrid cells

Before passaging, murine ES or iPS cells were dissociated using 0.25% Trypsin/EDTA. Medium was aspirated from the 6 cm cell culture plate and washed once with 5 ml pre-warmed PBS. Cells were then incubated with 1 ml of trypsin for 2-3 minutes at 37°C. Cells were uniformly dispersed into single cells by gentle trituration. Trypsin was inactivated by the addition of four volumes of ES/iPS cell medium. Cells were collected by centrifugation at 1000 rpm (300x*g*) for 5 minutes at RT in table top centrifuge. Supernatant was discarded and the pellet was resuspended in the fresh ES/iPS cell medium. The cells were counted using Neubauer counting chamber and the viability of cells was determined by Trypan blue dye exclusion method. The cells were seeded at a density of 0.3-0.5 x 10^6 cells per 6 cm plate containing irradiated MEFs.

3.2.2.3 Freezing and thawing of cells

ES or iPS cells, fusion hybrid cells and MEFs were trypsinized, washed, centrifuged and resuspended in ES/iPS cell medium. The cell concentration was adjusted to $1-2x10^6$ cells per ml and 0.9 ml of this suspension was mixed with the 0.9 ml of ice-cold cell freezing medium containing 80% FBS and 20% DMSO. Aliquots of cells (1-2 x 10^6 cells/vial/1.8 ml) were frozen at -80°C overnight and for long-term storage transferred

to liquid nitrogen. For thawing, frozen cells were quickly transferred from liquid nitrogen into a 37°C water bath, thawed, washed with 10 ml of pre-warmed medium and seeded on a dish coated with 0.1% gelatin or seeded with irradiated MEFs (see above) depending on the requirements of the experiment.

3.2.2.3. Mycoplasma assay

Supernatant from a 24 hour culture of the various cell lines was withdrawn and was routinely checked for mycoplasma contamination by a PCR based protocol using Venor GeM Mycoplasma Detection Kit.

3.2.2.4. Isolation of murine bone marrow cells and spleenocytes

The animals were sacrificed according to standard method of sacrifice and then dissected under sterile conditions. The spleen was then collected in a dish containing 1x PBS. A 70um filter is placed at the mouth of a 50 ml falcon tube and rinsed with 10% RPMI. The spleen is then transferred on the filter. With the help of the piston of 5ml syringe the spleen is pressed against the filter to dissociate the cells and continuously rinsed with medium. This collects all the cells after dissociating from spleen in the falcon.

Femoral bone was also collected by dissection of the thigh region to isolate bone marrow cells. With the help of 24G needle, a small hole is punctured into the head of the bone. Another hole is punched through the other end of the bone. Sterile medium (10% RPMI) is taken into the syringe, a 24G needle is fixed to the syringe which is then inserted into one of the holes made. The other end of the bone is directed toward the mouth of a sterile falcon. The medium is then flushed through the bone in a way that the bone marrow cells get collected in the falcon tube.

3.2.2.4 Reprogramming by fusion

$5x10^6$ HM-1 ES cells were mixed with $5x10^6$ of either murine bone marrow cells or spleenocytes (ratio of 1:1) from DBA/2JxBL6 mice and washed in PBS. The mixture was centrifuged in 50 ml conical tubes at 130x*g* for 5 minutes. After removal of the supernatant, 1 ml of a prewar med 50% polyethylene glycol (PEG 1500**)** was added to the cell pellet drop by drop. 24 ml of complete medium (DMEM supplemented with

15% FBS, 1x non essential amino acids, and 50μM beta mercaptoethanol) was added up to the final volume of 25 ml over 5 minutes time, with constant stirring at 37°C and 300 rpm in a Thermomixer (Eppendorf) equipped with a 50 ml tube adapter. The cells were centrifuged at 130x*g* for 10 minutes at RT in swing-out rotor, washed gently with DMEM, resuspended in a cell culture medium containing 1000 U/ml LIF and seeded onto inactivated MEFs. Selection for fused cells was started 48 hours later by addition of 50x HAT supplement (Invitrogen) into the medium to a final concentration of 1x (0.1mM hypoxanthine, 0.4μM aminopterin 0.016mM thymidine). The ES cell colonies rescued by fusion with wild-type somatic cells were detected 5-7 days after fusion and subcloned by culturing in the presence of HAT in ES/iPS cell medium.

3.2.2.5 *In vitro* cardiac differentiation of ES, iPS or fusion hybrid cells

Cardiac differentiation of transgenic iPS and ES cells was performed in spinner flasks. A confluent culture of ES/iPS cells was trypsinized and cell number was determined. $1x10^6$ cells were suspended in a total of 14 ml differentiation medium (IMDM/Glutamax supplemented with 20% FBS, 100 μM β-mercaptoethanol and 1x non-essential amino acids) to a final concentration of $7x10^4$ cells/ml and cultured for two days in non-adherent plates (10 cm in diameter) under continuous agitation (50 agitations per minute) on a horizontal shaker. Embryoid bodies (EBs) were formed in two days after beginning of differentiation. At day 2, EBs were counted in 30 μl drops and 28000 EBs were transferred into a spinner flask (Cell Spin 250, IBS Integra Biosciences) containing 200 ml of the differentiation medium. The spinner flask was equipped with stirrers and cells were agitated at 40 rpm. Medium was not changed until day 9 of differentiation when a sample of approximately 10 ml was withdrawn from the spinner flask and checked for appearance of beating or GFP-positive areas within the EBs. With the help of a florescence microscope the occurrence of GFP-positive areas in EBs arising as a result of cardiac differentiation of transgenic ES/iPS cell lines was also checked. At the sight of beating and green florescence in EBs, drug selection was initiated by adding puromycin (8 μg/ ml). This led to death of non cardiac cells and enrichment of cardiac cell types. On day 11, the surviving cardiac clusters (CCs) were transferred into 10 cm non-adherent culture dishes for further purification leading to highly purified cardiac clusters. Medium was changed with fresh puromycin every

second day. Analyses were performed with cardiac clusters that were collected on day 16 of differentiation after 7 days of puromycin treatment.

Differentiation of FH cells and HM-1 ES cells was performed using a hanging drop method. The cells were suspended in Iscove´s modified Dulbecco´s medium (IMDM), supplemented with 20% fetal calf serum, 100 units/ml penicillin, 100µg/ml streptomycin, 1% non-essential amino acids, and 100 µM β-mercaptoethanol. Then, 20µl drops containing 500 cells each were placed on the lids of cell culture dishes. The drops were made to hang by inverting the lid on the base of the dish. After 2 days of cultivation in hanging drops, the embryoid bodies (EB) were transferred into 20% IMDM and cultivated for further 5 days in suspension. On day 7, EB were plated onto 0.1% gelatine-coated adherent dishes. Fresh medium was replaced. The EBs attaches in 24hours to the surface and starts growing horizontally and undergoes multilineage differentiation. Appearance of cardiac cells (usually from day 8 onwards) is marked by beating areas within EBs.

3.2.2.6 Detection of contaminating undifferentiated ES or iPS cells in purified cardiomyocytes

In order to detect rare contaminating ES or iPS cells within the puromycin-selected cardiac clusters, 0.1-0.3x10^6 dissociated single cells were seeded on irradiated MEFs and cultured for 7-14 days in complete ES/iPS cell medium. During this period of time any surviving ES or iPS cells, which resisted puromycin in cardiac clusters, will appear. When colonies of undifferentiated cells were formed in plates, crystal violet staining was performed to visualize and count the number of colonies indicative of the number of contaminating ES/iPS cell in purified CMs. Crystal violet staining was performed by washing the cells two times with $PBS^{-/-}$ and then adding a 3% solution of crystal-violet (prepared in Methanol) to the plate and incubating for 10 min at Room temperature. The stained plates were then washed with tap water 4-5 times until the background was colorless. The plates were air dried and the number of blue stained colonies was determined.

Contaminating pluripotent cells in preparations of puromycin-selected CMs were also detected by monitoring teratoma formation. After 7 day of puromycin selection, day 16 cardiac clusters were dissociated into single cells by Trypsin/EDTA treatment and 0.5-2.0x10^6 CMs were injected subcutaneously into immunodeficient 6-8 weeks old $Rag2^{-/-}\gamma_c^{-/-}$

mice and monitored for a period of 3 months. Experimental animals were obtained from Dr. Mamoru Ito (Central Institute for Experimental Animals, Japan). Studies with experimental animals were approved by the regional government authority of the state North Rhine-Westphalia. Animals was sacrificed by cervical dislocation when teratomas reached appropriate size, tumors were explanted, measured and fixed using 4% buffered paraformaldehyde (pH 7.5) for 12 hrs at 4°C for H&E staining and histological analysis.

3.2.2.7 Alkaline phosphatase staining

ES, iPS or fusion hybrid cells were plated at a clonal density of 0.5-1 x 10^4 cells per 6 cm dish and cultured for 5-7 days. Cells on the dish were washed twice with PBS, fixed in 100% methanol for 10 min at RT and air-dried. Cells expressing alkaline phosphatase (ALP), a pluripotency marker, were determined by staining for 15-30 min at RT with 1:10 dilution of Napthol AS-MX phosphate (200 g/ml, Sigma) in Fast Red TR salt TM (1 mg/ml, Sigma) that was prepared in Tris/HCl (pH9.2) as described in section above3.1.10. The reaction was stopped by rinsing the cells with distilled water and air-drying. Alkaline phosphatase positive colonies were stained red and photographed using the phase-contrast microscope equipped with 4x and 10x lens (Zeiss).

3.2.2.8 Ploidy determination

To measure the content of DNA in fusion hybrid cells and to determine their ploidy, pluripotent cells were trypsinized and counted.1x10^6 cells were washed with PBS and then fixed overnight in 70% ethanol prepared with tdH_2O at -20°C. Next day cells were centrifuged at 130x*g*, resuspended in 1ml PBS and treated with RNAse (exact type, manufacturer) at the final concentration of 100 µg/ml for 15-20 minutes at 37°C. The suspension was then equally distributed into two 1.5 ml micro centrifuge tubes. One tube received propidium iodide (PI) at a final concentration of 50 µg/ml and the other was maintained as a control without PI. The samples are incubated on ice for 15 minutes. The DNA content of cells was measured using a flow cytometer FACScan. Tetraploid fusion hybrid clones and diploid parental ES cells (HM-1) and somatic cell (spleenocytes) were included in the analysis. HM-1 ES cells and spleenocytes were used as a diploid and dividing cell control. On the first dot plot FSC Vs SSC was acquired. A gate was drawn on the most dense population thereby eliminating cell debris. On the second dot plot FL3-A on X axis and FL-3W on the Y axis were set along with gate of

the first plot. During acquisition the cell aggregates would be seen on the extreme right of the FL3-AVs FL3-W plot. The cell aggregates were gated out for acquisition of a histogram with FL-3A as Y-axis.

3.2.2.9 Coating of cell culture plates

0.1% gelatin prepared in sterile PBS was layered over adherent tissue culture dishes of the required size and place in incubator at 37^0C for a minimum of 1 hour.
Fibronectin was diluted in sterile PBS to reach a final concentration of 2.5µg/ml This solution was coated on either ibidi plates (for immunostaining) or on coverslips placed in 3.5cm adherent dishes (for electrophysiology) and stored in incubator at 37^0C for 4-12 hours These plates were then ready for growing CMs. If not used immediately they could be stored at 4^0C until use. Care was taken to prevent drying.

3.2.3 Immunoassaying, microscopy and flow cytometry

3.2.3.1 Immunofluorescence staining

Immunostainings were performed on undifferentiated ES or iPS cells as well as differentiated cells. Undifferentiated ES/iPS cells were cultured on MEFs and after 2-3 days of culture they were fixed in 4% buffered paraformaldehyde (pH 7.5) for 15 min at RT for immunostaining. Single ES or iPS cell-derived cardiac muscle cells (CMs) were plated on fibronectin-coated µ-dishes (Ibidi) at $0.3x10^6$ cells /plate in differentiation medium containing 8µg/ml puromycin. After 2-3 days adherent CMs were fixed in 4% buffered paraformaldehyde (pH 7.5), permeabilized by Triton X-100 and then blocked against unspecific antibody binding with 10% fat free milk powder dissolved in PBS (blocking solution) for 1 h at RT. After blocking, cells were washed with PBS and incubated with primary antibody diluted in blocking solution overnight at 4°C. Cells were rinsed with 0.01% TritonX-100 in PBS and incubated with species-specific secondary antibodies diluted in blocking solution for 1 h at RT. Nuclei were counterstained with Hoechst 33342 (2µg/ml) at 37°C for 20-30 minutes. Finally cells were washed and imaged on an Axiovert 200M fluorescence microscope (Zeiss).

3.2.3.2 Flow cytometric analysis

Single cells were prepared by Trypsin/EDTA treatment as described above. The

dissociated cells were centrifuged, washed and filtered through a 40 μm cell strainer (BD Pharmingen). Cell count was then determined and cells were resuspended in appropriate volume of staining buffer (PBS supplemented with 0.1% FBS) to obtain a cell density of 0.5 x10^6 cell/ 50 μl. Cells were then dispensed into 1.5 ml micro centrifuge tubes (50 μl/tube) and stained either with isotype control or antigen-specific antibodies diluted in the staining buffer. Cell-surface antigen expression was detected using fluorescently conjugated antigen-specific antibodies (direct staining) or labeling the cells with unconjugated primary antibody followed by fluorescently-tagged secondary antibody (indirect staining) by incubating at 4°C for 30 minutes. The cells were washed 2-times with and 1 ml of Cell Wash. Unstained samples were used as a negative control. The cell suspension was transferred into the FACS tubes and data was acquired on flow cytometer (FACScan, BD Pharmingen). Cells were gated on forward and side scatter dot plots. 10,000 events per sample were acquired and the data were analyzed with CellQuest Pro software (Beckton Dickinson). To assess the percentage of GFP-positive cells in the purified cardiac clusters, single cell suspension of puromycin-selected cardiac clusters was prepared by dissociation with 0.05% Trypsin/EDTA and analyzed by flow cytometry. Propidium iodide was used for dead cell staining.

3.2.3.3 Electrophysiology

Puromycin-purified cardiac clusters from aPIG-ES and aPIG-iPS and beating areas from derived from FH clones were dissociated into single CMs by treating with collagenase B (1mg/ml) for 20-30 minutes. CMs were plated on fibronectin-coated glass cover slips (22x22 mm square glass cover slips in 3.5 cm dishes) and cultured for 24-48 hours before measurements were performed. The cover slips were placed into a recording chamber at RT and cells perfused continuously with extracellular solution. Cell membrane capacitance was determined on-line PULSE software (HEKA Elektronik). Action Potential (AP) of spontaneously beating CMs were recorded by the whole-cell current-clamp technique using an EPC-9 amplifier and the PULSE program. Response of CMs to hormonal regulation was assessed by administering isoproterenol (Iso) and carbachol (CCh). The standard whole-cell patch-clamp recording technique in the voltage-clamp mode was used for recording voltage-gated Na^+-, L-type Ca^{2+}-, and depolarization-activated outward K^+-channel currents. Data are presented as the mean ± standard error of the mean (SEM). Student's *t* test was applied for statistical evaluation.

4 RESULTS

4.1 Reprogramming by fusion

4.1.1 Generation of fusion hybrid clones

In order to test the reprogramming ability of ES cells and to determine the cell fate change brought about by fusion based reprogramming, we induced fusion between mouse ES cells and somatic cells like spleenocytes and bone marrow cells.

HM-1 ES cells were derived from 129/ola and are HPRT1-deficient. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) encoded by HPRT1 gene plays a central role in the generation of purine nucleotides through the purine salvage pathway. As a result, these cells are sensitive to medium containing the HAT (hypoxanthine, aminopterin, and thymidines) supplement. HAT inhibits the enzyme dihydrofolate reductase (DHFR), which is necessary in the *de novo* synthesis of nucleic acids. Thus, the cell is left with no other option but to use the alternate purine salvage pathway, which utilizes HGPRT. But due to the lack of HPRT in HM-1 cells, nucleic acids cannot be synthesized and this leads to cell death. When HM-1 cells are fused with $HGPRT^+$ bone marrow cells or spleenocytes, the resulting fused cells can be selected based on their resistance to HAT. In the HAT medium $HGPRT^-$ HM-1 cells will die and only the $HGPRT^+$ fused cells will survive. The bone marrow cells or spleenocytes eventually die under the ES cell culture conditions, thus only hybrid cells with ES cell-like morphology are left surviving.

HM-1 ES cells (haplotype b) were fused using polyethylene glycol (PEG) with either adult mouse bone marrow or spleen cells derived from DBA2J mice (haplotype d). After fusion the cell mixture was plated on MEFs and grown in ES culture conditions in the presence of HAT. After a few days of selection with HAT, the ES cells like colonies were obtained. The emerging colonies were indicative of successful reprogramming of somatic cells by fusion with ES cells. Each colony was picked up, individually expanded and termed as a fusion hybrid (FH) clone. These clones were routinely maintained under ES cell conditions in the presence of HAT. Four FH clones (FH1.2; FH2.1; FH3.1 and FH4.2 have been used in the present study **(Fig. 10A-C)**. FH1.2 and FH2.1 resulted by reprogramming of bone marrow cells and FH3.1 and FH4.2 were the result of reprogramming of spleenocytes by fusion with HM-1 cells. The FH clones

obtained resembled very closely in their morphology to ES cells and therefore presumed to be pluripotent.

The pluripotent nature of the FH clones was demonstrated by *in vitro* differentiation of FH cells. When differentiation was started, FH2.1 and FH4.2 formed embryoid bodies (EBs) similar to but comparatively smaller than those formed by HM-1 ES cells **(Fig. 10D-F).** EBs when allowed to spread on gelatin surface formed multicelluar structures similar to those formed by conventional HM-1 ES cells representing differentiation into multiple lineages **(Fig. 10G-I).**

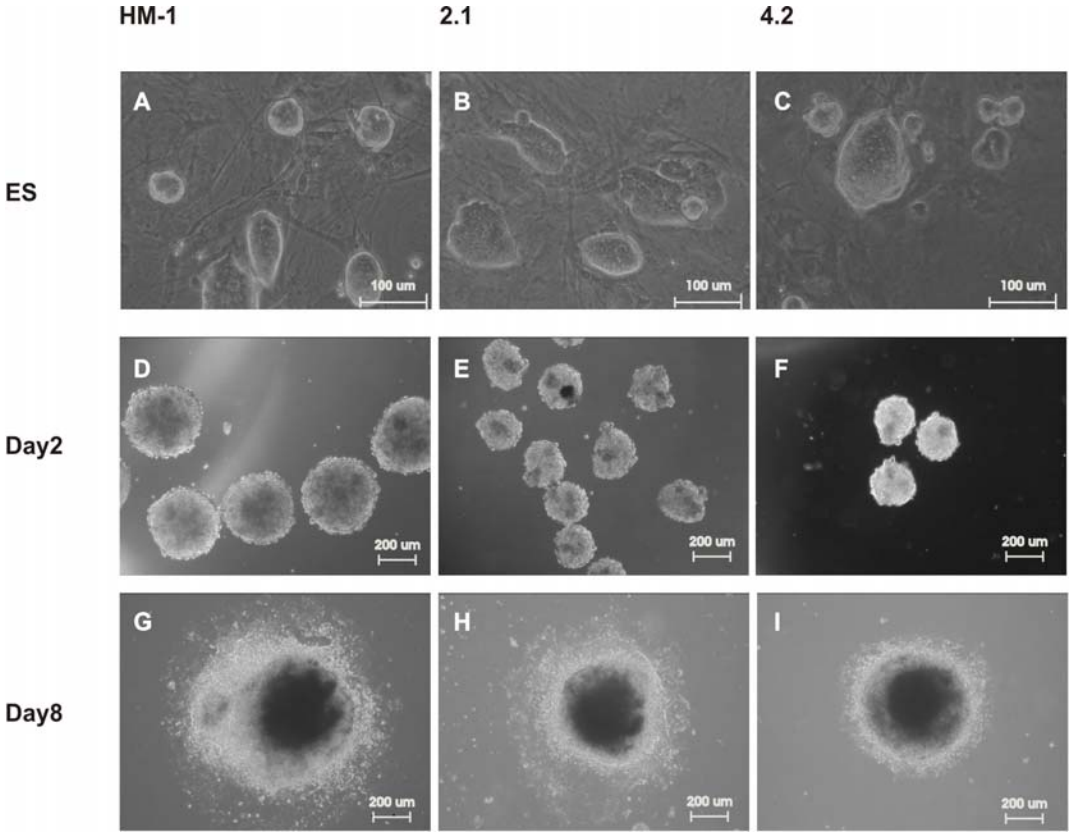


Figure 10. Pluripotency of HM-1 ES and FH cells. (A-C) Pluripotent colonies of HM-1ES (A) FHclone 2.1(B) and FH clone 4.1(C).Scale=100um. EBs at day 2 and day 8 of differentiation of HM-1 (D and G) FH clone2.1 (E, H) and FH 4.2(F, I).Scale bar=200µm. The fusion clones were provided by our collaborators Martin Breitbach (AG Bernd Fleshcmann) and Tobias Cantz (AG Hans R.Schöler).

4.1.2 Ploidy of fusion hybrid cells

The fusion between the two diploid cells leads to formation of a tetraploid cell. In order to confirm that the resulting fusion-derived pluripotent cell lines contain double amount of DNA, the DNA content of undifferentiated FH cells and HM-1 ES cells was determined by propidium iodide staining. Dead cells and aggregates were gated out based on FL3-A and FL3-W parameters Flow cytometric analysis of stained cells revealed that the cells of the fusion clones carry a tetraploid genome (**Fig. 11**). This was demonstrated by the predominant 4n peak and 8n peak in a histogram plot of the cells after uptake of propidium iodide. HM-1 ES cells showed only the expected 2n (cells in G0/G1 phase of the cell cycle) and 4n peaks (cells in the G2/M phase of the cell cycle). These results demonstrate that FH cells maintain tetraploid genome and do not loose their hybrid character during prolonged culture.

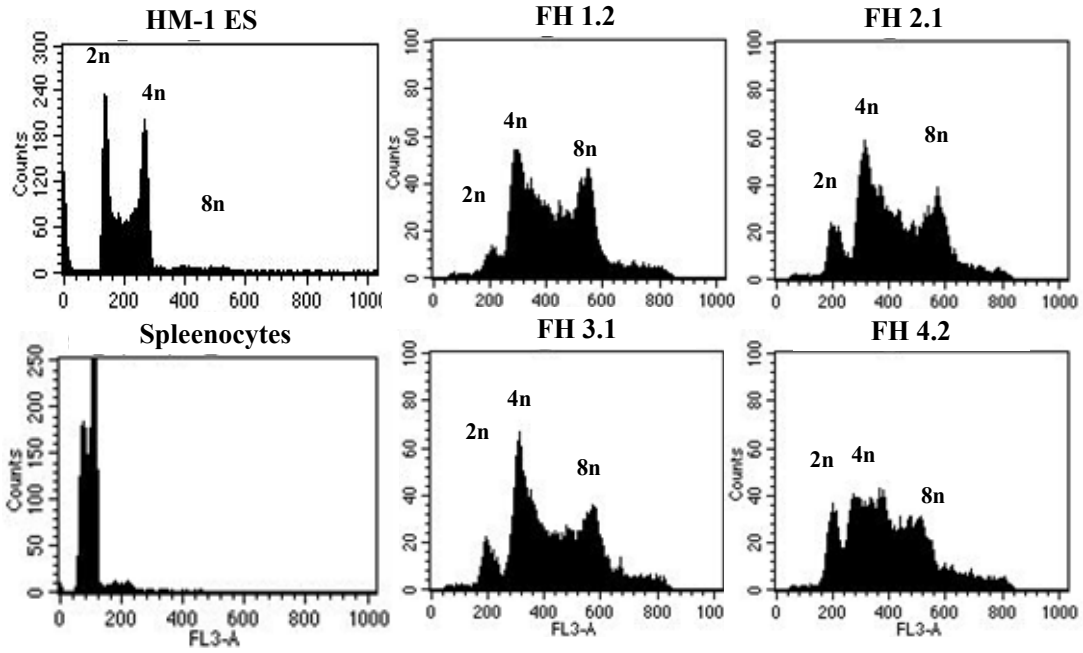


Figure 11. Cell cycle analysis of spleenocytes and undifferentiated cells of HM-1 ES and FH clones. Single cells were fixed and stained with Propidium iodide before analyzing on a flow cytometer 10,000 events were acquired for each sample on a histogram with FL3-A on Y-axis.

To test whether the tetraploid chromosome set in FH cells remains stable when the cells undergo differentiation, the ploidy of FH cells was determined at various time points of

spontaneous differentiation (day 4, 6, 8 and 10). FH2.1 EBs at all days of differentiation retained a DNA cotent similar to that of their undifferentiated counterparts with a high 4n and 8n peaks. Thus it seems that the tetraploid state of the FH cells was stably maintained in the course of prolonged passaging of undifferentiated FH cells as well as during the course of their differentiation.

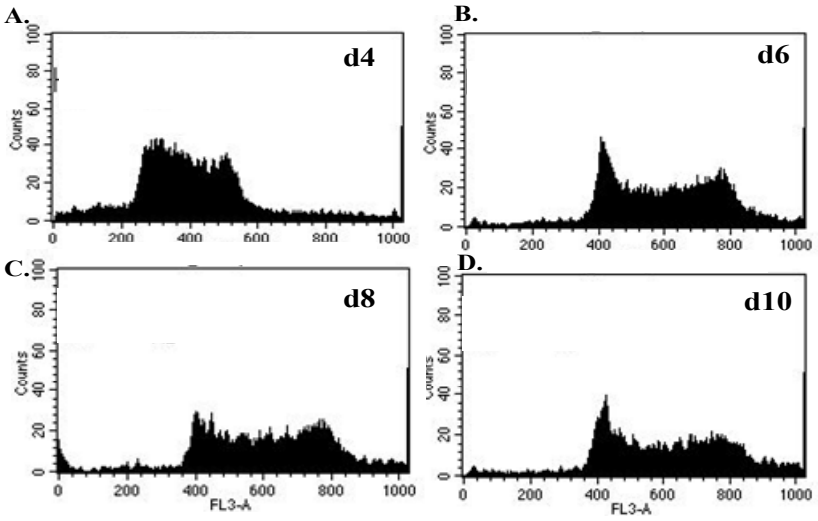


Figure 12. Fusion hybrid cells maintain tetraploidy in the course of differentiation. Ploidy state of fusion hybrid clone 2.1 in EB cells obtained at day 4, (A), day 6 (B), day 8 (C) and day 10 (D) of differentiation. EBs were dissociated by Trypsin/EDTA, stained with propidium iodide and analyzed by flow cytometry as described in the methods section.

4.1.3 Confirmation of tetraploid character of fusion hybrid clones by SNP genotyping

To enable the tracing of chromosomes from both fusing cells, we had chosen fusing partners from different strains of mouse that can be distinguished by their single nucleotide polymorphisms (HM-1 ES cells were from 129sv and somatic cells from DBA/2JxBL6 mouse). These strains also differ in their MHC class I haplotypes, enabling further confirmation of the hybrid character of fusion derived cells by flow cytometric assessment of MHC class I expression (see below chapter 4.1.4). A genome-wide screen of the SNP variation between these two strains of mice was performed to identify single nucleotide variations on each of chromosome. The reference variant alleles of the SNPs chosen for each chromosome are tabulated in **Table 1**. The presence

of both parental chromosomes in the fusion hybrid cell would be elucidated by the presence of two alleles at chosen SNP locus on a given chromosome.

Validation of the technique of identification of the variant alleles was performed by sequencing of HM-1 ES cell and somatic cell (spleenocytes) DNA mixed at predetermined ratios of 1:1 imitating the expected situation in fusion hybrid cells. **Figure 13** shows the result of sequencing of the SNP on chromosome 3 of this control setup demonstrating the presence of dual peak (nucleotides C and T) at position 170 in 1:1 mixed DNA. The pure DNA from HM-1 ES cells and somatic cells presents only the single nucleotide peak expected at this SNP position for each cell line (C for HM-1 ES cells and T for somatic cells).

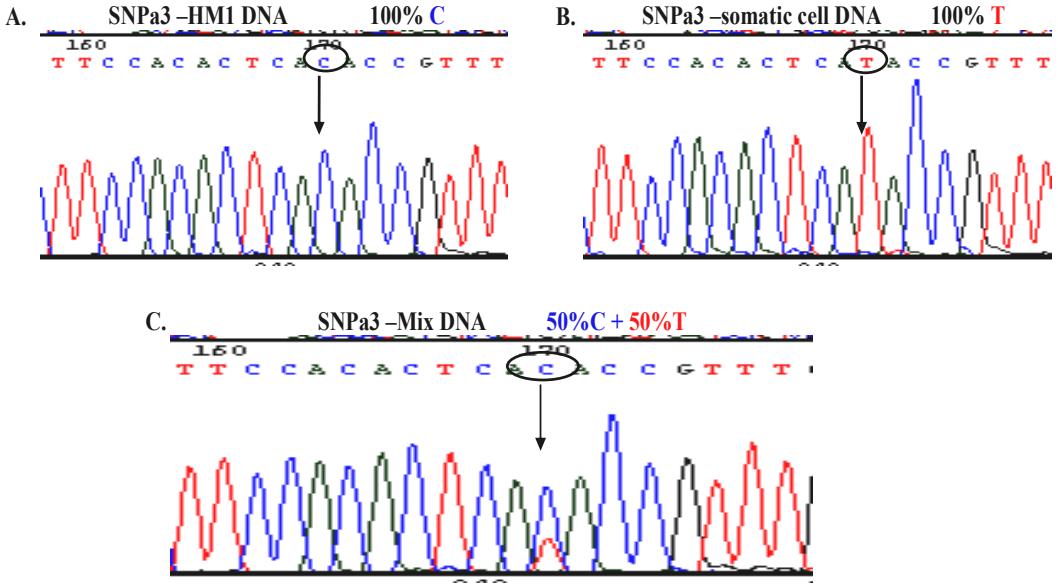


Figure 13. Validation of sequencing as a method for SNP genotyping. Identification of SNP on chromosome 3 with SNPa3 primer by sequencing of DNA isolated from HM-1 ES cells (A, expected variant allele: C) and spleenocytes (B, expected variant allele: T). The sequencing of DNA sample from both cell types mixed at ratio 1:1 revealed the presence of double peak for C/T variation at this SNP position.

Genomic DNA from parental cells and all four fusion hybrid clones (FH1.2; FH2.1; FH3.1 and FH4.2) was sequenced using the primers to identify one arbitrarily selected SNP variation on each of 21 murine chromosomes (19 autosomes and 2 sex chromosomes). These analyses confirmed that all fusion hybrid clones contained the variant alleles of their parental cell lines on all chromosomes tested **(Fig. 14 and Table**

1). These findings were confirmed in at least two independent experiments. Only clone 1.2 did not show two alleles on chromosomes 5 and 10 **(Table 1).** The presence of both alleles in all fusion clones demonstrated that the fusion clones retained both parental nuclei and at least one of each pair of parental autosomes, which were not lost during cell division and differentiation**.**

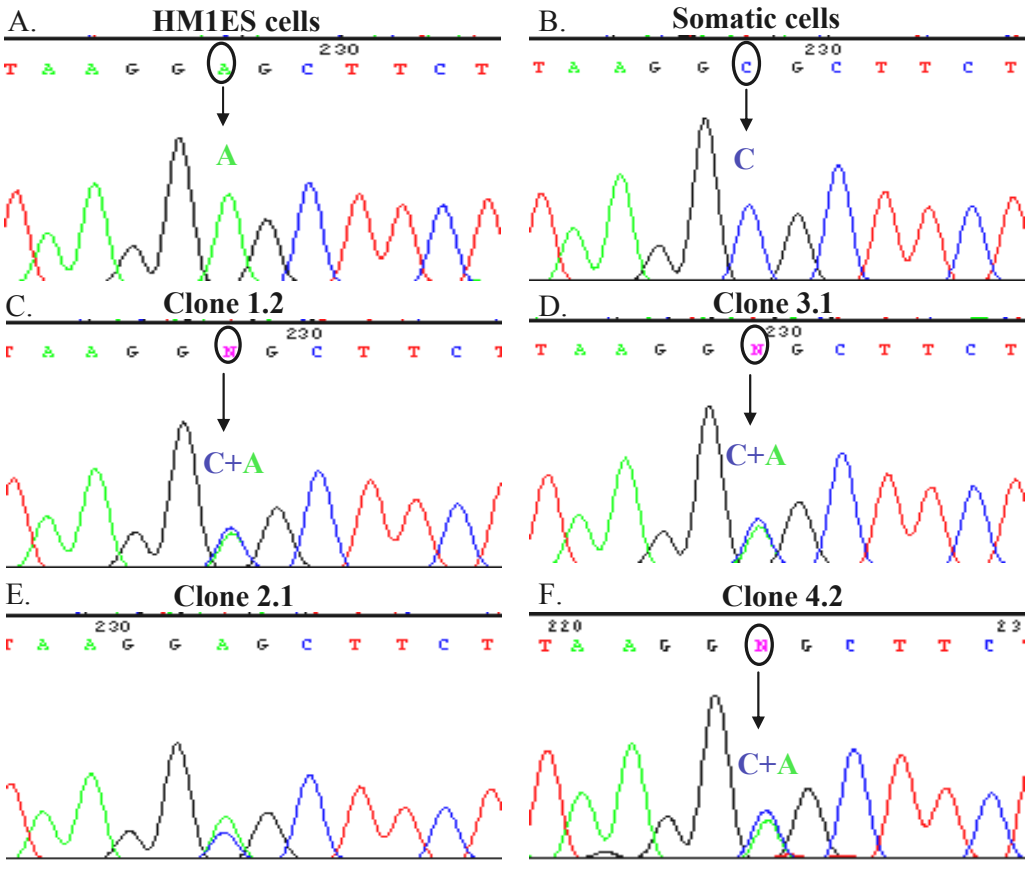


Figure 14. A representative example of identification of both parental SNPs in fusion hybrid clones. Sequencing of selected SNP on chromosome 7 with SNPa7 primer shows the presence of only one polymorphic allele in HM-1 ES cells (adenine) and somatic cells (cytosine). All four fusion hybrid cell lines contain both alleles (C/T, arrows) at this SNP position, suggesting the existence of both parental genomes in these cells.

Table 1. The results of SNP genotyping of parental HM-1 ES and cells (129sv strain) somatic cells (DBA2J strain) and fusion hybrid cells.

Chromosome number	SNP name	129 SV	DBA/2J	Presence of both alleles in fusion hybrids			
				Clone 1.2	Clone 2.1	Clone 3.1	Clone 4.2
1	SNPa1	C	T	CT	CT	CT	CT
2	SNPb2	C	T	CT	CT	CT	CT
3	SNPa3	C	T	CT	CT	CT	CT
4	SNPa4	G	T	GT	GT	GT	GT
5	SNPb5	A	G	A	AG	AG	AG
6	SNPa6	A	C	AC	AC	AC	AC
7	SNPa7	A	C	AC	AC	AC	AC
8	SNPb8	C	T	CT	CT	CT	CT
9	SNPa9	A	C	AC	AC	AC	AC
10	SNPa10	G	T	G	GT	GT	GT
11	SNPa11	C	T	CT	CT	CT	CT
12	SNPa12	A	G	AG	AG	AG	AG
13	SNPa13	C	T	CT	CT	CT	CT
14	SNPa14	A	T	AT	AT	AT	AT
15	SNPa15	C	T	CT	CT	CT	CT
16	SNPa16	A	G	AG	AG	AG	AG
17	SNPa17	C	T	CT	CT	CT	CT
18	SNPa18	C	T	CT	CT	CT	CT
19	SNPa19	A	G	AG	AG	AG	AG

4.1.4Expression of both parental MHC class I haplotypes on fusion hybrid cells

The SNP genotyping allows for accurate determination of the hybrid character of fusion hybrid cells. However, this method is time consuming and can not be used for a quick monitoring of cells. To enable easy monitoring of a fusion cell character, fusion cell partners were chosen from mouse strains expressing different MHC class I haplotypes, the expression of which can be easily determined by cell surface staining and flow cytometry. Reports from our and other groups have demonstrated that ES cells in their undifferentiated state do not express detectable levels of MHC class I molecules on their surface and their expression can not be enhanced by interferon γ (IFNγ) treatment (Abdullah et al. 2007; Frenzel et al. 2008). In contrast, differentiated cells derived from ES cells express MHC class I molecules at low but detectable levels and this expression

can be increased with IFNγ. We have postulated that somatic cells (such as spleenocytes) reprogrammed to pluripotency by fusion with ES cells would down regulate their normally very high MHC class I expression on the cell surface once their genomes in FH cells are successfully reprogrammed to a pluripotent state. To assess whether this is true we first assessed the expression of MHC class I molecules on fusion hybrid cells. As expected, fusion hybrid cells in their undifferentiated state expressed very low levels (1.5-1.7%) of MHC class I molecule of both haplotypes **(Fig. 15).** IFNγ treatment also could not enhance the expression of MHC class I molecules on FH cells in their undifferentiated state **(Fig. 15A).** However, when their progeny at day 4 of differentiation were analyzed, these cells expressed both MHC class I haplotypes. 2.6 % of FH 2.1derived day4 EBs expressed H-2K^b which was enhanced upto 43% after IFNγ induction. 11.9% of the same cells expressed H-2K^d antigen the expression of which was enhanced upto 70% upon IFNγ induction. **(Fig. 15B left panels).** 3% of FH clone 4.2 derived EBs, at day 4, expressed H-2K^b which was enhanced upto 30% after IFNγ induction. 21% of the same cells expressed H-2K^d antigen whose expression was enhanced upto 60% upon IFNγ induction. **(Fig. 15B, right panels)**. This response of day4 EB derived from fusion hybrid cells is similar to that of conventional murine ES cells and their differentiated derivatives. Day 11 EBs derived from fusion hybrid cells had relatively low expression of MHC class I molecules but still could be induced with IFNγ (28 % and 16% induction of H-2K^b and H-2K^d respectively in FH clone 2.1 derived day11EBs **(Fig. 15C left panels);** 17% and 40% induction of H-2K^b and H-2K^d respectively in FH4.2 derived day11EBs **(Fig. 15C, right panels)**, this also in agreement with the expression of these molecules on conventional ES cell derivatives at this stage of differentiation (Abdullah *et al.* 2007). HM-1 ES cells did not express H-2K^b and H-2K^d antigens in the undifferentiated state but when induced with IFNγ, day 4 EBs and day 11 EBs derived from HM-1 ES cells expressed H-2K^b antigen but not H-2K^d according to their origin from the 129sv mouse strain. The expression of both MHC class I haplotypes on fusion hybrid clones suggest that these cells contain chromosome 17 encoding for MHC class I heavy chain from both parental cell types. In addition, the ES cell-like expression pattern of MHC class I molecules on the surface of fusion hybrid cells further confirms that somatic cells, normally expressing high levels of these molecules, were successfully reprogrammed to a pluripotent state by fusion with ES cells.

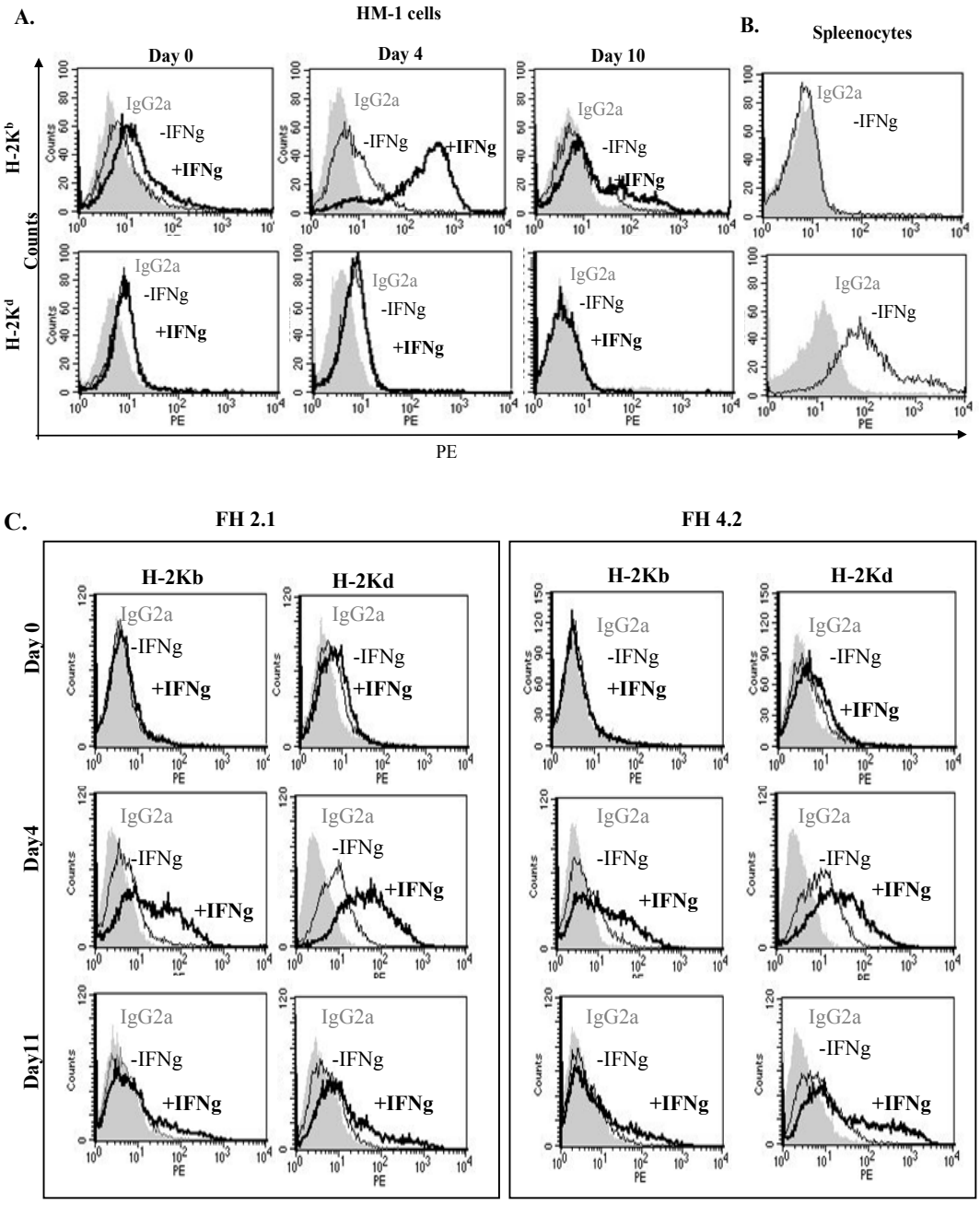


Figure 15. Expression of MHC class I molecule on the surface of fusion hybrid clones with and without induction. (A) HM-1 ES cells and their derivatives at day 4 and day10 and (C) Undifferentiated FH cells and their derivatives at day 4 and day11with and without IFNγ induction were dissociated into single cells, stained with antibodies specific for H-2K^b and H-2K^d molecules and the percentage of positive cells was determined by flow cytometry (B) Spleenocytes derived from DBA/2JxBL6 mice were checked for expression of H-2K^b and H-2K^d molecules.

The above results suggested that although a fraction of cell expresses one of the MHC class I haplotypes but still it was unclear if these molecules are expressed on a single cell. To rule out the possibility that two populations of cells (one H-2K^b expressing and the other H-2K^d) exist in these cultures, it was necessary to confirm that both types of molecules are expressed on one single cell. Therefore double staining was performed to provide a final proof for their fusion character.

Co-staining of H-2K^b and H-2K^d antigens was performed on FH 4.2 derived day 4 EBs after induction with IFNγ. Single stained cells were first analyzed for both haplotypes. 73% of FH4.2 derived day4 EBs were induced to express H2-K^d molecule whereas only 26% of the same cells expressed H-2K^b haplotype **(Fig. 16A)**. In control experiments, 90% of HM-1 ES derived day4 EBs were induced for H-2K^b but H-K^d could not be induced in these cells **(Fig. 16B)**.

Analysis of double stained cells revealed that out of the 73% cells that expressed H-2K^d. only upto 28% of cells were also positive for H-2K^b. Therefore only 28% of the cells expressed both H-2K^b and H-2K^d. 48% of the cells expressed only H-2K^d. All cells (26%) that expressed H-2K^b also expressed H-2K^d but not vice versa.

This lack of expression could be attributed to loss of one sister chromosome but this possibility is ruled out due to the fact that both alleles of chromosome 17 were detected in SNP analysis. We are unable to explain this result but it is likely that technical difficulties of low staining intensity of FITC conjugated H-2K^b antibody could not detect weaker signals. The presence of at least 28% of dual positive cells expressing both H-2K^b and H-2K^d molecules demonstrates the co-existence and stable transcription of two genomes in single fusion hybrid cell.

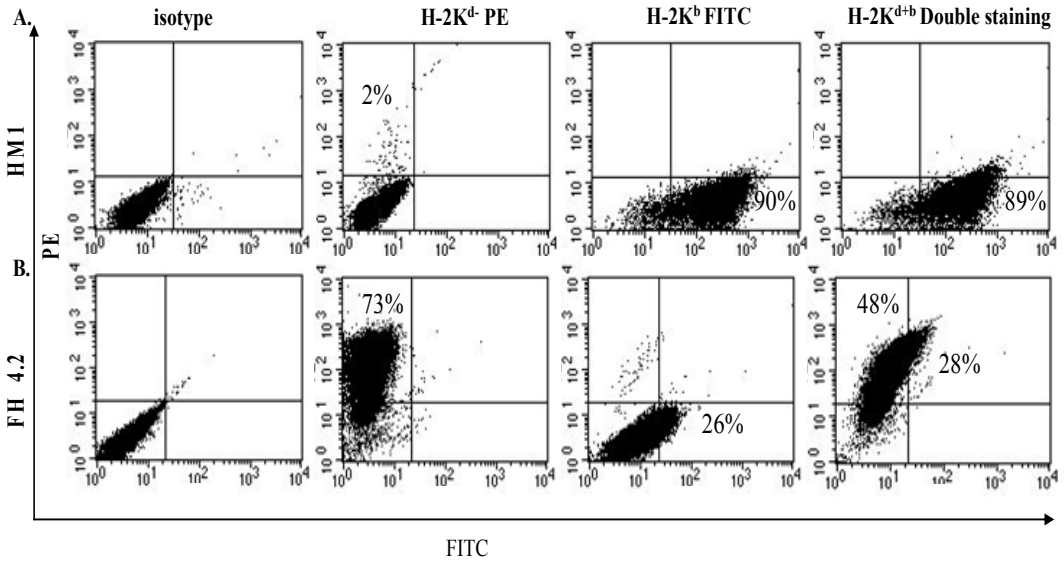


Figure 16. Dual staining of HM-1 ES and FH cell derived day 4EBs to perform simultaneous check for expression of H-2K^{b} and H-2K^{d} MHC-I haplotypes. A) HM-1 ES derived day 4 EBs stained with either H-2K^{b} FITC or H-2K^{d} PE antibody or both antibodies together. B) FH clone 4.2 derived day 4 EBs stained with either H-2K^{b} FITC or H-2K^{d} PE antibody or both antibodies together.

4.1.5 Expression of somatic CD markers on fusion hybrid cells

Upon reprogramming somatic cell is expected to loose its somatic marker as a result of unidirectional reprogramming towards pluripotency depending on the extent of reprogramming that has occurred in fusion hybrid cells. To elucidate this, we checked the expression of markers of the lymphoid lineage that are usually expressed on spleenocytes and bone marrow cells (somatic fusing partners). Using spleenocytes as positive control cells, we found that 97%, 35%, 57% and 39% f these cells expressed antigens specific for leukocytes (CD45), monocytes/macrophages (CD11b), B lymphocytes (CD19) and T lymphocytes (CD3a), respectively **(Fig. 17A).** Undifferentiated HM-1 ES cells did not express any of these markers **(Fig. 17B**). The fusion hybrid cells in undifferentiated state also did not express significant levels of CD45, CD11b and CD19 antigens, but low levels of T cell antigen CD3a was expressed on 2.4% of cells (**Fig. 17C)**. This could be attributed to the actively transcribing somatic genome coexisting with ES cell genome within the small fraction of fusion hybrid cells due to incomplete epigenetic reprogramming of specific regions of the somatic genome. These results demonstrate that the somatic genome has been dominated by chromatin

modifying factors expressed by the ES cell genome leading to silencing of the somatic cell genes in the FH cells.

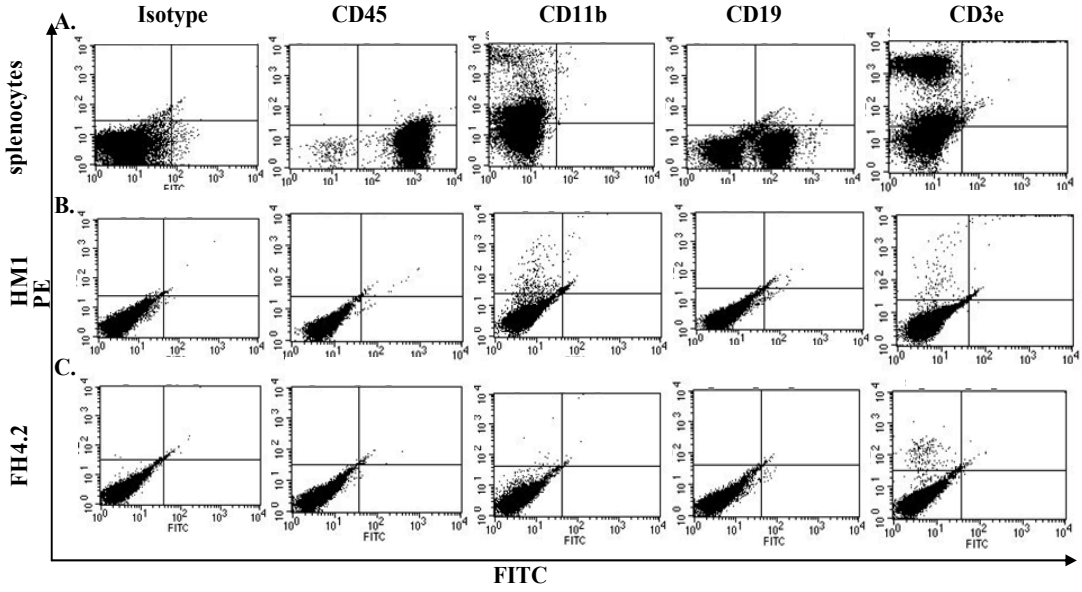


Figure 17. Expression of markers somatic cell markers (lymphoid lineage) on fusion hybrid cells in undifferentiated state. Flow cytometric analysis of the expression of surface antigens CD45, CD11b, CD19 and CD3a on spleenocytes (A) undifferentiated HM-1 ES cells (B) and undifferentiated fusion clone 4.2 cells (C) .

4.1.6 Cardiac differentiation of fusion hybrid clones

Mouse ES cells can bring about reprogramming of somatic cells and thus lead to the generation of hybrid cells that harbour a dominating ES nucleus in the tetraploid genome. These reprogrammed ES like FH cells could now be used as model systems to study the developmental processes in line with those on conventional ES cells.

First question that is addressed here is can the tetraploid cells differentiate into cardiac lineage as normal ES cells do. Secondly what are the physiological characteristics of the cardiac cells derived from tetraploid cells and will they exhibit any altered physiology. What will be the maturation state of the myocytes.

The cardiac differentiation potential of the FH cell was tested by subjecting the FH cells to spontaneous differentiation using hanging drop method. We routinely differentiated

clone 2.1 and clone 4.2 and used these clones in our study. The cardiac cells derived form fusion cells expressed cardiac specific genes αMHC, Mlc2v and cardiac troponin T (cTnT), which were strongly enriched in EBs on day 16 of differentiation **(Fig. 18A).** Immunocytochemical stainings for cardiac specific proteins α-actinin and cTnT revealed the cross striation pattern typical for CM derived from conventional ES cells **(Fig. 18B).**

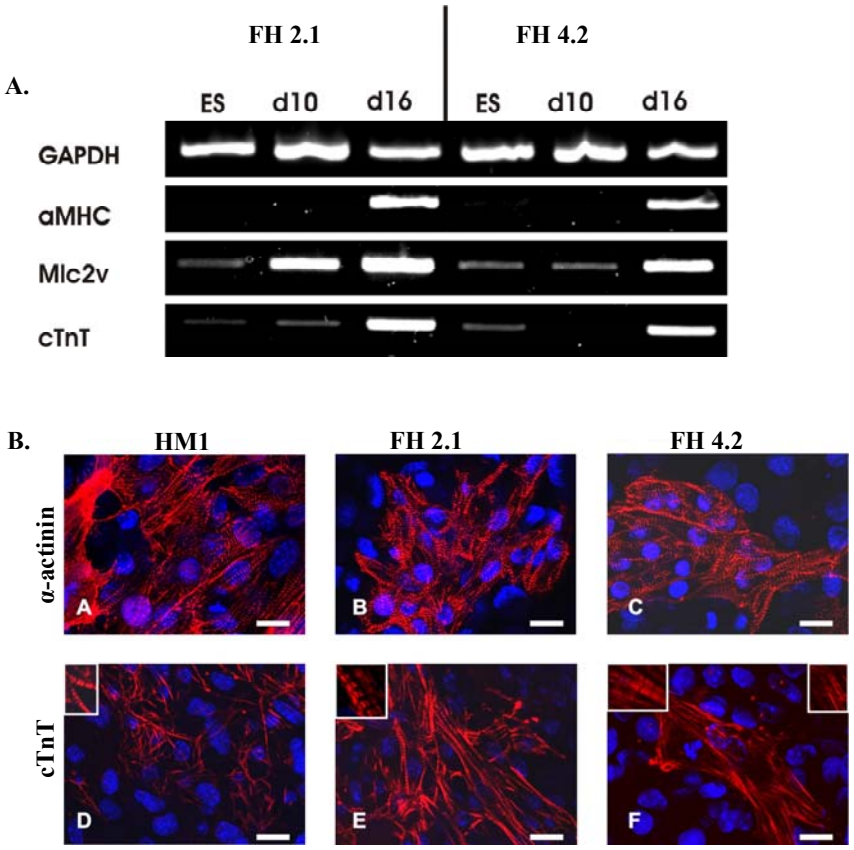


Figure 18. Cardiac differentiation of fusion hybrid cells. RT-PCR analysis for expression of cardiac genes and a house keeping gene GAPDH in FH clones 2.1 and 4.2 (A). Immunostainings for α-actinin and cardiac troponin T in HM-1 ES cell- and fusion hybrid derived-CMs at day 16 of differentiation. Scale bars=100 μm.

4.1.7 Electrophysiological properties of fusion hybrid-derived cardiomyocytes

Characteristics of spontaneous action potential (AP) in FH derived CM

In order to understand the physiology of CMs derived from FH cells, Action potential (AP) recording on single cardiac cells (obtained by dissociation of beating and plating on fibronectin coated dishes) was performed. Two stages of differentiation were assessed, early differentiation stage (EDS- between day 16 and day 20 of differentiation.) and late differentiation stage (LDS- between day 30 and day 40). Fusion-derived CM and ES-CM were characterized into different phenotypes, by analyzing of spontaneous action potentials (AP) at EDS and LDS **(Fig. 19**). In all CM types, all AP types were found at EDS and LDS **(Fig. 19)** their distribution, however, changed during the differentiation. At EDS, most CM of all types displayed pacemaker-like AP, the amount was 77% of ES-CM, 66% of 2.1-CM, and 67% of 4.2-CM (**Table 1**). Minor differences in AP parameters observed between EDS and LDS reflect these changes in AP distribution (**Table 1**). AP parameters were similar between fusion-derived CM and ES-CM, when corresponding AP types are compared.

Effect of muscarinic and β-adrenergic receptor agonists on AP frequency.

Carbachol (CCh), a synthetic acetylcholine analog, was applied to investigate the muscarinic signaling. CCh evoked a significant reduction of AP frequency by 53±12% in ES-CM ($n=9$, $p<0.01$), by 61±9% in FH2.1-CM ($n=10$, $p<0.001$), and by 44±7% in FH4.2-CM in EDS ($n=10$, $p<0.05$; **Fig. 20 A).** In ES-CM and FH 4.2-CM and FH2.1 CM CCh impact was similar at EDS. Negative chronotropic effects of CCh were reversible upon washout **(Fig. 20A right panels).**

We also examined the CM response to β-adrenergic regulation with isoproterenol (Iso). When 1 μM Iso was administered to EDS **(Fig. 20B)**, an increase of AP frequency was observed. At EDS, Iso increased the AP frequency by 69±22% in ES-CM ($n=9$, $p<0.01$), by 111±28% in FH 2.1-CM ($n=14$, $p<0.001$), and by 39±19% in FH 4.2-CM ($n=18$, $p>0.05$; **Fig. 20B).** At LDS, Iso increased the AP frequency by 60±20% in ES-CM ($n=12$, $p>0.05$), by 181±77% in FH 2.1-CM ($n=7$, $p<0.05$), and by 52±13% in FH 4.2-CM ($n=13$, $p<0.01$). Positive chronotropic effects of Iso were reversible upon washout **(Fig. 20, right panels).**

The action potentials recordings suggest that FH cell differentiate and form all three cardiac subtypes, atrial ventricular and pacemaker type in similar way like ES cells.

Response to β-adrenergic and muscarinic agonists suggests the presence of intact receptors as in fully functional cardiac cells. The response observed in FH derived CM was the same as observed for ES-CM. These observations suggests that FH derived CM have normal physiology despite originating from chromosomally abnormal cells.

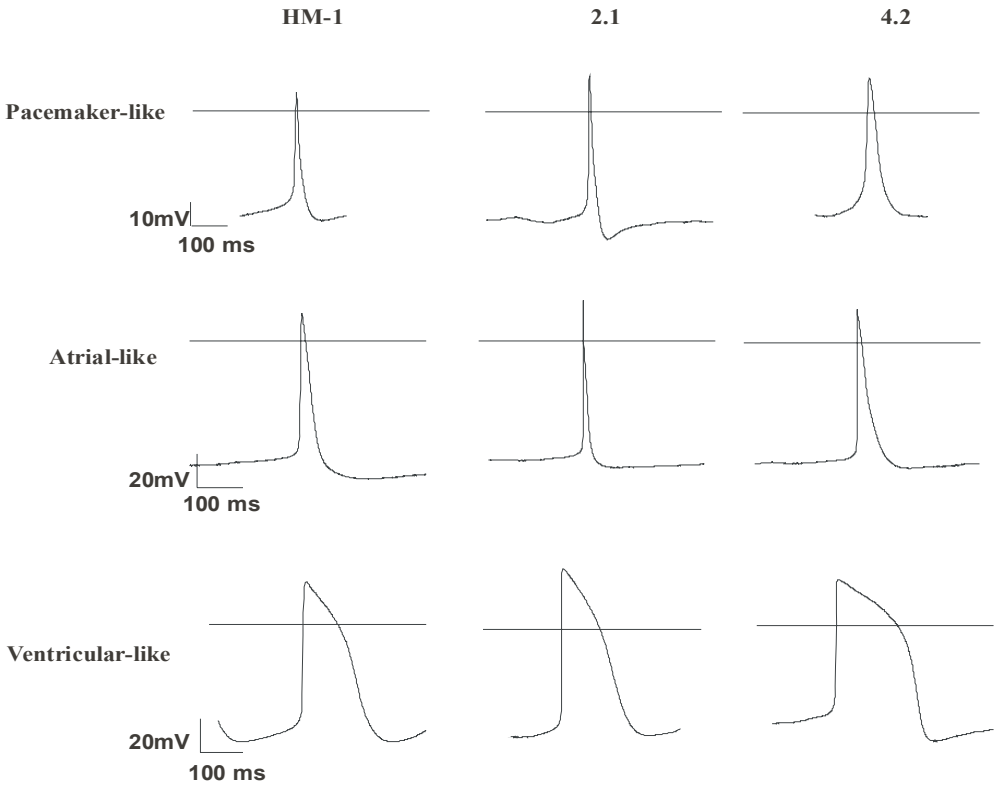


Figure 19. Cardiac subtypes derived form *in vitro* differentiation of HM-1, F2.1 and FH4.2. Three cardiac subtypes–atrial like, pacemaker like and ventricular like cells could be obtained at day16 of differentiation.

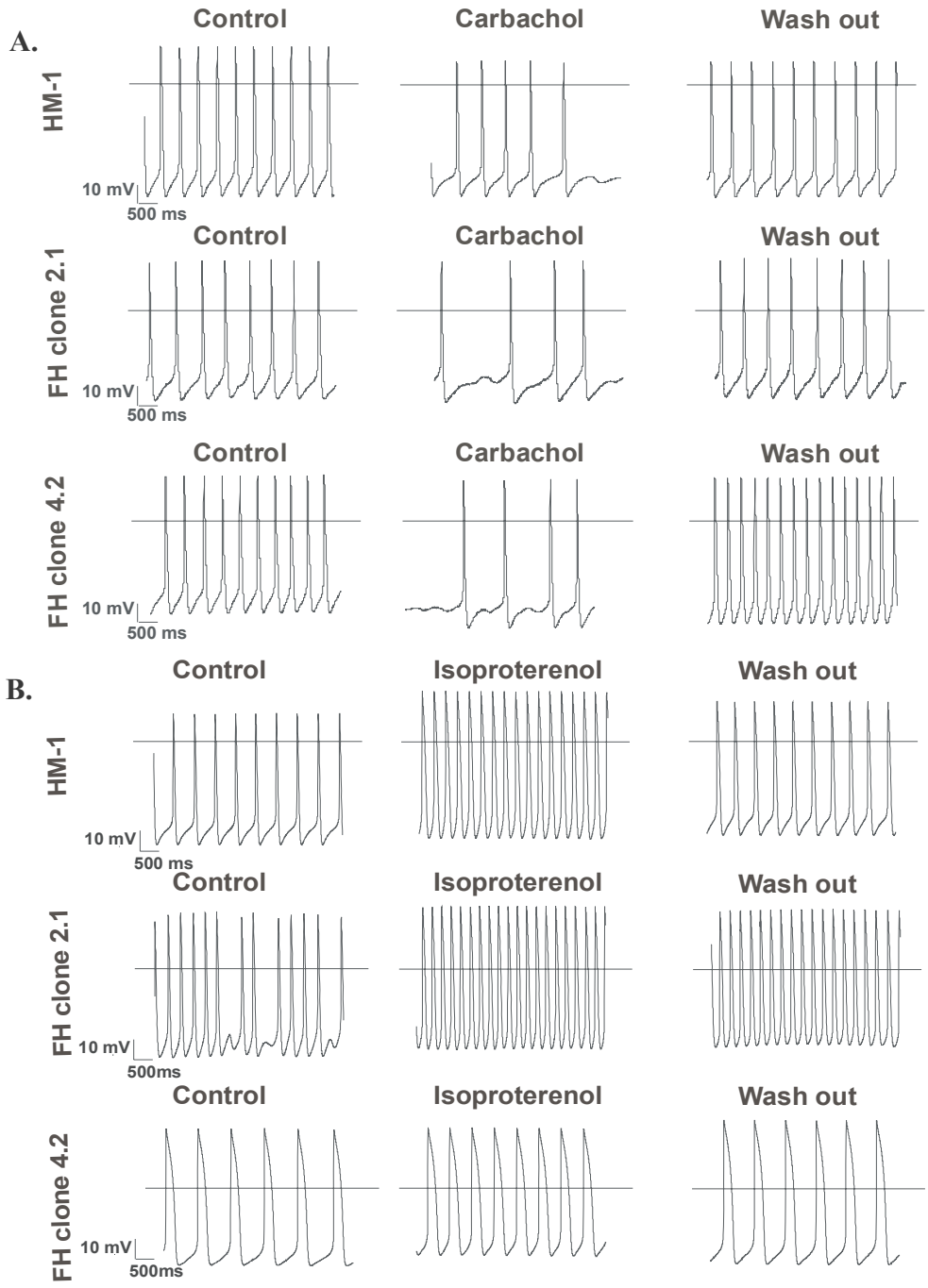


Figure 20. Chronotropic response of HM-1-ES and FH clone 2.1 and 4.2 derived EDS CM. (A) Negative chronotropic response to carbachol (B) Positive chronotropic response to isoproterenol.

The results of my studies on FH cells and the CMs derived from them suggest that:

1. Fusion between somatic cells and ES cells leads to a reprogrammed pluripotent state in the FH cells.
2. The FH cells carry a tetraploid genome through many passages but retain the same cardiac developmental potential as that of ES cells.
3. The FH derived EBs also maintain a tetraploid state during differentiation.
4. Expression of MHC class-I molecules corresponding to both fusing partners elaborated on the fusion nature of FH cells.
5. FH cells failed to express somatic markers relative to their somatic fusion partner demonstrating the dominance of ES cell genome in the FH cells.
6. The CMs derived from FH cells demonstrated normal cardiac structure and mature into three functional cardiac subtypes of atrial, ventricular and pace maker like cells as demonstrated by their AP parameters.
7. The CMs demonstrated intact β-adrenergic and muscarinic signaling thus suggesting normal function of CMs.
8. The ploidy state of the cardiac cell genome could not be determined due to heterogeneous cell population obtained in spontaneous differentiation by EB formation.

In the light of developments taking place in the filed of stem cells during 2006, another source of cardiac cells emerged from a very efficient way of reprogramming. The iPS cells were fast proving to be developmentally similar to ES cells. Cardiac cells derived from fully reprogrammed cells like iPS present a platform to study the stability of the differentiated state of cells during a developmental process. iPS cells have advantage over pluripotent FH cells, as iPS cells meet all the criteria for pluripotency, including germline transmission and live pups after tetraploid complementation (Meissner *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007). Fusion hybrid cells, however, may not develop into the germ cells after blastocyst injection, due to the tetraploidy of the cells.

4.2 Advancement of methods for iPS cell generation

4.2.1 Instability of the genetically unmodified iPS cell line TiB7-4

The iPS cell lines TiB7-4 were generated by Alexander Meissner and Marius Wernig at the laboratory of Rudolf Jaenisch at the Whitehead Institute of Technology, MA, USA. Retroviral transduction of wild type murine tail tip fibroblasts with Oct4, Sox2, cMyc and Klf4 was performed to generate genetically unmodified iPS cell line TiB7-4 (Meissner *et al.* 2007; Wernig *et al.* 2007). This cell line more closely fulfilled the criteria requirement of no genetic modification as our intention was to study the impact of reprogramming imposed on the properties of differentiated derivatives like cardiac cells.

TiB7-4 iPS cells were cultivated on MEFs in the presence of LIF. Interestingly, observations on the morphology of the colonies at various passages showed that this iPS cell line was not stably maintaining its morphology and thus its pluripotency. In early passages (p7) some colonies resembled an elliptical, sharp-bordered morphology typical for undifferentiated murine ES cells, but there were few colonies that lost this typical colony shape and appeared as loose colonies breaking into single cells. With increase in passage number this morphology turned more heterogeneous and all compact colonies disappeared. **(Fig. 21A).** The visual observation was confirmed by determining the percentage of cells that express pluripotency marker SSEA1. Flow cytometric analysis was performed on cells stained with SSEA1 at passages 7, 9 and 14 **(Fig. 21B)**. 54% of the cells expressed SSEA1 at passage 7. A strong reduction in this percentage was noticed in later passages with only 38% positive cells at passage 9 and 41% at passage 14. This implies that the cells lost pluripotency with each passage and therefore TiB7.4 cells were unstable pluripotent cells.

4.2.2 *UTF1*-Neo selection of pluripotent iPS cell sub clones from TiB7.4

To enrich for pluripotent cells from the non-homogenous population of TiB7-4 cells, we hypothesized the insertion of pluripotency marker driving antibiotic selection into the iPS cells (Tan *et al.* 2007). Hence *UTF1*-Neo was used as a selection vector. In this

vector, the neomycin resistance gene is under the control of the promoter of the *UTF1* gene, which is specifically expressed in pluripotent ES cells and is rapidly down regulated upon ESC differentiation). Hence, iPS cells stably expressing the neomycin resistance gene only in pluripotent state should be stabilized in an undifferentiated state in the presence of Neomycin (also known as G418). To test this hypothesis, TiB7-4 cells were electroporated with circular *UTF1*-Neo plasmid and selected with G418 beginning 24 hours after the transfection. During the G418 selection several colonies arose. Out of these colonies three were isolated two weeks after the electroporation and expanded as sub clones UTF-1, UTF-2 and UTF-3. No further selection with G418 was applied. UTF-1, -2 and –3 were subculture for 40 passages and displayed homogenous colonies with elliptical shape and sharp borders **(Fig. 22A)**. The expression of Stage Specific Embryonic Antigen 1 (SSEA1) was checked as a marker of pluripotency. At passage 4 and above, the SSEA1 surface antigen was expressed on 91%, 95% and 87% of UTF-1, UTF-2 and UTF-3 cells, respectively and remained so at later passages **(Fig. 21B).** For example, at passage 11, the fraction of SSEA1 positive cells for UTF-1, UTF-2 and UTF-3 clones was 95%, 94% and 98%, respectively.

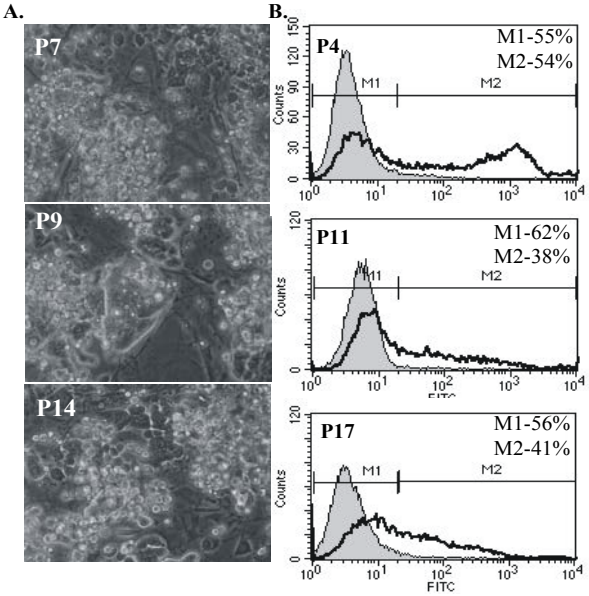


Figure 21. Morphology and SSEA 1 expression of wild type TiB7-4 iPS cells. (A) Morphology of TiB7-4 iPS cells at different passages in cell culture. (B) Expression of SSEA1 at different passages as determined by flow cytometry. M1 and M2 is the percentage of cells stained negative and positive respectively for SSEA1. Figures taken from own publication No. 2 (Pfannkuche *et al.* 2010).

These data suggest that *UTF1* selection generates pluripotent clones of TiB7-4 iPS cells that could be maintained stably for up to 40 passages even in the absence of drug selection.

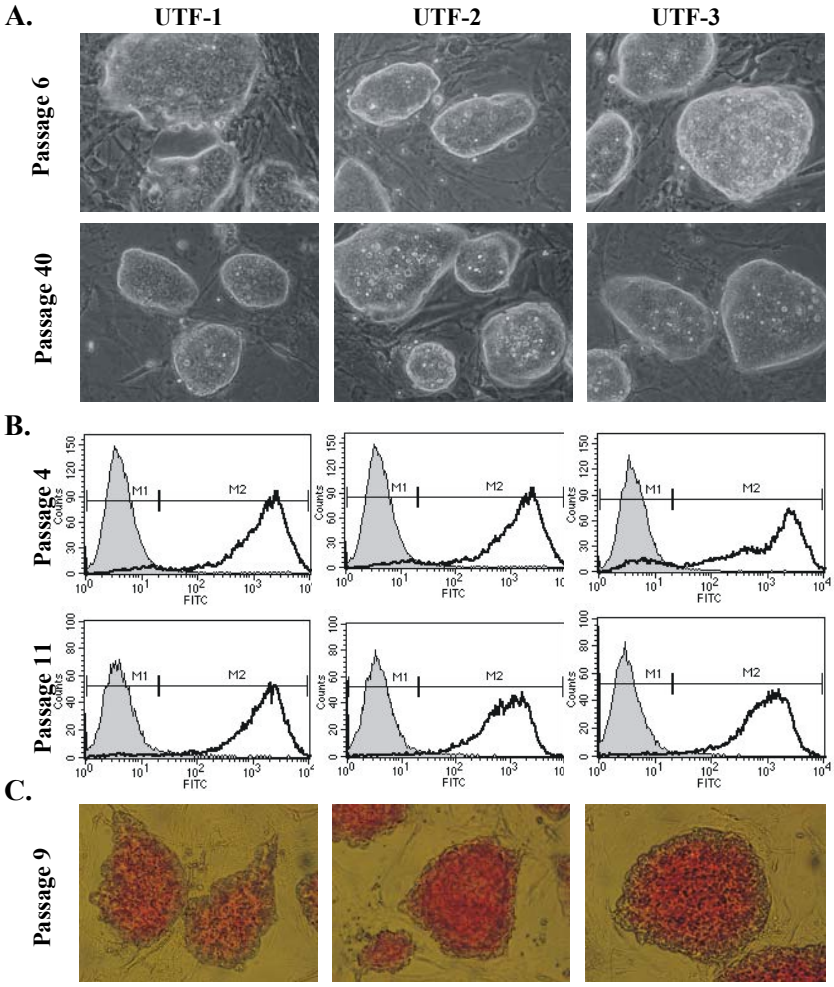


Figure 22. Characterization of three individual, *UTF1*-Neo selected iPS cell clones UTF-1, -2 and -3 derived from TiB7-4 cells. (A) Morphology of UTF-Neo selected iPS cells six and forty passages after transfection. Selection with G418 was performed only until initial isolation of colonies. (B) Expression of SSEA1 of UTF-1, -2 and –3 as indicated by flow cytometry four and eleven passages after transfection. (D) Alkaline phosphatase staining of UTF-1, -2 and –3 iPS cells sub clones at passage 4, 11 and 9. M1 and M2 is the percentage of cells stained negative and positive respectively for SSEA1. Figures taken from own publication No. 2 (Pfannkuche *et al.* 2010).

4.2.3 Analysis of TiB7-4 sub clones derived from TiB7-4 with SV40-Neo selection

To rule out effects generated by electroporation or by selection for highly proliferative sub clones, a control SV40-Neo vector was transfected into TiB7-4 parental iPS cells. The SV40-Neo vector expresses resistance against G418 and is driven by the ubiquitously active early SV40 promoter. G418 selection then resulted in a variety of clones. In a first experiment, ten clones of the SV40-Neo transfected TiB7-4 were randomly selected and isolated. Morphology of these clones varied, ranging from ES cell-like to differentiated colonies. Flow cytometric analysis of these clones at passage 3 resulted in different fractions of cells that were positive for the pluripotency marker SSEA1. In a second experiment, TiB7-4 cells were again transfected with SV40-Neo and subsequently selected with G418. Six clones with a typical morphology for murine ES cells were isolated and expanded. Stable integration of the SV40-Neo selection marker was confirmed by PCR amplification using genomic DNA of sub clones and primers for the SV40 promoter and the Neomycin resistance gene. The morphology of the colonies was analyzed at passages 3, 7 and 12 after transfection **(Fig.23A, B)**. At early passage, all six clones formed colonies that resembled the typical shape of mouse ES cells but spontaneous differentiation set in after further passaging and resulted in a fragmentation of the colonies at passage 8 in the SV40-Neo transfected clone 1 **(Fig. 23A)** and clones 2, 3 and 6. The observed loss of regular colony morphology was even more pronounced at passage 12. Remarkably, there was no such change in colony morphology seen in clone 4 (**Fig. 23B).**

To determine whether the loss of typical ESC-like colony morphology in majority of stable SV40-Neo transfectants was related to loss of pluripotency marker SSEA1, the expression of this marker was analyzed at different passages using six different sub clones **(Fig. 23C-H)**. All six clones show similar SSEA1 expression ranging from 80% to 89% at passage 3. At passage 7 even higher fraction of cells expressed SSEA1. However, at passage 12 only clones 4 and 5 retained high fraction of SSEA1 positive cells (88% and 87%, respectively), while the remaining four clones had almost lost SSEA1 expression. These data indicate that *UTF-1* Neo selection allows for enrichment of pluripotent cells in a specific manner and it is not a result of random effect of

electroporation procedure or selection of highly proliferative sub clones as the clones generated.

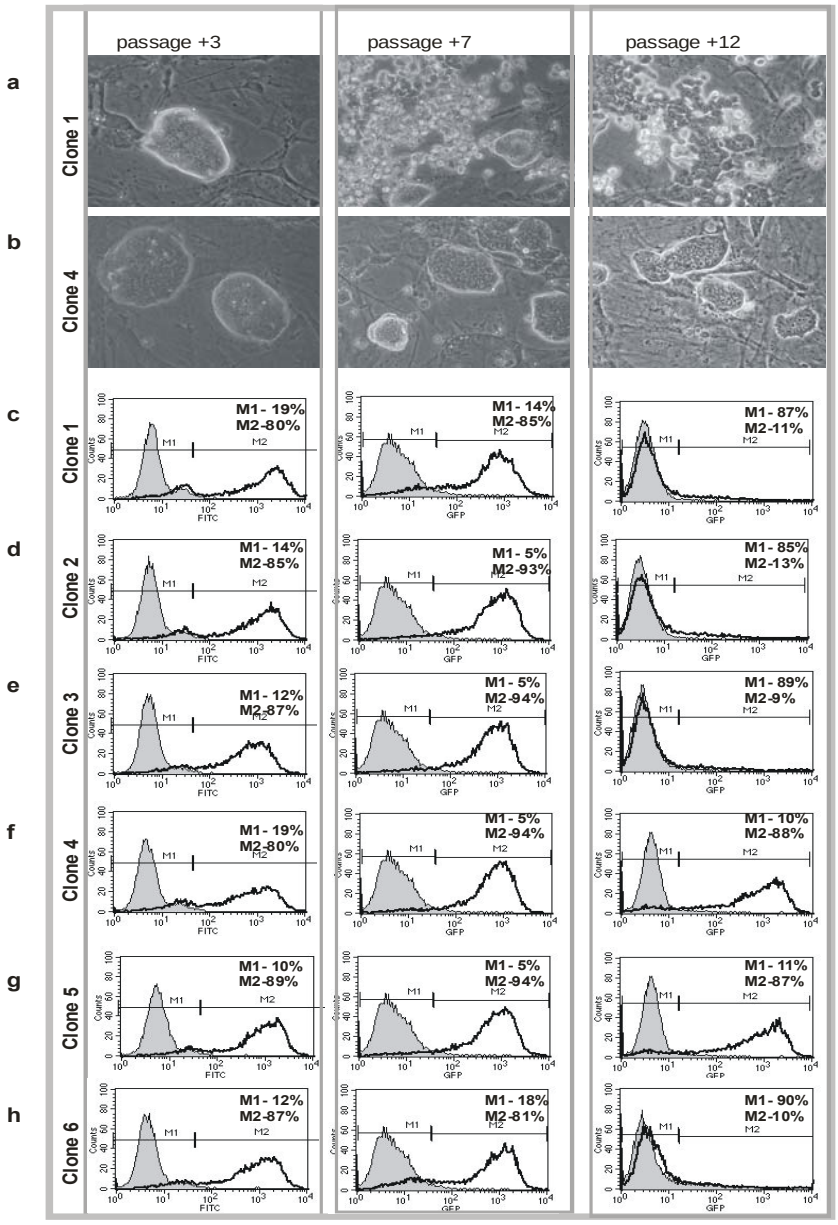


Figure 23. Morphology and SSEA 1 expression of SV40-Neo transfected control clones.
(a-b) Colony morphology of SV40-Neo selected clone 1 and 4 at passage 3, 7 and 12 after transfection. (c-h) SSEA1 expression of SV40-Neo selected clones 1-6 at the three different passages after transfection as determined by flow cytometry. M1 and M2 is the percentage of cells stained negative and positive respectively for SSEA1. Figures taken from own publication No. 2 (Pfannkuche *et al.* 2010).

Thus, selection of iPS cells based on colony morphology is not sufficient to prove the fully reprogrammed state of the cells and a stable pluripotent phenotype.Thus, the morphological observation of the iPS cell line TiB7.4 led to the improvisation in the method to effectively isolate fully reprogrammed clones of iPS cells based on *UTF-1* Neo selection.

4.3 Generation of transgenic iPS cells that will allow purification of cardiomyocytes

In depth analysis of the differentiated progeny from iPS cells is crucial before concluding on the similarity of ES and iPS cells. Research on iPS cells promises major application including *in vitro* developmental studies, for understanding disease pathologies and physiological pathways, toxicological testing, and for epigenetic studies. For such application of iPS cells, CMs as a differentiated derivative of iPS cells can serve as a model system. This study was aimed at performing high throughput molecular, phenotypic, physiologic and genomic characterization of cardiac cells derived by invitro differentiation of iPS cells as a pure population. To implement all the applications of iPS cells, there is requirement for robust supply of differentiated derivatives of iPS cells in a pure and homogenous form. Homogenous cell population enables a controlled analysis on various physiological and transcriptional processes.

iPS cells exhibit pluripotency and can differentiate *in vitro* into multiple lineages including CMs. When iPS cells are allowed to differentiate *in vitro*, they form cells of all three lineages, the ectoderm, mesoderm and endoderm. CMs can be easily identified during the differentiation of iPS cells as spontaneously beating areas within spherical embryoid bodies (EBs). But these beating EBs also contain cells of other lineages and thus make it difficult to obtain CMs in high purity. Therefore, we aimed at generating a transgenic iPS cell line to be able to purify CMs. We used the αPIG vector, in which cardiac α-myosin heavy chain (αMHC) promoter drives the expression of puromycin N- acetyltransferase (PAC) and the internal ribosomal entry site (IRES) flanked enhanced green fluorescent protein (eGFP), to genetically modify murine iPS cells to stably express PAC and eGFP specifically in CMs.

TiB7-4 iPS cells were electroporated with αPiG vector as described in the Methods

section. Resistant colonies were selected by addition of neomycin at a concentration of 500µg/ml. About 30 colonies were picked and sub cultured as separate clones. 8 clones were randomly chosen for cardiac differentiation and the others were frozen in multiple batches. Five clones could reproducibly differentiate into EBs containing spontaneously beating, green fluorescing areas, which first appeared on day 8-9 of differentiation. Three clones, αPIG-iPS clone 25, αPIG-iPS clone 15 and αPIG-iPS clone 11 were routinely used for cardiac differentiation. All data described here was generated with αPIG-iPS clone 25. The transgenic murine D3 ES cell line αPIG-ES (clone 44) was generated with the same αPIG vector previously (Kolossov *et al.* 1998) and was used for comparative analyses.

4.3.1 Transgenic iPS cells retain their pluripotential characteristics

When cultured on MEFs, undifferentiated murine iPS cells formed colonies very similar to murine ES cells **(Fig. 24A).** Undifferentiated ES and iPS cells appeared as tightly packed colonies with high nucleus/cytoplasm ratios. Within the colonies, iPS and ES cells had distinct margins and were positive for Oct4 **(Fig. 24B**) and ALP **(Fig. 24C),** and more than 90% of cells expressed SSEA1 on their surface (**Fig. 24D).**

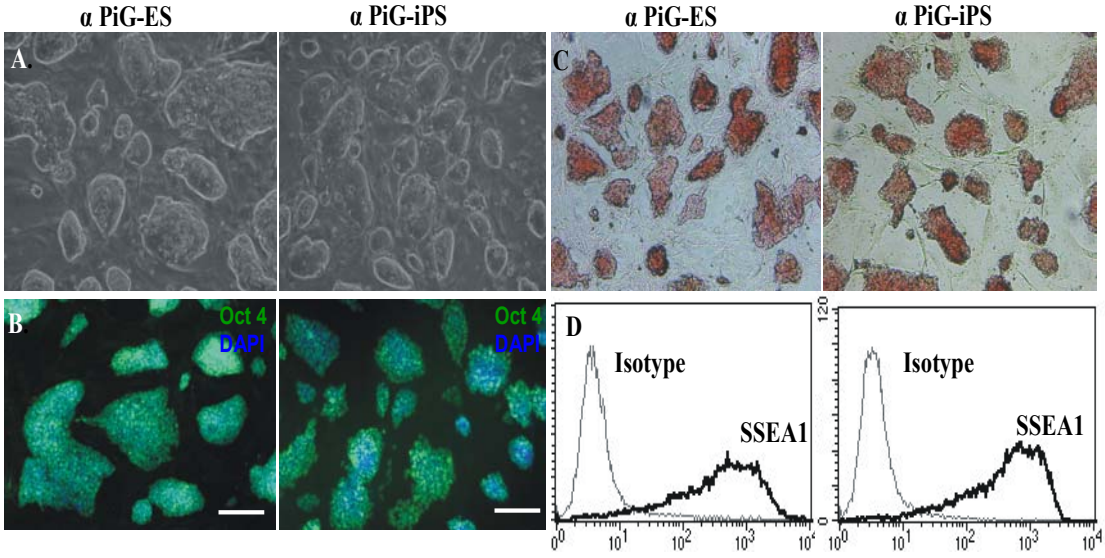


Figure 24. Expression of pluripotency markers on undifferentiated transgenic murine iPS and ES cells. A) Undifferentiated colonies on mitotically inactivated MEFs. Colonies were immuno-positive for Oct4 (B) and ALP (C). D) Flow cytometric analysis of expression of SSEA1 on ES and iPS undifferentiated cells. Scale bars=100 µm.

4.3.2 Transgenic iPS cells efficiently form EBs with a kinetic similar to that of ES cells

The iPS cells when differentiated by withdrawal of LIF and cultured in non-adherent bacterial dishes on an orbital shaker readily formed EBs. At day 2 of differentiation, these EBs were transferred to a 200 ml spinner flask where they differentiated further to cells of different lineages. The kinetics of the growth of EBs was monitored by withdrawing a small number of EBs from the spinner flask at various intervals (day 2-16) and acquiring images through a light microscope and determining their diameter using the Axiovision Software. These analyses revealed that iPS cells differentiated to multi-cellular EBs with kinetics similar to that of ES cells **(Fig. 25).**

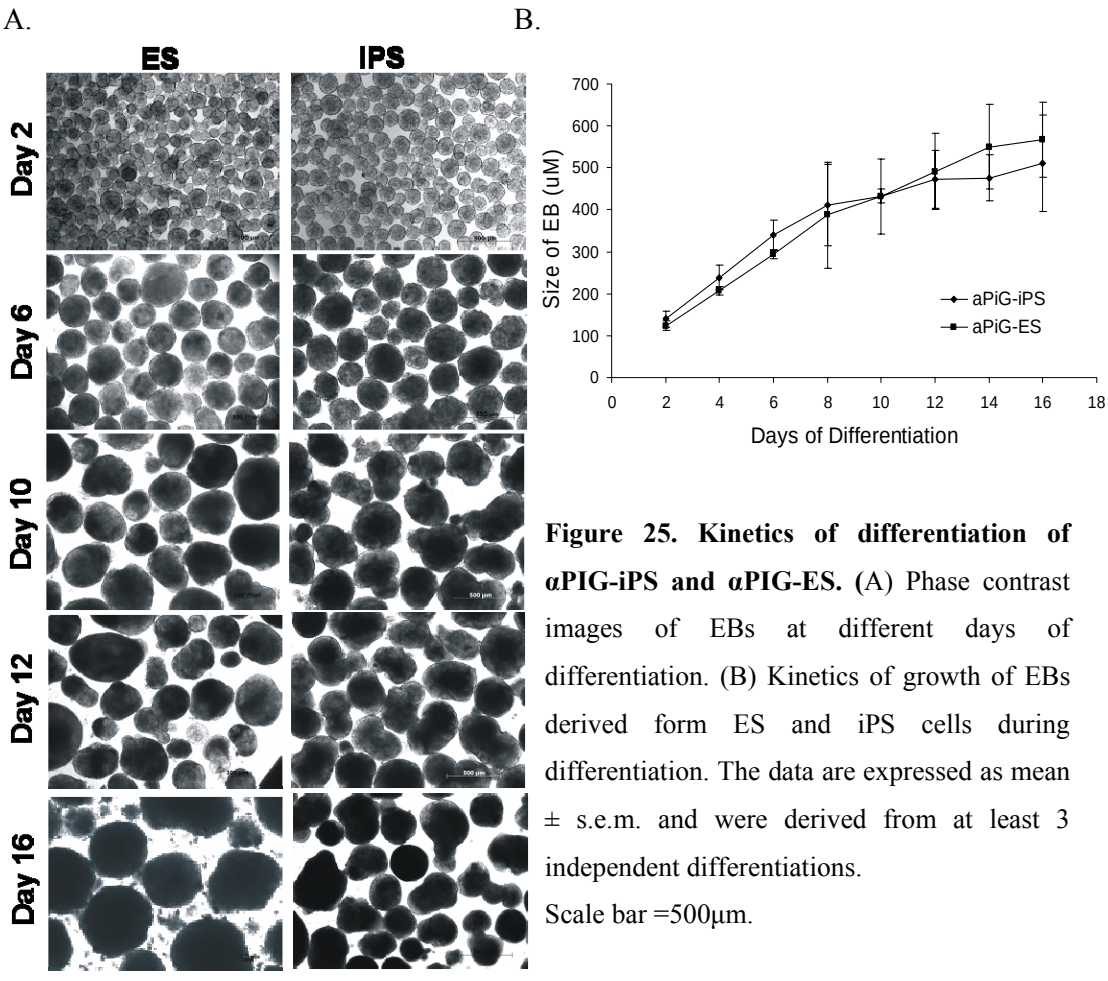


Figure 25. Kinetics of differentiation of αPIG-iPS and αPIG-ES. (A) Phase contrast images of EBs at different days of differentiation. (B) Kinetics of growth of EBs derived form ES and iPS cells during differentiation. The data are expressed as mean ± s.e.m. and were derived from at least 3 independent differentiations.
Scale bar =500µm.

4.3.3 Drug selection yields highly purified iPS-CMs

During the course of spinner flask differentiation of ES and iPS cells, multiple lineages are formed within the EBs. At day 9, the formation of cardiac lineage was identified by appearance of contracting areas within EBs. When visualized under a florescence microscope the contracting areas showed GFP florescence **(Fig. 26A).** Purification of the cardiac cells was initiated by addition of puromycin at day 9. After 7 days of puromycin treatment, all non-cardiac cells in EBs were eliminated and the resistant cells survived as GFP-positive clusters exhibiting vigorous spontaneous contractile activity **(Fig. 26B, C).**

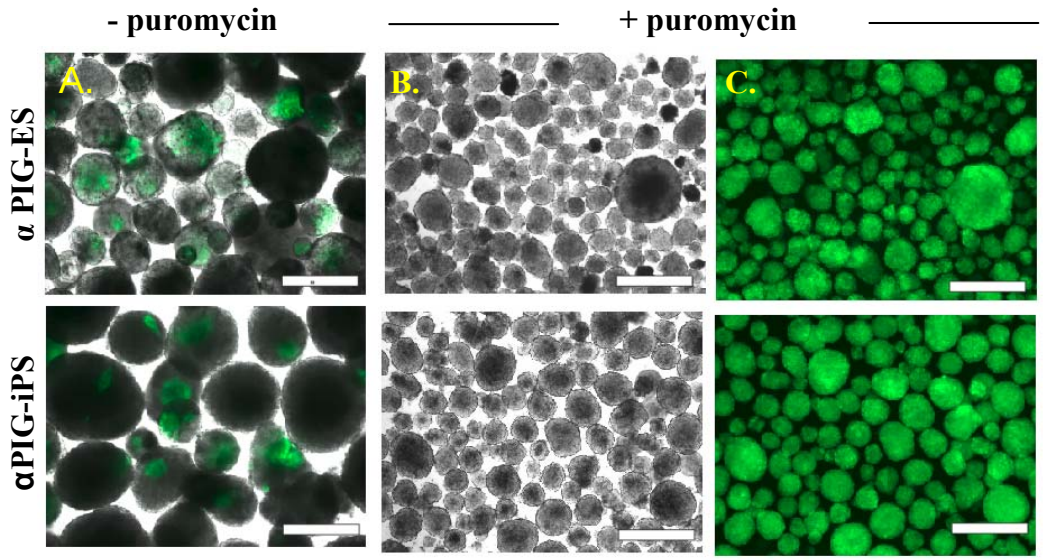


Figure 26. Embryoid bodies formed from transgenic ES and iPS cells and purification of cardiac clusters. (A) Phase contrast images of day 16 EBs derived from ES and iPS cells without puromycin treatment. (B) Phase contrast images of day 16 cardiac clusters derived from ES and iPS cells after puromycin treatment. (C) Florescence images of GFP fluorescing purified cardiac clusters. Scale bars=100 μm.

To assess the purity, after 7 days of puromycin treatment cardiac clusters were dissociated to single cells. Flow cytometric analysis revealed that cardiac clusters consisted almost exclusively of CMs (>97%, **Fig. 27A, B).** After drug selection, αPIG-iPS and αPIG-ES cells yielded comparable number of CMs (2.7±1.4 vs 2.0±1.6 CMs per each ES or iPS cell initially taken for differentiation, **Fig. 27C).**

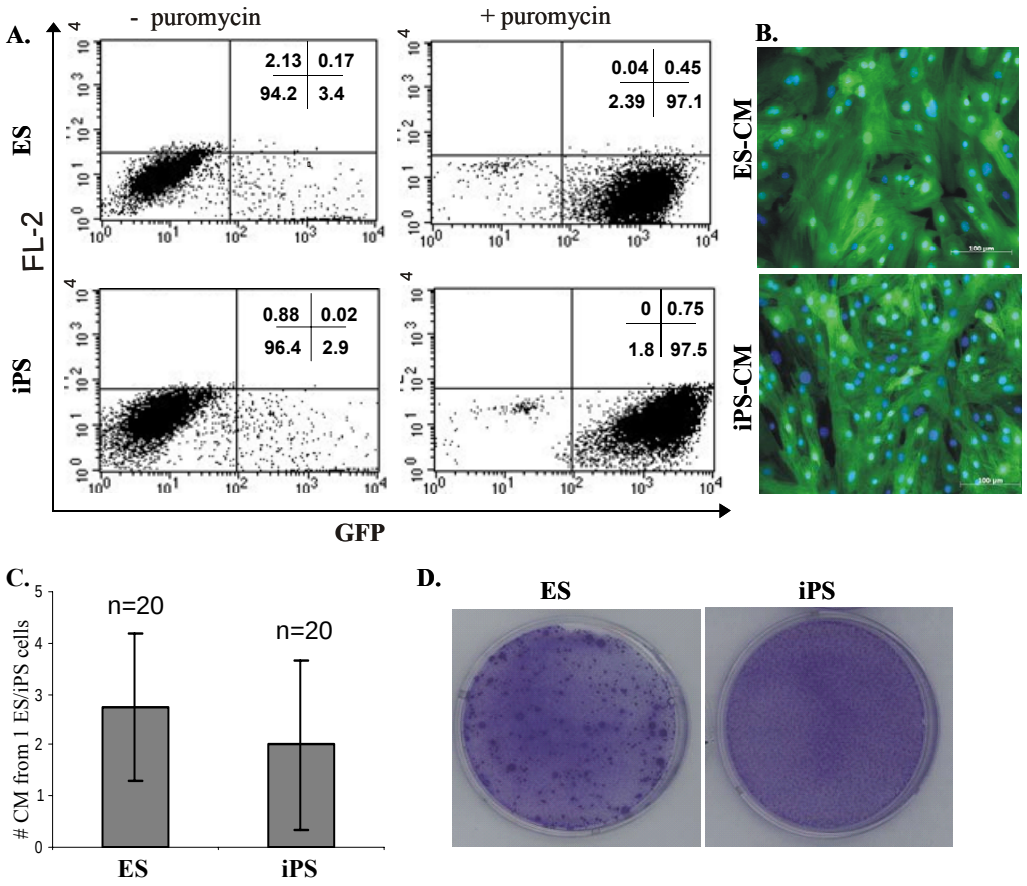


Figure 27. Purity of iPS-CMs and ES-CMs. (A) Flow cytometric estimation of the purity of cardiac clusters at day 16 of differentiation. Left panels show percentage of GFP positive cells in EBs before purification and right panels show percentage of GFP positive cells after purification with 8 μg/ml puromycin for 7 days. (B) Florescence image of plated purified CMs after dissociation into single cells. Scale bars=100 μm. (C) The yield of purified CMs obtained after differentiation of ES/iPS cells. (D) Pluripotent stem cell colonies obtained after plating of purified CMs derived from ES and iPS cells on MEFs.

To further determine the presence of any contaminating undifferentiated cells that had survived the selection procedure, purified cardiac clusters were also subjected to teratoma formation assay. Purified iPS-CMs and ES-CMs obtained after 7 days of puromycin treatment were injected subcutaneously into immunodeficient $Rag2^{-/-}\gamma_c^{-/-}$ mice and teratoma formation was monitored over a period of three months. The experiments were controlled by injecting undifferentiated iPS and ES cells in parallel animals. No teratomas were detected in animals injected with iPS-CMs, suggesting that

these cells were highly pure. However, teratoma was formed in animals that received ES-CMs, which is indicative of their significant contamination with ES cells.

Table 2. Assessment of purity of iPS cell- and ES cell-derived cardiomyocytes by teratoma induction assay *in vivo*

Cell type*	# of cells injected s.c.	# of animals injected	# of tumours detected
iPS	0.4x10E6	1	1
iPS	1.0x10E6	4	4
iPS	2.0x10E6	3	3
ES	1.0x10E6	2	2
ES	1.0x10E6	2	2
ES	2.0x10E6	1	1
iPS-CM	0.4x10E6	2	0
iPS-CM	1.0x10E6	3	0
iPS-CM	2.0x10E6	3	0
ES-CM	0.4x10E6	1	1

* The indicated number of undifferentiated αPIG-iPS or αPIG-ES cells or puromycin-selected day 16 αPIG-iPS-CMs or αPIG-ES-CMs was injected subcutaneously in $Rag2^{-/-}\gamma_c^{-/-}$ immunodeficient animals and teratoma occurrence was monitored over the period of three months.

In order to quantify the number of contaminating pluripotent stem cells in CM preparations, single cardiac cells were plated on irradiated MEFs in ES cell medium containing LIF but no puromycin. This culture facilitates the growth of remnant undifferentiated cells. Presence of one contaminating ES cell leads to the formation of one colony. The number of ES cell colonies was monitored after 2 weeks of culture using crystal violet staining. The results of this colony forming assay are tabulated in **Table 2**. iPS-CMs were found to be highly pure and devoid of any contamination form undifferentiated cells. In contrast, the purity of ES-CMs differed between batches sometimes with no colonies and sometimes with as high as 30 colonies per one million of seeded CMs.

To further assess the purity of iPS-CMs, dissociated CMs were cultured on MEFs for 14 days under ES cell culture conditions to detect rare contaminating pluripotent stem cells. In seven independent experiments no pluripotent stem cell colonies were detected

after seeding 0.1-0.5x10^6 drug-selected iPS-CMs/ plate **(Fig. 27D**, right panel). However, ES cell-derived CMs still contained some undifferentiated ES cells, although the contamination level was very low (20ES cells per 1x10^6 CMs) **(Fig. 27D**, left panel). Thus, puromycin selection allows for generation of highly purified αPIG-iPS-CMs.

Table 3. iPS or ES cell colony forming assay

Differentiation*	Cell type	# of cells plated	# of iPS cell colonies detected	Observation time
1	iPS-CM	0.1x10^6	0	2 weeks
		0.1x10^6	0	2 weeks
2	iPS-CM	0.5x10^6	0	2 weeks
		0.5x10^6	0	2 weeks
3	iPS-CM	0.3x10^6	0	2 weeks
		0.3x10^6	0	2 weeks
4	iPS-CM	0.3x10^6	0	2 weeks
		0.3x10^6	0	2 weeks
5	iPS-CM	0.8x10^6	0	2 weeks
6	iPS-CM	0.5x10^6	0	2 weeks
7	iPS-CM	0.5x10^6	0	2 weeks
		# of colonies:	0±0 per 1x10^6 CMs, n=11	

Differentiation*	Cell type	# of cells plated	# of ES cell colonies detected per plate	Observation time
1	ES-CM	0.2x10^6	6	2 weeks
	ES-CM	0.2x10^6	1	2 weeks
	ES-CM	0.2x10^6	3	2 weeks
	ES-CM	0.1x10^6	2	2 weeks
	ES-CM	0.1x10^6	2	2 weeks
2	ES-CM	0.2x10^6	0	2 weeks
	ES-CM	0.1x10^6	0	2 weeks
3	ES-CM	0.2x10^6	2	2 weeks
4	ES-CM	0.3x10^6	0	2 weeks
5	ES-CM	0.3x10^6	9	2 weeks
	ES-CM	0.3x10^6	6	2 weeks
		# of colonies:	19±9 per 1x10^6 CMs, n=8	

Finally, we have also determined the purity of CMs by highly sensitive RT-PCR assay. We found that the transcript levels for cardiac genes *Mef2c, GATA4, NKx2.5, troponin T* and C and *RyR2* were highly enriched in both the ES and iPS cell-derived puromycin-selected clusters as compared to intact EBs **(Fig. 28).** In contrast, the transcripts for endodermal (*AFP*) and ectodermal markers (*Sox17, CD31*) were not detected **(Fig. 28)** in purified CMs. These data indicate that drug selection yields highly pure populations of molecularly indistinguishable CMs from both transgenic iPS and ES cell lines.

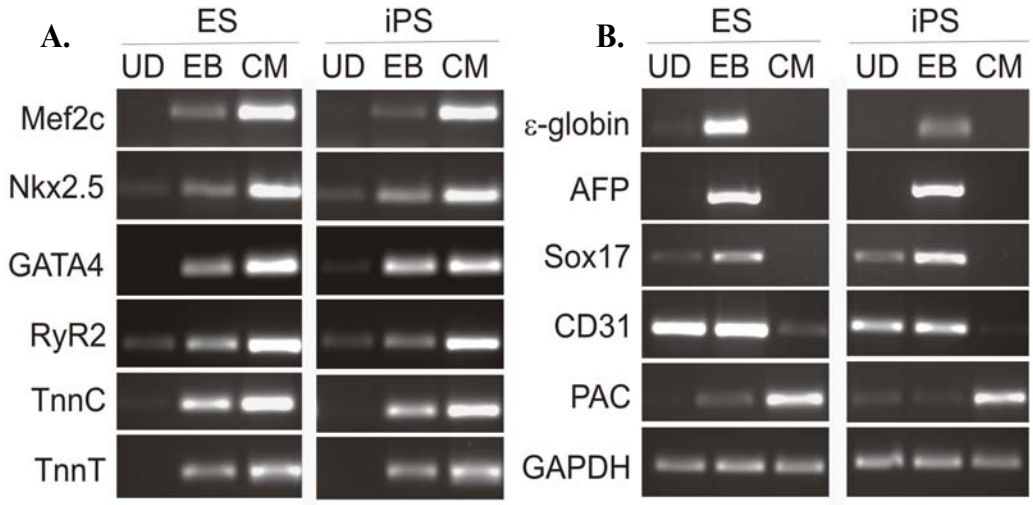


Figure 28. RT-PCR analysis of the genes expressed in αPIG-ES and αPIG-iPS. Expression of cardiac genes (A) and non cardiac genes (B) in undifferentiated state (UD), embryoid bodies (EB) and in purified CMs (CM) derived from ES and iPS cells.

4.3.4 Virally-encoded Oct4 is incompletely silenced in iPS-CMs

Since the iPS cells used in this study contain stably integrated retroviral expression cassettes, we asked whether any residual expression of viral transgenes can be detected in purified cardiac clusters. To distinguish between endogenous and viral transcripts, we used primers specific for virally encoded sequences to determine the viral transcripts and primers in the 5' untranslated region to determine the endogenous gene expression. The endogenous *Oct4*, *Sox2*, *Klf4* and *c-Myc* transcripts were strongly expressed in undifferentiated ES and iPS cells and EBs **(Fig. 29A)**. The transcripts for endogenous *Klf4* and *c-Myc* were also expressed in iPS- and ES-CMs but the endogenous *Sox2* was only barely detectable in CMs. The endogenous *Oct4* mRNA was weakly expressed only in iPS-CMs but not in ES-CMs. As expected, virally encoded transcripts were not detectable in ES cells. However, they were expressed at low levels in undifferentiated iPS cells and iPS-EBs with exception of *Sox2,* which was not detected in any of the samples. In purified iPS-CMs the viral *Klf4* and *c-Myc* were detectable at very low levels. However, viral *Oct4* was expressed in iPS-CMs at levels similar to those in undifferentiated iPS cells. Hence, retrovirally encoded Oct4 appears to be incompletely silenced in iPS-CMs. To firmly exclude the possibility that endogenous *Oct4* and *Nanog*

were expressed in purified CMs, we determined the methylation status of *Oct4* and *Nanog* promoters in undifferentiated cells and in CMs. We found that *Oct4* and *Nanog* promoters were highly methylated in iPS- and ES-CMs and strongly hypo ethylated in their undifferentiated counterparts **(Fig. 29B).** This data support the conclusion that the expression of *Oct4* in iPS-CMs is purely due to incomplete silencing of viral *Oct4* and not due to endogenous *Oct4* activation or undifferentiated cell impurity.

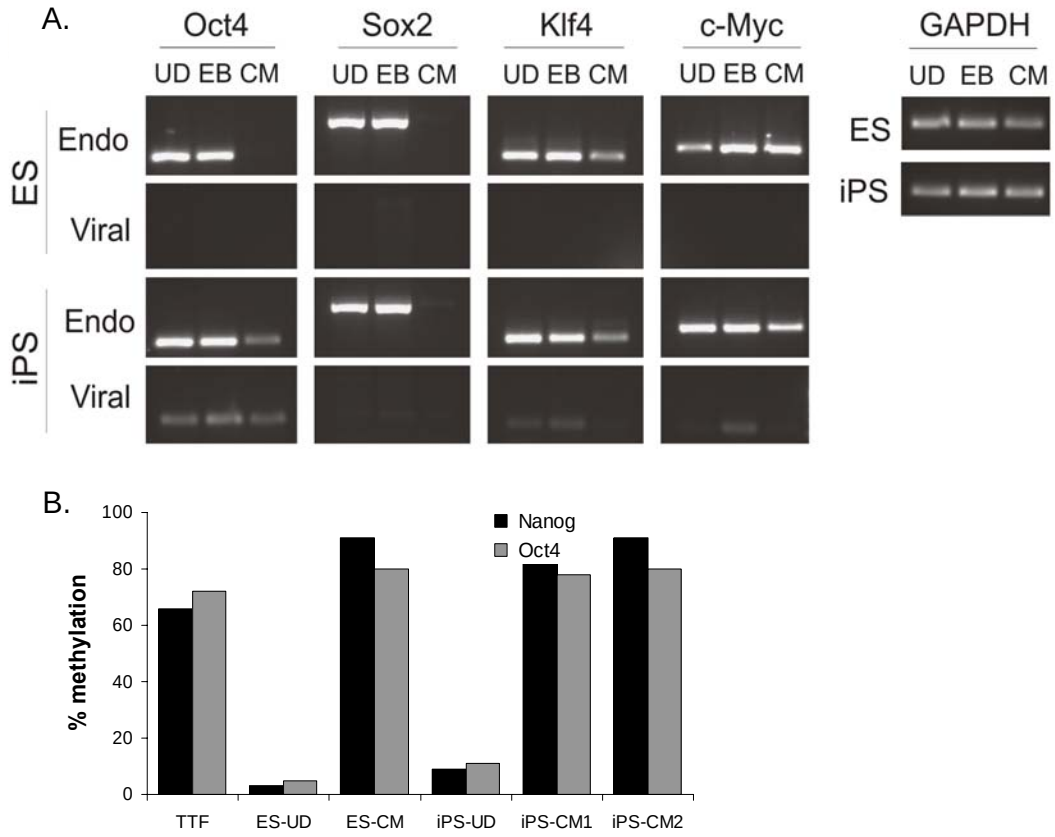


Figure 29. Expression of viral transcripts in αPIG-ES and αPIG-iPS. (A) Expression of viral and endogenous transcripts of the four reprogramming genes Oct4, Sox2, Klf4 and cMyc in undifferentiated (UD) ES and iPS cells, and in purified CMs derived from ES and iPS cells (clones 1 and 2). The expression levels are compared with that of housekeeping gene GAPDH (right panel). (B)% methylation of endogenous promoters of Nanog and Oct4 loci.

4.3.5 Structure of cardiomyocytes derived from iPS and ES cells

When plated CM cultures were immuno-stained with cardiac specific antibodies, both iPS and ES derived CMs demonstrated well-organized cross-striations of sarcomeric α-actinin, cardiac troponin T, and stained positive for MLC2v **(Fig. 30).** This proves the structural integrity of the CMs derived from both transgenic ES and iPS cells.

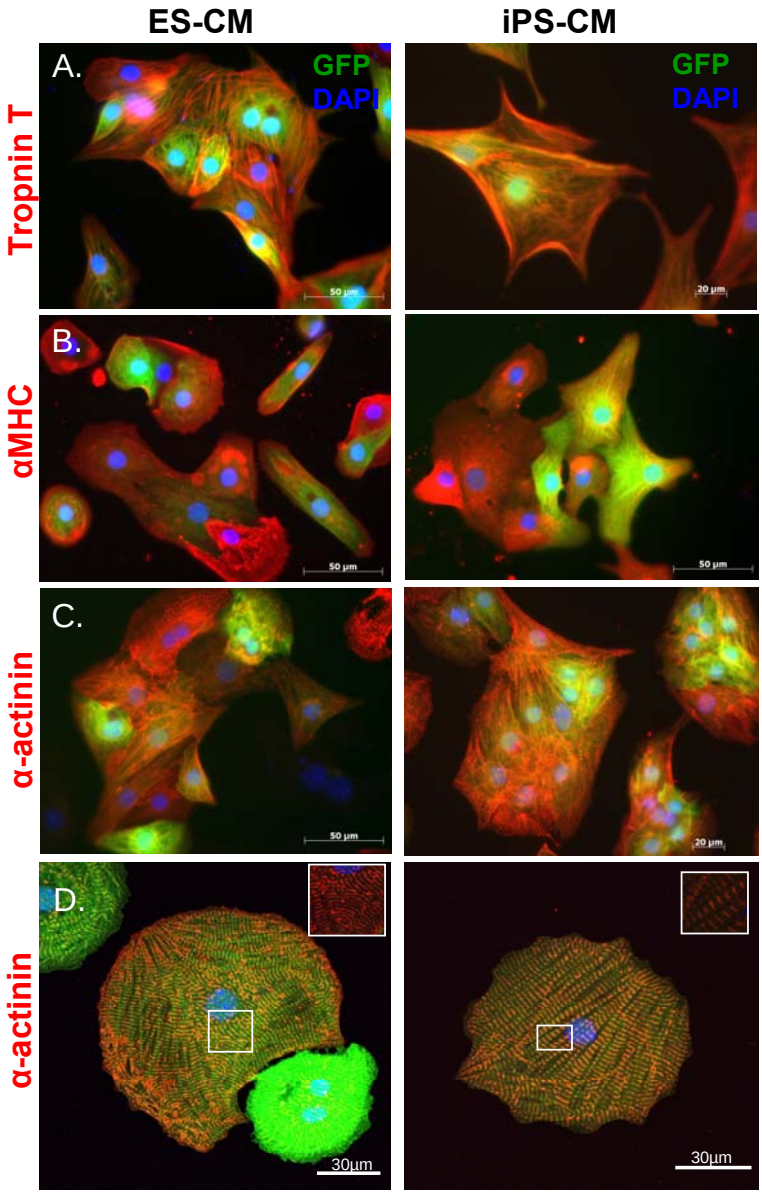


Figure 30. Immunocytochemistry of ES and iPS derived cardiomyocytes. (A) GFP-positive ES-CMs and iPS-CMs immunoassayed for (A) Troponin T (B) α-MHC and (C, D) α-actinin. Nuclei are stained with Hoechst 33342 (blue). Expression of cardiac genes in ES and iPS derived CMs. Scale Bar=50µm (A-C) and 30µm (D).

4.3.6 Cardiomyocytes derived from iPS cells are not fully mature

We compared the structural organization of iPS derived CMs with that of the mouse embryonic heart and adult heart. Scanning electron microscopy reveals that the organization of microfilaments and contractile apparatus in iPS derived CMs is highly disintegrated and less organized in comparison to highly ordered and dense pattern of the actin myosin filaments in adult heart. The murine heart at embryonic stage E11.5 and E16.5 also showed similar disorganized pattern. Therefore the structural pattern reveals that the day 20 CMs derived from iPS cells resembles an immature state.

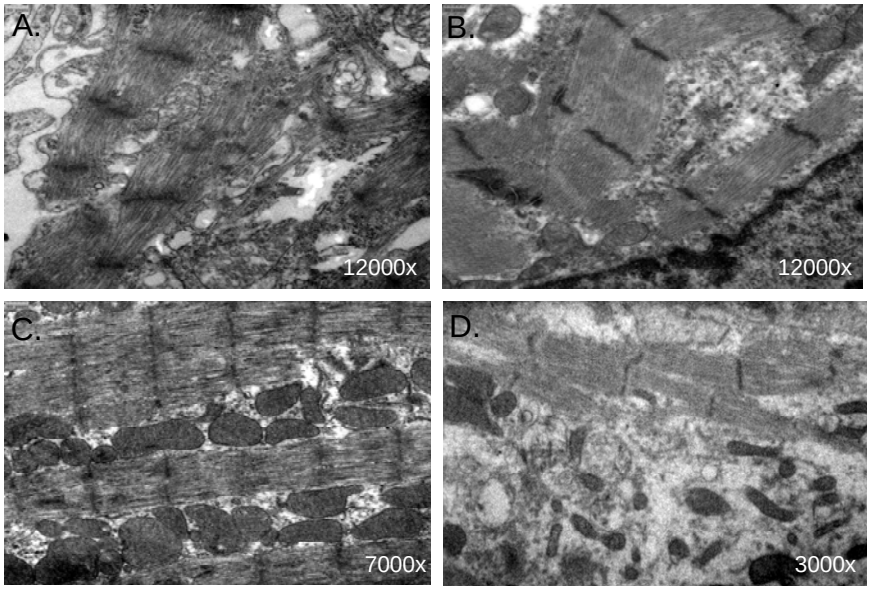


Figure 31. Ultra structural analysis of iPS-CMs. Scanning electron microscopic images of fetal heart at embryonic stage day 11.5 (A), 16.5 (B), adult heart (C) and iPS derived CMs (D). The electron microscopy was perfomed by Nadine Lange and Petra Muller in the lab of Wolfram Friedrich Neiss.

4.3.7 Intact β-adrenergic and muscarinic signalling in drug-selected iPS-CMs

To characterize the hormonal regulation of iPS-CMs compared to ES-CMs, β-adrenergic receptor agonist isoproterenol (Iso) and muscarinic receptor agonist carbachol (CCh) were applied **(Fig. 32A)**. In both cell types, Iso (1 μM) significantly increased the spontaneous AP frequency whereas CCh (10 μM) caused a decrease **(Fig. 32B**, **left panel**). Additionally AP duration at 90% of repolarization (APD90) was

shortened and prolonged in the presence of Iso and CCh, respectively **(Fig. 32B**, **right panel**). Small differences observed between iPS- and ES-CMs were not significant, suggesting the existence of intact β-adrenergic and muscarinic signaling cascades in lineage-selected αPIG-iPS-CMs.

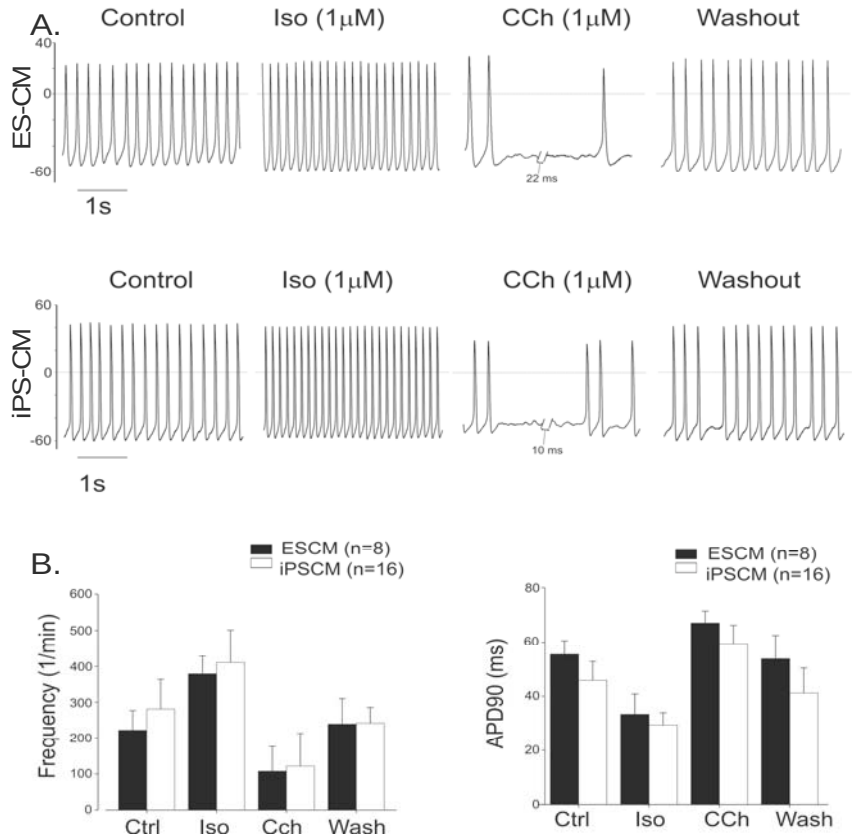


Figure 32. Current-clamp characterization of murine αPiG-ES and αPiG-iPS cell-derived cardiomyocytes. (A) Representative action potential (AP) traces showing the effect of Iso (1μM) and CCh (1μM) on ES cell- (top panel) and iPS (bottom panel) cell-derived CMs. (B) Statistical analysis of the effect of Iso and CCh on the spontaneous AP frequency (left) and on the AP duration at 90% of repolarization (APD90).

4.3.8 Functional voltage-gated ion channels in iPS-CMs

To quantify the expression of functional ion channels in the plasma membrane of iPS-CMs and ES-CMs, we used the whole-cell voltage-clamp and determined current densities by normalizing the maximal current amplitude to the cell size **(Fig. 33A)**. There was no significant difference in Na^+- and L-type Ca^{2+}-current density between iPS-CMs and ES-CMs Potassium currents in our experiments represent the sum of all

depolarization-activated outward components comprised of the transient-outward (K_{to}) and delayed-rectifier K^+-currents (ultra-rapidly activating, K_{ur}, rapidly activating, K_r, and slowly activating, K_s) as well as leak K^+-channels. We measured peak currents (I_{peak}), and late currents (I_{sus}) at the end of the 500-ms depolarizing pulses and found no significant difference in I_{peak} and I_{sus} densities between iPS- and ES-CMs**.** These data suggest that K^+-channels in iPS-CMs are functional and that the contribution of different K^+-channels to the net depolarization-activated outward K^+-current in drug-selected iPS-CMs and ES-CMs is comparable. We also determined the current-voltage relationships of the Na^+-, L-type Ca^{2+}-, and K^+-currents, and found that these parameters were also indistinguishable between iPS-CMs and ES-CMs for all channels tested **(Fig. 33B).**

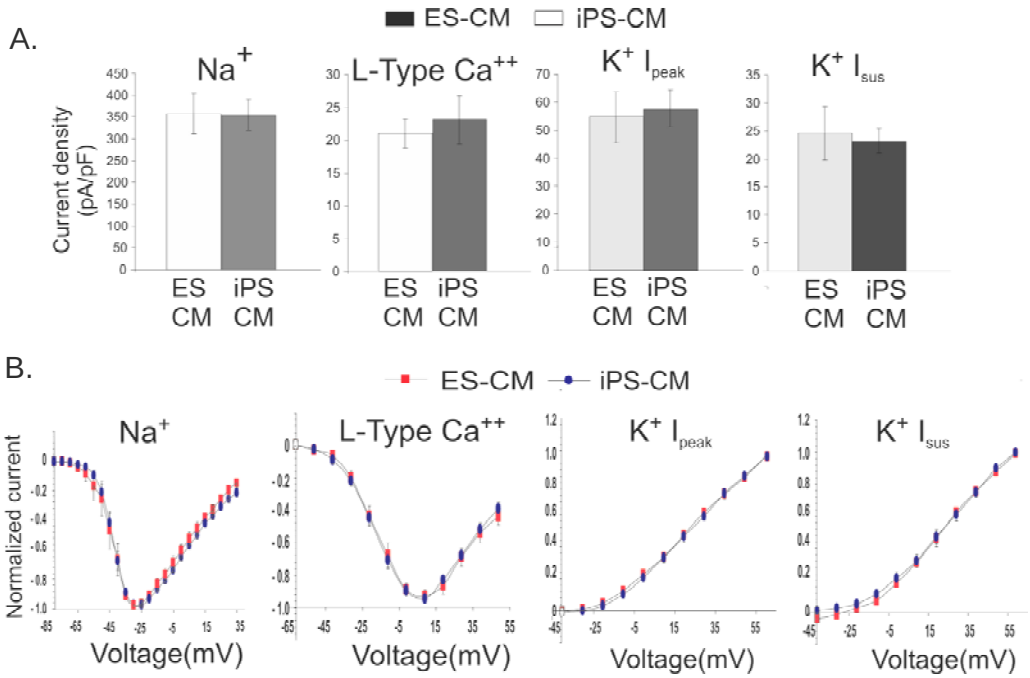


Figure 33. Functional expression of voltage-gated ion channels in αPIG-ES-CM and αPIG-iPS-CM. (A) Density values of Na^+, L-type Ca^{2+} , and K^+ I_{peak} and I_{sus} currents in ES-CMs and iPS-CMs. (**B**) Current-voltage relationships of Na^+, L-type Ca^{2+}, I_{peak}, and I_{sus} K^+ currents in ES-CM and iPS-CM.

4.4 Global transcriptional profiling of iPS and ES cell-derived cardiomyocytes

4.4.1 iPS-CMs and ES-CMs are transcriptionally highly similar

Functional analyses presented above indicate that iPS-CMs are electrophysiologically highly similar to those of their ES cell counterparts. Gene expression profiling is one important technology to take a global view on gene expression and it might be suited to check novel iPS cell lines with respect of their similarity to ES cells. To establish whether this functional similarity is mirrored at the molecular level, we compared the global gene expression pattern of iPS cells with that of ES cells at various developmental stages.

For global gene expression profiling, cRNAs from undifferentiated ES and iPS cells, EBs derived from day 16 ES and iPS cells, puromycin-selected cardiac clusters (CCs) derived from ES and iPS cells at day 16 of differentiation and tail tip fibroblasts (TTF) of adult mice were hybridized to mouse whole genome Illumina bead chips. Experimental samples are summarized in **Table 4.** Three biological replicates from independent experiments were used. All data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE18514.
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nhqtfgmousswodu&acc=GSE18514

In these analyses, 18339±1358 (40.5±3.0%) probe sets received intensity signals above background and among expressed transcripts 5689 (31.0%) were identified as variable among samples (coefficient of variation: 0.5-10). We classified all the above samples into four distinct developmental stages - undifferentiated stem cells, differentiated cells (mixture of different cell types in unselected EBs), pure CMs at early maturation stage and adult somatic cells (TTFs). Hierarchical clustering of all variable genes revealed that CMs derived from iPS and ES cells cluster closely together and are distinct from undifferentiated cells, EBs and fibroblasts (**Fig. 34A).** Likewise, principal component analysis (PCA) with this gene set showed that iPS-CMs and ES-CMs cluster together. Distinct clusters were also formed by undifferentiated ES and iPS cells, iPS-EBs and ES-EBs, and by TTFs **(Fig. 34B**). In addition, scatter-plot analysis further emphasized a

close correlation of gene expression levels between iPS and ES cells (r^2=0.98±0.005, n=9), iPS-EBs and ES-EBs (r^2=0.97±0.009, n=9), iPS-CMs and ES-CMs (r^2=0.97±0.003, n=9) but not between iPS cells and fibroblasts (r^2=0.82±0.014, n=9), **(Fig. 34C).**

Table 4. List of samples used for global gene expression profiling on Illumina platform.

No.	Sample name	Description	Cell line	Passage #
1	D3αPIG-ES _undiff_rep1	murine ES cells (D3αPIG-ES), undifferentiated	D3αPIG-ES	7
2	D3αPIG-ES _undiff_rep2	murine ES cells (D3αPIG ES), undifferentiated	D3αPIG-ES	9
3	D3αPIG-ES _undiff_rep3	murine ES cells (D3αPIG ES), undifferentiated	D3αPIG-ES	10
4	D3αPIG-d16- EB_rep1	mESC-derived embryoid bodies, day 16 of *in vitro* differentiation	D3αPIG-ES	5
5	D3αPIG-d16 -EB_rep2	mESC-derived embryoid bodies, day 16 of *in vitro* differentiation	D3αPIG-ES	15
6	D3αPIG-d16 -EB_rep3	mESC-derived embryoid bodies, day 16 of *in vitro* differentiation	D3αPIG-ES	21
7	D3αPIG-d16- CM_rep1	mESC-derived cardiac clusters, day 16 of *in vitro* differentiation	D3αPIG-ES	5
8	D3αPIG-d16 -CM_rep2	mESC-derived cardiac clusters, day 16 of *in vitro* differentiation	D3αPIG-ES	15
9	D3αPIG-d16 -CM_rep3	mESC-derived cardiac clusters, day 16 of *in vitro* differentiation	D3αPIG-ES	21
10	αPIG-TIB-iPS_undiff_rep1	murine iPS cells (αPIG-TIB-iPS), undifferentiated	iPS clone 25	5
11	αPIG-TIB-iPS_undiff_rep2	murine iPS cells (αPIG-TIB-iPS), undifferentiated	iPS clone 25	7
12	αPIG-TIB-iPS_undiff_rep3	murine iPS cells (αPIG-TIB-iPS), undifferentiated	iPS clone 25	8
13	αPIG-TIB-iPS-d16 EB_rep1	iPS-derived embryoid bodies, day 16 of *in vitro* differentiation	iPS clone 25	39
14	αPIG-TIB-iPS-d16 EB_rep2	iPS-derived embryoid bodies, day 16 of *in vitro* differentiation	iPS clone 25	20
15	αPIG-TIB-iPS-d16 EB_rep3	iPS-derived embryoid bodies, day 16 of *in vitro* differentiation	iPS clone 25	21
16	αPIG-TIB-iPS-d16 CM_rep1	iPS-derived cardiac clusters, day 16 of *in vitro* differentiation	iPS clone 25	39
17	αPIG-TIB-iPS-d16 CM_rep2	iPS-derived cardiac clusters, day 16 of *in vitro* differentiation	iPS clone 25	20
18	αPIG-TIB-iPS-d16 CM_rep3	iPS-derived cardiac clusters, day 16 of *in vitro* differentiation	iPS clone 25	21
19	Adult 129/B6 TTF1_rep1	Tail Tip fibroblasts derived from tail of adult mice of strain 129xB6	Primary cells	2
20	Adult 129/B6 TTF1_rep2	Tail Tip fibroblasts derived from tail of adult mice of strain 129xB6	Primary cells	3
21	Adult 129/B6 TTF1_rep3	Tail Tip fibroblasts derived from tail of adult mice of strain 129xB6	Primary cells	4

These data demonstrate the close similarity at the global transcriptional level between the various developmental stages of iPS and ES cells.

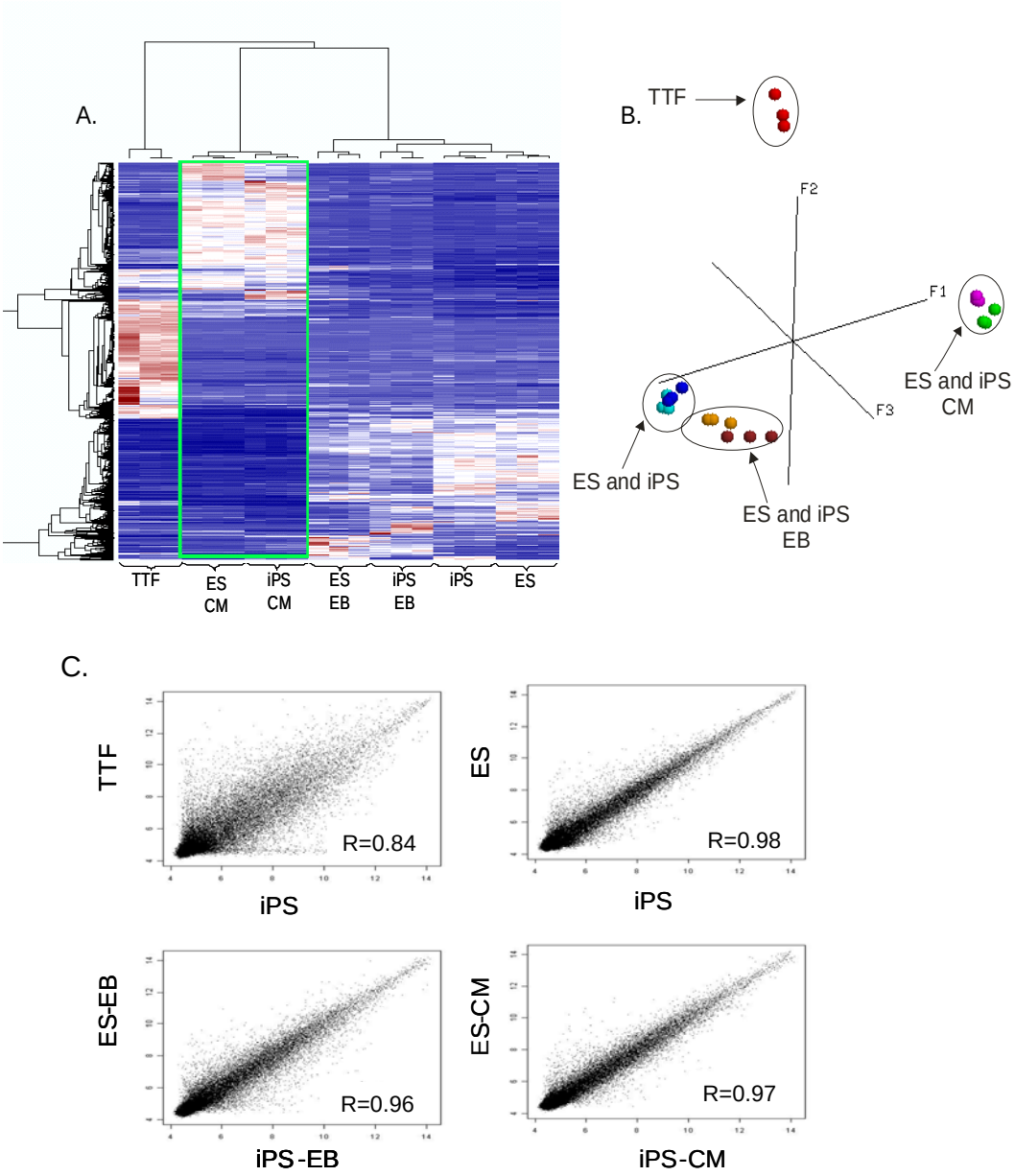


Figure 34. Global Transcriptional profile of αPIG-iPS cells and αPIG-ES cells. A) Hierarchical cluster analysis of variable genes. (B) Principal component analysis. (C) Scatter plots showing comparison of global gene expression between αPIG-ES and αPIG-iPS cells; αPIGES-EBs and αPIGiPS-EBs; and αPIGES-CMs and αPIGiPS-CMs. Comparison was also made with TTF against αPIG-iPS cells.

Although globally similar, small groups of genes comprising 1.4-2.8% of present transcripts were differentially expressed between iPS and ES cell groups at each

developmental stage (264 transcripts between undifferentiated iPS and ES cells, 508 between iPS-EBs and ES-EBs and 400 between iPS-CMs and ES-CMs) **(Fig. 35).** However, these differences were not significantly greater than the difference between undifferentiated iPS and ES cells or between different ES cell or different iPS cell lines as reported by others (Chin et al. 2009).

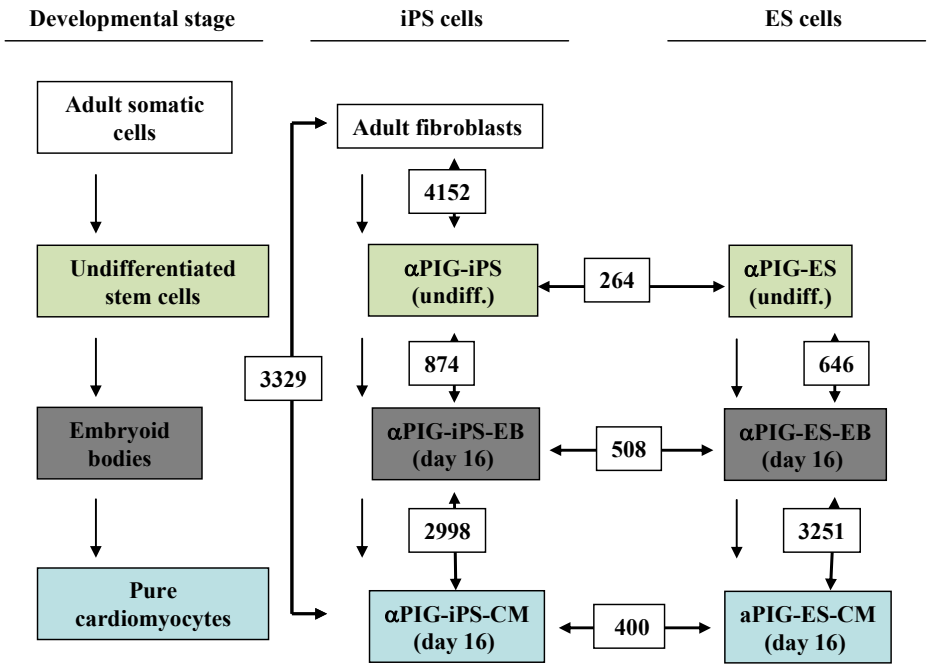


Figure 35. Differential gene expression chart of different developmental stages of ES and iPS cells.

4.4.2 Enriched functional categories in purified cardiac clusters

In order to determine which biological terms are enriched in iPS-CMs compared to ES-CMs. About 3000 probe sets were found to be differentially expressed (p-value adjusted for multiple testing using Benjamini-Hochberg correction smaller than 0.05 and fold change above ±2) between CMs and EBs and between CMs and fibroblasts. A total of 1478 genes were upregulated in iPS-CMs compared to iPS-EBs. In addition, comparison of iPS-CMs and TTFs revealed 1666 upregulated genes in iPS-CMs. In order to identify the most important biological processes, molecular functions, cellular components and pathways that are significantly enriched in drug-selected CMs, we performed the functional annotation clustering analysis using DAVID (Huang et al. 2009). Using the enrichment score of >3 as a threshold for significantly enriched categories we obtained 16 overrepresented annotation clusters that belonged into two

major groups (**Table 5**). The first group consists of 10 clusters containing GO terms of biological processes such as mitochondrion, oxidative phosphorylation, tricarboxylic acid cycle, and mitochondrial electron transport chain and glucose metabolism. The second group contained clusters with GO terms contractile fiber, myofibril assembly, sarcomere, heart development and cardiac cell differentiation. All these categories are known essential components of the functional cardiac phenotype and were similarly enriched in both iPS- and ES-CMs.

Thus, by generating highly purified CMs from transgenic murine iPS and (ES) cell lines, we derive the following information:

1. iPS and ES cells differentiated into CMs at comparable efficiencies yielding highly purified CMs after drug selection.
2. Purified iPS- and ES-CMs exhibited indistinguishable structural properties, similarly responded to pharmacological agents, expressed functional voltage-gated sodium, calcium and potassium channels and possessed comparable current densities.
3. Global transcriptional profile and gene ontology signature of transgenic iPS-CMs were very similar to that of ES-CMs but clearly distinct from fibroblasts used to generate iPS cells and differentiated cells in iPS or ES cell-derived embryoid bodies.
4. After 7 days of puromycin selection, iPS-CMs did not contain any residual pluripotent cells nor formed teratoma *in vivo,* while ES-CMs still showed contamination with undifferentiated cells and occasionally gave rise to teratoma after transplantation.

Thus, drug selection allows for production of large quantities of iPS-CMs that are functionally and molecularly indistinguishable from their ES cell counterparts. The availability of pure iPS-CMs will permit assessment of their utility for myocardial repair *in vivo*, development of improved protocols for cardiac differentiation of iPS cells, and validation of their applicability for pharmacological and toxicological applications.

Table 5. Functional annotation clustering performed in DAVID of genes upregulated in cardiomyocytes in iPS-CM vs. iPS-EB, ES-CM vs ES-EB and iPS-CM vs TTF comparisons.

		Enrichment score		
Annotation cluster	**Descriptor functional categories**	**iPS-CM vs iPS-EB**	**ES-CM vs ES-EB**	**iPS-CM vs TTF**
1	Mitochondrion, oxidative phosphorylation, respiratory chain	43,41	32,24	49,93
2	Cytoplasm, intracellular organelle	28,91	25,59	n.d.
3	Tricarboxylic acid (TCA) cycle, cellular respiration, catabolic process	14,12	8,54	13,46
4	Contractile fiber, myofibril, sarcomere, I band, Z band	13,77	14,61	11,05
5	Transmembrane transporter-, oxidoreductase-, cytochrome c oxidase- and hydrogen ion transmembrane transport-activity	12,97	11,83	14,63
6	NADH dehydrogenase (ubiquinon) activity	10,24	6,35	12,44
7	Cardiomyopathy	7,71	6,42	n.d.
8	Glucose/hexose metabolic process, monosaccharide catabolic process	6,77	5,81	4,55
9	Mitochondrial matrix and lumen, mitochondrial ribosome	6,41	4,98	5,85
10	Heart development and morphogenesis, myofibril assembly, actomyosin structure organisation,	4,66	4,65	5,43
11	Proton transporting ATP-synthase complex, energy coupled proton transport	4,31	3,59	5,88
12	Cellular polysaccharide-, energy reserve- and glucan-metabolic process	4,27	n.d.	n.d.
13	Mitochondrial respiratory chain, NADH dehydrogenase complex	3,87	n.d.	4,03
14	M band, A band	3,29	3,11	n.d.
15	Iron-sulfur- and metal-cluster binding	3,03	n.d.	4,35
16	Sarcoplasmic reticulum, sarcoplasm	n.d.	n.d.	3,12

n.d. – not detected; TTF – adult tail tip fibroblasts

5 DISCUSSION

Although reprogrammed cells are seemingly similar to ES cells, an in depth comparison of the pluripotent cell types is obviously essential. Accordingly CMs derived from diverse pluripotent cells. CMs can serve as a model system to understand the developmental process of the heart. Elucidating the role of structural proteins, understanding the signaling process of the maturation of the cardiac cells, functional regulation of proteins in the heart are important biological studies that can be undertaken when there is robust supply of CMs at hand. The rapid *in vitro* differentiation of mouse pluripotent cells into functional CMs provides a renewable source of CMs for such studies.

5.1 Reprogramming by fusion

In the present study, we have shown that somatic cells (bone marrow cells and spleenocytes) could be successfully reprogrammed by fusion with $HGPRT^{-/-}$ HM-1 ES cells. We have shown that PEG could efficiently induce the fusion of ES cells with somatic cells and that the resulting hybrid cells could be selected based on resistance to HAT. Hybrid lines expressed characteristics of normal ES cells, including apparent immortality in culture, ES cell-like colony morphology, and pluripotency by formation of differentiated cells derived from the three embryonic germ layers *in vitro*. The resulting hybrids carried a near tetraploid genome, suggesting existence of genome from both parental cells together. We confirmed the existence of both parental genomes by comprehensive analysis of the SNP variations in the genome of the hybrid cells. The presence of two individual alleles on each chromosome indicated the presence of two genomes in the hybrid cells. In 3 out of four clones tested, 19 SNP sites consisted of two alleles of parental types. Only one clone (clone 1.2) carried only one allele of SNP5 and SNP10 on chromosome 5 and 10 respectively. This could be due to loss of sister chromatid in FH clone 1.2 as represented by near tetraploid genome. The lack of expression of tissue specific surface markers on FH cells in their undifferentiated state and the fact that the hybrid cells exhibit the phenotype of ES cells and not that of the spleenocytes or bone marrow cells illustrates the dominance of the ES genome over the differentiated cell genome. Therefore pluripotency seems to behave as a dominant trait in the hybrid cell genome. These findings show the remarkable capability of ES cells to

override the adult somatic cell program, as seen after SCNT (Campbell *et al.* 1996). Our results are similar to prior findings by Ying *et al.* who also detected tetraploid cells after cell fusion with ES cells (Ying *et al.* 2002).

The coexistence of both parental genomes was confirmed in FH cells by detecting the MHC class I molecules specific for each parental cell type. As shown in Fig. 15 and 16, the EBs derived from FH cells at day 4 of differentiation, when induced with IFNγ, expressed MHC class I molecules of both haplotypes H-2K^b and H-2K^d on the surface of positive cells. Only 28% of the cells expressed both MHC class I molecules whereas 48% cells expressed only H-2K^d. All cells that expressed H-2K^b also expressed H-2K^d but not vice versa. The expression of somatic genes in differentiated derivatives of FH cells clearly suggests 'somatic memory' of the reprogrammed genome in a fraction of the cells. Speculation of the lack of expression of somatic genes in a majority of the cells can be based on dominance of fully reprogrammed genome but before we make any conclusions the technical difficulties of staining two surface antigens together on a single cell need to be resolved.

In this study we addressed the question of characteristics and functionality of CMs derived from FH cells which have been demonstrated to be near tetraploid. Tetraploid FH cells have been shown to contribute to chimeras after blastocyst injection (Matveeva *et al.* 1998), but only limited data exists on their differentiation ability *in vitro*. In our experiments, sspontaneous differentiation of hybrid cells in hanging drops lead to the appearance of beating areas in plated EBs. The onset of beating in hybrid cells was relatively delayed compared to what is routinely observed in conventional ES cells. This could be attributed to the ploidy state or strain of reprogramming imposed on FH cells. Late onset of beating has also been reported for certain iPS cell lines (Pfannkuche *et al.* 2009)**.** Although cardiac differentiation is delayed in fusion hybrid cells, they can efficiently differentiate to form functional CMs. CMs derived from FH clone 2.1 and 4.2 demonstrated typical molecular and structural characteristics of CMs. FH derived CM demonstrated intact β-adrenergic and muscarinic regulation and exhibited action potential parameters resembling that of three subtypes of CMs including ventricular, atrial and nodal phenotype. The atrial phenotype was the predominant one in fusion derived CMs whereas HM-1 ES derived CM mostly consisted of ventricular-like phenotype. There is also a difference between CM derived from the fusion-generated

clones and iPS-derived CM, as latter mostly reveals AP of ventricular phenotype (Kuzmenkin *et al.* 2009). This different cell fate might result from different reprogramming approaches and might reflect a different manifestation of somatic memory in FH derived CM reflecting the origin of cell.

ES cell-derived factors are capable of reverting the somatic cell genome into a pluripotent state in FH cells containing a near tetraploid genome but demonstrating an equivalent cardiac differentiation potential as that of ES cells. EBs derived from FH cells maintain tetraploid and contain CMs. But ploidy of CMs could not be determined in this study because of the heterogenous nature of spontaneous *in vitro* differentiation of FH cells. Thus, reprogramming does not affect the cardiac differentiation potential of hybrid cells and does not lead to any aberration in the molecular structural and functional characteristics of cardiac derivatives. The cell fusion approach is useful as a tool for investigating the mechanism of cellular plasticity and reprogramming. This method has also proven to be very powerful for investigating factors required for reprogramming of somatic nucleus into a pluripotent state (Lluis *et al.* 2008; Wong *et al.* 2008).

5.2 Advancement of the method for iPS cell generation

The establishment of homogenous cell populations is of importance for a variety of purposes. Lines of ES as well as iPS cells that contain a mixture of pluripotent and pre-differentiated cells might be less suited to perform *in vitro* differentiations to study aspects of differentiation or to form specific derivatives for detailed analysis. This is particularly true for human ES cell cultures that usually contain a subset of less pluripotent and pre-differentiated cells (Amit *et al.* 2000; Eiges *et al.* 2001). Reprogramming of somatic cells to pluripotency leads to generation of heterogeneous mix of iPS cells which have achieved varying levels of pluripotency. It is therefore highly desired to generate homogeneous populations of iPS cells since heterogeneous cultures will potentiate obscure findings.

It was demonstrated that an *UTF1* promoter-driven Neomycin-gene is a sensitive selection marker in human ES cells to enrich pluripotent cells and to eliminate pre-differentiated cell types from the culture (Tan *et al.* 2007). In addition, *UTF1* is down-regulated faster than other pluripotency markers upon differentiation of ES cells (Tan *et al.* 2007). For this reason, we hypothesized that UTF1-Neo selection might help to

select for high-quality pluripotent cells that exists within heterogenous TiB7-4 iPS cell population.

We established three sub clones of TiB7-4 after transfection of TiB7-4 with *UTF1*-Neo and selection for two weeks with Neomycin. These clones displayed homogenous morphologies without signs of spontaneous differentiation for up to 40 passages indicating the presence of high-quality pluripotent cells within the TiB7-4 population. Flow cytometric analysis for SSEA1 clearly indicated a substantial improvement of the culture after selection for *UTF1*-Neo with 97% to 98% SSEA1 positive cells in all three clones. With respect to the fact that the residual 2% of cells might represent feeder fibroblasts one can assume that all iPS cells in this culture are positive for SSEA1. Remarkably, this effect could be observed without continuous selection. We speculate that the complete removal of pre-differentiated cells leads to a terminal stabilization of the *UTF1*-Neo selected clones. While pre-differentiated cells might influence the cell signalling and induce differentiation of neighbouring cells, populations that are depleted of pre-differentiated cells might exhibit a more stable pluripotent phenotype. The described selection, however, does not influence the ability of the *UTF1*-Neo selected cells to form EBs and to undergo differentiation within these spheroids giving rise to all three germ layers, which was also demonstrated earlier for *UTF1*-selected human ES cell lines.

Control transfections with a Neomycin resistance under the control of the ubiquitous active SV40 early promoter led to the identification of cell clones that partially resembled a perfect ES cell-like elliptical colony shape with sharp borders. Closer analysis of six control clones resulted in the surprising finding that four out of these six clones spontaneously underwent differentiation after some passages what was first obvious by disturbed colony formation and down regulation of *UTF1* and *Nanog* and up regulation of *Oct4*, and later by a loss of SSEA1 surface antigen. The remaining two clones, however, seemed to be stably pluripotent at least during the observation period of 12 passages. The reason for the instability is not clear and it might be speculated that it results from incomplete reprogramming or reactivation of the reprogramming virus.

The discussed findings underline that a *UTF1*-based selection is more efficient in deriving fully reprogrammed cells and a stable pluripotent phenotype. Fully reprogrammed cells within an iPS colony can be effectively isolated by selection with

UTF1-Neo. However, a selection based on the colony morphology may also give rise to stable pluripotent sub clones but requires the culture of the cells over a prolonged period of time and the surveillance of the pluripotency markers Nanog and *UTF1* by real time PCR. Further studies will address the duration of *UTF1*-Neo based selection needed to achieve homogenous pluripotent cells. It can be speculated that shorter selection intervals than the two weeks employed here might be sufficient to obtain *UTF1* selected cultures, perhaps even without stable integration of the selection cassette into the iPS genome. The application of methods like transient nucleofection, adenoviral or baculoviral transduction to transiently introduce the *UTF1*-Neo vector into the iPS cell population might ultimately combine the benefit of selection with the advantage of transient transgene delivery.

Alternative selection approaches utilized reactivation of the endogenous Nanog or Oct4 locus to indicate induction of pluripotency. While selection of reprogrammed fibroblasts, in which neomycin resistance is driven by endogenous Nanog promoter, was reported to result in a higher number of colonies compared to *Oct4*-Neo selection, only 11.5% of the resistant colonies with ES-like appearance revealed pluripotency and 22% of the Oct4-selected clones with ES-like appearance were found to be pluripotent (Wernig *et al.* 2007). Based on these report it can be assumed that the Nanog-driven selection is less suitable to generate high quality iPS cells. A detailed characterization of a *Oct4*-Neo selected as well as a Nanog-Neo selected iPS cell line indicated a delay in the *in vitro* differentiation of both cell lines when compared to the ES lines R1 and D3 (Pfannkuche *et al.* 2009).

5.3 Functional cardiomyocytes derived from iPS cells

The importance of derivation of homogenous cell population is not limited to pluripotent state but also to the mature cells types like CMs. Purification of CMs from the differentiating cultures of pluripotent stem cells is especially important to study cardiac specific developmental, functional and transcriptional process.

Genetic enrichment strategies using drug selection markers expressed under the control of cardiac specific promoters have been successfully applied for purification of murine and human ES-CMs (Kolossov *et al.* 2006; Anderson *et al.* 2007; Doss *et al.* 2007; Xu *et al.* 2008; Kita-Matsuo *et al.* 2009). These approaches permitted genetic and

physiological studies of pure CMs without the potentially confounding influence of other cells present in differentiating EBs. These cell lines represent excellent tools for identification of new agents that direct differentiation of pluripotent stem cells to CMs and for optimization of scalable production of pure CMs in controlled bioreactors. In addition, the ability to produce high numbers of well characterized CMs that will facilitate progress in basic research leading to cell based therapy, disease modeling, drug screening and drug discovery.

The transgenic αPIG-iPS cell line generated in this study is the first that enables selection of contractile iPS-CMs free of other cell lineages and allows for detailed comparison of their functional and molecular properties with their ES cell-derived counterparts. A FACS-based isolation of cardiac progenitors from murine iPS cells expressing GFP under the control of Nkx2.5 promoter has been reported (van Laake *et al.* 2010; van Laake *et al.* 2010). In this study, the genome-wide transcriptional profiles of undifferentiated iPS and ES cells as well as isolated cardiac progenitors have been analyzed and shown that the gene expression profiles of iPS and ES cell-derived cardiac progenitors were even more similar than those seen between corresponding undifferentiated iPS and ES cell lines. In culture, these progenitors could be induced to form beating CMs and, upon transplantation, the progenitors engrafted into the infarcted mouse myocardium without formation of teratomas. Our analyses with iPS-CMs are in agreement with these findings, although we are unable to conclude, due to comparison of only single iPS and ES cell lines, whether the gene expression variance between iPS-CMs and ES-CMs is significantly smaller than that found between undifferentiated iPS and ES cells. Studies with additional transgenic cell lines will be necessary to provide clear answer to this question.

Our data show that the difference in transcriptome between iPS-CMs and ES-CMs is not significantly greater than the difference shown here by us and previously by others to exist between undifferentiated iPS and ES cells (Chin *et al.* 2009). This small difference in gene expression profile does not seem to affect the functional properties of iPS-CMs, which were undistinguishable from those of ES-CMs. Analysis of spontaneous APs of single cells revealed that the iPS-CMs and ES-CMs were similar with respect to their maturity. The difference observed in AP-frequencies of intact drug-selected cardiac clusters derived from iPS and ES cells may not necessarily be

attributed to the normal biological variability of the lines used in this study. In our previous functional assessment of micro dissected beating areas derived from two different iPS cell lines (lines N10 and O9) we also observed significantly slower beating frequencies in iPS-CMs compared to ES-CMs (Pfannkuche *et al.* 2009). These differences may have been caused by different proportions of different cardiac muscle cell types (ventricular, atrial, pacemaker) in multicellular iPS cell- and ES cell-derived beating areas. It is also possible that small difference in purity of iPS and ES cardiac clusters in the present study may have affected the AP-frequency of a cardiac tissue. Indeed, we have observed no contaminating pluripotent cells in iPS-cardiac clusters but found that rare ES cells (19 ES cells per $1x10^6$ ES-CMs) still contaminate ES-cardiac clusters after seven days of puromycin selection and occasionally lead to teratoma formation in animals injected with these cells. Here also, additional transgenic iPS and ES cell clones must be compared to accurately assess the differences between these cell types and implications for their functional performance in 3D tissue *in vivo* and long-term safety.

Although free of contaminating undifferentiated iPS cells, iPS-CMs potentially carry the risk of tumor formation due to residual expression of virally encoded *Oct4* transcripts. It is unclear whether this incomplete silencing of *Oct4* may have caused some of the differences in gene expression profiles detected between ES- and iPS-CMs. However, the comparable efficiencies of cardiac differentiation of iPS and ES cells and their similar functional properties demonstrate that the incomplete silencing of transgenes did not interfere with these processes. This is in accordance with the finding that ectopic Oct4 expression in the heart of adult mice did not lead to any detectable alterations of cell phenotype (Hochedlinger *et al.* 2005). However, iPS cells generated by non-integrating reprogramming methods were shown to be transcriptionally more similar to ES cells than those generated with stably integrating viral vectors (Wang *et al.* 2010). Therefore, it can not be excluded that minor transcriptional differences observed in our study were due to use of virally reprogrammed iPS cells.

Our study demonstrates the generation of highly purified populations of iPS-CMs using drug selection in a mass culture system and confirms that the cardiac derivatives of iPS and ES cells are highly similar in their structural, morphological and electrophysiological properties as well as global gene expression patterns. The ability to

produce homogenous population of CMs will facilitate further comparative analyses of iPS- and ES-CMs, assessment of the therapeutic potency of pure iPS-CMs in animal disease models, optimization of cardiac differentiation protocols and search for cardioactive drugs and toxic substances.

In conclusion, functional, structural and transcriptional assessment of the cardiac cells obtained as a result of invitro differentiation of ES cells and reprogrammed cells suggests that switching of cell fate from a differentiated state to pluripotent state and then again into cells of cardiac lineage does not have a major influence on the functional characteristics of the cardiac cells. This work proves reprogrammed cells as a renewable source of cardiac cell thus demonstrating potential of the reprogramming technology which holds great promise for scientific research and medicine in the years to come.

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7 ERKLÄRUNG

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Don't worry about your heart, it will last you as long as you live…

W.C Fields