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MARI PEKKANEN-MATTILA

Cardiomyocyte Differentiation from Human Pluripotent Stem Cells

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 1,
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ACADEMIC DISSERTATION
University of Tampere, Regea Institute for Regenerative Medicine
Tampere Graduate School in Biomedicine and Biotechnology (TGSBB)
Finland

Supervised by
Adjunct Professor Katriina Aalto-Setälä
University of Tampere
Finland
Erja Kerkelä, PhD
University of Tampere
Finland

Reviewed by
Professor Lior Gepstein
Technion - Israel Institute of Technology, Haifa
Israel
Professor Heikki Ruskoaho
University of Oulu
Finland

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 40 190 9800
Fax +358 3 3551 7685
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Abstract

The rapid development of stem cell technology has raised hopes for new and even revolutionary treatments for cardiac and other disorders with tissue damage. The adult human heart has very limited capability to regenerate and undergo extensive repair which is needed, for example, after myocardial infarction. Pluripotent stem cells, human embryonic stem cells (hESC) and human induced pluripotent stem (iPS) cells can be differentiated into cardiomyocytes by multiple methods. In spite of this development, therapeutic use of stem cell-derived cardiomyocytes is in its infancy.

However, functional cardiomyocytes can be differentiated from stem cells and they are themselves very useful as a cardiac cell model. Development of human iPS technology has raised the hope for the potential use of differentiated cardiomyocytes even further. By this method, patient specific stem cell lines can be derived and therefore disease models for genetic illnesses can be obtained.

The present thesis describes the differentiation of cardiomyocytes from pluripotent stem cells. The differentiation potential of several hESC lines and iPS cells was evaluated and the differentiated cells were characterized. Furthermore, the differentiation potential of hESC and iPS cells cultured on mouse and human feeder cells was monitored. Differentiation was performed by two differentiation methods, spontaneously in embroid bodies (EBs) and in co-culture with mouse visceral-endoderm-like cells (END-2 cells). In addition to the cardiac aspect, the formation of EBs and the differentiation of germ layers were evaluated in general. Differentiated cells were characterized by multiple molecular biology methods and their electrophysiological properties were also determined.

Pluripotent stem cells can be differentiated into functional cardiomyocytes even though the differentiation efficiency is low and cell lines differ in their cardiac differentiation potential. The differentiated cells beat spontaneously and expressed specific cardiac markers. The populations of the differentiated cardiomyocytes were heterogenous, containing mainly ventricular cardiomyocytes with varying maturation states. However, some of the differentiated cells had relatively mature characteristics, resembling adult human cardiac phenotype.

Tiivistelmä

Kantasolutekniikan odotetaan luovan uusia hoitomuotoja vaikeisiin, kudostuhosta johtuviin sairauksiin, kuten sydänsairauksiin. Aikuisen ihmisen sydämen uusiutumiskyky on hyvin rajallinen, eikä sydän pysty korjautumaan itsestään. Täten esimerkiksi sydäninfarktin jälkeen sydämeen jää vaurioituneelle alueelle sydämen toimintaa heikentävä arpi. Ihmisen alkion kantasolut ja ihmisen uudelleenohjelmoidut kantasolut (iPS- solut) ovat pluripotentteja kantasoluja, jotka pystyvät erilaistumaan periaatteessa kaikiksi kehon soluiksi. Näitä soluja voidaan monin eri menetelmin erilaista myös sydänlihassoluiksi, eli kardiomyosyyteiksi.

Huolimatta siitä, että erilaistettuja sydänlihassoluja ei pystytä vielä kliinisesti hyödyntämään, ovat ne käyttökelpoisia käytettäväksi ihmisen sydänsolumallina. IPS-solujen käyttö mahdollistaa myös potilasspesifisten kantasolulinjojen ja täten myös potilasspesifisten sydänlihassolujen aikaansaamisen. Täten vaikeiden geneettisten sydänsairauksien mallintaminen solutasolla on mahdollista.

Väitöskirjatutkimuksessani kuvataan sydänlihassolujen erilaistaminen sekä ihmisen alkion kantasoluista että ihmisen iPS-soluista. Tutkimuksessani verrattiin eri kantasolulinjojen erilaistumiskykyä sekä myös eri kantasoluviljelyssä käytettävien tukisolutyyppien vaikutusta erilaistumiseen. Sydänerilaistumisen lisäksi, embryoid bodien (EB) muodostumista tutkittiin myös yleisemmällä tasolla. Tämän lisäksi, erilaistetut sykkivät sydänlihassolut karakterisoitiin molekyylibiologisin ja elektrofysiologisin menetelmin.

Tämän väitöskirjatutkimuksen tulokset osoittavat, että pluripotentit kantasolut erilaistuvat toiminnallisiksi sydänlihassoluiksi. Erilaistuneet solut sykkivät spontaanisti ja ilmentävät kardiomyosyyteille spesifisiä geenejä ja proteiineja. Erilaistumistehokkuus on kuitenkin matala ja erilaistumistehokkuus vaihtelee eri kantasolulinjojen välillä. Erilaistetut sydänlihassolut ovat pääosin kammioperäisiä sydänlihassoluja, mutta erilaistunut solupopulaatio sisältää myös eteisperäisiä ja johtoratajärjestelmän solutyyppejä. Tämän lisäksi, erilaistettujen solujen kypsyysaste vaihtelee. Kuitenkin osa soluista todettiin olevan aikuisen ihmisen sydänlihassolujen kaltaisia elektrofysiologisilta ominaisuuksiltaan.

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II **Pekkanen-Mattila M**, Kerkelä E, Tanskanen JM, Pietilä M, Pelto-Huikko M, Hyttinen J, Skottman H, Suuronen R, Aalto-Setälä K. Substantial variation in the cardiac differentiation of human embryonic stem cell lines derived and propagated under the same conditions - a comparison of multiple cell lines. Ann Med. 2009, 41(5):360-70.

III **Pekkanen-Mattila M**, Ojala M, Rajala K, Skottman H, Kerkelä E and Aalto-Setälä K. The effect of human and mouse fibroblast feeder cells on cardiac differentiation of human pluripotent stem cells. Submitted.

IV **Pekkanen-Mattila M**, Chapman H, Kerkelä E, Suuronen R, Skottman H, Koivisto AP, Aalto-Setälä K. Human embryonic stem cell-derived cardiomyocytes: demonstration of a portion of cardiac cells with fairly mature electrical phenotype. Exp Biol Med (Maywood). 2010, 235(4):522-3.

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List of abbreviations

ABR	average beating rate
AFP	alpha-fetoprotein
AP	action potential
APD	action potential delay
ASC	adipose tissue stem cell
bFGF	basic fibroblast growth factor
BM	bone marrow
BMP	bone morphogenic protein
BSA	bovine serum albumin
CICR	calcium induced calcium release
cDNA	complementary deoxyribonucleic acid
CM	cardiomyocyte
cTnT	cardiac troponin T
Cx	connexin
GFP	green fluorescent protein
DAPI	4’, 6 diamidino-2-phenylindole
DKK1	dickkoptf homolog 1
DNA	deoxyribonucleic acid
dV/dt_{max}	maxium rate of rise of the action potential
EB	embryoid body
EC- cell	embryonal carcinoma cell
END-2	mouse visceral-endoderm-like cell line
ES-cell	embryonic stem cell
FA	forced aggregation
FBS	fetal bovine serum
FGF	fibroplast growth factor
FOX C1	forkhead box C1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA-4	GATA-binding protein 4
Hand1	heart and neural crest derivatives expressed 1 protein
hESC	human embryonic stem cell
hESC-CM	human embryonic stem cell-derived cardiomyocytes
hERG	human ether-to-go-go-related gene
hFF	human foreskin fibroblast
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
ICM	inner cell mass
I_{Kr}	delayed rectifier potassium current

IGF-1R	insulin-like growth factor-1 receptor
iPS cell	induced pluripotent stem cell
ISL-1	islet-1, the LIM homeodomain transcription factor
KDR	kinase insert domain receptor
Klf4	krupple-like family transcription factor 4
MAP	mitogen-activated protein
MDP	membrane diastolic potential
MEA	micro electrode array
MEF	mouse embryonic fibroblast
Mef2C	myocyte enhancer factor 2C
MESP	mesoderm posterior
MHC	myosin heavy chain
MLC	myosin light chain
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
Nanog	Nanog homeobox
NCX	Na/ Ca^{2+}exchanger
Nkx2.5	NK2 transcription factor related gene, locus 5
Oct-4	Octamer-4, POU domain, class 5, transcription factor 1
PBS	phosphate-buffered saline solution
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PPIG	peptidyl-prolyl isomerase G
PGI2	prostaglandin I_2
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
RPLP0	Ribosomal protein large P0
RyR	ryanodine receptor
SCNT	somatic cell nuclear transfer
SD	standard deviation
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SP	side population
SOX	SRY sex determining region Y-box
SR	serum replacement
SSEA4	stage specific embryonic antigen 4
Tbx	T-box transcription factor
TGF-β	transforming growth factor β
TdP	Torsades de Pointes
TRA	tumor-related antigen
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor 2 (FLK-1)
vMHC	ventricular myosin heavy chain
Wnt3A	wingless-type MMTV integration site family, member 3A

1. Introduction

The adult human heart has limited capability to regenerate and undergo extensive repair such as that needed, for example, after myocardial infarction. The rapid development of stem cell technology has raised hopes for new and even revolutionary treatments for cardiac and other disorders with tissue damage. Pluripotent stem cells, human embryonic stem cells (hESC) and induced pluripotent stem (iPS) cells, have the ability to differentiate into functional cardiomyocytes by multiple differentiation methods (Kehat et al., 2001, Mummery et al., 2003, Zhang et al., 2009, Freund et al., 2010). By contrast, the cardiac differentiation capability of adult, multipotent stem cells found in fetal and adult tissues is unresolved. The only adult stem cells that clearly have the capability to differentiate into beating cardiomyocytes are cardiac progenitor cells (Oh et al., 2003, Goumans et al., 2007).

Even though cardiomyocytes can be differentiated, their therapeutic use is in its infancy. However, functional cardiomyocytes can be differentiated from stem cells and they are themselves very useful as a cardiac cellular model. In addition, the differentiating event is valuable for developmental studies. Even though hESC derived cardiomyocytes resemble fetal cardiomyocytes exhibiting immature functional and structural characteristics compared to adult cardiomyocytes, they possess many promising capabilities for the pharmaceutical industry and for basic academic research. The development of human iPS technology (Takahashi et al., 2007, Yu et al., 2007) has raised the potential use of differentiated cardiomyocytes even more. By this method, patient specific stem cell lines can be derived and therefore disease models for severe illnesses can be obtained.

However, cardiac differentiation is still uncontrolled and inefficient. Even though new better defined differentiation methods have been published, spontaneous differentiation in embryoid bodies and slightly more directed differentiation in co-culture with END-2 cells are still widely used. In addition, hESC lines vary in their capabilities to differentiate towards cardiac linage.

The objective of present thesis was to evaluate the differentiation of pluripotent stem cells (hESC and iPS cells) to cardiomyocytes and to characterize the differentiated cells. In addition, the differentiation potential of several hESC lines cultures on mouse and human feeder cells was evaluated. Differentiation was performed by two differentiation methods and in addition to multiple molecular biology characterisation methods, the electrophysiological properties of differentiated cardiomyocytes were determined.

2. Review of the literature

2.1 Stem cells

Stem cells are unspecialized cells capable of renewing themselves through cell division or they can be differentiated to become tissue- or organ-specific cells with special functions (Wobus and Boheler, 2005). Classification of stem cells is represented in Figure 1. The cells in the embryo until the 8-cell morula stage are ***totipotent***; they can produce the whole organism. Stem cells derived from the inner cell mass (ICM) of the blastocyst are no longer totipotent but ***pluripotent***, having the ability to differentiate basically into all cell types of the body. In addition, certain organs have ***multipotent*** stem cells which have even more limited differentiation ability (Wobus and Boheler, 2005).

Stem cells enable diverse developmental studies e.g. early human lineage commitment, cell differentiation and maturation. They can serve as a unique human cell model for academic research as well as for the pharmaceutical industry. In addition, the progress in stem cell research has raised hopes for novel cell therapy treatments of severe diseases entailing tissue and cell damage like diabetes, neurological disorders, and cardiac failure. These hopes and developments are building on the successes of bone marrow hematopoietic stem cell (HSCs) transplants that have more than 30 years of patient applications in blood diseases and cancer (Thomas et al., 1959, Weissman, 2000).

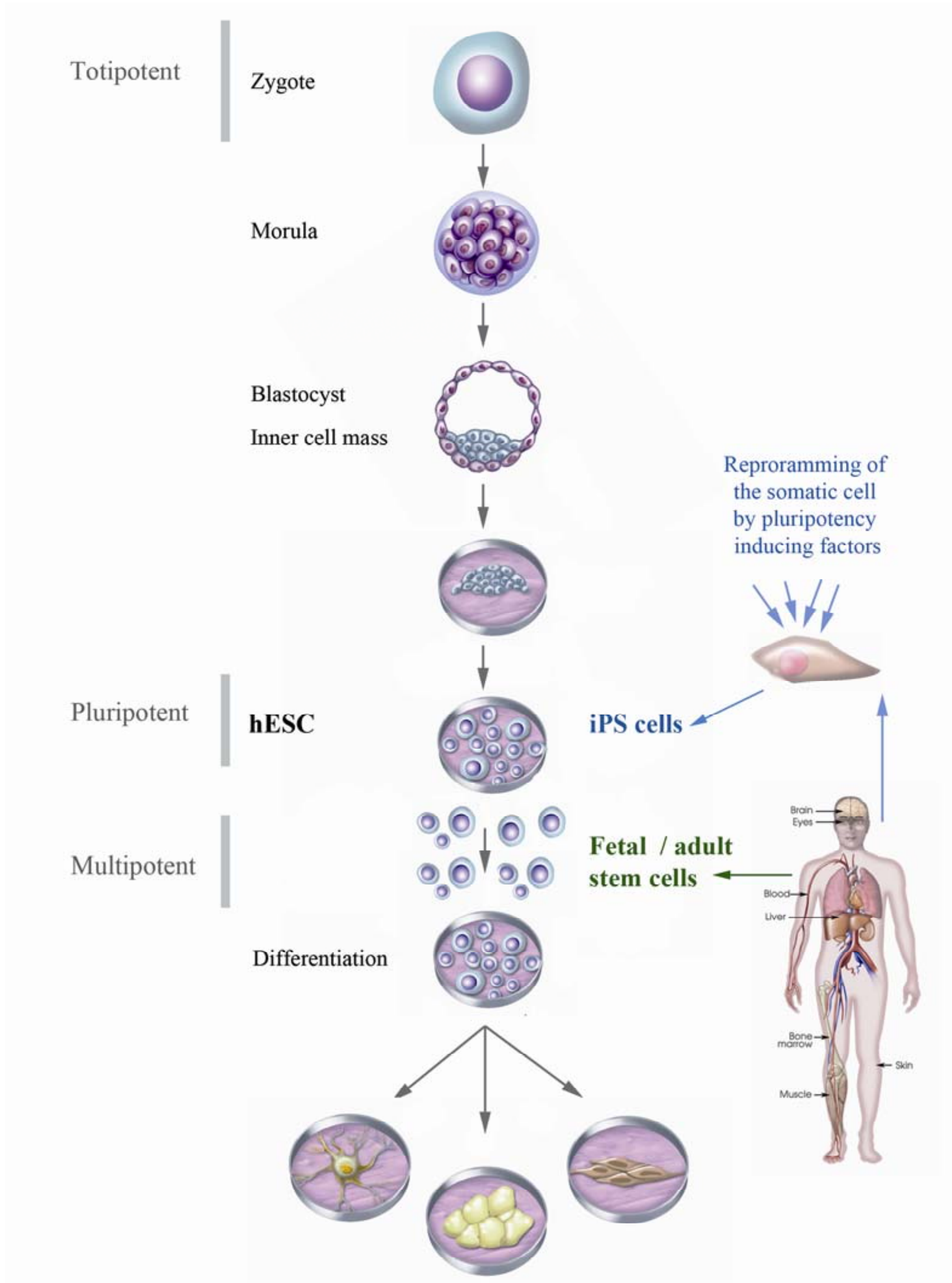


Figure 1. Origin and classification of stem cells. Zygote is totipotent, it can form any type of cells and the whole organism. Pluripotent human embryonic stem cells (hESC) are derived from the inner cell mass of the blastocyst-staged embryo. Induced pluripotent cells (iPS cells) are also pluripotent and are derived from human somatic cells by reprogramming with pluripotency inducing factors. Multipotent stem cells can be isolated from fetus or adult tissues. Figure is modified from pictures by Catherine Twomey (http://www.nationalacademies.org/stemcells).

2.1.1 Pluripotent stem cells

2.1.1.1 Human embryonic stem cells

The term “embryonic stem (ES) cell” was introduced in 1981 to distinguish embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells (Martin, 1981). First ES cells were derived from mouse ICM in the same year (Evans and Kaufman, 1981) and in 1994 Bongso and co-workers reported the successful isolation of human ICM cells and their continued culture for at least two passages *in vitro* (Bongso et al., 1994). The first permanent human embryonic stem cell (hESC) lines were derived more than a decade ago by Thomson and co-workers (Thomson et al., 1998) and these lines are still widely used. hESC have been derived from ICM of blastocyst (Thomson et al., 1998), morula (Strelchenko et al., 2004) or even from late stage (7-8 days) preimplantation embryos (Stojkovic et al., 2004). Human embryos have been donated for research by couples undergoing *in vitro* fertilisation treatments and they would have otherwise discarded because being excess or poor quality embryos.

hESCs are capable of proliferating extensively at undifferentiated state *in vitro* and have the ability to differentiate towards all three germ layers and furthermore can, in principle, give rise to all cell types of the body. hESC express transcription factors and surface markers associated with undifferentiation, such as Octamer-4, POU domain , class 5, transcription factor 1 (Oct4), Nanog homeobox (Nanog), Sex determining region Y-box 2 (Sox2), Stage specific embryonic antigen 4 (SSEA-4), Tumor-related antigen 1-60 (TRA-1-60) and TRA-1-81(Hoffman and Carpenter, 2005). Telomerase and alkaline phosphatase activity of hESCs is high and the karyotype should be normal and remain unaltered during extended culture periods (Hoffman and Carpenter, 2005).

2.1.1.2 Induced pluripotent stem cells

The cloning of the first mammal, “Dolly” the sheep, demonstrated that nuclei from a differentiated cell can be reprogrammed into undifferentiated state (Wilmut et al., 1997). The cloning of Dolly was achieved by a technique called somatic cell nuclear transfer (SCNT), where the oocyte nucleus is replaced by a nucleus derived from a somatic cell. In principle, embryonic stem cells can also be derived from embryos produced by SCNT enabling the production of patient specific hESC lines. However, this technique has major ethical reservations, since human embryos would be produced only for ES cell production and a large number of human oocytes would be needed. In addition, many countries have prohibited human cloning by law (Yamanaka, 2008). SCNT is represented in Figure 2.

hESC contain factors that can induce reprogramming of somatic cell nucleus (Cowan et al., 2005, Allegrucci et al., 2007). Therefore somatic cell fusion with ES cell regenerates pluripotent cells. However, pluripotent cells obtained by fusion contain both chromosomes from the ES cell and from the somatic cell resulting in rejection if implanted (Yamanaka, 2008). Cell fusion is represented in Figure 2.

Nevertheless, the above-mentioned findings led researchers to search for factors that induce reprogramming. Finally, in 2006 Takahashi and Yamanaka introduced four pluripotent genes; Oct4, Sox2, c-myc and Krupple-like family transcription

factor 4 (Klf4), that could reprogram mouse embryonic as well as adult fibroblasts into pluripotent stem cells (Takahashi and Yamanaka, 2006). The following year the same factors were used to make induced pluripotent cells (iPS cells) from human fibroblasts (Takahashi et al., 2007). Human iPS cells were also obtained by Thomson and co-workers by using Oct4 and Sox2 in combination with Nanog and Lin-28 homolog (Lin28) instead of c-myc and Klf4 (Yu et al., 2007). Ever since, the development in this field has been very intensive and this technique has been designated as a major breakthrough in stem cell research. The iPS cells are illustrated in Figure 1 and 2.

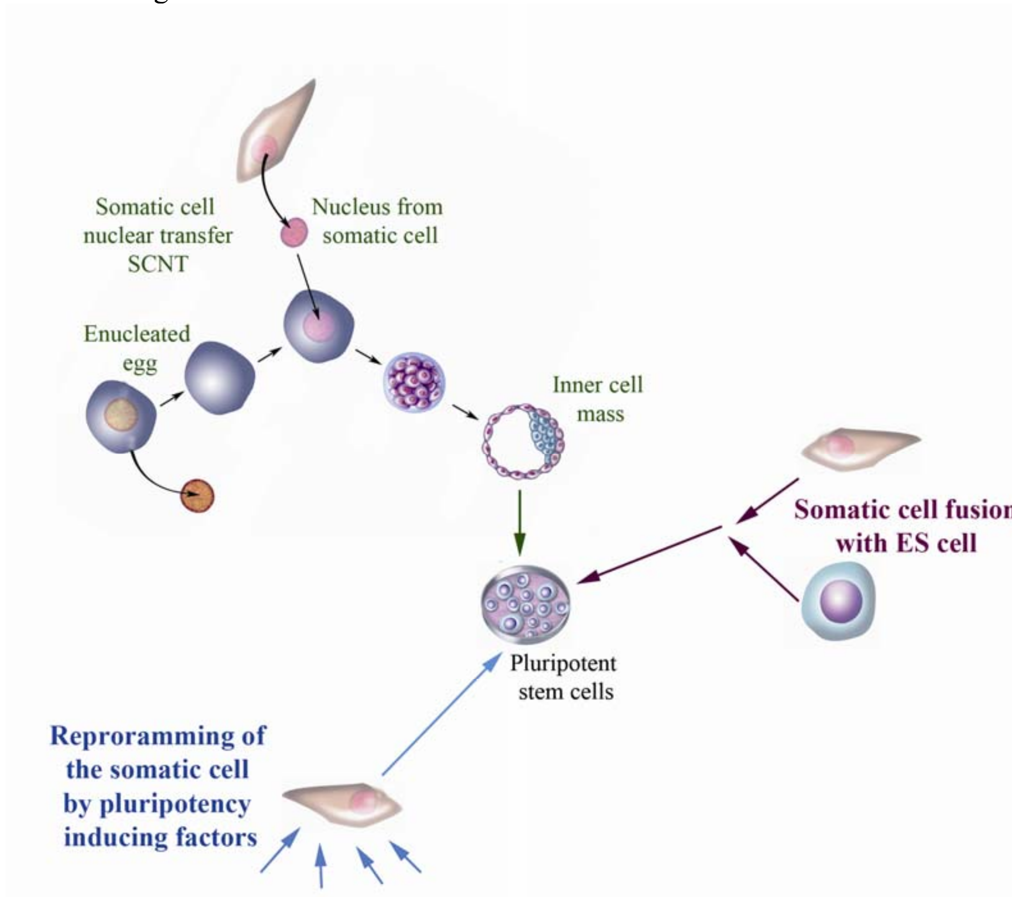


Figure 2. Pluripotent hESC lines can, in principle, be derived from embryos obtained by somatic cell nuclear transfer (up), where the nucleus from the somatic cell is transferred to an enucleated egg. Pluripotent hESCs can also be obtained by fusing a somatic cell with an ES cell (right). The somatic cell can also be reprogrammed to plutipotent state by pluripotency inducing factors. Figure is modified from pictures by Catherine Twomey (http://www.nationalacademies.org/stemcells).

The iPS cells have the same genome as the person whose cells have been reprogrammed and this makes it possible to obtain patient specific stem cells. These cells can be differentiated and they can serve as a cell or disease model or even may lead to stem cell therapies in the future (Dimos et al., 2008, Park et al., 2008).

The iPS cells share the characteristics of hESCs, such as expression of pluripotency markers, differentiation capability and need for supporting matrix of

the feeder layer. In addition, the cell culture and differentiation methods are similar for both stem cell types (Takahashi et al., 2007).

2.1.2 Multipotent stem cells

2.1.2.1 Fetal stem cells

Fetal stem cells can be obtained either from fetus or from extra-embryonic structures like umbical cord blood, amniotic fluid, Wharton's jelly, the amniotic membrane and the placenta (Hemberger et al., 2008, Pappa and Anagnou, 2009). The use of fetus itself as a source of stem cells has major ethical reservations whereas extra embryonic structures can be named as an ideal stem cell source. These tissues are dispensable after birth and have a large mass of tissue making the stem cells easy to harvest. In addition, stem cells of fetal origin express stem cell markers similar to hESCs, whereas their differentiation potential resides between the hESCs and adult stem cells (Guillot et al., 2007, Pappa and Anagnou, 2009).

2.1.2.2 Adult stem cells

Adult stem cells are undifferentiated cells found in differentiated tissues that have limited self-renewal and differentiation capacity, usually restricted to cell types of the tissue in which they originate (Choumerianou et al., 2008). Even though adult stem cells have limited differentiation and self-renewal capability, they are well suited for therapeutic purposes; patients' own stem cells can be used and therefore rejection is circumvented. In addition, adult stem cells have no ethical reservations and are easy to isolate (Choumerianou et al., 2008).

As mentioned, probably the best known source of stem cells is bone marrow (BM). Autologous BM transplants have been used in many patients with cancers, including those of the hematolymphoid system (lymphomas and leukemias), of plasma cells (multiple myeloma), and breast cancer (Thomas et al., 1959, Thomas, 1999, Weissman, 2000). BM contains two types of multipotent stem cells. Hematopoietic stem cells (HSC) give rise to all cell types of blood (Orkin, 2000) and mesenchymal stem cells (MSCs) can be differentiated into multiple mesenchymal tissue cell types such as bone, cartilage, adipose and muscle cells (Pittenger et al., 1999). In addition to BM, MSCs can be found in virtually all postnatal organs and tissues, e.g. from adipose tissue (da Silva Meirelles et al., 2006). Adipose tissue-derived stem cells (ASC) are easy to harvest in large numbers and they are reported to have similar properties and differentiation potential as BM-MSCs (Zuk et al., 2001, Zuk et al., 2002, Lindroos et al., 2009).

2.1.2.3 Cardiac stem cells

Classically, the heart has been classified as a post-mitotic organ. However, support for the endogenous regenerative ability of the heart has come from studies determining the age of human cells. The lifespan of human cardiomyocytes has been

successfully studied by utilizing ^{14}C levels of cardiac cells and according to the results, over an average human lifespan, half on the cardiomyocytes are replaced (Bergmann et al., 2009). Further evidence for the regenerative ability of the heart has come from recent studies of human cardiac progenitor cells. Human cells with the ability to differentiate into cardiomyocytes have been obtained from myocardial biopsies (Goumans et al., 2007). Several types of cells with stem cell characteristics have been discovered from the heart including cells expressing stem cell factor receptor (c-Kit) (Bearzi et al., 2007) or stem cell antigen-1 (Sca-1) on their cell surface (Oh et al., 2003). In addition, cells expressing the homeodomain transcription factor islet-1 (Isl-1) (Laugwitz et al., 2005), side population cells (SP) (Pfister et al., 2005) and cells able to grow in cardiospheres have been found (Messina et al., 2004). Cardiac progenitor cells have been nominated as a candidate cells for cardiac regeneration and intensive work is ongoing to activate these cells to proliferate and differentiate *in situ*. However, the origin and specific capabilities forming functional cardiac cells of these progenitors need to be more thoroughly determined (Gonzales and Pedrazzini, 2009).

2.2 Characteristics of human embryonic stem cells

2.2.1 Stem cell lines

At the end of the year 2009, the total number of hESC lines worldwide was estimated to be 1 071 (Loser et al., 2010). Even though the number of hESC lines has markedly increased since 2005, the estimation being 414 lines at that time, two lines H1 and H9 (WiCell Research Institute) have been the most used hESC lines in stem cell research (Guhr et al., 2006, Scott et al., 2009, Loser et al., 2010). In spite of the large number of lines, the number of available and well-characterized lines is probably considerably lower. Due to the poor characterization data published, it remains an open question whether the cell lines reported in the scientific literature manifest the characteristics of human pluripotent cell lines and are available for research (Loser et al., 2010).

The wide use of WiCell lines is partly due to polices and legislation on the use of hESC in certain countries, such as in the USA. On August 2001, President Bush introduced a law which proscribed the federal funding for research made with hESC lines derived after that date (Murugan, 2009). Even though President Obama revoked Bush's policy in 2009 enabling the use of hESC lines derived after 2001, federal funding for the derivation of new hESC lines is still prohibited (Murugan, 2009). Nevertheless, due to the differences between stem cell lines, the dominance of few lines in hESC research may reduce the universal applicability of the results and therefore even limit the development of the field (Loser et al., 2010).

If hESCs are to be used in clinical applications, there should be enough stem cell lines to cover the spectrum of transplant antigens and further to avoid immune rejection problems. It has been estimated that in the UK, 150 randomly obtained hESC lines would provide a worthwhile human leukocyte antigen (HLA) match for most potential recipients (Taylor et al., 2005). In addition, genetic variation of hESC lines would also be preferable for drug screening and safety pharmacology

applications of the pharmaceutical industry (Ingelman-Sundberg and Rodriguez-Antona, 2005, Allegrucci and Young, 2007).

Derivation and culture techniques of hESCs vary between laboratories (Allegrucci and Young, 2007). In addition, the blastocyst stage, the stage when hESCs are derived, is characterized by high levels of epigenetic activity, including DNA methylation, X chromosome inactivation, and dynamic chromatin remodelling (Bibikova et al., 2006). Therefore it is understandable that hESC lines differ in terms of epigenetics, for example H7 hES cells do not express the marker for X-chromosome inactivation as do H9 and hES 25 (Hoffman and Carpenter, 2005). In addition, it is not known how stable the epigenetic profile of ES cells is during long-term culture, nor how it may change as the cells differentiate along different developmental pathways (Bibikova et al., 2006).

Therefore variations in derivation and maintenance combined with the genetic variation of the human samples leads to hESC lines with different properties (Allegrucci et al., 2005, Allegrucci and Young, 2007). However, in spite of the known possibilities of genetic or environmental influences on the phenotype of the hESC lines, many of the lines have been published without detailed characterization data (Adewumi et al., 2007). In addition, probably due to the laborious and costly maintenance of several hESC lines, only few studies have been published comparing the characteristics of several hESC lines. According to these comparison studies, hESCs have been shown to be similar in regard to expression of pluripotency markers but after cells start to differentiate, the expression of differentiation markers and further differentiation propensity varies between different hESC lines (Adewumi et al., 2007, Kim et al., 2007, Osafune et al., 2008). Due to this observation it has been suggested, that the most suitable hESC line should be chosen according to its propensity to differentiate towards the linage of interest (Osafune et al., 2008).

In addition to changes due to derivation and culture techniques, prolonged culturing and passaging of hESC may alter them for adaptive changes such as karyotypic changes, increased growth rate or reduced apoptosis (Draper et al., 2004a, Draper et al., 2004b, Enver et al., 2005, Hanson and Caisander, 2005, Baker et al., 2007, Hovatta et al., 2010, Narva et al., 2010). According to karyotype studies based on G-banding, during prolonged culture the most frequent karyotype changes observed are gains of chromosomes 12, 17 and X, which are also seen in germ cell tumors (Baker et al., 2007). In addition, more similarities between hESC and tumor cells have been found in studies using higher resolution DNA analysis (Hovatta et al., 2010, Narva et al., 2010).

2.2.2 Cell culture

hESCs need specialized culture conditions to maintain their pluripotency and stable karyotype and phenotype. In addition to specialized culture media, feeder cells are needed for attachment, nourishment and to keep hESCs undifferentiated. Originally hESC were derived and cultured on top of mouse embryonic fibroblast (MEF) feeder cells in a culture media consisting fetal calf serum (FBS) (Thomson et al., 1998, Reubinoff et al., 2000). Later on, human based feeders have been used to replace MEFs (Hovatta et al., 2003, Inzunza et al., 2005, Skottman and Hovatta, 2006) and KnockOut Serum Replacement (SR) (Invitrogen, Carlsbad, CA, USA)

has replaced FBS in hESC culture medium. FBS is a problematic reagent because it contains unknown components and different serum batches vary in their capability to maintain pluripotency or even differentiate hESCs. Although SR still consists of many animal based components, it is more defined and also beneficial effects on hESCs' proliferation (Koivisto et al., 2004).

Much effort has been invested in replacing living feeder-cells by some other substrates to develop feeder-free hESC culture systems (Akopian et al., Xu et al., 2001, Thomas et al., 2009). Commercially available Matrigel (BD Biosciences), laminin and fibronectin have been reported to maintain pluripotent state of hESCs (Amit et al., 2000, Amit et al., 2004, Rosler et al., 2004).

Passaging of hESC is another challenging step in hESC production. Stem cells grow in colonies (Figure 3) and these colonies have to be broken either mechanically or enzymatically during passaging. Mechanical cutting of the colonies into smaller pieces does not expose the cells to xenogenic enzymes (e.g. trypsin or collagenase IV) which dissociate them in a more uniform way but at the same time disrupt their cell surface adhesion molecules and communication with other cells. In addition, dissociation of hESC to single cell stage may predispose cells to karyotypic changes (Brimble et al., 2004). Either way, passaging is a laborious and also critical step in the production of hESCs.

A more defined culture system is needed to fulfil the needs of clinical applications and also for hESC research. To avoid xenogenic materials and problems caused by lot-to-lot variation of FBS, growth factors and enzymes used in passaging would enhance the hESC production and make it more standardized and consistent.

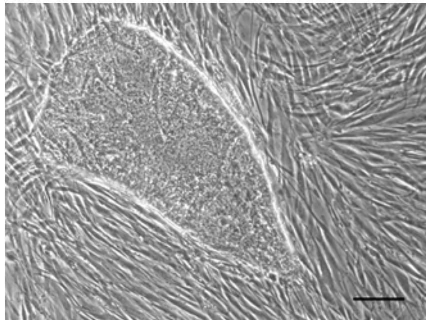


Figure 3. hESC line H7 colony cultured on human foreskin fibroblast feeders. Scalebar 200µm.

2.3 Development and differentiation markers of the heart

In vertebrates, the heart is the first organ to form and its circulatory function is essential for the viability of the embryo (Buckingham et al., 2005). Myocardial cells are derived from mesoderm, a germ layer which emerges during gastrulation from the primitive streak. The initial form of the heart is the heart tube which then undergoes multi-phased looping and finally forms the four-chambered heart (Buckingham et al., 2005).

Studies of early stages of heart differentiation are hampered by the lack of early stage cardiac cell markers (Lough and Sugi, 2000). Transient expression of Brachyury T is widely used to depict mesoderm and furthermore cardiac lineage formation (Kispert and Herrmann, 1994). According to present knowledge, there are two cardiac progenitor cell populations, called the heart fields, that contribute to the formation of the heart (Buckingham et al., 2005). One lineage contributes to the formation of the left ventricle, partly the right ventricle, the atrioventricular canal and atria. The other lineage is responsible for the formation of the outflow tract as well as the all right ventricle and atria. The latter field, called the secondary or anterior heart field is marked by Islet-1 (Isl-1), the LIM homeodomain transcription factor. This field forms two thirds of the embryonic heart, including the cardiac muscle, smooth muscle and endothelial cells (Cai et al., 2003). Other early markers for cardiac progenitors are mesoderm posterior 1 and 2 (MESP1 and MESP2), which are transiently expressed in newly formed mesoderm at the primitive streak (Kitajima et al., 2000). In mammals, bone morphogenic proteins (BMPs), transforming growth factor β superfamily (TGF-βs) and the fibroblast growth factors (FGFs) have been found to be essential for heart development and these factors regulate the activation of myocardial regulatory genes such as NK2 transcription factor related gene, locus 5 (Nkx 2.5) and GATA binding protein-4 (GATA4) (Brand, 2003). The developmental steps in heart formation are illustrated in Figure 4.

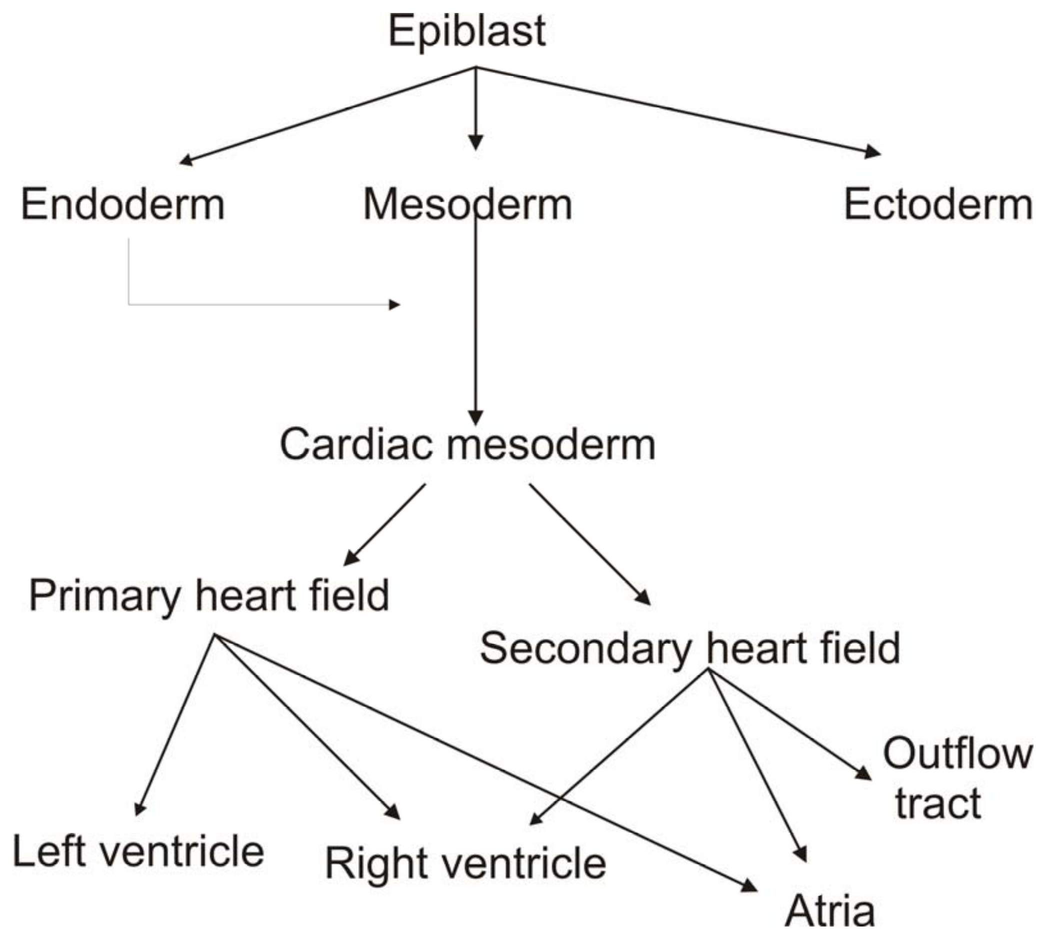


Figure 4. A simple schematic of developmental steps in heart formation. Embryonic stem cells have the potential to differentiate into cell types of all three germ layers, endoderm, mesoderm or ectoderm. Mesoderm is the origin of cardiac cells and it has been shown that cardiac differentiation inducing signals are to a large extent of endoderm origin. Two cardiac progenitor cell populations, the heart fields, contribute to the formation of the heart. The left ventricle is formed only from the primary heart field, whereas the atria and the right ventricle are formed from both of the progenitor cell populations.

2.4 Production of cardiomyocytes

2.4.1 Cardiac differentiation potential of stem cells

Pluripotent stem cells, hESC and iPS cells have the ability to differentiate into functional cardiomyocytes by multiple methods (Kehat et al., 2001, Mummery et al., 2003, Zhang et al., 2009, Freund et al., 2010). By contrast, the cardiac differentiation capability of adult, multipotent, stem cells is controversial. The only adult stem cells that clearly have the capability to differentiate into beating cardiomyocytes are cardiac progenitor cells (Oh et al., 2003, Goumans et al., 2007).

Adult human bone marrow mesenchymal stem cells have been shown to differentiate *in vitro* into cardiomyocyte-like cells with expression of cardiac specific genes (Rangappa et al., 2003, Antonitsis et al., 2007, Antonitsis et al., 2008). However, differentiation was induced by 5-azacytidine, a cytosine analog that can reduce DNA methyltransferase activity in the cells (Tsuji-Takayama et al., 2004) or in co-culture with cardiomyocytes (Wang et al., 2006). Similar methods have been reported to produce cells with cardiomyocyte phenotype from human adipose-derived stem cells and spontaneously beating cells were moreover obtained after co-culture with neonatal rat cardiomyocytes (Choi et al., 2010). When bone marrow–derived hematopoietic cells were transplanted directly into the hearts of mice subjected to acute myocardial infarction, no transdifferentiated bone marrow–derived cardiomyocytes were found in the damaged myocardium. However, cell fusion has been found to occur at very low levels, where bone marrow–derived cells have fused with host cardiomyocytes outside the infarction area (Nygren et al., 2004).

Many clinical studies have evaluated the therapeutic potential of human bone marrow derived stem cells (e.g. mesenchymal stromal cells or mononuclear cells) to improve cardiac function after myocardial infarction (Gonzales and Pedrazzini, 2009, Mathiasen et al., 2009, Wei et al., 2009, Miettinen et al., 2010). No evidence of cardiac regeneration characterised by differentiation of implanted stem cells into cardiomyocytes and other cardiac cell lineages has been reported. Some studies, but not all, report beneficial effects on heart function and on symptoms (Wollert et al., 2004, Janssens et al., 2006, Lunde et al., 2006, Schachinger et al., 2006). These benefits have been suggested to be short-term and according to five-year follow-up treatment with BMC was not able to achieve sustained improvements on heart function (Meyer et al., 2009). Nonetheless, the congruent results of these studies show that therapeutic use of human bone marrow stem cells is apparently safe.

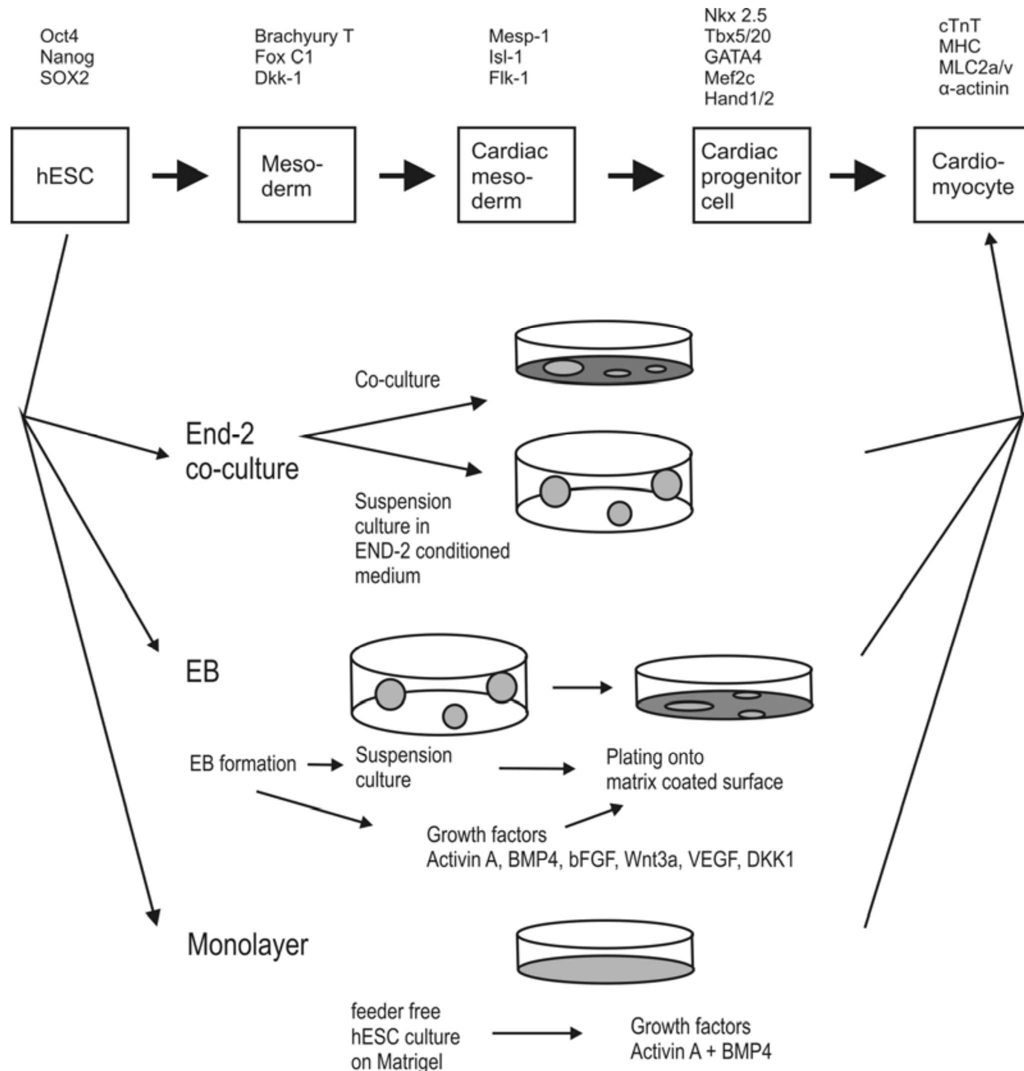


Figure 5. Cardiac differentiation cascade and the differentiation methods. From the top; Markers for different stages of cardiac differentiation, steps in cardiac differentiation and the differentiation methods. ***END-2 differentiation*** has two variables, hESC are either plated on top of END-2 cell layer or hESC are cultured as EBs in suspension in END-2 conditioned medium. In ***EB method*****,** differentiation can be performed spontaneously or with differentiation inducing growth factors. ***Monolayer differentiation*** is initiated with feeder free hESC cultures. Culturing of hESC and differentiation with activin A and bone morphogenic protein-4 (BMP-4) is preformed on top of Matrigel. By this method, beating monolayer can be obtained whereas END-2 and EB method produce thee dimensional beating areas.

2.4.2 Differentiation methods

2.4.2.1 Spontaneous differentiation in embryoid bodies

hESC and iPS cells can be differentiated spontaneously as embryoid bodies (EB) (Figure 5). In principle, during EB formation the culture condition for stem cells is changed from two-dimension into three-dimensional structure. First pluripotent stem cells are either enzymatically or mechanically dissociated to small cell clusters. Secondly cells are allowed to form aggregates in suspension and after a few days the formed EBs are normally plated down on matrix coated cell culture plates (Kurosawa, 2007). After hESCs have been removed from the environment which supports the undifferentiated state, they start to differentiate towards three germ layers in the cell aggregates (Itskovitz-Eldor et al., 2000). During the early stages of suspension culture, the cell aggregate transforms into a cystic body and a trilayer shell composed of extra cellular proteins forms around EB (Sachlos and Auguste, 2008). The paracrine and endocrine signalling determine the fate of the stem cell. Similarly as in embryo this signalling may lead to the formation of concentration gradient in the EBs and further influence the cell differentiation (Sachlos and Auguste, 2008).

EB formation has characteristics similar to those of embryonal development (Keller, 1995) and therefore the interplay of different germ layers and their influences on cell differentiation can be studied in EB cultures. EB differentiation, such as cardiac differentiation, is particularly well documented with mouse ES cells (Hescheler et al., 1997, Boheler et al., 2002). However, the EB formation and spontaneous differentiation from hESC has proven to be more difficult and inefficient if compared to mouse counterpart (Wobus et al., 1991, Kehat et al., 2001). When mouse ES cells are differentiated in EBs, beating areas appear 1 day after plating, and, within 2–10 days, 80–90% of EBs reveal beating areas (Wobus et al., 1991). In the hESC differentiation beating areas are observed later and the differentiation efficiency is much lower, usually under 10% (Kehat et al., 2001).

Cardiomyocytes can be obtained from hESC and iPS cells by spontaneous differentiation in EBs (Itskovitz-Eldor et al., 2000, Kehat et al., 2001, Zhang et al., 2009). EB differentiation is also widely used in the production of other cell types such as neuronal cells, hematopoietic cells, adipocytes and chondrocytes (Pera and Trounson, 2004). For the whole existence of hESC, EB differentiation has been a widely used differentiation method due to its relatively simple and inexpensive nature.

There are multiple methods for EB formation (Kurosawa, 2007). Suspension culture in bacterial-grade cell culture dishes was first developed for mouse ES cells (Doetschman et al., 1985) and was later used in cardiomyocyte differentiation from hESCs (Itskovitz-Eldor et al., 2000, Kehat et al., 2001). In this method enzymatically dissociated cells aggregate when cultured unattached in the culture medium. hESCs are vulnerable to dissociation to the single cell stage (Thomson et al., 1998, Amit et al., 2000, Kehat et al., 2001, Xu et al., 2002) and therefore hESCs have been dissociated into small aggregates of cells to retain the cell-to-cell contact (Amit et al., 2000, Pyle et al., 2006). To scale up EB formation in suspension cultures, bioreactors and spinner flasks have also been used (Messina et al., 2004, Kurosawa, 2007, Yirme et al., 2008).

Cardiomyocytes have also been differentiated by the hanging drop method, where single cell suspension is pipetted in small drops onto a petri dish cover and the cover is then inverted on top of a dish (Takahashi et al., 2003, Burridge et al., 2007). The drop hangs because of the surface tension and provides a good environment for the cells to aggregate and form the EB. The hanging drop-method is not suitable for long term EB differentiation because medium change is impossible (Kurosawa, 2007). Overall hanging drop method is very laborious and therefore not suitable for large scale experiments.

Recent studies indicate that EB size has an effect on cardiomyocyte differentiation as well as on differentiation in general (Burridge et al., 2007, Bauwens et al., 2008, Mohr et al., 2010). Therefore the number of cells should be measurable in order to optimize differentiation. The hanging drop method makes it possible to standardize the initial amount of hESC. However, Ng and co-workers developed a more robust method than hanging drops, a forced aggregation (FA) system for hematopoietic differentiation and this has also been used in cardiomyocyte differentiation (Ng et al., 2005, Burridge et al., 2007). FA mimics the hanging drop method; the cells are forced to aggregate by centrifugation in round bottomed, low-adherence 96-well plate wells. The medium change is possible to the wells and therefore longer culture times can be used and differentiation inducing agents can also be added to the culture medium (Burridge et al., 2007). Two-dimensional cell pieces can also be produced by microprinting technique, where standard-size colonies are formed and then scraped into suspension culture (Bauwens et al., 2008, Niebruegge et al., 2009). EB differentiation techniques are summarized in Table 1.

Table 1. Summary of the EB differentiation techniques.

<table>
<tr><th colspan="7"><u>EB differentiation techniques</u></th></tr>
<tr><th><u>Method description</u></th><th>Hanging drop</th><th>Forced aggregation (FA)</th><th>Suspension culture</th><th>Microprinting technique</th><th>Manual</th></tr>
<tr><td><u>hESC colony dissociation</u></td><td colspan="3">Enzymatic dissociation</td><td>Detachment of microprinted colonies</td><td>Manual cutting</td></tr>
<tr><td><u>EB formation</u></td><td>Single cells/small aggregates form EB in a hanging drop</td><td>Cell suspension is aggregated to EB by centrifuging in a 96-well plate</td><td>Spontaneous aggregation in suspension</td><td colspan="2">One cell colony or cell colony piece forms an EB in suspension</td></tr>
<tr><td rowspan="2"><u>EB culture</u></td><td colspan="2">Formed EBs transferred for suspension culture</td><td colspan="3">Suspension culture continues</td></tr>
<tr><td colspan="5">After suspension culture EBs are plated on a coated cell culture plate</td></tr>
<tr><td><u>Advantages</u></td><td>Gentle EB formation in a drop because of gravity</td><td>Scalable, straightforward, cell number per EB easy to standardize</td><td>Straightforward</td><td>Cell number per EB easy to standardize</td><td>Gentle, non-enzymatic hESC colony dissociation</td></tr>
<tr><td><u>Disadvantages</u></td><td>Laborious, non-scalable</td><td>hESC colonies have to be dissociated to single cell stage</td><td>Forming EBs randomly sized</td><td>Need for microprinting technique for colony formation</td><td>Laborious, non-scalable</td></tr>
<tr><td><u>Reference</u></td><td>(Takahashi et al., 2003)</td><td>(Ng et al., 2005)</td><td>(Doetschman et al., 1985)</td><td>(Bauwens et al., 2008, Niebruegge et al., 2009)</td><td></td></tr>
</table>

2.4.2.2 Differentiation in mouse visceral-endoderm-like cell co-cultures

A more controlled way to differentiate cardiomyocytes from hESCs is in co-culture with mouse endodermal-like cells (END-2) (Figure 5), particularly in the absence of serum and with ascorbic acid (Mummery et al., 2003, Passier et al., 2005). The differentiation inducing factors are secreted from END-2 cells and therefore the END-2 conditioned medium can also be used in cardiomyocyte differentiation (Graichen et al., 2008). END-2 cells support the differentiation towards endodermal and mesodermal derivatives (Mummery et al., 2003, Passier et al., 2005, Beqqali et al., 2006) and according to embryonal development studies, anterior visceral endoderm is essential in normal heart development (Lough and Sugi, 2000). It has therefore been suggested that cardiomyocyte differentiation could be mediated by END-2 cells directly or by hESC derived endodermal cells (Passier et al., 2005).

However, the mechanism or the specific factors inducing cardiac differentiation by END-2 cells are not clearly known. Systematic testing of END-2 conditioned medium revealed that END-2 cells were able to clear insulin from the medium (Xu et al., 2008a). Insulin has been shown to inhibit cardiac differentiation by suppressing endoderm and mesoderm formation and favouring ectoderm differentiation (Freund et al., 2008). Insulin acts via the insulin-like growth factor-1 receptor (IGF-1R) and phosphatidylinositol 3-kinase (PI3K/Akt) pathway and has been suggested to inhibit epithelial to mesenchymal transition by elevated levels of E-cadherin (Freund et al., 2008). In addition, IGF/PI3K/Akt has been shown to have a role in the proliferation of immature cardiomyocytes (McDevitt et al., 2005) which suggests that this pathway has a dual role in cardiomyogenesis.

END-2 cells were not the only type of cells which depleted insulin from the culture media. Similar phenomenon was observed with MES1-cells (Mummery et al., 1986) and mouse embryonic fibroblasts (MEFs) which do not have the cardiac inducing effect (Xu et al., 2008a). Therefore insulin depletion is likely not the cardiac inducing factor of END-2 cells. On the contrary, prostaglandin I_2 (PGI_2) was found to be secreted by END-2 cells at elevated levels if compared to other types of mouse cells lacking cardiac inductive effect. Including PGI2 in differentiation medium without END-2 conditioning resulting a similar level of cardiac differentiation as END-2 conditioned medium (Xu et al., 2008a).

In addition to the PGI_2, inhibition of p38 mitogen activated protein kinase (MAPK) increases the cardiac differentiation rate (Graichen et al., 2008). Selective MAPK inhibitors (molecules SB203580 and SB202190) (Cuenda et al., 1995) were found to increase the differentiation rate when added to END-2 conditioned medium. However, the inductive effect of these molecules was concentration dependent, at high concentrations (>15 μM) cardiomyocyte formation was decreased and finally inhibited (Xu et al., 2008a). The use of p38 inhibitor PD169316 also causes mouse ES cells to differentiate towards neural linage meanwhile the cardiac mesoderm formation is inhibited (Aouadi et al., 2006). Therefore the inhibition of MAPK has a partially opposite effect on mouse and human cells.

Even though some factors of END-2 cells that affect cardiac differentiation have been identified (Graichen et al., 2008, Xu et al., 2008a), the role of END-2 cells as a

whole in cardiac differentiation remains a mystery. The END-2 differentiation techniques are compared in Table 2.

Table 2. Summary of the END-2 differentiation techniques.

END-2 differentiation techniques		
Method description	END-2 co-culture	END-2 conditioned medium
END-2 culturing	Culturing on END-2 cells, mitomycin-C- treatment and preparation of END-2 cell layers on cell culture plates or flasks	
Preparation of the differentiation media	Normal cell culture medium	Production of conditioned medium (culture medium on END-2 cell layer for 4-5 days)
hESC colony dissociation	Manual cutting	Enzymatic dissociation
Differentiation procedure	Plating hESC colony pieces onto END-2 cell layer	EB formation in suspension
		EBs to be cultured in the conditioned medium
	Culturing with medium change after every 3-5 days	
Beating areas observed	~7 days after initiation of co-culture	~12 days after exposure to conditioned medium
Advantages	Simple, fast, no enzymatic dissociation	Conditioned medium can be stored, no plating of EBs necessary, scalable
Disadvantages	END-2 cell layer production needed right before differentiation	Preparation of conditioned medium (possible lot-to-lot variation)
Reference	(Mummery et al., 2003, Passier et al., 2005)	(Xu et al., 2008a)

2.4.2.3 Differentiation with defined growth factors

Cardiac differentiation consists of complex signalling network and currently there is no single factor to direct stem cells to differentiate effectively towards cardiac lineage. Laflamme and co-workers used a combination of activin A and BMP-4 in cardiomyocyte differentiation (Laflamme et al., 2007) (Figure 5). This cascade of factors enhances mesoendoderm formation, an early precursor cell lineage which gives rise to mesoderm and endoderm. Mesoderm is the origin of cardiac cells and it has been shown that cardiac differentiation inducing signals are to a large extent of endoderm origin (Lough and Sugi, 2000). Therefore mesoendoderm induction would yield more efficient human embryonic stem cell-derived cardiomyocyte (hESC-CM) differentiation.

A stepwise differentiation protocol was also developed by Yang and co-workers (Yang et al., 2008). This protocol involves the induction of primitive streak-like population, in addition to the formation of cardiac mesoderm and expansion of

cardiac lineages. The protocol is based on EB differentiation and comprises three stages. Growth factors BMP-4, FGF, activin A, vascular endothelial growth factor (VEGF) and dickkoptf homolog 1 (DKK1) were used in varying combinations.

Mesoendoderm formation has also been induced by Wnt3A, an activator of the canonical Wnt/β-catenin signalling pathway (Tran et al., 2009).

Taken together, even though the use of growth factors may enhance cardiac differentiation, pure populations of cardiomyocytes can not currently be produced and enrichment methods are still needed. Due to multi-phased differentiation protocols and the high costs of growth factors, the simple and functional EB differentiation method is still a widely used method in cardiomyocyte production. However, as in hESC culturing, more defined differentiation system should be developed for cardiomyocyte differentiation to enhance reproducibility and purity of the differentiated cardiomyocyte population.

2.4.3 Enrichment of differentiated cardiomyocytes

Due to the inefficient differentiation, the resulting cell populations are a mixture of different cell types and the yield of hESC-CM cultures is very low. EB differentiation in serum containing medium yields < 1% and the more defined activinA/BMP-4 protocol yielded > 30% of cardiomyocytes (Laflamme et al., 2007). Therefore differentiation methods need considerable upscaling and effective enrichment and purification methods should be developed before hESC-CM can undergo testing in large animal models and clinical use in the future.

For certain research purposes, it is usually adequate to enrich the hESC-CM by mechanically dissecting beating areas from the differentiation cultures (Kehat et al., 2001, Mummery et al., 2003). However, only 5-20% of the cells in the beating areas are positive for cardiac α-actinin (Passier et al., 2005).

Percoll™ gradient separation based on density gradient separation has been used in combination with the generation and maintenance of cardiac bodies (Xu et al., 2006). After separation and 7 days of suspension maintenance, 50 % of the cultured EBs contained beating areas. However, this method has been difficult for others to reproduce (van Laake et al., 2006).

Transgenic selection is one technique to enrich cardiomyocytes from hESC differentiation cultures. This method utilizes transgenic hESC lines where a gene of green fluorescent protein (GFP) or an antibiotic resistance gene is located under the control of a cardiac specific promoter (e.g. myosin light chain promoter) (Kolossov et al., 2005, Anderson et al., 2007, Huber et al., 2007, Xu et al., 2008b, Kita-Matsuo et al., 2009). Although this method is efficient, genetic modification is neither feasible for hESC or human iPS cell lines nor suitable for possible future clinical use (Mummery, 2010, Vidarsson et al., 2010).

A recent study sorted cardiomyocytes from mixed cell populations by utilizing the endogenously expressed surface marker activated leukocyte cell-adhesion molecule, CD166 (ALCAM) (Rust et al., 2009). However, there is a lack of cardiac specific surface proteins and therefore a lack of antibodies to make sorting possible (Mummery, 2010). Nevertheless, fluorescence-activated cell sorting (FACS) was successfully used in selection by utilizing the high mitochondria content of cardiomyocytes (Hattori et al., 2010).

2.5 Characterization of differentiated cardiomyocytes

2.5.1 Functional and structural analysis

hESC-CM have the capacity to beat spontaneously (Kehat et al., 2001, Mummery et al., 2003). Beating cells are at an early stage relatively small and round and situated in circular accumulations in the EBs. At later stages, EBs gradually develop to be larger and the cells turn to be more elongated in shape and tend to accumulate in strands. Electron microscopy studies reveal that cardiomyocytes contain myofibrils which are first randomly and in a varying manner distributed throughout the cytoplasm. However, organized sarcomeric structures occur at later stages of differentiation with A, I, and Z bands. Close to the sarcomeres, mitochondia are also present. In addition, cells have intercalacted disks with gap junctions and desmosomes (Kehat et al., 2001, Snir et al., 2003).

2.5.2 Expression of cardiac markers

The gene expression profiles of the hESC during cardiac differentiation (Beqqali et al., 2006, Synnergren et al., 2008a) and the differentiated hESC-CM have been studied by DNA microarray (Cao et al., 2008, Synnergren et al., 2008b, Kita-Matsuo et al., 2009, Xu et al., 2009). These studies reveal that the molecular signature of hESC-CM resembles the cardiomyocytes from the human heart (Vidarsson et al., 2010).

hESC-CM differentiation can be predicted by the transient expression of the early mesodermal marker Brachyury T. Brachyury T expression peak is detected at the time point of 3 days in END-2 co-cultures (Beqqali et al., 2006) and a day later in EBs (Bettiol et al., 2007). Brachyury T belongs to the family of transcription factors which are encoded by the T-box genes (Showell et al., 2004). This protein family functions in many developmental processes and has a sequence similarity with the DNA-binding domain, the T-domain (Showell et al., 2004). The phenotype of heterozygous Brachyury T mutant mice was first described by Nadine Dobrovolskaïa-Zavadskaïa in 1927, in these mice the axial development was not completed and they had a truncated tail (Dobrovolskaia-Zavadskaïa, 1927). Homozygous mice, however, display many mesodermal abnormalities and die shortly after gastrulation (Gluecksohn-Schoenheimer, 1938, Gluecksohn-Schoenheimer, 1944). Brachyury T can be nominated as a classic transcription factor, it is localized in the nucleus and is an endogenous activator of mesodermal genes (Conlon et al., 1994, Kispert et al., 1995, Showell et al., 2004). In the embryo, Brachyury T expression is suggested to be induced by TGFβ and FGF signalling (Hemmati-Brivanlou and Melton, 1992, Amaya et al., 1993). Overall, very few direct targets for T-box genes have been identified. However, embryonic FGF (eFGF) (Casey et al., 1998), Brachyury-induced homeobox Bix4 (Tada et al., 1998) and XWnt11 (Tada and Smith, 2000) have been suggested as downstream targets for Brachyury T.

Differentiation cascade can be further followed by the expression of cardiac regulatory transcription factors such as Islet-1 (Isl-1), Mesp 1, GATA-4, Nkx2.5 and T-box transcription factor 6 (Tbx6) (Graichen et al., 2008, Yang et al., 2008).

Cardiac troponin T (cTnT) is encoded by the TNNT2 gene (Thierfelder et al., 1994), is the tropomyosin-binding subunit of the troponin complex and can therefore be used for characterizing hESC-CM. Troponin complex is located on the thin filament of striated muscles and regulates muscle contraction in response to alterations in intracellular calcium ion concentrations as reviewed (Farah and Reinach, 1995, Tobacman, 1996). In addition to cTnT, other cardiac specific structural proteins are used for confirming cardiac phenotype of the beating hESC-CM such as cardiac troponin I, myosins or cardiac α-actinin (Kehat et al., 2001, Mummery et al., 2003).

In addition proteins of contractile apparatus, proteins of gap junctions and ion channels can be used in characterization of hESC-CM. Gap junctions are formed from connexin proteins and have an important role in signal transduction. Connexin 43 (Cx43) is the most common form in the ventricle, Cx40 predominates in the atria and Cx45 is found in both atria and ventricle and also from Purkinje fibres (Gaborit et al., 2007).

2.5.3 Electrophysiology

hESC as well as iPS cell-derived cardiomyocytes exhibit heterogenic action potential (AP) morphologies which can be divided into nodal, atrial and ventricular subtypes according to the shape of AP as represented in Figure 6 (He et al., 2003a, Zhang et al., 2009). If compared to the human neonatal or adult atrial or ventricular cardiomyocytes, hESC-CM have relatively positive maximum diastolic potential (MDP) and slow maximum rate of rise of the AP (dV/dt_{max}) and are therefore called embryonal atrial- and ventricular like cells (He et al., 2003a).

Differentiated beating cells exhibit spontaneous APs and contractile activity and therefore express cardiac structural proteins and ionic currents (Kehat et al., 2001, He et al., 2003b, Mummery et al., 2003). During differentiation the expression of some ion channel genes increases suggesting that hESC-CM reach a more mature state with time in culture (Sartiani et al., 2007).

Traditionally a patch clamp has been used in analyzing the action potential and also the electrophysiological properties of cardiomyocytes. Micro-electrode array (MEA) technology provides another useful platform to study cell electrophysiology, especially ES-derived cardiomyocytes (Hescheler et al., 2004, Reppel et al., 2004). In MEA, cells are plated on top of electrodes in a cell culture well-type platform and can be cultured and measured repeatedly for long periods of time. In addition, MEA can be utilized for testing the effects of pharmaceutical agents on hESC-CM (Braam et al., 2010).

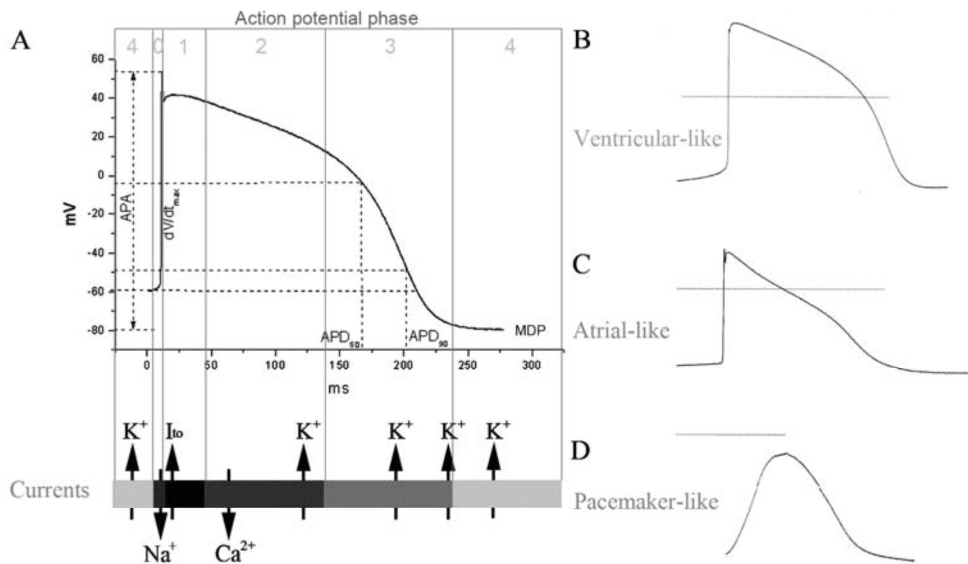


Figure 6. Action potential phases and cardiomyocyte subtype specification A. Action potential (AP) parameters: Action potential amplitude (APA), maximum rate of rise of the action potential (dV/dtmax), action potential delay (ADP) and membrane diastolic potential (MDP). AP phase 0 is a rapid depolarization phase when the sodium channels are activated and membrane permeability is increased to Na^+. Rapid depolarisation is followed by rapid repolarization phase 1 and plateau phase 2, where Ca^{2+} ions are entered to the cell throught L-type calcium channels. At phase 3, calcium channels are inactivated and repolarization is caused by outward potassium currents. Repolarization is due to the currents carried mainly by the slow Iks and rapid Ikr components of the delayed rectifier potassium channels. The Ikr current is produced by hERG channel (encoded by the human ether-à-go-go-related gene). By contrast, inward potassium current contributes to the maintenance of the resting membrane potential, phase 4. B-D. Classification of ventricular (B), atrial (C) an pacemaker-like (D) action potentials. Ventricular action potential has a prominent plateau phase whereas atrial action potential is more triangularly shaped. Pacemaker-like cells are characterized by slower upstroke velocity and amplitude if compared to ventricular and atrial type of cells.

2.5.4 Excitation-contraction coupling

The calcium handling properties of hESC-CM have not been studied intensively. However, due to the few existing reports, hESC-CM possesses functional, albeit immature, calcium handling components when compared to adult cardiomyocytes (Dolnikov et al., 2006, Liu et al., 2007, Satin et al., 2008). For clinical applications, the calcium system should be functioning properly to hESC-CM to integrate properly after transplantation. Poor integration to the host myocardium could pose a threat of serious arrhythmias. In any case, a better understanding of calcium properties of the differentiated hESC-CM is needed.

2.6 Applications for human embryonic stem cell or induced pluripotent stem cell derived cardiomyocytes

2.6.1 Human cardiac cell/tissue model

Since the establishment of the first permanent hESC line (Thomson et al., 1998) there has been a great hope of replacing damaged heart tissue by hESC derived cardiomyocytes. However, many major problems need to be solved before hESC-CM are usable in clinics. Before clinical use becomes a reality, it is likely that the hESC-CM will be applicable for drug discovery and safety pharmacology applications (Braam et al., 2009). Nevertheless, cardiac differentiation and the beating cells are already a useful tool for developmental biology and to study the pathophysiology of human cardiac diseases. In addition, iPS technology enables the production of patient specific cell lines which extends the potential use even further.

2.6.1.1 Pathophysiology of cardiac diseases

Many cardiac diseases are caused by gene mutations or gene-enviroment interactions. So far, these severe diseases have been studied in animal models, especially with transgenic mice. Even though mouse models can yield valuable information, differences between human and mouse physiology limit the applicability of the results, for example the much faster beating rate of the mouse may override the effects of arrhythmias which would be severe for humans (Freund and Mummery, 2009).

Cardiomyocytes derived from genetically modified hESC could be used as a disease model. To construct a mutated hESC line and the disease model, the hESC line needs to be genetically manipulated. However, genetic manipulation of hESCs has proven to be more challenging if compared to mouse ES cells and only a limited number of reports of successful gene targeting and manipulation have been published (Braam et al., 2008, Giudice and Trounson, 2008).

To obtain disease specific lines, the genetic manipulation step can be circumvented by deriving the iPS cell lines from patients with genetic diseases (Park et al., 2008, Ebert et al., 2009, Freund et al., 2010). The differentiation of these model iPS cells to the desired cell type makes it possible to study the development and the pathophysiology of the disease. In addition, the factors affecting the development and the progress of the disease can be studied (Freund et al., 2010). However, iPS cell technology is still in its infancy and it remains to be seen if differentiated cells really manifest the disease phenotype of the mutation they carry and serve as a real disease model (Freund and Mummery, 2009).

2.6.1.2 Safety pharmacology and drug discovery

The heart has been proven to be very sensitive to the side effects of pharmaceutical compounds. Severe reactions, such as syncope, arrhythmia and

sudden death, related polymorphic ventricular tachycardia, torsade de pointes (TdP), have led to the refusal of approval or the withdrawal from the market of many pharmaceutical agents (Roden, 2004). In the absence of a complete understanding and direct analysis of TdP, the regulatory authorities have adopted the QT prolongation as a marker for the possible development of drug-induced TdP even though it is not a perfect marker for arrhythmogenesis (Finlayson et al., 2004). Prolongation of the QT interval resulting from a delay in ventricular repolarization, whether drug-induced or, for instance, congenital arising from mutation of genes (to date LQT1-12), may be associated with TdP (Roden, 2004, Zareba and Cygankiewicz, 2008), although the relationship is complex (Shah and Hondeghem, 2005). However, the QT interval is the cornerstone of the guidelines for the assessment of new chemical compounds in regard to proarrhythmic potential (ICH, 2005b, ICH, 2005a). Delayed rectifier potassium current (I_{Kr}) is responsible for the repolarization of the action potential and the channel protein is encoded by the human ether-to-go-go-related gene (hERG) (Vandenberg et al., 2001, Pollard et al., 2008). Inhibition of this hERG channel ($K_V11.1$) and the subsequent inhibition of the I_{Kr}, is the predominant basis of drug-induced QT prolongation and TdP (Redfern et al., 2003, Hancox et al., 2008). Currently a number of preclinical models and assays have been employed by pharmaceutical companies (Carlsson, 2006, Pollard et al., 2008). These assays include *in vivo* QT assays, such as ECG telemetry of conscious dogs (Miyazaki et al., 2005), and *in vitro* assays, such as repolarization assay, which detects changes in the action potential delay (APD) of cardiac tissues (isolated animal Purkinje fibres, papillary muscles or cardiac myocytes) or the hERG channel assay where hERG current expressed in heterologous cell system (such as CHO- or HEK293-cells) or native I_{Kr} is characterized (Finlayson et al., 2004, Martin et al., 2004).

Current methods are not fully adequate (Redfern et al., 2003, Lu et al., 2008). In addition, they are costly and the *in vivo* assays are ethically questionable because of the large number of animals used. Therefore there is a need for an *in vitro* method based on human cardiac cells that would bring additional value and reliability for testing novel pharmaceutical agents.

Cardiomyocytes derived both from hESC and iPS cells have many potential applications in the pharmaceutical industry including target validation, screening and safety pharmacology. These cells would serve as an inexhaustible and reproducible human model system and preliminary reports of the validation of hESC-CM system already exist (Braam et al., 2010). However, much optimization and development remains to be done, especially because of the immature phenotype of these cells and problems due to the differentiation efficiency, heterogeneous hESC-CM populations and enrichment methods (Braam et al., 2009).

2.6.2 Regenerative medicine

In principle, it would be possible to restore the function of the damaged heart by transplanting differentiated hESC or iPS cells. However, this may be one of the most challenging tasks to put into practice. The needed number of transplantable cells is high and they should be immunocompatible. In addition, the transplanted graft should integrate into the host myocardium and receive blood flow to remain vital, couple with host myocardium and contract in synchrony in response to the conduction system (Braam et al., 2009).

Using iPS cells as a cell source, immunomatched cells can be produced but current methods for reprogramming entail infecting the somatic cells with multiple viral vectors (Takahashi et al., 2007, Yu et al., 2007), which precludes consideration of their use in transplantation medicine at this time.

hESC-CM have been transplanted into healthy myocardium of rodents. The cells were reported to survive, form myocardial tissue and proliferate but they were usually separated from the rodent myocardium by a layer of fibrotic tissue (Laflamme et al., 2005, van Laake et al., 2007). When transplanted into infracted rat or mouse hearts, some beneficial effects for the function of the heart occurred (Laflamme et al., 2007, van Laake et al., 2007). However, after longer follow-up the positive effects were no longer present (van Laake et al., 2007, van Laake et al., 2008, van Laake et al., 2009). It is questionable whether these temporary benefits are due to the formed myocardium or paracrine effects, as has been proposed for adult stem cells.

Even though some information concerning transplantation can be obtained by using rodent models, studies with larger animals (pigs, goats and sheep) are warranted to give more accurate results regarding safety issues, electrical coupling and cardiac function. Usage of the iPS cells or ESC from the same species would eliminate the xeno barriers (Braam et al., 2009).

In addition to the above-mentioned issues, the timing of cell therapy and the delivery methods still needs to be determined. It is likely that cells need supportive material during transplantation and therefore biomaterial research is also needed before clinical studies can be properly designed (Passier et al., 2008).

3. Aims of the study

The objective of present thesis was to evaluate the differentiation of hESC and iPS cells to cardiomyocytes and to characterize the differentiated cells. In addition, the differentiation potential of several hESC lines was evaluated with two differentiation methods and the electrophysiological properties of the differentiated cardiomyocytes were determined. The specific aims of this study were the following:

I. To evaluate the cardiac differentiation capabilities of several hESC lines with two differentiation methods.
II. To study the spontaneous differentiation of different hESC lines into three germ layers
III. To study the effect of different human and mouse feeder cells used in hESC and iPS cell culture on cardiac differentiation
IV. To characterize the electrophysiological properties and maturation state of the differentiated cardiomyocytes

4. Materials and methods

4.1 Cell culture

4.1.1 Origin of cell lines and ethical approval

HS lines (HS181, HS237, HS293, HS346, HS360, HS362, HS368 and HS401) derived at the Fertility Unit of Karolinska University Hospital, Huddinge (Karolinska Institutet, Stockholm, Sweden) (Hovatta et al., 2003, Inzunza et al., 2005) were used in Studies I, II and IV. The derivation team had an approval from the Ethics Committee of the Karolinska Institutet for the derivation, characterization and differentiation of hESC lines. Regea Institute of Regenerative Medicine, University of Tampere, Finland has the approval of the Ethical Committee of Pirkanmaa Hospital District to culture hESC lines derived at the Karolinska Institute (Skottman R05051).

The Regea 06/015 and 06/040 cell lines (Rajala et al., 2010) used in Studies I and IV and Regea 08/017 cell line (Skottman, 2010) used in Study III were derived at Regea. The National Authority for Medicolegal Affairs has given the permission for Regea to perform research with human embryos (Dnro 1426/32/300/05). In addition, Regea has approval from Ethical Committee of Pirkanmaa Hospital District to derive, culture, characterize and differentiate new hESC lines (Skottman R05116). The embryos used in hESC line derivation were surplus embryos donated by couples undergoing *in vitro* fertilization treatments. Both partners have signed an informed consent form after receiving oral and written descriptions of the research. The donors did not receive any financial compensation.

H7 line used in Study III was purchased from WiCell Research Institute (Madison, WI, USA). The iPS cell line h106 used in Study III was derived at Regea from human foreskin fibroblasts (hFF) (American Type Culture Collection, Manassas, VA, USA). The Ethical Committee of Pirkanmaa Hospital District has granted approval for iPS cell research at Regea (Aalto-Setälä R0708).

4.1.2 Human embryonic stem cell culture (I-IV)

The hESC were cultured on a feeder cell layer of irradiated human fibroblasts (American Type Culture Collection, Manassas, VA, USA) in a medium (hES medium) consisting of Knockout Dulbecco's modified Eagle medium (KO-DMEM) (Invitrogen, Carlsbad, CA, USA), 20% Serum Replacement (SR) (Invitrogen), 2 mM GlutaMax (Invitrogen), 1% non-essential amino acids (Cambrex Bio Science Inc., Walkersville, MD, USA), 50 U/ml penicillin/streptomycin (Cambrex Bio Science Inc), 0.1 mM 2-mercaptoethanol (Invitrogen), and 8 ng/ml basic fibroblast

growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA). Colonies were passaged mechanically on a weekly basis.

In Study III, the Regea 08/017 and H7 lines were also cultured in similar culture medium as mentioned above on mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) treated mouse embryonic fibroblast (MEF) feeder cell layers (Millipore Billerica, MA, USA) and passaged enzymatically by collagenase IV (Invitrogen) on a weekly basis.

4.1.3 Human induced pluripotent cell culture (III)

h1/06 cell line has been derived from human foreskin fibroblasts (hFF) (American Type Culture Collection) using lentivirus infection followed by retrovirus infection of Oct4, Sox-2, Klf4 and c-Myc (Takahashi et al., 2007). In Study III, h1/06 cells were cultured both on hFF and MEF feeders similarly as described above.

4.2 Cardiomyocyte differentiation

4.2.1 Spontaneous differentiation in embryoid bodies (I, IV)

EB formation was performed by mechanically cutting the undifferentiated hESC colonies into small pieces and placing them on a U-shaped low attachment 96-well plate (Nunc, Roskilde, Denmark), one piece per well, in EB-medium (200 µl per well) consisting of KO-DMEM (Gibco Invitrogen, USA) supplemented with 20 % foetal bovine serum (FBS) (Gibco Invitrogen, USA), 1 % non-essential amino acids (Cambrex BioSciences, Verviers, Belgium), 1% L-glutamine (Invitrogen, USA), and 50 U/ml penicillin/streptomycin (Cambrex BioSciences, Verviers, Belgium). The hESC colonies were cut and detached in the same way as with normal passaging of hESCs, only the pieces were cut bigger, one cell colony split into 2-4 pieces. EBs cells were cultured on the 96-well plate for seven days and the EBs formed were plated onto 0.1 % gelatine type A coated (Sigma-Aldrich, Germany) cell culture plates in EB medium. The EBs were allowed to attach to the bottom of the well and the medium was changed three times a week. The cell cultures were checked daily for beating areas under a phase contrast microscope (Olympus, Tokyo, Japan).

4.2.2 Co-culture with mouse visceral-endoderm-like cells (II-IV)

Cardiomyocyte differentiation was carried out by co-culturing hESC with mouse visceral-endoderm-like (END-2) cells, which were kindly donated by Prof. Mummery, Humbrecht Institute, Utrecht, Netherlands. END-2 cells were cultured in END-2 medium consisting 1:1 Dulbecco’s modified Eagle medium and Ham’s F12 medium DMEM-F12 (Invitrogen) with 7.5 % fetal calf serum (FBS) (Mummery et

al., 1991). To initiate cardiomyocyte differentiation, undifferentiated hESC colonies were dissected mechanically into aggregates containing a few hundred cells and placed on the top of mitomycin C (Sigma-Aldrich) treated END-2 cells in hESC culture medium (described above) with 3 mg/ml ascorbic acid and without serum, serum replacement or bFGF (Passier et al., 2005). Differentiating cell colonies were monitored by microscopy daily (Olympus, Tokyo, Japan) and the medium was changed after 5, 8 and 12 days of culturing. After 16 days the 10% SR was added to the medium.

4.2.3 Estimation of cardiac differentiation efficiency (I-III)

In Studies I and II, the differentiation efficiency was calculated as a percentage of beating areas per total number of hESC colony pieces plated on END-2 cells or per total number of EBs.

In Study III, undifferentiated cell colonies were dissociated by scraping from the hFF feeders or from the cell culture plate after MEF feeder removal. The number of cell pieces plated on END-2 cells was 30. However, the plated material also included smaller colony pieces and single cells which could not be quantified. Therefore the total number of plated cell colony pieces could not be quantified as precisely as when cell colonies were cut into pieces and the same efficiency determining method could not be used. Instead, from all the six variable cell lines, similar numbers of colonies were plated onto END-2 cell plates and the differentiation efficiency was determined by the average number of beating areas per 12-well plate well. In addition, to support the differentiation efficiency data, the percentage of troponin T positive cells versus the total cell number (4',6-diamidino-2-phenylindole (DAPI) staining of nuclei) was determined after 21 days of END-2 differentiation. The cells from three wells of a 12-well plate were trypsinized (20 min. at +37ºC), resuspended in 5 ml of EB-medium with 15 % FBS. To standardize the analysis, the same wells were selected for dissociation from each cell line. The total number of cells was determined and 500 000 cells resuspended in a total volume of 12 ml with PBS were spun at 800 rpm for 5 minutes onto polysine slides (Thermo Scientific) by cytospin system (Cyto-Tech, Sakura). The cells were fixed and stained with anti-troponin T.

4.3 Morphology and size analysis of embryoid bodies (I)

The growth and morphology of the EBs was examined daily under a phase contrast microscope (Olympus, Tokyo, Japan). Pictures were taken from 10 EBs and the sizes were determined from the pictures during the suspension phase (seven days from the start of the differentiation protocol). The diameter of the EBs was measured manually, if the EBs were not round in shape, the average diameter was measured. To support this measurement, the cross-sectional area of each EB in the picture was determined using Cell^D imaging software (Olympus Soft Imaging

Solutions GmbH, Japan). The overall EB size for each cell line and each day was determined from the mean value of ten EBs.

4.4 Gene expression studies

4.4.1 RNA isolation and cDNA synthesis (I-III)

Total-RNA was isolated in Studies I, II with the RNAeasy mini plus kit including a DNAse treatment (Qiagen, Valencia, CA, USA) and in study III with NucleoSpin® RNA II kit including a DNAse treatment (Machery-Nagel, Duren, Germany). The concentration and quality of RNA was monitored spectroscopically (Nanodrop, Wilmington, DE, USA) and 0.2-1 µg of total RNA was transcribed to complementary DNA either by Sensiscript reverse transcriptase (Qiagen) for reverse transcriptase polymerase chain reaction (RT-PCR) or for quantitative PCR (qPCR) purposes by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

4.4.2 Reverse transcriptase-polymerase chain reaction (II)

The expression of β-actin (control), α- myosin heavy chain, atrial myosin light chain, ventricular myosin light chain, troponin T type 2, GATA-4, and connexin-45, Sox1, Brachyury T, BMP-4, AFP and KDR was determined by RT-PCR. Commercial heart RNA (Ambion, Austin, TX, USA) was used as a positive control. PCR reaction was performed using Phusion DNA-Polymerase (Finnzymes, Espoo, Finland) with 0.2 µM of primers and 1 µl of cDNA as a template. The amplification program included the initial denaturation step at 98˚C for 30 seconds followed by 32 cycles of 10 seconds at 98˚C, 30 seconds at 63˚C and 30 seconds at 72˚C. The PCR end products were separated in agarose gel containing ethidium bromide and visualized under UV light.

4.4.3 Quantitative polymerase chain reaction (I-III)

The gene expression levels of the pluripotency, germ layer and differentiation markers in differentiating hESC in END-2 co-cultures (Studies II and III) and during EB differentiation (Study I) were assessed by quantitative polymerase chain reaction (qPCR). qPCR was performed according to standard protocols on an Abi Prism 7300 instrument (Applied Biosystems, Foster City, CA, USA) by either Taqman or SYBR-green chemistry. C_τ values were determined for every reaction and the relative quantification was calculated with the $2^{-\Delta\Delta C_\tau}$ method (Livak and Schmittgen, 2001). The data were normalized to the expression of the housekeeping gene Peptidyl-prolyl isomerase G (PPIG), Ribosomal protein large P0 (RPLP0) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and one sample was

nominated as the calibrator. At least two biological replicates from each timepoint and line were analyzed as triplicates or duplicates. Results are shown as the average values of biological replicates.

4.5 Protein expression studies

4.5.1 Tissue multi-array (I)

In the Study I, the protein expression in the tissue sections from the differentiating EBs was assessed. The expression of pluripotency marker Oct4, different germ layer markers (mesodermal Brachyury T, endodermal AFP and ectodermal Sox1 and Paired box gene 6 (Pax6)) and the cardiac lineage marker Nkx2.5 were analyzed. In addition, caspase-3 was used to assess the number of apoptotic cells. EBs at the age of 2, 4, 6, 8, 10 and 12 days were collected (n=8) and fixed with 4 % paraformaldehyde for 2 hours. In addition, older EBs with and without beating areas were prepared for immunocytochemisty in a similar way. The samples were cryoprotected with 20% sucrose in PBS for several days. A tissue multiarray was prepared by punching 1mm holes in an inert medium as originally developed by Pelto-Huikko (Parvinen et al., 1992). The EBs were individually transferred to the wells filled with OCT compound. The multiarray was frozen on dry ice and 6µm frozen sections were cut throughout the chuck and thaw mounted on Polysine glass slides (Menzel, Braunschweig, Germany). Sections were stored at -70ºC until used.

Immunocytochemistry was performed using the *N*-Histofine® Simple Stain MAX PO staining method (Nichirei Biosciences Inc., Tokyo, Japan). Antibodies used were mouse anti-Oct4 1:200, goat anti-Sox1 1:500, goat anti-AFP 1:500 (Santa Cruz Biotech) and rabbit anti-Brachyury T 1:300 (Abcam), Nkx2.5 1:200 (R&D Systems), caspase-3 1:500 (Cell Signalling Tech., Danvers, MA, USA) anti-Pax6 1:300 (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA) and anti-cardiac Troponin T 1:50 (Abcam).

The sections were incubated overnight at 4°C with primary antibodies followed by appropriate *N*-Histofine staining reagent for 30 min. ImmPACT™ (Vector Laboratories, Burlingame, CA, USA) diaminobenzidine-solution was used as the chromogen. All antibodies were diluted in PBS containing 1% BSA and 0.3% of Triton X-100. The sections were briefly counterstained with hematoxylin, dehydrated and embedded in Entellan. Controls included omitting the primary antibodies or replacing them with non-immune sera. No staining was seen in the controls.

4.5.2 Immunocytochemistry (I-IV)

For the immunocytochemical stainings, the beating areas from the cell colonies (from END-2 or EB differentiations) were first cut with a scalpel and then dissected by collagenase II treatment (Mummery et al., 2003). Dissociated cells were plated on 0.1% gelatin coated 12- or 24-well tissue culture plates in a medium containing 7.5 % (FBS). Beating areas were dissociated from co-cultures 14-25 days after plating and fixed 4-7 days after dissociation with 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 20 min at room temperature (RT). Fixed cells were washed 2 x 5 min by PBS and permeabilized and blocked with 0.1% Triton X-100, 1 % bovine serum albumin (BSA) and 10 % normal donkey serum in PBS for 45 min at RT. The primary antibodies used (listed in the Table 3) were diluted to 0.1% Triton X-100, 1 % bovine serum albumin (BSA) and 1 % normal donkey serum in PBS. The cells were incubated with primary antibodies overnight (12-16 hours) at +4ºC. The primary antibody solution was removed and the cells were washed with 1% BSA in PBS.
Secondary antibodies used were either Rhodamine Red (Jackson Immuno Research Laboratories Inc., West Grove, PA) or Alexa Fluor-488 or -568 (Invitrogen) conjugated anti-mouse, anti-rabbit or anti-goat antibodies. Secondary antibodies were diluted into 1% BSA in PBS 1:400 or 1:800 and incubated for 1 hour at RT. Omitting the primary antibody and using only secondary antibodies in the immunocytochemical protocol resulted in the disappearance of all positive staining.
After secondary antibody incubation, the cells were washed in PBS 3-5 x 5 min and mounted in Vectashied mounting medium with DAPI. The immunostained cells were analyzed and documented by Olympus IX51 phase contrast microscope with fluorescence optics and with Olympus DP30BW camera.

4.5.3 Western blot (I)

EBs were collected at the age of 0, 3, 7, 10 and 20 days for protein isolation. Proteins were isolated by M-PER reagent (Pierce, Rockford, IL, USA) and the protein concentration was determined by BCA method (Pierce). Proteins were separated by 12% SDS-PAGE gel and transferred to PVDF-membrane (Hybond-P, GE-Healthcare, www.ge.com). Membrane was blocked with 2 % BSA (Sigma-Aldrich) overnight at +4 ºC. Primary antibodies used were anti-Oct4 1:100 (Santa Cruz Biotech.), anti-Brachyury T 1:400 (Abcam), anti-AFP 1:200 (Santa Cruz Biotech), anti-Sox1 1:400 (Abcam) and they were diluted to TBS-Tween. Beta-actin 1:1000 (Santa Cruz) was used as an endogenous control. Primary antibodies were incubated overnight at +4 ºC. Peroxidase-conjugated antibodies (1:4000) (Zymed, Invitrogen) were used for one hour at room temperature and ECLplus-kit (GE Healthcare) was used as the detection reagent. Exposure was done by CCD camera with Quantity One software (Biorad, Hercules, CA, USA).

4.6 Electron microscopy and immunoelectron microscopy (II)

For electron microscopy, the dissociated beating cells were fixed with 2.5% glutaraldehyde overnight and subsequently postfixed with 1% osmium tetroxoide for one hour. Cells were dehydrated and embedded in Epon. Ultrathin sections were counterstained with 1% uranyl acetate (30 minutes) and with lead citratate (5 minutes). For immunoelectron microscopy 22-day-old cells were fixed with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in PBS (0.2M) for 15 minutes. Immunocytochemistry was performed using the ABC-method (Vectastain ABC Elite Kit, Vector Laboratories). The cells were incubated overnight with a mouse monoclonal antibody to Troponin-I (Chemicon) (diluted 1:100 in PBS containing 1% BSA and 0.1% Saponin) followed by incubation with a biotinylated sheep anti-mouse antibody (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) and the ABC-complex for 60 minutes each. Diaminobenzidine was used as a chromogen to visualize the sites expressing Troponin I-immunoreactivity. Cells were postfixed with 2.5% glutaraldehyde (15 minutes) and 1% osmium tetroxide (30 minutes). The samples were dehydrated and embedded in Epon. Ultrathin sections were examined with a Jeol 1200EX electron microscope (Jeol USA, Paabody, MA).

Table 3. List of primary antibodies used in immunocytochemical studies.

Marker type	Antibody	Origin	Dilution rate	Supplier
Pluripotency	OCT3/4	goat	1:300	af1759,R&Dsystems
Pluripotency	Nanog	goat	1:200	af1997,R&Dsystems
Cardiac	Troponin I	goat	1:500	SC8118,Santa Cruz Biotech
Cardiac	Troponin T	mouse goat	1:500 1:1500	ab33589, Abcam ab64623, Abcam
Cardiac	α-actinin (sarcomeric)	mouse	1:1500	A7811, Sigma
Cardiac(ventricular)	ventricular-MHC	mouse	1:100	mab1552, Chemicon
Cardiac (atrial)	MLC-2a	mouse	1:300	311 011, SynapticSystems
Cardiac progenitor	Isl-1	mouse	1:500	DSHB
Mesoderm	Brachyury T	rabbit	1:100	ab20680, Abcam
Endoderm	α -fetoprotein (AFP)	goat	1:100	SC8108, Santa Cruz Biotech
Ectoderm	Sox1	goat	1:100	SC17318, Santa Cruz Biotech
Ectoderm	Pax 6	mouse	1:300	DSHB
Gap junction	Connexin 43	mouse	1:1500	mab3068, Chemicon
Gap junction	Connexin 40	rabbit	1:1000	ab1726, Chemicon
Proliferative cells	Ki67	rabbit	1:800	ab9260, Chemicon

4.7 Electrophysiological methods

4.7.1 Patch clamp (IV)

In Study IV, action potentials (APs) were recorded from dissociated beating cells using the whole-cell configuration of the patch-clamp technique with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and data acquisition and analysis was performed with pClamp 9.2 software (Molecular Devices). A coverslip with the adhering cells was placed in the recording chamber and perfused with extracellular solution consisting of 143 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES (pH 7.4 with NaOH; osmolarity adjusted to 301 ± 3 mOsm). The osmolarity was measured with an Osmostat OM-6020 osmometer (DIC Kyoto Daiich Kagagu Co. Ltd, Japan). Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Kent, UK) and had resistances of 1.5 to 3 MΩ when filled with a solution consisting of 130mM KCl, 7 mM NaCl, 1 mM $MgCl_2$, 5 mM Na_2ATP, 5 mM EGTA, 5 mM HEPES (pH 7.2 with KOH; osmolarity adjusted to 290 ± 3 mOsm).

To measure calcium currents the extracellular solution consisted of 137 mM TEA-Cl, 5.4 mM CsCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES (pH 7.4 with TEA-OH; osmolarity adjusted to 298 mOsm) and the intracellular solution consisted of 115 mM Cs methanesulfonate, 20 mM CsCl, 2.5 mM $MgCl_2$, 2 mM MgATP, 11 mM EGTA, 10 mM HEPES (pH 7.2 with CsOH; osmolarity adjusted to 273 mOsm). These recordings were undertaken in voltage-clamp mode with cell capacitance and series resistance compensated the latter by ≥70%. Calcium currents were elicited from a holding potential of –60 mV by 500 ms voltage steps from -50 to +40 mV.

Noradrenaline (Research Biochemicals Inc., Natick, Ma, USA) and E-4031 (Alomone Labs, Jerusalem, Il) were dissolved in water while veratridine (Sigma, St Louis, MO, USA) was dissolved in dimethyl sulphoxide, all to obtain stock solutions of 10 mM from which the final concentrations were prepared daily by dilution with extracellular solution. Experiments were conducted at 35.7 ± 0.1°C. The concentrations of the drugs were: 100 nm noradrenaline, 100 nM E-4031 (selective hERG inhibitor) and 10 µM veratridine.

4.7.2 Microelectrode array (II)

The electrical activity of dissociated beating cells was monitored using the Micro Electrode Array (MEA) system (Multi Channel Systems MCS GmbH, Reutligen, Germany). Dissociated cells were plated on FBS and gelatin coated MEA chambers (type: 500/30iR-Ti) and measurements were done at +37ºC. Signals were recorded for four minutes via every microelectrode. The sampling frequency was 20 kHz, and the signal frequency band was limited to 1 Hz – 8 kHz.

Chronotropic response characterization was performed using dissociated beating cells. The twenty-two-day-old cells were in a MEA chamber for 14 days before the addition of the pharmacological agents. The effect of verapamil, a specific L-type Ca^{2+} channel blocker (Verpamil, Orion, Espoo, Finland), was tested at a concentration of 5 µM (2.5 µg/ml). To increase the beating rate, a β-adrenoreceptor system activator, isoprenaline (Isuprel, SA Abbot NW, Ottignies, Belgium), was added 0.07 µM (0.017 µg/ml) to the cells. Finally, a cardioselective β-antagonist, esmolol (Breviblock, Baxter Healthcare Group, Deefield, IL, USA) at concentrations of 0µM, 43µM or 75 µM (0 µg/ml, 14.3 µg/ml, or 25.0 µg/ml) was tested without washing off the isoprenaline.

4.8 Statistical Analysis (I-IV)

From EB size analysis (I), qPCR (II and III), differentiation efficiency (III) and electrophysiological measurements (IV) the data is given as mean ± standard deviation (SD) of experiments. The statistical significance was ascertained using either one-way ANOVA test with Bonferroni correction or by the Student's t-test. The statistical analysis was performed with SPSS software (version 14-17) (SPSS Inc., Chicago, IL, USA).

5. Results

5.1 Analysis of undifferentiated pluripotent stem cells (I-IV)

Before differentiation, the undifferentiated state of pluripotent stem cells was verified by visual observation and by the expression of the pluripotency markers. In the Study III, after prolonged adaptation of H7 line to the hFF feeders, the expression level of germ layer markers (Brachyury T, AFP and Sox1) was elevated. Nevertheless, according to the immunocytochemistry, the cell colonies of these lines were still positive for pluripotency markers.
All hESC lines had a normal karyotype but iPS cell line h1/06 had an inversion on chromosome 12.

5.2 Spontaneous differentiation in embryoid bodies (I)

5.2.1 Formation and growth of embryoid bodies

The EB formation was performed by the similar method as for the hESC passaging, i.e. mechanical cutting of hESC colonies. The EBs were allowed to form in the U-shaped, low attachment, 96-well plate wells for seven days. In addition to a round-shaped EB, a thin cell layer formed on the bottom of the wells. On day 7, the EBs formed were transferred and allowed to attach to the gelatine coated cell culture plates.

The cross-sectional area (mm^2) of the forming EBs was determined for the first seven days of differentiation. The area of the EBs increased significantly during this time period in all the hESC lines studied (Study I, Table 1). After plating, the size of the original EBs did not change but rather asymmetric three dimensional outgrowth structures originating from them were formed. In addition, a layer-like structure formed finally covering (> 35 days) the whole 12-well plate well. No relevant differences in the EB morphology between hESC lines were observed before plating onto gelatine, but thereafter there was considerable variation in the cell proliferation and growth of the EBs (Study I, Supplementary Figure 2). Some hESC lines formed thick cell layers and outgrowths whereas some did not grow at all, only forming a thin cell layer on the bottom of the cell culture well. Occasionally three-dimensional cystic structures started to occur after plating to gelatine. Beating areas were observed only in well-growing EBs.

5.2.2 Pluripotency, germ layer and differentiation marker expression during embryoid body development

Protein expression in the tissue sections from the differentiating EBs was assessed by immunocytological stainings with pluripotency marker Oct4, with different germ layer markers (mesodermal Brachyury T, endodermal AFP and ectodermal Sox1) and with the cardiac lineage marker Nkx2.5. Because fragmented nuclei were seen in many developing EBs (Figure 7), caspase-3 staining was performed to assess apoptosis in the developing EBs. Additionally, RT-qPCR was used to assess the mRNA expression levels of the same markers and to confirm the immunocytochemical data, Oct4 and Brachyury T were also studied by Western blot analysis.

The protein expression of Oct4 started to decrease after the initiation of EB differentiation, but it was occasionally still clearly visible on day 8 or 10 and was also present in some EBs even on day 12 (Study I, Figure 1 and Supplementary Figure 4). Generally the staining decreased regularly from day 8. The expression could be seen in the middle of the EBs as well as on the edges of EBs (Figure 7). Overall, the expression pattern for Oct4 was similar in all the hESC lines studied. Western blot analysis confirmed that Oct4 was present at all time points studied, decreasing only on day 20. At the mRNA level, Oct4 was strongly expressed in the undifferentiated cells and the amount decreased so that on day 20 the expression level was very low.

The protein expression of Brachyury T was remarkably low in all the EB sections. Sparse stained cells were detected between days 4 and 12 in most of the EBs, while there were several Brachyury T stained-cells in only a few sections (Study I, Figure 3). Unexpectedly, hardly any Brachyury T positive cells were seen in the hESC line with the best differentiation efficiency (HS346). Confirmation of the protein expression of Brachyury T by Western blot showed fairly low expression at all time points (Study I, Figure 2). Brachyury T gene expression increased during the first week of differentiation and reached its highest level on day 7, decreasing steadily thereafter (Study I, Figure 2).

AFP protein was present in EBs of all ages. AFP protein expression was sporadic in some samples and abundant in others, but did not correlate with EB age or the hESC line (Study I, Figure 3). AFP staining was already detected on day 4 and did not markedly increase over time. AFP was expressed both in the middle and on the periphery of EBs (Figure 7). Expression was not clearly overlapping with Sox1 or Brachyury T but in the multiple EBs the same areas and even overlapping cells were labelled for AFP and Oct4 (Figure 7). AFP gene expression was detected on day 3 and was most highly expressed on day 20 (Study I, Figure 2).

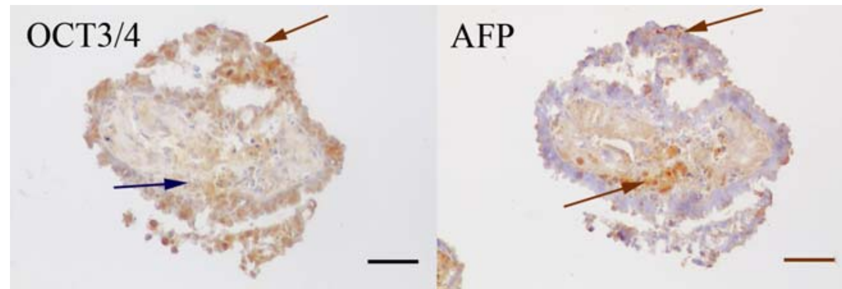


Figure 7. Oct4 and AFP protein expression in EB (brown arrows). Oct4 was partly overlapping with AFP. Fragmented nuclei were seen in many EBs (blue arrow). Scale bar 50µm.

Sox1 protein expression could first be detected in 4 to 6-day-old EBs as single positive cells (Study I, Figure 3). Clear positive areas were seen in the EBs from day 8 onwards. Some differences in the amount of Sox1 could be detected between the lines. The strongest positive staining was seen in HS362 and HS181 lines. In HS181 a small number of immunoreactive cells were already detected on day 4. In the hESC lines with the highest cardiac differentiation potentials, such as HS346, Sox1-positive cells were sporadic and no clearly positive areas were detected. The labelled areas did not overlap with any other germ layer marker or with Oct4 and the positive areas were located both at the edges of the EBs and in the middle of them. At the mRNA level, Sox1 was also most abundant around day 20 (Study I, Figure 2).

Protein expression of cardiac lineage marker Nkx2.5 was already detected in a few cells in the 4-day-old samples and the expression continued until day 12. No differences between hESC lines were observed (data not shown). Nkx2.5 gene expression increased during the first week and peaked around day 11 (Study I, Figure 2).

Caspase-3 immunoreactivity was observed throughout the study period. At the initial stage of EB development, staining was seen in the inner parts of EBs (Study I, Figure 4). In the older EBs caspase staining was not so intense and at the same time cavities begin to occur inside the EBs. However, caspase-3 labelling or cavity formation was not seen in all EBs (Study I, Figure 4 and Supplementary Figure 4). The cavity formation was not specific to any hESC line or age of EBs.

Older beating and non-beating aggregates (aged 30 to 60 days) were stained with Oct4, Brachyury T, Sox1, AFP, cardiac troponin T (cTnT), Pax6, and Nkx2.5 antibodies. Single Brachyury T-positive cells but no Oct4 or Sox1-positive cells were detected in these older aggregates (data not shown). Obvious distinct areas containing AFP, cTnT, and Pax6-immunoreactive cells were detected in beating aggregates (Study I, Figure 5). If no beating was detected, no cTnT positive cells could be observed either. AFP staining was relatively abundant and co-localized with cTnT-positive areas in some of the EBs but AFP also stained areas that were not cTnT-immunoreactive. Ectodermal Pax6 -positive cells formed separate areas without overlapping with other markers. Early cardiac marker Nkx2.5 was expressed sporadically and partly overlapped with cTnT-immunoreactive area. The size of the beating areas varied considerably between samples, some areas

containing only tens while others contained hundreds of cells. Clear striated patterns in the cells could be seen with cTnT staining (Study I, Supplementary Figure 5).

5.3 Differentiation in mouse visceral-endoderm-like cell co-cultures (II and III)

5.3.1 Morphology of differentiating cell aggregates (II)

The morphologies of the undifferentiated colonies of all eight hESC lines were similar. However, when differentiated, cell lines with good or intermediate cardiac differentiation efficiency formed more compact three dimensional structures on END-2 cells when compared to lines with lower efficiency (Study II, Supplementary Figure 1). This concurs with earlier results, as sharp-edged three dimensional structures have been reported to be conducive to the formation of beating areas (Passier et al., 2005).

5.3.2 Pluripotency, germ layer and differentiation marker gene expression levels during mouse visceral-endoderm-like cell co-culture

To investigate the gene expression of germ layer markers in lines HS181, HS293, HS346 and HS368, the mRNA levels of differentiation and germ layer markers were compared (Brachyury T, MESP1, Nkx2.5, Isl-1, BMP-4, KDR, AFP, Sox17 and Sox1) (Study II, Figure 1 and Supplementary Figure 2). Differentiating cell aggregates were pooled from four or six 12-well late wells at timepoints of 3 and 6 days. For every cell line, two cell pools were collected from separate differentiation experiments.

The expression of mesoderm marker Brachyury T was transient, peaking on day 3 in all four lines (Study II, Figure 1). The highest expression on day 3 was observed in HS346 when compared to HS293 and HS181 ($p<0.01$). The expression of cardiac mesoderm marker MESP1 and Brachyury T had similar expression patterns. In all lines MESP1 expression was higher on day 3 than on day 6.

In HS346, the Nkx2.5 mRNA level increased vastly from day 3 to day 6 and was more than three times higher on day 6 in HS346 compared to the other lines ($p<0.00005$) (Study II, Figure 1). mRNA levels of Isl-1, a cardiac progenitor marker, behaved differently from the other markers reportedly associated with cardiac differentiation. On day 3 Isl-1 was significantly more expressed in HS293 than in the other lines ($p<0.0005$) (Study II, Figure 1).

The expression of the mesodermal marker BMP-4 increased in all four lines from day 3 to day 6 and KDR, another mesoderm marker, was decreased in HS181, but also in HS346 (Study II, Supplementary Figure 2).

AFP expression increased significantly from day 3 to day 6 in HS293, HS346 and HS368 ($p<0.05$) similarly as was observed with HS181 cells. The expression of

another endodermal marker Sox17 did not differ between the four lines (Study II, Supplementary Figure 2).

The expression of the ectoderm marker Sox1 was lowest in HS346 on both day 3 and 6 and highest in HS181. There was also a significant difference in the expression of Sox1 between HS293 and HS346 on day 6 ($p<0.05$) (Study II, Supplementary Figure 2).

The expression of pluripotency marker Oct4, germ layer markers (Brachyury T, AFP, Sox1) were also evaluated during END-2 differentiation with the hESC lines Regea 08/017, H7 and iPS cell line h106 (Study III, Figure 2). To assess the effect of feeder cells on the cardiac differentiation, these lines were cultured both on hFF and MEF feeder cell layer.

Mesodermal marker Brachyury T expression was significantly higher on day 3 for H7 cultured on MEF than for H7 cultured on hFF ($p=0.021$). Similarly, the expression was at higher level for Regea 08/017(MEF) than Regea 08/017(hFF) ($p=0.002$). h1/06 behaved differently, having significantly higher expression when cultured on hFF than MEF feeders ($p=0.001$). H7(hFF) had a Brachyury T expression peak later if compared to H7(MEF) day 6 (Figure 8). A similar kind of expression curve was seen for Regea 08/017(MEF), having still relatively high expression at the same time point (Study III, Figure 2).

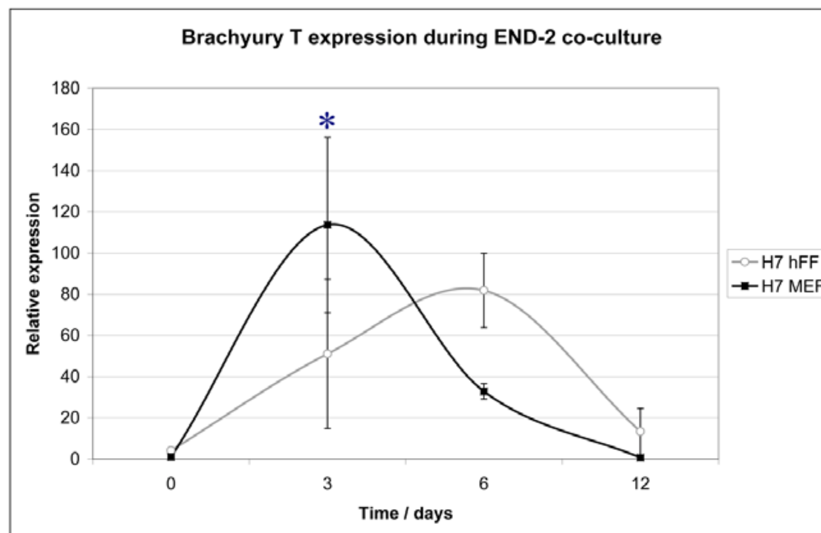


Figure 8. Brachury T mRNA levels during END-2 co-culture for H7 cultured on human feeder cells (hFF) (open circles) and cultured on mouse feeder cells (MEF) (closed circles). Expression peak for H7(hFF) is delayed when compared to H7 (MEF cultures. Expression difference was significant between samples as marked with *.

5.4 Cardiomyocyte differentiation efficiency (I, II, III)

In the Studies I and II, cardiac differentiation was performed with the same cell lines and by two differentiation methods. The differentiation efficiency varied between hESC lines with both the methods used. Overall, the efficiency was relatively low, the HS346 formed beating areas with the highest efficiency rate of 9.4% in END-2 co-cultures and 12.5% with EB differentiation. The line HS293 did not form any beating areas with either of the differentiation methods. The differentiation efficiencies are summarized in Table 4. Substantial variation in cardiac differentiation within individual lines was also observed, but this variation did not correlate with the passage number.

Table 4. Cardiac differentiation efficiency of hESC with END-2 co-culture and EB-method.

Cell line	**Karyotype**	**END-2 Differentiation efficiency %** (total number of beating areas/ total number of cell aggregates)	**EB Differentiation efficiency %** (total number of beating areas/ total number of EBs)
HS181	46 XX	**2.9** (18/625)	**1.6** (3/192)
HS237	46 XX	**2.7** (13/480)	**n.a**
HS293	46 XY	**0** (0/420)	**0** (0/192)
HS346	46 XX	**9.4** (299/2433)	**12.5** (24/192)
HS360	46 XY	**1.5** (7/480)	**1.0** (2/192)
HS362	46 XY	**3.2** (11/349)	**2.1** (4/192)
HS368	46 XY	**4.8** (46/968)	**n.a**
HS401	46 XY	**2.8** (24/867)	**7.3** (14/192)
Regea 06/015	46 XY	**3.3** (21/629)*	**7.3** (14/192)
Regea 06/040	46 XX	**5.1** (41/800)*	**12.0** (23/192)

*(Pietilä, 2008)

In the Study III, the cardiac differentiation potential of three lines (H7, Regea 08/017 and iPS cell line h1/06) cultured on both hFF and MEF feeder cells was compared. The H7 line had the highest differentiation rate and also the iPS cell line h1/06 formed more beating areas than the Regea 08/017 line. The number of Troponin T positive cells was significantly higher for H7(MEF) (36 122 cells) than H7(hFF) (14 482 cells) (p=0.008). For Regea 08/017 the number of cells in total and Troponin T positive cells was notably lower than for H7. In addition, the number of Troponin positive cells was slightly but not significantly higher for MEF (2 226) than for hFF (1 024) cultures (p=0.087). The differentiation data of this study is summarized in Table 5.

Table 5. Summary of the differentiation data of the lines Regea 08/017, H7 and iPS cell line h1/06. * The total number of cell aggregates could not be precisely determined. Therefore the estimation, 30 cell aggregates, was used to determine the differentiation efficiency.

Cell line	**Regea 08/017**		**H7**		**h1/06**	
Feeder type	MEF	hFF	MEF	hFF	MEF	hFF
Average number of beating areas/well	0.84	0.17	3.4	0.67	1.4	0.83
Differentiation efficiency % (total number of beating areas / total number of cell aggregates)	2.53*	0.56*	11.28*	2.22*	4.54*	2.78*
Average number of Troponin T positive cells/ in a well (SD)	2 226 (381)	1 024 (585)	36 122 (7023)	14 482 (7023)	9 021 (4014)	6 766 (1460)

5.5 Characterization of the differentiated cells (I-IV)

5.5.1 Expression of cardiac specific genes (II)

Differentiated cells expressed cardiac marker genes such as α- myosin heavy chain, atrial myosin light chain, ventricular myosin light chain, cardiac troponin T, GATA-4, and connexin-45 at levels comparable to commercial adult heart RNA (Study II, Supplementary Figure 3).

5.5.2 Structural characteristics (II)

In electronmicroscopy, the cardiac cells showed a variable degree of differentiation. In a large number of cells, bundles of myofibrils could be seen (Figure 9). In most of the cells bundles were randomly distributed, however in some cells, clearly differentiated sarcomeres with Z lines were seen (Figure 9). Intercalated discs were also seen between adjacent cells. Cells contained several

mitochondria, which were often situated close to sarcomeres and numerous polyribosomes. In addition, immunoelectron microscopy revealed troponin I-immunoreactivity in the myofibrils (Study II, Supplementary Figure 4).

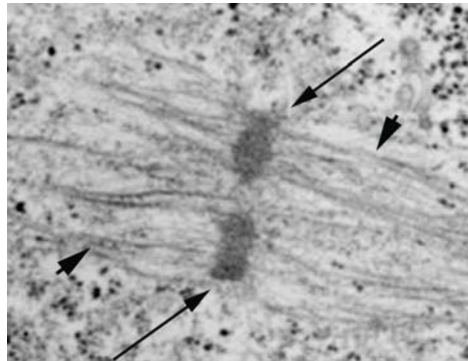
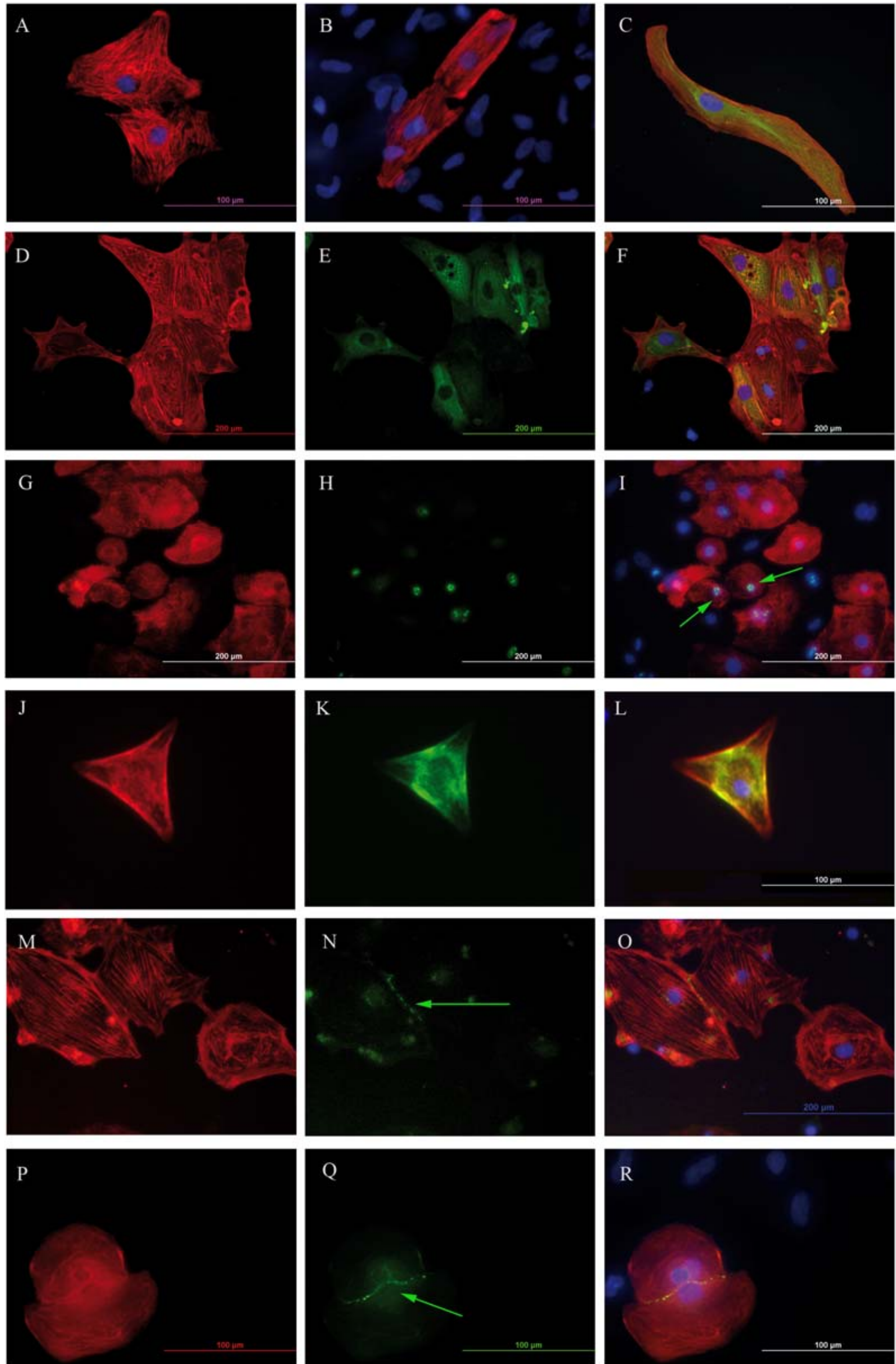


Figure 9. Electon microscopy figure of the hESC-CM. In the electron microscopy clearly differentiated sarcomere structures (arrowheads) with Z lines (arrows) could be seen.

5.5.3 Expression of cardiac specific proteins (I-IV)

The differentiated cardiac cells stained positively with several cardiac markers, including cardiac α-actinin, cardiac troponin T and I, ventricular α-myosin heavy chain, myosin light chain A, connexin-43 and connexin-40 (Figure 10). Connexin-43 and 40 were localized between the actinin or troponin positive cells, indicating that the beating cells have gap junctions between them. According to immunostaining with a Ki67 antibody (a marker for proliferating cells) some of the cardiac α-actinin positive cells were in the cell cycle. In addition, cTnT staining revealed a striatern pattern indicating sarcomere structures.

Figure 10. Characterization of differentiated and dissociated cells by immunocytochemistry. **A-B** Cardiac Troponin T (cTnT) (red) and nuclei stain dapi (blue), **C** cTnT (red), ventricular α-myosin heavy chain (green) and dapi (blue), **D-F** cTnT (red), α-myosin heavy chain (green) and dapi (blue), **G-I** cardiac α-actinin (red), Ki67 (green) indicating cardiac α-actinin-positive cells that are still in the cell cycle and dapi (blue), **J-L** cTnT (red), myosin light chain A (green) and dapi (blue), **M-O** cTnT (red), connexin 43 (green) revealing gap junction structures between the Troponin T positive cells, and dapi (blue) **P-R** cTnT (red), connexin 40 (green) again revealing gap junction structures between the Troponin T positive cells and dapi (blue). Differentiated beating cells have a striatern pattern typical for sarcomeric structures (A and B).


A
B
C
100 µm
D
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5.5.4 Functional characteristics of the differentiated cells (II and IV)

5.5.4.1 General action potential properties of human embryonic stem cell-derived cardiomyocytes (IV)

The basic electrical properties of cardiomyocytes obtained from different hESC lines were very similar and thus the results from different lines were pooled. The spontaneous electrical activity of hESC-CMs mostly exhibited a continuous pattern where AP rates were relatively constant throughout the recording period, with frequencies ranging from 24 to 156 beats per minute (bpm), mean 72 ± 6 bpm (0.4 – 2.6 Hz, mean = 1.2 ± 0.1 Hz). EB-derived cardiomyocytes were slightly more homogeneous since 97% of them were beating continuously, while 89% of END2-derived cardiomyocytes exhibited continuous firing pattern. The remaining hESC-CMs displayed firing patterns either of more than one frequency or episodic in nature (Study IV, Figure 2).

Based on the AP morphology and parameters, such as AP duration at 90% repolarization (APD_{90}), maximum rise of the AP upstroke (dV/dt_{max}), AP amplitude (APA) and maximum diastolic potential (MDP), the AP phenotype was classified as nodal-like, atrial-like or ventricular-like. Atrial-like APs were defined as those with triangular in shape whereas those APs with a significant plateau phase were categorized as ventricular-like (He et al., 2003b, Cao et al., 2008). None of the APs (from 69 hESC-CMs patched) fulfilled the criteria adopted for classification as nodal-like i.e. slow upstroke, prominent phase 4 depolarization, relatively depolarized MDP and small APA. Atrial-like APs were few as ventricular-like predominated (Study IV, Table 1), distinguished respectively by their triangular shape and significant plateau phase (Study IV, Figure 4). The beating rate of atrial-like cardiomyocytes was on average 48/min while the rate of ventricular-like cardiomyocytes was on the average 78 at least partly explaining the observed longer AP duration in atrial-like cardiomyocytes versus ventricular-like APs. All the atrial-type cardiomyocytes were obtained by END-2 differentiation method, while none of the EB-derived cardiomyocytes demonstrated atrial-like AP. The prevalence of ventricular type cardiomyocytes was 80% (28/35) and 100% (34/34) with END-2 and EB differentiation methods respectively.

Within the ventricular-like AP category there was heterogeneity in AP shapes and properties (Study IV, Figure 4). For instance the dV/dt_{max} ranged from 15.8 to 302.5 V/s. The APs exhibiting a fast upstroke ($dV/dt_{max} \geq 200V/s$) were associated with a significantly more hyperpolarized MDP compared to APs with low upstroke ($dV/dt_{max} \leq 100V/s$). The average MPD was less than -70 mV ranging from -55 mV to over -80 mV. The dV/dt_{max} was similar whether differentiation was via END-2 co-culture or spontaneously within the EB and between the various times in culture (Study IV, Figure 5). A difference was evident between the differentiation methods in terms of the MDP with the EB groups exhibiting a significantly more hyperpolarized MDP. Time in culture had no effect on MDP. For APD_{90} it appeared the culture time was a factor, though not significant, with the longest time for each differentiation method giving rise to shorter APD_{90} values.

5.5.4.2 *Human embryonic stem cell derived cardiomyocytes as cellular models of QT prolongation and proarrhythmia (IV)*

Addition of the selective hERG inhibitor E-4031 resulted in a marked prolongation of the hESC-CM AP (Study IV, Figure 6), especially for phase 3 as evident from the greater effect on APD_{90} compared to APD_{50}. Such slowing of repolarization leads to triangulation of the AP. APD_{90} prolongation was also observed with the inhibition of sodium channel inactivation by veratridine, which mimics the sodium current (I_{Na}) defect of congenital LQT3 and is another mechanism for drug-induced QT prolongation (Milberg et al., 2005). Furthermore the APD_{90} evolved from changes between APs in control, with E-4031 exposure, to successive lengthening and later to deviation from unity as successive large changes occur, corresponding to beat-to-beat variability i.e. APD instability.

The presence of the L-type Ca^{2+} current and EADs during prolonged E-4031 exposure in hESC-CMs was readily demonstrable. Another form of triggered activity, delayed afterdepolarizations, which take place after full repolarization, occurred on occasion spontaneously.

5.5.4.3 *Chronotropic response of human embryonic stem cell-derived cardiomyocytes (II and IV)*

Chronotropic response of hESC-CM was monitored with dissociated beating cells with the micro-electrode array (MEA) (Study II, Figure 3). Beating in the cardiomyocyte culture ceased approximately two minutes after the adding of 5 μM verapamil. Thereafter, the culture was washed with medium, and a resumption of consistent beating was observed both visually and electrically. Adding 0.07μM isoprenaline increased the average beating rate (ABR) by about 50% and this effect could be reversed by adding esmolol in a dose dependent manner. After the measurements, the culture was washed, and the reversibility of the drug effects was confirmed. According to on the MEA measurements, we also observed conduction of electrical activation between beating colonies.

Patch clamp studies revealed increase in the frequency of AP firing after exposure of hESC-CMs to noradrenaline (Study IV, Figure 3), concomitant with which was an increase in the diastolic depolarization rate and a decrease in AP duration.

6. Discussion

The aim of present thesis was to evaluate the differentiation of pluripotent stem cells (hESC and iPS cells) to cardiomyocytes and to characterize the differentiated cells. In addition, the differentiation potential of several hESC lines cultures on mouse and human feeder cells was evaluated. Differentiation was performed by two differentiation methods and in addition to multiple molecular biology characterization methods, the electrophysiological properties of differentiated cardiomyocytes were determined.

Pluripotent stem cells can be differentiated into functional cardiomyocytes, even though the differentiation potentials of cell lines differ from each other. Overall, the differentiation rate of the hESC lines used was low. However, commercially available line H7 differentiated more effectively likewise human iPS cell line derived in our laboratory. Differentiated cells from all the lines used were beating spontaneously and expressed specific cardiac markers.

6.1 Evaluation of cardiac differentiation capability of pluripotent stem cell lines (I-III)

In the Studies I-III the cardiac differentiation potential of several individual hESC lines and one human iPS cell line was evaluated and compared. The cardiac differentiation was performed using the END-2 co-culture and spontaneous differentiation method in EBs. Some of the lines were clearly more efficient than others in forming beating areas, but variation in cardiac differentiation potential within individual lines was also observed. One of the hESC lines, HS293, completely lacked the ability to form beating areas.

In the Studies I and II considerable variation was found in cardiomyocyte differentiation between hESC lines derived in the same laboratory and maintained with similar culturing and passaging methods. The differentiation efficiencies of the hESC lines used ranged from 0% to 9.4% in END-2 co-culture and 0% to 12.5% in EBs. The variation between lines was uniform with both of the differentiation methods used. It is noteworthy that line HS293 did not form beating cells with either of the differentiation methods. A similar finding was reported earlier with EB differentiation; hESC line hES2 (NIH code ES02) failed to produce beating areas spontaneously. However, this line produced cardiomyocytes when differentiated in co-culture with END-2 cells (Mummery et al., 2003, Moore et al., 2008).

Variation in the differentiation potential of individual hESC lines and iPS cell lines has been reported in numerous studies (Lee et al., 2005, Mikkola et al., 2006, Kim et al., 2007, Zhang et al., 2009). In addition, hESC-CM differentiation has been analyzed in many reviews comparing individual studies (Devine et al., 2001, Mummery, 2007), but usually only one hESC line has been used in the study (Xu et

al., 2002, He et al., 2003a, Pal and Khanna, 2007). The cardiac differentiation efficiency in these studies has varied enormously (8.1%-70%). In a recent report of 17 individual hESC lines (Osafune et al., 2008) the variation in cardiac differentiation potential was analyzed by comparing the expression levels of cardiac markers (Nkx2.5 and cTnT). The percentage of EBs containing cells positive for either cardiac marker was 10% (for the cell line with the lowest differentiation efficiency) and 44% (for the line with the highest differentiation efficiency), while the frequency of beating EBs was only 2.9% and 13.6% respectively (Osafune et al., 2008).

Another study compared the cardiac differentiation of four different hESC lines derived in three different laboratories and maintained with two different passaging methods (Burridge et al., 2007). The percentage of beating EBs varied between 1.6% and 9.5%. and the authors concluded that the variation was due to derivation in different laboratories (Burridge et al., 2007). Our results differ from these results. All our hESC lines were derived in the same laboratory and six of them were initiated with the same derivation method (Hovatta et al., 2003). Two of the lines (HS181 and HS237) were derived in FBS, but then transferred to serum replacement medium. Culture conditions cannot explain the differences either, since the all cells lines were cultured and passaged identically. It is noteworthy that HS293 (which lacked the ability to form beating cells) and HS346 (the line with the highest differentiation efficiency) were derived using exactly the same method, but showed the greatest difference in their cardiac differentiation potential.

The estimation and comparison of differentiation efficiencies is problematic, especially with END-2 co-culture, due to the technical dissimilarities between different laboratories. When undifferentiated hESC colonies were dissociated mechanically, in the present study the number of beating areas per total number of cell aggregates plated onto END-2 cells was compared. However, some of these aggregates are lost during medium change because they do not attach to the bottom of the cell culture well. One solution to this would be to count the attached cell aggregates after a few days of plating. This is very laborious and also rather difficult because some of the aggregates may be attached to each other, form flat cell areas on the bottom of the well or grow so that is impossible to accurately count the cell areas. For these reasons, to standardize differentiation 15 cell colony pieces were plated onto END-2 cells and this number was used in estimation the differentiation efficiency in the Study II.

In Study III another approach was used in the dissociation of undifferentiated cell colonies. Cell colonies were dispersed either by cell scraper or by scalpel because the enzymatically passaged undifferentiated cell colonies were much thinner making the cutting of colonies impossible. The colony pieces were not similarly sized and the cell suspension also contained smaller cell colony pieces and even single cells in addition to pieces similar to those used in Study II. Therefore the number of plated cell colony pieces could not be quantified as precisely as when the cell colonies were cut into pieces. To enable comparison of differentiation efficiencies between Studies II and III, the efficiencies were counted using the number of large cell colony pieces (30 cell pieces) to determine differentiation efficiency. However, a similar number of colonies were plated onto END-2 cells for all the six cell lines used in Study III and the differentiation efficiency was determined by the average number of beating areas per 12-well plate well. In addition, the percentage of troponin T positive cells from the total number of cells was determined.

The differentiation in co-culture with END-2 cells as originally described by Mummery and co-workers (Mummery et al., 2003) was reported to produce on average 32.7 beating areas per 12-well plate (2.7 beating areas per well) and corresponding to 16 600 cardiomyocytes (Passier et al., 2005). Since the starting numbers of cells was not stated in their work a comparison of results is difficult as discussed above. It is, however, evident that we could not achieve corresponding differentiation efficiencies with HS hESC lines and Regea lines. The best differentiation efficiency (HS346) was 9.4% corresponding to 17 beating areas per 12-well plate and 1.4 per well. The corresponding numbers for Regea 08/017 line cultured on hFF feeder cells were 0.56% corresponding to 2.04 beating areas per 12-well plate, 0.17 beating areas/well and 1024 cardiomyocytes/well. By contrast, H7 differentiated most efficiently on END-2 cells in the present study, having an efficiency of 11.28%, forming 3.4 beating areas per well. The number of cardiomyocytes was also in line with the report by Passier and co-workers mentioned above, H7 line formed 36 122 cardiomyocytes per well (3.3 % troponin T positive cells in a well of 1 100 000 cells in total).

Passier and co-workers reported a wide variation in the number of cells in the beating areas, ranging from 1-2500 cells (Passier et al., 2005). Therefore the number of beating areas per plated cell aggregates might give misleading results about the differentiation efficiency. Cytospin analysis has been used in the estimation of percentage of cardiac marker positive cells per total number of cells (Graichen et al., 2008, Xu et al., 2008a). This method gives a more accurate number of differentiated cells but unfortunately has its own weaknesses. Before spinning the cells to the class slides the cells have to be enzymatically dissociated into single cell stage. Older co-cultures especially contain solid cell aggregates which cannot be dissociated thoroughly and some cell aggregates remain and are lost before staining and analysis.

One major difference between many other cardiac differentiation reports and the present study is that here hFFs were used as feeder cells for hESC while others have used MEFs. Therefore we evaluated whether the culturing on MEF feeders increased the cardiac differentiation rate. The Regea 08/017 usually maintained on hFF feeder cells was also adapted to be cultured on MEF feeders and hESC line H7 and iPS cell line were adapted to be cultured on hFF feeders in addition to MEFs. Indeed, the adaptation and culturing on MEFs increased the differentiation rate of Regea 08/017 line slightly but not significantly. However, the differentiation efficiency of H7 was decreased with statistical significance after hFF culturing. The number of troponin T positive cells decreased from 36 122 MEF cultures to 14 482 on hFF. Therefore it can be suggested that feeder cells affect the differentiation efficiency.

Activin A may be one key factor affecting this phenomenon, as it is reported to be expressed at higher levels from MEF than hFF feeder cells (Eiselleova et al., 2008) and furthermore it is used in cardiac differentiation protocols (Laflamme et al., 2007, Yang et al., 2008). Activin A in combination with BMP-4 induces the formation of primitive streak-like population and mesoderm formation (Yang et al., 2008). Therefore, cells cultured on MEF feeder cells may be more prone to differentiate towards mesoderm and further towards cardiac lineage than hFF cultured cells. In addition, culturing on MEF feeders may also adapt hESC to mouse cells and therefore the plating on END-2 cells does not cause as much stress for the cells at the beginning of differentiation.

The gender and the karyotype of the hESC line have also been thought to play a role in determining differentiation capacity (Mikkola et al., 2006, Adewumi et al., 2007, Pal and Khanna, 2007). An hESC line having an altered karyotype (trisomy 12, 17 and XXX) has been reported to differentiate normally into cardiomyocytes (Pal and Khanna, 2007) while in another study, an altered karyotype was shown to affect differentiation in general (Mikkola et al., 2006). Neither gender nor karyotype can explain our results. We used both XX and XY cell lines with no correlation with cardiac differentiation potential. In addition, the karyotypes of our hESC lines were checked regularly and found to be normal. The iPS cell line h1/06 had an abnormal karyotype (inversion in chromosome 12) but the line differentiated normally into beating cells.

Our results support the suggestion that in addition to the culturing conditions, individual cells of the inner cell mass are the most critical factor for the basic differentiation capacity. Mechanical splitting of a single inner cell mass has been reported to produce multiple embryonic stem cell lines with dissimilar differentiation capacities (Lauss et al., 2005). Furthermore, two hESC lines have been shown to differ in their gene expression profiles of mesoderm and early cardiac markers at the undifferentiated state, but after cardiac differentiation both expressed cardiac markers and engrafted into mouse myocardium (Tomescot et al., 2007). In our cell lines the basal expression levels of germ layer markers were undetectable or low and did not differ between the lines. Thus, our results confirm the earlier report that basal germ layer expression levels do not explain the variation in differentiation potential. However, the expression of differentiation markers in the line H7 was higher after adaptation and culturing on hFF feeders. This may be due to the inefficiency of hFF feeders to maintain the pluripotent state of H7 compared to MEF feeders. The line 08/017 maintained the low expression of differentiation markers. The differentiation of H7 occurred at the stage where elevated expression was not yet seen, so therefore the effect of elevated differentiation marker expression on differentiation cannot be presumed.

The differentiation of human iPS cell line h1/06 emulated the differentiation of hESC lines. Differentiation efficiency was at a similar level, beating areas occurred on the same timescale and were morphologically alike. This is consistent with earlier reports where iPS cells have been differentiated to cardiomyocytes with END-2 and EB method (Zhang et al., 2009, Zwi et al., 2009, Freund et al., 2010).

6.2 Formation and structure of the embryoid bodies (I)

Many methods have been developed to control the number of cells at the initiation of EB formation and to scale up the differentiation process (Zandstra et al., 2003, Burridge et al., 2007, Bauwens et al., 2008, Niebruegge et al., 2009, Mohr et al., 2010). In the present study, enzymatic dissociation in combination with forced aggregation (FA) (Burridge et al., 2007) was not successful in producing viable EBs. The breaking of hESCs into a single cell stage is usually avoided in hESC handling (Thomson et al., 1998, Amit et al., 2000, Kehat et al., 2001, Xu et al., 2002). The hESC lines used have always been passaged manually and thus these lines may be more prone to the stress caused by enzymatic dissociation and subsequent centrifugation. Thus, EB formation was commenced by manual dissection of hESC, which is a laborious method and may produce more

heterogeneous EBs of varying sizes. However, the EBs were cut by the same researcher and in fact the size did not vary as much as with the enzymatic method (Bettiol et al., 2007, Burridge et al., 2007). The manual method mimics the microprinting technique (Bauwens et al., 2008), where the undifferentiated cells are at first printed into 2-dimensional colonies with a standard number of cells and then detached from the surface to form the three dimensional aggregate. Bauwens and co-workers found the status of initial cell population pivotal for cardiomyocyte differentiation (Bauwens et al., 2008). This is supported by our finding that the individual hESC line is more decisive for cardiomyocyte differentiation efficiency than the size of the EBs.

In addition to the input hESC population, EB size and morphology affect the differentiation pathways by influencing the spatial signalling, cell-cell interactions or the niche, of the cells. The shape and morphology of the EBs varied considerably, but no clear correlation between EB morphology and differentiation patterns was seen. However, all the germ layer markers were present in the EBs of all the hESC lines studied, but no clear consistent pattern in organization of germ layers was observed during these early stages.

Before the plating of EBs onto cell culture plate, there was no significant difference in EB size between the hESC lines. However, at later stages EBs from some lines were observed to grow better and looked more viable. Beating areas were only seen to form in these well-growing EBs. A similar phenomenon has been reported earlier when different culture methods have been used in cardiac differentiation. Methods that produced more viable and proliferative EBs also produced more cardiomyocytes (Yirme et al., 2008).

Due to the large number of fragmented nuclei seen in the EBs, caspase-3 staining for the samples was performed. Caspase-3, a key player in apoptosis, was already expressed in the 2-day-old EBs and continued through all the time period studied. Programmed cell death is a fundamental process throughout mammalian development (Jacobson et al., 1997) and, interestingly, caspase-3 has been shown to be crucial for mouse ES cell differentiation by inactivating nanog (Fujita et al., 2008). Expression of caspase-3 may also be related to cavity formation, which has been reported to occur due to apoptosis inside the EBs (Joza et al., 2001). Cavity formation, on the other hand, may be related to the regionalized differentiation of hESC (Itskovitz-Eldor et al., 2000, Conley et al., 2004). This is in line with results of present study, as cavity-like structures began to occur inside the EBs on day 4.

6.3 Expression of pluripotency, germ layer and differentiation markers during cardiac differentiation (I, II and III)

Many transcription factors and other proteins controlling germ layer and cardiac differentiation could serve as markers for identifying cardiac mesoderm formation and developing cardiomyocytes in a differentiating cell cultures. They could also be useful in studying the kinetics of differentiation. Hence, we hypothesized that the lines, which had a better intrinsic cardiac differentiation capacity, would have higher levels of the endoderm and mesoderm/cardiac mesoderm markers expressed during early differentiation stages. The expression of these markers was studied at mRNA level during END-2 differentiation and at messenger RNA (mRNA) and protein level in the EBs.

Brachyury T is an early mesoderm marker and has been reported to have an expression peak at day 3 during cardiac differentiation (Bettiol et al., 2007, Graichen et al., 2008, Osafune et al., 2008). Delayed mRNA expression peak of Brachyury T has been suggested to indicate the absence of early endodermal and mesodermal cell population, which could then lead to poor cardiac differentiation (Bettiol et al., 2007). During EB differentiation in our study, the gene expression of Brachyury T reached its peak around day 7 being therefore slightly delayed. In addition, the protein expression of Brachyury T was surprisingly low in all the hESC lines studied and there was no obvious difference in the expression between the hESC lines with different cardiogenic potential. Therefore it is possible that all our lines (Regea and HS-lines) resemble the poor mesodermal/cardiac lines, with low Brachyury T immunoreactivity, as suggested in the earlier report (Kim et al., 2007). This phenomenon is further supported by the fairly modest cardiac differentiation potential of our lines, line (HS346) with the highest differentiation efficiency having only about 10 % or less of the EBs with beating areas.

However, during END-2 differentiation Brachyury T expression was significantly induced in the line (HS346) compared to the other lines studied. Moreover, Brachyury T expression was higher on MEF cultures than hFF cultures of H7 and Regea 08/017 lines which was in line with the cardiac differentiation efficiency of these lines. As discussed, delayed Brachyury T expression could indicate poor cardiac differentiation ability (Bettiol et al., 2007) and the comparison of MEF and hFF feeder cells supports these results. H7 cultured on MEF feeder cells, had the highest differentiation efficiency and a sharp Brachyury T peak at day 3 whereas H7 on hFF gave a wider expression curve peaking later, at day 6, and also has less effective cardiac differentiation. Brachyury T expression was also increased for Regea 08/017 line after MEF adaptation in line with improved cardiac differentiation rate. However, the expression peak was not sharp, staying at the same level until day 6. Nevertheless, it can be concluded that Brachyury T expression level and the shape of the expression curve could serve as a predictive indicator for cardiac differentiation efficiency.

As the use of END-2 cells in cardiac differentiation indicates, visceral endoderm has an important regulatory role in cardiac development (Hosseinkhani et al., 2007). In the Study II, the levels of endodermal markers, especially AFP, were low in the

best cardiac line, HS346 contradicting to previous findings (Beqqali et al., 2006, Bettiol et al., 2007). However, day 6 was the last time point studied and according to Studies I and III, the AFP expression was markedly elevated after this time point. The expression level of AFP at day 12 for Regea 08/017 line cultured on MEF was significantly higher than when cultured on hFF feeder cells and the difference also reflected the differentiation efficiency.

In the EBs, the number of AFP expressing cells was relatively large at all time points and, interestingly, some co-localized expression of AFP and pluripotency marker Oct4 was detected. Expression of Oct4 in primitive endodermal cells has been reported earlier in mouse (Palmieri et al., 1994) as well as induction of primitive endoderm differentiation by increased OCT4 expression (Niwa et al., 2000). Thus it is possible that the endodermal cells in the EBs also expressed Oct4. In many older (>30 days) samples AFP was expressed in the vicinity of cardiac Troponin T-positive areas suggesting a possible interaction. Whether these areas with co-localized Oct4 and AFP protein expression could direct cardiomyocyte differentiation cannot be determined.

Even though the amount of Oct4 protein decreased slowly in the EBs and some cells were still positive on day 20, the mRNA level of Oct4 decreased during EB and END-2 differentiation rapidly as has been earlier reported (Adewumi et al., 2007).

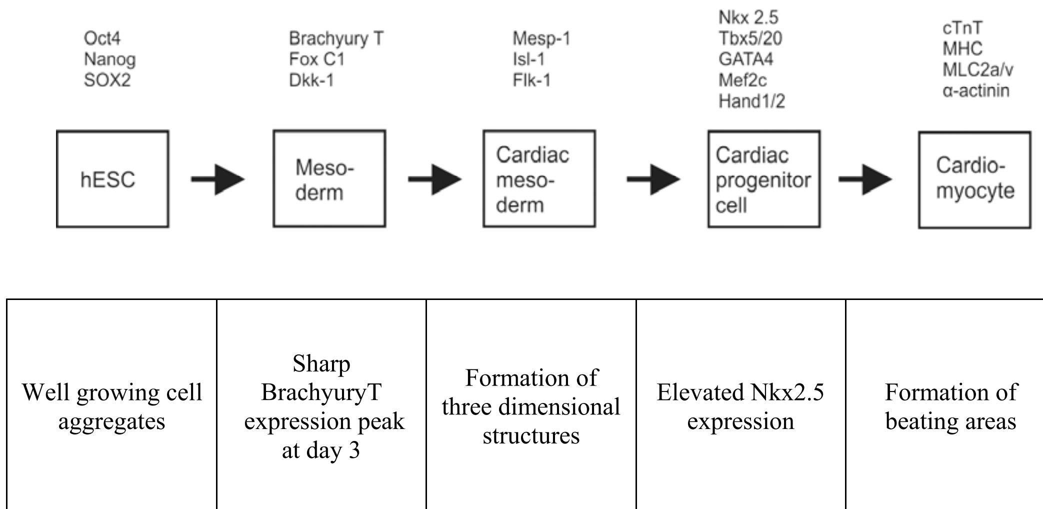
Ectoderm differentiation was studied with Sox1, which was the only marker showing obvious differences in the level of protein expression between the hESC lines during EB differentiation. In line HS181, which had relatively poor cardiac differentiation capability, Sox1-positive cells were seen in larger amounts during EB differentiation when compared to other lines with better cardiac differentiation capability. Interestingly, this strongly Sox1-positive line also has a good neurogenic differentiation ability (Lappalainen et al., 2010). Similarly, elevated Sox1 expression of HS181 was seen in END-2 differentiation (Study II). Taken together, these results, in combination with others, suggest that certain circumstances could support ectoderm or cardiomesoderm formation exclusively. These circumstances include medium composition, EB size or the intrinsic differentiation potential of individual hESC line (Bettiol et al., 2007, Kim et al., 2007, Graichen et al., 2008, Xu et al., 2008a).

By contrast, Sox1 gene expression does not explain the inability of HS293 to differentiate into cardiac cells since it was expressed similarly, at low levels, in HS293 and in HS346 (the line with highest differentiation efficiency) in the Study II. In addition, H7 had an exceptional expression curve in this respect. Even though H7 has the highest cardiac differentiation rate compared to all other lines studied here, Sox1 was significantly more expressed in H7 than in other lines during END-2 differentiation (unpublished data).

The expression of the cardiac lineage marker Nkx2.5 increases after 4 days of differentiation (Graichen et al., 2008, Hsiao et al., 2008) and this was clearly observed with both differentiation methods used in the present thesis. Nkx2.5 expression was significantly higher in HS346 on day 6 of END-2 differentiation when compared to the HS293, indicating more efficient cardiac lineage differentiation. However, at the protein level we saw no correlation with the cardiac differentiation capability. Osafune and co-workers have reported that Nkx2.5 expression could not necessarily determine the cardiac differentiation potential of their cell lines (Osafune et al., 2008). In addition, non-cardiac cells have also been shown to express Nkx2.5 (Hsiao et al., 2008) which was also supported by our

findings because in the older EBs (>30 days), Nkx2.5 overlapped only partly with cTnT expressing cells.

Isl-1 has been suggested to identify a cardiac progenitor population contributing to the majority of the cells in the heart (Cai et al., 2003). The expression of Isl-1 was unexpectedly highest on day 3 in line HS293, which did not form beating areas. However, the difference was no longer significant on day 6. It has been shown that Isl-1 expression increases continuously from day 3 to day 12 during END-2 co-culture (Beqqali et al., 2006) and thus it is possible that our timeframe was not sufficient to detect the highest levels in Isl-1 expression for HS293 and HS346. To support this, in lines Regea08/017, H7 and h1/06 the Isl-1 mRNA level increased after day 6 and reached highest levels at day 12 (unpublished data). The characteristics during the END-2 differentiation of the hESC line with a good cardiac differentiation potential are summarized in Figure 11.



Well growing cell aggregates	Sharp BrachyuryT expression peak at day 3	Formation of three dimensional structures	Elevated Nkx2.5 expression	Formation of beating areas

Figure 11. The cardiac differentiation cascade and the characteristics during cardiac differentiation in END-2 co-cultures of the hESC line with a good cardiac differentiation potential.

6.4 Characterization of differentiated cardiomyocytes (I-IV)

6.4.1 Molecular and structural characterization

Multiple molecular biology methods were used in analyzing the beating cells obtained from hESC and iPS cells. In addition to the markers for cardiac commitment (Nkx2.5 and Isl-1) mentioned above, differentiated cells were expressing cardiac specific genes and proteins such as cardiac troponin, α-actinin and myosin. Cardiomyocytes were connected to each other via gap-junctions as demonstrated by the presence of connexin-43 and 40 between cells. The striatern pattern of the cells could be seen with immunocytochemistry similarly in hESC and iPS cell-derived cells obtained by both differentiation methods used.

Organized and clear striatern sarcomeric structure was also evident from the presence of Z-bands as seen in electron microscopy. Various degrees of myofibrillar organization could be observed, which is characteristic of early stage cardiomyocytes (Kehat et al., 2001, Snir et al., 2003). These results indicate that regardless of the variation in the differentiation efficiency of hESC and iPS cell lines and the differentiation method, differentiated cells share characteristics similar to those of functional cardiomyocytes.

According to immunocytochemistry for Ki-67 (marker for proliferating cells), some of differentiated cardiomyocytes were still in the cell cycle and therefore not yet fully matured, as has been reported earlier (Snir et al., 2003). The observation that cardiomyocytes differentiated *in vitro* have not matured completely could be beneficial for future cell therapy experiments, since the immature cells might survive better and could still have the capacity to divide and be influenced by the surroundings in the recipient heart (Mummery, 2007).

6.4.2 Electrophysiological characterizations

The differentiated beating cells, in line with earlier studies (Kehat et al., 2002, Mummery et al., 2003), acted as a functional syncytium with cell-to-cell coupling between the hESC-CMs as was evident from the homogeneity of the AP frequency within beating areas and from connexin stainings. These findings were the same with both differentiation methods used (spontaneous differentiation in EBs and END-2 co-culture). The AP firing was usually constant, but in a few cells different firing patterns were observed confirming earlier findings (He et al., 2003b) which may be due to intermittent conduction block caused by structural discontinuities within the cellular network (Kehat et al., 2002).

According to the immunocytochemisty and action potential studies, most of the differentiated cardiomyocytes were ventricular type cells. A slight variation in subtype content was seen between different cell lines as has been reported by others (Moore et al., 2008) and a difference between the differentiation methods was also observed (80% and 100% of END-2 and EB-derived cardiomyocytes, respectively). The differentiation methods had affected AP properties. However, both of the methods resulted in cardiomyocytes with a great heterogeneity in their electrical properties with as many as about one third of them presenting a fairly mature phenotype. Unlike an earlier report (Sartiani et al., 2007) the AP-phenotype and maturity of the hESC-CM did not correlate with the age of the hESC-CM.

The ventricular phenotype and its heterogeneous properties are concur with earlier reports (He et al., 2003b, Mummery et al., 2003, Cao et al., 2008). The ventricular-like AP properties of hESC-CM previously reported have been mostly comparable to those of cultured human fetal myocytes, with MDPs of ~-50 mV and upstroke velocities of <30 V/s (Mummery et al., 2003, Sartiani et al., 2007, Cao et al., 2008), even though more mature cardiac phenotypes in hESC-CMs have also been reported (Satin et al., 2004). However, in present study as many as one third of hESC-CM exhibited a more mature phenotype with MDPs of < -70 mV and upstroke velocities >140 V/s. The demonstration of the presence of cardiomyocytes with dV/dtmax of over 150 and MDP of close to -80 is very important and suggests that mature human cardiac cells can be produced from hESCs, but the optimal conditions are still to be defined.

Corroborating earlier studies (Kehat et al., 2001, Xu et al., 2002, Mummery et al., 2003, Norstrom et al., 2006), the electrical activity and chronotropic response characterization revealed that the functional adrenergic mechanisms as well as L-type Ca2+ channels were present in cardiomyocytes derived from hESCs. In addition, according to the results of studies made with hERG blocker E-4031 and veratridine, hESC-CM could provide a sensitive cellular model to detect signals for proarrhythmic toxicity and antiarrhythmic efficacy.

Differentiated hESC-CM express endogenously the repertoire of cardiac ion channels, unlike the heterologous expression in transfected cells currently used in long QT assays by the pharmaceutical industry (Redfern et al., 2003, Pollard et al., 2008). The effects of new chemical compounds could therefore be investigated with those cells having native cardiac electrophysiological phenotype. However, the heterogeneous nature of differentiated hESC-CMs is an important limitation at the

moment. Therfore more defined and targeted differentiating methods are needed for obtaining more homogenous cardiomyocyte populations.

6.5 Future perspectives

The ultimate goal for stem cell research is to cure patients with diseases caused by the loss of functional tissue such as in chronic heart disease. However, it has become clear that pluripotent stem cell derived cardiomyocytes are not ready for the clinical use in the near future because a lot of development and basic research is still needed. Recent developments in the derivation of induced pluripotent cells in addition to more defined culture and differentiation methods, has turned the focus of research towards pluripotent stem cell derived cardiomyocytes to be used as a disease model and in the pre-clinical drug discovery and safety pharmacology applications.

Even though this goal is easier to achieve, much work is still needed. The cardiac differentiation is still inefficient and uncontrolled. Therefore effective methods for differentiation that supply homogenous populations of cardiomyocytes of sufficient quality, reproducibility and in large quantities are prerequisite for applications in the pharmaceutical industry as well as for clinical use.

The differentiated cardiomyocytes are a mixed population consisting of non-cardiac cells and cardiomyocytes with several subtypes and maturation stages. For studies of the pathogenesis disease or for testing new potential drug molecules the cardiomyocyte population should be of one subtype (e.g. ventricular) and of the mature, adult-like phenotype. In addition, for standardized testing and reliable research of the pathology of diseases, this is necessary because many diseases do not manifest the fetal staged cardiomyocytes.

Moreover, other non-cardiac cell types are present in the differentiated cell populations. This is not only a disadvantage because the target is a cardiac tissue model, other cell types such as fibroblasts and endothelial cells are needed. However, the population should again be standardized and composed of desired cells with right proportions. It is likely that cells cannot form three-dimensional tissue model structure by themselves and some biomaterial is needed to give cells support, attachment surfaces and nutrition.

The invention of induced pluripotent cells was a revolutionary step in stem cell research. However, iPS cell technology is in its infancy and needs a lot of development, for example in the induction step as well as in culture conditions. Defined culture conditions are still also under development for hESC. Due to the similarities of these cells, the development of defined culture conditions is beneficial for both pluripotent cell types.

Even though the pluripotent stem cell derived-cardiomyocytes are not suitable for clinical use in the foreseeable future and many obstacles have also to be overcome before they can be used in drug discovery, they offer tremendous opportunities for basic research, the pharmaceutical industry and for regenerative medicine.

7. Conclusions

The aim of this work was to evaluate the differentiation of hESC and iPS cells to cardiomyocytes and to thoroughly characterize the differentiated cells. Based on the four studies presented the following conclusions can be drawn:

- Significant differences between the cardiac differentiation potentials of different hESC lines were found even though the cell lines were derived and maintained in a similar fashion. This suggests that the embryo or the part of inner cell mass that contributes to the cell line could already be committed to certain lineages.
- Culture conditions have an effect on the cardiac differentiation capability of pluripotent stem cells. hESC maintained on MEF feeder cells had better cardiac differentiation potential than cells maintained on hFF cells. Furthermore, MEF feeder cells support the undifferentiated state of cells originally derived and maintained on MEF better than hFF feeder cells.
- The EB formation as well as the temporal and spatial organization of germ layers took place in a fairly similar manner during early EB differentiation. All germ layer markers were present in all lines, but the EB structure was not very organized and no obvious distinct trajectories were formed. The cardiac differentiation potential of hESC lines could not be predicted by the number or localization of early germ layer marker proteins or by the morphology of EBs in the early stages of differentiation.
- Despite the wide variation in the cardiac potential of hESC lines, cardiomyocytes produced from these lines with two different differentiation methods express proper cardiac markers and have properly functioning L-type Ca^{2+} channels and a β-adrenoreceptor system indicating that these cells could be used as a cell model for human cardiomyocytes in pharmacological, toxicological and cell therapy trials in the future.
- hESC-CMs exhibited electrophysiological heterogeneity, but fairly mature adult cardiac phenotype could also be detected. The study using two differentiation methods produced similar cardiac cell heterogeneity. The demonstration of cells with fairly mature electrical phenotype, however, suggests that with more specific and detailed differentiation methods, mature and more homogenous cardiomyocyte cultures could be obtained.

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