

Influence of pluripotent stem cell culture conditions on cardiac differentiation

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PREFACE

This research project was carried out at the Institute of Biomedical Technology and Institute of Biosciences and Medical Technology (BioMediTech), University of Tampere. I would like to thank my supervisors Marisa Ojala and Kristiina Rajala for all the help. I would also like to thank my family, mom and dad, and Eetu Raivio for supporting me and being my side throughout the whole time.

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ABSTRACT

Background and aim: In culture the aim is to keep stem cells in an undifferentiated state as round-shaped with uniform quality and to prevent spontaneous differentiation. Feeder cells that are generally used in stem cell culture provide important factors which induce stem cell proliferation and prevent spontaneous differentiation. Three different culture conditions were used in this study: 1) mouse embryonic fibroblast cells (MEFs) feeder cells with KSR medium, 2) SNL feeders clonally-derived from a STO cell line expressing both G418 resistance and leukemia inhibitory factor (LIF) with KSR medium and 3) Matrigel matrix which is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells with commercial mTeSR1 medium. The stem cell lines were cultured in these different conditions and then subjected to cardiac differentiation which was repeated three times. The aim of the study was to evaluate the differentiation of pluripotent stem cells (hESC and iPS cells) to cardiomyocytes and to characterize the differentiated cells.

Methods: The efficiency of cardiac differentiation is defined by quantifying troponin T positive cardiomyocytes using Cytospin method, by counting the beating areas and by evaluating the size of cardiomyocyte clusters. In addition the morphology of cardiomyocytes is evaluated by immunocytochemical staining. Further the expression of certain genes typical for either stem cells or cardiomyocytes was determined on samples collected on days 0, 3, 6, 13, 30 during cardiac differentiation by quantitative PCR analysis.

Results: The differentiation rate of the cell lines used was low. Commercially available line H7 differentiated more effectively than UTA.00112.hFF and UTA.04602.WT lines. The analysis with q-PCR showed positive gene expression of stem cell markers and some expression of cardiac markers in the differentiated cardiomyocytes with each tested culture conditions and cell lines.

Conclusions: Pluripotent stem cells cultured on MEF feeder cells were most efficiently differentiated to cardiomyocytes. Pluripotent stem cells from Matrigel matrix produced less beating cardiomyocytes. After this study, the understanding of the effects of culture condition in the differentiation of cardiomyocytes can be used in future in studies to improve cardiomyocyte differentiation.

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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Soluviljelyssä tavoitteena on pitää kantasolut erilaistumattomina. Kantasolujen tulisi kasvaa yhtenäisen näköisinä ja pyöreäreunaisina kolonioina ja niiden spontaani erilaistuminen tulisi estää. Kantasolujen viljelyssä yleensä käytettävä tukisolukko erittää tärkeitä tekijöitä, jotka aikaansaavat kantasolujen jakautumisen ja lisääntymisen ja estävät erilaistumista. Tässä tutkimuksessa käytettiin kolmea erilaista kasvatusmenetelmää: 1) hiiren embryonaaliset fibroblasti-solut (MEF) tukisolukkona KSR soluviljelynestessä, 2) SNL-tukisolukko, joka on klonalisesti STO-solulinjasta erilaistettu ja joka ilmentää sekä G418 resistenssiä että leukemia inhibitorista tekijää (LIF) KSR soluviljelynestessä ja 3) Matrigel alusta, joka on gelatiinia sisältävä Engelbreth-Holm-Swarm (EHS) hiiren sarkooma solujen erittämä proteiini-sekoitus. Matrigel alustan kanssa soluviljelynesteenä käytettiin kaupallista mTeSR1:tä. Kantasolulinjoja viljeltiin näissä eri olosuhteissa kunnes ne erilaistettiin sydänlihassoluiksi. Sydänlihassolu-erilaistus suoritettiin kaikille olosuhteille ja linjoille kolme kertaa. Tutkimuksen tarkoituksena oli arvioida pluripotenttien kantasolujen; ihmisen alkion kantasolujen (hESC-solut) ja indusoitujen kantasolujen (iPS-solut), erilaistumista sydänlihassoluiksi. Lopuksi erilaistettujen sydänlihassolujen ominaisuuksia tutkittiin eri tavoin.

Tutkimusmenetelmät: Sydänlihassoluksi erilaistamisen tehokkuutta arvioitiin troponiini T-positiivisia sydänlihassoluja laskemalla käyttäen cytospin-menetelmää. Lisäksi laskettiin kasvatusalustalta sykkivät alueet, arvioitiin niiden kokoa ja morfologiaa. Erilaistamattomia ja erilaistettuja soluja värjättiin immunosytokemiallisin menetelmin. Tyypillisten kantasoluissa sekä sydänlihassoluissa esiintyvien geenien ilmentymistä määritettiin päivinä 0, 3, 6, 13 ja 30 sydänlihassolu-erilaistamisen aikana käyttämällä kvantitatiivista PCR-analyysiä (qPCR).

Tutkimustulokset: Käytettyjen solulinjojen erilaistumisaste oli matala. Kaupallinen H7-solulinja erilaistui tehokkaammin kuin solulinjat UTA.00112.hFF ja UTA.04602.WT. qPCR-analyysissä ilmentyi kantasolu-geenejä. Sydänlihassolumarkkerien ilmentymistä oli kaikilla solulinjoilla kaikista olosuhteista vaihtelevissa määrin.

Johtopäätökset: MEF-solujen päällä viljeltyt pluripotentit kantasolut erilaistuivat parhaiten sydänlihassoluiksi. Matrigeelillä viljeltyt pluripotentit kantasolut taas muodostivat vähemmän sykkiviä sydänlihassoluja. Tutkimuksesta saatavaa tietoa kasvatusolosuhteiden vaikutuksesta sydänlihassolujen erilaistamiseen voidaan käyttää jatkotutkimuksissa parantamaan sydänlihassolujen erilaistamisen tehokkuutta.

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ABBREVIATIONS

Ct	Cycle threshold
bFGF	basic fibroblast growth factor
BM	bone marrow
BMP	bone morphogenic protein
BSA	bovine serum albumin
CM	cardiomyocyte
c-Myc V-myc	myelocytomatosis viral oncogene homolog
cTnT	cardiac troponin T
GFP	green fluorescent protein
DAPI	4,6 diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMEM/F12	Dulbecco's modified Eagle's medium: nutrient mixture F-12
DMSO	Dimethyl sulfoxide
EB	embryoid body
EDTA	Ethylenediaminetetra acetic acid
END-2	mouse visceral-endoderm-like cell line
FBS	Fetal bovine serum
FDA	Unites States Food and Drug Administration
ES	embryonic stem cell
hESC	human embryonic stem cell
hESC-CM	human embryonic stem cell-derived cardiomyocytes
hFF	human foreskin fibroblast
HSC	hematopoietic stem cell
ICM	inner cell mass
iPS cell	induced pluripotent stem cell
Klf4	kruppel-like family transcription factor 4
KO-DMEM	Knockout™ -Dulbecco's modified Eagle's medium
KO-SR	Knockout™ serum replacement
LIF	Leukemia inhibitor factor
MAP-2	Microtubule-associated protein 2
MEF	Mouse embryonic fibroblast
MHC	myosin heavy chain
MSC	mesenchymal stem cell
Nanog	Nanog homeobox
NEAA	Non-essential amino acids
NF-68	Neurofilament 68
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
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
SNL	feeder cells clonally-derived cells from a STO cell line
SOX	SRY sex determining region Y-box
SR	serum replacement
TGF-β	transforming growth factor β
TRA	Tumor-related antigen, keratan sulfate-related antigen
VEGF	vascular endothelial growth factor
vMHC	ventricular myosin heavy chain
WT	Wild type

1 INTRODUCTION

Ischemic heart disease is the leading cause of mortality in the western world. Cardiomyocytes derived from human pluripotent stem cells (hPSCs) could be useful in restoring heart function after myocardial infarction or in heart failure (Mummery et al., 2003). The ultimate goal for stem cell research is to cure patients with diseases caused by the loss of functional tissue such as myocardial infarction (Pekkanen-Mattila et al., 2009). Regenerative medicine aims to repair the heart by cardiomyocyte replacement but this cannot be reached in the near future. First challenge is to develop strategies to differentiate and isolate cardiomyocytes more efficiently in vitro (Mummery et al., 2003).

Before clinical use of differentiated cardiomyocytes becomes reality, it is likely that the pluripotent stem cell derived cardiomyocytes would be applicable for disease modeling, drug discovery and safety pharmacological applications (Pekkanen-Mattila et al., 2009). Before pluripotent stem cells can be applied clinically, it is important to control their growth and differentiation. Mouse embryonic stem cells respond to cues within the mouse embryo to differentiate to all somatic tissues. If these cues and the signal transduction pathways they activate could be identified, this knowledge could be used to control differentiation of stem cells in culture and in vivo (Mummery et al., 2003).

Human stem cells are classified according to their origin and their differentiation potential. Human stem cells can be classified into four different groups: embryonic- (ESCs), fetal-, adult- and induced pluripotent stem cells (iPSCs). ESCs are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro (Thompson et al., 1998). Human ESCs and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. One major difference between adult and ESCs is their different abilities in the number and type of differentiated cell types they can become. ESCs can become all cell types of the body because they are pluripotent. Adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin. iPSCs are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of ESCs (Winslow, 2001).

Undifferentiated human pluripotent stem cells have the potential for unlimited self-renewal with normal karyotype. They also have the ability to differentiate into cells of all three germ layers – endoderm, mesoderm and ectoderm. Human cells with pluripotent characteristics were initially derived from the inner cell mass of pre-implantation blastocysts and termed human embryonic stem cells (hESCs) (Takahashi et al., 2007). Pluripotency pertains to the cells of early embryos that can generate all of the tissues in the organism. ESCs are embryo-derived cell lines that retain pluripotency and represent invaluable tools for research into the mechanisms of tissue formation. The discovery that human somatic cells can be reprogrammed by the transient overexpression of small number of genes into iPSCs revolutionized the whole field. iPSCs resemble functionally and phenotypically hESCs. This raises the possibility that cellular therapies using patient-specific cells may be a reality in the future (Park et al., 2008).

hESCs have been successfully derived from the inner cell mass (ICM) of blastocysts and can be maintained *in vitro* for prolonged periods of time. Culturing of hESCs still requires MEF cells, either as a feeder layer or as a source of conditioned medium when the cells are grown on Matrigel-coated plastic (Richards et al., 2002). The culturing of hESCs and hiPSCs is demanding and requires daily media changes, observation of growth and passaging at least once a week.

In this study the goal was to compare the effect of different stem cell culture conditions on cardiac differentiation. It is hypothesized that different culture conditions may affect the efficiency of cardiac differentiation and properties of the resulting cardiomyocytes. Three pluripotent stem cell lines used in this study were: human embryonic stem cell line H7 and human induced pluripotent stem cell lines UTA.00112.hFF and UTA.04602.WT.

2 Review of the literature

2.1 Stem cells

Human stem cells are classified according to their origin and their differentiation potential into four different groups: embryonic stem cell- (ESC), fetal-, adult- and induced pluripotent stem cells (iPS cells) (Thomson et al., 1998).

The fertilized egg, the zygote, is defined as totipotent, because it gives rise to a complex organism. At the blastocyst stage, only cells of the inner cell mass (ICM), ESCs derived from the ICM, and iPS cells derived from somatic cells retain their capacity to differentiate into all three primary germ layers, the endoderm, mesoderm, and ectoderm, and are defined as pluripotent (Twomey, 2007). The cells of each primary germ layer are committed to generating cells from multiple, but still a limited number, of lineages. These cells are called multipotent.

The three embryonic germ layers include the endoderm which forms the gastrointestinal tract. The mesoderm gives rise to the connective tissue, smooth and striated muscles, bones, cartilage, blood, and the most of the internal organs. The ectoderm gives rise to the nervous system and the epidermis including neural epithelium, embryonic ganglia, and stratified squamous epithelium (Wobus and Boheler, 2005; Choumerianou et al., 2008; Thomson et al., 1998).

2.1.1 Fetal and adult stem cells

Fetal stem cells have been isolated from fetal tissues or from supportive extra embryonic structures of fetal origin like amniotic membrane or placenta (In 't Anker et al., 2003). Fetal stem cells exhibit several features of ESCs, such as the expression of stem cell markers and their ability for self-renewal (Guillot et al., 2007).

Adult stem cells can be isolated from somatic tissues. The major advantage of adult stem cells is that they can be used in autologous therapies (Mimeault et al., 2007). Bone marrow contains two types of stem cells. In addition to haematopoietic stem cells, the most characterized and widely-used types of adult stem cells are the stromal mesenchymal stem cells (MSCs). MSCs can give rise to a large variety of specialized

mesenchymal tissues including bone, fat and connective tissues (Pittenger et al., 1999). Within the last decade adult stem cells have been identified also in other organs and tissues like in brain, dental pulp and heart (Mimeault et al., 2007). Adult and fetal stem cells are both multipotent stem cells but adult stem cells have even more restricted differentiation potential.

Comparing human pluripotent and adult stem cells, they both have advantages and disadvantages regarding potential use for cell-based regenerative therapies. One major difference between adult and pluripotent stem cells is their different abilities in the number and type of differentiated cell types they can become. Pluripotent stem cells can become all cell types of the body. Adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin (Doss et al., 2004).

Pluripotent stem cells can be grown relatively easily in culture. Adult stem cells are rare in mature tissues, so isolating these cells from an adult tissue and the large scale expansion in culture is challenging. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies. Tissues derived from embryonic and adult stem cells may differ in the likelihood of being rejected after transplantation. Adult stem cells, and tissues derived from them, are currently believed less likely to initiate rejection after transplantation. This is because a patient's own cells could be expanded in culture, differentiated, and then reintroduced into the same patient (Doss et al., 2004).

2.1.2 Human embryonic stem cells

Human ESCs are generally derived from ICM of 5-day-old blastocyst of mammalian embryo and are capable of unlimited, undifferentiated proliferation *in vitro* (Thomson et al., 1998, Reubinoff et al., 2000). Couples undergoing *in vitro* fertilization treatments donate excess or poor quality embryos for research and these are used to create hESC lines (Skottman et al., 2006). Human ESC lines differ from each other a lot. This is because of the differences of how and when isolated and of individual allelic differences. Each hESC line has different growth rate, differentiation capacity and karyotypic stability (Carpenter et al., 2009).

The undifferentiated state of hESCs can be monitored based on the morphological characteristics of the cells. The basic characteristics are high nucleus to cytoplasm ratio, prominent nucleoli and a distinct colony morphology (Carpenter et al., 2009). The human ESC lines express high levels of telomerase activity. Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length, which plays an important role in replicative life-span. This explains ESCs nearly unlimited self-renewal (Thomson et al., 1998).

The hESC lines express characteristic cell surface markers including stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumor-related antigen (TRA)-1-60, TRA-1-81, and alkaline phosphatase. Also several transcription factors, including POU domain transcription factor Oct3/4, HMG-box transcription factor SOX2, and homeobox protein Nanog, which are functioning in the maintenance of pluripotency in both early embryos and ESCs. Several genes that are frequently upregulated in tumors, such as Stat3, c-myc, Klf4 and b-catenin, have been shown to contribute to the long-term maintenance of the ESC phenotype and the rapid proliferation of ESC in culture (Takahashi and Yamanaka, 2006). In addition hESC-specific characteristics include unique cell cycle with a shortened G1 phase (Stead et al., 2002) and pluripotency.

Human ESC may be used to treat diseases, such as Parkinson's disease, spinal cord injury, and diabetes (Thomson et al., 1998). However, there are ethical difficulties regarding the use of human embryos, as well as the problem of tissue rejection following transplantation in patients. Also the technical limitations of hESC culture, efficient differentiation of hESCs, the risk of teratoma formation and the potential immunogenicity of hESCs are problems yet to be solved before clinical application of hESCs. After transplantation, pluripotent stem cell based therapies involve the risk of tumor formation, such as teratomas which comprise all three germ layers and arise from an undifferentiated population of the transplanted cells (Carpenter et al., 2009). Teratoma formation can be tested *in vivo* when transplanted into severe combined immunodeficient (SCID) mice (Thomson et al., 1998). Also the immunosuppressive capacity of the adult stem cells may favor the growth of the tumor cells (Carpenter et al., 2009).

2.1.3 Human induced pluripotent stem cells

Generating pluripotent stem cells directly from cells obtained from patients has been one of the ultimate goals in regenerative medicine. These iPS cells are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of ESCs. Mouse iPS cells were first reported in 2006 (Takahashi and Yamanaka, 2006), and human iPS cells were first reported in late 2007 (Takahashi et al., 2007). Although these cells meet the defining criteria for pluripotent stem cells, it is not known if iPS cells and ESCs differ clinically in significant ways. Pluripotent stem cells can be directly generated from somatic cells by the addition of only a few defined factors (Takahashi and Yamanaka, 2006). iPS cells are an appealing option of regenerative medicine, as no embryos or oocytes are required for their generation (Yamanaka, 2007).

Somatic cells can be reprogrammed by transferring their nuclear contents into oocytes or by fusion with ES cells indicating that unfertilized eggs and ES cells contain factors that can confer totipotency or pluripotency to somatic cells. The factors that play important roles in the maintenance of ES cell identity also play important roles in the induction of pluripotency in somatic cells. Oct3/4, Klf4, Sox2, and c-Myc play important roles in the generation of iPS cells. Oct3/4, Sox2, and Nanog have been shown to function as core transcription factors in maintaining pluripotency (Boyer et al., 2005). Among these three, Oct3/4 and Sox2 are essential for the generation of iPS cells. Nanog, on the other hand, is dispensable. Also tumor-related c-Myc and krupper-like family of transcription factor Klf4 have been identified as essential factors. The c-Myc protein has many downstream targets that enhance proliferation and transformation (Adhikary and Eilers, 2005), many of which may have roles in the generation of iPS cells. Klf4 has been shown to repress *p53* directly (Rowland et al., 2005). iPS cells show levels of p53 protein lower than those in MEFs (Takahashi and Yamanaka, 2006). In addition to MEFs also other cell types have been used to produce iPS cells. Human dermal fibroblasts (hDFs) can be reprogrammed to iPS cells (Takahashi et al., 2007). Transcription factors Oct-4, Sox2, Nanog and Lin-28 also induce pluripotency in human foreskin fibroblasts (hFFs) (Yu et al., 2007). Other starting cell types tried so far around the world include T cells, keratinocytes, adipose stem cells, mesenchymal stem cells, dental pulp stem cells, hepatocytes, amniotic fluid cells, cord blood cells, neural stem

cells and germline cells (González et al., 2011). The pluripotency induction efficiency may differ between cell types and the degree of differentiation. Cell types with endogenously high expression levels of one or more of the factors that induce pluripotency, may be reprogrammed with only a subset of factors (de Souza, 2010).

Any reprogramming experiment is determined by a number of preliminary choices regarding the donor cell type to be reprogrammed, the factors to use and the mode of their delivery. Delivery modes include MMLV-derived retrovirus which is moloney murine leukaemia virus and composed of an ssRNA genome replicating through a DNA intermediate that integrates into the host genome. MMLV infects only actively dividing cells. Other modes of delivery include lentivirus, adenovirus, RNA, transposon, episomal vector or some small molecule or protein. The vector, in which the reprogramming cDNA is cloned, provides a viral packaging signal, as well as transcription and processing elements. On transfection into a packaging cell line that expresses a specific viral envelope protein and which determines the range of cell types that can be infected, and also replication-defective viruses are produced; they can infect donor cells with efficiencies of up to 90%. Although they are efficient and reproducible, reprogramming using viruses entails the production of potentially harmful viral particles that express potent oncogenes such as MYC (González et al., 2011).

In **Figure 1**, one way of reprogramming somatic cell has been shown schematically. Such induced pluripotent human cell lines could be useful in addition of the production of new disease models and in drug development, also as well for applications in transplantation medicine, once technical limitations (for example, mutation through viral integration) are eliminated (Yu et al., 2007).

Human iPS cells exhibit many features characteristic of hESCs including morphology, marker expression and differentiation potential, therefore also the culture requirements are similar (Takahashi and Yamanaka, 2006).

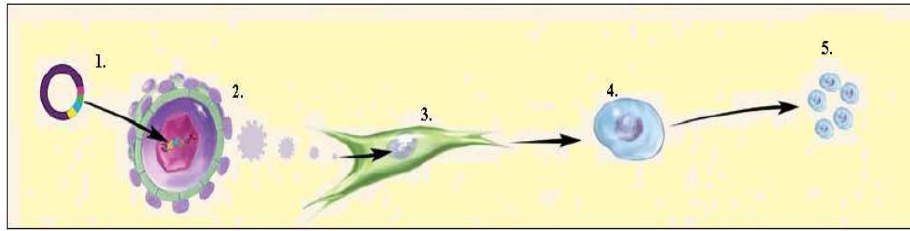


Figure 1. Generating induced pluripotent stem cell (iPS cell). General method for creating iPS cell is to use a circular DNA plasmid which is carrying reprogramming factors (1.). These factors are carried into the nucleus of a somatic cell (2.). This somatic cell is then reprogrammed and it changes from an elongated fibroblast-like shape to a rounded, immature type of cell (3.-4.). Finally, if the cell is cultured like hESC, a pluripotent iPS cell line is produced (5.). Picture modified from the picture of Terese Winslow, 2008, *The Promise of Induced Pluripotent Stem Cells (iPSCs)* by Charles A. Goldthwaite.

2.2 Culture of stem cells

Basic techniques to culture hESCs and hiPS cells are well established, although limitations remain in many of these procedures. In particular, many existing cell lines have been cultured using mouse embryonic fibroblast (MEF) feeder cells or human feeder cells and serum (Thompson et al., 1998, Xu et al., 2001, Amit et al., 2003).

An important advancement in the field is the definition of culture conditions that support the proliferation and culture of hESCs and hiPS cells without the need for feeders or animal-derived components (Ludwig et al., 2006). An optimal culture system would include an appropriately buffered medium that contains metabolites, cytokines and growth factors required for self-renewal and survival of pluripotent cells as well as a culture matrix that supports cell growth (Lu et al., 2006).

Members of the transforming growth factor-beta (TGF- β) super-family and the fibroblast growth factors (FGFs) have emerged as important regulators of pluripotent stem cell self-renewal (Xu et al., 2001). The FGF family, consisting of at least 22 ligands, mediates its effects through various isoforms of four distinct cell-surface FGF receptors (FGFRs1–4). Exogenous FGF-2 is routinely used for the culture of undifferentiated pluripotent stem cells suggesting an important role for FGFs in the regulation of stem cell fate (Amit et al., 2004).

Despite the addition of exogenous factors that manipulate the activation of the FGF and TGF- β pathways, the local cellular microenvironment and hence the signaling inputs

varies significantly in stem cell maintenance cultures. In fact, it is likely that stem cells are exposed to a wide range of signaling environments by virtue of the properties of stem cell colonies (size, distribution and culture condition-specific associated differentiated cells), and individual stem cell position in a particular colony (Peerani et al., 2007).

Most culture conditions result in some level of unwanted spontaneous differentiation of hESCs. While some spontaneously differentiated cells usually appear at the margin and at the centre of hESCs colonies, an ideal culture condition provides growth support with minimal amounts of differentiated cells.

2.2.1 Feeder cell-dependent culture conditions

The first animal-free culture system for hESCs replaced traditionally used MEFs with human feeder cells of fetal or adult fallopian tube-epithelial cell origin. However, the culture medium used was supplemented with 20 % human serum and had issues with batch-to-batch variability due to its undefined nature (Richards et al., 2002). Feeder cells support self-renewal either by providing necessary factors or by removing inhibitory factors. Human feeder cells secrete transforming growth factor beta 1 (TGF β 1), Activin A, basic fibroblast growth factor (bFGF), and low levels of bone morphogenic protein 4 (BMP4), while mouse feeder cells secrete comparable levels of TGF β 1 and BMP4, higher levels of Activin A, and no bFGF (Prowse et al., 2007).

Primary cultures are usually established using FBS-containing media and passaged with enzymes such as trypsin. In the culture of MEFs or other feeder cells such as SNLs, fetal bovine serum (FBS) or knock-out serum replacement (KO-SR) containing culture medium are used (Lu et al., 2006). The development of a commercially available serum replacement, KO-SR was a major advancement in the establishment of a serum alternative (Price et al., 1998). KO-SR supplemented with bFGF supports the prolonged growth of hESCs in an undifferentiated state, with a higher growth rate and cloning efficiency than in FBS-containing medium (Amit et al., 2003). MEF-conditioned medium, produced by harvesting spent culture medium from MEF cell cultures, also support self-renewal of stem cells.

Human pluripotent stem cells can be propagated extensively in culture while retaining the ability to differentiate into somatic cell types. In recent years, extensive

investigation into improving culture systems for stem cells has yielded three main advances: 1) the ability to grow cells under serum-free conditions; 2) the maintenance of the cells in an undifferentiated state on Matrigel matrix; and 3) the use of either human embryonic fibroblasts, adult Fallopian tube epithelium, or foreskin fibroblasts as feeder layers. Exposure to animal pathogens through MEF-conditioned medium or Matrigel matrix is a possibility; human feeder layer-based culture systems require the simultaneous growth of both feeder layers and hES cells; and the culture system cannot be accurately defined due to differences between the various feeder layer lines or the use of conditioned medium (Amit et al., 2003).

More efficient feeder systems, such as different types of human feeders (human fetal and adult fibroblast feeders) or immortalized mouse feeders, have been developed and tested for replacement of the laborious and inconsistent preparation of traditional MEF feeders (Améena et al., 2008).

SNL feeder cells are established by Dr. Allan Bradley, and are clonally derived from a mouse fibroblast STO cell line transformed with neomycin resistance and murine leukemia inhibitory factor (LIF) genes. SNLs can be used as a feeder cells for ES cell growth and in mouse or human iPS cell culture. The feeder cells must be mitotically inactivated prior to the addition of ES or iPS cells, such as treatment with mitomycin C (2-4 hr, 10 µg/mL) or by irradiation.

2.2.2 Feeder cell-free maintenance method

Several groups have developed culture conditions for hESCs that are, to various degrees, serum- and feeder-free. The first feeder cell-free maintenance method for existing hESC lines were cultures on Matrigel using MEF conditioned medium consisting of animal component-containing serum replacement and bFGF that allowed hESCs to be cultured without direct contact with feeders (Xu et al., 2001). Other feeder-independent culture has been reported using extracellular matrix and a combination of transforming growth factor beta (TGFβ) and bFGF (Amit et al., 2004).

mTeSR2 is a serum-free medium that supports derivation and long-term feeder-independent culture of hESCs and hiPSCs. The formulation of mTeSR2 includes high levels of bFGF together with transforming growth factor beta (TGFβ), aminobutyric

acid (GABA), pipecolic acid, and lithium chloride. The original publication describes the use of cell support matrix composed of four human components (collagen IV, fibronectin, laminin, and vitronectin). In an effort to reduce the cost of this defined system for everyday research, Ludwig and colleagues formulated Matrigel suitable medium mTeSR1, which does include some animal-sourced proteins yet retains the advantages of being fully-defined and serum-free and supports the self-renewal of hESCs without requiring feeder cells (Ludwig et al., 2006).

FGF signaling has a central role in sustaining hESCs. bFGF is less stable in serum-free medium compared to medium conditioned by fibroblasts, which in part explains the use of the high concentrations of bFGF present in mTeSR1. Heparan sulfate proteoglycans modulate the stability and activity of FGFs by forming high affinity binding complexes, but at high concentrations, FGF proteins can signal through direct interaction with FGF receptors, and the requirement for these complexes can be circumvented. In the future, the addition of appropriate heparan sulfate proteoglycans to mTeSR1 may further improve the culture of hESCs and reduce the required bFGF concentrations. At lower concentrations of FGFs, both activin and TGF-beta have clear strong effects on hESCs and based on inhibitor studies, it has been suggested that TGF-beta/Activin signaling is essential for hESCs self-renewal.

hESCs cultured in TeSR1 or mTeSR2 are smaller than cells cultured on fibroblasts. Four to five million cells per well of a 6-well plate is not uncommon just before splitting, and because of this increased density, cultures must be fed daily to assure adequate nutrition. This increased density (and growth rate) may also account for the increased importance of passage timing. If cells are split just a day too late, they can differentiate at passage and cannot be rescued. When culturing hESCs on fibroblasts or conditioned medium, cells can be passaged once a week, maintaining a consistent split regimen, and the exact day of passage is less critical. Using TeSR1 or mTeSR1, however, cells must be observed daily and split when the appropriate density is achieved, generally every 4–5 days (Ludwig et al., 2006).

2.3 Cardiac differentiation

Pluripotent stem cells have the ability to differentiate towards all three germ layers and furthermore can, in principle, give rise to all cell types of the body. There are several different methods described for cardiomyocyte differentiation. Currently cardiomyocytes can be differentiated from pluripotent stem cells by 1) spontaneous embryoid body (EB) differentiation in suspension, 2) co-culture with mouse endoderm-like cells (END-2 cells) or 3) guiding the cardiac differentiation with defined growth factors either in suspension or in monolayer culture. Recently it has been indicated that different cardiac differentiation methods produce cardiomyocytes having distinct properties (Rajala et al., 2011).

Cardiomyocytes can be differentiated spontaneously as cell aggregates called embryoid bodies (EB) (Kurosawa, 2007). During EB formation the culture condition for stem cells is changed from two-dimension into three-dimensional structure. Pluripotent stem cells are either enzymatically or mechanically dissociated to small cell clusters and allowed to form aggregates in suspension. Soon these aggregates start to differentiate towards three germ layers. The paracrine and the endocrine signaling determine the fate of the stem cell. Similarly as in embryo this signaling may lead to the formation of concentration gradient in the EBs and further influence the cell differentiation (Sachlos and Auguste, 2008). Functional properties of cardiomyocytes can be detected within mixed EB population. Interactions between the cells stimulate the expression of markers for mesodermal and early cardiac cell lineages (Tran et al., 2009). The spontaneous differentiation in embryoid bodies is rather inefficient, usually under 10%, and is also highly cell line dependent (Kehat et al., 2001).

More directed way to differentiate cardiomyocytes from pluripotent stem cells is in co-culture with mouse endodermal-like cells (END-2), particularly in an absence of serum and with ascorbic acid (Goumans et al., 2007). Co-cultures are then grown for up to 4 weeks and counted for the presence of areas of beating cardiomyocytes (Xu et al., 2006). Beating cardiomyocytes usually begin to emerge within 12-20 days of co-culture with END-2 cells (Mummery et al., 2003). END-2 cells secrete mostly unknown cardiac differentiation inducing factors. Molecular pathways leading to specification and terminal differentiation of cardiomyocytes from embryonic mesoderm during

development are still unclear. Data derived from chick and amphibian suggested that cardiac progenitors require interaction with anterior endoderm and possibly the organizer for myocardial differentiation to take place. Visceral endoderm has been identified as a cellular source of signals that result in hESC differentiating to cardiomyocytes with characteristics of fetal ventricular, atrial, or pacemaker cells (Xu et al., 2006). Based on phenotype and electrophysiology, generally pluripotent stem cell-derived cardiomyocytes resemble human fetal ventricular cardiomyocytes (Mummery et al., 2003). However, the cardiac differentiation efficiency from standard END-2 co-culture experiments is usually fairly low.

Combinations of BMP4, Wnt3a and Activin A induce gastrulation-like events and meso-/endoderm development in pluripotent stem cells (Xu et al., 2006). It has been suggested that signals from the endoderm, such as bone morphogenic proteins (BMPs), fibroblast growth factors, and repressors of Wnt signaling, may be important for cardiac development (Mummery et al., 2003). Use of these factors is called the guided cardiac differentiation. In this differentiation approach, undifferentiated pluripotent stem cells are cultured under feeder cell-free conditions. Cardiac differentiation is induced with growth factors, such as BMP2, BMP4, Activin A, bFGF, and Wnt3a. At the moment new cellular signaling molecules which induce pluripotent stem cell cardiogenesis are tried to find. Examples of these include cardiogenols, ascorbic acid, isoxazolyserines, sulfonyl hydrazones and DMSO (Willems et al., 2009). All of these molecules were found to upregulate late-stage markers of cardiogenesis, even though the reason for this is not always understood. Some of the molecules have clear effects on cells such as DMSO and ascorbic acid, others are more selective. A guided cardiac differentiation has been carried out for example using Activin A and BMP4. In the study which is based on a high-density monolayer, pluripotent stem cells were cultured in a feeder cell-free system. Cardiomyocyte differentiation was induced by a defined serum-free medium and supplemented sequentially with BMP4 and Activin A. Spontaneously contracting areas were generally observed approximately 10 days after induction with Activin A, and typically in the final dissociation step there were >30% cardiomyocytes of all the cells (Laflamme et al., 2007).

In theory iPS cells can be directed to differentiate into a specified lineage that will support treatment or tissue regeneration. Somatic cells from a patient with

cardiovascular disease could be used to generate iPS cells that could be directed to give rise to functional adult cardiac muscle cells i.e. cardiomyocytes that could replace diseased heart tissue. However, much have to be studied concerning the differentiation process before any clinical experiments can be done. iPS cells created from human fibroblasts can give rise to functional cardiomyocytes that display hallmark cardiac action potentials.

2.3.1 Characterization of differentiated cardiomyocytes

Morphology of pluripotent stem cell-derived cardiomyocytes can be used to characterize the cells. Differentiated cardiomyocytes from pluripotent stem cells have less defined rod shape compared to their mature adult counterparts. In addition, pluripotent stem cell-derived cardiomyocytes display multinucleation under 1 % frequency compared to 20 % of adult human cardiomyocytes (Snir et al., 2003). In general, cardiomyocyte induction from pluripotent stem cells results in mixtures of ventricular-like, atrial-like, and pacemaker-like cells defined by intracellular electrophysiological measurements of action potentials (Mummery et al., 2003). The cardiac phenotype of iPS cell-derived cardiomyocytes seems to be comparable to that of hESC-derived cardiomyocytes (Takahashi et al., 2007).

Oct-3/4 and Nanog are gene markers at the ES cell state. Their expression should be high on day 0 samples (undifferentiated stem cells). SOX-17 is an endoderm marker. Mesoderm markers include Brachyury T and cardiac progenitor cell markers for example Nkx2.5. hESC-CM differentiation can be predicted by the expression of the early mesodermal marker Brachyury T which has an expression peak at the time point of 3 days in END-2 co-cultures (Beqqali et al., 2006). Brachyury T is a transcription factor encoded by the T-box genes. Brachyury T functions in many developmental processes, is localized in the nucleus and is an endogenous activator of mesodermal genes (Showell et al., 2004). Differentiation cascade can be further followed by the expression of cardiac regulatory transcription factors such as Nkx2.5. Nkx2.5 is a cardiac marker and so it should be expressed during and after differentiation. Cardiac Troponin T is the tropomyosin-binding subunit of the troponin complex and can therefore be used for characterizing cardiomyocytes. Troponin complex regulates muscle contraction in response to alterations in intracellular calcium (Tobacman, 1996). Other cardiac specific structural proteins include for example troponin I, myosins and

cardiac alfa-actinin with which cardiac phenotype can be confirmed (Mummery et al., 2003).

Different pharmacological and electrophysiological approaches have been used to examine functional characteristics of cardiomyocytes. 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 (Zhang et al., 2009). Differentiated beating cells exhibit spontaneous APs and contractile activity and therefore express cardiac structural proteins and ionic currents (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. Little is known about the functional maturation of human cardiac cells over time. The shape of APs results from the ordered integration of several functional ionic currents. During cardiac development, expression and function of diverse relevant channel types occur over time. Indeed, studies on animal models show that channels undergo fetal and postnatal developmental changes, a complex process leading to the acquisition and maintenance of a mature cardiac electrophysiological phenotype (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 (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.

The analysis of the sequential excitation of cardiac tissue is of high relevance, both for clinical pathophysiological purposes; detection of sustained ventricular arrhythmias, as well as for experimental electrophysiology. Clinically, different technical approaches such as single electrode measurements and bipolar mapping electrode catheters have been used. In experimental setups several techniques to record cardiac activity have been proposed. Beside the well-established intracellular current-clamp recordings of action potentials, recent studies have performed extracellularly activation sequence mapping or simultaneous multichannel action potential electrode array measurements. Measurement of extracellularly recorded field potentials (FPs) hereby especially provides detailed information about the origin and spread of excitation in the heart. A

similar analytical approach for cardiac FPs advanced the analysis of excitation spread and arrhythmic activity in multicellular preparations like developmental differentiation tissue of mouse embryonic stem cells or multicellular preparations of isolated native embryonic cardiomyocytes. The use of substrate-integrated MEAs with 60 electrodes of 10–30 μm diameters on a 100–200 μm grid, coated with porous titanium nitride to minimize the impedance allows recording of FPs at a high signal to noise ratio. The possibility to electrically stimulate the tissue further expands the range of applications and bioassays. It may thus facilitate the evaluation of drug research providing detailed information about the interplay of the complex cardiac network, and might improve the predictability of physiological and pathophysiological conditions or drug effects in embryonic heart tissue (Reppel et al., 2004). Efficient, reproducible differentiation of hESC to ventricular cardiomyocytes, preferably phenotypically similar to adult human cardiomyocytes, is of critical importance for implementing hESC-CM technologies in drug cardiotoxicity screening. END-2 coculture system is a very efficient and reproducible method for cardiac lineage differentiation, in particular to ventricular-like cells. These cardiomyocyte populations are usually phenotypically homogeneous, have strong electrophysiological responses, and are suitable for drug safety pharmacology. hESC-CM technology can be used in a medium-throughput screening system using microelectrode arrays (MEAs) to determine action potential duration or QT interval by measuring extracellular field potentials. Validation of this system has been made using a selection of drugs previously associated with QT prolongation and/or TdP during clinical use in humans. hESC-CM can recapitulate drug toxicity mediated through human ion channels and therefore represent an opportunity for preclinical evaluation (Braam et al., 2010).

In cardiomyocytes, the trigger for contraction is a rise in intracellular calcium (Mummery et al., 2003). Differentiated cardiomyocytes can be maintained in culture for even months without losing their spontaneous contractile capacity.

3 AIM OF THE RESEARCH

The aim of this study was to optimize the cardiomyocyte differentiation of human pluripotent stem cells using one human embryonic stem cell line H7, two human induced pluripotent stem cell lines UTA.00112.hFF and UTA.04602.WT and three different culture conditions: MEFs, SNLs and Matrigel. The comparison between these conditions was carried out by characterizing the differentiated cardiomyocytes. The ultimate goal was to find a best suited culture condition for cardiomyocyte differentiation.

4 Materials and Methods

4.1 Ethical approval

The study was conducted with the permission of National Authority for Medicolegal Affairs Finland (TEO) to study human embryos (Dnro1426/32/300/05) and in accordance with the Ethical Committee of Pirkanmaa Hospital District to derive, culture, and differentiate hESC lines from surplus human embryos (R05116) and to produce new hiPSC lines (R08070).

4.2 Culture conditions

Three pluripotent stem cell lines were used in this study. H7 is a hESC line initially derived in WiCell Research Institute (Madison, WI, USA) and UTA.00112.hFF (H1/12), a hiPSC line has been derived from human foreskin fibroblasts, and hiPS cell line UTA.04602.WT from healthy adult skin fibroblasts. Human iPSC lines have been produced in Institute of Biomedical Technology, University of Tampere, Finland.

The stem cell lines were maintained in HERA cell 150 Thermo ELECTRON CORPORATION -incubator at +37 °C in 5% CO₂. Mitotically inactivated mouse embryonic fibroblast cells (MEFs, Millipore Corporate, Billerica, MA, USA) were used as feeder cells, before this study began, during passages 1-43 for H7, 1-54 for UTA.00112.hFF and 1-32 for UTA.04602.WT. MEFs are generally used in culturing and for this study these lines were transferred also to grow on SNL feeders and Matrigel matrix which is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. The standard KSR culture medium was used with MEF and SNL feeder cells consisting of Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) and is supplemented with 20% Knock-Out serum replacement (KO-SR), 2 mM Glutamax, 0.1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA), 1% MEM (Minimum Essential Medium) non-essential amino acids (from Cambrex Bio Science, Walkersville, MD, USA), 50 U/ml Penicillin/Streptomycin (from Lonza), and 4 ng/ml recombinant human basic fibroblast growth factors (bFGF, from R&D Systems).

The third culture condition was MatrigelTM (BD Biosciences) coating. Commercial mTeSR1 (Stem Cell Technologies) medium was used with Matrigel coating. This is a widely used commercial feeder cell-free culture method for pluripotent stem cells.

The culture media was changed six times a week, except for the cells on MEFs three times a week. The stem cell lines were passaged once a week on MEFs and between 5-7 days on SNLs and Matrigel, generally when the cultures were confluent. MEF and SNL feeder cells were prepared by first coating a 6 well plate with 0.1 % gelatin (1mL/well) and incubated for an hour at room temperature (RT). Mitomycin C treated MEF or irradiated SNL cells were thawed and added to 15 ml MEF medium consisting of Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) supplemented with 20% fetal bovine serum (FBS, PAA Laboratories GmbH, Pasching, Austria), 2mM Glutamax (Invitrogen) and 50 U/ml Penicillin/Streptomycin (from Lonza). 2,5 ml per well of this solution was plated on a 6 well plate which was incubated over night at +37 °C. 250 000 MEF and 280 000 SNL feeder cells were plated per well of area of 9,5 cm². The next day MEF and SNL feeders were ready for stem cells to grow on. When passaging, differentiated regions and feeder cells surrounding stem cell colonies were first removed by scraping with a pipette tip. The MEF feeder cells were removed manually or enzymatically with 1mg/ml Collagenase IV (Invitrogen) and stem cells were transferred onto new MEF feeder cell layers. SNL feeders were removed enzymatically with CTK consisting of 10x Trypsin, 1 mg/ml Collagenase IV, 0,1M CaCl₂, KSR medium without bFGF and milli-Q H₂O. Stem cell colonies were scraped off with a pipette tip in KSR medium and plated onto new SNL feeder cells.

Matrigel (12 µl/well) was thawed on ice and added to cold DMEM/F-12 (1mL/well, Invitrogen) and the solution was spread evenly across the surface of a 6-well plate and incubated at RT at least one hour. Stem cells were ready to be passaged when colonies were large, began to merge and had centers that were dense and phase-bright compared to their edges, usually 5-7 days after seeding. When passaging, differentiated regions were first removed by scraping with a pipette tip. 1 mL of dispase (1 mg/mL, from Stemcell technologies, USA) was added into well and plate was incubated for 7 minutes at + 37 °C. Dispase was washed away by rinsing the wells two times with 2 ml of DMEM/F-12. The colonies were scraped off with a pipette tip in mTeSR1 medium.

Finally aggregates with 2,5 ml of mTeSR1 per well were plated on 6-well plates from which Matrigel solution was removed just before.

4.3 Cardiac differentiation

To initiate cardiac differentiation 175 000 Mitomycin C inactivated END-2 cells which were a kind gift from Professor Mummery, Humbrecht Institute, The Netherlands were plated per well of a 12 well plate and cultured over night. MEF feeder layer was removed by pulling. SNL feeder layer was removed by using CTK like in passaging. Differentiated stem cells were removed with the tip of pipette. Large cell colonies were cut smaller with cell scraper. Then cell colonies were detached with cell scraper to the culture medium. Approximately 30 cell colony pieces / well were transferred onto END-2 cells in KSR medium supplemented with 3 mg/ml ascorbic acid without KO-SR or bFGF. The medium was changed on days 6, 9 and 13. On day 5-6 the attached areas were counted by using a microscope. On day 15 KSR medium containing 10% KO-SR without ascorbic acid or bFGF was changed to the cells. This medium was changed for 3 times a week (Monday, Wednesday and Friday). The cardiac differentiation was repeated three times for each pluripotent stem cell line cultured in three different conditions. The cardiac differentiation was characterized by q-RT-PCR and by immunocytochemical stainings. The cardiac differentiation efficiency of stem cell lines was evaluated by cytopsin experiments on day 19-20 and by counting the number of beating areas in the end of differentiation on day 30-32. Figure 2. shows the timetable of differentiations. Cardiac differentiation was performed for 36 wells of each stem cell line from each three differentiations. 6 wells were intended for cytopsin analysis, 12 wells for qPCR samples which were collected on day 0 from two wells of undifferentiated 6-well plate and on days 3, 6, 12 and 30 from the beginning of differentiation from 12-well plate. Beating cells were counted from 18 wells.

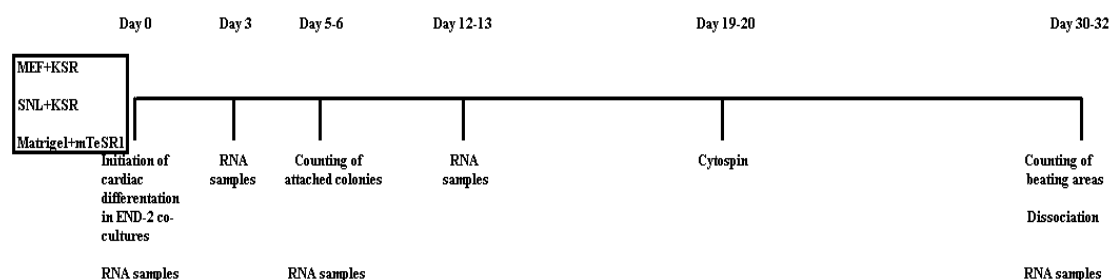


Figure 2. Timetable of cardiac differentiation on END2-coculture. In the box left side there are the culture conditions before differentiation. Above the line there are the days after the initiation of differentiation and under the line there are the tasks made on each timepoint.

4.4 Cytospin

The cardiac differentiation efficiency was determined by analyzing the expression of Troponin T from cytopsin samples on day 19-20. In the method 1 x Trypsin (Lonza) was used for detaching the cell aggregates from the bottom of the well. 0,5 ml trypsin was pipetted in wells and incubated for 45 minutes at 37 °C after washing them twice with DPBS (0,0095 M Dulbecco's Phosphate Buffered Saline from BioWhittaker, Lonza, Verviers, Belgium). Cell aggregates were dissociated to single cells by pipetting up and down. 0,5 ml EB medium containing KO-DMEM supplemented with 20% FBS (PAA Laboratories GmbH, Pasching, Austria), 1% NEAA, 2 mM GlutaMax and 50 U/ml penicillin/streptomycin per well was added. Two replicates consisting of 3 wells each were collected into the same 50 ml falcon tube with additional 1 ml EB medium which was used to rinse the wells. Cells were counted with a Neubauer glass chamber. Then 500 000 – 1 000 000 cells were cytocentrifuged 800 rpm for 5 minutes in SAKURA Cyto-Tek cytopsin machine onto a glass slide. Fixation and Troponin T - staining were performed according to the normal protocol as described below in section Immunocytochemical staining. Pictures of the cells on the glass slides were randomly selected and taken with a fluorescence microscope Olympus DP30BW (Olympus, Tokyo, Japan). About 30 pictures per sample were taken so, that 1500 nuclei could be counted from them.

4. 5 Dissociation protocol of beating areas of cardiomyocytes

For immunocytochemical staining the beating areas were dissociated into single-cell suspension by collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) treatment as described before (Mummery et al. 2003). Beating areas of cardiomyocytes were isolated on day 29-32 by cutting the aggregates manually with a sterile scalpel under a Nikon SMZ800 ThermoPlate TOKRI HIT -microscope and transferred into one well in 24-well plate in KSR culture medium. Beating areas were then washed in three different dissociation buffers: A for 30 min (RT), B for 45 min (+37 °C) and C for 1 h (RT). Reagents of dissociation buffers A, B and C are listed in **Table 1**. Then cell aggregates were carefully resuspended in EB medium. Cells were then let to attach to gelatine coated (0.1 %) 24-well plate at +37 °C until they were used for immunocytochemical staining.

Table 1. Dissociation buffers.

Dissociation buffers	A	B	C
Reagent	100ml	100ml	100ml
NaCl	12ml(1M)	12ml(1M)	-
CaCl ₂			-
K ₂ HPO ₄	-	3ul(1M)	3ml(1M)
KCl	0,54ml(1M)	0,54ml(1M)	8,5ml(1M)
Na ₂ ATP	-	-	2mmol/L
MgSO ₄	0,50ml(1M)	0,50ml(1M)	0,50ml(1M)
EGTA	-	-	0,1ml(1M)
Na Pyruvat	0,50ml(1M)	0,50ml(1M)	0,50ml(1M)
Glucose	2ml(1M)	2ml(1M)	2ml(1M)
Creatine	-	-	5ml(1M)
Taurine	20ml(1M)	20ml(1M)	20ml(1M)
Collagenase A	-	1mg/ml	-
HEPES	1ml(1M)	1ml(1M)	-
pH corr.	NaOH	NaOH	NaOH
pH	7	7	7

4.6 Immunocytochemical staining

Undifferentiated colonies and dissociated cardiomyocytes from each three cell lines cultured in three different conditions were characterized by immunocytochemical stainings. Cells were washed two times with 1 x PBS for 5 minutes and fixed with 4 % paraformaldehyde (PFA, Sigma-Aldrich) for 20 minutes. Again cells were washed two times with 1 x PBS for 5 minutes. Fixed cells were permeabilized and blocked by incubating in 10 % normal donkey serum (NDS), 0,1 % TritonX-100, 1 % BSA in PBS –solution at RT for 45 minutes. Wells were washed once and primary antibodies were diluted in 1 % NDS, 0,1 % TritonX-100, 1 % BSA in PBS –solution. Undifferentiated stem cells were stained with Oct-3/4, Nanog and TRA-1-60 antibodies for undifferentiated cells; nestin for neural precursor cells and MAP-2 (microtubuline associated protein-2) for neuronal cells. Neural markers were used to identify spontaneously differentiated neural cells from the cultures. Differentiated cardiac cells were stained with connexin-43, α -actinin, Troponin T and ventricular myosin heavy chain antibodies. In **Table 2**. primary antibodies, their binding target and their dilutions used are listed. Primary antibodies were incubated over night at +4 °C.

Next day wells were washed 3 times with 1 % BSA in PBS for five minutes. Secondary antibody used was diluted in 1 % BSA in PBS and incubated light protected 1 hour at RT. In **Table 3**. secondary antibodies and their dilutions used are listed. Wells were washed light protected two times with 1 x PBS for 5 minutes and two times with PB working solution for 5 minutes. Finally wells were mounted with Vectashield containing DAPI and glass slide. Pictures were taken with Olympus BX60 microscope (Olympus, Tokyo, Japan).

4.7 Sample collection and RNA isolation

For q-RT-PCR, RNA samples were collected from cardiac differentiation cultures on days 0, 3, 5-6, 12-13 and 30-32. Four wells were washed with DMEM/F-12 and scraped with a cell scraper. Two wells were collected to the same falcon tube to get two biological replicates. Cell debris was spinned down in two 15 ml falcon tubes at 300 rpm for 5 minutes with Biofuge primo Heraeus CF0507. DMEM/F-12 was discarded and 350 μ l of RA1 buffer (Macherey-Nagel) with 1 % 2-mercaptoethanol

Table 2. Primary antibodies. In the columns there are the binding targets in which the antibodies attach, animal of origin, dilution used in this study and manufacturing information.

	Primary antibody	Binding target	Origin	Dilution	Information
1	Troponin T	heart muscle cells	goat	1:1500	ab64623, abcam,
					Cytospin
				1:2500	ab64623, abcam,
					Immunocytochemistry
2	anti hOCT-3/4	undifferentiated pluripotent cells	goat	1:400	R&Dsystems 0,2 mg/ml
3	anti hNanog	undifferentiated pluripotent cells	goat	1:200	R&Dsystems 0,1 mg/ml
					AF1997
4	MAP-2	neural cells	rabbit	1:400	Millipore AB5622
5	Nestin	neural stem cells	mouse	1:100	MAB5326 1 mg/ml
6	TRA 1-60	undifferentiated pluripotent cells	mouse	1:200	MAB4360 1 mg/ml
7	Connexin-43	Ca ²⁺ channels in muscle cells	rabbit	1:1000	C6219 Sigma
8	Myosin heavy chain	ventricular muscle cells	mouse	1:100	mab1552 Chemicon
9	alpha-actinin	Z-discs (thin filaments in striated muscle cells)	mouse	1:1500	A7811 Sigma

Table 3. Secondary antibodies. In the columns there are the color of the antibody under fluorescent light and wavelength, dilution used in this study and manufacturing information.

	Secondary antibody		color, nm		Dilution	Information
1	anti-Goat		red, 568		1 : 800	Invitrogen, Alexa fluor
2	anti-Goat		green, 488		1 : 800	Invitrogen, Alexa fluor
3	anti-Rabbit		green, 488		1 : 800	Invitrogen, Alexa Fluor
4	anti-mouse		green, 488		1 : 800	Invitrogen, Alexa Fluor
5	anti-mouse		red, 568		1 : 800	Invitrogen, Alexa Fluor

(Sigma-Aldrich) was added to lyse the two parallel cell samples. Eppendorf tubes were stored in -70 °C until RNA isolation step.

Total RNA was extracted using NucleoSpin RNA II Total RNA purification –kit (Macherey-Nagel) according to the manufacturer’s instructions. All the centrifugation steps were performed with eppendorf Centrifuge 5415 R 11 000 x g at +4 °C. The lysate was first filtrated with a NucleoSpin Filter and centrifuged for 1 minute. RNA binding conditions were adjusted by mixing properly 350 ul 70 % ethanol to the homogenized lysate. Lysate was transferred to NucleoSpin RNA II Column and centrifuged for 30 seconds. Silica membrane was desalted by adding 350 ul Membrane Desalting Buffer and membrane was dried by centrifuging for 1 minute. DNA digestion was made by applying 95 ul rDNase reaction mixture, which hydrolyses the phosphodiester bonds of DNA strands. Sample was incubated for 15 minutes. Washing of the silica membrane was made by adding first 200 ul Buffer RA2 which inactivated the rDNase and centrifuged for 30 seconds. Second and third washes were made by adding 600 ul and 250 ul Buffer RA3 and centrifuged for 30 seconds and 2 minutes. RNA was eluted in 60 ul RNase-free water. The RNA concentration and quality of the samples were measured by NanoDrop ND-1000 Spectrophotometer, ND-1000V3.7.1 using Nucleic Acids setting and Sample type RNA-40. The purity of the samples was analyzed with RNA

concentration ratios from different wavelengths. RNA was considered pure when 260nm/280nm was over 2 and 260nm/230nm was between 1,8-2,2. Samples were stored in nuclease-free Collection Tubes in -70 °C until complementary DNA reverse transcription step.

4.8 Complementary DNA reverse transcription

500 ng of RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions in the presence of RNase inhibitor. Samples were kept on ice before TC0501 eppendorf –PCR machine was used. Duplicate samples were combined so that the final concentration of 500 ng/μl was obtained; 250 ng/μl from each parallel samples. Appendix shows the concentrations and concentration ratios of every RNA sample, and the counted amounts of samples and water pipetted to cDNA samples. The desired cDNA sample amount was 10 μl in which 10 μl Master mix solution was added. Master Mix solution and –RT-control were prepared according to **Table 4**. PCR-program used is in **Table 5**. Samples were stored at -20 °C until the q-PCR run.

4.9 Quantitative polymerase chain reaction

Synthesized cDNA samples were diluted 1:3 in RNase-free water. 7 different gene markers were used for the characterization. Reaction was performed on 96 well plates in which 2 genes were pipetted. These gene pairs with different chemistry are listed in **table 6**. **Table 7**. shows how the working solutions for the q-PCR reactions were made. 14 μl per well of working solution was pipetted on 96-well plate and 1 μl of the diluted sample on an ice block. Samples were analyzed in triplicates. Sterile water was used as control and marked as H2O (PCR) in the pipetting charts. Other water control was from cDNA reaction and marked as H2O (cDNA). –RT control samples were taken from day 0 samples of each line. In each reaction there were controls –RT1. (H7), –RT2. (UTA.04602.WT) and –RT3. (UTA.00112.hFF). –RT controls did not contain any enzyme and so there should not emerge any DNA in the reactions. After pipetting the 96-well plate was covered with a plastic sheet and centrifuged for 2 minutes at 2000 rpm with MULTIFUGE 1 S-R Heraeus. The thermal program was premade with the

Table 4. Master Mix solution and –RT-control.

Component	Volume/Reaction (μ l)	
	MasterMix	-RT
10 X RT Buffer	2,0	2,0
25 X dNTP Mix		
(100mM)	0,8	0,8
10 X RT Random		
Primers	2,0	2,0
MultiScribe Reverse		
Transcriptase	1,0	0
RNase inhibitor	0,5	0,5
Nuclease-free H ₂ O	3,7	4,7
Total per Reaction	10,0	10,0

Table 5. cDNA PCR-program.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

AB Applied Biosystems 7300 Real Time PCR System –machine. **Tables 8.** and **9.** show the thermal program of q-PCR reaction for TaqMan and for SYBR assays. The results of the q-PCR runs were analyzed with 7300 System Software and exported to excel. Relative quantification was calculated with the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). RPLP0 was used as an internal control to normalize added RNA amount and cell line H7 from MEFs was used as a calibrator.

Table 6. Gene markers of qPCR. In the columns there are the visualization technique used in this study and gene marker property.

Gene	Visualization technique	Gene property
OCT-4 (POU5F1)	Taqman	pluripotency marker
RPLP0	Taqman	ribosomal protein, internal control
NF-68	Taqman	neuronal marker
Brachyury T	SYBR	mesodermal marker
Nkx2,5	SYBR	cardiac lineage marker
SOX-17	SYBR	endodermal marker
Troponin I	SYBR	cardiac lineage marker

Table 7. Working solution of the q-PCR.

TAQMAN MM	1x (μl)
TaqMan mastermix	7,5
H2O	5,75
Assay	0,75
Total	14
SYBR MM	1x (μl)
Power SYBR mastermix	7,5
H2O	5,6
Forward primer	0,45
Reverse primer	0,45
Total	14

Table 8. Thermal program of q-PCR reaction for TaqMan assay.

TaqMan	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	50	95	95	60
Time (min)	2	10	15 sec	1
Cycles	1	1	40	

Table 9. Thermal program of q-PCR reaction for SYBR assay.

					Dissociation stage		
SYBR	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
Temperature (°C)	50	95	95	60	95	60	95
Time (min)	2	10	15 sec	1	15 sec	1	15 sec
Cycles	1	1	40		1	1	1

5 Results

5.1 Morphology of undifferentiated colonies

Before differentiation, the undifferentiated state of pluripotent stem cells was verified by visual observation and by the expression of the pluripotency markers.

Undifferentiated hESCs and hiPSCs grew as compact, multicellular colonies having distinct borders. They exhibit a high nuclear-to-cytoplasm ratio and prominent nucleoli. Spontaneous differentiation of stem cells was characterized by loss of border integrity, gross non-uniformity of cell morphology within a colony, and the emergence of obvious alternate cell types. There were little, neuro-like cells surrounding colonies growing on Matrigel, inhibiting the normal growth of the stem cells as shown in **Figure 3**. H7 Matrigel. If these neuro-like cells were not properly removed before passaging, the normal growth of next passage was hindered. These neuro-like cells emerged in every cell line throughout culturing. Often colonies on Matrigel looked star-like right after passaging but grew nicely and got more round-shaped before next passaging as shown in **Figure 3**. UTA.00112.hFF. Few times especially cell line UTA.00112.hFF colonies could not be passaged to more wells because they were not growing properly and had differentiated spontaneously before being confluent enough for passaging. These times good colonies were picked and passaged to fewer wells.

An increased rate of differentiation occurred on Matrigel if the cell aggregates were too large when passaging. If the cell aggregates were too small with many single cells present, cell survival, and resulting number and health of colonies was compromised. We detected that many colonies in the Matrigel dish should be just beginning to touch each other at the time of passaging i.e. plate is approximately 75% confluent. This way colonies grew well and without excess differentiation before next passaging.

On MEFs stem cells grew well and usually colonies were round-shaped without excess differentiation. Stem cell colonies cultured on MEFs were passaged only once a week. These colonies grew slower than the colonies on Matrigel or SNL feeder cell layer but

after a week of growing resulted in adequate size colonies as shown in **Figure 3**. MEF colonies.

Colonies grown on SNL feeders grew normally well but sometimes they were small-sized even after growing for 5-6 days. These times good colonies were picked and passaged to fewer wells. Both, MEFs and SNL feeder cells, were sometimes hard to remove without removing colonies from the bottom of the well. Important also when using MEFs and SNLs, was to passage right-sized colonies, which were little smaller than the colonies on Matrigel.

5.2 Cardiac differentiation and beating areas

Beating areas were counted after 30-32 days from the initiation of differentiation. The effect of culture condition used before differentiation was clear with MEFs because the cardiac differentiation of stem cells cultured on MEFs was the most efficient in all the three passages in general. The smallest beating areas were in general faster beaters than big branched areas. All in all beating areas cultured on MEFs were really different to each other in their size, fastness and morphology. Beating areas cultured on SNLs before cardiac differentiation were usually bigger than from Matrigel and also beat slower. Also the passage had an effect on the amount of beating areas found from differentiations. The last and the oldest passage 15 had less beating areas compared to earlier two passages 5 and 10. Between these two passages there was not much difference. In **Figure 4**. and **5**. there are pictures of differentiated cells on day 5 (**Figure 4**.) and on day 30 (**Figure 5**.) of cardiac differentiation.

Differentiated cell lines with good cardiac differentiation efficiency, like cell line H7, formed compact three dimensional structures on END-2 cells when compared to cell lines with lower cardiac differentiation efficiency. Cell line UTA.04602.WT formed a lot of bubbles full of medium on END-2 cells as shown in **Figure 5**. UTA.04602.WT SNL. These bubbles grew during differentiation and were troubling when counting the beating areas because they were hindering the visual observation of the wells.

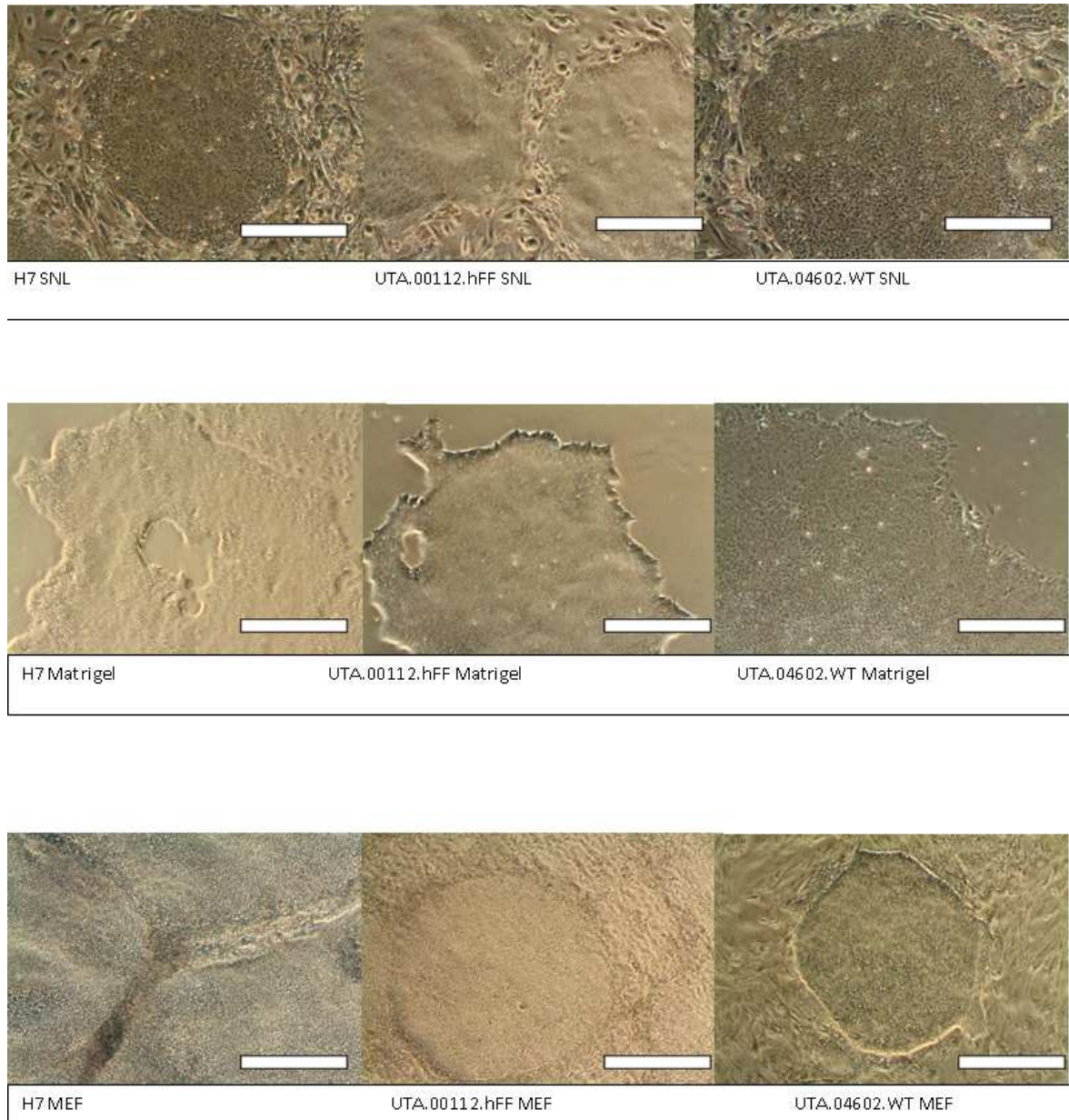


Figure 3. Undifferentiated stem cell colonies on day 4. There is the cell line and culture condition used in this study under each picture. Scale bars 500 μm .

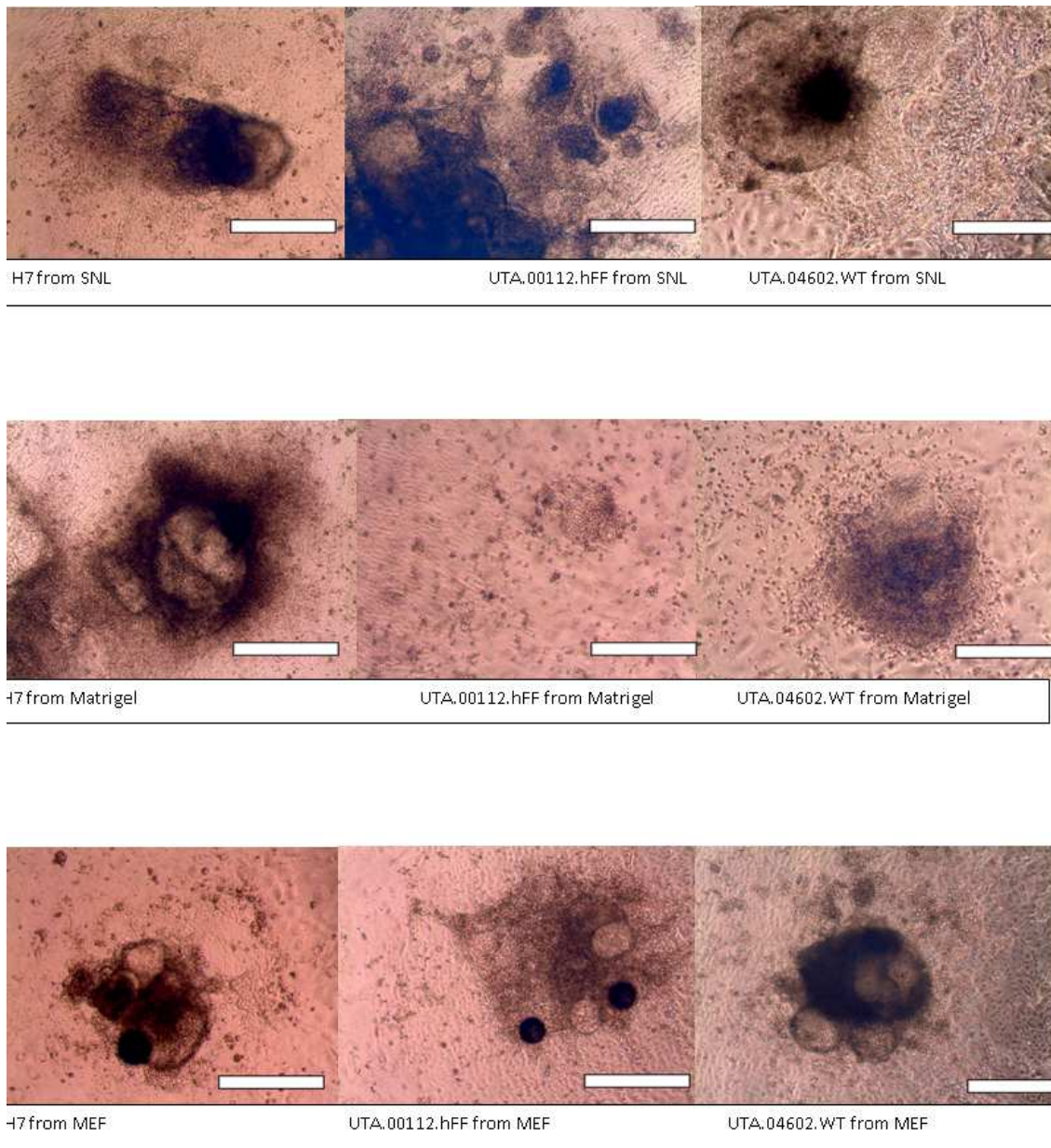


Figure 4. Differentiated cells on day 5 of cardiac differentiation on END2-cells. There is the cell line and culture condition used in this study under each picture. Scale bars are 500 μ m.

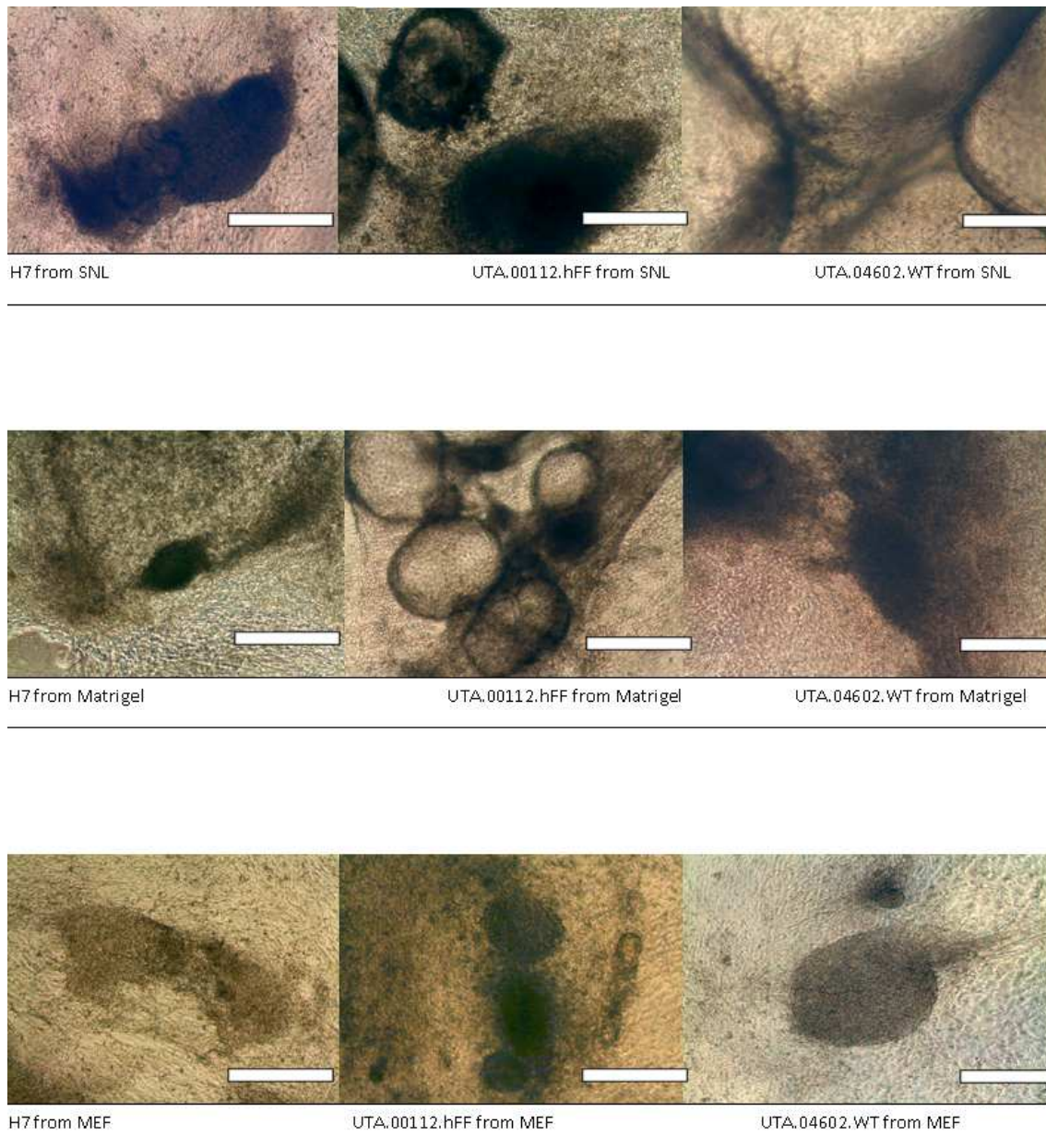


Figure 5. Beating cardiomyocytes on day 30 of cardiac differentiation on END2-cells. There is the cell line and culture condition used in this study under each picture. Scale bars are 500 μm .

The amount of attached areas and beating areas, counted beating area average and differentiation efficiency per one well is shown in APPENDIX 1. Cell line H7 on MEFs and SNLs has the best beating area average per one well through all three passages. Also H7 Matrigel has more beating areas than the rest of the cell lines. Cell line H7 differentiated most efficiently to beating cardiomyocytes compared to the other two cell lines used in this study. When only taken to account the cell line H7, the culture

conditions can be organized in order of the best differentiation result. The stem cells from MEFs were the best to differentiate to cardiac beating cells and worse were the stem cells from Matrigel in every three passage. Cell line H7 stem cells from SNLs were between these two in their differentiation efficiency. The cell lines UTA.00112.hFF or UTA.04602.WT from all three culture conditions had beating area average per one well under two i.e. very weak in all three passages. The amount of attached areas on day 6 does not clearly correlate in all of the cell lines to the amount of beating areas on day 30 but END-2 differentiation efficiency % was however counted by dividing total number of beating areas divided by total number of attached areas from each cell line. These results confirm also that H7 cell line had the highest differentiation rate. Cell line UTA.04602.WT from Matrigel shows also pretty good differentiation efficiency in every three passages. **Figure 7. (A.) – (C.)** shows the counted beating areas per well from each three differentiation time (passages 5, 10 and 15).

5.3 Cytospin

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., 2008). Total of 1500 cells were counted shown blue in **Figure 6.** and of them the troponin T positive cells shown red in the **Figure 6.**

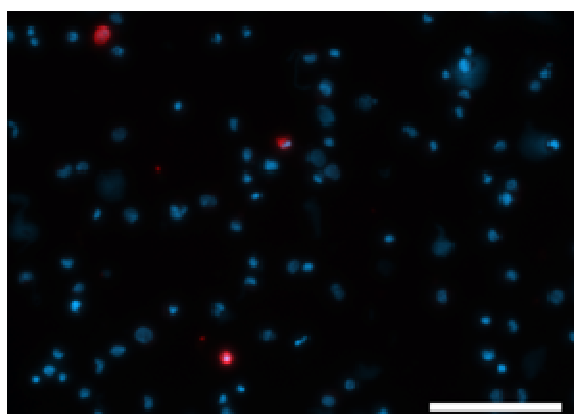
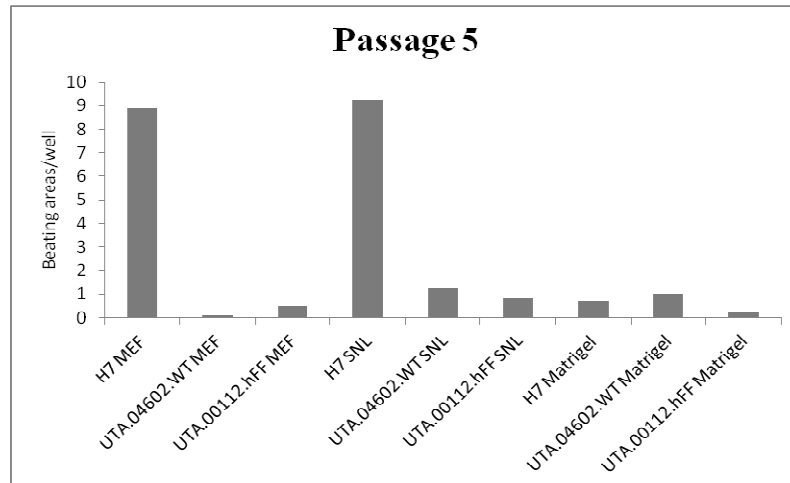
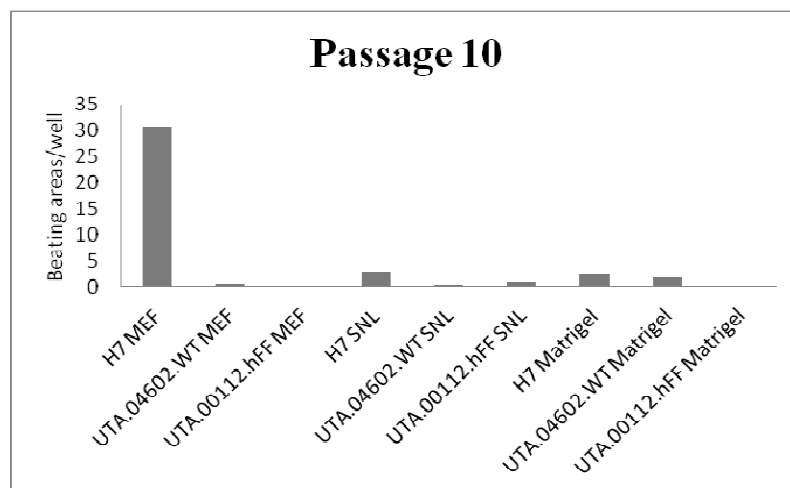


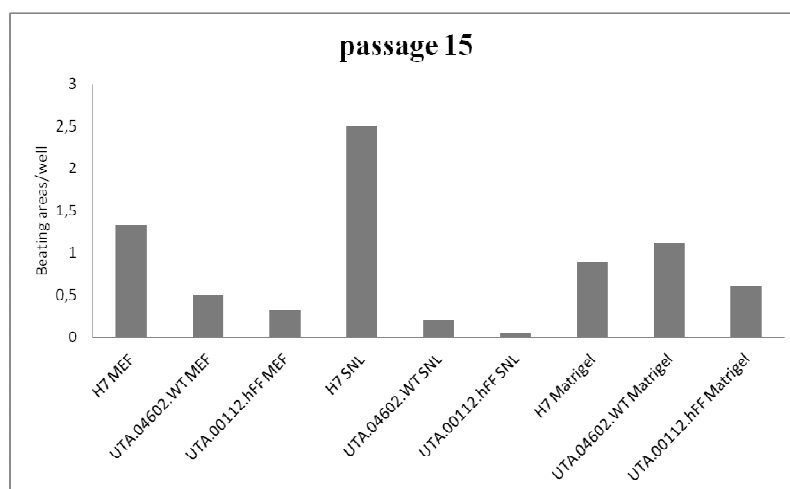
Figure 6. Example of picture from which the results of Cytospin method were counted. Blue color are the nuclei of the cells (DAPI) and red the troponin T positive cells.



(A.)



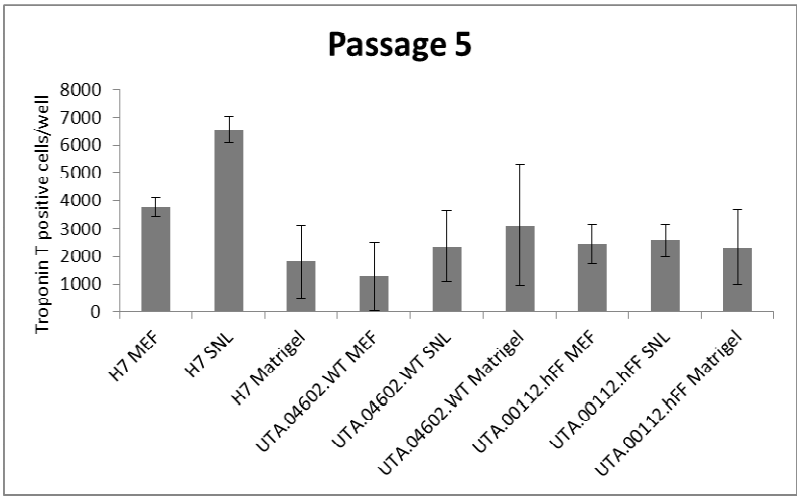
(B.)



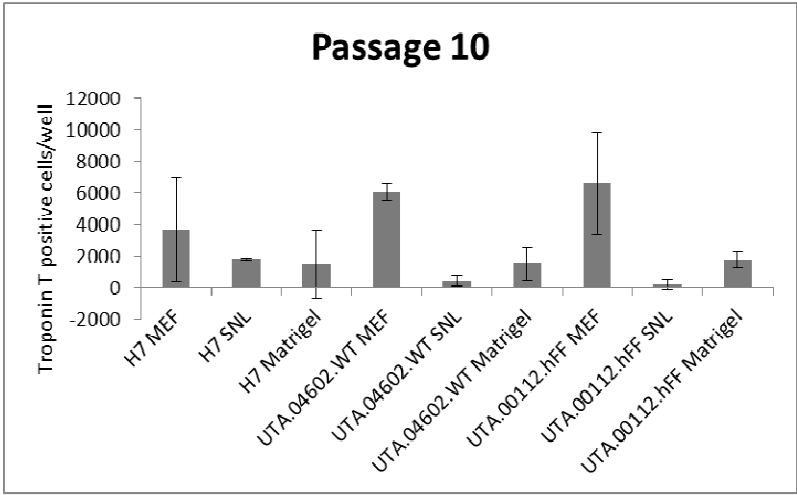
(C.)

Figure 7. (A.) – (C.) On day 30-32 counted beating areas per well. Y-axis is the counted beating areas per well and x-axis is the cell line and the culture condition.

From these results the percent of troponin T positive cells could be counted. Then troponin T positive cells per well was counted for sample 1 and 2 by dividing the total cell count by 3 (ml) and then multiplied with the percent of troponin T positive cells in that sample. APPENDIX 2. shows the combined results of the parallel samples 1 and 2 in the columns “average”. The amount of troponin T positive cells per well ranges enormously from 240 (UTA.00112.hFF SNL passage 10) to 6 660 (UTA.00112.hFF MEF passage 10). H7 MEF had the most cardiomyocytes per well through all three differentiations. **Figure 8. (A.) – (C.)** shows cytopsin results combined in three charts; one from each passage.



(A.)



(B.)

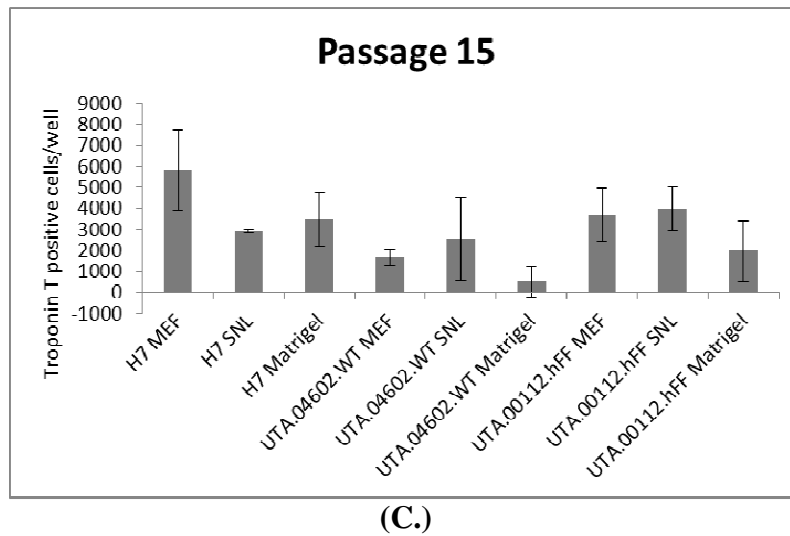
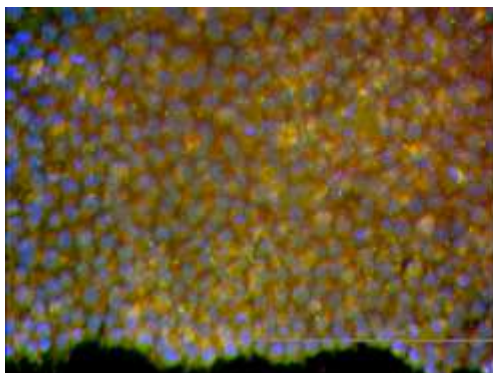


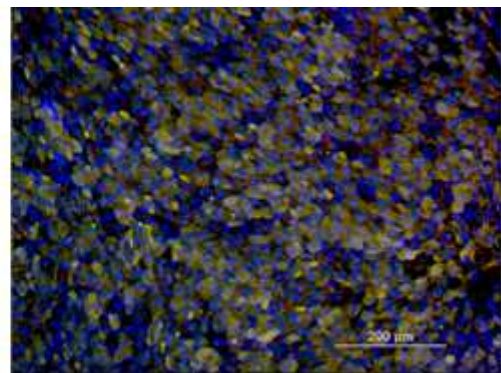
Figure 8. (A.) – (C.) Cytospin results. Y-axis is the counted troponin T positive cells per well and x-axis is the cell line and the culture condition.

5.4 Immunocytochemical staining

Immunocytochemical staining was used to confirm the cardiac differentiation. Antibodies used bound to their target area successfully. Both undifferentiated and dissociated differentiated beating cells were stained. Undifferentiated cell colonies were stained with undifferentiated pluripotent stem cell markers in **Figure 9**. Nanog (green), OCT3/4 (green) and TRA-1-60 (red). Nestin (red) was used to stain neural stem cells and MAP-2 (red) mature neural cells. The cell nuclei are stained blue (DAPI) in all of the pictures.



(A.)



(B.)

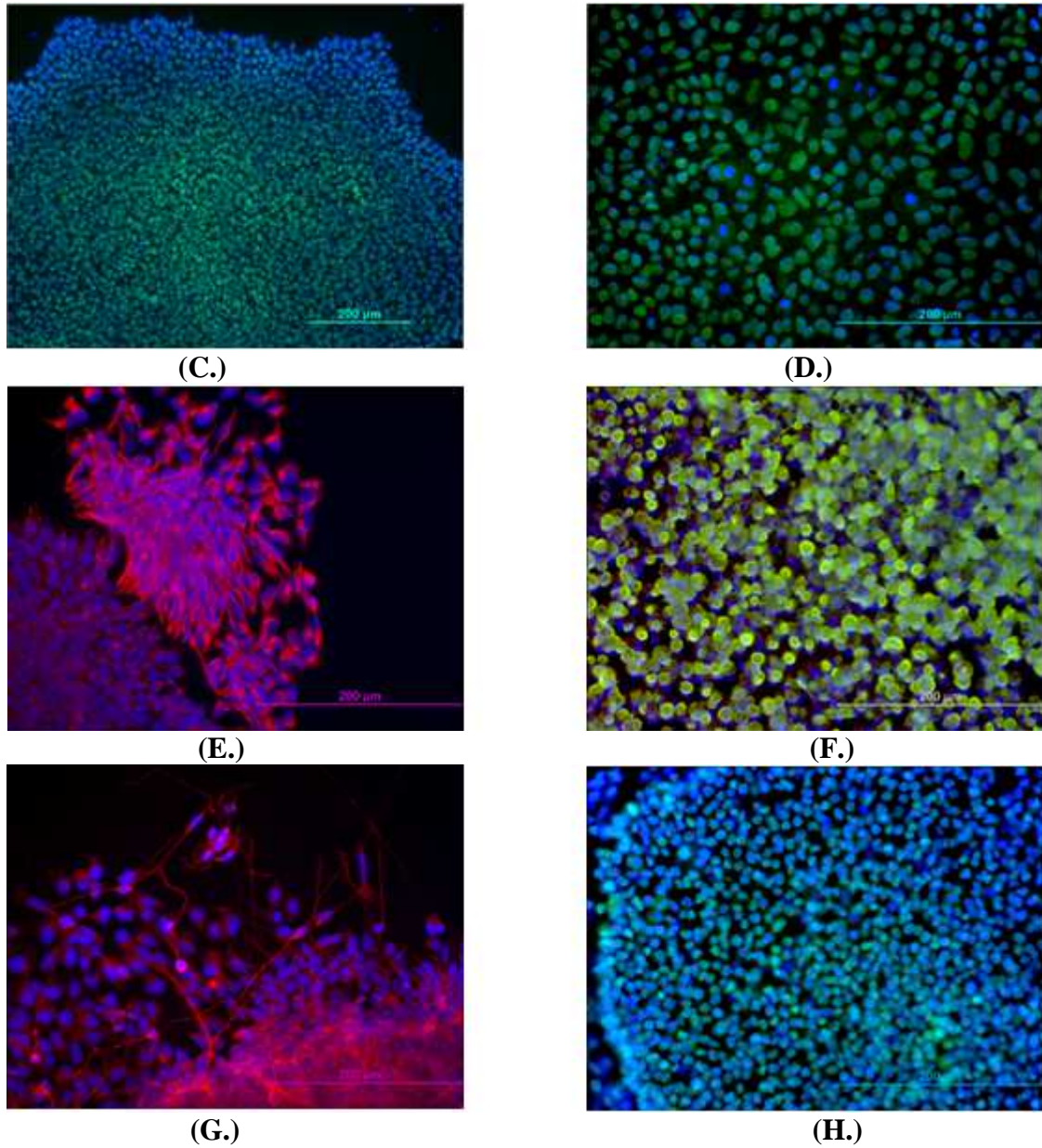


Figure 9. Immunocytochemical staining of undifferentiated stem cells.

Scale bars are 200 μm.

- (A.) UTA.04602.WT Matrigel: OCT3/4 / TRA-1-60,
- (B.) UTA.04602.WT SNL: OCT3/4 / TRA-1-60 / DAPI,
- (C.) UTA.04602.WT Matrigel: Nanog / DAPI,
- (D.) UTA.04602.WT SNL: Nanog / DAPI,
- (E.) UTA.04602.WT Matrigel: Nestin / DAPI,
- (F.) UTA.04602.WT MEF: OCT3/4 / TRA-1-60 / DAPI,
- (G.) UTA.04602.WT Matrigel: MAP-2 / DAPI,
- (H.) UTA.04602.WT MEF: Nanog / DAPI.

The differentiated cardiac cells stained positively with several cardiac markers as shown in **Figure 10.**, including cardiac α -actinin (red), cardiac Troponin T (red) and ventricular α -myosin heavy chain (vMHC) which stained the ventricular cardiomyocytes with green. Ca²⁺-channels and Z-disc filaments stained red with α -actinin, and Troponin T stained all cardiomyocytes with red. The cell nuclei are stained blue (DAPI) in all of the pictures. Connexin-43 was localized between the α -actinin or troponin T positive cells, indicating that the beating cells had gap junctions between them but is not shown in the **Figure 10**. Dissociated beating cells in single cell state looked similar when stained between different cell lines.

5.5 RNA extraction

Almost every sample yielded the needed amount of RNA (500 ng/combined samples 1 and 2). Significantly low RNA concentration was only in few samples. The purity of the RNA was pretty good, the 260/280 ratio was near or over 2 in most samples. However the 260/230 ratio was less than 1 in many samples but RNA was still considered pure enough for cDNA and qPCR steps. APPENDIX 3. shows the RNA concentrations from each cell line and time points from passages 5 and 15.

5.6 Q-RT-PCR

qPCR was used to assess the mRNA expression levels of different gene markers and to confirm the immunocytochemical data. It was carried out on all the samples from passages 5 and 15. Each cell line's undifferentiated sample from day 0 from the culture condition MEF was given the value 1 and was used as a calibrator in the calculations. Ribosomal protein RPLP0 was used as an internal control. Ct values which differed from the other two parallels over 1 unit were discarded from the calculations. Pluripotency marker OCT3/4 (POU5F1), mesodermal marker Brachyury T and cardiac lineage markers Nkx2.5 and Troponin I and SOX-17 which is a endodermal marker were also used in the analysis. **Figure 11. (A.) – (F.)** summarizes the results of the analysis.

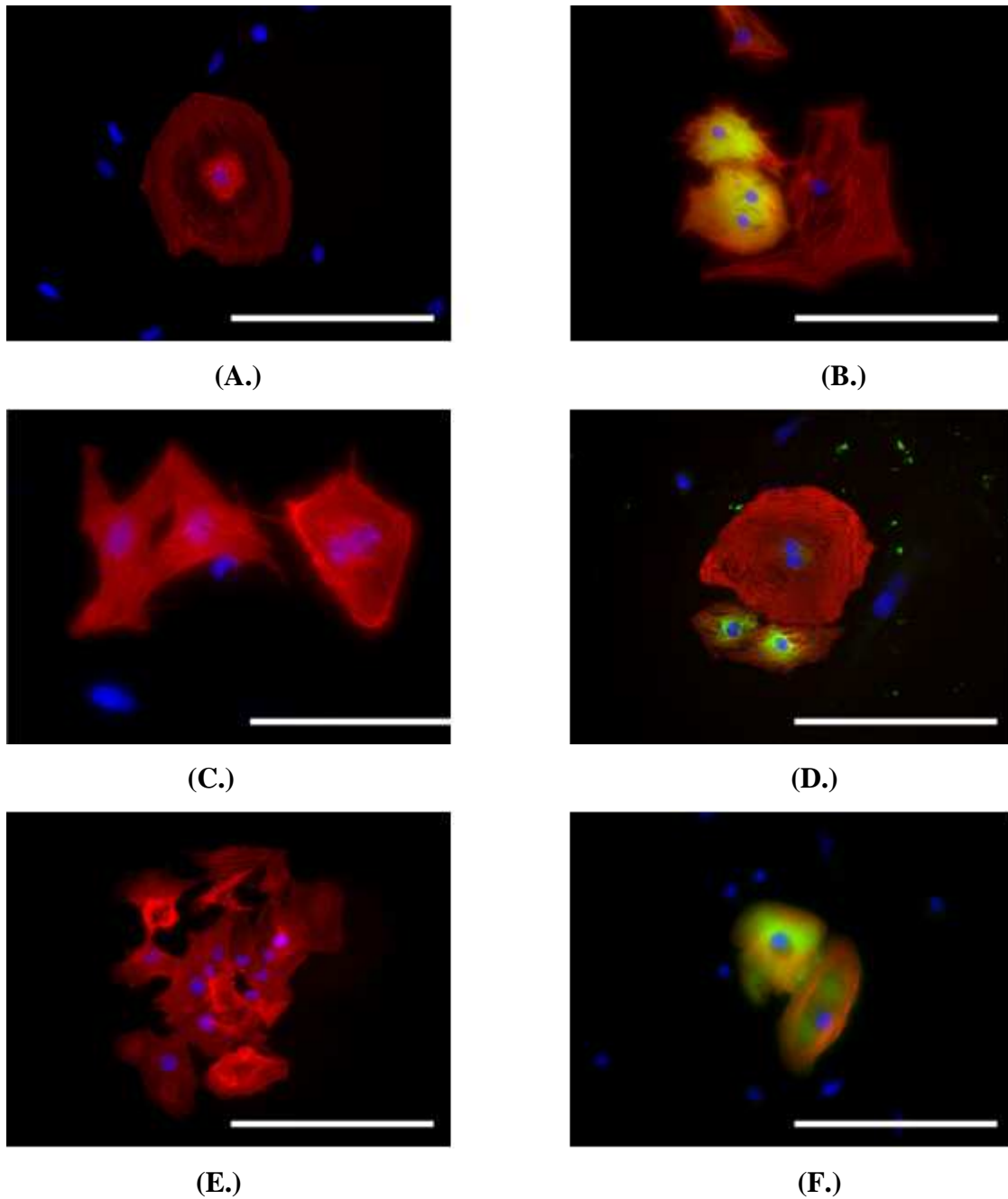


Figure 10. Characterization of differentiated and dissociated cells by immunocytochemistry. Scale bars are 100 μm.

- (A.) H7 MEF: α-actinin / DAPI,
- (B.) UTA.04602.WT MEF: Troponin T / vMHC / DAPI,
- (C.) H7 Matrigel: α-actinin / DAPI,
- (D.) UTA.04602.WT Matrigel: Troponin T / vMHC / DAPI,
- (E.) H7 SNL: α-actinin / DAPI,
- (F.) UTA.04602.WT SNL: Troponin T / vMHC / DAPI.

The method showed that the expression of stem cell marker OCT3/4 was highest in undifferentiated cell samples taken on day 0 in both passages and decreased so that on days 13 and 30 there is hardly expression at all. Results of the marker OCT3/4 were consistent throughout all the cell lines and both passages as shown in **Figure 11. (A.)**.

The gene expression of mesodermal marker Brachyury T was high on day 0 samples of cell lines UTA.00112.hFF and H7 from SNL in both passages. Cell line UTA.04602.WT from Matrigel was showing also a gene expression peak on day 0 but only in passage 5. From day 6 forward the expression started to decrease in all the cell lines, conditions and both passages as shown in **Figure 11. (B.)**.

Endodermal marker SOX17 has low expression in undifferentiated samples on day 0 and differentiated samples on day 30. In-between the expression is higher. This trend can be seen in both passages with some minor exceptions as shown in **Figure 11. (C.)**.

There was variation between cell lines and passages in the expression of cardiac lineage marker Nkx2.5. Expression was detected already in some amounts in the day 0 samples especially in cells originating from SNLs. Some cells differentiate towards cardiac lineage already when culturing. Throughout results expression was highest on day 3 and started to decrease already on day 12. Passage 5's cell line H7 looked logical so that the Nkx2.5 gene expression increased during differentiation when more cardiomyocytes matured as shown in **Figure 11. (D.)**.

Cardiac marker Troponin I expression levels should be rising until complete differentiation of day 30 samples when the expression should be highest. Troponin I expression could be seen however through the days 0-30 which is contradicting. The MEF condition shows especially high expression in both passages as shown in **Figure 11. (E.)**.

Neural cell differentiation was noticed during culturing and this spontaneous differentiation into neural cell direction was verified by the expression of neuronal marker NF-68. NF-68 expression was higher in cells from Matrigel culture condition than in cells from other culture conditions. Results from cell line UTA.04602.WT differed between passages 5 and 15 so that in passage 5 neural cell marker expression

was decreasing and in passage 15 increasing in cells from Matrigel. This depends of the amount of neural cells in the culture when cardiac differentiation was initiated as shown in **Figure 11. (F.)**.

There was variation in the marker expressions between cell lines, passages and conditions. However all the cell lines expressed both the pluripotency and cardiac markers as desired. Each cardiac marker was expressed throughout the days 0-30.

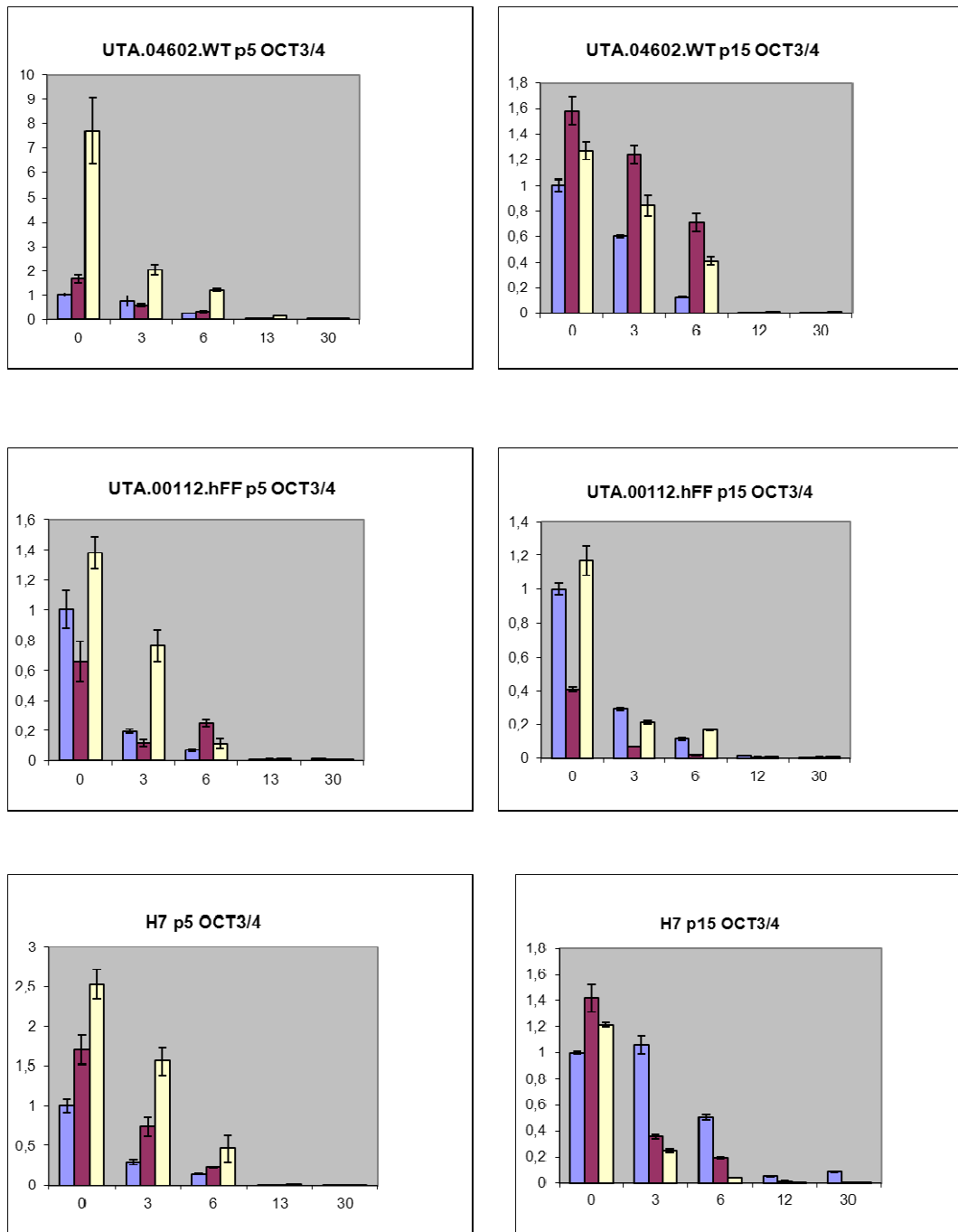


Figure 11. (A.) OCT3/4. Y-axis is the fold change and x-axis the day the sample was taken.

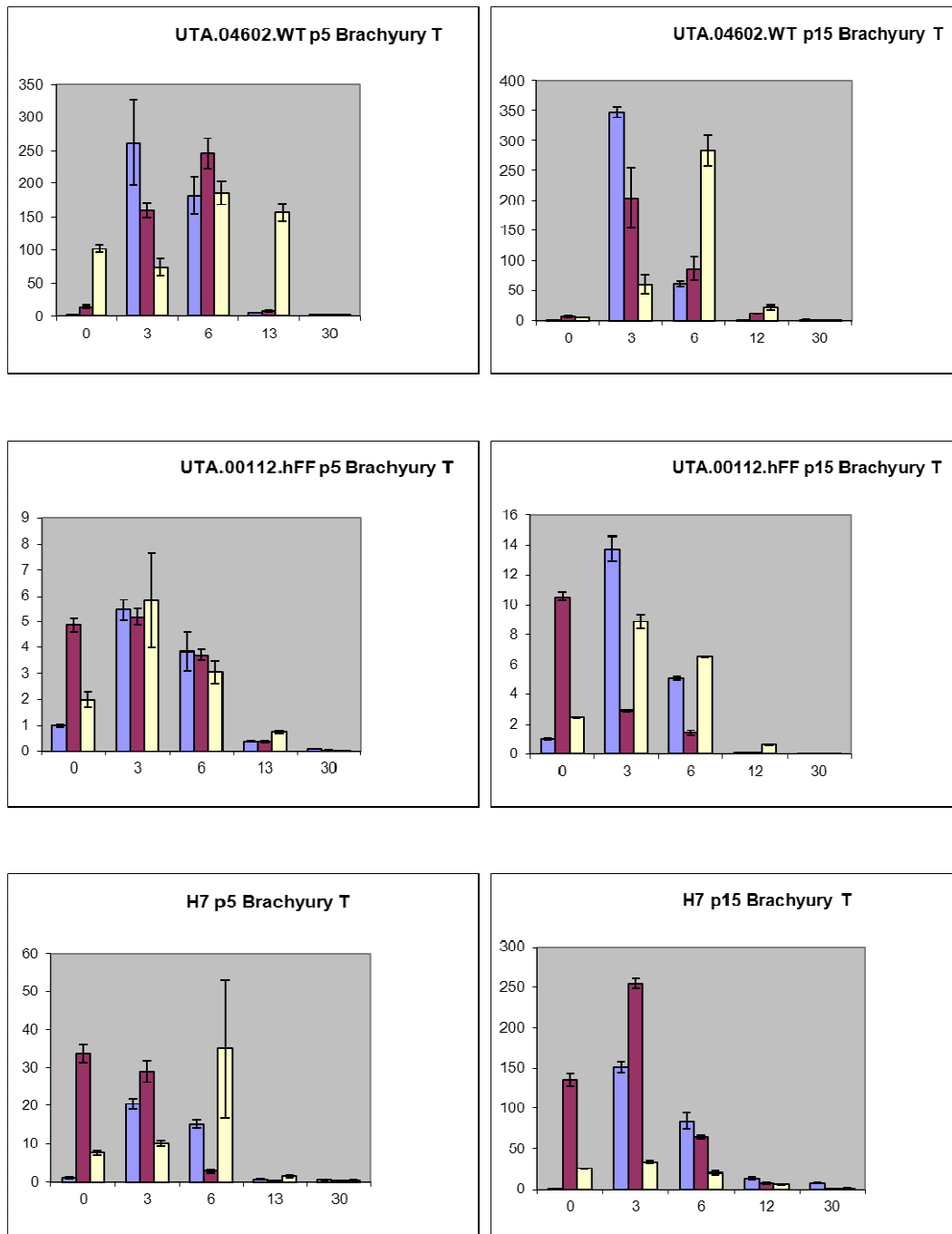


Figure 11. (B.) Brachyury T. Y-axis is the fold change and x-axis the day the sample was taken.

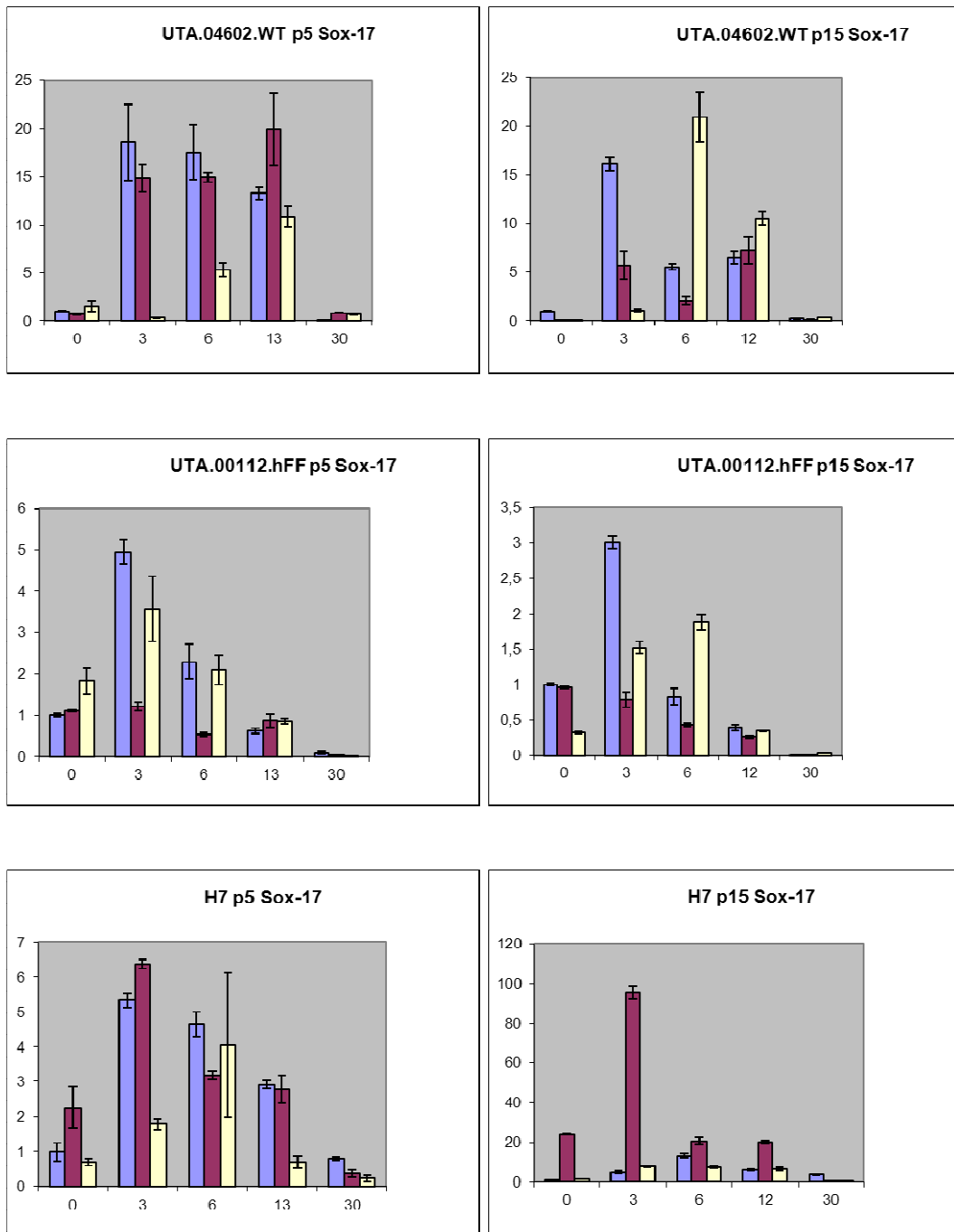


Figure 11. (C.) Sox-17. Y-axis is the fold change and x-axis the day the sample was taken.

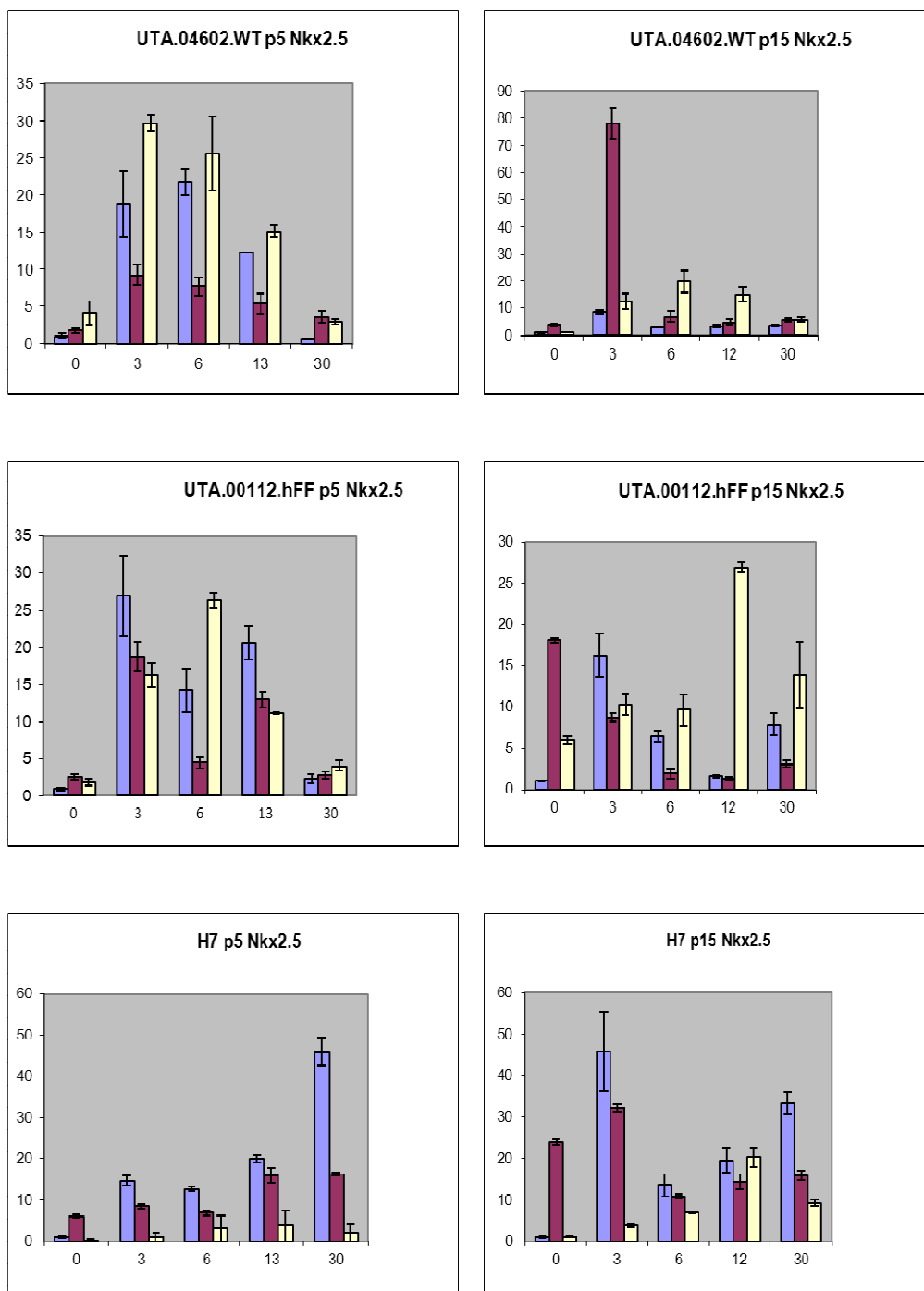


Figure 11. (D.) Nkx2.5. Y-axis is the fold change and x-axis the day the sample was taken.

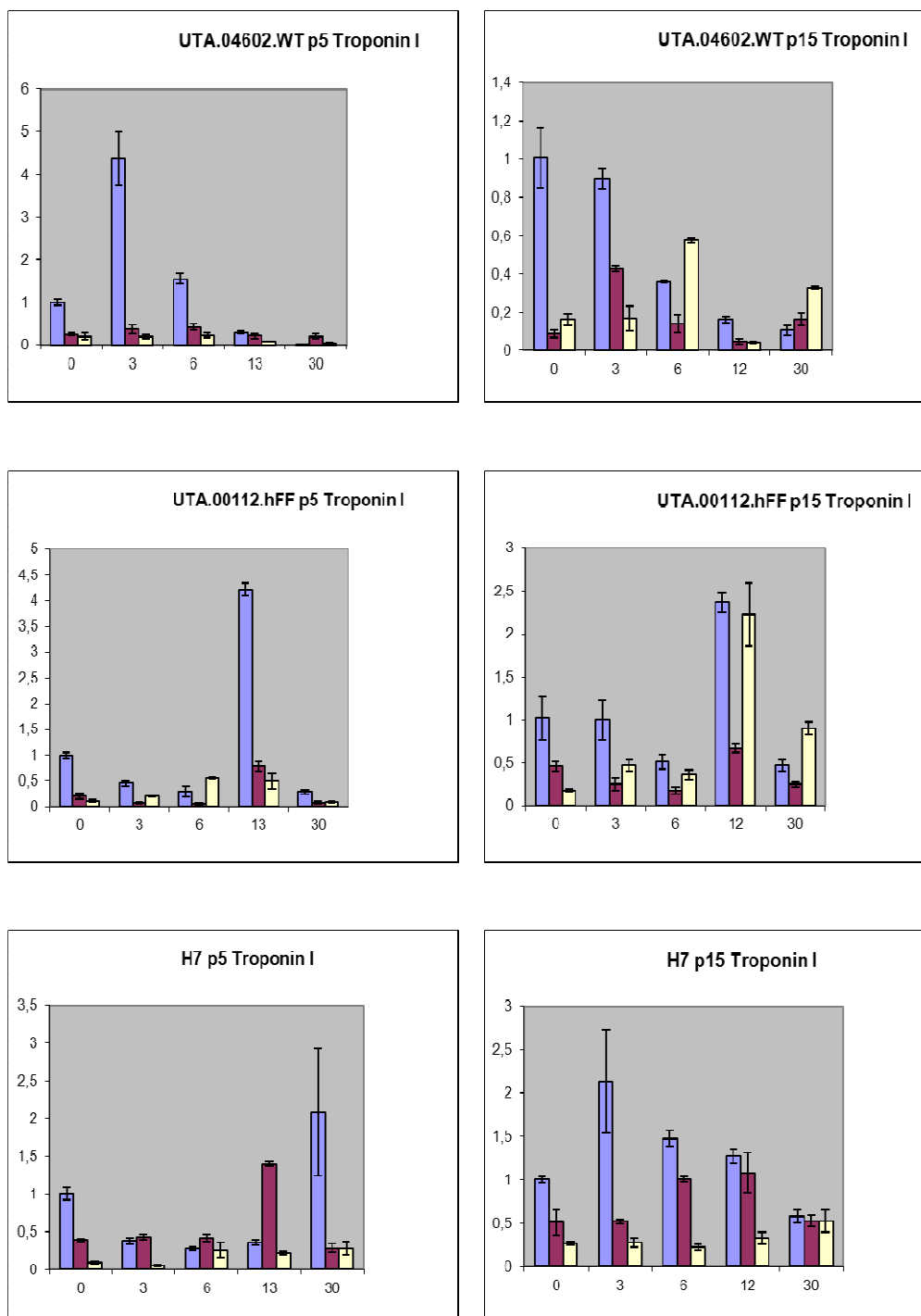


Figure 11. (E.) Troponin I. Y-axis is the fold change and x-axis the day the sample was taken.

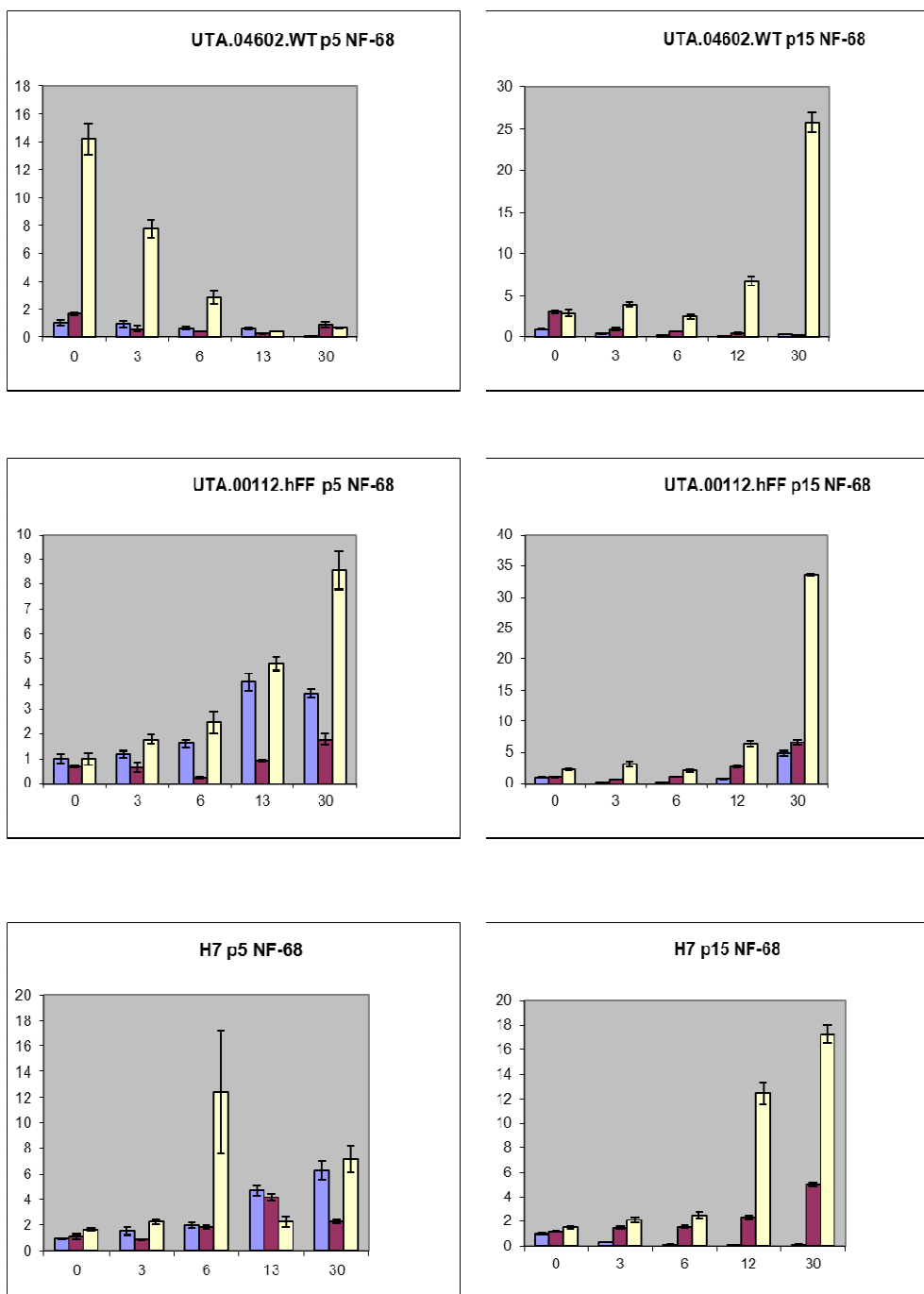


Figure 11. (F.) NF-68. Y-axis is the fold change and x-axis the day the sample was taken.

Figure 11. (A.) – (F.) Relative gene expressions of gene markers.
 OCT3/4 (A.), Brachyury T (B.), Sox-17 (C.), Nkx2.5 (D.),
 Troponin I (E.), NF-68 (F.).
 Y-axis is the fold change and x-axis the day the sample was taken.
 Left side there is passage 5 and right side passage 15.



6 Discussion

The aim of the study was to evaluate the impact of different culture conditions on differentiation of pluripotent stem cells to cardiomyocytes and to characterize the differentiated cells. Both hESC and hiPS cell lines were used in this study and the hypothesis was that both would act similarly in different culture conditions. Morphology, marker expression and differentiation potential which were studied are similar between hESCs and hiPSs. Culturing and differentiating three different cell lines in three culture conditions enabled the comparison of different cell lines and culture conditions to each other. The main goal was to find the best culture condition which would give the most efficient cardiac differentiation results. This culture condition could then be more advanced with some additives like growth factors and used to produce cardiac cells more efficiently than before.

Since the discovery of stem cells, different kinds of culture conditions have been used for different types of stem cells. Most existing stem cell lines have been exposed to undefined xenogeneic products during their lifetime. This exposal makes these cell lines undesirable for clinical applications for humans. Furthermore, these kind of undefined products, such as FBS or human serum, show significant variability passage after passage that may affect reproducibility of the results. The most widely used culture conditions are KO-SR media employed with either human or mouse feeder cells and feeder cell-free culture utilizing Matrigel as a substrate and mTeSR1 medium. Pluripotent stem cells can be differentiated into functional cardiomyocytes, however the differentiation potential of different cell lines differ from each other. Commercially available cell line H7 differentiated more effectively than UTA.00112.hFF and UTA.04602.WT cell lines. Our results support the hypothesis that culture conditions and individual cell lines are the main things affecting to 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).

It has been reported earlier that feeder cell-free derivation and culture of hESCs using defined medium TeSR1 and ECM mix coating from human sources can be carried out successfully (Ludwig et al., 2006). Human iPS cells have similar culture requirements

with hESCs and culture media developed for hESCs was as efficient also for the culture of human iPS cells. In our study colonies grew mainly well on Matrigel. Matrigel matrix used with mTeSR1 medium made the cells form round-shaped and in general undifferentiated. Unfortunately however, a lot of neural cells formed on Matrigel. Overall cardiac differentiation was less from cells originating from Matrigel than cells originating from SNL or MEF feeder cells. This happened because neural cells took place from the wells from the normal growth of stem cells. Most of the colonies attached to the feeder cells or Matrigel matrix on days 1 to 2 after passaging, and on days 6 to 7 the colonies had reached a size where they needed to be passaged. Some spontaneous differentiation of undifferentiated colonies could be noticed after the attachment on days 5-7. Some cell lines have tendency to differentiate more spontaneously than others. Small, neural cells were disturbing the growth of the colonies, especially on Matrigel like mentioned, when not properly removed when passaging. For example cell line UTA.04602.WT grew well on Matrigel. In general can be thought that Matrigel may not be suitable for cell lines that have tendency to spontaneously differentiate towards neuronal lineage. In conclusion Matrigel is not suitable for cardiac differentiation; it may be more suitable for neuronal differentiation.

Very important was also to change the medium at least 6 times a week. If not changed, the growth of the colonies was again hindering because there were no left nutrients for cells to use. Throughout the experiment the size of the passaged cell aggregates was maintained the same, to minimize the variation of the size in the resulting colonies so that the passaging could always be done after 6-7 days and not earlier or later. This way the growth is stable and cells healthier when it is time to start the cardiac differentiation on END-2 cells. Also, hESC and iPS lines exhibit natural variation in growth rates and in their tendency to differentiate spontaneously in culture (Carpenter et al., 2009), which complicates the interpretation of the best culture condition.

The cardiac differentiation was performed using the END-2 co-culture. 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). The number of beating areas per total number of cell aggregates plated onto END-2 cells was counted and differentiation efficiencies determined. The cardiac differentiation efficiencies of the cell lines used varied enormously from 0,5 % to 102, 0 % in END-2 co-culture.

Counting the attached colonies after six days of plating is 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 them. Variation in the differentiation potential of individual hESC and hiPS cell lines has been reported in numerous studies (Mummery et al., 2003, Lee et al., 2005, Zhang et al., 2009). However, it can be suggested from the results that culture condition affects the cardiac differentiation efficiency in addition to the individual characteristics of the cell line. For example, cell lines have different tendencies towards cardiac lineage. Furthermore, the individual characteristics change during culture from passage to passage. When the cell lines get older, there may be more mutations which affects overall to the behavior of the cells. In addition, the freezing of the cell lines may affect to the tendency to differentiate also because of the mutations. This could be the reason to the cell line UTA.00112.hFF less effective differentiation than in the studies conducted to the same cell line before in the same study group. Perhaps there is not only one universal and ideal culture condition for all the cell lines of the world because to the other cell lines other culture condition or differentiation method could just fit better than to the others.

In this study a successful differentiation in form of beating areas was acquired with each culture condition and cell line even though differentiation was not very efficient, and only few beating areas was found in most differentiation experiments. The beating areas varied a lot in size and shape even within the same culture conditions and cell line. Sharp-edged, three dimensional structures have been reported to be conducive to the formation of beating areas (Passier et al., 2005) and this was the case also in our study. Also varying was the speed of beating which was detected under the microscope, even though the functional characteristics were not studied in this study. H7 from MEF had the most of stable beating areas overall. It was hypothesized that the H7 human embryonic control stem cell line would yield best differentiation results. The other cell line UTA.00112.hFF, an iPS cell line derived from neonatal fibroblasts, has also differentiated in earlier studies efficiently to cardiomyocytes. In this study, however, UTA.00112.hFF differentiated even less than UTA.04602.WT, an iPS cell line derived from adult somatic stem cell line. UTA.00112.hFF cell line was thawed from older passage than in earlier studies conducted in Heart group, and this might have an effect on the poor result due to mutations that might have taken place in thawing. The best differentiation efficiency was with cell line H7 cultured on MEFs in passage 10; 102,0

% corresponding to 550 beating areas in 18 wells of 12 well plates and 31 beating areas per well. This H7 cell line formed 3700 cardiomyocytes per well (0,43 % troponin T positive cells in a well of 4 780 000 cells in total). UTA.00112.hFF MEF on passage 10 differentiated most inefficiently on END-2 cells in this study, having an efficiency of 0,5 %, forming 0,1 beating areas per well. This UTA.00112.hFF cell line formed 6 700 cardiomyocytes per well (0,73 % troponin T positive cells in a well of 5 340 000 cells in total). The number of beating areas per plated cell aggregates might give misleading results about the differentiation efficiency.

In cytopspin method before spinning the cells to the class slides the cells have to be enzymatically dissociated into single cell stage. We tested that trypsin was the best of dissociating the cells of available enzyme solutions but even after 45 minutes of incubation in trypsin some wells contained solid cell aggregates which were not dissociating thoroughly even harshly tried. Cell aggregates which remain were either lost before staining or could not be counted in the analysis under the fluorescence microscope because single cells could not be detected from the aggregate. All differentiated cells were expressing in some amounts of cardiac specific genes and proteins such as cardiac troponin, α -actinin and myosin heavy chain. The immunocytochemical staining showed differentiated cells from each cell line and medium.

Quantitative PCR tested the samples for their expression of certain genes. It was hypothesized that the cell lines, which had a better intrinsic cardiac differentiation capacity, would have higher levels of the endoderm and cardiac mesoderm markers expressed during early differentiation stages. Comparing passages 5 and 15, it is obvious that a lot of variation can be found between the results. There are many reasons for this. In passages 5 and 15 there was different pipettor which can cause variation in the final results. Every sample was tried to handle through all the steps the same way but single samples can still get ruined from some unintentional mishandling.

Pluripotency marker Oct-3/4 was detected to decrease towards day 30 samples like expected when the pluripotency decreases towards more differentiated cells. In the day 30 samples the peak is already really low. Oct-3/4 results are pretty similar between both passages. From passage 5 results can be seen that Matrigel condition had the most

Oct-3/4 gene expression, this is not so clearly seen in the charts of passage 15 in which Matrigel condition had the most gene expression only in cell line H7. Without these exceptions, it can be verified that Oct-3/4 gene expression is throughout the samples like expected and decreases towards day 30 in both passages.

An early mesoderm marker Brachyury T has been reported to have an expression peak at day 3 during cardiac differentiation (Bettioli et al., 2007, Graichen et al., 2008) and this can be seen also in our result-charts of Brachyury T. Delayed 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 (Bettioli et al., 2007). On day 6 samples there is also pretty high expression which could indicate the poor differentiation which was true in our study if thinking the small amount of beating areas found. In this study Brachyury T expression was higher on MEF cultures than other cultures of cell line H7. In general the results of Brachyury T are hard to interpret because of the great variation which could be caused of the used gene marker itself. Through all the Brachyury T charts there is no expression on day 30 samples which is logical because it is an early mesoderm marker and the cells of the day 30 of differentiation should already be differentiated to cardiac direction. Endoderm marker SOX-17 gene expression decreases towards day 30 in all the charts with few exceptions. In general there is more expression in cells originating from MEFs and Matrigel than from SNLs which is in line with the results from beating cell counting.

The expression of the cardiac lineage marker Nkx2.5 increases after 4 days of differentiation (Graichen et al., 2008). There is high expression on day 30 with UTA.00112.hFF and UTA.04602.WT cell lines, also high expression can be found from day 0 samples which could be caused of the differentiation happened already when culturing or by the marker itself. However as expected, the expression of Nkx2.5 and Troponin I in differentiated cardiomyocytes was higher than in stem cell samples (Schwartz et al., 1999). The only chart in which the gene expression of all the conditions is clearly increasing is passage 5 of cell line H7. In both passages Matrigel condition has the most Nkx2.5 gene expression like which indicates that this gene expresses better when cells are grown on Matrigel matrix before cardiac differentiation same like Oct3/4 and also troponin I. This indicates that cells from Matrigel condition express these genes better than cells from other conditions and their cardiac

differentiation is proved to be better than cells from SNL which confirms the results of the beating cells counted. Neural cell marker NF-68 is also expressed more in the samples from Matrigel condition than in the others. This was expected because neural cells were seen on Matrigel already when culturing before differentiation. Higher expression is found in the end of differentiation, so more cells have differentiated towards neural cells when time passed, even though cardiac cells were aimed. The qPCR results in part also confirm the differences between the cell lines, showing slightly different gene expression between cells lines with the same conditions.

Differentiated cardiac cells from all the cell lines used were beating spontaneously and expressed specific cardiac markers. From the results obtained, the following conclusion can be drawn. The best culture condition before cardiomyocyte differentiation is clearly the long studied MEF with KSR medium by producing the most beating areas with good morphology and beating. Cell line H7 from the studied cell lines from MEF culture condition differentiated most efficiently to cardiomyocytes. MEFs were also clearly the best for the cells to grow undifferentiated, comparing to SNLs and Matrigel culture conditions. Activin A is reported to be expressed at high levels from MEFs. It induces the formation of primitive streak-like population (Yang et al., 2008). Activin A also enhances mesoendoderm formation which is an early precursor which gives rise to mesoderm and endoderm. Therefore this induction yields probably more efficient pluripotent stem cell derived cardiomyocyte differentiation (Laflamme et al., 2007). So it can be suggested that Activin A may be one key factor affecting the growing and efficient differentiation of cells from MEFs because it is expressed at higher levels from MEFs than SNL feeder cells, and in Matrigel culture condition it does not exist at all. Other key factor of the cardiac differentiation might be insulin. mTeSR medium used with Matrigel culture condition contains 100 ng/ml insulin which may have a hindering effect on the cardiac differentiation and might induce neuronal cell to form. Because of the possible effects of insulin on the cardiac differentiation, KO-SR medium was added in our study not until the day 15 of differentiation because it also contains insulin even though it only contains half of the amount of insulin that mTeSR medium contains.

The goal for stem cell research is to help patients with diseases caused by the loss of functional tissue such as in chronic heart disease. Pluripotent stem cell derived cardiomyocytes are not ready for the clinical use before a lot of development and basic

research is done but the understanding and optimization of the mechanism of cardiomyocyte differentiation from pluripotent stem cells is step towards the solution of treating cardiac diseases. The cardiac differentiation is still inefficient and mostly uncontrolled. The differentiated cardiomyocytes are a mixed population consisting of noncardiac cells and cardiomyocytes with several subtypes. The development of defined culture conditions is beneficial for both hESCs and hiPSCs applications. MEF feeder cells support the undifferentiated state of cells originally derived and maintained on MEF better than SNL feeder cells or Matrigel matrix. After this study, the understanding of the effects of culture condition in the differentiation of cardiomyocytes can be used in future studies to improve cardiomyocyte differentiation. Further studies should be conducted with even more passages to see the variation from passage to passage even clearer.

7 Conclusions

In culture stage neural cells surrounding colonies growing on Matrigel inhibited the normal growth of the stem cells. On MEFs stem cells grew well and usually colonies were round-shaped without excess differentiation. Colonies grown on SNL feeders grew normally well but sometimes they were small-sized even after growing for 5-6 day. Counting of the beating areas was made under a microscope on days 30-32. The cardiac differentiation of stem cells cultured on MEFs was the most efficient in all the three passages. In general the last and the oldest passage 15 had less beating areas compared to earlier two passages 5 and 10. Cell line H7 differentiated most efficiently to beating cardiomyocytes compared to the other two cell lines used in this study.

Quantifying the cardiomyocytes by Cytospin method was conducted on days 19-20 and the amount of troponin T positive cells per well ranged enormously from hundreds to thousands. In this analysis also H7 cell line originating from MEF condition was the most efficient and had the most troponin T positive cardiomyocytes per well through all three differentiations. Both undifferentiated and after the day 30 dissociated differentiated beating cells were immunocytochemically stained to confirm the cardiac differentiation and all the antibodies used bound to their target area successfully.

qPCR was used to assess the mRNA expression levels of different gene markers. It was carried out on all the samples from passages 5 and 15. It was hypothesized that the cell lines, which had a better intrinsic cardiac differentiation capacity, would have higher levels of the endoderm and cardiac mesoderm markers expressed during early differentiation stages. Comparing passages 5 and 15, it is obvious that a lot of variation can be found between the results. Neuronal marker NF-68 is expressed more in the samples from Matrigel condition than in the other conditions like expected because of the culture condition had already a lot of neural cells.

Cardiac differentiation is still inefficient and mostly uncontrolled. Differentiated cardiomyocytes are a mixed population consisting of noncardiac cells and cardiomyocytes with several subtypes. The best culture condition before cardiomyocyte differentiation of all the methods tested in this study is the MEF feeder cells with KSR

medium by producing the most beating areas with good morphology and beating. Perhaps one key factor for this may be Activin A which enhances mesoendoderm formation and through this cardiac differentiation and is reported to be expressed at high levels from MEFs.

In the future the demand for undifferentiated pluripotent stem cells and their differentiated progenies for research and regenerative medicine is expected to increase massively. The culture methods for pluripotent stem cells available today do not allow for the production of cell quantities to meet this demand. In addition, the protocols for the transformation of undifferentiated stem cells to specific functionally differentiated cells are rather inefficient, thus large amounts of the undifferentiated stem cells as a starting material are required. Considerable effort should now be put into the industrial scale-up of stem cell production. Large scale production of stem cells requires new culture technologies which are more robust, efficient and more cost-effective (Améena et al., 2008). After this study, the understanding of the effects of culture condition in the differentiation of cardiomyocytes can be used in future in studies to improve cardiomyocyte differentiation.

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APPENDIX 1. Differentiation results. In the columns from left to right passage time (5, 10, 15), cell line and culture condition (feeder), real passage number, total counted attached areas and number of wells they were counted from, total counted beating areas and number of wells they were counted from and beating areas per one well and differentiation efficiency counted from attached areas and beating areas.

Pas sag e	Cell line	Feede r	Real pass age	Attached areas/wel l	Beatin g areas/ well	Beating area average/one well	END-2 Differentiation efficiency %
p5	H7	MEF	p47	641/18	160/18	8,89	25,0
p5	H7	SNL	p49(6)	268/18	166/18	9,22	61,9
p5	H7	Matri gel	p48(5)	490/18	13/18	0,72	2,7
p5	UTA.0 4602. WT	MEF	p35	244/18	2./18	0,11	0,8
p5	UTA.0 4602. WT	SNL	p37(5)	343/18	22/18	1,22	6,4
p5	UTA.0 4602. WT	Matri gel	p37(5)	136/18	18/18	1,00	13,2
p5	UTA.0 0112.h FF	MEF	p15	162/12	9./12	0,50	5,6
p5	UTA.0 0112.h FF	SNL	p16(5)	96/12	10./12	0,83	10,4
p5	UTA.0 0112.h FF	Matri gel	p23(12)	52/18	4./18	0,22	7,7
p10	H7	MEF	p51	539/18	550/18	30,56	102,0
p10	H7	SNL	p53(10)	233/18	51/18	2,83	21,9
p10	H7	Matri gel	p53(10)	559/18	48/18	2,67	8,6
p10	UTA.0 4602. WT	MEF	p40(10)	405/15	8./15	0,53	2,0
p10	UTA.0 4602. WT	SNL	p48(16)	171/18	8./18	0,44	4,7
p10	UTA.0 4602. WT	Matri gel	p47(15)	133/9	17./9	1,89	12,8
p10	UTA.0	MEF	p18	425/18	2./18	0,11	0,5

	0112.h FF						
p10	UTA.0 0112.h FF	SNL	p21(10)	161/12	12./12	1,00	7,5
p10	UTA.0 0112.h FF	Matri gel	p26(15)	123/18	1./18	0,06	0,8
p15	H7	MEF	p57	368/18	24/18	1,33	6,5
p15	H7	SNL	p63(20)	142/12	30./12	2,50	21,1
p15	H7	Matri gel	p60(17)	832/18	16/18	0,89	1,9
p15	UTA.0 4602. WT	MEF	p44	666/18	9./18	0,50	1,4
p15	UTA.0 4602. WT	SNL	p52(20)	76/10	2./10	0,20	2,6
p15	UTA.0 4602. WT	Matri gel	p51(19)	61/18	20/18	1,11	32,8
p15	UTA.0 0112.h FF	MEF	p22	693/18	6./18	0,33	0,9
p15	UTA.0 0112.h FF	SNL	p25(14)	434/18	1./18	0,06	5,6
p15	UTA.0 0112.h FF	Matri gel	p29(18)	455/18	11/18	0,61	2,4

APPENDIX 2. Cytospin results. In the columns left to right passage time (5, 10, 15), cell line and culture condition (feeder), samples one and two and their percent of troponin T positive cells and average percent of the samples. Counted troponin T positive cells per well from samples one and two and the average of these.

Pas sag e	Cell line	Feeder	TropT positi ve %		Sampl e 1 and 2	TropT positive cells/ well		Sampl e 1and2
			sampl e 1.	sampl e 2.	avera ge %			avera ge
p5	H7 MEF	MEF	0,2	0,2	0,2	3 520	4 000	3 760
p5	H7 SNL	SNL	0,533	0,467	0,5	6 893	6 227	6 560
p5	H7 Matrigel	Matrigel	0,067	0,2	0,134	893	2 760	1 827
p5	UTA.04602. WT MEF	MEF	0,067	0,333	0,2	406	2 153	1 280
p5	UTA.04602. WT SNL	SNL	0,333	0,867	0,6	1 443	3 237	2 340
p5	UTA.04602. WT Matrigel	Matrigel	0,267	0,533	0,4	1 584	4 655	3 120
p5	UTA.00112. hFF MEF	MEF	0,667	0,6	0,634	2 979	1 960	2 470
p5	UTA.00112. hFF SNL	SNL	0,867	0,4	0,634	3 006	2 160	2 583
p5	UTA.00112. hFF Matrigel	Matrigel	0,533	0,467	0,5	3 269	1 370	2 319
p10	H7 MEF	MEF	0,667	0,2	0,434	6003	1386, 667	3 694
p10	H7 SNL	SNL	0,4	0,267	0,334	1 867	1 762	1 814
p10	H7 Matrigel	Matrigel	0,133	0	0,067	3 068	0	1 534
p10	UTA.04602. WT MEF	MEF	1,133	1	1,067	5 665	6466, 667	6 066
p10	UTA.04602. WT SNL	SNL	0,4	0,067	0,234	693	259	476
p10	UTA.04602. WT Matrigel	Matrigel	0,333	0,133	0,233	2 287	789	1 538
p10	UTA.00112. hFF MEF	MEF	0,533	0,933	0,733	4 371	8 957	6 664
p10	UTA.00112. hFF SNL	SNL	0	0,133	0,067	0	479	239
p10	UTA.00112. hFF Matrigel	Matrigel	0,467	0,333	0,4	2148,2	1 421	1 785
p15	H7 MEF	MEF	0,533	0,867	0,7	4 442	7 167	5 804

p15	H7 SNL	SNL	0,4	0,467	0,434	2986,6 67	2864, 267	2 925
p15	H7 Matrigel	Matrigel	0,133	0,2	0,167	2 571	4 387	3 479
p15	UTA.04602. WT MEF	MEF	0,2	0,267	0,234	1 413	1 958	1 686
p15	UTA.04602. WT SNL	SNL	0,733	0,467	0,6	3 958	1 121	2 540
p15	UTA.04602. WT Matrigel	Matrigel	0,133	0	0,067	1 011	0	505
p15	UTA.00112. hFF MEF	MEF	0,333	0,467	0,4	2 797	4 577	3 687
p15	UTA.00112. hFF SNL	SNL	0,4	0,4	0,4	3 253	4693, 333	3 973
p15	UTA.00112. hFF Matrigel	Matrigel	0,07	0,2	0,135	943	2 973	1 958

APPENDIX 3. RNA Concentrations. Total RNA purification from cultured cells. Measured concentrations from samples one and two after purification and the amounts (µl) pipetted on the q-RT-PCR analysis based on their concentrations.

NanoDrop ND-1000 Spectrophotometer, ND-1000V3.7.1, Nucleic Acids, Sample type RNA-40

Concentrations							
Cell line			DAY 0	DAY 3	DAY 5-6	DAY 12-13	DAY 30-32
H7	Feeder		ng/uL	ng/uL	ng/uL	ng/uL	ng/uL
	MEF	1. sample	242,7	177,8	138,0	191,1	141,1
H7		2. sample	305,0	149,7	165,5	183,1	166,1
	SNL	1. sample	405,5	215,9	92,2	109,0	172,5
H7		2. sample	361,1	174,2	105,6	162,4	127,5
	Matrigel	1. sample	98,2	31,4	54,5	47,1	91,6
UTA.04602.WT		2. sample	105,7	89,2	81,8	165,5	106,7
	MEF	1. sample	366,7	98,4	51,4	74,8	106,0
UTA.04602.WT		2. sample	408,5	98,5	37,7	94,6	266,7
	SNL	1. sample	243,6	124,9	21,5	60,3	140,9
UTA.04602.WT		2. sample	233,9	132,5	38,5	25,8	128,6
	Matrigel	1. sample	37,3	46,2	41,3	22,2	120,1
UTA.00112.hFF		2. sample	52,6	56,3	21,1	35,5	142,5
	MEF	1. sample	86,0	24,7	91,0	43,7	86,6
UTA.00112.hFF		2. sample	83,3	51,4	89,8	84,1	34,7
	SNL	1. sample	74,0	46,9	37,9	113,2	212,7
UTA.00112.hFF		2. sample	145,9	74,4	73,7	99,9	241,3
	Matrigel	1. sample	298,5	76,8	37,9	15,0	249,3
Pipetting		2. sample	376,3	105,5	88,7	116,5	222,5
250ng/(ng/uL)=			DAY 0	DAY 3	DAY 5-6	DAY 13	DAY 30-32
H7			RNA ul	RNA ul	RNA ul	RNA ul	RNA ul
	MEF	1. sample	1,03	1,41	1,81	1,31	1,77

H7		2. sample	0,82	1,67	1,51	1,37	1,51
	SNL	1. sample	0,62	1,16	2,71	2,29	1,45
H7		2. sample	0,69	1,44	2,37	1,54	1,96
	Matrigel	1. sample	2,55	7,96=7,5	4,59	5,31	2,73
UTA.04602.WT		2. sample	2,37	2,80 2,5	3,06	1,51	2,34
	MEF	1. sample	0,68	2,54	4,86 = 4	3,34	2,36
UTA.04602.WT		2. sample	0,61	2,54	6,63 = 6	2,64	0,94
	SNL	1. sample	1,03	2,00	11,63= 7	4,15=2	1,77
UTA.04602.WT		2. sample	1,07	1,89	6,49= 3	9,69=8	1,94
	Matrigel	1. sample	6,70 =6	5,41	6,05=3	11,26=7	2,08
UTA.00112.hFF		2. sample	4,75 =4	4,44	11,85=7	7,04 =3	1,75
	MEF	1. sample	2,91	10,12 = 8	2,75	5,72	2,89 =2,80
UTA.00112.hFF		2. sample	3,00	4,86 = 2	2,78	2,97	7,20
	SNL	1. sample	3,38	5,33	6,60	2,21	1,18
UTA.00112.hFF		2. sample	1,71	3,36	3,39=3,40	2,50	1,04
	Matrigel	1. sample	0,84	3,26	6,60	16,67=9	1,00
		2. sample	0,66	2,37	2,82	2,15=1	1,12
Concentrations							
Cell line			DAY 0	DAY 3	DAY 5-6	DAY 12-13	DAY 30-32
H7	Feeder		ng/uL	ng/uL	ng/uL	ng/uL	ng/uL
	MEF	1. sample	170,9	32,4	51,3	61,8	230,8
H7		2. sample	256,2	37,1	81,9	37,6	213,9
	SNL	1. sample	23,0	293,3	118,6	155,8	129,8
H7	8.7.2011	2. sample	47,4	155,0	117,2	22,2	188,1
	Matrigel	1. sample	344,9	126,9	187,5	69,3	170,9
UTA.04602.WT		2. sample	220,2	141,7	241,7	28,9	201,5
	MEF	1. sample	262,9	76,1	141,7	136,3	185,8
UTA.04602.WT		2. sample	342,6	73,3	199,2	144,4	24,5

		sample					
	SNL	1. sample	15,4	37,6	33,5	199,9	180,7
UTA.04602.WT		2. sample	25,8	28,9	25,8	38,2	102,0
	Matrigel	1. sample	64,9	36,3	10,4	18,3	286,1
UTA.00112.hFF		2. sample	51,6	100,7	34,5	21,8	173,8
	MEF	1. sample	129,4	49,7	78,0	223,8	175,1
UTA.00112.hFF		2. sample	180,0	49,7	81,6	199,8	135,5
	SNL	1. sample	199,1	200,2	169,7	101,5	140,4
UTA.00112.hFF		2. sample	273,3	127,9	236,6	169,8	109,0
	Matrigel	1. sample	134,1	60,8	61,2	162,6	
Pipetting		2. sample	120,1	52,8	52,2	133,6	
250ng/(ng/uL)=			DAY 0	DAY 3	DAY 5-6	DAY 12-13	DAY 30-32
H7			RNA ul	RNA ul	RNA ul	RNA ul	RNA ul
	MEF	1. sample	1,46	7,72=5,5	4,87	4,05=4	1,08
H7		2. sample	0,98	6,74=4,5	3,05	6,65=6	1,17
	SNL	1. sample	10,87=8	0,85	2,11	1,6=1	1,93
H7		2. sample	5,27=2	1,61	2,13	11,26=9	1,33
	Matrigel	1. sample	0,72	1,97	1,33	3,61=2	1,46
UTA.04602.WT		2. sample	1,14	1,76	1,03	8,65=8	1,24
	MEF	1. sample	0,95	3,29	1,76	1,83	1,35=1
UTA.04602.WT		2. sample	0,73	3,41	1,26	1,73	10,2=9
	SNL	1. sample	16,23=6	6,65=4,5	7,46=4	1,25	1,38
UTA.04602.WT		2. sample	9,69=4	8,65=5,5	9,69=6	6,54	2,45
	Matrigel	1. sample	3,85	6,89	24,04=8	13,66=6	0,87
UTA.00112.hFF		2. sample	4,84	2,48	7,25=2	11,47=4	1,44
	MEF	1. sample	1,93	5,03=5	3,21	1,12	1,43
UTA.00112.hFF		2. sample	1,39	5,03=5	3,06	1,25	1,85
	SNL	1.	1,26	1,25	1,47	2,46	1,78

		sample					
UTA.00112.hFF		2. sample	0,91	1,95	1,06	1,47	2,29