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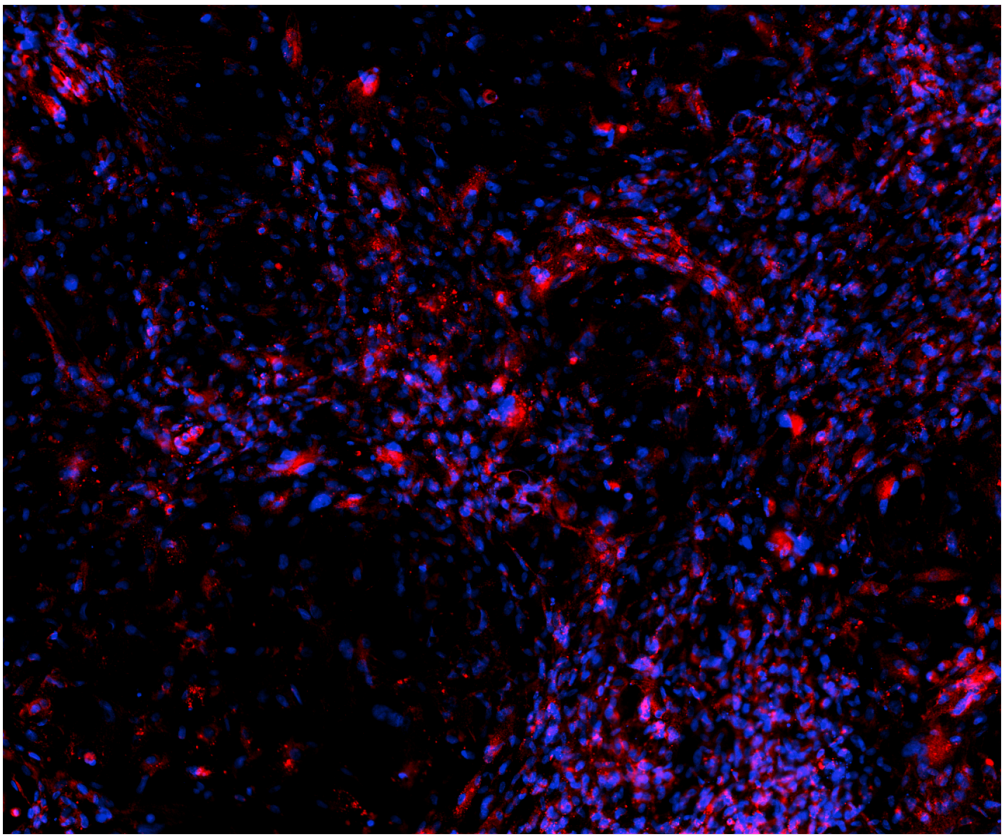

Optimizing culturing conditions using factorial experimental design for culturing of SSEA4+/CD34- cells isolated from human cardiac tissue

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Cover: iCell CPCs cultured on LN-521 in N61 medium stained with Hoechst and cTNNI3 SmartFlare Probes.

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Abstract

Cardiac disease is one of the major causes of death worldwide and current treatment aims at reducing further loss of viable tissue and treating symptoms rather than replenish lost cardiomyocytes in response to cardiac pathology. Therefore, finding an alternative method for treating cardiac disease is of essence. Recently cardiac progenitor cells were found residing in the adult human heart capable of cardiac regeneration. Isolation and *in vitro* cardiomyocyte differentiation of such cells would provide a potential platform for cardiac regeneration. The SSEA4+/CD34- cardiac progenitor cell population have previously shown signs of cardiac commitment, but no stable culture system have yet been suggested. The major aim of this Master's Thesis project was therefore to establish a stable culture system for primary isolated SSEA4+/CD34- cells present in the adult human heart. Real time quantitative PCR was used to confirm the cardiac commitment of the SSEA4+/CD34- population, based on expression of cardiac-specific genes and to determine the medical relevance of such cells in comparison to differentiated induced pluripotent stem cells. It was also determined that short-term culturing of all isolated cells before sorting the population of interest using fluorescence-activated cell sorting, rather promoted endothelial commitment amongst cultured cells. Initial differentiation of SSEA4+/CD34- cells using the GiWi protocol published by Lian *et. al.* did not yield cardiomyocytes, but rather CD31 mRNA specific for endothelial cells was slightly increased while cardiac specific mRNA was maintained. Factorial experimental design was used to determine the relevance of each medium component to maintain primary isolated SSEA4+/CD34- cells. Statistical analysis in MODDE 10.1 showed the model to be unfitted and of low significance, both when using primary isolated cells and pre-ordered iCell CPCs, making all obtained results uncertain. A great variance could also be seen between samples, which could be limited by reducing model size through limiting the number of factors included in the model for future experiments. Thereby the relevance of each factor can be determined with certainty, allowing implementation of a stable culture system for primary isolated SSEA4+/CD34- cells, which potentially could be beneficial for future cardiac regeneration strategies.

Keywords: Cardiac regeneration, cardiac progenitor cells, tissue dissociation, fluorescence-activated cell sorting, real time quantitative PCR, cardiomyocyte differentiation, factorial experimental design, mammalian cell culture, induced pluripotent stem cells

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Märta Jansson, Gothenburg, December 2015

Abbreviations

APC	Adenomatous polyposis coil. Part of the destruction complex
BMP4	Bone morphogenic factor 4
Bry	Brachyury. A transcription factor within the T-box
CD34	Hematopoietic progenitor cell antigen. Cluster of differentiation
CH	CHIR99021, Gsk3 inhibitor
c-Kit	Tyrosine-protein kinase Kit, CD117 or SCFR
cTNNT2	Cardiac muscle troponin T, the tropomyosin-binding subunit of the troponin complex
cTNNI3	Cardiac muscle troponin I, part of the troponin complex
Dsh	Dishevelled. Axin binding, part of the destruction complex
DMEM/F12	Dulbecco's Modified Eagle Medium with Nutrient Mix F-12
ESC	Embryonic stem cell
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FHF	First heart field
FRET	Fluorescence resonance energy transfer
FED	Factorial experimental design
FACS	Fluorescence-activated cell sorting
GATA	Zink finger transcription factor binding 'GATA' sequence
Gsk3	Ser/Thr kinase
HGF	Hepatocyte growth factor
HS	Human serum
IGF-1	Insulin-like growth factor-1
iPSC	Induced pluripotent stem cell
IWP2/4	Inhibitor of the Wnt pathway by prevention of palmitylation of Wnt proteins by Porcupine
LDL	Low density lipoprotein
Lrp5/6	Low density lipoprotein receptor-related protein 5/6. Co-receptor to Frizzled
LN	Laminin
MCP	Multipotent cardiovascular progenitor
Mesp1	Mesoderm posterior 1 Homolog. Transcription factor
MI	Myocardial infarction
Nkx2.5	Homeobox protein Nkx2.5 important for regulation of tissue-specific gene expression
Oct3/4	Octamer-binding transcription factor-3/-4. Expressed in stem cells
PEST	Penicillin/Streptomycin
ROCKi	ROCK inhibitor
RT-qPCR	Real time quantitative PCR
SSEA4	Stage specific embryonic antigen 4
SHF	Secondary heart field
SCM	Suspension culture medium
TGFβ3	Transforming growth factor β3
Tcf/Lef	T-cell factor/Lymphoid enhancer-binding factor
TBX5	T-box transcription factor

Contents

List of Figures

List of Tables

1
Introduction

The human heart has previously been described as a post-mitotic organ, unable to regenerate damaged tissue or lost cardiomyocytes in response to cardiac disease. Measurements of ^{14}C incorporation, remaining in the atmosphere from nuclear testing, to cardiomyocytes have revealed that during a life span, nearly 50% of all cardiomyocytes present in the heart had formed after birth, conflicting with the previous definition (Bergmann O. *et. al.* 2009). Endogenous cardiac progenitor cell populations have been suggested to be the driving force behind cardiomyocyte regeneration, a turnover at an annual rate of 1-2% at the age of 25 which gradually decreases with age (Sandstedt J, 2014; Schade D, Plowright A. T, 2015). One strategy for cardiac regeneration to date is to induce an increased turnover of such endogenous cardiac progenitor cells into cardiomyocytes *in vitro* or *in vivo*, or even re-entry to the cell cycle of preexisting cardiomyocytes. Such strategies are necessary, since ischemic heart disease is considered as one of the major causes of death worldwide (Schade D, Plowright A. T, 2015; World Health Organization, 2014). The need is further increased by the fact that current methods for treatment of cardiac disease only focuses on reducing the loss of viable cells and maintaining functional tissue following cardiac disease rather than replacing lost or damaged tissue. Current methods does therefore not treat the actual disease, only reducing its chronic effects (Lin Z, Pu W.T. 2014; Mummery C. *et. al.*. 2014; Xin M. *et. al.*. 2013).

A group at the Clinical Chemistry Department at Sahlgrenska University Hospital in Gothenburg is currently working on mapping such cardiac progenitor cell populations present in the adult human heart and their distribution. Along with several other scientific groups worldwide, a variety of protocols and growth conditions considered as selective for human cardiac progenitor cell populations have been suggested (Beltrami AP *et. al.*. 2003; Goumans M. J. *et. al.*. 2008; Ott H. C. *et. al.*. 2006). Cardiac progenitor cells will during this project be isolated from dissociated cardiac tissue coming from patients undergoing routine cardiac surgery (coronary artery bypass or valve replacement surgery) at the Cardiothoracic Surgery Department and sorted using Fluorescence-activated cell sorting (FACS) based on expression of progenitor-specific surface markers. According to previous findings SSEA4+CD34- progenitor cells located in the right atrium show signs of cardiac commitment (Sandstedt J, 2014). Consequently, SSEA4+ cardiac progenitor cells will be focused on during this Master's Thesis project.

At AstraZeneca in Mölndal an in-house cardiomyocyte population derived from human induced pluripotent stem cells (iPSCs) are being evaluated as a potential car-

diac disease model as well as a human-specific *in vitro* model for early drug discovery, enabling the possibility of identifying drug candidates lacking efficacy or acting as a toxic agent earlier in the process. As iPS-cell derived cardiomyocytes of human origin more or less can correlate to the *in vivo* milieu it would also circumvent the requirement of animal testing, beneficial in an ethical point of view. Animal testing, which currently is necessary in the drug discovery process and understanding of human diseases, provides an adequate and rather easily accessible *in vivo* model, but it cannot overcome the obvious genetical difference between for example mice and men (Mummery C. *et. al.*. 2014; Xin M. *et. al.*. 2013). Directly isolated primary cells of human origin could therefore provide a suitable complement to animal models, if properly tested and evaluated. It is therefore of interest to both AstraZeneca and Sahlgrenska University Hospital to evaluate cardiac progenitor cells. In this Master's Thesis project growth conditions will be evaluated for a SSEA4+ cardiac progenitor cell population isolated from cardiac biopsies and be examined on a gene expression level using RT-qPCR to evaluate if the expected cardiac progenitor features can be maintained in culture. The gene profile of selected genes will also be examined to enable comparison between the primary isolated cell line and the differentiating iPSCs. This comparison would not only determine the degree of cardiac commitment of the primary cells but also reveal the medical relevance of the differentiated iPSCs.

1.1 Aim

Heart disease is one of the major causes of death worldwide. As an attempt of finding a permanent solution for diseased and failing hearts with reduced cardiac capacity and loss of functional cardiomyocytes, both AstraZeneca and Sahlgrenska University Hospital put a lot of effort into cardiovascular research. This Master's Thesis project, as a part of that research, is aiming at determining the possibility of establishing a stable *in vitro* culture system for the endogenous SSEA4+/CD34- cardiac progenitor cell population present in the adult human heart, as well as evaluating potential changes in genetic expression amongst cultured cells. Establishing a stable culture system would provide a human specific *in vitro* model for early drug development applications, where drug-mediated toxicity could be avoided in early stages, as well as it might provide a potential source of endogenous cells capable of cardiomyocyte regeneration following cardiac disease, such as myocardial infarction. Also, this project might contribute to increased knowledge of endogenous cardiac progenitor cell populations and their behavior in culture.

1.2 Limitations

Due to the highly limited amount of available material as well as time limitations, some aspects will not be included in this thesis. Some of these limitations include

- Cardiac progenitor cells will only be isolated from right atrium appendage biopsies, since such biopsies are readily available as surgical waste from routine cardiac surgery. Therefore it is not possible to draw any conclusions

about the distribution of SSEA4+/CD34- cells in the adult human heart. Ideally cells would be isolated from multiple locations in the heart to enable such comparisons, but will not be possible during this Master's Thesis project.

- Cardiac tissue is obtained from routine coronary artery bypass or heart valve replacement surgery at Sahlgrenska University Hospital, meaning that the tissue originates from diseased hearts. Whether the diseased status has an impact on the availability of or gene expression of endogenous cardiac progenitor cells or not remains unknown. It is also unknown how and if gene expression and distribution of cardiac progenitor cell populations differ between patients and what factors that in such cases are responsible for the differences, as it is known that life style and individual health may induce epigenetic changes. Biopsies used in this thesis are pooled as far as possible, even though no more than two biopsies can be obtained simultaneously due to timing and logistic limitations.

- Due to limited amount of starting material and equipment, isolated cardiac progenitor cells are here cultured in 2D monolayer format. 3D cultures allows for increased mimicking of *in vivo* conditions, but will not be possible in this project.

- Visual observation of spontaneous contraction through a light microscope will be used as functional assessment of cardiomyocyte differentiation of iPSCs. More thorough investigational methods such as measuring changes in action potential using scanning electrochemical microscopy, whole-cell patch clamping or voltage sensitive dyes (Hirano Y. *et. al.* 2014), will not be included in this thesis due to time and resource limitations.

- RT-qPCR will in this thesis be used for assessment of gene expression analysis of iPSC-derived cardiomyocytes and cultured SSEA4+/CD34- progenitor cells. A complete genetic expression analysis lies outside the scope of this thesis, why only chosen genes correlated with progenitor state and cardiac commitment will be included.

- Whether the different medium compositions have an effect on cardiac commitment or induce differentiation towards the cardiac lineage or not is dependent on relatively high cell densities, something that is restricted for SSEA4+/CD34- cells due to the highly limited amount of available material. Such studies will therefore be done using iCell Cardiac Progenitor Cells delivered from Cellular Dynamics International (CDI), which are readily differentiated into spontaneously contracting cardiomyocytes.

1.3 Scientific questions

It is hypothesized that SSEA-4+/CD34- cells may provide an endogenous cell population capable of cardiomyocyte regeneration. It is also believed that such cells

resemble iPSC-derived cardiomyocytes and are possible to culture *in vitro* for further studies on their biological and medical relevance. During this thesis a number of scientific questions will be attempted to be answered. These include

- Can the culture systems routinely used at AstraZeneca for culturing of an in-house iPSC line of human origin be applied on SSEA4+/CD34- cells isolated from human cardiac biopsies? Will such culture systems allow for maintained gene expression pattern in isolated cells or will the cells spontaneously differentiate, resulting in altered gene expression?

- What type of medium would provide a stable culture system for SSEA4+/CD34- cells directly isolated from cardiac tissue?

- How is cardiac progenitor cell associated genes affected in SSEAA4+/CD34- cells by sorting after 5-7 days of culturing of the whole-cell isolate compared to being directly sorted following dissociation? Is it by such means possible to reduce the work load during dissociation without changing the culture morphology of the cells, by sorting the cells at a later time point?

- Is the expected cardiac progenitor features of SSEA4+/CD34- cells maintained in culture? Can the degree of development in SSEA4+/CD34- cardiac progenitor cells be determined when compared to differentiating iPSCs?

- Does FED-model generated media induce cardiomyocyte differentiation in cultured iCell CPCs, and if so, to what extent? Which factors or components in the media promotes cell survival and which have a large effect on the differentiation capacity?

2

Background

The main function of the fully developed mammalian heart is to provide all organs of the body with sufficiently oxygenated blood. It consists of four chambers, right and left atrium and right and left ventricles and is located in the thoracic cavity surrounded by the pericardium (Tortora G. J and Derrickson B, 2013). Both atrium and ventricle cavity pairs are separated by valves that prevent backflow of the blood. The heart itself is provided with blood through two large coronary arteries descending directly from the aorta near the point where the aorta meets the left ventricle. Poorly oxygenated blood is pumped to the lungs for oxygenation from the right side of the heart, while the left side is responsible for providing the rest of the body with sufficiently oxygenated blood. Each cardiac cycle is initiated by depolarization of the sinoatrial node located in the upper right area of the right atrium. The spontaneously arisen action potential is distributed through the atria down to the ventricles through cardiomyocyte to cardiomyocyte transmission and via Purkinje fibers, giving rise to the characteristic rhythmical contracting movement of the heart. Such cells are located in the myocardium, which is the middle layer of the heart wall. The other two layers are the external epicardium and the inner endocardium (Sandstedt J, 2014; Tortora G. J and Derrickson B, 2013; Wilcken D. L, 2006).

Smooth muscle cells, endothelial cells, fibroblasts and cardiomyocytes are the main cell types found in the heart, each contributing to the specialized structural and mechanical features of the postnatal heart. More than 50% of all cells in the heart are fibroblasts, but it is proliferation of the cardiac muscle cells, the cardiomyocytes, that enables the heart to grow during embryogenesis. This event is terminated shortly after birth, after which cardiomyocytes undergo DNA synthesis without cytokinesis, resulting in polyploidity of the cells. Postnatal heart growth is thereby not mediated through cell proliferation but rather by hypertrophy of cardiomyocytes, leading to limited regeneration capacity of the postnatal heart (Xin M. *et. al.*. 2013). Therefore, damaged or lost cardiomyocytes due to injury or cardiac disease cannot be fully regenerated and cardiac function is in many cases impaired, potentially leading to the death of the patient. As the malfunctioning cardiomyocytes are gradually replaced by fibroblasts and scar tissue is eventually formed, the contractile capacity of the heart will be significantly reduced. Tissue damage due to myocardial infarction can be as great as 25% loss of the total myocardial mass of cardiomyocytes (Ptaszek L. M. *et. al.*. 2012). The severity of cardiac damage generally depends on the extent and time period of hypoxia caused by impaired blood flow. Current treatments seeks only to reduce the effect of cardiac disease and to maintain the functionality of non-diseased cardiac tissue, but none of the current methods are able to restore

lost cardiomyocytes. If damage is too great heart transplantation may be necessary, despite the risk of highly limited availability of donor hearts, patient rejection and requirement of life-long intake of immunosuppressant drugs. Since cardiac disease is the major cause of death worldwide, it is of great importance of finding alternative ways of treatment. One of the methods that have been suggested is cardiac regeneration through endogenous hCPCs residing in the adult heart (Mummery C. *et. al.*. 2014; Xin M. *et. al.*. 2013).

2.1 Cardiac embryology

During mammalian embryonic development, all cells that eventually will give rise to the definitive structures of the fetus are derived from the inner cell mass of the blastocyst. Cells of the inner cell mass are pluripotent rather than totipotent, meaning that they are capable of developing into all cells that eventually will constitute the organism but not into the cell types required for maintaining and developing the embryo. The inner cell mass is triploblastic, thus giving rise to three cell types or germ layers during gastrulation, see figure 2.1. These are ectoderm, mesoderm and endoderm. Ectoderm is the outer layer and it undergoes neurulation, a process resulting in the formation of three new types of cells; neural tube cells, neural crest cells and epidermis. The neural tube and neural crest cells give rise to the central nervous system, brain and facial cartilage while the epidermis cells will develop into skin, sebaceous glands and mouth, anus and olifactory epithelium. The cells of the endoderm get pushed into the interior of the blastocyst during gastrulation. Here it will eventually give rise to the gut, inner linings of the respiratory and digestive system, the pancreas and lungs as well as the tonsils and thymus. In between the outer and the inner basal layers the mesoderm is located. The mesoderm gives rise to muscles, connective tissue, blood cells and to the cells of the first functional organ of the developing embryo, the heart. Signals, such as bone morphogenic protein 4 (BMP4), will also be secreted from surrounding developing cell types during early embryogenesis resulting in the formation of the germ cells (Gilbert S. F. , 2010; Mummery C. *et. al.* 2014; Alberts *et. al.* 2008).

Embryonic cardiac development has previously been extensively studied and it involves a series of molecular and morphogenic events, and includes several tissue specific transcription factors and signaling molecules influencing different pathways. The cells which are to build up the presumptive heart as well as neural crest cells, originate from the early primitive streak. A crescent migration of cells occurs resulting in two distinct groups of mesodermal cells, called cardiogenic mesoderm. These two groups of multipotent cardiovascular progenitors (MCPs) can be further characterized as first heart field (FHF) and second heart field (SHF) MCPs. MCPs are tripotent, meaning that they can be further differentiated only into three cell types constituting the final heart, namely mesothelium, endothelium and myocardium (Gilbert S.F., 2000). It has been shown that tripotent MCPs express *Brachyury* (Bry), while some bipotent MCPs express Nkx2.5 and c-Kit (Bondue *et. al.* 2011; Kattman *et. al.* 2006). Further, SHF MCPs express Isl1 (Bruneau B. G. 2015; Henning R. J. 2011), one of the earliest markers for cardiac commitment along with

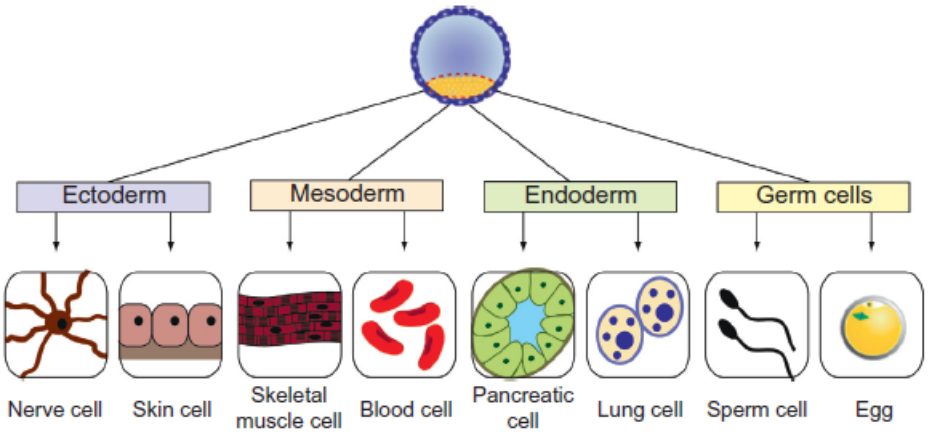


Figure 2.1: The germ layers formed through gastrulation of the blastocyst (Mummery C. *et. al.* 2014)

Mesp1 (Saga *et. al.* 2000; Bondue *et. al.* 2011; Bondue *et. al* 2008; Bruneau B.G. 2015). From the flat disc of tripotent cells two cardiac tubes will form in the shape of a crescent, seen in figure 2.2 (Srivastava D., Olson E.N. 2000; Bruneau B.G. 2015). Cells originating from the FHF are responsible for right and left atria as well as left ventricle development. The origin of the right ventricle has been debated but has been suggested to be developed from SHF MCPs (Bondue *et. al.* 2011).

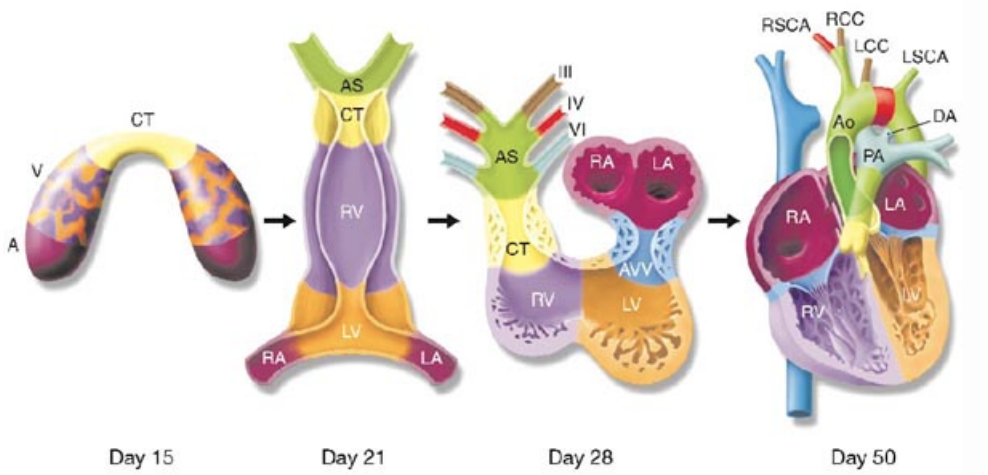


Figure 2.2: Embryonic cardiac development. AS, aortic sac. CT, conotruncal valve. AVV, atriuventricular valve. A, atrium. Ao, aorta. DA, ductus arteriosus. LA, left atrium. LCC, left common carotid. LSCA, left subclavian artery- LV, left ventricle. PA, pulmonary artery. RA, right atrium. RCC, right common carotid. RSCA, right subclavian artery. RV, right ventricle. V, ventricle. (Srivastava D., Olson E.N. 2000)

Around three to four weeks of embryogenesis the cardiac tubes start to fuse into a single linear tube as a result of migrating cells. Spontaneous contraction occurs around this time, due to the intrinsic contractile ability of cardiac muscle cells or

cardiomyocytes, enabling blood to pump through the early heart even without complete valve structures. It has been shown that transcription factors of the GATA family play an important role in this process (Gilbert S.F., 2000; Hill M.A. 2015, Sandstedt J. 2014). By late fourth or early fifth week of embryogenesis the developing heart consists of two chambers, one atria and one ventricle. During this time looping of the heart tube occurs. The directionality of the looping is dependent on certain proteins. The Hand1 and Hand2 transcription factors are regulated by Nkx2.5, and have been shown to have a crucial impact in looping of the cardiac tube along with Pitx-2. After looping is complete, Hand1 becomes confined to the left and Hand2 to the right portion of the developing heart. Blocking any of these proteins would result in a hampered looping process and disables normal formation of the ventricles. Nkx2.5 along with MEF2C, Xin, activates initiation of cytoskeletal changes required for the looping to occur (Gilbert S.F.; Hill M.A. 2015; 2000, Wang *et. al.* 1999). A complex rotation occurs and endocardial cells accumulate, resulting in formation of an endocardial cushion that separates right and left atrioventricular channels and thus initiates the definition of the four cardiac chambers. The formation of the endocardial cushion is believed to be triggered by members of the TGFβ-family such as TGF-β3 and BMPs. Late cardiac development continues, terminally defining the four cardiac chambers as well as formation of heart valves and the trabeculations at the luminal surface of the heart. The development of the embryonic heart is completed around day 50 (Gilbert S.F., 2000; Hill M.A. 2015; Sandstedt J. 2014). An overview of central transcription factors and other proteins during embryonic cardiac development is given in figure 2.3.

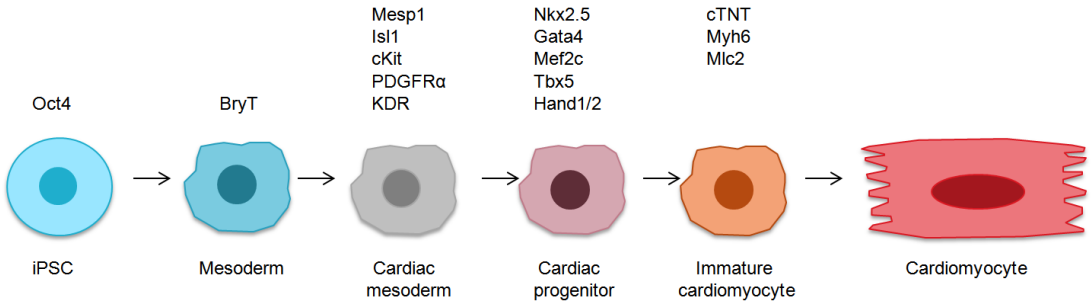


Figure 2.3: Central transcription factors and other proteins in cardiac development. Picture adapted from (Yi B. A. *et. al.* 2010)

2.2 Cardiac progenitor cells in adult hearts

The human heart has previously been recognized as a post-mitotic organ with limited regenerative capacity. Recent publications however show the presence of stem cells and cardiac progenitor cells both in adult murine and human hearts (for example Beltrami A.P. *et. al.* 2003).

Cardiac progenitor cells can be described as resident cardiac committed, or mesodermal induced cells of unknown origin. Constituting only around 1% of the entire cell population of the heart, cardiac progneitor cells are believed to be the reason for cardiomyocyte and vascular endothelial turnover observed within the adult heart. Such cells are categorized based on expression of surface proteins or transcription factors, such as c-Kit, Sca1 and Isl1 (Beltrami A.P. *et. al.*. 2003) (Sandstedt J. 2010) (Henning R. J. 2011). c-Kit is a tyrosine receptor that is activated by binding of either membrane bound or soluble stem cell factor, which induces dimerization of the receptor and thus activates it to allow further intrinsic tyrosine kinase activity. c-Kit expression have been characterized as specific for hematopoietic stem cells, but is lost during hematopoietic differentiation. In the murine heart, a subpopulation of c-Kit+ cells negative for hematopoietic markers has been identified, which is capable of differentiating into the three major cardiac cell lineages. These cells have an essential role in the cardiac response to myocardial injury and are important in the chemoattraction of stem cells to the injured tissue, which is why they are of interest for future cardiac regeneration strategies (Beltrami A.P. *et. al.*. 2003; Sandstedt J. 2014; Henning R. J. 2011; Sandstedt J. *et. al.*. 2010). Other cardiac progenitor cell populations found within the adult human heart includes cells expressing Isl1 and Nkx2.5, both important in early cardiogenesis, as discussed in section 2.1. Isl1 and Nkx2.5 expressing cells are also negative for c-Kit and Sca1, a marker found in murine hematopoietic stem cells, commonly co-expressed with c-Kit (Holmes, 2007). Sandstedt J *et. al.* presented the finding of a cardiac progenitor cell population expressing SSEA4. SSEAs, or stage specific embryonic antigens are superficial carbohydrate structures, recognized by certain monoclonal antibodies and exists in many varieties (Hamburger Shevinsky L. *et. al.* 1982). SSEA-1, -3 and -4 have all been linked to differentiation status in embryonic stem cells (ESCs). In human ESCs, SSEA-3 and SSEA-4 are expressed and during differentiation SSEA-1 is highly expressed while SSEA-3 and SSEA-4 is down regulated. Therefore, analysis of SSEA expression would offer a convenient method of evaluating differentiation status in immature cardiac cells (Sandstedt *et. al.* 2014).

2.3 Cardiac regeneration strategies

In 2009 Bergmann *et. al.* showed evidence of cardiomyocyte renewal by 0.5-1% annually in adult hearts by measurements of incorporated ^{14}C in response to the increased atmospheric levels caused by above-ground nuclear testing. (Bergmann *et. al.* 2009). An increased cardiomyocyte turnover in response to cardiac injury has been seen in rodent models. As many as 3.2% of the cardiomyocytes in the myocardial infarct border zone in adult rodents had re-entered the cell cycle and divided over an 8-week period, compared to 0.76% annual turnover in healthy adult mice (Senyo S.E. *et. al.* 2013). Such findings increase the anticipation of cardiac regeneration. To date, there are four general regeneration strategies, namely i. repopulation of isolated cardiac progenitor cells to diseased cardiac areas after *in vitro* expansion, ii. reentry of pre-existing cardiomyocytes to the cell cycle, iii. enhancing cardiomyocyte turnover by activating resident cardiac progenitor cells and iv. inducing non-cardiomyocyte cell turnover into new cardiomyocytes (Lin Z, Pu W.T.

2014; Senyo *et. al.* 2013). Each strategy is presented in more detail below.

i. **Cell therapy using resident cardiac progenitor cells**

Cardiac regeneration has also been suggested to benefit from transplantation of various cell types capable of cardiomyocyte differentiation. Skeletal myoblasts were in early studies transplanted with the hope that they would differentiate into cardiomyocytes in response to the kinases and other factors present in the cardiac environment. Unfortunately it was shown that the transplanted skeletal myoblasts did not differentiate into cardiomyocytes, but rather into myotubes without electrical and contractile properties and did not integrate with surrounding cardiac tissue. When tested in clinical trials with patients it was shown that the lack of proper integration into the myocardium caused an increased risk of arrhythmia, why the idea of transplanting skeletal myoblasts was discarded (Sanganalmath S. L, Bolli R, 2013). More recent studies have shown that other cell types with cardiac activity can be utilized in the field of cardiac regeneration. Pluripotent ESCs represent such a cell type. Differentiated ESCs have been shown to increase cardiac function after MI upon delivery into diseased cardiac areas in rodents, but when transplanted in their pluripotent state teratoma formation was observed. Along with the oncogenic issue the use of ESCs are connected to ethical issues concerning the embryonic origin. To circumvent the ethical issues iPSCs may in many cases offer a substitute for the use of ESCs. iPSCs were first produced by Takahashi and Yamanaka in 2006 (Takahashi K, Yamanaka S. 2006) , when their discovery of the "Yamanaka factors", namely OCT3/4, Sox2, c-Myc and Klf4 allowed them to redefine the cell fate, or "reprogram" adult mouse fibroblasts into ESC-like morphology and growth characteristics. Reprogramming of autologous fibroblasts would therefore circumvent the otherwise required of immunosuppression. Despite the obvious benefits from using iPSCs, one cannot circumvent the time requirement for producing such cells, disabling the use of iPSCs when treating acute conditions such as MI. The pluripotent state and rapid proliferation of iPSCs may also induce tumorgenesis. Also, the Yamanaka factors are known to be oncogenic, often causing teratomas. These are issues that needs to be circumvented before iPSCs can be implemented as a therapeutic agent for treatment of acute cardiac failure (Sanganalmath S. L, Bolli R, 2013; Lin Z, Pu W. T. 2014).

Endogenous cardiac progenitor cells provide another source of cells with potential for cardiac regeneration, as they have been suggested to be the driving force behind the slow cardiomyocyte turnover in the adult human heart. Several populations have been characterized, such as c-Kit+, Sca1+ in rodent hearts, side population and Isl1+ cells, all reported to have some potential for cardiac regeneration. The ability of c-Kit+ cells to serve as cardiac progenitor cells have though been questioned, and the distribution of progenitor populations is hard to predict as the individual-specific variability is great. Isl1+ populations have been shown not to exist in postnatal ventricular myocardium. Therefore the use of endogenous cardiac progenitor cells might be limited, even if such

cells are readily available as well as circumventing the need for immunosuppression (Sanganalmath S. L, Bolli R, 2013; Weinberger F. *et. al.* 2012). To date, two phase-1 studies on re-populating the human heart with cardiac progenitor cells have been reported, the SCIPIO and the CADUCEUS trials. In the SCIPIO trial c-Kit+ cardiac progenitors was administered to human heart following ischemic injury resulting in the reduction of infarct size, improved ventricle functionality and an increase in viable cardiac tissue compared to untreated patients, but these findings have been questioned (Chugh A.R *et. al.* 2012). The second phase-1 study is the CADUCEUS trial, where cardiosphere-derived cells were administered to the ischemic human heart. Similar findings was seen in the CADUCEUS trial as in the SCIPIO trial, with reduced infarct size and improved ventricle functionality (Makkar R. J *et. al.* 2012).

ii. **Cell cycle re-entry**

Unlike the neonatal heart, the adult human heart is allowed to grow mostly through hypertrophy of pre-existing cardiomyocytes rather than proliferation, resulting in its strictly limited regeneration capability (Xin M. *et. al.* 2013; Mollova *et. al.* 2013). In rodents, cardiomyocyte proliferation is terminated one week after birth but the precise timing for cardiomyocyte cell cycle arrest in humans is to date unknown even though some studies have indicated that cardiomyocyte proliferation is continued for some time after birth. The approach where cardiomyocytes are stimulated to reenter the cell cycle is an attractive strategy in regenerative medicine and one strategy for increasing understanding of such mechanisms is to study the native mechanisms of myocardial growth. Cell cycle arrest is under transcriptional and epigenetic control, as certain cell cycle genes in rodent cardiomyocytes have been characterized. *Cyclin A, cyclin B* and CDC2 represents some of the cell cycle genes in rodent cardiomyocytes being strongly down-regulated upon cell cycle arrest, with significantly reduced cardiomyocyte proliferation as result, whereas cyclin-dependent kinase inhibitors are highly expressed, a driving force in cell cycle arrest. Cell cycle re-entry of rodent cardiomyocytes has therefore been showed to be induced by repression of such cell cycle genes. Such behavior has also been shown to be induced by transcriptional control by increasing the activity of transcription factors or introduction of microRNAs (miRNAs) acting as transcription factors. Delivery of miRNAs has previously been mediated using adeno-associated virus, a method not yet applicable in humans due to the inherent risks using virus as delivery agents. There is also a risk for unwanted off-target effects, such as oncogenesis as a result. Thus further studies are required before induction of cardiomyocyte proliferation can be applied on human hearts (Mollova *et. al.* 2013; Lin Z, Pu W. T. 2014).

iii. **Enhancing resident cardiac progenitor cell activity**

Another strategy for replenishing lost cardiomyocytes in response to cardiac disease is to enhance the activity of endogenous cardiac progenitor cells *in situ*.

Some regenerative activity in the adult heart has been seen, but whether resident cardiac progenitor cells are responsible for this cardiomyocyte turnover or not is to date unknown. Several cardiac progenitor cell populations have been found within the adult human heart. One of these population is located in the epicardium and is important during cardiac development, by transitioning from epithelial to mesenchymal cells. Thereby they offer a source of epicardium-derived cells capable of differentiating into cardiac fibroblasts, coronary smooth muscle cells and to some extent also cardiomyocytes. Injection of thymosin β to diseased rodent hearts resulted in the generation of *de novo* cardiomyocytes, although not to a significant extent capable of improving cardiac function following cardiac disease (Lin Z, Pu W.T. 2014; Smart N. *et. al.* 2011). Activation of resident cardiac progenitor cells requires an inducing agent. Paracrine factors have been suggested to induce cardiomyocyte turnover of cardiac progenitor cells but the delivery is crucial, as well as the dosing, timing and duration. DNA plasmids, viral vectors or peptides have been suggested as delivery vessels for paracrine factors but have not yet yielded any promising results. Modified RNA (modRNA) was evaluated for delivery of inducing factors in rodent models, which after injection to infarcted areas resulted in effective delivery with reduced infarction size and improved cardiac function. ModRNA is believed to have good translatability to humans, and are immunocompatible, reducing the requirement for immunosuppressants. The safety of using modRNA in humans requires further studies, as potential off-target effects might be present (Zangi L. *et. al.* 2013) .

iv. **Non-cardiomyocyte cell turnover**

In 2008 Liu *et. al.* showed that treating fibroblasts with the myogenic regulatory factor (MyoD) alone or in combination with connexin 43 (Cx43), the major gap junction protein in cardiomyocytes, induced transdifferentiation into myocyte-like cells. By showing the possibility of transdifferentiating fibroblasts into cardiomyocytes opened up for a new area in cardiac regeneration, where scar tissue consisting mainly of fibroblasts following MI could theoretically be transdifferentiated into cardiomyocytes, thus reducing scar size and increasing cardiac function following cardiac injury (Liu *et. al.* 2008; Davis RL *et. al.* 1987; Hochedlinger K 2010). The discovery of the Yamanaka factors allowed reprogramming of fibroblasts into iPSCs capable of *in vitro* cardiomyocyte differentiation (Takahashi K, Yamanaka S. 2006). Later it was showed that transcription factors important during cardiac development such as Mef2c, Gata4 and Tbx5 directly transdifferentiated fibroblasts into myocyte-like cells, expressing cardiac-specific genes and surface markers, without passing through a pluripotent intermediate. The factors were delivered by viral vectors acting through integration, not suitable for use in humans. Also the degree of reprogramming efficiency is to date unknown as well as the functional integration efficiency of *de novo* cardiomyocytes to the native environment. Thus, more studies on rodent models is required before the transdifferentiation of fibroblasts into cardiomyocytes can be applied for treatment of MI in human patients

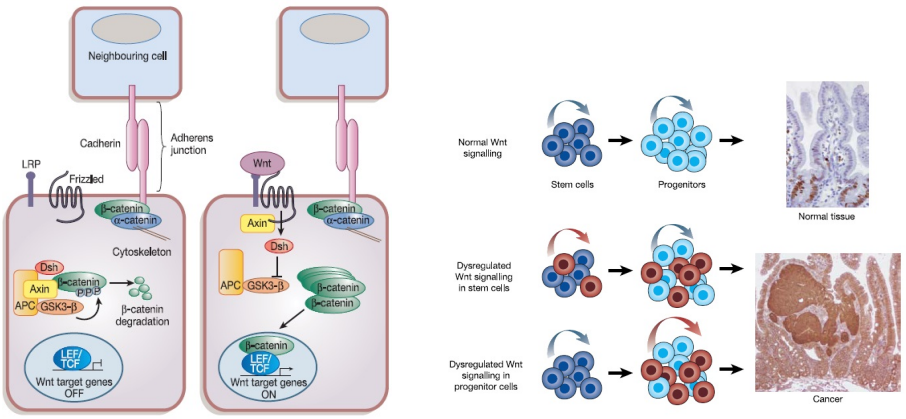
(Ieda M. *et. al.* 2010; Lin Z, Pu W.T. 2014).

2.4 *In vitro* cardiac differentiation through the canonical Wnt pathway

There are several publications and protocols for *in vitro* cardiac differentiation from ESCs or iPSCs. Mummery *et. al.* showed that ESCs could be differentiated to cardiomyocytes in a 2D fashion by co-culturing with the pro-cardiac endodermal cell line END2 (Mummery *et. al.* 2003). Laflamme *et. al.* showed that cardiac differentiation can be induced by addition of activin A and BMP4 to the culture medium, which have been found to be important for mesodermal differentiation. They reported a 30% increased cardiomyocyte yield compared to previous results (Laflamme *et. al.* 2007). Another approach for cardiac differentiation was described by Lian *et. al.*, where inhibition of the canonical Wnt pathway was used for induction of cardiac differentiation, see figure 2.4(a) (Reya T and Clevers H. 2005; Rattis F.M. *et. al.* 2004). The canonical Wnt pathway is a key regulator in cardiogenesis and is highly up regulated during early embryogenesis and highly down regulated at later stages in embryogenesis (Lian *et. al.* 2013; Lian *et. al.* 2012). There are approximately 20 Wnt genes in the human genome that trigger the signaling cascade upon binding to the transmembrane Lrp 5/6 receptor of the LDL family (Reya T. and Clevers H 2005; Clevers H 2006). Binding of Wnt protein induces a conformational change allowing formation of a complex with the seven-transmembrane domain receptor Frizzled. The key role in Wnt signaling is played by the cytoplasmic protein β-catenin.

Without a bound Wnt protein, the Lrp 5/6 receptor is apart from Frizzled, which allowes the destruction complex to bind and degrade cytoplasmic β-catenin. The destruction complex consists of the tumor suppressors APC and axin as well as the kinase Gsk3 and the axin-binding Dishevelled (Dsh). Gsk3 phosphorylates β-catenin, which attracts a β-TrCP-containing E3 ubiquitin ligase that degrades β-catenin. Binding of a Wnt protein to the Lrp 5/6 receptor hinder axin and/or Dsh to bind to the destruction complex through translocation of the negative Wnt regulator axin, thus preventing degradation of β-catenin. β-catenin is allowed to accumulate and is transported to the nucleus, where it triggers gene expression by binding to DNA-binding proteins of the Tcf/Lef family. Uncontrolled Wnt signaling or mutations in *Tcf/Lef* genes causes uncontrolled cell proliferation and tumors in several murine models. Reya and Clevers showed that mice with one mutated *Tcf/Lef* gene allele developed hair follicle tumors, see figure 2.4(b) (Reya T, Clevers H 2005; Clevers *et. al.* 2014). The Wnt pathway thus regulates cell fate and directs proliferation through binding of ligands that inhibits Gsk3. Controlling the Wnt pathway thus allow for controlled *in vitro* differentiation towards the cardiac lineage (Lian *et. al.* 2013; Lian *et. al.* 2012).

In 2012 Lian *et. al.* showed that iPSCs can be robustly and efficiently differentiated towards the cardiac lineage by inhibiting the Wnt pathway, an approach yielding 82-



(a) Canonical Wnt signalling pathway

(b) Uncontrolled Wnt signalling can result in tumor formation

Figure 2.4: Wnt signaling (Reya T and Clevers H. 2005; Rattis F. M. *et. al.* 2004)

98% more cardiomyocytes than previous protocol. In the GiWi-protocol published in 2013 by the same group, iPSCs are initially cultured in defined RPMI 1640x supplemented with B27 without insulin, as insulin is known to inhibit early cardiac differentiation, see figure 2.6. At day 0 of cardiac differentiation the Gsk3 inhibitor CHIR99021 (CH) is added, resulting in accumulation of β-catenin in the nucleus as Gsk3 no longer is capable of phosphorylating the protein. CH also activates TCF/LEF promotor activity, promoting up regulated gene expression of Wnt target genes.

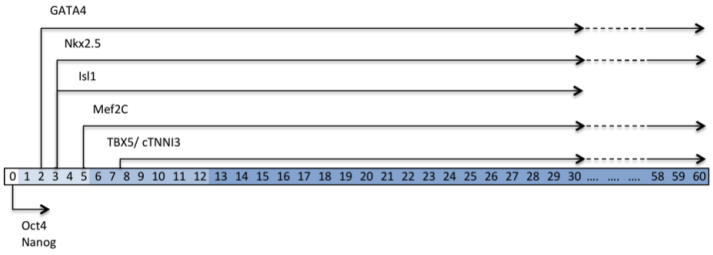


Figure 2.5: Gene expression profile during GiWi-cardiomyocyte differentiation. Adapted from Lian *et. al.* 2012

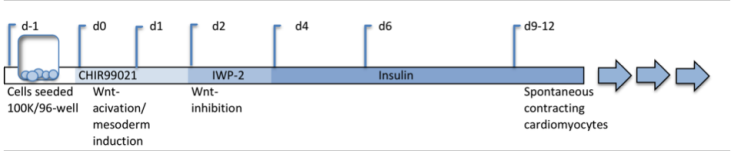


Figure 2.6: Cardiomyocyte differentiation by modulating Wnt/β-catenin signaling. Adapted from Lian *et. al.* 2013

After inducing cells with CH cells have been shown to express Isl1 and Nkx2.5, indicating an early mesoderm induction. It has also been shown that if β-catenin is down regulated, expression of stem cell-specific genes, such as Oct4, Nanog and

SSEA4 is maintained. Inhibitor of Wnt production 2 or 4 (IWP2/4) is added at day 2, resulting in inactivation of Wnt signaling by prevention of palmitylation of Wnt proteins by Porcupine. At day 6 the medium is changed to RPMI 1640x supplemented with B27 containing insulin, as this effectively triggers cardiac mesoderm induced cells to develop into spontaneous contracting cardiomyocytes within 8-12 days (Lian *et. al.* 2013, Lian *et. al.* 2012).

3
Materials and Methods

In this Master's thesis project focus is laid on establishing a stable culture system allowing for maintenance of primary isolated SSEA4+/CD34- cardiac progenitor cells outside the natural environment, without inhibiting expression of progenitor specific genes while adapting culturing morphology. Cells originating from patients undergoing routine cardiac surgery at the Thoracic Surgery Department at Sahlgrenska University Hospital were isolated using FACS sorting and cultured for two to four weeks. A variety of media and coating compositions were evaluated using a factorial experimental design (FED) model for optimal cell viability and proliferation. Gene expression of cultured primary cardiac progenitor cells were characterized by RT-qPCR and compared with iPSC-derived cardiomyocytes, a cell type routinely used at AstraZeneca. In the following section the experimental layout of this Master's thesis project will be discussed in more detail. Some brief information about included methods will also be presented.

3.1 Choice of culturing medium

Initially a minor number of media and coating compositions were evaluated for primary cardiac progenitor cell viability based on previous publications and experiences from the cardiac group at Clinical Chemistry at Sahlgrenska University Hospital (Sandstedt J, 2014; Noseda M. *et. al.* 2015). Isolated human cardiac progenitor cells were cultured for a maximum of four weeks in Dulbecco's Modified Eagle Medium/ Nutrient Mix F-12 (DMEM/F12) (*Life Technologies*, art 21331-020) supplemented with 200mM L-glutamine (*Sigma*, Art. No.G7513), 100x PEST (*Thermo Scientific*, Cat. No. SV30010), 10% human serum (HS; collected from pooled donor blood according to ethical consent given by *Regionala etikprövningsnämnden i Göteborg*), NutriStem (*Stemgent*, art 01-0005) and a medium originally used by Tateishi *et. al.* (Tateishi *et. al.* 2007) for suspension culture (DMEM/F12 with L-glutamine and PEST, 5% HS, 16ng/ml EGF, 40ng/ml FGF), here called suspension culture medium (SCM).

Conditioned medium was produced by collecting DMEM/F12 from mono layer cultures of whole cell isolates of left ventricle tissue from a cardiac explant during 5 days. The pooled medium aliquots were sterile filtered and used either alone or in combination (1:1 mixture) with 50% SCM. Cells were cultured in 96-well format, coated with laminin (LN)-521, -211, and -221 (*Biolamina*, recommended for cardiomyocyte culturing) compared to no coating at all. LN-211 and LN-221 was

chosen since both LN types are expressed in in the developing human heart (Roediger M. *et. al.* 2010). Laminin solutions were prepared in a ratio of 1:3 LN-521 and LN-211 or LN-221 to a final concentration of 10μg/ml. It has previously been shown that addition of 5μM Rho-associated kinase (ROCK) inhibitor Y-27632 (here ROCKi, *Calbiochem* art 688000) has the potential of increasing cell viability after dissociation (Watanabe K *et. al.* 2007), an effect also seen during splitting of cultured iPSCs at AstraZeneca. Therefore the addition of 10μg/ml ROCKi to the dissociation medium as well as the culturing medium was evaluated in this project. As the initial experiments revealed an increased yield of cells from dissociation and increased viability of cultured cells, 10μg/ml ROCKi was included in all following experiments.

To enable screening of a larger quantity of media types and additives such as growth factors, a FED model was later created using the Umetrics software MODDE 10.1. Factorial experimental design is convenient when studying the interaction effect two or more factors. The factor contains different levels, which all contribute to the characteristics of the factor. Changing one level in a factor causes a change in the effect of the factor and thus its interactions with other factors. For example, if changing one level a contained in the factor A, the response of factor A is changed along with its interaction with factor B containing the levels b. In such a design each single replicate contains all possible treatment combinations of ab. A FED model containing two factors is a two-factor factorial design, but far more than two factors may be included. If factors $A, B, \ldots, n$ is included in the model a general factorial design can be formulated with $abc \ldots n$ observations. Each design must contain n replicates where $n \geq 2$ to enable determination of the sum of squares of the errors in the model.

M199 (*Thermo Fisher Scientific*, Cat. No. 11150-059) and the basal medium EBM-2 (*Lonza*, Cat. No. CC-3156) were chosen based on promising results from other groups (A. M. Smits *et. al.* 2009; M. J. Goumans *et. al.* 2008) and human serum was added to promote cell viability. It was hypothesized that FGF (*Gibco*, Art 13256-029) and EGF (*Sigma-Aldrich*, Art. E9644) are essential for culturing cardiac progenitor cells based on previous publications (Goumans M.J.*et. al.* 2008; Bolli R. *et. al.* 2011). IGF-1 (*Thermo Fisher Scientific*, Art. PHG0078), HGF (*Thermo Fisher Scientific*, Art. PHG0324) and Dexamethasone (*Sigma-Aldrich*, Art. D4902-25MG) was also included in the FED-model, based on previous publications. Levels of all factors was also set based on previous publications (Smits A.M. *et. al.* 2009; Goumans M.J. *et. al.* 2008; Bolli R. *et. al.* 2011), see table 3.1. The precision of each factor is given as the limits of the 95% confidence interval (a $\pm$ value.) The FED model was run in 3 blocks due to the limited amount of available material. For each block two biopsies weighing 20-400mg originating from two patients undergoing either ACB-surgery or ACB- and heart valve replacement surgeries were pooled to reduce patient-associated variability, except for block 2 where only one biopsy could be obtained. Since it was not possible to predict the SSEA4+/CD34- cell yield after isolation, "number of cells" was also included in the FED-model as a quantitative factor. To reduce the model size chosen interaction terms were excluded from the model. All interactions with FGF and EGF were excluded since it was hypothesized

that these growth factors are essential for culturing of cardiac progenitor cells *in vitro*. The same was hypethesized for all interaction terms including the medium why they were excluded. The same FED-model was applied on iCell Cardiac progenitor cells for evaluation of cardiomyocyte differentiation potential in the different medium compositions, with the exception that the model was run in 2 blocks due to higher cell seeding density compared to isolated human cardiac progenitor cells.

Table 3.1: FED design generated in Umetrics MODDE 10.1. Levels are presented as low; centre point; high

Factor	Type	Levels	Precision
FGF	Quantitative	10; 55; 100 ng/ml	2,5
EGF	Quantitative	5; 27,5; 50 ng/ml	1,13
IGF-1	Quantitative	5; 27,5; 50 ng/ml	1,13
HGF	Quantitative	0; 5; 10 ng/ml	0,25
Dexamethasone	Quantitative	0; 10; 20 ng/ml	0,5
Number of cells	Quantitative	-	-
Laminin	Qualitative	LN521; LN521/-211; LN521/-221	-
HS	Formulation	5%; 12,5%; 20%	0,00365
EBM-2	Formulation	0%; 43,75%; 95%	0,025
M199	Formulation	0%; 43,75%; 95%	0,025

Summary of fit was calculated in MODDE 10.1.1 to determine model significance, where 1, or 100% represents a perfect model fit. In general, summary of fit shows how well fitted the model is, meaning how well the model can predict future responses based on the response of other variables included in the model. Here the model was fitted using partial least squares (PLS) and the summary of fit was presented as four parameters; R^2, Q^2, model validity and reproducibility. R^2 is calculated according to equation (1) and represents the model fit and should not be less than 0.5, as R^2<0.5 is considered as a poorly fitted model with rather low significance. R^2 overestimates the goodness of fit, meaning that the model complexity is not enough to fit the data.

$$R^2 = SS_{\mathrm{REG}}/SS \tag{1}$$

where

SS_{REG} = the sum of squares of Y corrected for the mean, explained by the model

SS = the total sum of squares of Y corrected for the mean

Q^2 is an estimate of the precision of future predictions made by the model and is calculated according to equation (2). As the most sensitive parameter Q^2 should be greater than 0.1 for the model to be significant and greater than 0.5 for the model to be considered as good. Q^2 underestimates the goodness of fit, indicating that the model is too complex to fit the data. The model complexity should be determined based on each experiment, as increasing the model complexity by increasing

the number of parameters included in the model can help fitting the model but can simultaneously increase the work load drastically.

$$Q^2 = 1 - PRESS/SS \tag{2}$$

where

$PRESS$ = the prediction residual sum of squares.

SS = the total sum of squares of Y corrected for the mean.

In an ideal model R^2 and Q^2 are similar in size and close to 1. If the difference is greater than 20% the model is considered unfitted with low significance.

The model validity parameter is a measure of the validity of the model, *i.e.* it describes the lack of fit, and is defined according to equation 3. Lack of fit shows how the model error based on estimations compares to the pure error. If the model validity is greater than 0.25 there is no lack of fit, meaning that the model errors are within the same range as the pure error. If the model validity on the other hand is less than 0.25 there is a lack of fit, indicating that the error generated by the model is significantly larger than the pure error, which is the error within the same condition, usually at the center point(s).

$$Validity = 1 + 0.57647 * log(p_{\text{lof}}) \tag{3}$$

where

p_{lof} = p for lack of fit.

The reproducibility of the model, calculated according to equation 4, shows the response variation within the same condition in comparison to the total variance of the response. This condition is usually represented by the center point(s). Here only one center point was included and defined as a mixture of 50% M199 and 50% EBM-2 and the mean level of all factors included in the model. The greater the variability within the same condition is, the lower the reproducibility of the model will be (Umetrics AB MODDE 10.1 Help File, 2014).

$$Reproducibility = 1-(\text{MS(Pure error)/MS(total SS corrected)}) \tag{4}$$

where

MS = Mean squares (Variance)

3.2 Isolation of human cardiac progenitor cells

Cardiac progenitor cells were obtained from right atrium biopsies from male and female patients between the ages of 53-83, undergoing routine cardiac surgery at Sahlgrenska University Hospital, see table 3.2 for detailed information about biopsies. Patients were informed and consent was given according to *Regionala etikprövningsnämnden i Göteborg* standards. Two biopsies were pooled as far as possible, but due to timing and cancelled surgeries for some experiments only one biopsy was obtained. Biopsies of approximately 20mg-6.7g were dissociated according to protocol MS84, for complete protocol see Appendix I. Tissue samples were mechanically digested with scissors into small pieces in ice cold PBS. Liberase TH (*Roche*, Art. No. 05401135001) was used for dissociation and the incubation time was prolonged from 2.5h to 4h to ensure proper dissociation and to increase the final yield of isolated cardiac progenitor cells. To further evaluate the possibility of increased cell viability the effect of 5μM ROCKi (*Calbiochem* Art. No. 688000) addition during dissociation and culturing of cardiac progenitor cells was evaluated. The cell suspension was treated with 10ml Trypsin-EDTA to resolve aggregates and 10ml 1x Lysis buffer to remove erythrocytes and other blood cells.

Table 3.2: Detailed information on cardiac biopsies. ACB= Artery Coronary Bypass surgery, MAZE= surgical treatment of atrial fibrillation, HVR= Heart valve replacement surgery

Biopsy	Sex	Surgery	Weight (mg)
Patient 1	Male	ACB	363
Patient 2	Male	MAZE	912
Patient 3	Female	Cardiac explant	6712
Patient 4	Female	ACB	249
Patient 5	Female	HVR	370
Patient 6	Female	ACB/HVR	20
Patient 7	Male	ACB	397
Patient 8	Male	ACB	80
Patient 9	Male	ACB	170

3.3 FACS analysis

To allow isolation of the cardiac progenitor population of interest from the whole-cell suspension, cells were labeled with antibodies for FACS sorting based on SSEA4 expression against lack of CD34 expression. FACS, or fluorescence-activated cell sorting, is a specialized type of flow cytometry used for identification and sorting of live cells based on light scattering or fluorescent character of the sample. The heterogenic cell suspension is labeled using antibodies tagged with fluorochromes, which emits light of a specific wavelength upon being exposed to high energy lasers. Cells are carried through the system in a single-cell fashion by a fast flowing liquid stream. After intense irradiation the stream is exposed to a high frequency vibrating

mechanism that causes the stream to break into electostatically charged droplets, where ideally each droplet confines one single cell. The cell-carrying droplets are passed between voltage plates that deflect the droplets into a separate container. In that way single cells can be sorted based either on size or fluorescent response (Herzenberg L.A. *et. al.* 1976; Herzenberg L.A. *et. al.* 2002). Isolated human cardiac progenitor cells were labelled with anti CD45-PE-Cy7 for hematopoietic membrane bound tyrosine phosphatises (*BD Biosciences* art. 557748), anti SSEA4-PE (*eBioscience* art. 12-8843-42) for Stage-specific embryonic antigen-4 protein, anti CD34-APC (*BD Biosciences* art. 345804) for hematopoietic cells, CD90-BV421 (*BD Biosciences*, Art. No. 555593, Lot. 28970) for fibroblasts and 7-AAD as a viability marker, according to table 3.3. Cells for isotype controls were labeled with Mouse IgG1 PE-Cy7 (*eBioscience*, Art. No. 25-4714-80, Lot. E07625-1634), Mouse IgG3 PE (*eBioscience*, Art. No. 12-4742-42, Lot. E13926-105), Mouse IgG1 APC (*BD Biosciences* , Art. No. 554681, Lot. 45520), Mouse IgG1 BV421 (*BD Biosciences*, Art. No. 562438, Lot. 3338910) and 7-AAD as a viability marker according to table 3.4.

Table 3.3: General antibody panel

Antibodies	Volume per tube(μl)
Anti CD45-PE-Cy7	2
Anti SSEA4-PE	5
Anti CD34-APC	2
CD90-BV421	5
7-AAD	1
TOTAL	**l5**

Table 3.4: General antibody panel for isotype controls

Antibodies	Volume per tube(μl)
Mouse IgG1 PE-Cy7	0.5
Mouse IgG3 PE	0.6
Mouse IgG1 APC	1
Mouse IgG1 BV421	5
7-AAD	1
FACS buffer	1
TOTAL	**l5**

Cells were sorted using FACS (*BD Biosciences* FACSAria II Cell Sorter, USA) and results were compiled using BD FACSDiva 8.0.1 software. For cells sorted directly to the culture plate and cells sorted after attachment in culture (5-7 days after seeding) different compensations were made. CompBeads Plus Anti-Mouse Igκ (*BD Biosciences*, Art. No. 560497) were labeled with CD45-PE-Cy7, SSEA4-PE, CD34-APC and CD90-BV421 and used for automatic calculation of laser emission spillover between the fluorophores. Compensation allows for correction of the spillover caused by the primary signal on the signal from a secondary channel. This is illustrated in

figure 3.1, where the emission spectra for 7-AAD (PerCP-Cy5.5) and PE is shown. The emission spectra of PE spills over in the emission spectra of 7-AAD (PerCP-Cy5.5), which would result in detection of double-positive stained particles if not compensated for. During automatic calculation of the compensation, the response signal from the primary signal, here PE, which is responsible for the spillover is subtracted from the response signal from the secondary signal, which here is PerCP-Cy5.5. Thereby the risk of detecting double-positive particles is reduced (Herzenberg L. A. *et. al.* 2006).

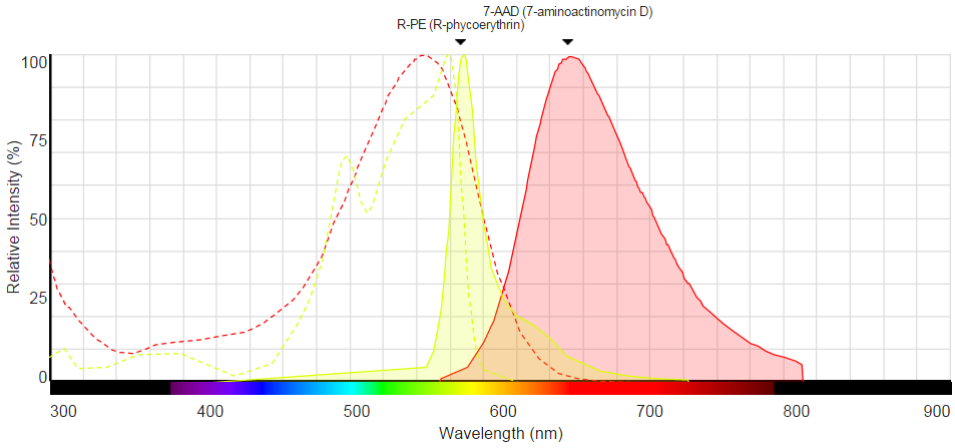


Figure 3.1: Spillover in the emission spectra for 7-AAD (PerCP-Cy5.5) and PE. Generated via Thermo Fisher Scientific Fluorescence SpectraViewer

Un-specific binding of primary antibodies is necessary to compensate for. This is done using isotype controls, which are designed to estimate non-epitope-driven binding of antobodies, as primary antibodies may un-specifically bind to FC-receptors naturally found on cell surfaces (Herzenberg L. A. *et. al.* 1972; Herzenberg L. A. *et. al.* 2002; Herzenberg L. A. *et. al.* 2006). Here approximately one third of all cells obtained from dissociation was used for isotype control. Dead cells and other cellular debris was excluded by a polygon gate on the FSC vs. SSC plot. Selected cells were gated based on 7-AAD, only allowing live cells *i.e.* 7-AAD- cells. CD45+ cells were excluded and further a four-way gate was applied to the FSC vs. SSC plot for SSEA4-PE and CD34-APC cells to define the SSEA4+/CD34- population. The gating was set to reduce the amount of false positives. A small fraction of false positives were accepted due to the low amount of SSEA4+/CD34- progenitor cells present in the adult human heart.

3.4 Initial culturing of SSEA4+/CD34- cells

Human cardiac biopsies were dissociated and sorted based on SSEA4+ vs. CD34- labelling for evaluation of potential increased cell viability by ROCKi addition (Patient 1-2) directly to LN-521/-211, LN-521/-221 coated or un-coated 96-well Primaria

plates (*Corning*, Art. #353872) and cultured for three weeks in DMEM/F12 supplemented with 10% HS, SCM or NutriStem, see figure 3.2 for experimental layout. Half of the cells were cultured with 5μM ROCKi. Cells were left for attachment for 5 days before changing medium every 4 days. Cells were observed in phase contrast microscope (*Nikon DIAPHOT 300, Japan*) and photographed using *Nikon Digital Sight, Japan* and analyzed in the NIS-Elements F 2.30 SP2 Nikon Imaging Software.

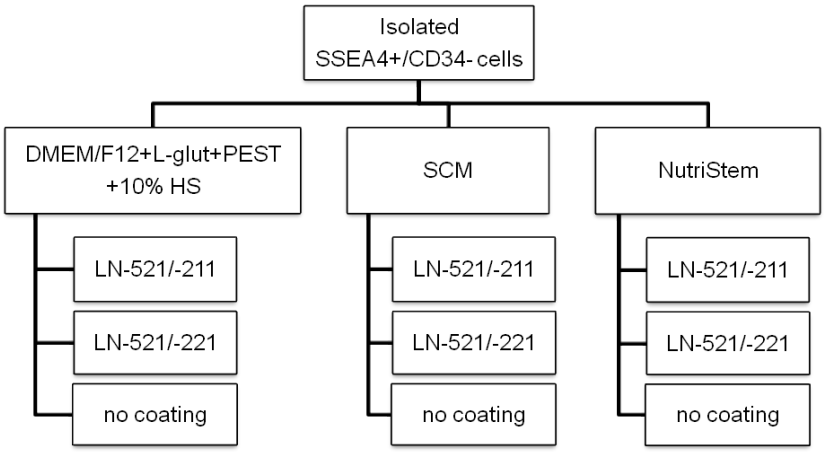


Figure 3.2: Experimental layout for H224, H225 and Ex12 biopsies

3.5 Cardiomyocyte differentiation

To evaluate the viability of SSEA4+/CD34- cells in response to cardiomyocyte differentiation medium an initial cardiomyocyte differentiation experiment was conducted using the GiWi protocol (Lian *et. al.* 2013). iPSCs were also differentiated for comparison of gene expression during the differentiation process.

3.5.1 Cardiomyocyte differentiation of iPSCs

To enable characterization of isolated human cardiac progenitor cells, the RiPSC-1J line (Sjogren AK *et. al.* 2014) was differentiated into spontaneous contracting cardiomyocytes using the GiWi-protocol (Lian *et. al.* 2012), see Appendix A.1.3. Cells were seeded at 312K/cm^2 on LN-521, LN-521/-211 or LN-521/-221 in NutriStem. Mesoderm induction by the addition of CHIR99021 was initiated two days after seeding, when cells had reached a confluency degree of 95-100%, as it was shown in previous experiments that initiating differentiation at lower degree of confluency increased the risk of cell detachment around day 5 of differentiation. Spontaneous contracting behavior was seen for some populations at day 9, and more pronounced at day 12-13. Cells were harvested for RT-qPCR at day 0, 1, 2, 4, 6, 9, 13 and 16. mRNA levels of OCT4, NANOG, ISL1, NKX2.5, GATA4, TBX5 and cTNNT2, see

figure 3.7, were determined and compared to published data, see figure 2.5.

3.5.2 Initial differentiation of SSEA4+/CD34- cells

A whole cell isolate (Patient 3) originating from left ventricle instead of right atrium was seeded on un-coated T-25 flasks (*Corming*, Cat. No. CLS430639 SIGMA) for production of conditioned medium. Cells were left for attachment during 5 days after which medium was changed every day for five days. At the time of medium change conditioned medium was harvested, sterile filtered and stored at -20°C until use. A fraction of the cells were instead seeded onto un-coated T-75 flask-format (*Corning*, Cat. No. CLS430641 SIGMA). After 7 days of attachment cells were harvested for sorting. 3ml Trypsin-EDTA was added per T-75 flask and incubated in 37°C for 5 min. Cells were spun down at 400g for 5 min and resuspended in FACS-buffer (PBS, 5% FCS, 1% BSA, 2mM EDTA) containing 5μM ROCKi. SSEA4+CD34- cells were sorted using FACS to LN-521/-211, LN-521/-221 coated or un-coated 96-well Primaria plates and cultured for 2 weeks in DMEM/F12 supplemented with 10% HS, SCM or NutriStem before changing medium to cardiomyocyte differentiation medium given in the GiWi protocol (Lian *et. al.* 2013) to evaluate cell viability and potential initial cardiomyocyte differentiation potential of isolated SSEA4+/CD34- cells. The GiWi protocol was started at day 2, with IWP-2 (*Calbiochem*, Art. No. 681671) addition. Mesoderm induction by CHIR99021 addition was skipped, as SSEA4+/CD34- already are committed to the cardiac lineage, see figure 2.6 and Appendix A.1.3 for detailed protocol. In general, the differentiation was initiated by adding 100μl "LC2" (RPMI GlutaMAX, 20μl/ml B-27 minus insulin, 5μl/ml IWP-2) and 100μl "M2" (RPMI GlutaMAX, 20μl/ml B-27 minus insulin) per well. At day 3 medium was changed to 200μl "M2" per well and at day 5 to "M3" (RPMI GlutaMAX, 20μl/ml B-27). Medium "M3" was changed every three days. Differentiation was continued for 20 days after which cells were harvested for RT-qPCR by resuspension in RLT Plus with DTT (20μl 2M DTT per mL of Buffer RLT Plus) (*Qiagen*, Cat. No. 1053393) after washing with PBS-/-.

3.6 Evaluating potential of postponed sorting

As the process of sorting cells directly following dissociation of cardiac tissue is extremely time consuming, seeding the whole-cell isolate before sorting would reducing work load tremendously. Cardiac tissue was dissociated (Patient 4) and a fraction of the cells were sorted directly to RLT Plus with DTT and stored in -80°C until RT-qPCR analysis. The other fraction of cells were seeded onto LN-521/-211 or LN521/-221 coated 6-well Primaria plates (*Corning*, Art. No. 353846) and cultured in 100% SCM, 50% SCM and 50% conditioned medium or 100% conditioned medium for 7 days without medium change, after which cells were harvested by incubating with 2ml Trypsin-EDTA at 37°C for 5 min. The trypsin was stopped with 2ml DMEM/F12 with 10% FCS, spun down at 400g for 5 min and resuspended in FACS-buffer containing 5μM ROCKi. Cells were sorted based on SSEA4+ vs.

CD34- expression to RLT Plus with DTT and stored in -80°C until further gene expression analysis by RT-qPCR.

3.7 Culturing of cells in FED model generated medium

To enable testing of a larger quantity of medium and additives a FED-model was generated including M199, EBM-2, HS, EGF, FGF, IGF-1, HGF and Dexamethasone. Cells were cultured in 384-well format on LN-521 or in combination with LN-211 or LN-221. Directly sorted SSEA4+/CD34- cells were cultured in the FED model generated medium for evaluation of the ability of the medium to maintain the cells in culture. To evaluate the cardiomyocyte differentiation in cultured iCell CPCs in response to the different media, cells were cultured for 12 days and evaluated using ICC.

3.7.1 Culturing of SSEA4+/CD34- cells

The FED model was designed in 3 blocks due to the limited amount of starting material. Cardiac biopsies (Patient 5-9) were dissociated according to protocol MS84 using Liberase TH and the addition of 5μM ROCKi to the dissociation medium, and cells were left for dissociation during 4h at 37°C. Two biopsies were pooled for all experiments except for block2, where only one biopsy was obtained. After dissociation cells were resolved in FACS-buffer and labeled using the same antibodies and isotype controls as in previous experiments. Cells were sorted based on SSEA4+ vs. CD34- expression directly to 384-well plates (*Corning*, Art. No. 353962) predispensed with 50μl of each medium per well. Medium was changed after 4 days of cell attachment with following medium change every two days. 5 μM ROCKi was added fresh to each medium before medium change but was forgotten when seeding cells from Patient 5-6. SSEA4+/CD34- cells were cultured for 12 days. At day 12 cells were studied in bright field microscope and attached cells were labeled with 1μl/10μl medium NucRed Live 647 ReadyProbes Reagent (*Thermo Fisher Scientific*, Art. No. R37106) for 30 min and imaged using Nikon Eclipse fluorescence microscope equipped with Andor Zyla sCMOS and Andor iXon x3 cameras and analyzed using NIS Elements Software (Nikon Corporation, Tokyo, Japan). After imaging attached cells were washed with PBS -/- and harvested for RT-qPCR by resuspending in RLT Plus with DTT.

3.7.2 Culturing of iCell cardiac progenitor cells

iCell cardiac progenitor cells were used instead of isolated and sorted human cardiac progenitor cells due to the limited availability of human cardiac tissue to evaluate the potential ability of the different media to induce spontaneous cardiomyocyte

differentiation. The FED-model was modified to suit culturing of iCell CPCs. The model was identical to the previous design, except that it was designed in 2 blocks instead of the 3 blocks used for human cardiac progenitors. iCell CPCs (*Cellular Dynamics International*, Cat. No. CPC 301-020-001-PT, Lot. CPC 101242) were thawed according to CDI Users Guide and seeded on LN-521, LN-521/211 or LN-521/-221 coated 96-well plates (*Corning*, Cat. No. 353219) at a cell density of 280K/cm^2. As only around 40% of all seeded cells have been reported to actually attach (Cellular Dynamics International, 2015), the actual seeding density was assumed to approximately 100K/cm^2. Four control-wells were included, one where cells were seeded in recommended conditions, *i.e.* on 5μg/ml fibronectin (*Roche Applied Sciences*, Ar. No. 11051407001) in Williams E Medium (*Life Technologies*, Art. No. A12176-01) supplemented with 4ml Cocktail B (*Life Technologies*, Art. No. CM4000) per 100ml medium. One control well coated with each laminin combination was also included using the recommended medium. iCell CPCs were cultured for 2 weeks with medium change every two days. At day 14 cells were labeled with SmartFlare Probes for cTNNI3 (*Merck Millipore*, Art. No. SF-3222), GAPDH (*Merck Millipore*, Art. No. SF-136) as housekeeping gene and Hoechst for detection of cell nuclei. Controls were labeled with Scramble (*Merck Millipore*, Art. No. SF-102).

SmartFlare RNA Detection Probes are nanoparticles that allow detection and imaging of mRNA in live cells without the requirement of fixating cells prior to imaging and eliminating the need of pre-amplification of recovered RNA from lysed cells. This allows for further culturing and analysis after imaging, such as live-sorting of labeled cells using FACS. SmartFlare Probes consist of a colloidal gold nanoparticle about 10-15nm in size with short oligonucleotide duplexes, or capture strands, on its surface, see figure 3.3. The duplexes bind fluorochrome-tagged Flares, short oligonucleotides complementary to the specific mRNA sequence of interest. The hybridization to the duplexes and its proximity to the gold-nanoparticle quenches the Flare, or reporter strand, thus permitting it from fluoresce through FRET upon exposure to high energy light such as Cy-3 or Cy-5. The more mRNA of interest that is expressed in the cell, the higher the response signal and the more reporter strands gets translocated away from the quencher. The gold-nanoparticles enter the cell via receptor-mediated endocytosis into the cytoplasm, where the reporter strand is allowed to release from the capture strand and hybridize to complementary mRNA strands, thus allowing detection of relative RNA levels in live cells. Entry of the probes through natural cellular processes is beneficial for some cell types, such as pluripotent stem cells, which are sensible to cellular perturbations. Since the endocytosis is receptor-mediated, the nanoparticles cannot be taken up by cell types lacking the specific receptors. Some cells exhibit low uptake rates, and therefore low uptake rates of the SmartFlare probes. To determine the specific uptake efficiency in specific cell types a specific uptake control is used. The uptake control contains fewer fluorochromes per particle but does not need to hybridize to any specific gene sequence to activate its bound fluorochrome. Instead each oligonucleotide bound to the gold particle carries a fluorochrome that never is quenched. Since the molecule is always fluorescent it is therefore possible to determine the relative uptake, and thus

determine if the cell type of interest is suitable for SmartFlare RNA Detection Probe assays. Another control used in SmartFlare assays is the Scrambe Control for detecting background fluorescence. The Scramble Control carries oligonucleotides which are not reactive to any mRNA present in the cell, enabling it to detect background fluorescence, which is always expected. Each target response obtained while using SmartFlare Probes should therefore always be compared to the Scramble Control response. The nanoparticles are naturally excreted by the cells within a few days, allowing for further culturing and future analysis of the cells (Yuzuki D. 2013; Merck Millipore, 2015; Seferos D. S. *et. al.* 2007).

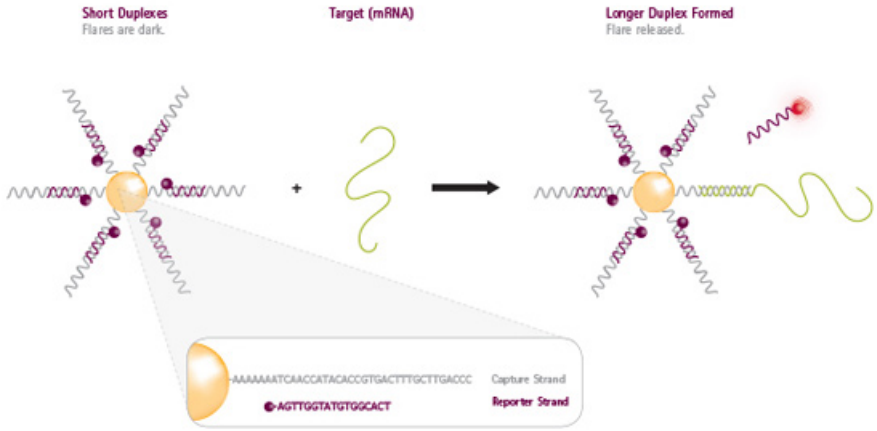


Figure 3.3: Principle of the SmartFlare RNA Detection Probe technology (Merck Millipore, 2015)

All SmartFlare Probes were reconstituted according to recommendations prior to use and stored at room temperature. Working solutions of 200pM SmartFlare Probes were prepared and 4μl of 1:20 diluted cTNNI3 (*Merck Millipore*, Art. No. SF-3222) detected by Cy5 and GAPDH (*Merck Millipore*, Art. No. SF-136) detected by Cy3 was added to each well 24h prior to imaging along with 10mg/ml 16.2mM Hoecht 33342 (trihydrochloride, trihydrate, *Life Technologies*, Art. No. H3570, Lot. 1689875) for detection of cell nuclei. The Scramble (*Merck Millipore*, Art. No. SF-102) detebted by Cy5 was added to control wells to enable characterization of background fluorescence. No Uptake control was used in this experiment as no control wells were seeded and available for this purpose. Only one of the duplicates were labeled using SmartFlare Probes due to the high cost of the probes. SmartFlare Probe labeled cells were taken for ICC imaging using ImageXpress (*Molecular Devices*, Sunnyvale, California, USA) after 24h of incubation in 37°C. The results were analyzed in MetaXpress v5.1 (*Molecular Devices*, Sunnyvale, California, USA), where Hoecht stained nuclei and cTNNI3 labeled cytoplasms were counted using the Multi Wavelength Scoring function. The results were statistically analyzed in MODDE 10.1.

3.8 Gene expression analysis

To determine the potential loss of progenitor-specific gene expression in SSEA4+/CD34- cells sorted after culturing of whole-cell isolate during 5-7 days and to evaluate potential differences in gene expression caused by short term culturing, real-time quantitative PCR (RT-qPCR) was used. Gene expression of cells harvested after differentiation of Ex12 was also evaluated using RT-qPCR. To determine which of the 81 medium generated in the FED-model that is able to maintain SSEA4+/CD34- cells in culture and to determine if the SSEA4 expression along with other progenitor-specific markers are maintained, attached cells after 12 days of culturing was harvested for RT-qPCR. RT-qPCR is a specific and sensitive method that can be used for detecting mRNA levels and characterize its patterns in cell populations. Since RNA cannot be used as template, the RNA must be reversely transcribed to cDNA by using reverse transcriptase initiated by the binding of random primers (Bustin S.A. 2000; Alasaad *et. al.* 2011). TaqMan Gene Expression Assay was used for RT-qPCR analysis. The TaqMan system includes two unlabelled primers and a specialized probe consisting of a FAM reporter dye label on the 5'-end and a non-fluorescent quencher on the 3'-end. The probe binds to the cDNA strand between the two primers. In addition to the quencher, the 3'- end carries a minor groove binding (MGB) sequence, which increases the specificity of the reaction. The quencher hinders the reporter dye to be excited by light. Instead the energy is transferred from the reporter to the quencher through fluorescence resonance energy transfer (FRET) and no fluorescent signal will be recorded. Binding of the probe to the single cDNA strand initiates extension of the strand until the Taq-polymerase reaches the probe. The exonuclease activity of the enzyme allows it to separate the fluorescent reporter dye from the probe, thus separating it permanently from the quencher, allowing it to be excited by incoming light. Quantification is possible based on the intensity of the fluorescence, since each doubling will produce more unquenched reporter molecules (Life Technologies, 2015).

RNA was isolated from cells resuspended in RLT Plus with DTT using RNeasy Plus Micro Kit (50) (*Qiagen* Art. No. 74034) as the number of cells harvested for RT-qPCR was highly limited. The included protocol was followed and genomic DNA contaminations were removed by passing the samples through included gDNA Eliminator spin columns carried by a high-salt buffer. Ethanol was added to the flow-through to ensure removal of other contaminants and to ensure proper conditions for RNA-binding to the membrane of the spin column. 5μl 20mM poly-A carrier RNA was added to samples containing less than 500 cells for increased yield of total-RNA. Total-RNA was eluted in 15µl RNAse free water, by adding the water to the center of the column filter and left for incubation for 3 min before the final centrifugation for increased recovery. The RNA concentrations were measured using NanoDrop 2000 (*Thermo Scientific*).

cDNA was synthesized using the High Capacity cDNA Synthesis Kit (*Applied Biosystems*, Cat. No. 4374967) capable of synthesizing a maximum of 2μg total-RNA per 20μl reaction mixture, according to table 3.5. To each reaction 10µl master mix was

added along with 10µl of purified total-RNA. Newly synthesized cDNA was stored in 4°C until further use.

Table 3.5: High Capacity cDNA syntesis master mix

Component	Volume per tube(μl)
ddH20	3.2
10x RT buffer	2
10x RT Random hexamer	2
25x dNTPs mix (100mM)	0.8
RNase inhibitor	1
MultiScribe RTase	1
TOTAL	**l0**

PCR was run using the following standard program.

25°C 10 min
37°C 120 min
85°C 5 min
4°C Hold

cDNA pre-amplification was necessary since low amounts of total-RNA was recovered from cultured cells. cDNA was pre-amplified using the TaqMan PreAmp Master Mix Kit (*Applied biosystems*, Cat. No. 4384267, Lot. 1411057). The included protocol was followed, see figure 3.6.

Table 3.6: TaqMan PreAmp master mix

Component	Volume per tube(μl)
TaqMan preAmp master mix (20x)	5
Pooled assay mix (0,2x)	2.5
cDNA (1-250ng)	2.5
TOTAL	**l0**

Pre-amplification PCR was run according to the following standard program. 14 cycles was used since the starting material was highly limited.

95°C 10 min

$$\left.\begin{array}{l}95^\circ\text{ C } 15\text{ sec}\\ 60^\circ\text{ C } 4\text{ min}\end{array}\right\} x14$$

4°C Hold

Newly pre-amplified cDNA was diluted 1:20 and stored at 4°C until further use.

RT-qPCR was run according to standard protocol and settings. CREBBP was used as reference gene, RNAse free water as blank and CMix3 as internalcalibrator. Samples were run in duplicates to reduce variability within each sample. TaqMan Gene expression master mix (*Applied biosystems*, Cat. No. 4369016 Lot. No. 1406207/1504227) was used along with the TaqMan assays listed in table 3.7. The mean of the obtained Ct-values was calculated for each sample and the mean Ct-value of the reference gene was subtracted, yielding ΔCt. To each mean Ct-value the internal calibrator was subtracted to obtain $\Delta\Delta$Ct, which was used for calculating the fold change as $2^{-\Delta\Delta CT}$.

Table 3.7: Used TaqMan assays

TaqMan assay	Cat. No.	Lot. No.
OCT-4/POU5F1	Hs0185061_u1	1221911
NANOG	Hs02387400_g1	1127809
ISL-1	Hs00158126_m1	1122918
MESP-1	Hs00251489_m1	920385
NKX2.5	Hs00231763_m1	1049587
GATA4	Hs00171703_m1	954553
TBX5	Hs01052563_m1	936354
cTNNT2	Hs00165960_m1	1070965
CD31/PECAM1	Hs00169777_m1	1090349
CREBBP	Hs00231733_m1	1285135/1252057

4
Results

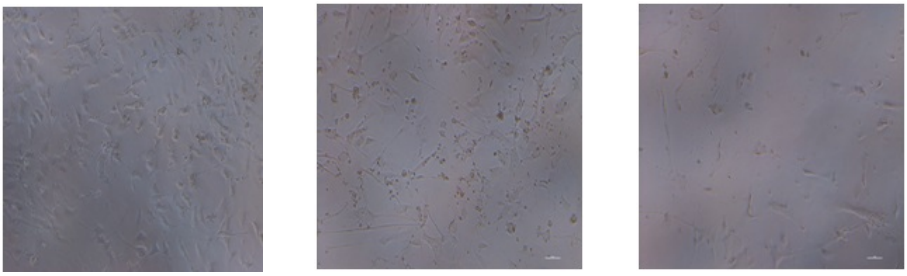
To enable characterization of SSEA4+/CD34- cells and establishing of a stable culture system for the chosen cell population several experiments was done. RT-qPCR was used for evaluating gene expression of directly sorted cells in comparison to sorted cells after attachment in culture to determine the possibility of postponed sorting. The effect of cardiac differentiation medium previously used on iPSCs for cardiomyocyte differentiation on SSEA4+/CD34- cells was also determined by evaluating gene expression using RT-qPCR. Further a FED model was designed for culturing of iCell CPCs as well as SSEA4+/CD34- cells isolated from human cardiac biopsies. Here the compiled results of each experiment included in this Master's Thesis will be presented in more detail.

4.1 Culturing of SSEA4+/CD34- cells

Human cardiac progenitor cells (Patient 1-2) were cultured in DMEM/F12 supplemented with L-glutamine, PEST and 10% HS, SCM or NutriStem with and without the addition of 5μM ROCKi, to evaluate the potential increase in cell viability caused by the known effect of reduced dissociation-associated apoptosis with using ROCKi. Cells were dissociated with and without ROCKi addition, after which cells were counted in Bürker chamber. A higher cell density was obtained when dissociating cardiac tissue with added ROCKi than dissociating without ROCKi addition. Cell viability after short-term culturing of isolated cells were also increased when adding ROCKi to the medium (data not shown). Therefore ROCKi was included in all following experiments, as ROCKi was shown to increase viability of cultured SSEA4+/CD34- cells.

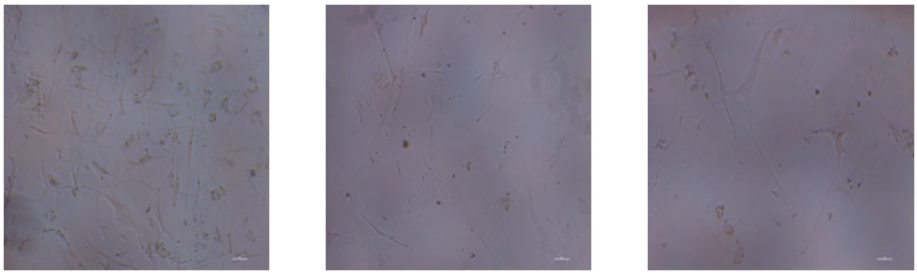
SSEA4+/CD34- cells (from Patient 3) were initially cultured in DMEM/F12 supplemented with L-glutamine, PEST and 10% HS, SCM or NutriStem on LN-521/-211, LN-521/-221 or un-coated 96-well plates. Cells were sorted 5 days after attachment in culture and medium was changed every 4 days. ROCKi was added fresh to the medium at each medium change. At day 7 of culturing cells were taken for imaging using phase contrast microscope. No pronounced difference in cell density could be seen in progenitor cells cultured on LN-521/-211 or LN-521/-221, see figure 4.1 (a)-(c) and 4.2 (a)-(c). A more pronounced difference could be seen in cells cultured on coated than un-coated surfaces, especially for cells cultured in DMEM/F12 (supplemented with L-glutamine and PEST), something also seen in previous experiments (Patient 1-2; data not shown). It was therefore concluded that SSEA4+/CD34- cells

should preferentially be cultured on coated plates, as the amount of attached cells seems to be promoted by the coating. Cells cultured in NutriStem were not maintained in culture, see figure 4.3 (a)-(c), why NutriStem was excluded from further experiments. No further conclusions could be drawn, as any pronounced differences between DMEM/F12 and SCM could not be seen.



(a) (b) (c)

Figure 4.1: SSEA4+/CD34- cells cultured 1 week in DMEM/F12 supplemented with L-glutamine, PEST, 10%HS and 5μM ROCKi. (a) LN-521/-211 (b) LN-521/-221 (c) No coating



(a) (b) (c)

Figure 4.2: SSEA4+/CD34- cells cultured 1 week in SCM supplemented with 5μM ROCKi. (a) LN-521/-211 (b) LN-521/-221 (c) No coating

(a) (b) (c)

Figure 4.3: SSEA4+/CD34- cells cultured 1 week in NutriStem supplemented with 5μM ROCKi. (a) LN-521/-211 (b) LN-521/-221 (c) No coating

4.2 Cardiac differentiation

iPSCs and isolated SSEA4+/CD34- cells were differentiated and analyzed using RT-qPCR to enable comparison between gene expression. The degree of translatability between iPSCs and SSEA4+/CD34- cells were also evaluated.

4.2.1 Differentiation of iPSCs

To enable further characterization of isolated human cardiac progenitor cells, iPSCs were differentiated into cardiomyocytes. Prior to differentiation iPSCs were cultured on LN-521, LN-521/-211 or LN-521/221 in NutriStem, previously shown to yield the most efficient differentiation. LN-521 was here included to evaluate the effect of LN-521 alone and in comparison to the cardiac specific combinations. At chosen time points cells were harvested for RT-qPCR and mRNA of chosen genes were detected. Results are presented in figure 4.4 as a timeline. If comparing figure 4.4 to figure 2.5, representing published gene expression data from cardiomyocyte differentiation of iPSCs, it can be seen that the obtained data agrees with published data with only a few exceptions. Stem cell markers are reduced by day 2-3, which agrees with published data. GATA4 was detected at day 1 compared to published data where GATA4 mRNA was detected at day 2. Detection of Isl1 mRNA agrees with published data, but remarkably TBX5 and cTNNT2 mRNA was detected at day 4 and 6, which could be seen first at day 7-8 in published data. It should be noted that in published data cTNNI3 was evaluated and not cTNNT2, but as these proteins are interconnected and part of the same troponin complex comparisons can be made. The time difference between cTNNT2 and cTNNI3 detection is therefore not surprising. It was also noted that NKX2.5 mRNA was detected at day 6 along with cTNNT, where in published data NKX2.5 was detected at day 3.

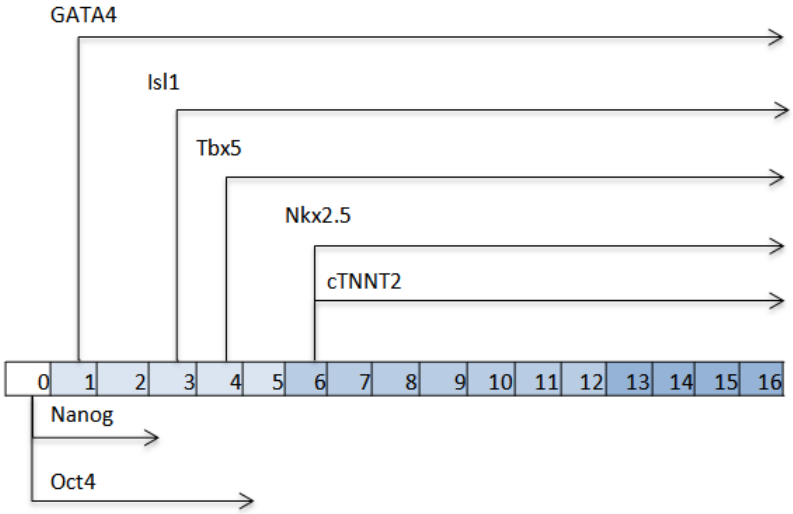


Figure 4.4: Gene expression during cardiomyocyte differentiation of iPSCs

4.2.2 Initial differentiation of SSEA4+/CD34- cells

Cardiac progenitor cells isolated from human left ventricle cardiac tissue (Patient 3) were cultured in DMEM/F12 with L-glutamine and PEST, SCM or NutriStem supplemented with 5μM ROCKi. Cells were maintained in culture and at day 14 cells were taken for an initial differentiation experiment, by application of the GiWi protocol (Lian *et. al.* 2012) seen in appendix A.1.3. The differentiation was initiated at differentiation phase 2 by addition of 50% LC2 medium and 50% M2, as the isolated cells already are mesoderm committed, with the aim of evaluating the viability of human cardiac progenitor cells after being cultured in differentiation medium. After 20 days of differentiation live cells were harvested for RT-qPCR. OCT4, ISL1, MESP1, GATA4, NKX2.5, TBX5, cTNNT2 and CD31 mRNA was detected, and the results are presented in figure 4.5(a)-(h). No ISL1 mRNA was detected in any sample except for cells cultured in DMEM/F12 on coated plates. OCT4 mRNA was detected in most samples. According to published data of cardiomyocyte differentiation of iPSCs seen in figure 2.5, which agrees with the differentiation of iPSCs presented in figure 4.4, OCT4 is down-regulated the latest at day 4. TBX5 and cTNNT2 mRNA was detected in most samples, along with CD31 mRNA. CD31 mRNA levels were overall higher in cells cultured in DMEM/F12 compared to the other medium. cTNNT2 mRNA levels were relatively higher in cells cultured in SCM compared to others. No difference between type of coating could be seen based on obtained gene expression data. Further studies would therefore be required before any conclusions can be drawn on the differentiation capacity of isolated SSEA4+/CD34- cells.

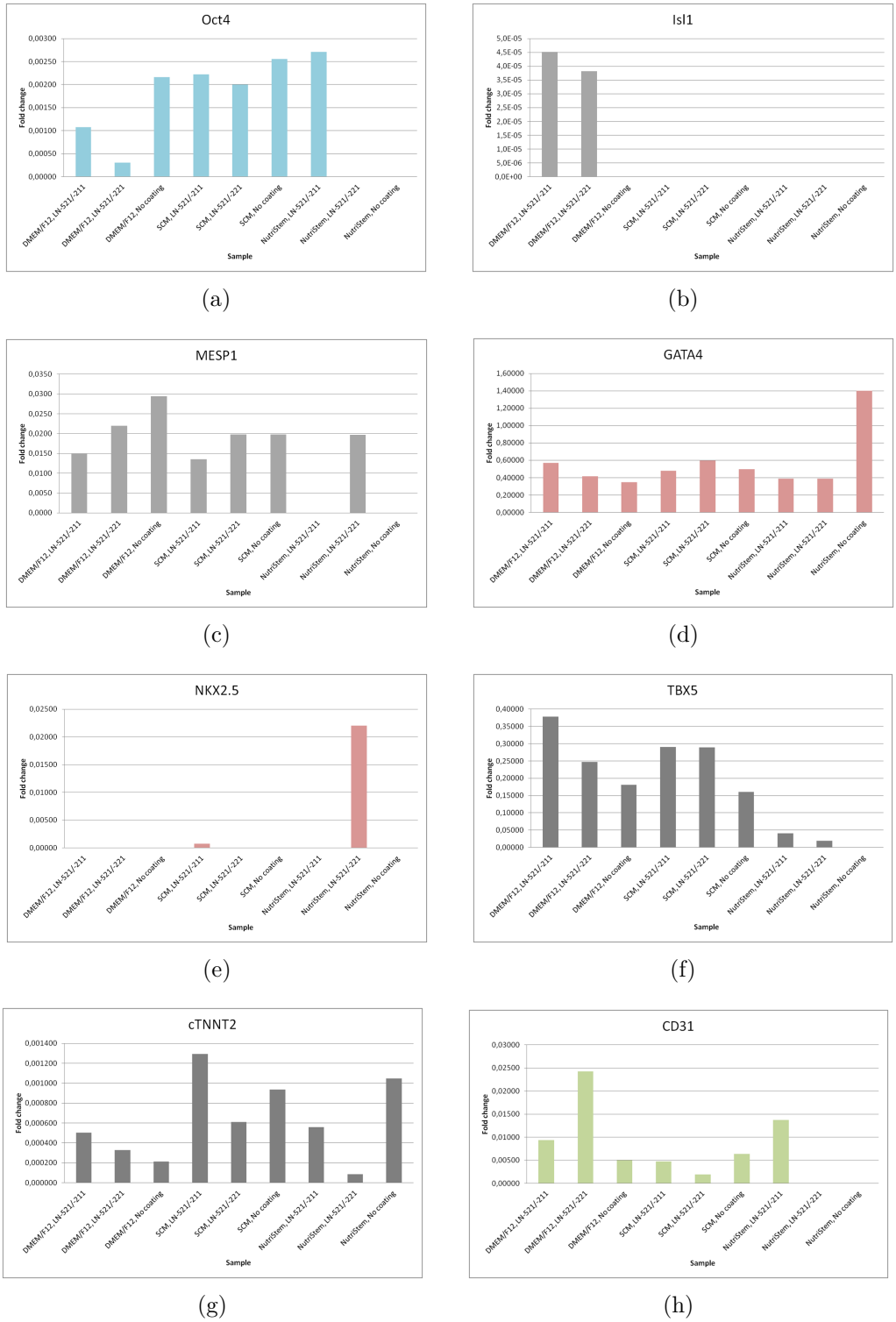


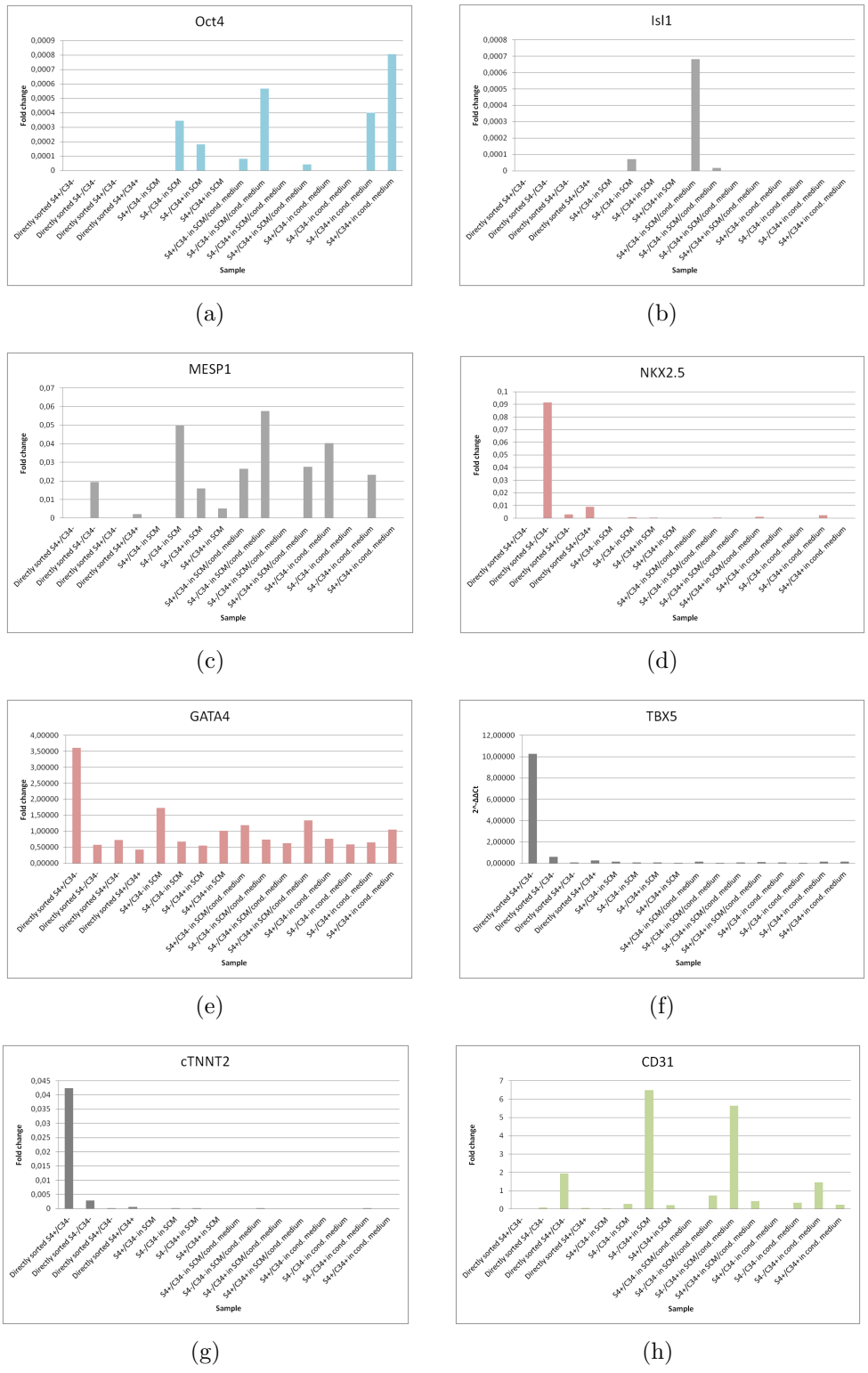
Figure 4.5: mRNA levels in SSEA4+/CD34- after 14 days of differentiation

4.3 Postponed sorting of SSEA4+/CD34- cells

To determine the possibility of postponing FACS-sorting of SSEA4+/CD34- cells dissociated from human cardiac tissue (Patient 4) a fraction of the whole-cell isolate was seeded on LN-521/-221 coated plates and sorted after 5 days in culture. Cells were cultured in SCM, conditioned medium or a 1:1 mixture of the two. The rest of the cells were directly sorted based on SSEA4 vs. CD34 expression into RLT Plus with DTT. Cultured cells were sorted after 5 days to RLT Plus DTT. mRNA was collected and cDNA was synthesized and pre-amplified and RT-qPCR was run according to standard protocols.

In figure 4.6 mRNA levels of analysed samples are presented. OCT4 mRNA levels were undetermined for all SSEA4+/CD34- cells except for SSEA4+/CD34- cells cultured in 1:1 mixture of SCM and conditioned medium produced by left ventricle cardiac cells for 5 days, as seen in figure 4.6 (a). A slight increase in Oct mRNA levels could be seen in the other sorted cell populations, *i.e.* SSEA4-/CD34-, SSEA4-/CD34+ and SSEA4+/CD34+. A peak in Isl1 mRNA levels was seen for SSEA4+/CD34- cells cultured in 1:1 mixture of SCM and conditioned medium, indicating that addition of 50% conditioned medium might have an effect on culturing of unsorted SSEA4+CD34- cells. This aligns with the increased MESP1 mRNA levels seen in figure 4.6 (c) for that sample. Culturing of SSEA4+CD34- cells in 100% conditioned medium induced an increase in MESP1 mRNA levels but did not have an effect on Isl1 mRNA levels. It is though not possible to draw any conclusions whether addition of conditioned medium would allow for culturing of un-sorted SSEA4+CD34- cells without a change in genotype.

NKX2.5 mRNA levels were in general constantly low, except for a more evident expression in directly sorted SSEA4-/CD34- cells, but since NKX2.5 mRNA levels were undetermined it is likely that something went wrong during the RT-qPCR process why measurements should be re-run. Therefore it is not possible to draw any conclusions from this finding. In figure 4.6 (e) a peak in mRNA levels for GATA4 was seen in directly sorted SSEA4+/CD34- cells, compared to the mRNA levels for samples sorted after short-term culturing, which decreased GATA4 mRNA. The same pattern could be seen for TBX and cTNNT2 mRNAin figure 4.6 (f)-(g). A slight increase in CD31 mRNA levels could also be seen in short-time cultured cells compared to directly sorted cells. In general, more pronounced CD31 mRNA levels could be seen in cultured SSEA4-/CD34+, which could be associated with the CD34 expression, as CD34 is a known marker in hematopoietic and vascular-associated tissue. Taken together, due to the uncertain reason for decreased GATA4, TBX5 and cTNNT2 mRNA levels after short-time culture and the slight increase in CD31 mRNA levels, further studies are required to determine the preferred timing of SSEA4+/CD34- cell sorting. Before that, it is recommended to sort SSEA4+/CD34- cells directly following dissociation to avoid potential loss of cardiac progenitor cell features.

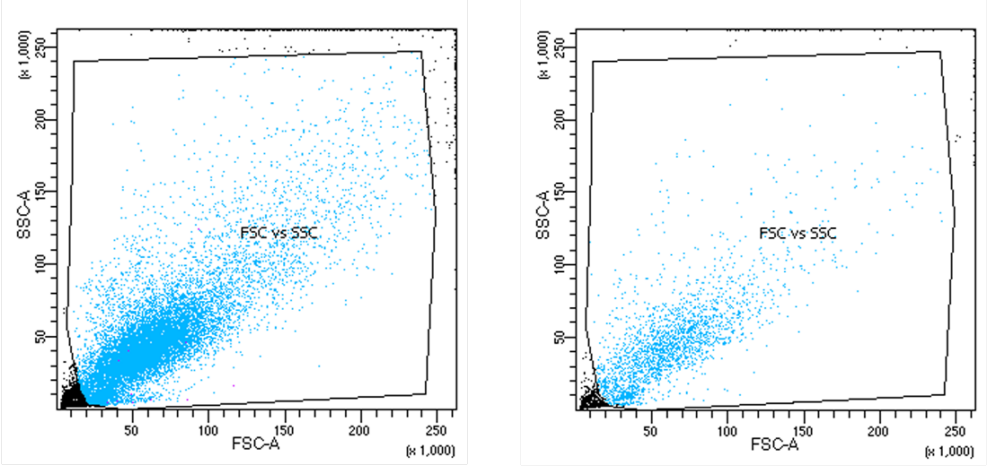


(a) (b) (c) (d) (e) (f) (g) (h)

Figure 4.6: mRNA levels in directly sorted cells vs. cells sorted after 5 days of culture

4.4 Culturing of SSEA4+/CD34- cells in FED model generated media

Isolated cells (from Patient 5-9) were labelled with fluorochrome-tagged antibodies and sorted using FACS. Cell debris and dead cells were excluded by drawing a polygon gate in the forwars scatter vs. side scatter plot, as seen in figure 4.7(a). In figure 4.7(b) the corresponding isotype control can be seen. Gates were applied to only allow 7-AAD- cells and further only allowing CD45- cells (data not shown). SSEA4+/CD34- cells were defined by applying a four-way gate to the bivariate histogram between the SSEA4 and CD34 event responses, as presented in figure 4.8 along with the isotype control.

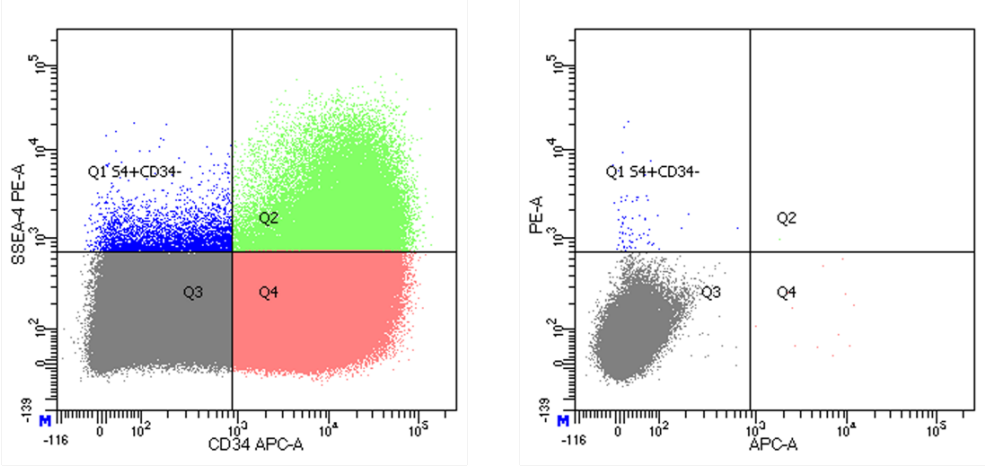


(a) (b)

Figure 4.7: Exclusion of cell debris and dead cells using a polygon gate in the FSC vs. SSC plot. (a) FSC vs. SSC plot for all events *i.e.* cells (b) FSC vs. SSC plot for isotype control. Data shown for sorting of LM45-LM46 biopsies

The gate was set to reduce the amount of false positives. When sorting cells isolated from Patient 5-6 the amount of false positives were 0.04%. In the same experiment the total amount of SSEA4+/CD34- cells were 0.44% of the parent population (*i.e.* all events or cells). A small amount of false positives were accepted in all experiments, as the total amount of SSEA4+/CD34- cells present in the adult human heart were expected to be low.

For cells isolated from cardiac tissue donated from Patient 5-6 CD90-BV421 was included, resulting in the finding of two distinct subpopulations when back-gating on CD90-BV421 labeling within the SSEA4+/CD34- population, seen in figure 4.9. In this way 61.2% of the SSEA4+/CD34- cells were defined as SSEA4+/CD34-/CD90-, corresponding to 23.9% in the isotype control meaning that a pronounced number of cells are considered as false positives. As seen by the location of the subpopulation in the scatter plots, it can be concluded that the SSEA4+/CD34-/CD90- population



(a) (b)

Figure 4.8: Definition of the SSEA4+/CD34- cell population by a four-way gate in the SSEA4 vs. CD34 bivariate histogram plot. (a) Bivariate histogram plot for all events *i.e.* cells (b) Bivariate histogram plot for isotype control. Data shown for sorting of cells dissociated from cardiac tissue donated by Patient 5-6

have a somewhat lower SSC than SSEA4+/CD34-/CD90+ cells, meaning that this population consists of cells with lower granularity as SSC represents cell granularity.

Cells were cultured in several media generated by a FED-model. In total, five additives were evaluated in three levels; low, medium and high based on previous publications. The model was run in 3 blocks due to the limited amount of material, the complete model can be seen in Appendix II, and the cells were cultured for 12 days. N6, N7, N8, N9, N14, N24, N29, N30, N34, N42 and N57 media maintained cells in culture until analysis, for medium description see Appendix II. Before harvesting attached cells for RT-qPCR, cells were labelled with NucRed Live 647 ReadyProbes and imaged in fluorescence microscope. As NucRed is a live-stain, staining only living cell nuclei, the goal was to determine the degree of cell attachment and proliferation, but upon imaging it was seen that NucRed Live 647 was not ideal for labelling SSEA4+CD34- cells in culture, despite prior testing on fibroblast originating from human cardiac tissue. It was also noticed that the imaged region of interest was not evenly lighted, resulting in a tremendous loss of detectable nuclei when setting the threshold for background fluorescence. Therefore the cells could not be further analysed using this technique. In figure 4.10 (a) a fraction of an image can be seen, where cells have been successfully labelled. Despite just seeing a fraction of the image, the uneven lightning is visible. Figure 4.10 (b) shows the same fraction imaged with phase contrast microscope.

In figure 4.11 mRNA levels for chosen genes are presented as fold change, calculated as $2^{-\Delta\Delta CT}$. As can be seen in figure 4.11 (a) and (b), some samples express early stem cell markers *i.e.* Oct4 and Nanog. For directly sorted SSEA4+/CD34+, SSEA4-/CD34+ and SSEA4-/CD34- cells as well as SSEA4+/CD34- cells cultured

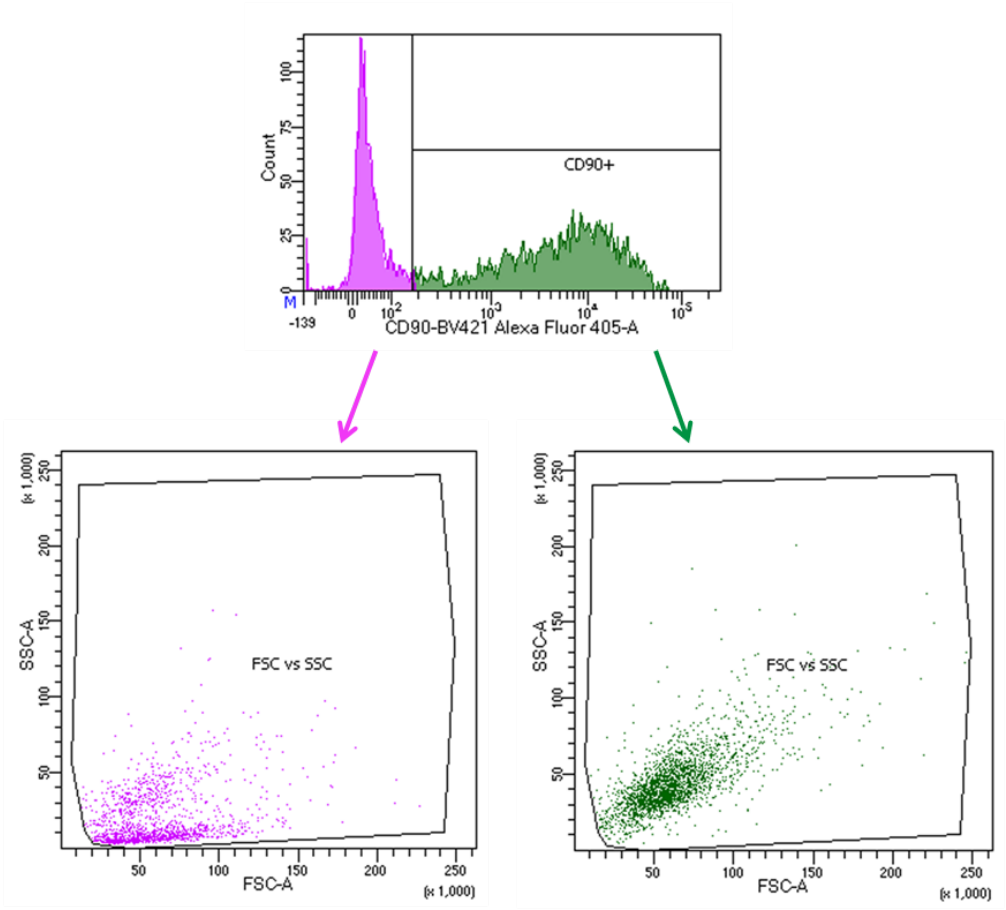
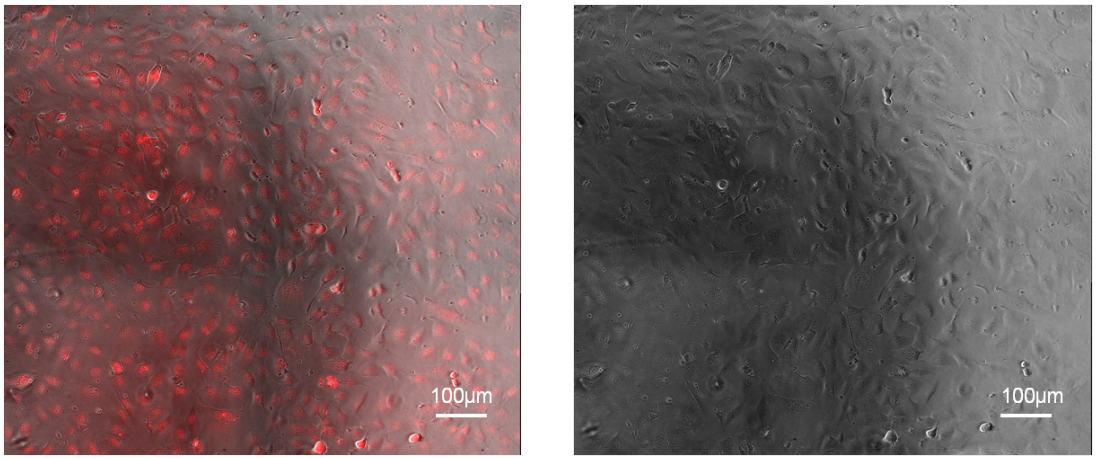


Figure 4.9: Back-gating on CD90-BV421 resulted in the finding of two distinct subpopulations within the SSEA4+/CD34- population. Purple represents the SSEA4+/CD34-/CD90- subpopulation, green represents the SSEA+/CD34-/CD90+

in N14 or N29 both OCT4 and NANOG mRAN was detected, indicating that these cells are in an early pluripotent stem cell-like state. Looking at TBX5 mRNA levels, it is obvious that directly isolated SSEA4-/CD34- cells also contain relatively high mRNA levels in comparison to other samples, despite the detection of stem cell-specific markers. This behaviour is also seen in other samples, with lower relative TBX5 mRNA levels compared to directly sorted SSEA4-/CD34- cells. Remarkably none of the chosen genes were detected in directly sorted SSEA4+/CD34- cells, which indicates that something went wrong during RT-qPCR preparation steps as GATA4, TBX5 and cTNNT2 mRNA was detected in directly sorted SSEA4+/CD34- cells seen in figure 4.6. Further it was seen that NKX2.5 mRNA levels for the SSEA4-/CD34- cell population is significantly higher compared to the other sorted populations, a finding that is consistent within the two experiments.

It has previously been suggested that the SSEA4+/CD34- population also express NKX2.5 and ISL1 (Sandstedt J, 2014). NKX2.5 mRNA was detected in some samples. In directly sorted SSEA4-/CD34- cells and SSEA4+/CD34- cells cultured in N42 NKX2.5 mRNA levels were high in comparison to mRNA levels in other samples



(a) (b)

Figure 4.10: NucRed labelled SSEA4+/CD34- cells isolated from LM45-LM46 cardiac biopsies after 12 days of culturing

as well as the internal calibrator CMix3, indicating that these culturing conditions might preserve gene expression of the SSEA4+/CD34- population in culture. Based on phase contrast microscopy images of cells cultured for 12 days in the FED-model generated media, it was seen that N57 (10ng/ml FGF, 5ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 20ng/ml dexamethasone, EBM-2, 20% HS, LN-521) yielded the highest density of attached cells (data not shown). Looking at the constituents of the media that maintained SSEA4+/CD34- cells in culture, it was seen that all levels of all parameters included in the FED-model were included, why it was not possible to draw any conclusions of the most optimal growth conditions for culturing of human cardiac progenitor cells based on medium constituent levels.

By culturing cells in FED model generated medium it was expected that the relative importance of each model factor could be determined, and thereby be able to characterize an optimal medium for stable culture of SSEA4+/CD34- cells. The idea was to import nuclei count obtained after NucRed Live 647 ReadyProbes staining to MODDE 10.1 for statistical analysis complemented by gene expression data from RT-qPCR analysis. Unfortunately NucRed Probes was not suitable for labelling of cardiac progenitor cells, why a statistical analysis was not possible to present. It was also noted that the variability between replicates was large, as only one of the two replicates maintained cells in culture for some media. Further testing is therefore required including more replicates. Based on the inconsistent gene expression data in combination with the large variability between replicates, it was not possible to determine the characteristics of isolated SSEA4+/CD34- cells in comparison to iPSC-derived cardiac progenitor cells.

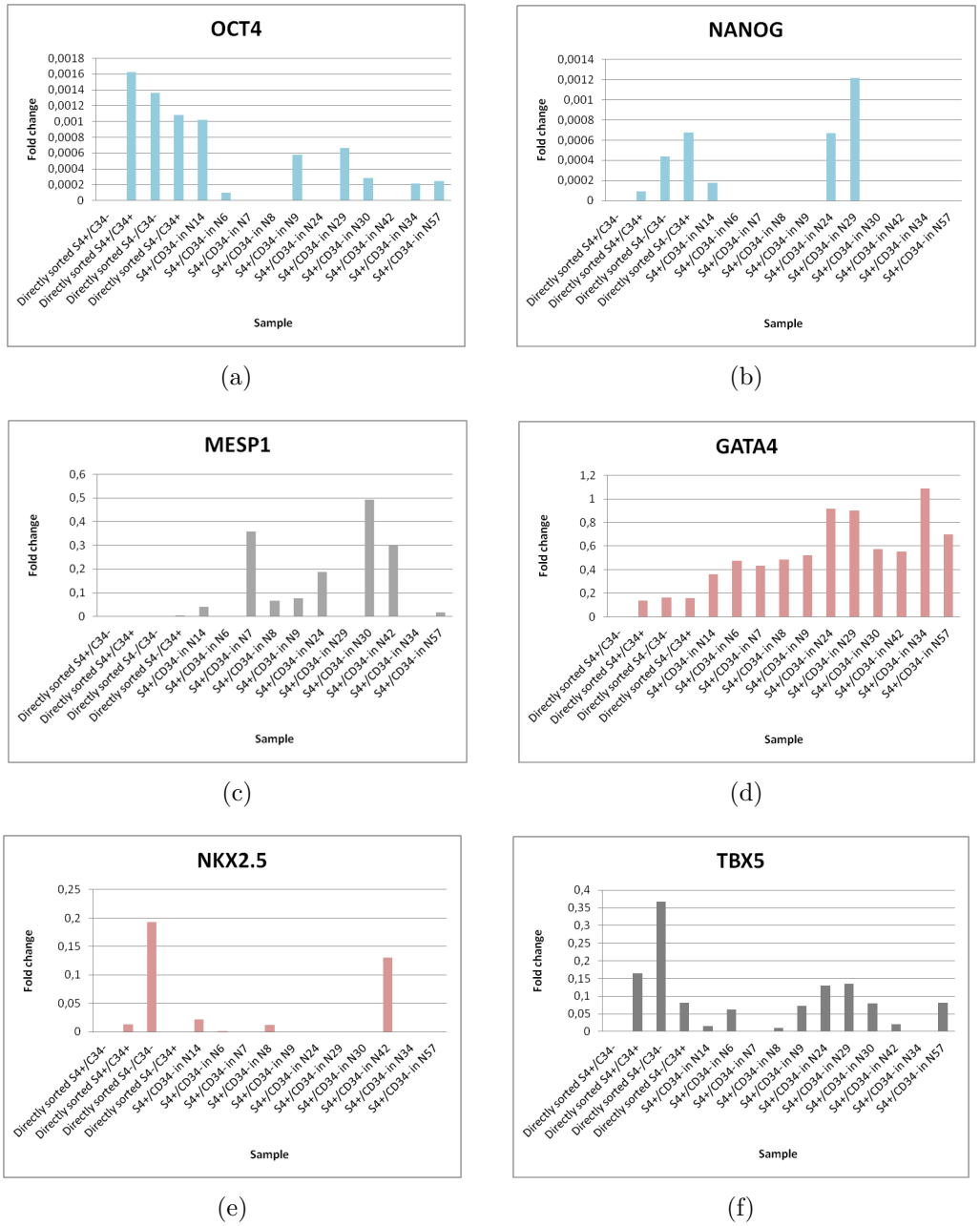
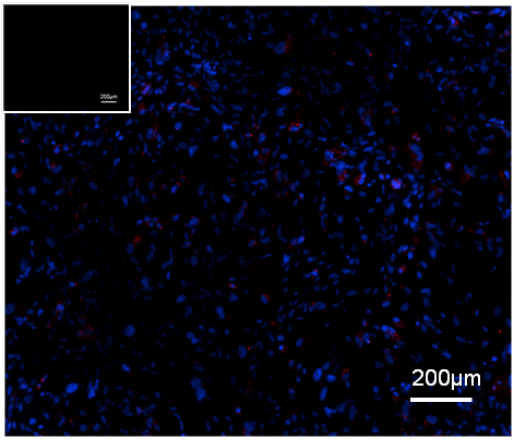


Figure 4.11: mRNA levels in SSEA4+/CD34- cells after 12 days of culture in FED model generated medium. Cells isolated from cardiac tissue donated from Patient 5-9

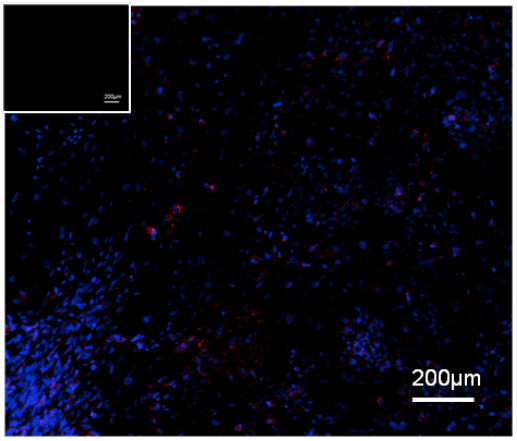
4.5 Culturing of iCell CPCs

iCell CPCs were cultured to determine the potential of the FED-model generated medium to induce cardiomyocyte differentiation. Cells were labelled with Hoechst 33342 for determination of cell proliferation and cTNNI3 SmartFlare Probes for determination of the degree of cardiomyocyte differentiation and taken for ICC. In figure 4.12 some representative images are shown, where blue colour represents Hoechst labelled cell nuclei and red areas represents the cytoplasm of cTNNI3 expressing cells. As most culture conditions yielded live cells to varying extent, all data is not shown. Cells cultured in media N61 (100ng/ml FGF, 50ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 0ng/ml dexamethasone, M199, 5% HS, LN-521) and N63(100ng/ml FGF, 50ng/ml EGF, 50ng/ml IGF-1, 0ng/ml HGF, 20ng/ml dexamethasone, M199, 5% HS, LN-521) yielded the highest degree of differentiated cells, with 80.5% and 66.6% differentiated cells respectively (*i.e* the amount of cTNNI3 labelled cytoplasms with Hoechst stained nuclei). In figure 4.12 (c) cells cultured in N61, yielding the highest degree of differentiated cells, is presented. The total number of nuclei stained with Hoechst and the number of cytoplasms labelled with cTNNI3 is presented in Appendix 2. N24, N28, N66 and N71, in figure 4.12 (a)-(b) and (e)-(f) represents average cTNNI3 expressing cells. When comparing the media inducing the most widespread cardiac differentiation it was noted that all media consists of high levels of FGF and IGF-1. EGF levels are overall high except in N65.

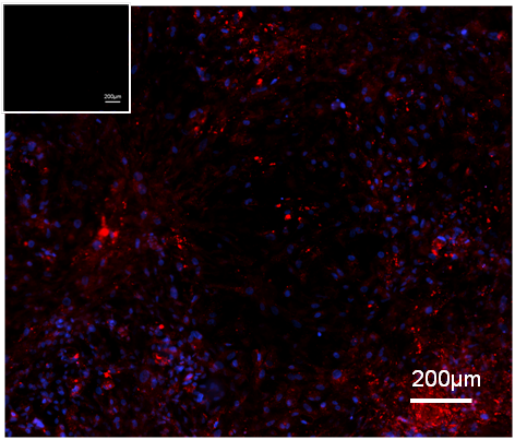
As all media were able to maintain cells in culture, it was concluded that none of the media constituent levels had a pronounced negative effect on cultured iCell CPCs resulting in reduced proliferation and cell death. Therefore it can be concluded that all factors may contribute to cell viability and proliferation, but it is not possible to determine to what extent. To further investigate each factor effect data was imported to MODDE 10.1 for statistical analysis.



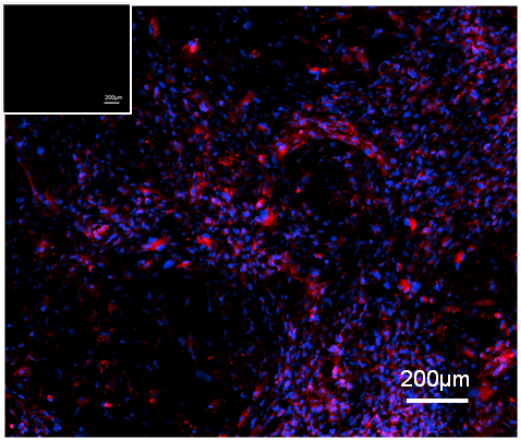
(a) N24 (100ng/ml FGF, 5ng/ml EGF, 5ng/ml IGF-1, 0ng/ml HGF, 20ng/ml dexamethasone, M199, 20% HS, LN-521)



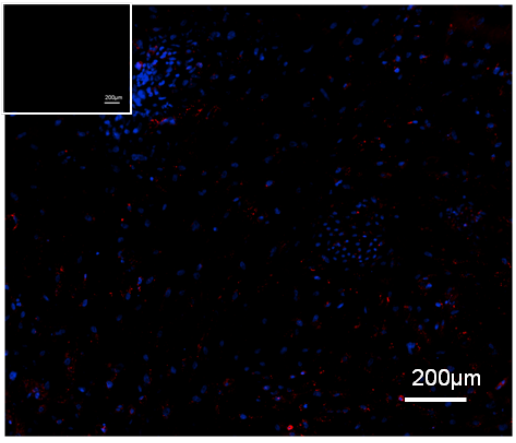
(b) N28 (100ng/ml FGF, 50ng/ml EGF, 5ng/ml IGF-1, 10ng/ml HGF, 0ng/ml dexamethasone, EBM-2,5% HS, LN-521/-211)



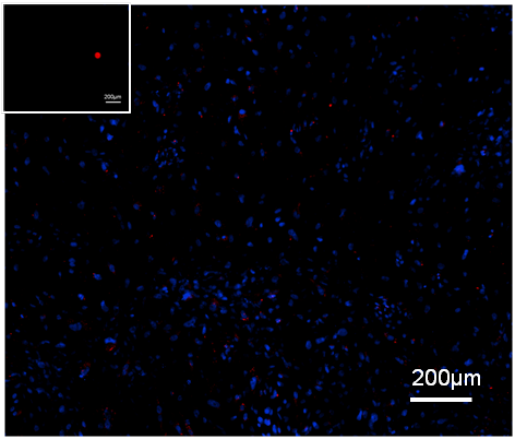
(c) N36 (55ng/ml FGF, 27.5ng/ml EGF, 27.5ng/ml IGF-1, 5ng/ml HGF, 10ng/ml dexamethasone, EBM-2/M199, 12.5% HS, LN-521/-221)



(d) N61 (100ng/ml FGF, 50ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 0ng/ml dexamethasone, EBM-2, 20% HS, LN-521)



(e) N66 (10ng/ml FGF, 5ng/ml EGF, 5ng/ml IGF-1, 0ng/ml HGF, 0ng/ml dexamethasone, EBM-2, 20% HS, LN-521/-211)



(f) N71 (100ng/ml FGF, 5ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 0ng/ml dexamethasone, EBM-2, 20% HS, LN-521/221)

Figure 4.12: ICC of Hoechst stained nuclei (blue) and cTNNI3 labelled cytoplasms (red) after 12 days of culture in FED model generated medium

4.5.1 Statistical analysis

The summary of fit for the model is presented in figure 4.13 for Hoechst stained nuclei and cTNNI3 labeled cell cytoplasms. The model precision was found to be low and of low significance based on the large difference between R^2 and Q^2, despite R^2 being larger than 0.5. Further it was seen that for Hoerchst stained nuclei, the model validity was negative, indicating that there is a lack of fit for the model meaning that the model error is greater than the pure error. The relative difference between R^2 and Q^2 for cTNNI3 labelled cytoplasms was also found to be large, further indicating a low significance and un-fitted model. As can be seen in figure 4.13 the model validity is for cTNNI3 labelled cytoplasms positive, indicating that there is no lack of fit of the model. The reproducibility was found to be 80% ± 5% for both Hoechst stained nuclei and cTNNI3 labelled cytoplasms.

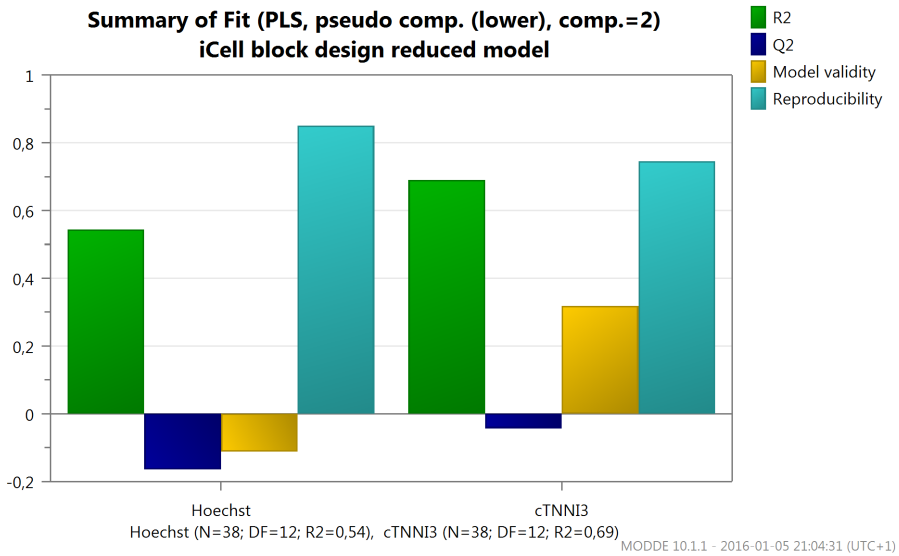


Figure 4.13: Summary of fit for Hoechst and cTNNI3 staining

Coefficient plots were generated in MODDE 10.1 for Hoechst stain and cTNNI3 labelling responses to graphically illustrate the relative significance of each term included in the model as well as included interaction effects, see figure 4.14. The longer the distance from y=0, the larger effect a factor has. A negative coefficient with long distance from y=0 means that this factor has a large negative effect. As the model was considered badly fitted and of low significance all results from the coefficient plot are highly uncertain. It was noted that the correlation effect between HGF and LN-521 had a positive effect on cell proliferation, *i.e.* more Hoechst stained nuclei. Further it was seen that the single effect of LN-521 affected cell proliferation positively while LN-521 in combination with LN-211 or LN-221 affected viability negatively. The same was seen for cTNNI3 labelled cytoplasms in figure 4.15. Here it was seen that all interaction effects with LN-521 affected cTNNI3 labelled cytoplasms positively while in combination with LN-211 or LN-221 the interaction effects with other factors were negative. The interaction effect between Dexamethasone and LN-521 had the most positive effect on cTNNI3 labelled cyto-

plasms.

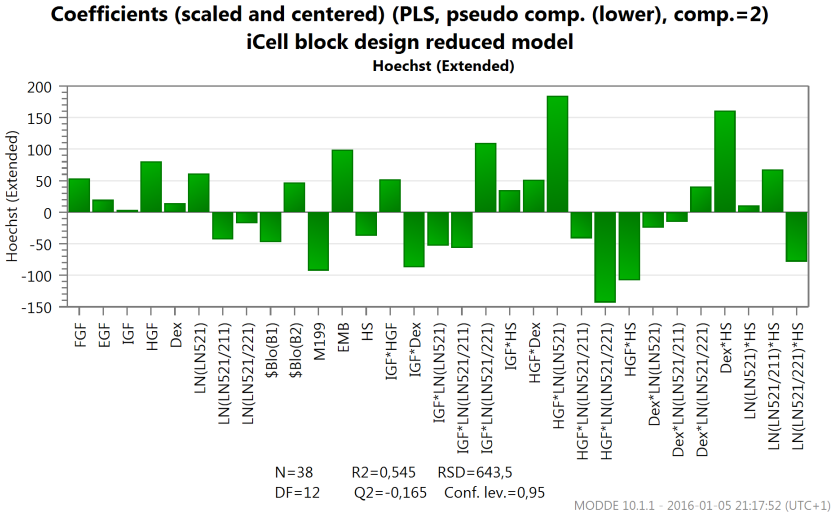


Figure 4.14: Coefficient plot for Hoechst stained nuclei

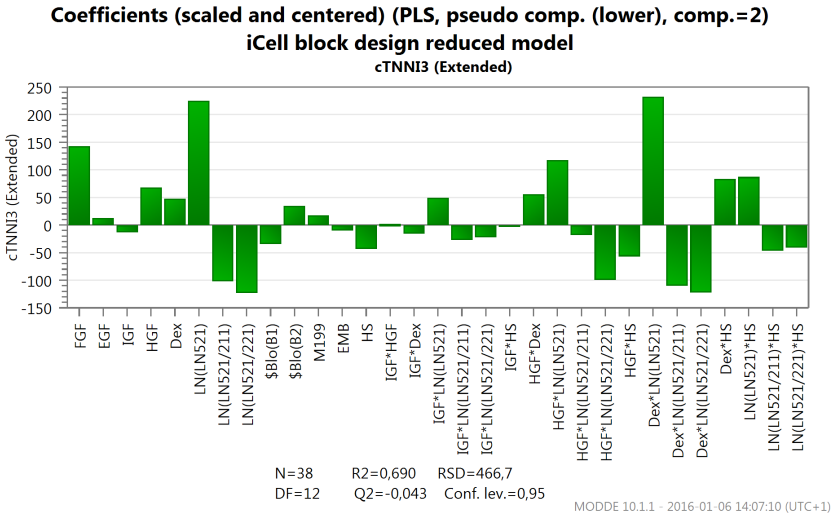
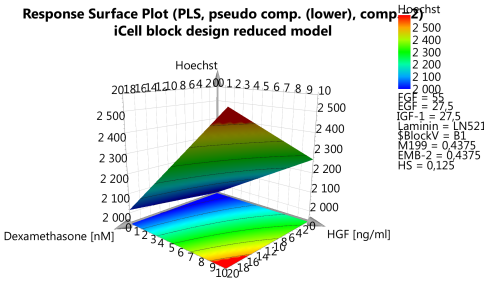


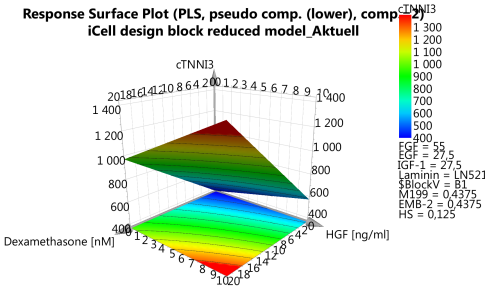
Figure 4.15: Coefficient plot for cTNNI3 Probe labelled cytoplasms

The response surface for HGF and Dexamethasone was plotted to further characterize the effect of the coating on Hoechst stained nuclei and cTNNI3 labelled cytoplasms. As seen in figure 4.16(a) high levels of both HGF and Dexamethasone generated more Hoechst stained nuclei than low levels when cultured on LN-521. The same was seen for cTNNI3 labelled cytoplasms. If instead coating with LN-521/-211 or -221 it was seen that the response surface indicated that high levels of HGF and low levels of Dexamethasone promoted cell proliferation and thus a high number of Hoechst stained nuclei. When generating the same response surfaces for cTNNI3 labelled cytoplasms it was seen that low levels of Dexamethasone and HGF yielded a higher degree of cardiomyocyte differentiation amongst cultured cells, *ie.*

more cTNNI3 labelled cytoplasms, compared to high levels. It is not possible to determine the definite importance and effect of each factor based on the generated model and its responses due to the high variability seen between replicates. The response surfaces for the interaction effect between Dexamethasone and HGF on the different Laminin combinations rather shows an example on how much a single factor can affect the model response as well as how it change drastically when combined with a second factor. Thereby it is not possible to determine the relative importance of each factor without further experiments.



(a)



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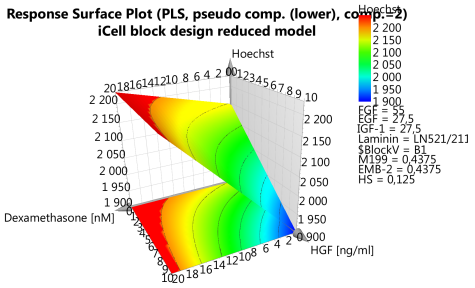

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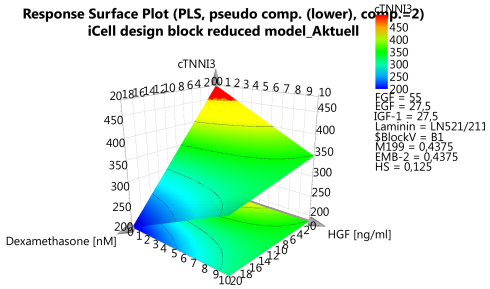

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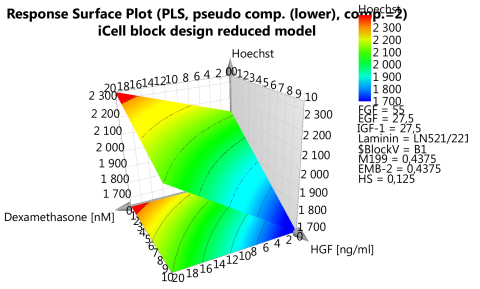

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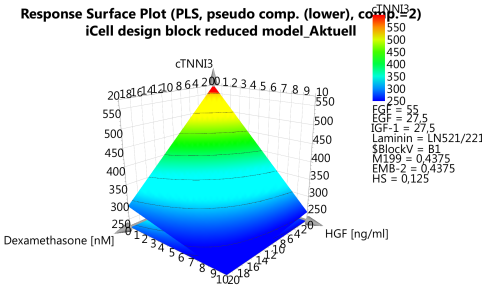

(f)

Figure 4.16: Response surfaces for HGF and Dexamethasone interaction effect. (a) Hoechst, on LN-521 (b) cTNNI3, on LN-521 (c) Hoechst, on LN-521/-211 (d) cTNNI3, on LN-521/-211 (e) Hoechst, on LN-521/-221 (f) cTNNI3, on LN-521/221

5
Discussion

SSEA4+/CD34- cardiac progenitor cells have in this Master's Thesis project been isolated from human cardiac biopsies originating from live patients undergoing routine cardiac surgery at the Thoracic surgery department at Sahlgrenska University Hospital. Several media and coating combinations were evaluated for maintenance of SSEA4+/CD34- cells in culture and a FED model was generated including M199, EBM-2, HS, FGF, EGF, IGF-1, HGF, Dexamethasone, LN-521, LN-521/-211 and LN-521/-221. Some FED model generated media were found to maintain SSEA4+/CD34- cells in culture and cells were harvested for gene expression analysis. iCell CPCs were also cultured in FED model generated media for evaluation of cardiac differentiation. It was seen that most media induced cardiac differentiation to varying extents. After short-time culturing cells were labelled with cTNNI3 SmartFlare Probes and Hoechst stained and taken for ICC. In this section obtained results will be discussed in more detail to increase the understanding of the results.

Addition of 5 μM ROCKi was found to increase cell viability during dissociation and culturing of SSEA4+/CD34- cells and was therefore included in all following experiments. It was also seen in the initial experiments that SSEA4+/CD34- cells attached better in culture on plates coated with LN-521/-211 or LN-521/-221 than on uncoated plates. This was expected, since Laminins are expressed during early cardiogenesis in the extracellular matrix of human cardiomyocytes (Roediger M *et. al.* 2010). Cells did not attach at all in NutriStem, which was expected since NutriStem is adapted for culturing pluripotent stem cells and SSEA4+/CD34- are believed to be mesoderm induced cardiac progenitor cells.

To minimize the patient specific variability between samples two biopsies were ideally pooled. Due to timing and postponed surgeries only one biopsy was sometimes obtained. This applied for experiments including cardiac tissue from Patient 1, 2, 4 and 7. Therefore these data should be considered with care, as the patient-specific variability still may have a pronounced effect but to unknown extent on the experiments, which may reduce comparability between samples. That could explain certain differences in gene expression found within these samples and between others and should be kept in mind. It is therefore important to reduce the patient-specific variability for future experiments, as the extent of effects are unknown. One approach to further reduce the patient-specific variability could be to separate biopsies originating from patients with different diseases, as disease might contribute to the variability between samples. To further differentiating between female and male patients might also reduce the patient-specific variability as it has previously been

seen that differences exists between sexes. For example it has been seen that female patients typically are older with maintained systolic cardiac function (Konhilas J. P, 2010). Separating between male and female patients might therefore reduce the chance of detecting effects caused by sex dimorphism. Most importantly a great variability was seen between replicates, where usually only one of the replicates did maintain cells in culture while the other did not. This might be explained by the low number of cells isolated in each experiment, and the low attachment in culture of seeded cells. To reduce this variability it is necessary to reduce the amount of conditions to allow an increased number of replicates with a lager amount of cells seeded to each condition, to decrease the variability seen between replicates. This is of high importance to enable characterization of culturing medium and important factors included in the medium. An increased number of replicates (with reduced variability) might also contribute to increased FED model significance and help fitting the model. The limited access of biopsies and the variety in biopsy size between patients also resulted in remarkably smaller starting material than expected, why in some cases the experimental size had to be reduced. Cells were also only dissociated from diseased hearts. Whether cardiac failure in some way affects SSEA4+CD34- cells or the overall viability of isolated cells remains unknown. Cardiac tissue from healthy hearts was not available during this Master's Thesis project why it is not possible to conclude whether this A cardiac explant was available for analysis, where left ventricle tissue was dissociated instead of right atrium tissue. The distribution of SSEA4+/CD34- cells in the human heart is to date unknown, but left ventricle tissue was here assumed to be translatable to tissue originating from right atrium based on assumptions.

iPSCs were differentiated into spontaneously contracting cardiomyocytes using the GiWi protocol. After 16 days of differentiation cells were harvested for gene expression analysis by RT-qPCR. Obtained gene data, presented in 4.4 did differ somewhat from published data (Lian *et. a.* 2012). For example OCT4 mRNA was detected during a longer period of time compared to published data, seen in figure 2.5. TBX5 and cTNNT2 mRNA was detected at day 4 and 6 of differentiation compared to published data suggesting that TBX5 and cTNNT2 mRNA should not be detected until day 7-8. Remembering that different cell lines and different culturing conditions may affect the timing of differentiation and that for example timing of spontaneous contraction differ between experiments, this difference in gene expression timing is not unexpected. The conclusion was therefore drawn that the gene expression data of differentiated iPSCs corresponds well to published data.

Initial cardiomyocyte differentiation of SSEA4+/CD34- cells isolated from cardiac tissue donated from Patient 4 did not yield spontaneously contracting cardiomyocytes. As this was an initial experiment to mainly investigate cell viability after exposure to cardiomyocyte differentiation medium spontaneous contraction was not expected. Cells were maintained in culture and were after 20 days harvested and analysed by RT-qPCR. Cardiac specific mRNA was detected for most samples as well as CD31, indicating that cells might have de-differentiated or differentiated towards another cell lineage. GATA4, TBX5 and cTNNT2 mRNA were detected in all cells

cultured in differentiation medium, but also in directly sorted SSEA4+/CD34- cells, seen in figure 4.6 (e)-(g). As these cardiac progenitor specific genes are expressed by directly isolated cells, it indicates that the SSEA4+/CD34- cell population indeed is cardiac committed and prone to differentiation into the cardomyocyte lineage. Though, it is not possible to compare relative levels of gene expression based on the obtained data, which otherwise could give an indication on the degree of differentiation amongst cultured cells. Increased cardiac progenitor specific mRNA levels would indicate that cells further differentiated towards the cardiac lineage and if such mRNA levels were decreased in response to culturing in differentiation medium would indicate that cells rather de-differentiated or differentiated towards another cell lineage. It could also indicate that a subpopulation within the SSEA4+/CD34- population is allowed to take over, such as the SSEA4+/CD34-/CD90+ population found during back-gating of FACS results, see figure 4.9. Heterogenic features and the presence of subpopulations within cardiac progenitor cell populations has been seen in previous studies, for example the C-kit+/CD45- population that has been showing signs of both cardiac and endothelial commitment, further complementing the argument for heterogenicity within the SSEA4+/CD34- population (Sandstedt J. *et. al* 2014.c-kit). Further studies are required to enable increased understanding of the differentiation capability of the SSEA4+/CD34- cells and the specific characteristics of the subpopulations within the potentially heterogenic cell population. Based on these findings it cannot be concluded that the SSEA4+/CD34- cells did differentiate towards the cardiac lineage, but it was found that there is a possibility that cardiac progenitor cell features to some extent might be preserved when cultured in differentiation medium, but further studies are required before this can be concluded with any certainty.

Cells isolated from cardiac tissue donated from Patient 4 were used for determining the possibility of postponed sorting of SSEA4+/CD34- progenitors until attachment of the cells in culture. In figure 4.6 mRNA levels are presented for directly sorted SSEA4+/CD34- cells in comparison to cells sorted after 5 days in culture. It should be noted that cells were cultured only on LN-521/-221 for practical reasons, why a different result might be obtained if changing the type of coating. No pronounced levels of NKX2.5 or ISL1 could be seen for most SSEA4+/CD34- cells. A slight increase in CD31 mRNA levels could be seen in the CD34+ subpopulation sorted after short-term culturing compared to directly sorted cells, potentially indicating an increased proliferation of endothelial lineages, and potential repression of other cells. In figure 4.6 it was seen that GATA4, TBX5 and cTNNT2 mRNA was decreased in response to short-term culturing, indicating that short-term culturing might contribute to the loss of cardiomyocyte progenitor specific gene expression, why the decision was taken that SSEA4+/CD34- cells should preferentially be sorted directly following dissociation to reduce the risk of loosing such features before further studies can prove otherwise.

It was seen that culturing cells in conditioned media produced by cells isolated from a left ventricle biopsy cultured in DMEM/F12 with HS induced an increase in mRNA levels for ISL1 and MESP1 in comparison to culturing human cardiac progenitor

cells in SCM. Adding 50% conditioned media to SCM resulted in a significant increase in ISL1 and MESP1 mRNA levels, while culturing in 100% conditioned media resulted in only increased MESP1 mRNA but no significant levels of ISL1 mRNA. Such findings indicate that the addition of conditioned media have an effect on the gene expression of isolated cells, potentially caused by kinases and other growth factors secreted by cultured cells from which the media was harvested. Therefore it would be of interest to further investigate the effect of conditioned medium, and if other cell types such as iPSC-derived cardiomyocytes could be used for conditioned medium harvesting.

A FED model was formulated to enable evaluation of a larger quantity of media additives for culturing directly sorted SSEA4+/CD34- progenitor cells. Conditioned medium was not included in the FED-model, despite showing some potential effect on cultured cells, since the content in such medium varies between different production sources, as well as the exact content is not known. SSEA4+/CD34- cells were dissociated and cultured in the FED-model generated media for 12 days before imaged and taken for RT-qPCR. In several samples early pluripotency markers *i.e.* Nanog and Oct4 were detected along with TBX5 or NKX2.5, early cardiac markers, as seen in figure 4.11. According to previous findings the SSEA4+/CD34- population express relative high levels of early and late cardiac-specific genes, TBX5, NKX2.5 and cTNNT2. The population has also been shown to express small amounts of OCT4, which could explain the co-expression of OCT4 and TBX5 or NKX2.5 found in some cells (Sandstedt J. *et. al.* 2014). The obtained gene expression data was based on only one replicate, meaning that the large variability between replicates may contribute to the obtained data. The absence of cells for certain conditions might rather be caused by cells not surviving the sorting and seeding process, rather than not being maintained in culture as a consequence of the medium. Therefore the experiment where SSEA4+/CD34- cells were cultured in FED model generated media should preferentially be re-run to ensure more reliable data. It was also noted that for directly sorted SSEA4+/CD34- cells no gene expression data was recorded. Remembering that during a previous experiment both GATA4, TBX5 and cTNNT2 mRNA was detected, see figure 4.6, this indicates that something went wrong during the RT-qPCR preparation steps, such as cDNA synthesis or pre-amplification of cDNA, which is further confirmed by the lack of expression for the reference gene CREBBP. The inconsistent gene expression data, the possible heterogenic features of the cell population and large variability seen between replicates limited the possibility of comparing SSEA4+/CD34- cells to iPSC-derived caridac progenitor cells. Based on phase contrast microscopy imaging of cultured SSEA4+/CD34- cells the N57 media (10ng/ml FGF, 5ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 20ng/ml dexamethasone, EBM-2, 20% HS, LN-521) was found to yield the highest degree of attached cells, but it is not possible to draw any conclusions on why since no specific media or additive level was found to promote survival of cultured SSEA4+/CD34- cells.

The FED model was further optimized for evaluating the potential to induce cardiomyocyte differentiation in iCell CPCs cultured in the FED model generated me-

dia. All media was found to induce cardiomyocyte differentiation to varying extent. The N61 (100ng/ml FGF, 50ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 0ng/ml dexamethasone, M199, 5% HS, LN-521) medium was found to induce the highest degree of differentiation, 80.5% of all the cultured cells. Further the results were statistically analysed in MODDE 10.1. In the summary of fit in figure 4.13 the R^2, Q^2, model validity and reprodicibility were presented. As the R^2 represents the model fit and should be greater than 0.5, it could be seen that both responses here indicated a well fitted model. But to draw any final conclusions of the overall model fit, other included parameters must be considered. Q^2 represents an estimate of the model precision of future predictions, a parameter found to be negative for both Hoechst staining and cTNNI3 labeling. As Q^2<0.1 is considered as a poor model, the model precision of estimating future predictions were determined to be low, most probably caused by lack of replicates in this experiment. Further the low model precision of future predictions could also be due to the large variability seen within the cTNNI3 and Hoechst assays or that some factors included in the model not have a pronounced effect on the model readout. Increasing the amount of replicates would potentially increase Q^2 and the model significance, if compensating for the variability seen during analysis. When comparing R^2 and Q^2 it was noted that the difference is larger than 20%, indicating that the model is badly fitted and of low significance. The model validity describes lack of fit of the model. If model validity is larger than 0.25 there is no lack of fit. For Hoechst stained nuclei, the model validity does not exceed 0.25 as the actual value is negative, meaning that there is a lack of fit in the model, which indicates that the model errors are significantly larger than the pure error. The model validity for cTNNI3 labelled cytoplasms is on the other hand larger than 0.25, indicating that there is no lack of fit in the model for this assay. Though, as the difference between R^2 and Q^2 is great, this rather indicate that the model is badly fitted and cannot estimate future predictions of high relevance. As the model validity is based on center points, an apporoach for increasing the model validity would therefore be to increase the number of center points, but most importantly to reduce the large variability between replicates.

The reproducibility of the model is rather high in the designed model. For Hoechst stained nuclei the reproducibility is over 80% and for cTNNI3 labelled cytoplasms slightly less than 80%. Here the reproducibility is based on center points (corresponding to the media containing 50% M199, 50% EBM-2, 12.5% HS, 55ng/ml FGF, 27.5ng/ml EGF, 27.5ng/ml IGF-1, 5ng/ml HGF, 10ng/ml Dexamethasone) as no replicates were not included in the model. Thus, the variability seen between replicates greatly affects the reproducibility, why increasing the amount of replicates and reducing the variability are essential for increasing the reproducibility of the model and would potentially help fitting the model to enable reliable predictions of future responses and in that way determine the components of a stable culture medium for SSEA4+/CD34- cells.

A coefficient plot was generated for Hoechst stained nuclei and cTNNI3 labeled cytoplasms. It is though important to remember that the model was found to be badly fitted with low significance, meaning that the following findings are indica-

tions rather than conclusive facts with large uncertainties. It was seen in figure 4.14 that each single factor have a more or less positive effect on iCell CPC proliferation, *i.e.* Hoechst stained nuclei. It was noted that the single effect of LN-521 was positive, but the single effects of LN-521/-211 and LN-521/-221 affected cell proliferation negatively. When comparing the single effects of the different types of Laminins, it was noted that the effect of LN-521 was drastically reduced when combined with IGF-1 or Dexamethasone but had an increased positive effect when in combination with HGF. In the coefficient plot for cTNNI3 labeled cytoplasms it was seen that the single effect of LN-521 was positive and the LN-521/-211 and LN-521/-221 effects were negative, as seen for Hoechst stained nuclei. When comparing the interaction effects between LN-521 to all other factors included in the model, it was seen that the positive effect on cTNNI3 labeling was maintained. The same was true for all interaction effects with LN-521/-211 and LN-521/-221, all being negative. This finding indicates that culturing iCell CPCs on LN-521 could potentially benefit cardiomyocyte differentiation amongst cultured cells. To evaluate the influence of type of coating on Hoechst stained nuclei and cTNNI3 labeled cytoplasms response surfaces were generated for the interaction effect between HGF and Dexamethasone. When comparing the response surfaces generated for Hoechst stained nuclei on different coatings, it was seen that high levels of Dexamethasome and HGF promoted iCell CPC proliferation *i.e.* more Hoechst stained nuclei when cultured on LN-521, while low levels of Dexamethasome and high levels of HGF promoted-cell proliferation when cultured on LN-521/-211 or LN-521/-221. Further it was seen that high levels of HGF and Dexamethasone also induced a more widespread cardiomyocyte differentiation amongst cells cultured on LN-521, *i.e.* more cTNNI3 labeled cytoplasms, while low levels of both HGF and Dexamethasone on LN-521/-211 or LN521-/-221 promoted cardiomyocyte differentiation. These findings indicate that iCell CPCs should preferentially be cultured on LN-521 to benefit cardiomyocyte differentiation, but further studies are required to be able to draw any definite conclusions.

6
Conclusion

During this Master Thesis project SSEA4+/CD34- cells were exclusively isolated from human cardiac tissue, showing that this cell type can be efficiently isolated. Several media were evaluated for maintenance of SSEA4+/CD34- cells in culture and it was seen that addition of 5μM ROCKi increased cell viability and culturing the cells on different combinations of Laminins were more beneficial than seeding cells on un-coated surfaces. iPSCs were differentiated into spontaneously contracting cardiomyocytes and found to correlate well to previously published data. It was not possible to compare SSEA4+/CD34- cells cultured in differentation medium to iPSC-derived cardiac progenitor cells due to inconsistent gene expression data and large patient-specific variability. Culturing of SSEA4+/CD34- cells did not yield contracting cardiomyocytes and based on genetic data cells seemed rather to have lost cardiac progenitor cell features and increased CD31 mRNA. Genetic data for directly sorted SSEA4+/CD34- cells were compared to genetic data collected from cells sorted after short-term culturing. Based on a remarkable loss in detectable GATA4, TBX5 and cTNNT2 mRNA levels, it was concluded that SSEA4+/CD34- cells should preferentially be sorted directly following dissociation of cardiac tissue to reduce the loss of cardiac progenitor cell features.

A FED model was generated to further evaluate medium components necessary for culturing SSEA4+/CD34- cells in a more systematic way. Some media were found to maintain SSEA4+/CD34- cells in culture ((N14, N6, N7, N8, N9, N24, N29, N30, N42, N34 and N57), where N57 (10ng/ml FGF, 5ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 20ng/ml dexamethasone, EBM-2, 20% HS, LN-521) yielded the highest degree of confluency in seeded cells. Some FED model generated media were also found to induce cardiomyocyte differentiation in iCell CPCs after 12 days of culturing, such as N61, N63 and N36, where N61 (100ng/ml FGF, 50ng/ml EGF, 50ng/ml IGF-1, 10ng/ml HGF, 0ng/ml dexamethasone, M199, 5% HS, LN-521) yielded 80.7% differentiated cells. During statistical analysis it was seen that the model was badly fitted and of low significance. All findings were therefore highly uncertain, but it was seen that the interaction effect of HGF and LN-521 effected the amount of Hoechst stained nuclei in the most positive way, despite being highly uncertain. Further studies are needed to draw any further conclusions on culturing conditions for SSEA4+/CD34- cells isolated from cardiac tissue.

7

Future aspects

Since it was not possible during this project to fully characterize the SSEA4+/CD34- cardiac progenitor cell population and its behaviour in culture, further studies are required to gain increased understanding of the cardiac progenitor cell population. As it was not possible to draw any definite conclusions on optimal culturing conditions for primary isolated human SSEA4+/CD34- cells, it would be of interest to repeat the experiments where SSEA4+/CD34- cells were cultured in FED model generated media. While repeating the experiment it would be of high importance to reduce the number of factors included in the model, to further allow sorting of a larger number of cells to each condition and thereby reducing the great variability seen within replicates. Reducing the variability between replicates, would probably help fitting the model and increase the model precision of future response predictions, thus allowing estimation of optimal culturing conditions for primary isolated human cardiac progenitor cells. By doing so, more reliable data could be generated, and the relative importance of each single factor effect could be estimated along with interaction effects with other factors included in the model. Another approach to further reduce the variability seen between samples would be to separate biopsies originating from patients of both sexes undergoing different types of surgeries as they have different types of cardiac diseases. Whether cardiac disease or sex dimorphism affect the distribution of cardiac progenitor cells and to what extent is to date unknown, why separating biopsies based on type of surgery and sex would reduce the risk of detecting patient-specific variability. The N57 medium was found to maintain the most cells in culture, why it would be of interest to further investigate this type of medium and whether it allows maintained cardiac progenitor cell features.

It would also be of interest to further investigate the media that induced the most widespread cardiac differentiation in cultured iCell CPCs. One approach would be to further culture iCell CPCs in media only containing the factors having the largest positive effect on culturing of SSEA4+/CD34- cells to further characterize optimal culturing conditions and to improve the statistical significance of the model. Another approach would be to optimize culturing conditions using these factors for iCell CPCs and then further evaluate the effect on the optimal medium on SSEA4+/CD34- cells isolated from human cardiac tissue. By doing so the overall knowledge of resident cardiac progenitor cells might be increased as well as the translatability between iCell CPCs (and thus iPSCs) and primary isolated cells could be determined.

Culturing SSEA4+/CD34- cells in conditioned medium produced by cells isolated form left ventricle biopsy was shown to induce an increase in ISL1 and MESP1 mRNA levels. This indicates that the conditioned media actually have an effect on SSEA4+/CD34- cells, potentially contributing to maintained cardiac progenitor cell features *in vitro*. Here cells used for conditioned medium production were isolated from the left ventricle, but in future experiments it would be valuable to evaluate other cell sources for production of conditioned medium, for example iPSC-derived cardiomyocytes or cells isolated from other localisations within the adult human heart. Another approach would be to culture SSEA4+/CD34- cells in feeder systems to further characterize the potential genotypic and phenotypic changes induced by such culture systems. Co-culturing SSEA4+/CD34- cells with cardiomyocytes or potentially fibroblasts could also contribute to increasesd understanding, as the presence of other cell types further resembles the *in vivo* conditions. 3D-scaffolds, such as de-cellularized tissue, have previously been suggested for culturing of *in vitro* expanded cells, to further resemble the *in vivo* conditions. Such scaffolds require a stable culture system for seeded cells, why further characterization of culture conditions for SSEA4+/CD34- is required before 3D-scaffolds can be implemented for SSEA4+/CD34- cells.

During back-gating of sorting data two distinct subpopulations were found, based on CD90 labelling. The SSEA4+/CD34-/CD90- population was found to be small in size and with low granularity, as seen in the FSC vs. SSC plot. The SSEA4+/CD34-/CD90+ on the other hand was found have a slightly higher SSC than the other population, indicating a somewhat higher granularity. The characteristics of the two distinct subpopulations and how they differ are to date unknown, but it is hypothesized that the SSEA4+/CD34-/CD90+ subpopulation rather is some type of fibroblastic subpopulation and that it is the SSEA4+/CD34-/CD90- subpopulation that is committed to the cardiac lineage. Further studies are though required before any definite conclusions can be drawn on the separate characteristics of the two subpopulations found within the SSEA4+/CD34- cell population present in right atrium tissue. An approach to increase the meaning of the CD90 expression would be to sort and culture the two subpopulations separately. By doing so, the complete knowledge could be increased of the SSEA4+/CD34- population and its role as cardiac progenitor cells present in the human heart.

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A

Appendix 1

Protocols

A.1.I Dissociation of cardiac tissue (MS84)

Solutions

- DNAse I solution:
  Prepare fresh DNAse I solution for each dissociation. Dilute to 10mg/ml (to 5mg add 0,5ml ddH_20) followed by 1mg/ml in medium (to 5mg add 4,5ml medium). Sterilize by filtration.
- Liberase solution: (50ml final volume)
  To 1ml Liberase TH (26 Wünschunits/ml stock solution) add 23,5ml DMEM/F12 supplemented with L-glutamine, PEST and 2500μl sterilized DNAse I solution.

1. Keep cells cold during step 1-3. Directly transfer the tissue sample to ice cold PBS (30ml). Transport to cell lab within 15 min.
2. Remove connective and adipose tissue. Wash with ice cold PBS to remove residual blood. Weigh the biopsy in a pre-weight Falcon tube. Cut the biopsy thoroughly into small pieces in minimal amount of cold PBS using a sterilized scissors on the lid of a Petri dish. Transfer to a 50 ml Falcon tube.
3. Spin down the tissue fragments at 400g for 5 min. Discard the supernatant and add 25ml Liberase solution. If necessary add ROCKi to the dissociation solution. Dissociate in Corning Erlenmeyer cell culture flasks with magnetic stirrer in 37°C for 2.5-4h.
4. Transfer the cell solution to 50ml Falcon tubes and spin down the cells at 400g for 5 min. Rinse the Erlenmeyer flask with additional DMEM/F12 supplemented with L-glutamine and PEST to avoid cell loss.
5. Transfer the supernatant to new 50ml Falcon tubes and spin again at 400g for 5 min to collect residual cells in solution. Meanwhile resuspend the original cell pellet in 10ml Trypsin-EDTA and incubate for 10 min at 37°C.
6. Discard the supernatant and resuspend the second cell pellet in 5ml Trypsin-EDTA and incubate for 10min at 37°C.
7. Stop the trypsin by adding an equal amount of DMEM/F12 supplemented with L-glutamine, PEST and 10% human or FCS. Pool the cell suspensions.
8. Filter the cells through a 100μm cell strainer to remove eventual aggregates. Rinse the filters with additional DMEM/F12 supplemented with L-glutamine,

PEST and 10% HS or FCS to avoid cell loss. Transfer the cell solutions to 15ml Falcon tubes and spin down the cells at 400g for 5 min.

For direct monolayer culture

1. Resuspend the cells in suitable amount of medium and seed cells at a concentration of ca 1mg/cm^2.

For FACS analysis

1. Resuspend the cells in 10ml Lysis buffer (1x) and incubate for 1min at room temperature to lyse erythrocytes and other blood cells that can interfere during FACS analysis.
2. Spin down the cells at 400g for 5 min and resuspend and pool the cells to a final amount of 10ml FACS buffer.
3. Filter the cells through a 40µm cell strainer to remove larger particles and aggregates-
4. Count the cells in a Bürker chamber. Spin down the cells at 400g for 5 min and resuspend to desired concentration in FACS buffer.

A.1.II FACS protocol

Following Dissociation of cardiac tissue (MS84) for FACS analysis:

1. Add 1μl 7-AAD per tube (final concentration of 10μg/ml)
2. Add antibodies at desired concentration. Keep antibodies dark.
3. Add cell suspension to the tubes. Cell suspension without antibodies and 7-AAD should be 100μl/tube if nothing else is specified.
4. Vortex briefly and incubate cells for 30 min at 4°C. Keep dark.
5. Wash with 1,5ml cold FACS buffer per tube. Pour through cell strainer and spin down at 400 g for 5 min. Resuspend in 1,5ml cold FACS buffer and resuspend in cold FACS buffer to desired volume to get a suitable cell concentration for acquisition
6. Acquire and analyze data. Keep cells on ice in the dark until acquisition.

Note: FACS buffer should be cold to prevent antibody capping and to reduce metabolic activity of cells.

A.1.III GiWi Protocol for cardiomyocyte differentiation

Day-1	Seeding of cells	Seed cells at 312K/cm^2
Day 0	Differentiation phase 1	Aspirate and change medium to L1
Day 1	Differentiation phase 2	Aspirate and change medium to 50% LC2 and 50% M2.
Day 2		No medium change (MC)
Day 3	Maintenance 2	Aspirate and change to M2
Day 4		No MC
Day 5	Maintenance 3	Aspirate and change to M3
Day 6		No MC
Day 7		No MC
Day 8		Aspirate and change to M3
Day 9		No MC
Day 10		No MC
Day 11		Aspirate and change to M3
Day 12		No MC
Day 13		No MC
Day 14		Aspirate and change to M3
Day 15		No MC
Day 16		No MC
Day 17		Aspirate and change to M3
Day 18		No MC
Day 19		No MC
Day 20		Aspirate and change to M3

Contracting behavior should be visible at day 14.

Table A.1: Maintenance 2 medium (M2)

Reagent	Working conc	Stock	Stock/ml media	Add for total vol
RPMI GlutaMAX				6000μl
B-27 minus insulin	1x	50x	20μl/ml	6000μl

Table A.2: Phase 2 Combined Medium 50% (LC2)

Reagent	Working conc	Stock	Stock/ml media	Add for total vol
RPMI GlutaMAX				6000μl
B-27 minus insulin	1x	50x	20μl/ml	6000μl
IWP-2	10μM*	2mM	5μl/ml	30μl

*Double working concentration (2*5μM) due to 50% dilution

Table A.3: Maintenance 3 medium (M3)

Reagent	Working conc	Stock	Stock/ml media	Add for total vol
RPMI GlutaMAX				6000μl
B-27	1x	50x	20μl/ml	120μl

B

Appendix 2

FED-model generated media for culturing of SSEA4+/CD34- cells

Exp No	Exp Name	Run Order	Incl/Excl	FGF	EGF	IGF-1	HGF	Dexametasone	Laminin	Cells per well	$BlockV	EBM-2	M199	HS
				ng/ml	ng/ml	ng/ml	ng/ml	ng/ml				%	%	%
1	N4	1	Incl	10	5	50	10	20	LN521		B1	0,95	0	0,05
2	N19	15	Incl	10	5	50	10	20	LN521		B1	0,95	0	0,05
3	N5	18	Incl	10	50	50	10	20	LN521		B1	0	0,8	0,2
4	N20	4	Incl	10	50	50	10	20	LN521		B1	0	0,8	0,2
5	N13	17	Incl	55	27,5	27,5		10	LN521		B1	0,4375	0,4375	0,125
6	N14	30	Incl	55	27,5	27,5	5	10	LN521		B1	0,4375	0,4375	0,125
7	N3	22	Incl	100	5	5	10	20	LN521		B1	0	0,95	0,05
8	N18	21	Incl	100	5	5	10	20	LN521		B1	0	0,95	0,05
9	N1	8	Incl	100	50	5	0	0	LN521		B1	0,95	0	0,05
10	N16	26	Incl	100	50	5	0	0	LN521		B1	0,95	0	0,05
11	N2	13	Incl	100	50	5	0	0	LN521		B1	0	0,8	0,2
12	N17	16	Incl	100	50	5	0	0	LN521		B1	0	0,8	0,2
13	N6	28	Incl	10	5	50	0	0	LN521/211		B1	0,95	0	0,05
14	N21	3	Incl	10	5	50	0	0	LN521/211		B1	0,95	0	0,05
15	N7	24	Incl	10	50	5	10	0	LN521/211		B1	0	0,8	0,2
16	N22	29	Incl	10	50	5	10	0	LN521/211		B1	0	0,8	0,2
17	N15	19	Incl	55	27,5	27,5	5	10	LN521/211		B1	0,4375	0,4375	0,125
18	N28	23	Incl	55	27,5	27,5	5	10	LN521/211		B1	0,4375	0,4375	0,125
19	N8	11	Incl	100	50	5	0	20	LN521/211		B1	0	0,8	0,2
20	N23	12	Incl	100	50	5	0	20	LN521/211		B1	0	0,8	0,2
21	N11	10	Incl	10	5	5	0	20	LN521/221		B1	0,95	0	0,05
22	N26	5	Incl	10	5	5	0	20	LN521/221		B1	0,95	0	0,05
23	N9	14	Incl	10	50	50	0	0	LN521/221		B1	0	0,8	0,2
24	N24	7	Incl	10	50	50	0	0	LN521/221		B1	0	0,8	0,2
25	N29	9	Incl	55	27,5	27,5	5	10	LN521/221		B1	0,4375	0,4375	0,125
26	N30	27	Incl	55	27,5	27,5	5	10	LN521/221		B1	0,4375	0,4375	0,125
27	N12	6	Incl	100	5	50	0	20	LN521/221		B1	0	0,95	0,05
28	N27	25	Incl	100	5	50	0	20	LN521/221		B1	0	0,95	0,05
29	N10	20	Incl	100	5	50	10	0	LN521/221		B1	0	0,95	0,05
30	N25	2	Incl	100	5	50	10	0	LN521/221		B1	0	0,95	0,05

Figure B.1: FED model Block1 for culturing SSEA4+/CD34- isolated from LM-biopsies. All media was supplemented with 5μM ROCKi

Exp No	Exp Name	Run Order	Incl/Excl	FGF	EGF	IGF-1	HGF	Dexamet asone	Laminin	Cells per well	$BlockV	HS	EBM-2	M199
1	N31	32	Incl	10	50	50	10	0	LN521		B2	0,05	0	0,95
2	N44	40	Incl	10	50	50	10	0	LN521		B2	0,05	0	0,95
3	N41	50	Incl	55	27,5	27,5	5	10	LN521		B2	0,125	0,4375	0,4375
4	N42	41	Incl	55	27,5	27,5	5	10	LN521		B2	0,125	0,4375	0,4375
5	N32	47	Incl	100	50	5	10	20	LN521		B2	0,2	0,8	0
6	N45	54	Incl	100	50	5	10	20	LN521		B2	0,2	0,8	0
7	N35	43	Incl	10	50	50	0	20	LN521/211		B2	0,05	0,95	0
8	N48	42	Incl	10	50	50	0	20	LN521/211		B2	0,05	0,95	0
9	N43	55	Incl	55	27,5	27,5	5	10	LN521/211		B2	0,125	0,4375	0,4375
10	N54	31	Incl	55	27,5	27,5	5	10	LN521/211		B2	0,125	0,4375	0,4375
11	N33	53	Incl	100	5	50	0	0	LN521/211		B2	0,2	0	0,8
12	N46	38	Incl	100	5	50	0	0	LN521/211		B2	0,2	0	0,8
13	N36	36	Incl	100	5	50	10	20	LN521/211		B2	0,05	0	0,95
14	N49	56	Incl	100	5	50	10	20	LN521/211		B2	0,05	0	0,95
15	N34	52	Incl	100	50	50	10	0	LN521/211		B2	0,2	0,8	0
16	N47	49	Incl	100	50	50	10	0	LN521/211		B2	0,2	0,8	0
17	N40	45	Incl	10	5	5	0	20	LN521/221		B2	0,2	0	0,8
18	N53	35	Incl	10	5	5	0	20	LN521/221		B2	0,2	0	0,8
19	N38	46	Incl	10	5	5	10	0	LN521/221		B2	0,05	0,95	0
20	N51	34	Incl	10	5	5	10	0	LN521/221		B2	0,05	0,95	0
21	N37	39	Incl	10	50	5	0	0	LN521/221		B2	0,05	0	0,95
22	N50	44	Incl	10	50	5	0	0	LN521/221		B2	0,05	0	0,95
23	N55	51	Incl	55	27,5	27,5	5	10	LN521/221		B2	0,125	0,4375	0,4375
24	N56	37	Incl	55	27,5	27,5	5	10	LN521/221		B2	0,125	0,4375	0,4375
25	N39	48	Incl	100	5	5	10	0	LN521/221		B2	0,2	0	0,8
26	N52	33	Incl	100	5	5	10	0	LN521/221		B2	0,2	0	0,8

Figure B.2: FED model Block 2 for culturing SSEA4+/CD34- isolated from LM-biopsies. All media was supplemented with 5μM ROCKi

Exp No	Exp Name	Run Order	Incl/Excl	FGF	EGF	IGF-1	HGF	Dexametasone	Laminin	Cells per well	$BlockV	HS	EBM-2	M199
1	N57	70	Incl	10	5	50	10	0	LN521		B3	0,2	0,8	0
2	N71	59	Incl	10	5	50	10	0	LN521		B3	0,2	0,8	0
3	N60	83	Incl	10	50	50	0	20	LN521		B3	0,05	0	0,95
4	N74	58	Incl	10	50	50	0	20	LN521		B3	0,05	0	0,95
5	N68	72	Incl	55	27,5	27,5	5	10	LN521		B3	0,125	0,4375	0,4375
6	N69	84	Incl	55	27,5	27,5	5	10	LN521		B3	0,125	0,4375	0,4375
7	N59	75	Incl	100	5	50	0	20	LN521		B3	0,2	0,8	0
8	N73	74	Incl	100	5	50	0	20	LN521		B3	0,2	0,8	0
9	N58	64	Incl	100	50	5	0	20	LN521		B3	0,05	0,95	0
10	N72	71	Incl	100	50	5	0	20	LN521		B3	0,05	0,95	0
11	N61	76	Incl	10	5	5	0	0	LN521/211		B3	0,2	0,8	0
12	N75	65	Incl	10	5	5	0	0	LN521/211		B3	0,2	0,8	0
13	N64	60	Incl	10	5	5	10	20	LN521/211		B3	0,2	0,8	0
14	N78	66	Incl	10	5	5	10	20	LN521/211		B3	0,2	0,8	0
15	N70	80	Incl	55	27,5	27,5	5	10	LN521/211		B3	0,125	0,4375	0,4375
16	N82	82	Incl	55	27,5	27,5	5	10	LN521/211		B3	0,125	0,4375	0,4375
17	N62	57	Incl	100	5	5	0	0	LN521/211		B3	0,05	0	0,95
18	N76	67	Incl	100	5	5	0	0	LN521/211		B3	0,05	0	0,95
19	N63	63	Incl	100	50	5	10	0	LN521/211		B3	0,05	0,95	0
20	N77	77	Incl	100	50	5	10	0	LN521/211		B3	0,05	0,95	0
21	N66	79	Incl	10	50	5	10	20	LN521/221		B3	0,05	0	0,95
22	N80	78	Incl	10	50	5	10	20	LN521/221		B3	0,05	0	0,95
23	N83	61	Incl	55	27,5	27,5	5	10	LN521/221		B3	0,125	0,4375	0,4375
24	N84	62	Incl	55	27,5	27,5	5	10	LN521/221		B3	0,125	0,4375	0,4375
25	N65	68	Incl	100	50	50	0	0	LN521/221		B3	0,05	0,95	0
26	N79	69	Incl	100	50	50	0	0	LN521/221		B3	0,05	0,95	0
27	N67	81	Incl	100	50	50	10	20	LN521/221		B3	0,2	0,8	0
28	N81	73	Incl	100	50	50	10	20	LN521/221		B3	0,2	0,8	0

Figure B.3: FED model Block 3 for culturing SSEA4+/CD34- isolated from LM-biopsies. All media was supplemented with 5μM ROCKi

FED-model generated media for culturing of iCell CPCs

Exp No	Exp Name	Run Order	Incl/Excl	FGF	EGF	IGF-1	HGF	Dexamethasone	Laminin	$BlockV	M199	EMB-2	HS
	#	#		ng/ml	ng/ml	ng/ml	ng/ml	ng/ml			%	%	%
1	N1	11	Incl	10	5	5	0	0	LN521	B1	0,95	0	0,05
2	N20	33	Incl	10	5	5	0	0	LN521	B1	0,95	0	0,05
3	N6	12	Incl	10	5	50	0	20	LN521	B1	0	0,95	0,05
4	N25	27	Incl	10	5	50	0	20	LN521	B1	0	0,95	0,05
5	N4	32	Incl	10	5	50	10	0	LN521	B1	0	0,95	0,05
6	N23	34	Incl	10	5	50	10	0	LN521	B1	0	0,95	0,05
7	N8	38	Incl	10	50	50	10	20	LN521	B1	0,8	0	0,2
8	N27	6	Incl	10	50	50	10	20	LN521	B1	0,8	0	0,2
9	N5	7	Incl	100	5	5	0	20	LN521	B1	0,8	0	0,2
10	N24	36	Incl	100	5	5	0	20	LN521	B1	0,8	0	0,2
11	N7	10	Incl	100	5	5	10	20	LN521	B1	0,95	0	0,05
12	N26	21	Incl	100	5	5	10	20	LN521	B1	0,95	0	0,05
13	N2	23	Incl	100	5	50	0	0	LN521	B1	0,8	0	0,2
14	N21	28	Incl	100	5	50	0	0	LN521	B1	0,8	0	0,2
15	N3	25	Incl	100	50	5	10	0	LN521	B1	0	0,8	0,2
16	N22	14	Incl	100	50	5	10	0	LN521	B1	0	0,8	0,2
17	N9	20	Incl	100	50	5	10	0	LN521/211	B1	0	0,95	0,05
18	N28	17	Incl	100	50	5	10	0	LN521/211	B1	0	0,95	0,05
19	N12	8	Incl	100	50	50	0	20	LN521/211	B1	0	0,8	0,2
20	N31	30	Incl	100	50	50	0	20	LN521/211	B1	0	0,8	0,2
21	N11	1	Incl	10	50	5	0	20	LN521/211	B1	0,95	0	0,05
22	N30	37	Incl	10	50	5	0	20	LN521/211	B1	0,95	0	0,05
23	N10	29	Incl	10	50	50	10	0	LN521/211	B1	0,8	0	0,2
24	N29	3	Incl	10	50	50	10	0	LN521/211	B1	0,8	0	0,2
25	N13	35	Incl	100	50	5	0	0	LN521/221	B1	0,8	0	0,2
26	N32	2	Incl	100	50	5	0	0	LN521/221	B1	0,8	0	0,2
27	N16	18	Incl	100	50	50	10	20	LN521/221	B1	0,95	0	0,05
28	N35	15	Incl	100	50	50	10	20	LN521/221	B1	0,95	0	0,05
29	N15	13	Incl	10	50	5	10	20	LN521/221	B1	0	0,8	0,2
30	N34	16	Incl	10	50	5	10	20	LN521/221	B1	0	0,8	0,2
31	N14	26	Incl	10	50	50	0	0	LN521/221	B1	0	0,95	0,05
32	N33	5	Incl	10	50	50	0	0	LN521/221	B1	0	0,95	0,05
33	N17	31	Incl	55	27,5	27,5	5	10	LN521/221	B1	0,4375	0,4375	0,125
34	N18	19	Incl	55	27,5	27,5	5	10	LN521/221	B1	0,4375	0,4375	0,125
35	N19	22	Incl	55	27,5	27,5	5	10	LN521/221	B1	0,4375	0,4375	0,125
36	N36	4	Incl	55	27,5	27,5	5	10	LN521/221	B1	0,4375	0,4375	0,125
37	N37	24	Incl	55	27,5	27,5	5	10	LN521/221	B1	0,4375	0,4375	0,125
38	N38	9	Incl	55	27,5	27,5	5	10	LN521/221	B1	0,4375	0,4375	0,125

Figure B.4: FED model Block 1 for culturing iCell CPCs

Exp No	Exp Name	Run Order	Incl/Excl	FGF	EGF	IGF-1	HGF	Dexamethasone	Laminin	$BlockV	M199	EMB-2	HS
	#	#		ng/ml	ng/ml	ng/ml	ng/ml	ng/ml			%	%	%
41	N41	52	Incl	10	5	5	10	0	LN521	B2	0,8	0	0,2
60	N60	53	Incl	10	5	5	10	0	LN521	B2	0,8	0	0,2
43	N43	67	Incl	10	50	5	0	20	LN521	B2	0	0,8	0,2
62	N62	50	Incl	10	50	5	0	20	LN521	B2	0	0,8	0,2
45	N45	76	Incl	10	50	5	10	20	LN521	B2	0	0,95	0,05
64	N64	72	Incl	10	50	5	10	20	LN521	B2	0	0,95	0,05
40	N40	47	Incl	10	50	50	0	0	LN521	B2	0	0,8	0,2
59	N59	59	Incl	10	50	50	0	0	LN521	B2	0	0,8	0,2
46	N46	49	Incl	100	5	50	10	20	LN521	B2	0	0,8	0,2
65	N65	75	Incl	100	5	50	10	20	LN521	B2	0	0,8	0,2
39	N39	46	Incl	100	50	5	0	0	LN521	B2	0	0,95	0,05
58	N58	68	Incl	100	50	5	0	0	LN521	B2	0	0,95	0,05
44	N44	70	Incl	100	50	50	0	20	LN521	B2	0,95	0	0,05
63	N63	66	Incl	100	50	50	0	20	LN521	B2	0,95	0	0,05
42	N42	54	Incl	100	50	50	10	0	LN521	B2	0,95	0	0,05
61	N61	61	Incl	100	50	50	10	0	LN521	B2	0,95	0	0,05
47	N47	51	Incl	10	5	5	0	0	LN521/211	B2	0	0,8	0,2
66	N66	55	Incl	10	5	5	0	0	LN521/211	B2	0	0,8	0,2
50	N50	45	Incl	10	5	50	10	20	LN521/211	B2	0	0,95	0,05
69	N69	41	Incl	10	5	50	10	20	LN521/211	B2	0	0,95	0,05
49	N49	73	Incl	100	5	5	10	20	LN521/211	B2	0,8	0	0,2
68	N68	44	Incl	100	5	5	10	20	LN521/211	B2	0,8	0	0,2
48	N48	57	Incl	100	5	50	0	0	LN521/211	B2	0,95	0	0,05
67	N67	43	Incl	100	5	50	0	0	LN521/211	B2	0,95	0	0,05
51	N51	48	Incl	10	5	5	10	0	LN521/221	B2	0,95	0	0,05
70	N70	58	Incl	10	5	5	10	0	LN521/221	B2	0,95	0	0,05
54	N54	63	Incl	10	5	50	0	20	LN521/221	B2	0,8	0	0,2
73	N73	64	Incl	10	5	50	0	20	LN521/221	B2	0,8	0	0,2
55	N52	71	Incl	100	5	50	10	0	LN521/221	B2	0	0,8	0,2
56	N71	39	Incl	100	5	50	10	0	LN521/221	B2	0	0,8	0,2
57	N55	74	Incl	55	27,5	27,5	5	10	LN521/221	B2	0,4375	0,4375	0,125
74	N56	40	Incl	55	27,5	27,5	5	10	LN521/221	B2	0,4375	0,4375	0,125
75	N57	60	Incl	55	27,5	27,5	5	10	LN521/221	B2	0,4375	0,4375	0,125
76	N74	42	Incl	55	27,5	27,5	5	10	LN521/221	B2	0,4375	0,4375	0,125
53	N75	62	Incl	55	27,5	27,5	5	10	LN521/221	B2	0,4375	0,4375	0,125
72	N76	69	Incl	55	27,5	27,5	5	10	LN521/221	B2	0,4375	0,4375	0,125
52	N53	56	Incl	100	5	5	0	20	LN521/221	B2	0	0,95	0,05
71	N72	65	Incl	100	5	5	0	20	LN521/221	B2	0	0,95	0,05

Figure B.5: FED model Block 2 for culturing iCell CPCs

Table B.1: Hoechst staining and cTNNI3 labeling responses

Medium	Hoecht stained nuclei	cTNNI3+ cells	% cTNNI3+ cells
N20	2121	695	32.8
N25	2202	467	21.2
N23	2284	372	16.3
N27	1617	749	46.3
N24	1797	872	48.5
N26	3083	1213	39.3
N21	2400	865	36.0
N22	1784	641	35.9
N28	2077	815	39.2
N31	1913	226	11.8
N30	2223	474	21.3
N32	2071	356	17.2
N35	2905	382	13.1
N34	2712	97	3.6
N33	2354	668	28.4
N18	2712	283	10.4
N36	1904	1166	61.2
N38	1884	278	14.8
N60	2655	293	11.0
N62	2238	313	14.0
N64	1918	242	12.6
N59	1562	459	29.4
N65	2224	374	16.8
N58	2476	1231	49.7
N63	3115	2074	66.6
N61	2591	2086	80.5
N66	2480	590	23.8
N69	2476	235	9.5
N68	2780	411	14.8
N67	2116	1285	60.7
N70	339	4	1.3
N73	2370	152	6.4
N71	2543	331	13.0
N56	2128	683	32.1
N74	2246	514	22.9
N76	2264	513	22.8
N72	2309	580	25.1