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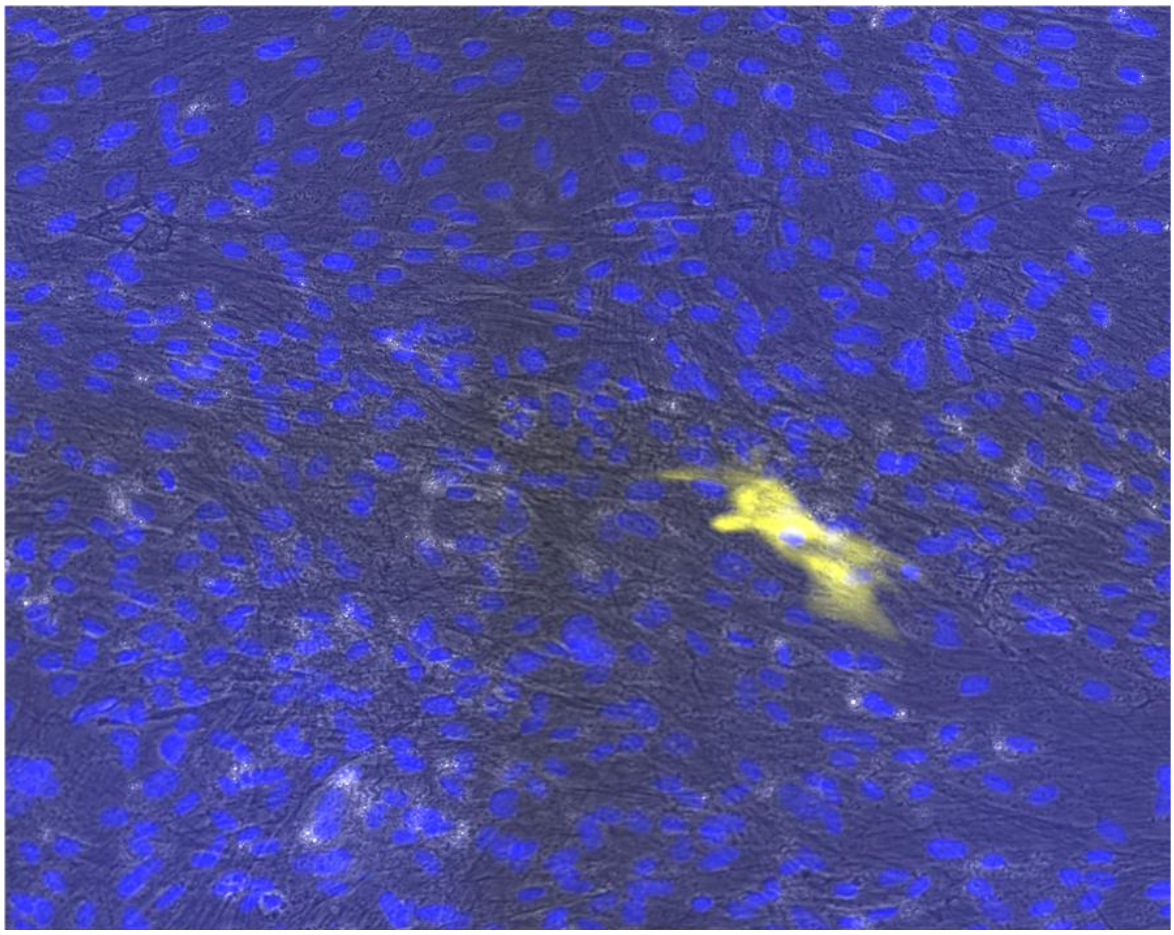

Optimization of Growth Conditions for Expansion of Cardiac Stem Cells Resident in the Adult Human Heart

Master's thesis in Biotechnology

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Master's Thesis 2016
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Cover: Heart biopsy cells cultured 7 days in expansion medium followed by 9 days in differentiation medium with nuclei stained in blue and cardiac troponin T in yellow.

Gothenburg, Sweden 2016

Abstract

Cardiovascular diseases such as myocardial infarction and heart failure are major causes of death all around the world. Today there is no way to restore correct function of the heart and the regenerative ability of the heart is limited. However, a number of cardiac stem cell populations have been identified in the adult human heart and can hopefully be utilized for regenerative strategies in the future. One of these populations has been identified based on C-kit+CD45- expression, but this population is committed toward endothelial fate when expanded *in vitro*. The main goal of the project was to evaluate if different culture conditions can make the cells committed to a cardiac fate instead. The SmartFlare™ technology was evaluated on induced pluripotent stem cells (iPSCs) as a possible method for live cell isolation of stem cell populations in the heart. A method like this is necessary to be able to expand the cells further after isolation, since cardiomyocytes markers are intracellular and sorting with methods such as Fluorescent-Activated Cell Sorting (FACS) requires cell lysis. SmartFlares™ enable live cell detection of gene expression since the probes enter the cells via receptor-mediated endocytosis, interact with the mRNA of interest and thereafter send out detectable fluorescent signals that can be used to isolate cells with FACS technology. An expansion phase of heart biopsy cells in NutriStem followed by the differentiation media DMEM:F12, 10% human serum and dexamethasone (10nM) resulted in most cells committed to cardiac lineage. The method used for evaluation of the different conditions was immunocytochemistry. The SmartFlare™ technology was shown to work well in iPSCs and could be used to detect cardiac troponin I expression in iPSCs differentiated to cardiomyocytes. SmartFlares were also used for sorting out a small population of cardiomyocytes, but analysis using real time quantitative polymerase chain reaction showed that the sorting step needs to be further optimized.

Acknowledgements

I would like to thank my supervisors Cecilia Boreström and Joakim Sandstedt for all your guidance, support and patience during this master's thesis. I would also like to thank Gabriella Brolén, Ryan Hicks and all other members of the IPSC team for input and scientific discussions that have been of great value. Enormous thanks also to my examiner Julie Gold for guidance regarding the report, Elin Pernevik for help concerning cardiac differentiation protocols, Henrik Andersson for assistance with the Image Express and image analysis, Mikael Sandstedt for advice about heart biopsies and Marianne Jonsson for all help with RT-qPCR. Thanks also to Linda Thimour-Bergstrom and the surgeons at the Department of Cardiothoracic Surgery, Sahlgrenska University Hospital, for skilful handling of cardiac biopsies. My greatest thanks to all of you for making this thesis possible!

Abbreviations

BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Surgery
CD31	Platelet endothelial cell adhesion molecule-1 or PECAM-1
C-kit	Tyrosine-protein kinase receptor kit
CSC	Cardiac Stem Cell
cTNT	Cardiac Troponin T, also known as TNNT2 and TnT
cTNI	Cardiac Troponin I, also known as TNNI3 and TnI
EDTA	Ethylenediaminetetreaacetic acid
EGF	Epidermal Growth Factor
FACS	Fluorescent Activated Cell Sorting
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
HS	Human Serum
HSC	Hematopoietic Stem Cells
HUVEC	Human Umbilical Vein Endothelial Cell
ICC	Immunocytochemistry
iPSC	induced Pluripotent Stem Cell
LN	Laminin
PEST	Penicillin-Streptomycin
PK	Protein Kinase
RI	Type I receptor
RII	Type II receptor
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
SF	SmartFlare™
SMC	Smooth Muscle Cells
vWF	von Willebrand Factor

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1. Introduction

Cardiovascular diseases such as chronic ischemic heart disease, myocardial infarction (MI) and heart failure are major causes of death all around the world. These three diseases are closely connected and often dependent of each other. Approximately 1 billion cardiomyocytes die following MI and are replaced by collagen-rich fibrotic tissue that severely disturbs the function of the heart, leading to heart failure. Today there is no way to restore correct function of the heart; instead symptoms are treated and preventive actions are taken to reduce the risk of cardiovascular disease (Schade and Plowright, 2015). The seemingly lack of regeneration of heart muscle tissue following MI has contributed to the opinion that the heart loses its ability to form new cardiomyocytes shortly after birth (Sandstedt, 2014). However, this point of view has in recent years been questioned based on measurements such as ^{14}C incorporation in cardiomyocytes (Bergmann *et al*, 2009). Regeneration from an existing population of multipotent stem cells present in the heart has been proposed and several studies indicate that such cells exist (Beltrami *et al*, 2003, Messina *et al*, 2004, Pfister *et al*, 2005, Goumans *et al*, 2008, Ott *et al*, 2007). Different strategies have been utilized for isolation of cardiac stem cells, including cultivation methods considered as selective for stem cells (Messina *et al*, 2004), functional assays such as the ability to efflux Hoechst 33342 (Pfister *et al*, 2005), and selection based on surface antigen profile. Surface antigens used for this purpose include C-kit (Beltrami *et al*, 2003), Sca-1 (Goumans *et al,* 2008) and Stage Specific Embryonic Antigens (SSEAs) (Ott *et al*, 2007). However, consensus has not been reached so far and doubts have been raised about physiological relevance of these minor cell populations (van Berlo and Molkentin, 2014). However, the existence of cardiac stem cell populations raises expectations that those cells might be utilized for regeneration of lost cardiomyocytes in the diseased human heart in the future.

Several of the above mentioned cell populations have been identified in the atrial tissue in the human heart (Sandstedt, 2014). In one study, a population of C-kit+CD45- cells were isolated and were shown to be predestined for endothelial development after expansion *in vitro* (Sandstedt *et al*, 2010). However, gene expression analysis of directly isolated single cells indicated that the C-kit+CD45- population harbors a small subpopulation of cells directed towards cardiac lineage (Sandstedt *et al*, 2014). Sandstedt concludes in his work that further studies is required to investigate if culture conditions can be optimized to favor expansion of the cardiac committed C-kit+ subpopulation. He proposes that factors such as coating of culture plastics, media composition and oxygen level can be of importance for expansion of the C-kit+ subpopulation (Sandstedt, 2014).

At AstraZeneca, protocols have been developed for differentiation of induced pluripotent stem cells (iPSCs) into a multipotent stem cell population with ability to develop into cardiomyocytes and other populations within the heart. These cell populations have been studied for their potential use regarding identification of pharmaceutical compounds that could potentially contribute to regeneration in the human heart.

1.1 Aim and research questions

The main goal for the project is to optimize culture conditions to favor expansion of the cardiac committed C-kit+CD45- subpopulation. A number of research questions have been formulated to clarify which steps that needs to be taken to reach the goal. First of all, an analysis method to quantify the number of cardiac committed cells is needed. A cardiomyocyte depleted population will be used for expansion of the C-kitCD45- subpopulation since previous experiences has shown that it is hard to expand the subpopulation from directly isolated cells. It is on this heterogeneous population that different culture conditions will be tested in order to promote cardiac development from the small number of cardiac progenitor cells present in the tissue. The amount of cardiac stem cells is very small and it is therefore crucial to use a method that can identify small numbers of positive cells. The method of choice is immunocytochemistry using antibodies targeting cardiac cells, and the following question needs to be evaluated:

- Can small number of positive cells be detected using small amounts of antibodies?

The motivation for this question is that a large cultivation format such as 6-well plates needs to be used to have a chance of localizing as much of these rare cells as possible. Antibodies are expensive and it is therefore not reasonable to use as much antibodies when it is known that few cells are positive. After finding out if the method is suitable for this purpose, the optimization step can be started which means cultivation of heart tissue in different setups.

- Is it possible to increase the number of cells committed to the cardiac lineage by using optimizing culture conditions such as media composition, coating, oxygen level and small molecule additives?

The heart tissue is grown as a heterogeneous mixture and it is therefore necessary to have an isolation method that can be used for sorting out live cells for further cultivation and observation. One such method is usage of RNA-probes named SmartFlares™ which is said to be non-toxic and able to stain live cells (Merck Millipore, 2015). A method like this is necessary for live-cell sorting of cells committed to cardiac lineage since markers for cardiomyocytes are intracellular and sorting with FACS therefore require cell lysis. This method is however relatively new and needs to be evaluated. The first step is to control if the cells of interest are able to take up the probes. The next step is to sort small numbers of positive cells to mimic the conditions that will be used for adult heart cells. The SmartFlare™ method will be evaluated on iPSCs and iPSCs differentiated to cardiomyocytes since these cells are easily accessible at AstraZeneca in large amount. A differentiation protocol is also available that will be used for the differentiation of iPSCs into cardiomyocytes.

- Can SmartFlares™ be used on iPSCs?
- Can SmartFlares™ be used on cardiomyocytes?
- Can SmartFlares™ be used for isolation of small numbers of cardiac committed cells?

1.2 Limitations

All cells will be cultivated in monolayers since evaluation of 3D-culture systems is not a part of this work. Regarding the cells from human hearts, the heart tissue will be obtained from three different types of surgeries performed at Sahlgrenska University Hospital, which limits the amount of available material and makes it difficult to know exactly when the biopsies will be available. These two factors influence the number and size of experiments that can be performed due to lack of suitable patients. The hearts that the biopsies are obtained from are not healthy, but the cells of interest should be vital enough for *in vitro* cultivation and analysis. It would be of interest to use healthy cardiac tissue as well for comparison, but that is not possible from an ethical as well as a medical point of view. iPSC-derived cardiomyocytes will be functionally assessed by visualization of contraction and no further functional assessment will be performed. Other methods could be used, such as measurement of calcium oscillation in response to different activating or toxic compounds, but that is beyond the scope of this master thesis. Drug screening will not be covered in this work. All work that includes cells from heart biopsies will be performed at Sahlgrenska University Hospital.

2. Background

Relevant theory is presented in this section and included are two main sections describing the heart and stem cells. The section about the heart includes theory about structure and function, cardiac cell types, cardiogenesis and signaling pathways in cardiomyocyte formation. The stem cell section provides theory about embryogenesis, induced pluripotent stem cells (iPSCs) and cardiac stem cells (CSCs). These theory sections are included to provide a deeper understanding about the field that the thesis is carried out within.

2.1 The heart

The following sections describe structure and function of the heart and the different cell types that are present within. These facts are included to get increased knowledge about the heart and the different cells that are responsible for the function of the heart, which is important since the long-term goal of cardiac stem cell research is to regain the normal function of the heart. Development of the heart, also called cardiogenesis, is included as well as an introduction to the complex signaling networks that are involved in the formation of cardiomyocytes.

2.1.1 Structure and function of the heart

The heart functions as a pump that circulates blood throughout the body. Poorly oxygenated blood is delivered from the body to the right side of the heart, which is pumped to the lungs for oxygenation. The oxygenated blood is delivered to the left side of the heart and pumped out to the whole body (Moore and Dalley, 2013). The heart is constituted of four chambers, the two upper chambers are named right and left atria and the two lower chambers are called right and left ventricles. The atria receive blood and pump it into the corresponding ventricle, which in turn pumps blood into the lungs (right ventricle) or out to the body (left ventricle). The heart has four valves that prevent backflow of blood and these are controlled by pressure differences and they are situated between the atria and the ventricles and at the outflow tract of the ventricles. The heart itself is supplied with oxygen and nutrients by the coronary arteries that originate as branches from the ascending aorta, which carries blood out to all parts of the body (Tortora and Derrickson, 2013).

The four cardiac chambers contract in a coordinate manner, which is a prerequisite for making the heart an efficient blood pump. The conduction system is controlled by so-called pacemaker cells that make up approximately 1% of cardiac muscle fibers, which have the ability to generate action potentials in a rhythmic pattern. Nerves regulate the beating rate, but stimulation of the heart beating rhythm is performed by pacemaker cells. The cells stimulate heartbeat even after the nerves are cut, as for example during heart transplantations. Cardiac excitation normally begins in the sinoatrial (SA) node that is located in the right atrial wall, which is conducted to the two atria. The action potential reaches the atrioventricular (AV) node, were the action potential slows down to give the atria time to deliver blood into the ventricles. The action potential finally reaches the Purkinje fibers that makes the ventricles contract and pump the blood out of the heart. Contraction is initiated by the SA node approximately 100 times per minute. Different hormones and neurotransmitters can speed up or slow down the rate by SA node fibers. Acetylcholine released by the parasympatic portion of the autonomic nervous system slows down the action potential initiation of the SA node to get a resting pulse of approximately 75 beats/minute (Tortora and Derrickson, 2013).

The pericardium is a protective membrane that surrounds the heart and holds it in place. The outer part, called the fibrous pericardium, is a connective tissue layer that is tough, inelastic, dense and irregular. The fibrous layer protects the heart and prevents it from overstretching as well as anchors it in the right position. The inner part of the membrane, named serous pericardium, is thinner, delicate and forms a double layer around the heart. The outer serous pericardium (called parietal layer) is fused to the fibrous pericardium, and the inner layer of the serous pericardium (called visceral layer) is tightly bound to the surface of the heart. Between the two membranes of the serous pericardium is a thin film of pericardial fluid that reduces friction between the membranes arising from heart movement (Tortora and Derrickson, 2013). The heart wall is constituted by three layers; the epicardium (outer layer), the myocardium (middle layer) and the endocardium (inner layer). The epicardium is the same as the visceral layer of the serous pericardium, and is composed of a thin layer mesothelium and connective tissue. The myocardium is the bulk of the heart and consists of cardiac muscle tissue that is responsible for the pumping motion of the heart. Cardiac muscle cells, also called cardiomyocytes, make up the myocardium. They are involuntary, striated, branched and arranged in interconnected fiber structures. The endocardium consists of a thin layer of epithelium that lines the inside of the heart cavity (Tortora and Derrickson, 2013).

2.1.2 Cell types in the heart

The heart is built of several cell types including cardiomyocytes, endothelial cells, smooth muscle cells, pericytes and fibroblasts. These cell types are described in this section along with common marker that can be used for identification of each of the cell types.

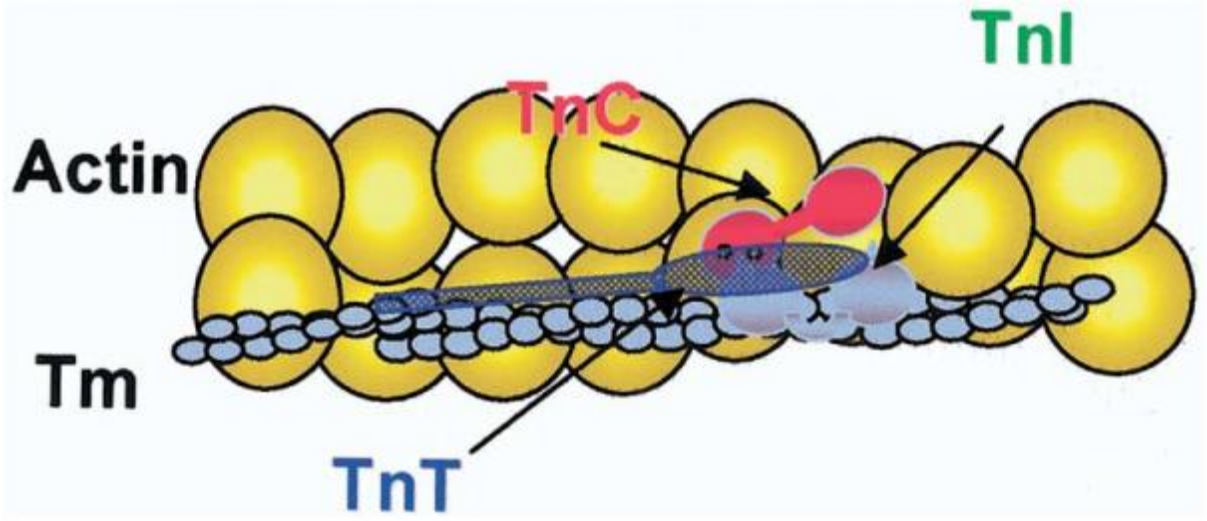

Figure 1. The cardiac troponin complex in cardiomyocytes. The troponin complex plays a critical regulatory role in the contracting motion of the heart. Tm, or tropomysin, is a double stranded helical protein located close to the grove of the actin filament. Troponin C (TnC) is the unit that Ca^{2+}-binds to. Troponin I (TnI) is the inhibitory part of the complex and Troponin T (TnT) is the subunit that connects the three troponin subunits to TM and to the actin double helix (Parmacek and Solaro, 2004).

Cardiomyocytes

Cardiac muscle cells, also called cardiomyocytes, make up the bulk of the heart. Cardiomyocytes are located in the myocardium and are a specialized cell type that cannot be found anywhere else in the body. Cardiomyocytes are the working unit of the heart, responsible for the contractive motion that transports blood throughout the body. The cells are branched, involuntary controlled, striated and the tissue is formed by interconnected fiber bundles. The

contracting unit is called myofilaments, which in turn are packed together to striated myofibrils. These fibers are connected by intercalating disks with gap junctions that enable conduction of action potential between cardiomyocytes. There are two separate networks of cardiomyocytes, one atrial and one ventricular. Each of the networks contract as one functional unit and they are independent of each other (Tortora and Derrickson, 2013, Severs, 2000). Markers of the contractile apparatus, such as cardiac troponin T (cTNT) and cardiac troponin I (cTNI), can be used for identification of mature cardiomyocytes (Mueller *et al*, 2013) (Figure 1). Transcription factors for the cardiac lineage, such as Nkx2.5 and GATA4, can be used for detection of early differentiation toward cardiomyocytes (Olson, 2006).

Smooth muscle cells

Cardiac smooth muscle cells (SMCs) are necessary for efficient performance of the vasculature. They contract and relax to regulate blood flow by alternating vessel diameter, ensuring appropriate blood pressure in the blood vessels. SMCs are also involved in regulation of structural remodeling by change of cell number and connective tissue composition after events such as vascular injury. SMCs can be identified by for example the marker alpha-smooth muscle actin (α-SMA) that is a protein in the contractile apparatus (Rensen, Doevendans, and van Eys, 2007).

Endothelial cells

The endocardium is composed of endothelial cells that form the inner lining of blood vessels and cardiac valves (Xin, Olson and Bassel-Duby, 2013). Their functions include regulation of blood coagulation, recruitment of inflammatory cells, regulation of smooth muscle cell contraction and vascular permeability. The cardiac endothelium also releases factors such as prostaglandin, nitric oxide and endothelins that affect the contractile force of cardiomyocytes (Celermajer, 1997). Endothelial cells can be identified with markers such as CD31 (Baldwin *et al*, 1994).

Pericytes

Pericytes are the second most abundant cell type in the heart (Nees et al, 2012) and are located outside endothelial blood vessels that are found throughout the myocardial wall. Cardiac pericytes displays a star-shaped morphology and are directly connected with gap junctions to nearby endothelial cells as well as to other pericytes. The function of pericytes includes regulation of blood vessel formation, coagulation and blood flow (Nees, Weiss and Juchem, 2013). To date, no specific markers for pericytes have been identified, but a combination of markers can be utilized to distinguish pericytes from other cell types (Armulik, Genové and Betsholtz, 2011).

Fibroblasts

Fibroblasts are in general classified based on morphology and/or proliferation capacity. These characteristics can vary depending on tissue and organism studied. But some guidelines are available, such as the general lack of basement membrane, the tendency to have sheet-like extensions, the oval nucleus with 1 or 2 nucleoli, an extensive rough endoplasmic reticulum and a rich amount of granular material in the cytoplasm (Camelliti, Borg and Kohl, 2005). Fibroblasts constitute more than 50% of the total cell number in the human heart (Xin, Olson and Bassel-Duby, 2013) and can be found throughout the cardiac tissue. They contribute to cardiac development, cell signaling and homeostasis of the extracellular matrix, and also to structure and electro-mechanical function of the myocardium (Camelliti, Borg and Kohl, 2005). Fibroblasts

are important following myocardial infarction in an event called fibrogenesis, during which fibroblasts infiltrate and proliferate in the infracted area. Fibrogenesis is initiated due to stretching of the myocardial wall and low oxygen levels (hypoxia), events that are typical for myocardial infarction and increases the levels of fibrogenic mediators (Kusachi and Ninomiya, 2003). Fibroblasts are often identified by Discoidin Domain Receptor 2 (DDR2), a marker for a collagen specific receptor tyrosine kinase that has not been found in other cell types in the heart (Goldsmith *et al*, 2004).

2.1.3 Cardiogenesis

Gastrulation occurs during third week of human embryogenesis, in which the three primary germ layers are formed. These layers are called ectoderm, mesoderm and endoderm, and they form the major embryonic tissues from which all tissues and organs develop (Tortora and Derrickson, 2013). Myocardial cells originate from the mesoderm germ layer. Most of the knowledge about mammalian cardiogenesis is based on model organisms such as the mouse. The mouse embryonic heart is formed from two progenitor populations that originate from one common progenitor in the mesoderm. The first population migrates to the heart forming region, also named primary heart field, to form the cardiac crescent. The primary heart field is later involved in formation of the left ventricle and the atria. The second population forms the secondary heart field which is located around the cardiac crescent and contributes to the formation of the right ventricle, the outflow tract and the atria. The cardiac crescent thereafter forms a beating heart tube comprised of cardiomyocytes and endothelial cells. The heart tube then grows unevenly and remodels to form the primitive chambers of the heart. The next step of cardiogenesis is called looping, which brings outflow and inflow tracts to the correct positions as well as places ventricles and atria in the correct position for continued development into a functional heart (Xin *et al*, 2013).

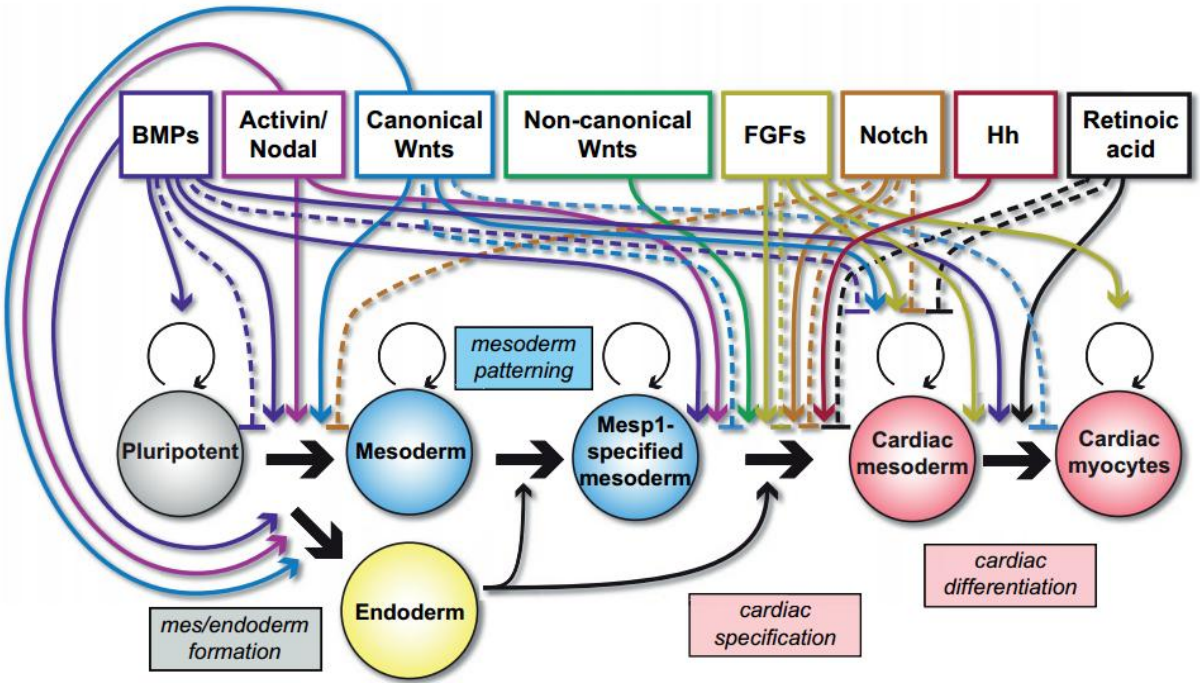

Figure 2. Signaling pathways involved in cardiomyocyte formation. Solid lines represent positive signals, dashed lines negative signals and circular arrows indicate self-renewal (Noseda *et al*, 2011).

Vertebrate cardiogenesis include a complex network of intrinsic and extrinsic signaling as well as crosstalk between the primary- and secondary heart field (Xin *et al*, 2013). Genes encoding transcriptional activators expressed in both heart fields can be activated by inductive signals such as bone morphogenetic protein (BMP), Notch, WNT and sonic hedgehog (Hh) (Figure 2 and Figure 3). The activated transcription factors are important for regulation of genes that are involved in events such as development, growth and patterning of the heart. Two central transcription factors in the heart fields are GATA4 and Nkx2.5. Tbx5 is a transcription factor only expressed in the primary heart field while Isl1 are a marker for cardiac progenitor cells in the secondary heart field (Bruneau, 2013).

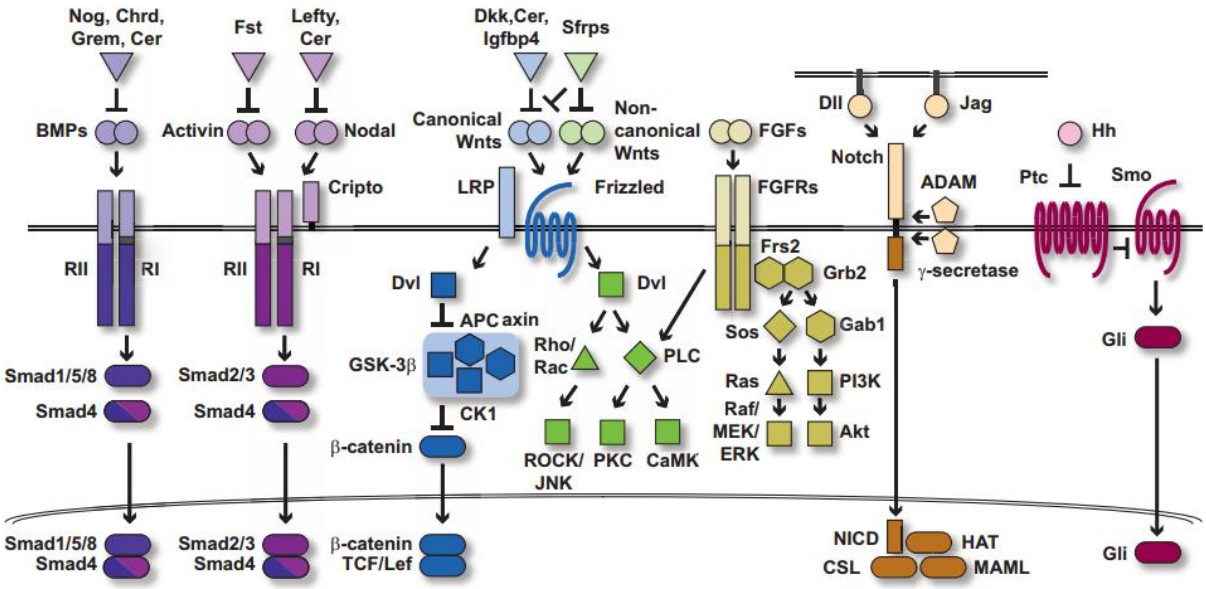

Figure 3. Summary of major signaling pathways involved in the formation of cardiomyocytes. From left to right are BMP, Activin/Nodal, Wnt, FGF, Notch and HH pathways displayed. Circle represent ligand, inverted triangle mean ligand antagonist, rectangle is receptor-ligand binding, square is protein kinase, triangle is G-protein, hexagon represent scaffold protein, pentagon indicate protease and oval is transcription factor (Noseda *et al*, 2011). See text for details of each pathway.

BMPs

The signal receptors in the BMP (bone morphogenetic protein) pathway consist of two membrane proteins that have intracellular serine-threonine kinase domains (Figure 3). The type II receptor (RII) phosphorylate the type I receptor (RI) upon BMP ligand binding, which activated the RI kinase domain. Activated RI then phosphorylates Smad transcription factors, which in turn forms a complex with Smad4 and translocates to the nucleus (Noseda *et al*, 2011).

Activin/Nodal

Activin ligands signals through the membrane bound receptor Activin type II receptor (ActRII) or ActRIIB in combination with the type I receptor ActRIA/Alk2 or Alk4. Nodal ligands also signal through ActRII/ActRIIB together with ActRIB/Alk4 or Alk7, but often require a co-receptor from the EGF-CFC family. The EGF-CFC receptor is named Cripto in mammalians (Figure 3). Ligand signaling of Nodal/Activin to the RII and RI receptors lead to a signaling cascade via Smad2/3 which forms a complex together with Smad4. The Smad complex promotes DNA binding together with FoxH1 (forkhead winged helix protein). FoxH1 is the main mediator of the effects for the Activin/nodal pathway (Noseda *et al*, 2011).

Wnts

Secreted Wnt proteins operate over short distances and Wnt signaling occurs typically between neighboring cells that are in contact to each other. Every Wnt protein has a covalent lipid modification, a palmite added by the enzyme Porcupine in the endoplasmatic reticulum that gives the protein a hydrophobic character (Clevers, Loh and Nusse, 2014). Wntless is a transmembrane protein that only binds Wnt proteins with added palmitate and thereafter helps transporting it to the plasma membrane for secretion and direct binding to the cell membrane (Najdi *et al*, 2012). After secretion, it is not fully understood how Wnt signals are delivered to the correct target cell (Clevers, Loh and Nusse, 2014). It is believed that some of the Wnt proteins may be enfolded by secretory vesicles (Gross *et al*, 2012) in which Wnt maintain the membrane bound connection via a Wls protein (Korkut et al, 2009).

Wnt ligands act by binding to the transmembrane receptor Frizzled and the core receptors Lrp5/6 (lipoprotein receptor-related protein) (Figure 3). Canonical Wnt ligands destroy the so called destruction complex that is present in the cytoplasm when Wnt ligands are absent. The destruction complex consists of Axin, adenomatous polyposis coli (APC) tumor suppressor protein, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK-3β) and target β-catenin degradation. β-catenin is destroyed via proteosomal degradation through phosphorylation by GSK-3β and CK1. Binding of canonical Wnt ligands recruits the Disheveled (Dvl) protein kinase and the destruction complex to the Frizzled receptor. This leads to stabilization of β-catenin since phosphorylation is reduced and β-catenin is therefore accumulated in the cytoplasm. β-catenin is thereafter relocated to the nucleus were it co-activates Tcf (T cell specific transcription factor) and Lef (lymphoid enhance binding factor 1) proteins. This event displaces repressors and recruits other co-activators (Noseda *et al,* 2011).

Noncanonical, or β-catenin independent, Wnt ligands activates two signaling pathways (Figure 3). The planar cell polarity pathway pathway is one of the cascades and includes the two G-proteins Rho and Rach that activates ROCK (Rho associated protein kinase) and JNK (Jun N-terminal kinase). The other cascade depends on PLC (Phospholipase C) which releases Ca^{2+} and activated PK (protein kinase) C and CaMKs (calmodulin dependent protein kinases) (Noseda *et al*, 2011).

FGFs

The Fibroblast growth factor (FGF) pathway is highly conserved and consists in mammals of circa 20 ligands and 4 transmembrane receptor tyrosine kinases (FGFRs). The outcome of FGF ligand binding to FGFRs is receptor dimerization and autophosphorylation, creating a binding place for adaptor proteins and downstream mediators (Figure 3). Three relevant pathways for cardiomyocyte formation are illustrated in Figure 3. In the first signaling cascade is adaptor substrate Frs2 recruiting Grb2 and Sos which activates Ras/Raf/MEK/ERK pathway. The second cascade involves recruitment of Gab (Grb2-associated binding protein) 1 which activates the PI3 (phosphatidylinositol-3-kinase)/Akt pathway. The third pathway is activated when phospholipase C (PLC) binds and catalyzes hydrolysis of phosphodylinositol 4,5-bisphosphate to inositol triphosphate and diacylglycerol that lead to Ca^{2+} release and activation of PKC (Noseda *et al*, 2011).

Notch

Notch receptor proteins span through the membrane and are receptors of type I. The pathway is initiated when a neighboring cell expressing a notch ligand sends signals that the cell with the Notch receptor retrieves (Pedrazzini, 2007). Ligand binding initiates cleavage of the Notch protein receptor by a disintegrin and metalloproteinase (ADAM) and by the γ-secretase complex (Figure 3). This event releases the intracellular part of the Notch receptor protein (NICD), which is then translocated to the nucleus. In the nucleus, NICD bind and activate CSL transcriptional regulators. This event removes co-repressors and instead recruits co-activators such as MAML (Mastermind-like). The formed complex activates transcription of notch target genes such as genes important for chamber specification in the heart. The mechanism of cell regulation used by notch is called lateral inhibition (a cell that adopts a certain fate inhibit equivalent cells from adopting the same fate) and also by asymmetric cell division (Noseda *et al*, 2011).

Hh

Patched (Ptc) 12-span transporter-like receptors inhibit signals from Smoothened (Smo) serpentine receptors (Figure 3). Hedgehog (HH) ligands bind to Ptc receptor and disinhibits the Smo receptor which causes accumulation of Smo in the primary cilium organelle. Gli transcriptor factors start gene regulation by this event which includes both activators and repressors (Noseda *et al* 2011).

Retinoic acid

The biologically active vitamin A derivate retinoic acid (RA) is produced by retinaldehyde dehydrogenase (Raldh). It is produced as a rate limiting step and limit gene expression directly via RA receptors and heterodimerization partners called retinoid X receptors (Noseda *et al*, 2011).

2.2 Stem cells

Stem cells are defined by their self-renewal capacity and by their ability to form other cell types in the body (differentiation). These properties make stem cells of great interest in research since it in theory provides an unlimited cell source that can differentiate into any cell type of interest. Stem cells can be divided into embryonic and adult/somatic stem cells. Embryonic- and fetal development and growth are controlled by embryonic stem cells, while somatic stem cells are responsible for growth, tissue maintenance, regeneration and repair of adult tissues (Blitterswijk, 2007). The following sections describe embryogenesis and the involved stem cells, the concept of induced pluripotent stem cells as well as cardiac stem cells. Knowledge in the stem cell field is required since main objective of this work is to investigate the population of cardiac stem cells resident within the human heart. A theory section about cardiac stem cells is included to inform about progress that have been made in the field during the recent years.

2.2.1 Embryogenesis

All mammalian tissues originates from a fertilized oocyte that during the first divisions is unaccompanied by cellular growth. The resulting cells are called blastomeres and are totipotent, which means that they are able to form a complete individual. The third division, i.e. at 8 cells, is called the morula stage and the first differentiation starts to occur at this point when the morula is transformed into a blastocyst. The outer cell layer of the blastocyst is called trophectoderm and mediates formation of extra embryonic structures such as the placenta. The blastocyst

contains a fluid filled cavity with an inner cell mass consisting of pluripotent cells that will make up the entire embryo. These cells can be cultured *in vitro* and are called embryonic stem cells. Generation of genetically modified animals using embryonic stem cells is an important tool in mouse genetics. After 13-15 days in the human embryo, or at 6.5 days in mice, an event called gastrulation occurs during which the three germ layers are established. Cells committed towards a specific germ layer are called multipotent stem cells. The germ layers are called ectoderm, mesoderm and endoderm. The ectoderm forms structures such as the outer part of the skin, brain cells and nerve cells. Mesoderm gives rise to skeletal muscle, the heart and blood vessels, bone marrow, blood and connective tissue etcetera, while the endoderm forms the lining of the gastrointestinal and respiratory tracts, liver, pancreas, thyroid gland, thymus and the lining of the bladder. As maturation continues, the stem cells will become unipotent and thus only able to differentiate into one specific cell type. Cells lose their differentiation potential when they are fully matured and represent a highly specialized cell type that makes up a certain tissue or organ (Blitterswijk, 2007).

2.2.2 Induced pluripotent stem cells

Induced pluripotent stem cells or iPSCs were first generated in 2006 by Takahashi and Yamanaka and awarded the Nobel Prize in Physiology or Medicine in 2012. This groundbreaking work consisted of reprogramming adult skin fibroblasts to pluripotent stem cells using viral transduction with four transcription factors; Oct4, Sox2, Klf4 and c-Myc. These reprogrammed cells are very similar to embryonic stem cells and can differentiate into all three germ layers, proving that they are truly pluripotent (Takahashi and Yamanaka, 2006, Takahashi *et al*, 2007). Delivery methods of the reprogramming factors have been developed since the discovery of the iPSC technique. The method with the highest reprogramming efficiency is based on a modified-RNA technique. The use of mRNA eliminates risks of genomic integration as well as instertional mutagenesis (Warren *et al*, 2010). The discovery of iPSCs revolutionized the field of human stem cell biology since it removed the need to sacrifice embryos for collection of pluripotent stem cells. A simple skin biopsy or blood sample can result in patient-specific stem cell lines that can be used for research in order to identify therapeutic applications of stem cells (Mummery *et al,* 2014).

2.2.3 Cardiac stem cells

Cardiomyocytes were for a long time considered as terminally differentiated. This means that they have lost the ability to replicate and generate cells with identical properties and this conclusion was drawn from lack of DNA replication and failure to identify mitotic cells. The number of cardiomyocytes must be fixed throughout life and maintained until death for this to be possible and the heart should therefore consist of a homogenous population of cells with the same age. A theory for the main mechanism for this to be achievable includes turnover of structural proteins in cardiomyocytes. However, the main argument for lack of regenerative potential of the heart is the apparent lack of repair after events such as myocardial infarction. On the other hand, the outcome of infarctions are the same even in self-renewing organs such as bone marrow, skin and kidneys – migration of stem cells to the damaged area do not normally occur (Leri *et al*, 2015).

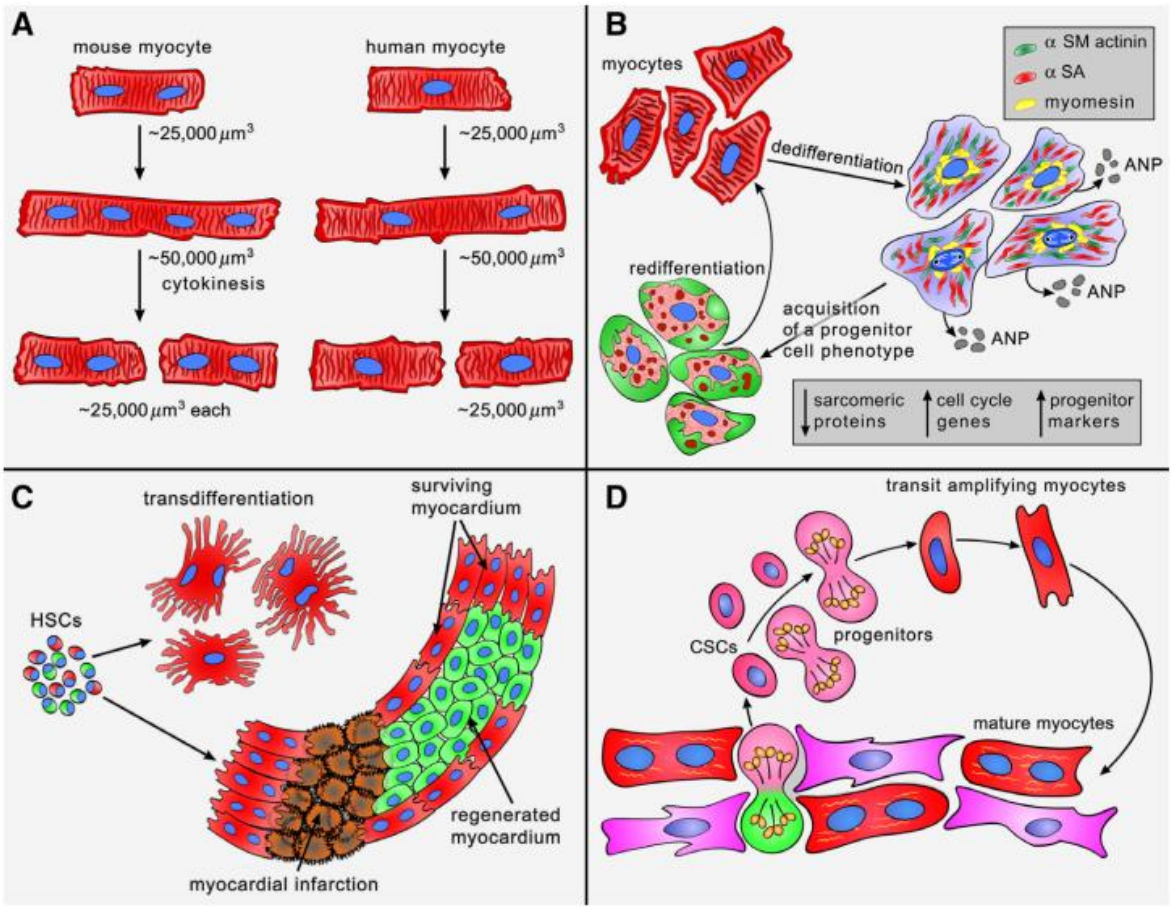

Figure 4. Proposed mechanisms for generation of cardiomyocytes in the adult heart (Leri *et al*, 2015). Five possible mechanism for cardiomyogenesis has been proposed, the fifth being a combination of (A)-(D). (A) Cardiomyocytes can re-enter the cell cycle. (B) Cardiomyocytes can dedifferentiate into primitive cells that in turn acquire a cardiac progenitor phenotype that can redifferentiate into cardiomyocytes. (C) Circulating hematopoietic stem cells (HSCs) can be committed towards a cardiac lineage and transdifferentiate into cardiomyocytes. (D) Cardiac stem cells (CSCs) exist in the cardiomyocyte population and are responsible for cellular turnover of cardiomyocytes.

The traditional point of view has in later years been questioned due to heterogeneity between cardiomyocytes as well as evidence of regeneration of the myocardium. The new perspective on cardiac regeneration raises questions about the origin of the newly formed cardiomyocytes in the adult heart. Five possibilities have been proposed for cardiomyocyte regeneration (Figure 4): i) cardiomyocytes are not terminally differentiated, but re-enter the cell cycle and divide into identical copies, ii) cardiomyocytes dedifferentiate into a more primitive cell phenotype, acquiring a progenitor cell phenotype to finally differentiate back into cardiomyocytes, iii) new cardiomyocytes form from circulating hematopoietic stem cells (HSCs) that undergo commitment towards the cardiac lineage, iv) the presence of a CSC population responsible for turnover of cardiomyocytes, v) a combination of the four described mechanisms contributing to the turnover of cardiomyocytes. The correct mechanism is yet to be established and such evidence is crucial for development of regenerative strategies such as cell therapy (Leri *et al*, 2015).

Several reports have suggested the fourth alternative, namely that a resident CSC population exists within the human heart. This cell pool has been identified and characterized based on the surface antigen C-kit in rats, mice, dogs and humans. The C-kit positive cells are multipotent and clonogenic *in vitro* and *in vivo* and transplantation assays have demonstrated their capability to

regenerate and repair damaged myocardial tissue.. Other classes of CSCs have been identified as well but C-kit CSCs has received most attention so far (Leri *et al*, 2015). However, CSCs have been questioned as well, especially regarding their physiological relevance since regeneration occurs at such low levels (van Berlo and Molkentin, 2014).

3. Methods and Materials

Methods used in this project include cell culturing methods and analytical methods. iPSCs have been differentiated to cardiomyocytes using a slightly modified published protocol and culture of heart tissue cells have been done based on knowledge from iPSC culturing and differentiation. Differentiated iPSCs were used to evaluate the SmartFlare™ technology and the result was analyzed using fluorescent activated cell sorting (FACS) technology and real-time quantitative polymerase chain reaction (RT-qPCR). Immunocytochemistry (ICC) was used to evaluate the different culture conditions used for heart tissue culture. Materials and reagents used are listed in appendix I.

3.1 *In vitro* cell culture

Many different cell types have been cultured in this project including iPSCs, Human Umbilical Vein Endothelial Cells (HUVECs), fibroblasts and heart biopsy cells. The following sections describe culture conditions and how the different cell types were used. L-glutamine (Sigma) where supplemented to DMEM:F12 (Life Technologies) in all cases where it was used as culture medium.

3.1.1 Dissociation of cardiac tissue

Biopsies from human hearts were used during this master thesis. The obtained tissue samples are removed on a routine basis and then discarded following surgery. It will therefore be no adverse effects for the patients participating in the study. The local ethical committee at University of Gothenburg has approved the study and an informed consent from each involved patient was obtained. All biopsies were obtained from the right atrium from patients undergoing three different types of surgeries. The first type is Coronary Artery Bypass Surgery (CABG), the second surgery being valvular replacement and the third Maze surgery, a procedure intended for treatment of atrial fibrillation (Cox *et al,* 1995). All biopsies were obtained from diseased hearts from both male and female patients 53-74 years old.

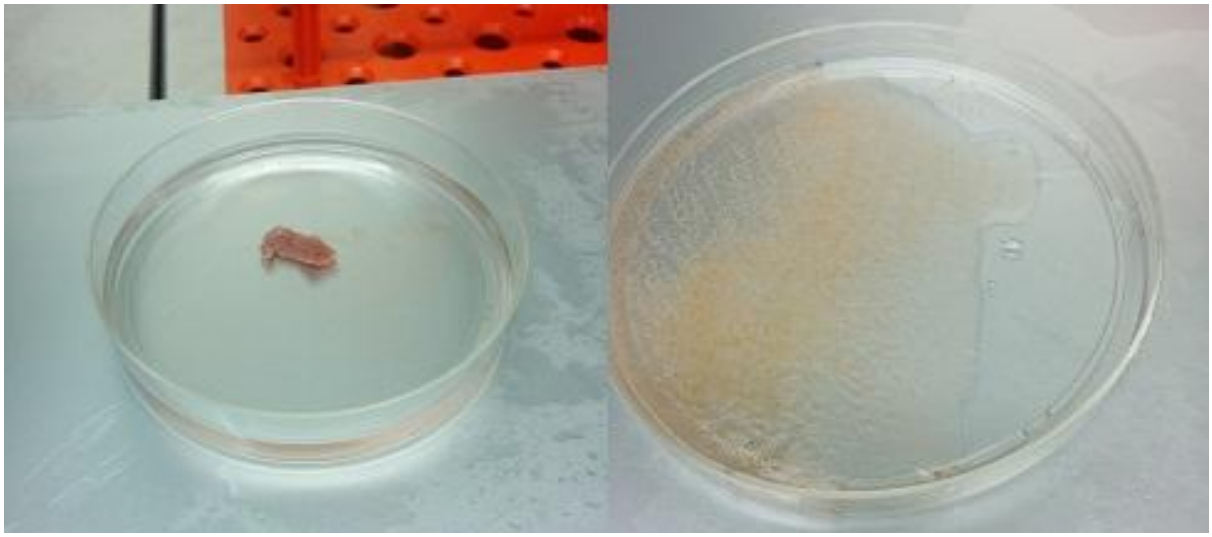

Figure 5. Mechanical dissociation of a heart biopsy. The biopsy is mechanically dissociated using a pair of scissors to enable efficient enzymatic dissociation into a single-cell solution.

The collected cardiac biopsy was put in cold PBS and kept on ice at all times to optimize viability. Connective tissue and epicardial adipose tissue was cut off after rapid transportation of the sample to cell culture lab and remaining blood was removed by washing with PBS. The biopsy was weighed and thereafter cut in minimal pieces to ensure efficient enzymatic dissociation (figure 5). The tissue fragments were centrifuged for 5min at 400g and the pellet was dissolved in warm DMEM:F12. Liberase type TH (26 Wünschunits/ml) and DNase (1mg/ml) was added and dissociation proceeded at 37°C for 4h while stirring. The cell suspension was thereafter centrifuged for 5min at 400g and the pellet dissolved in 0.05% trypsin-EDTA for 10min to obtain a single-cell solution. The dissociation was terminated by addition of DMEM:F12 supplemented with PEST and 10% Fetal Calf Serum (FCS). The cell solution was then filtered through a 100µm cell strainer to remove remaining aggregates and cardiomyocytes. The cells were centrifuged and dissolved in appropriate medium for seeding at a concentration of approximately 1mg/cm^2.

3.1.2 Culture and differentiation of iPSCs into cardiomyocytes

The iPSC line used in all experiments is named riPSC-1J. These cells are reprogrammed from foreskin fibroblasts using the Stemgent mRNA reprogramming kit which includes five mRNA reprogramming factors (c-Myc, Klf4, Lin28, Oct4 and Sox2). Using a non-integrative method such as mRNA reprogramming eliminates the risk of genomic integration and insertional mutagenesis while simultaneously enabling efficient reprogramming with high success rate (Sjogren *et al*, 2014). The NutriStem (Stemgent)/LN-521 (Biolamina) system was used for iPSC ongoing culture. The system is advantageously since it supports differentiation capacity, rapid cell expansion and maintenance of pluripotency (Stemgent, 2015). TrypLE Select was used for enzymatic passage when the cells have reached adequate confluency. This is performed every 3-4 day in order to keep the cells in an undifferentiated. iPSCs were differentiated into cardiomyocytes using a published protocol that have been slightly modified (Figure 6) (Lian *et al*, 2013, Pernevik, 2015). The starting point of the protocol is highly confluent iPSCs that are seeded into 96-wells coated with Matrigel (80µg/ml) at 312K/cm^2. The cells are seeded in NutriStem medium containing Rock inhibitor (also known as Y27632, 5µM).

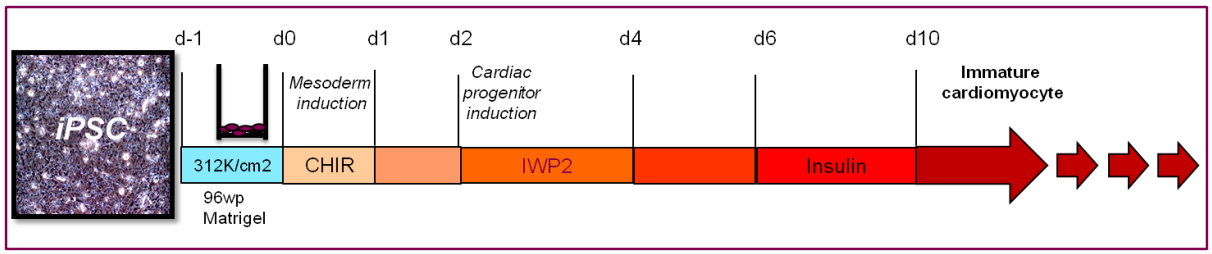

Figure 6. Differentiation protocol for iPSCs to cardiomyocytes. Original protocol from the Lian group (Lian *et al*, 2013) with small modifications for optimized use with the riPSC-1J line (Pernevik, 2015).

Differentiation was initiated d0 when the cells have reached 100% confluency by changing to RPMI 1640X GlutaMax media (differentiation media) supplemented with B27 without insulin and CHIR99021 (12µM). CHIR99021 is a GSK3β-inhibitor and induces activation of the Wnt pathway. At d1, media was changed to differentiation media supplemented with B27 without insulin. At d2, half of the media was changed to differentiation media supplemented with B27 without insulin and the Wnt-inhibitor IWP2 (10µM). At d4, media was changed to differentiation media supplemented with B27 without insulin. The media was changed to differentiation media containing B27 with insulin at d6. After d6, media was changed every third day for

cardiomyocyte maintenance. Insulin has been shown to inhibit cardiomyocyte yield during the first days of differentiation and are therefore only added from d6 (Lian et al, 2013).

3.1.3 Antibody evaluation and cell culture of HUVECs and fibroblasts

Only a small number of cultured cells from human hearts are expected to be committed to cardiac lineage and therefore positive for cardiac markers. To evaluate the amount of antibody necessary to detect these rare positive cells, fibroblasts were co-cultured with 3% cardiomyocytes (differentiated from iPSCs) in 6-well plates. Confluent fibroblasts were used to calculate the cell number per cm^2 obtained when culturing 100% fibroblasts, and that number was then used to get the number of cardiomyocytes needed to have a 3% co-culture. Cardiomyocytes and fibroblasts were co-cultured in cardiomyocyte maintenance medium for 2 days before fixation of cells. The analysis method used was immunocytochemistry, see section 3.2.1. Antibodies diluted in a 40µl volume according to Table 3 are sufficient to mark a 100% positive cell population in one 96-well. That amount of antibody should in theory be able to stain the same amount of positive cells in the larger 6-well plate. 40µl antibody solution (for dilutions, see Table 3) were therefore added to each 6-well along with 600µl of 4% FCS in PBS to cover the surface with fluid. The same approach was applied to test endothelial markers by co-culture of fibroblasts and 3% endothelial cells, in this case Human Umbilical Vein Endothelial Cells or HUVECs. HUVECs and fibroblasts were co-cultured in HUVEC-medium for 2 days before fixation of cells. C-kit antibodies were evaluated by the same method, but using cells from a biopsy cultured since it is known that heart tissue contains a few percentages of C-kit positive cells (Sandstedt *et al*, 2010). The heart biopsy was treated as previously described and thereafter seeded in DMEM:F12 supplemented with PEST and 10% human serum (HS). Percentage of C-kit positive cells were controlled using FACS-technology.

HUVECs were cultured in 10ml HUVEC-medium consisting of EBM-2, 5% FCS and EGM®-2 MV SingleQuots® supplements. The cells were grown in 75cm^2 flasks and passaged every fourth day to avoid over-confluency. Splitting of cells were done by washing cells with DMEM:F12 followed by addition of 3.7ml trypsin-EDTA to each flask. The flasks were incubated 4min in 37^0C. 3.7ml DMEM:F12 supplemented with 10% FCS were added to stop the reaction.. 1.5ml of the single cell suspension (1:5 split) together with 1.5ml DMEM:F12 were added to a 15ml tube and centrifuged for 5min at 400rpm. The supernatant was discarded and the cells resuspended in 10ml HUVEC-medium and transferred to a new 75cm^2 cell culture flask.

One frozen vial of fibroblasts (LGC Standards AB, Borås, Sweden) was seeded in 20ml DMEM:F12 supplemented with 10% FCS and PEST (penicillin-streptomycin). Medium was changed every fourth day and the cells were split every sixth day to avoid over-confluency. Cell passaging was performed by washing cells with DMEM:F12 supplemented with PEST followed by addition of 7ml trypsin-EDTA. The flask was incubated in 37^0C for 7min. The reaction was then stopped by adding 7ml DMEM:F12 supplemented with PEST and 10% FCS. 700µl of the cell suspension (1:20 split) and 1.3ml DMEM:F12 supplemented with PEST and 10% FCS were added to a 15ml tube. The tube was centrifuged for 5min at 400rpm and the supernatant was discarded. The cell pellet was resuspended in 20ml DMEM:F12 supplemented with PEST and 10% FCS and seeded in a new 162cm^2 cell culture flask.

3.1.4 Evaluating growth conditions for culture of heart biopsy cells to increase commitment towards the cardiac lineage

Heart biopsy cells were cultured in two phases, first an expansion phase and thereafter a differentiation phase. The same plates and the same cells were kept during both phases. In the first phase, different media, coatings and oxygen concentrations were evaluated in order to find the optimal conditions for *in vitro* expansion toward cardiac lineage. The cells were grown in monolayers on Primaria® 6-well tissue culture plates and 8 different conditions presented in table 1 were evaluated. This includes coating with collagen-1, laminins (521+211 and 521+211) as well as no coating. The three laminins used are important for development and maintenance of cardiac muscle tissue and Biolamina recommend a combination of the above laminins for cultivation of cardiomyocytes (Biolamina, 2015). Three different media were used for these 8 conditions, namely NutriStem, DMEM:F12 supplemented with PEST and 10% HS and so called suspension medium consisting of basal cell culture medium DMEM:F12 supplemented with PEST, 5% HS and the growth factors FGF (40ng/ml) and EGF (20ng/ml).

Table 1. Layout for growth condition of the *in vitro* expansion phase. 8 conditions were tested, each condition for an entire 6-well plate.

Plate	Medium	Coating	O_2 (%)
1	NutriStem	Laminin 521+211 and Laminin 521+221	21
2	NutriStem	-	21
3	Suspension	-	21
4	Suspension	-	5
5	DMEM:F12 +10%HS	-	21
6	NutriStem	Collagen-1	21
7	Suspension	Collagen-1	21
8	Suspension	Collagen-1	5

Three differentiation protocols were tested during phase 2 on each expansion condition to find the optimal combination of expansion and differentiation conditions. Change to differentiation medium was made after 7 days to allow proper attachment of the cells before changing the medium to avoid cell loss. The cells were kept in the same plates as during the expansion phase to avoid the cell loss that passage may cause and also to avoid *in vitro* artifacts by keeping the cells as close to the *in vivo* situation as possible. Each of the plates from table 1 were treated with three different differentiation protocols (table 2). In plate 5, 3 wells were grown in DMEM:F12 supplemented with 10%HS and medium was changed every third day to have undifferentiated cells as a control. The differentiation protocols tested were from van Berlo *et al* 2014, Lian *et al* 2013 and Kattman *et al* 2011. The van Berlo group differentiated mouse cells into beating cardiomyocytes by treating cells with 10nM dexamethasone in DMEM supplemented with 10% FCS. In this case, DMEM:F12 supplemented with PEST and dexamethasone were used as differentiation medium with addition of 10% human serum, since it is human cells that are being cultivated. The protocols from the Lian and Kattman groups were chosen since they have successful at AstraZeneca for differentiation of iPSCs into cardiomyocytes. The Lian protocol uses small molecules for differentiation into cardiomyocytes. Mesoderm induction with CHIR99021 was not performed in this case since the cardiac stem cells in the heart already are assumed to be mesoderm committed. Instead, the first step was cardiac specification using the

Wnt-inhibitor IWP2 in a differentiation medium consisting of RPMI 1640x with GlutaMax supplemented with B27 –insulin, since it has been shown that B27 with insulin inhibits cardiomyocyte yield during the first days of differentiation. From d3, cells were kept in maintenance medium RPMI 1640x with GlutaMax supplemented with B27 with insulin (Lian *et al,* 2013). The protocol from the Kattman group is described for EB-formats, but has also been tested for monolayer cultures. The protocol used is an adaption from the original Kattman protocol that has been used at AstraZeneca for differentiation of iPSCs. As with the Lian protocol, the mesoderm induction step were not performed and cardiac specification was initiated using Wnt-inhibitor IWP2 and Activin/Nodal/TGFβ-inhibitor SB431542 in differentiation media KO-DMEM supplemented with GlutaMax, phosphoascorbic acid, transferrin and selenium (TS). From d3 and forward, cells were kept in differentiation media only. Plate 4 and plate 8 were kept in 5% oxygen during the differentiation. Cells were fixated on d10 in 4% formaldehyde for 15min and kept in PBS in 4°C until being analyzed with immunocytochemistry.

Table 2. Differentiation phase. 2w/plates were treated with media combinations from each protocol. The cells were kept in the conditions from table 1 for day -8 until day 0 when differentiation was started.

Day	van Berlo protocol	Lian protocol	Kattman protocol
-7	Cell seeding according to table 1	Cell seeding according to table 1	Cell seeding according to table 1
0	DMEM:F12 +PEST +10%HS 10nM dexamethasone	RPMI + GlutaMax B27-insulin (50x) 5μM IWP2	KO-DMEM + GlutaMax TS 250μM Phospho-L-Ascorbic acid 5μM IWP2 5.4μM SB431542
1			
2		RPMI + GlutaMax B27-insulin (50x)	KO-DMEM + GlutaMax TS 250μM Phospho-L-Ascorbic acid
3	DMEM:F12 +PEST +10%HS 10nM dexamethasone		
4		RPMI + GlutaMax B27 (50x)	KO-DMEM + GlutaMax TS 250μM Phospho-L-Ascorbic acid
5			
6	DMEM:F12 +PEST +10%HS 10nM dexamethasone	RPMI + GlutaMax B27 (50x)	KO-DMEM + GlutaMax TS 250μM Phospho-L-Ascorbic acid
7			
8		RPMI + GlutaMax B27 (50x)	KO-DMEM + GlutaMax TS 250μM Phospho-L-Ascorbic acid
9	DMEM:F12 +PEST +10%HS 10nM dexamethasone		

3.2 Analytical methods

Analytical methods used in the project are described in the following sections. FACS analysis and sorting was mainly used for SmartFlare evaluation, ICC for evaluating culture conditions for heart biopsy cells and RT-qPCR for evaluation of FACS sorted cells.

3.2.1 Immunocytochemistry (ICC)

ICC is a method used for identification of proteins and other types of macromolecules in cells. ICC utilizes antibodies for localization of different proteins in the examined sample. Primary antibodies specific for the protein of interest are used and secondary antibodies specific for the primary ones with an attached fluorophore are thereafter added for visualization of the protein structure in fluorescent microscope. Blocking with serum minimizes unspecific binding and does not affect the binding of the primary antibody to the target antigen (Burry, 2011).

Cells were treated with 4% formaldehyde in room temperature for 15min followed by washing with PBS -/- for 3x5 minutes. PBS -/- were added to the wells and stored at 4°C until use. Blocking was performed with 10% Fetal Calf Serum (FCS) in PBS -/- for 1h. 0.5% Triton-X was added in case of intra cellular markers. Primary antibodies were diluted according to Table 3 in PBS -/- with 4% FCS and 0.5% triton if intracellular markers were used. Primary antibodies were added and incubated over night in 4°C. 3x5min washes with PBS+/+ was then performed. Secondary antibodies (table 3) were diluted 500x in PBS+/+with 4% FCS and incubated for 1h. For 6-well plates, 3x10min washes with PBS+/+ was made followed by addition of one drop of ProLong® Gold antifade reagent with DAPI per well. A cover glass was added to each well and plates were stored in 4°C until usage. For 96-wells, DAPI solution were incubated for 20min followed by 3x10min washing with PBS+/+. The plates were stored in PBS+/+ in 4°C until use.

Table 3. Evaluated antibodies for ICC in 6-well formats.

1° Antibody	Cat. No	Supplier	Dil.	Marker type	Cell type
anti-cTnt	Ab8295	Abcam	400x	Structural protein	Cardiomyocytes
anti-Nkx2.5	Ab35842	Abcam	300x	Transcription factor	CPCs
Nkx-2.5	B2609	Santa Cruz	150x	Transcription factor	CPCs
CD117/C-kit	PA5-16770	Thermo Fisher	100x	Surface receptor	CSCs
AntiCD117APC	A3C6E2	MiltenyiBiotec	-	Surface receptor	CSCs
AntiCD117APC	104D2	BD	-	Surface receptor	CSCs
anti human von willebrand factor	F3520	Sigma	100x	Blood glycoprotein produced by endothelial cells	Endothelial cells
CD31	303101, 25T	Biolegend	100x	Surface receptor	Endothelial cells

Table 4. Secondary antibodies used for ICC.

2° Antibody	Cat. No	Supplier	Conjugate
Goat anti rabbit	A11071	Life Technologies	Alexa 546
Goat anti mouse	A21237	Life Technologies	Alexa 647
Goat anti mouse	A11018	Life Technologies	Alexa 546
Goat anti rabbit	A21246	Life Technologies	Alexa 647

3.2.2 Evaluation of SmartFlare™ probes for detection of gene expression

Detection of gene expression is often performed using techniques such as RT-qPCR or ICC. These methods give relevant data but require cell lysis (RT-qPCR), cell fixation (ICC) and cell

permeabilization (ICC with intracellular antibodies). They are so called end-point methods and the cells cannot be further cultivated. The SmartFlare™ (SF) probe technology enables live cell detection of gene expression since the probes enter live cells via the natural cellular mechanism of receptor-mediated endocytosis. The probes are encapsulated in endosomes and are after a while released into the cytoplasm were they interact with the mRNA of interest. Fluorescent signals are best detected 16-24h after SF addition since the process of endocytosis takes some time (Merck Millipore, 2015).

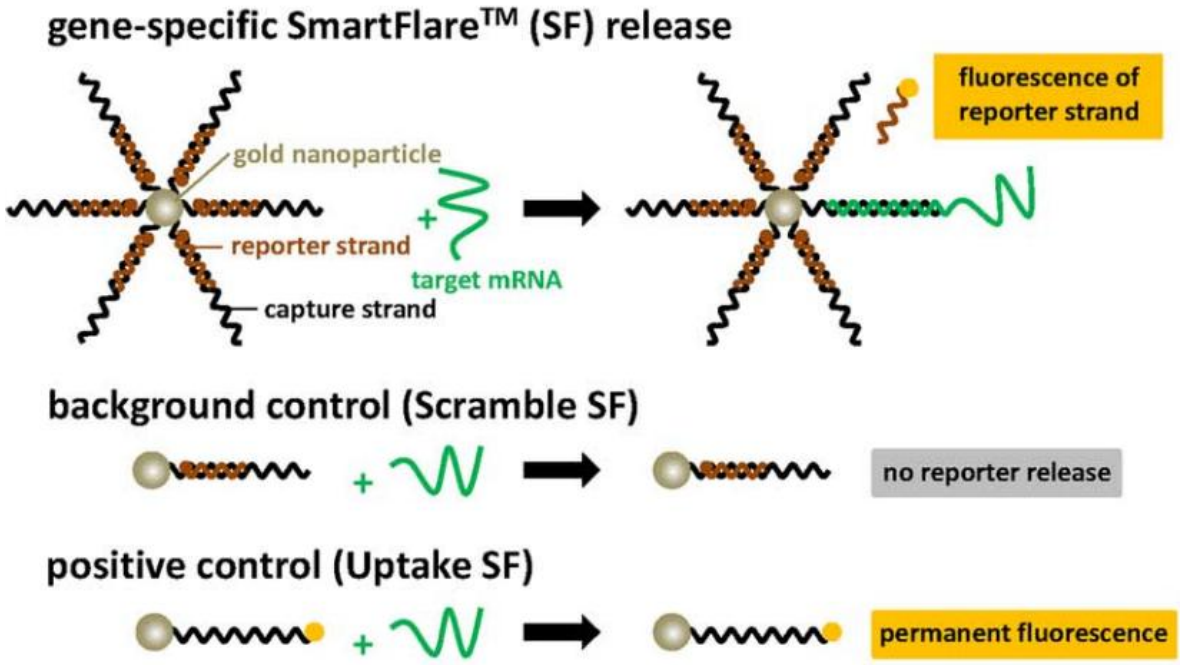

Figure 7. The principle of gene detection using SmartFlare™ (SF) probes. The SF probe consists of a gold nanoparticle core. Double-stranded DNA oligonucleotides specific to the gene of interest are attached to the gold particle. The oligonucleotides are built up of one reporter strand and one longer RNA capture strand. Binding of target RNA replaces the reporter strand, which is released and send out a fluorescent signal that can be detected using FACS or a fluorescent microscope. Background fluorescence level is determined by including a Scramble SF. Uptake SF have permanent fluorescence and are included to see that the cells are able to take up the SF probes (Lahm *et al*, 2015).

The SF probes are built up of a gold nanoparticle as core with several double-stranded DNA oligonucleotides attached to it (Figure 7). The oligonucleotide duplex is constituted of a reporter strand and a capture strand. Fluorescence is quenched by the gold particle when it is bound to the capture strand. The capture strand is complementary to its target RNA and detection is therefore initiated by sequence specific binding. The binding of target mRNA to the capture strand displaces the reporter strand, which after release emits fluorescence that can be detected. A higher amount of target RNA releases more reporter strands and result in a higher fluorescent signal. Uptake control SF probes should be included as positive control. These probes are not sequence-specific and are therefore suitable to use to determine if the cells of interest are able to take up the probes with endocytosis. Different cell types have different endocytosis rates and it is therefore important to test if the cells are suitable before initiating further SF experiments. SF probes for reference genes can also be included as positive control since they have a stable expression in the cell type used. Uptake control and housekeeping gene fluorescence levels should therefore be similar to each other. A background control should always be included to measure the background fluorescence. The background can vary between cell types or between culture conditions. It is also convenient to include unflared cells, i.e. cells without addition of SF,

which can be used to detect auto-fluorescent noise in the sample (Merck Millipore, 2015, Lahm *et al*, 2015).

iPSCs were seeded in 96-well plates with a seeding density of 60k/cm^2 at d0. Regular medium change was performed d1. Smart flare probes were diluted in 50µl nuclease-free water to obtain 100nM stock solution. At d2, working solutions of SF probes was made by diluting the stock solution 1:20 with PBS. This was done maximum 1h before addition to the cells. A regular media change was done so that each 96-well contained 200µl media. 4µl SF working solution was thereafter added to each well to get a final probe concentration of 100pM, 8µl for a 200pM concentration or 16µl to get a final SF concentration of 400pM. The medium containing SF was gently mixed by pipetting. The cells were then incubated for 16h or 24h. For FACS analysis, the cells were after incubation harvested by washing with 200µl PBS followed by addition of 50µl TrypLE Select which were incubated for 4min at 37^{0}C. 150µl HBSS + 2% FCS were added to each well and mixed several times to ensure a single-cell solution. Samples were transferred to eppendorf tubes for FACS analysis.

For iPSCs differentiated towards cardiomyocytes, SmartFlares were added d7 and d15 of differentiation in the same way described above. Cells were after incubation directly imaged and then put back in the incubator for continued growth. iPSCs differentiated to cardiomyocytes at d4 of differentiation were used to evaluate if SF can be used to sort out small number of positive cells. These cells were sorted using FACS technology and later analyzed using RT-qPCR. SF probes used for the experiments are displayed in table Table 5.

Table 5. SmartFlare™ probes used in the project.

SmartFlare™ probe	Fluorophore	Catalogue number	Target gene
Scramble	Cy3	SF-103	-
Scramble	Cy5	SF-102	-
Uptake control	Cy3	SF-114	-
Uptake control	Cy5	SF-137	-
GAPDH	Cy3	SF-126	Housekeeping
GAPDH	Cy5	SF-136	Housekeeping
OCT4	Cy3	SF-438	Stem cell
NANOG	Cy5	SF-875	Stem cell
TNNI3	Cy5	SF-3222	Cardiomyocyte

3.2.3 Fluorescence-activated cell sorting (FACS)

FACS technology can be used for both analysing and sorting of cells with different characteristics. A number of parameters can be analyzed simultaneously and these properties are relative size (forward scatter or FCS), granularity (side scatter or SSC) and fluorescence intensity of different wavelengths. Cells can be sorted out based on these properties if the machine has a cell sorting unit. Cells are sorted by giving each droplet that full fill sorting criteria a positive or a negative charge. The droplets are run through a high voltage field and that changes the direction of the droplets so that they are collected in different tubes or plates (Sandstedt, 2014). Cells can be sorted into tubes containing RLT buffer for later RT-qPCR analysis. RLT lyses cells and protects RNA from degradation (Qiagen, 2015). Cell sorting of cells

marked with SmartFlares was carried out on a BD FACSAria™ III and analysis of cells marked with SF was carried out on a BD LRSFortessa™.

Cells to be subjected for FACS analysis using antibodies (table 6) were treated with trypsin-EDTA (0.05%) for 7min and thereafter stopped by addition of media with 10% FCS. The single-cell suspension was centrifuged in 15ml tubes at 400g for 5min. Cells were resuspended in FACS-buffer (5% FCS, 1% BSA and 2mM EDTA dissolved in PBS). Cell number was counted. Cell solutions were centrifuged at 400g for 5 min and resuspended at desired concentration. Primary antibodies and 7-AAD were added at desired concentrations and incubated for 30min at 4°C. The cells were thereafter washed with FACS-buffer and poured through a 40µM cell strainer followed by centrifugation at 400g for 5min. The supernatant were removed and the cells resuspended in new FACS-buffer. The cells were spun down again and thereafter resuspended in 100-200µl FACS-buffer/1*10^6 cells. Data were acquired on a BD FACSAria™ II and thereafter analysed using the FACSDiva software version 6.1.4 (BD). Isotype controls were used to determine non-specific binding (table 7).

Table 6. Antibodies used for FACS analysis. The listed antibodies were used in this project to characterize the C-kit+CD45- stem cell population in right atrium of the adult human heart.

Antibodies	Target cell	Catalogue number	Company
C-kit-APC	CSCs	130-098-207	Miltenyi Biotec
SSEA-4-PE	CSCs	12-8843-42	eBioscience
CD45 FITC	Hematopoietic cells	555482	BD
C-kit-PE-Cy7	CSCs	339217	BD
CD34-BV421	Hematopoietic cells	562577	BD
CD34-APC	Hematopoietic cells	345804	BD
CD144 [VE-Cad]-PE	Vascular endothelial cells	12-1449-80	eBioscience
CD31-Alexa 488	Endothelial cells	303109 / 25T	Biolegend
CD90-BV421	Fibroblasts	562556	BD

Table 7. Isotype controls used for FACS analysis. Isotype controls are used in flow cytometry to determine non-specific binding.

Isotype controls	Catalogue number	Company
Mouse IgG1-APC	554681	BD
Mouse IgG3-PE	12-4742-42	eBioscience
Mouse IgG1-FITC	554679	BD
Mouse IgG1-PE-Cy7	25-4714-80	eBioscience
Mouse IgG1-BV421	552438	BD
Mouse IgG1-PE	554680	BD
Mouse IgG1-Alexa 488	400129/100T	Biolegend

3.2.4 Imaging

Images of cells stained with antibodies were acquired using a Nikon Eclipse Ti fluorescence microscope. The cameras used were an ANDOR Zyla sCMOS camera and in some case an ANDOR iXon x3 camera in combination with NIS elements software (Nikon Corporation, Tokyo, Japan). A motorized board was used for automatic scanning of the images and the "stitch images" option provided by the software was chosen. The images were analyzed using Fiji ImageJ software by

visual observation of positive cells and ranges were set to extinguish most of the background in relation to positive and negative controls. Brightfield images of these cells were taken using a Nikon DIAPHOT 300 (Japan) microscope with a Nikon Digital Sight camera before ICC was performed. For cells marked with SmartFlares, images were acquired using the ImageXpress (Molecular Devices, Sunnyvale, California, USA) and data analysis was done using the MetaXpress software. Brightfield images and movies of contracting of differentiated iPSCs were obtained using a Nikon Eclipse TE2000-U with a Zyla cMOS camera and the NIS elements software (Nikon Corporation, Tokyo, Japan). Cell morphology of differentiated iPSCs was observed on a daily basis during differentiation using a Nikon TMS microscope.

3.2.5 RT-qPCR

Real time quantitative polymerase chain reaction (RT-qPCR) can be used to quantify mRNA levels in cells and tissues. A reverse transcription step must first be performed to turn RNA into cDNA before the PCR reaction takes place. The four phases of the PCR reaction include; 1) the linear phase in which the fluorescence is lower than the background 2) the early exponential phase in which fluorescence increases and becomes much higher than the background (C_T) 3) the exponential phase in which product number doubles in every cycle 4) the plateau phase in which amplification slows down due to no remaining reaction components (Glick, Pasternak and Patten, 2010). The mRNA expression data is normalized against expression of mRNA level of one or several housekeeping genes (HKGs). Housekeeping genes are required for basic cellular maintenance and function and are therefore expressed at rather stable levels independent of growth conditions. Different housekeeping genes can be used and the use of for example glyceraldehyde-3- phosphate dehydrogenase (GAPDH) is normal for somatic cells. It has been shown that stem cells and early progenitors express different housekeeping genes compared to other cell type and HKGs such as CREBBP is therefore recommended for these cells (Synnergren *et al*, 2007).

RNA purification

For RNA purification from cells the protocol "Purification of Total RNA from Animal and Human Cells" from the RNeasy® Micro Handbook (Qiagen) was used. First of all, monolayer cultivated cells were sorted using FACS into tubes containing 350µl RLT with β-mercaptoethanol. The tubes were stored in -80^{0}C until use. RNA was prepared according to the manufacturer's instructions. RNA concentration of each sample was measured using a NanoDrop2000 (Thermo Scientific).

cDNA synthesis

Generation of cDNA from the purified RNA samples is required to enable quantitative PCR. The High Capacity cDNA Reverse Transcription Kits from Applied Biosystems were used for this purpose (Table **8**). 500ng RNA sample in 10µl volume were added to the tube containing 10µl master mix and thereafter put in the PCR-machine. For samples containing less than 10ng/µl, the purified RNA were not diluted. 10µl purified RNA sample were in that case added to the 10µl master mix in PCR-tubes. The PCR program was run on a 2720 Thermal Cycler (Applied Biosystems) and the program is show in Table 9.

Table 8. Master mix used for cDNA synthesis of purified RNA samples.

Master mix for cDNA synthesis	Volume/sample (µl)
RNAse free H_2O	3.2
10x RT Buffer	2
10x RT Random Primers	2
25x dNTP Mix (100 mM)	0.8
RNAse Inhibitor	1
MultiScribe™ Reverse Transcriptase, 50 U/µL	1
Total volume:	**10**

Table 9. PCR program used for reversed transcription of RNA into cDNA.

Temperature (°C)	Time (min)
25	10
37	120
85	5
4	∞

Pre amplification of target genes

A pre-amplification step of target genes were included for samples containing low concentration of RNA (<10ng/µl). TaqMan® PreAmp Master Mix Kit from Applied Biosystems was used (Table 10). The pooled assay mixed contain all TaqMan assays from table Table 11 diluted 100x in TE 1x buffert. The pre-PCR amplification protocol is displayed in Table 12 and was run on a 2720 Thermal Cycler from Applied Biosystems.

Table 10. Amplification mixture.

Pre amp mix	Volume (µl)
TgM PreAmp master mix (2x)	5
Pooled assay mix (0.2x)	2.5
cDNA (1-250ng)	2.5
Total volume/tube:	10

Table 11. TaqMan assays used for pre-amplification and for RT-PCR.

TaqMan Assay	Assay no	Lot no	Gene type
CD31/PECAM1	Hs00169777_m1	1090349	Endothelial
TNNT2	Hs00165960_m1	1070965	Cardiomyocyte
TNNI3	Hs00165957_m1	1339855	Cardiomyocyte
GATA4	Hs00171403_m1	954553	Cardiac progenitor
Nkx2.5	Hs00231763_m1	1049589	Cardiac progenitor
CYPA	Hs99999904_m1	1257262	Housekeeping gene
CREBBP	Hs0023173_m1	1252057	Housekeeping gene

Table 12. PCR protocol for amplification of target genes in samples containing low amount of cells.

Temperature (°C)	Time (min)	Cycles
95	10	-
95	15 sec	14
60	4	
4	∞	-

RT-qPCR

The RT-qPCR reaction was carried out using commercially available TaqMan assays for the gene of interests (Table 11). The assay consist of unlabeled PCR primers and a TaqMan® probe with a fluorescent dye on the 5' end (FAM™) and a minor groove binder (MGB) and a non-fluorescent quencher (NFQ) on the 3' end that quenches the fluorescent signal. Primers and probe bind to their complementary sequence in the cDNA template. A complementary DNA strand is synthesized by the Taq polymerase using the unlabeled primers and the cDNA template as guides. As the Taq polymerase reaches the TaqMan® probe, the probe is degraded and the dye released which gives a fluorescent signal that can be detected. Each PCR cycle releases more dye molecules and the fluorescence intensity is proportional to the amount of amplified DNA (Life Technologies, 2015).

A master mix consisting of water, TaqMan assay and TaqMan® Gene Expression Master Mix was mixed for each gene to be analyzed (table 13). 15µl master mix was then transferred to each well in a 96well-plate. After addition of master mix, 5µl cDNA (0.5ng/µl or 1:20 diluted preAmp material) sample were added to each well. Water was used as negative sample and a calibrator was used to relate data from different plates to each other. The PCR program for RT-qPCR is shown in Table 14 and was run in a 7900HT Fast Real-Time PCR system (Applied Biosystems).

Table 13. Master mix for each gene for RT-PCR.

Master mix	**1x (µl)**
H2O	4
TaqMan assay	1
2x TaqMan Gene expression master mix	10
Total volume/96-w	**15**

Table 14. PCR program for RT-qPCR.

Temperature (°C)	**Time (min)**	**Cycles**
50	2	-
95	10	40
95	15s	
60	1	-

The gene expression values were calculated based on the comparative C_T method. This means that the amount of target gene product is normalized to an endogenous reference (CREBBP) and relative to a calibrator. The fold change is given by equation 1 (Schmittgen and Livak, 2008).

$$Fold\ change = 2^{-\Delta\Delta C_T} \qquad (1)$$

4. Results

The results from the project are presented in the following sections. The first part of the project focused on evaluating if small amounts of antibodies could be used for detecting a small number of positive cells in a large culture format. Thereafter, culture conditions for expansion and differentiation of heart biopsy cells were evaluated. The purpose was to get more cells committed towards the cardiac lineage, which was analyzed using ICC and fluorescent imaging. The second part of the project was dedicated to evaluation of SmartFlare™ technology on iPSCs and iPSCs differentiated to cardiomyocytes. The results were analyzed using FACS analysis, fluorescent imaging and FACS sorting followed by RT-qPCR analysis.

4.1 Antibody evaluation

Three different C-kit antibodies were evaluated. Tissue biopsy cells were used as a model system (left in Figure 8) since it is known that the right atrium consists of a small population of C-kit positive cells (Sandstedt *et al*, 2010). Heart biopsy cells were cultured for 13 days followed by FACS analysis to confirm the percentage of C-kit positive cells. Two different C-kit antibodies were used to get cells truly positive for C-kit and the Q2 area in Figure 8 represents those cells which were 4.5% of the total population. Other antibodies were used as well to further characterize the cells in the biopsy (data not shown).

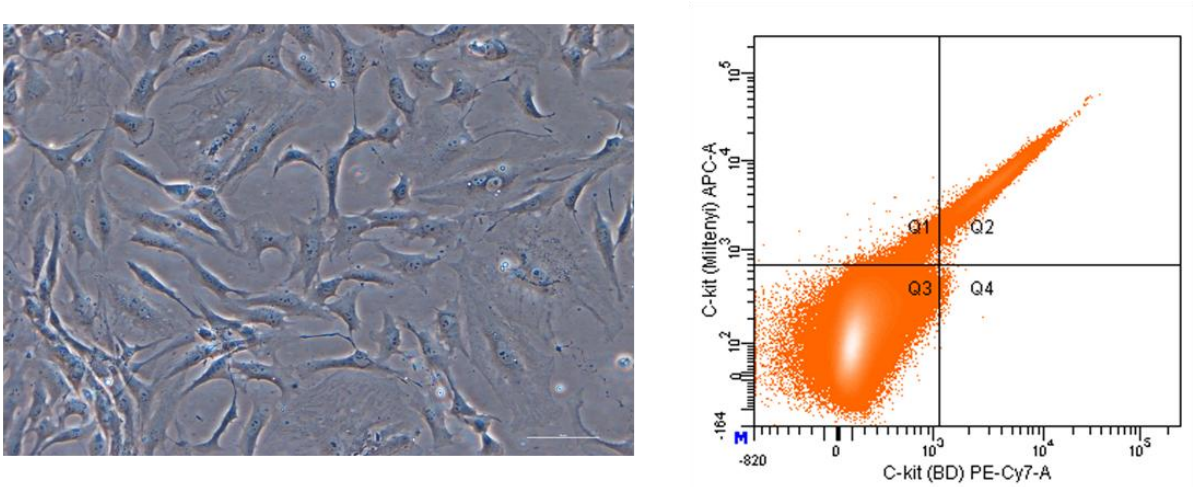

Figure 8. Left: Heart biopsy cells in culture after 7 days. Right: Percentage of C-kit positive cells present in heart biopsy cultured for 13 days. The Q2 population is cells positive for two different C-kit antibodies and represent 4.5% of the entire population.

The anti CD117/C-kit-APC antibody from BD was the antibody was the most successful one based on analysis on the results from ICC. The other two C-kit antibodies more non-specific and much weaker (data not shown). So based on comparison between the amount of marked cells and their morphology, the BD anti CD117 APC was determined to be the most promising one for this application (Figure 9). A negative control consisting of only fibroblasts was included in the experiment. Secondary antibodies were used in all cases to increase the fluorescent signal since directly conjugated antibodies normally only is used for FACS-analysis.

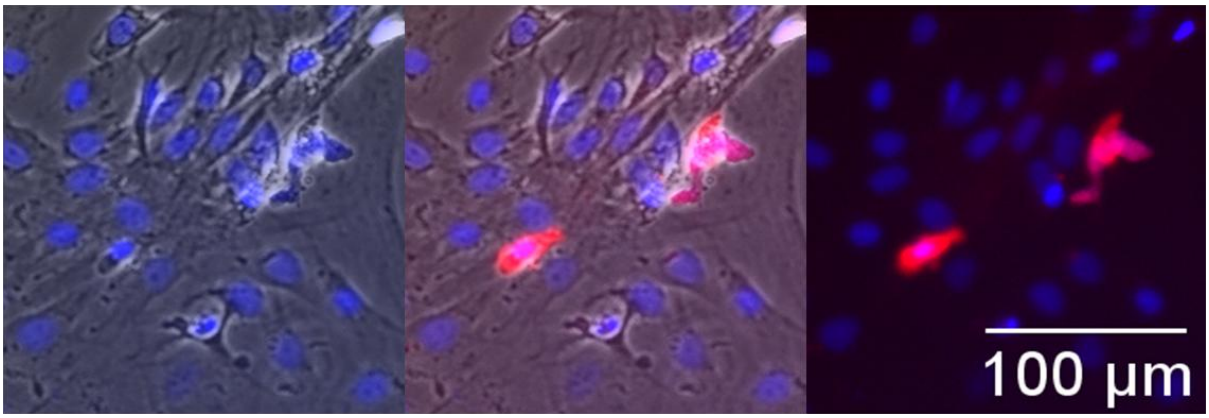

Figure 9. Heart biopsy cells stained with C-kit antibody from BD. All three images are obtained from the same location in the well. Cells are stained with DAPI (blue) and anti CD117 APC (red). The images were obtained using a 20x objective.

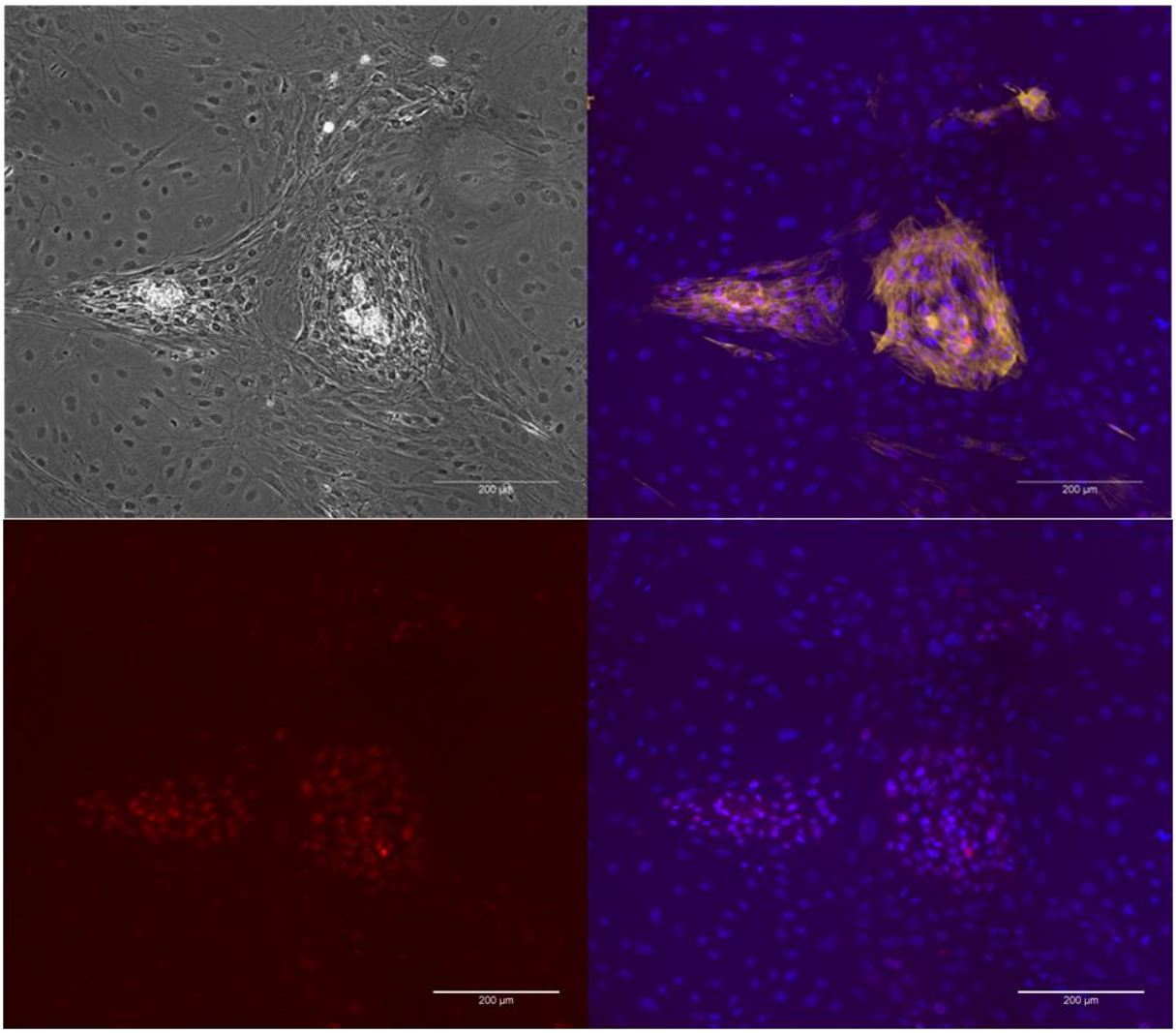

Figure 10. Co-culture of fibroblasts and 3% CMCs to evaluate cardiac antibodies. All images are obtained at the same location in the well. Cells are stained with cTNT (yellow), Nkx2.5 (abcam, red) and DAPI (blue). The image was obtained using a 20x objective**.**

cTNT and Nkx2.5 were evaluated for antibodies targeting cells committed to cardiac lineage. All markers showed promising results, but Nkx2.5 from Santa Cruz had a much higher level of unspecific binding than Nkx2.5 from Abcam (data not shown). Due to difficulties with achieving a single-cell solution while passaging cardiomyocytes differentiated from iPSCs, the cardiomyocytes often appeared in clusters in the fibroblast co-culture. Such a cluster is shown in Figure 10 and the cTNT cytoplasm structures can clearly be seen in these cells and also in some of the surrounding cells. The cTNT marking was very specific and promising since it easy to detect positive cells by looking at the cTNT structures in the cytoplasm. The images were analyzed by comparing with intensity ranges set after positive controls constituted of solely cardiomyocytes differentiated from iPSCs (data not shown). A negative control consiting of only fibroblasts was also included in the experiment. The Nkx2.5 expression was weak in both the positive control (data not shown) and the co-culture, probably due to maturity of cells since Nkx2.5 is a progenitor marker.

vWF and CD31 were evaluated in a co-culture model system with fibroblasts and a small percentage of endothelial cells (HUVECs). The cell types cultured separately are shown in Figure 11 and in co-culture in Figure 12.

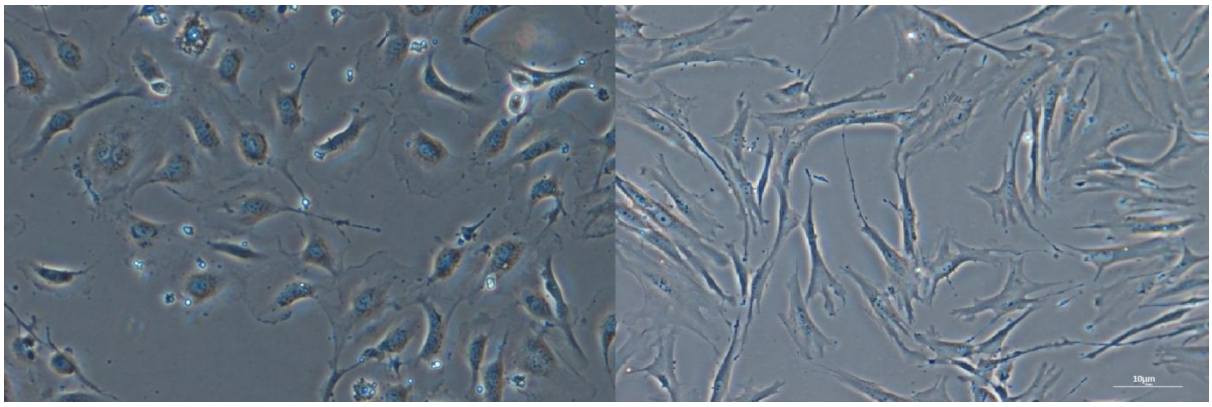

Figure 11. Culture of HUVECs (left) and fibroblasts (right). The brightfield images were obtained using a 10x objective and are of equal size and scale with a scale bar of 10μm.

The endothelial antibodies (Figure 12) stained mostly the same cells which indicated that both antibodies were able to detect positive cells. It can also be noted that it is impossible to identify rare cell types based on just morphology by comparing the brightfield image with the fluorescent one. The images were analyzed by comparing with intensity ranges set after positive controls consisting of solely HUVECs (data not shown). A negative control consiting of only fibroblasts was also included in the experiment.

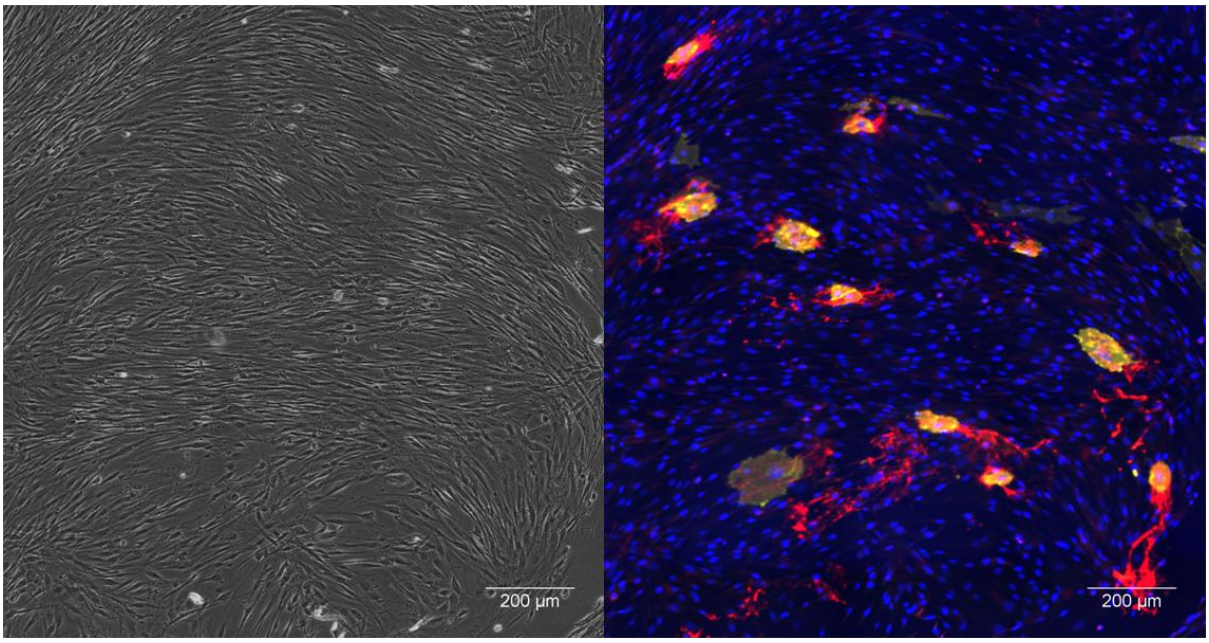

Figure 12. Co-culture of fibroblasts and 3% HUVECs to evaluate antibodies for endothelial cells. The images are obtained from the same location in the well. Cells are stained with vWF (yellow), CD31 (abcam, red) and DAPI (blue). The image was obtained using a 20x objective**.**

4.2 Evaluation of growth conditions and differentiation protocols

Cells from a cardiac biposy was dissociated and cultured in different conditions. The cultivation occurred in two phases, the first being an expansion phase and the second a differentiation phase. Several of the conditions resulted in endothelial structures. Some of the conditions gave cells growing in vascular structurs than can be seen both in brightscale images and in DAPI and CD31-stained versions (Figure 13). Other conditions resulted in cells positive for endothelial marker CD31, but where vascular structures cannot be visulized in brightfield (Figure 14). The CD31 antibody used is directly conjugated to its fluorophore and was therefore weaker. A directly conjugated antibody was used to be able to mix three different antibodies in one well. This was confirmed with a positive HUVEC control (data not shown).

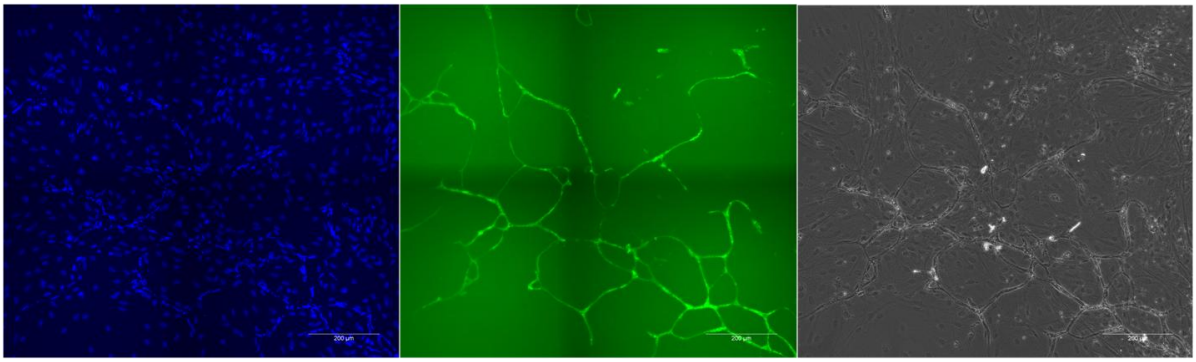

Figure 13. Example of cells displaying a vascular morphology that can be visualised both in the nuclear staining (blue), CD31 staining (green) and in brightfield images (grey). All three images are obtained in the same position in the well and are obtained using a 20x objective. Scale bars are 200µM.

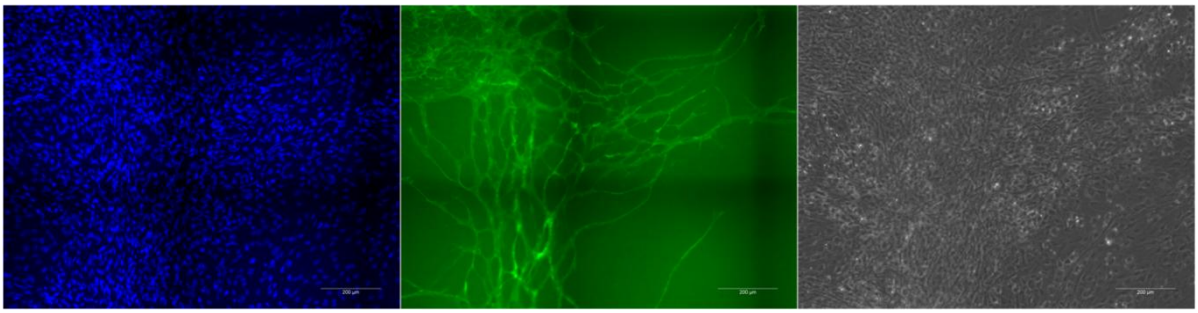

Figure 14. Example of cells displaying a vascular morphology that can only be visualised with CD31 staining (green). Nuclear staining with DAPI in blue and brightfield in grey. All three images are obtained in the same position in the well and are obtained using a 20x objective. Scale bars are 200µM.

The images were analyzed by comparing with intensity ranges set after positive controls consisting of HUVECs and cardiomyocytes differentiated from iPSCs. A negative fibroblast-contol was also included in the experiment. The result is shown in table 15. Region of interest (ROI) was used for cTNT, Nkx2.5 and C-kit antibodies. CD31 positive cells grew in networks and it was therefore impossible to decide a number of ROIs. The majority of the evaluated conditions resulted in no or one single cell positive for either cTNT, Nkx2.5 or C-kit staining when analyzed with fluorescent microscope. Most conditions resulted in cells positive for CD31 growing in vascular networks. The only condition resulting in cells positive for cardiac marker cTNT was plate 2, where the cells were grown in NutriStem for the expansion phase and the van Berlo protocol (DMEM:F12 supplemented with 10% HS and 10nM dexamethasone) for the differentiation phase (Figure 15).

Table 15. **Results from the evaluation of different expansion and differentiation conditions.** ROIs stand for Region of Interests since it often is hard to decide the number of cells positive in a certain region. Some wells were not stained with CD31 due to only one replicate (marked with not analysed (NA)). All other conditions had 2 replicates.

Plate	Differentiation Protocol	CD31 positive	C-kit ROIs	Nkx2.5 ROIs	cTNT ROIs
1	Van Berlo	NA	-	-	-
	Lian	NA	-	-	-
	Kattman	NA	-	-	-
2	Van Berlo	-	-	1	3
	Lian	-	-	-	-
	Kattman	-	-	-	-
3	Van Berlo	Yes	-	-	-
	Lian	Yes	1	-	-
	Kattman	Yes	1	-	-
4	Van Berlo	Yes	-	-	-
	Lian	Yes	-	-	-
	Kattman	Yes	-	-	-
5	Van Berlo	NA	-	-	1
	Lian	NA	-	-	-
	Kattman	NA	-	-	-
	Undifferentiated	Yes	-	-	-
6	Van Berlo	Yes	-	-	-
	Lian	Yes	-	-	-
	Kattman	-	-	-	-
7	Van Berlo	Yes	-	-	-
	Lian	Yes	-	-	-
	Kattman	Yes	-	-	-
8	Van Berlo	Yes	-	-	-
	Lian	Yes	-	-	-
	Kattman	Yes	-	-	-

As can be seen in Figure 15, all three regions of interest display a morphology that might be cells committed to the cardiac lineage when comparing the corresponding bright scale images. The only Nkx2.5 positive cell was found in the core of one of these regions (Figure 15, image in the bottom).

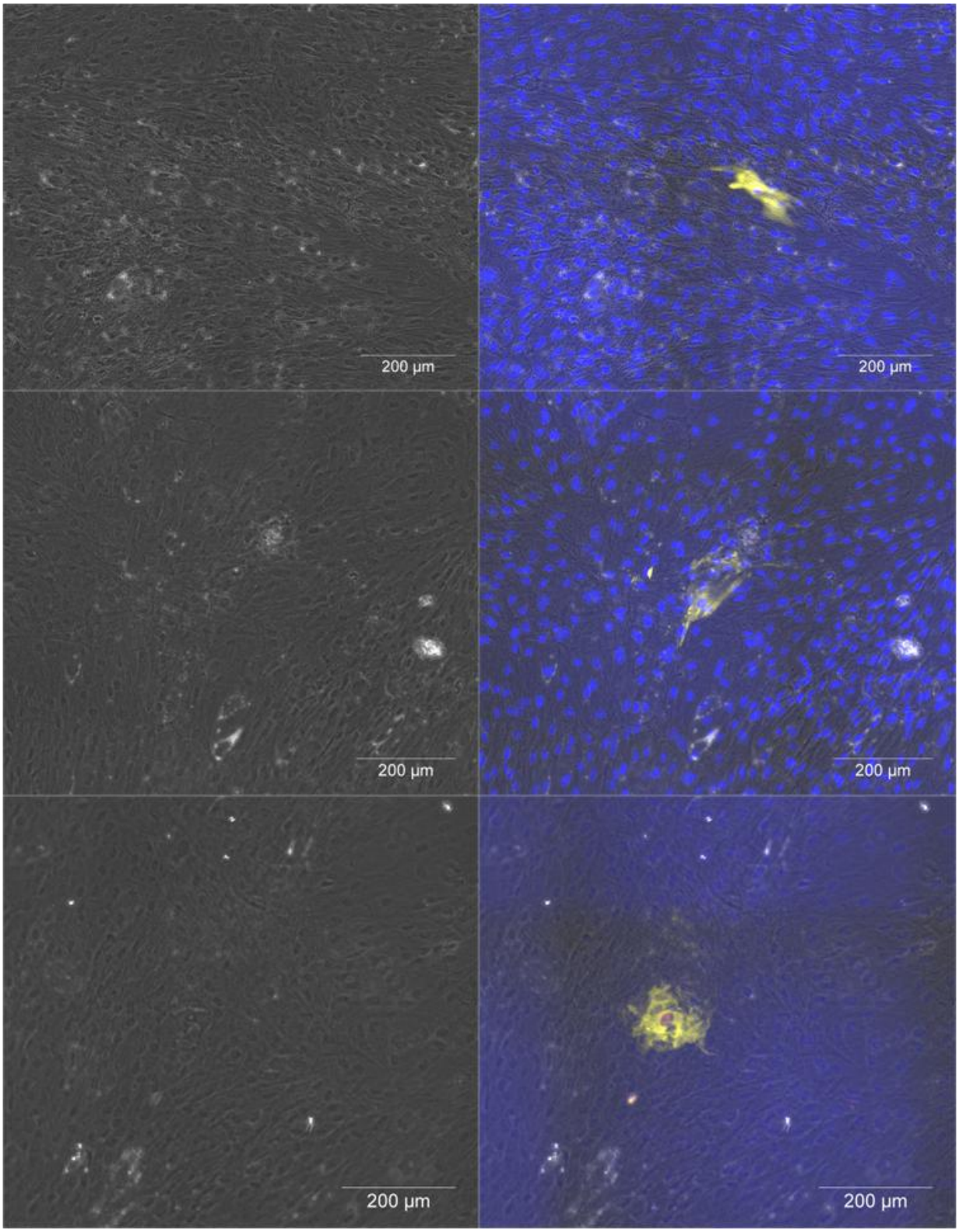

Figure 15. Heart biopsy cells expanded in NutriStem and differentiated with DMEM:F12 supplemented with 10% HS and 10nM dexamethasone. Images on the same row are from the same location in the well, but displayed in brightfield (grey) to the left and DAPI (blue), cTNT (yellow) and Nkx2.5 (red) to the right. A 20x objective was used to capture the images.

4.3 SmartFlare™ evaluation

The SmartFlare™ (SF) technology was evaluated in iPSCs and the initial experiment evaluated if iPSCs are capable of SF uptake. The iPSCs were incubated with SFs for 24h and then analyzed with FACS technology. OCT4 expression is compared to Uptake control in Figure 16. OCT4 is known to be highly expressed in the RiPSC-1J line (in house data, not shown). The result indicates that iPSCs have SFs uptake since the uptake control and OCT4 peaks are similar in intensity. SF probes at a concentration of 400pM was also evaluated regarding uptake control versus OCT4 but resulted in a similar result but with a larger overlap with the scramble signal (Figure 17). 200pM was therefore used as SF concentration for the following experiments to minimize probe volumes and thus reduce cost.

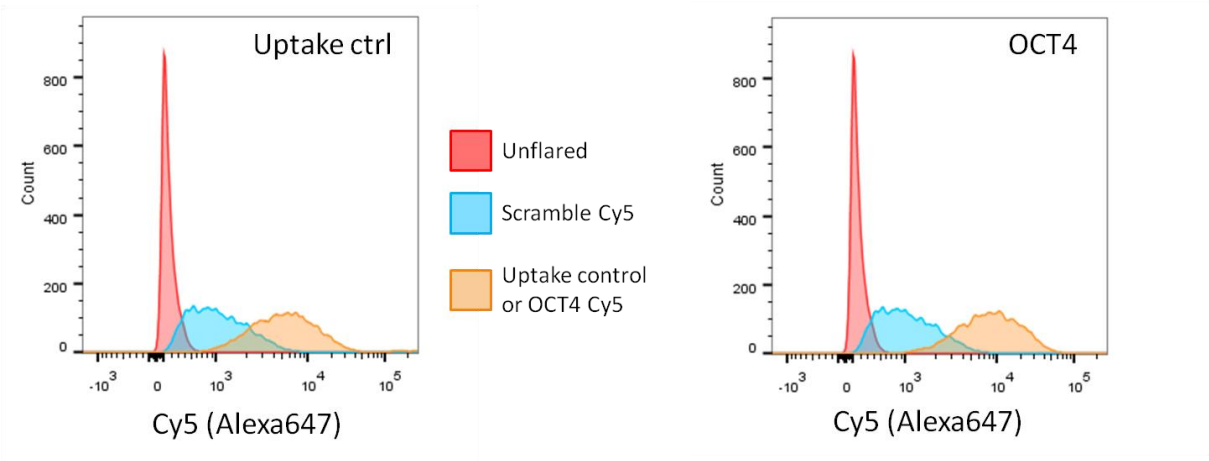

Figure 16. Uptake control versus OCT4 SmartFlare™ expression (200pM) in iPSCs. The cells were incubated with 200pM SFs for 24h followed by analyze using FACS technology.

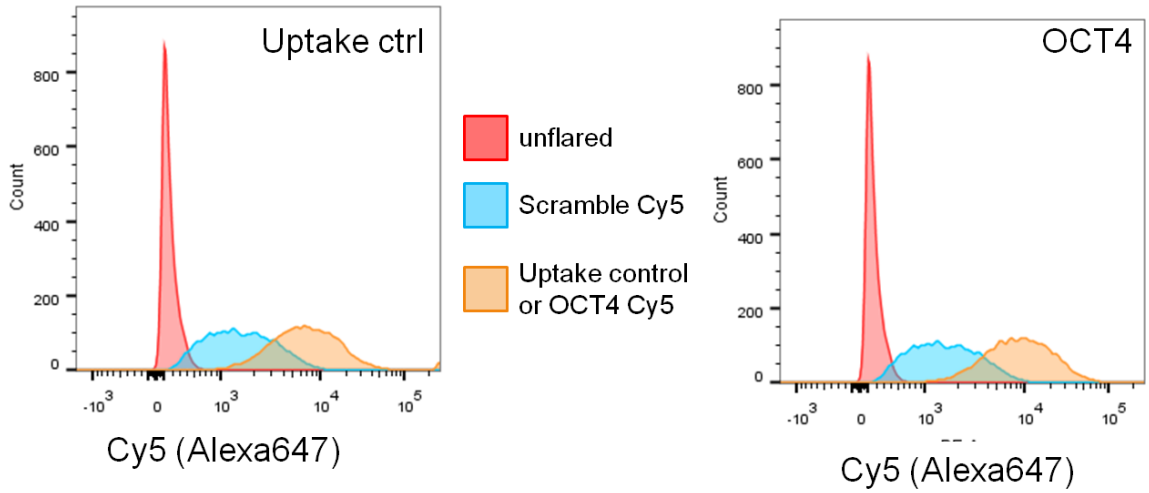

Figure 17. Uptake control versus OCT4 SmartFlare™ expression (400pM) in iPSCs. The cells were incubated with 400pM SFs for 24h followed by analyze using FACS technology.

Incubation times 16h versus 24h was also evaluated (figure 18). A slightly better separation of scramble and OCT4 peaks can be seen for 24h incubation, and that incubation time was thereafter used for following experiments.

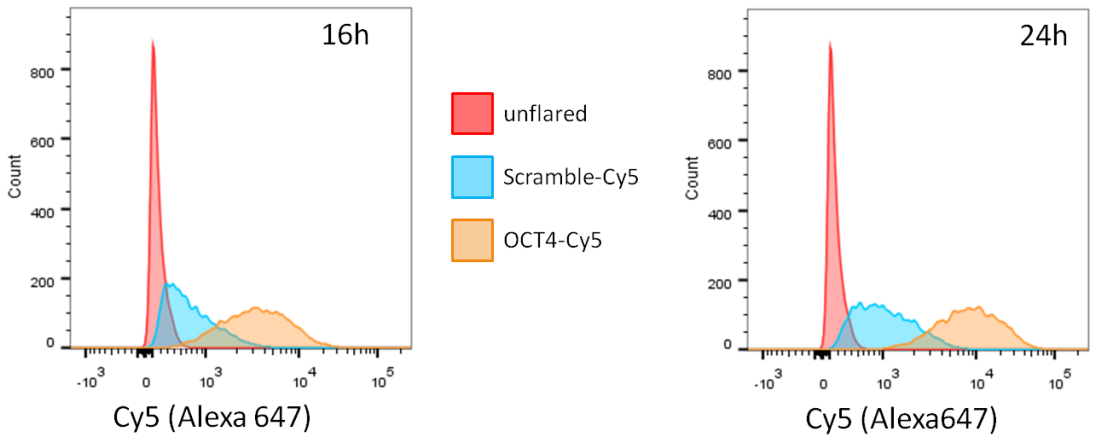

Figure 18. OCT4 expression after 16h and 24h incubation with 200pM SmartFlares™. A better separation between scramble and OCT4 peaks (smaller intersection-area) can be seen after the longer incubation time.

The expectation is that SFs can be used in the future to isolate populations of interest from human biopsies. The next experiment was therefore conducted on iPSCs differentiated towards cardiomyocytes. Cardiomyocytes was differentiated using the Lian protocol (Figure 19).

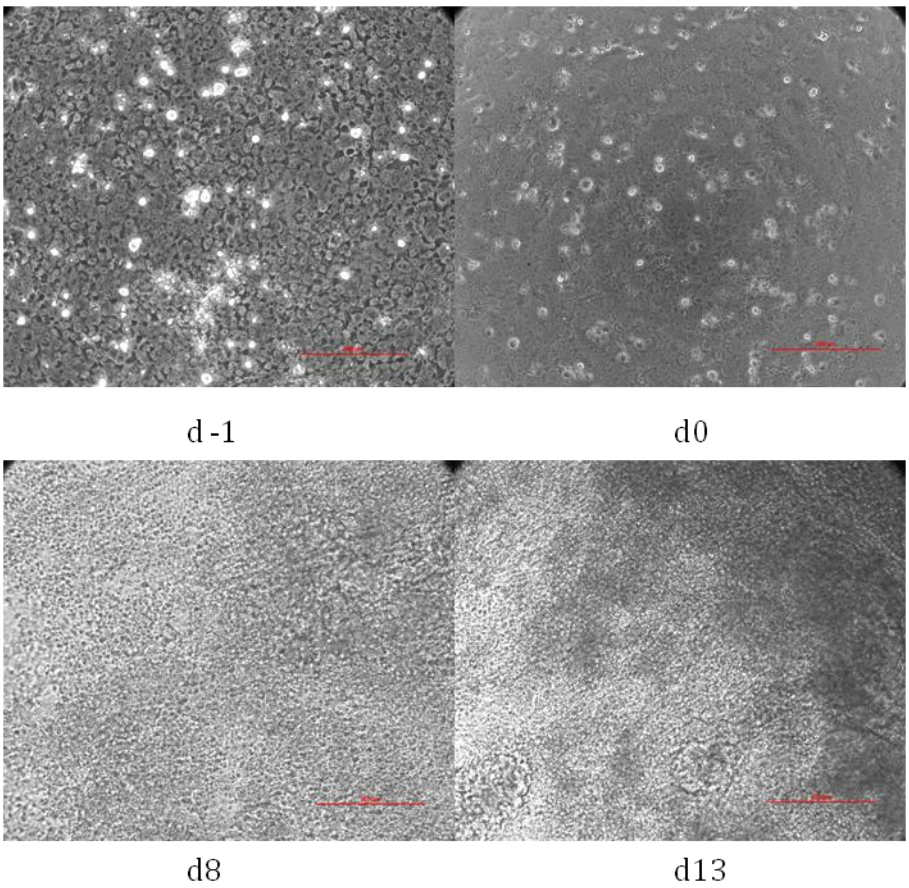

Figure 19. Differentiation of iPSCs to cardiomyocytes. The slightly modified Lian protocol was used for differentiation. The protocol starts with highly confluent iPSCs (d-1) which are seeded at 312k/cm² in 96-wells. Differentiation is initiated d0 when the cells are confluent. D8 and d13 of differentiation is showed as well and a morphological difference can be seen, which is due to initiation of spontaneous beating (often d9) when the beating cells are aggregated into tightly dense aggregates. The scale bar is 200µM and the images were obtained using a 20x objective.

SF probes were added to the differentiated cells on d7 and d15 followed by 24h incubation and imaging of live cells on d8 and d16. Cells from the same differentiation were used for both incubations, but in different wells to avoid remaining probes from the previous incubation. Both 100pM and 200pM of probes were tested, but 100pM wells showed the same pattern as 200pM wells, but in much lower intensities (data not shown). Figure 20 indicates that cardiomyocytes differentiated from iPSCs are able to take up SF probes (highly positive for uptake control SFs) and also that SFs can be used to see increased gene expression of cardiac troponin I.

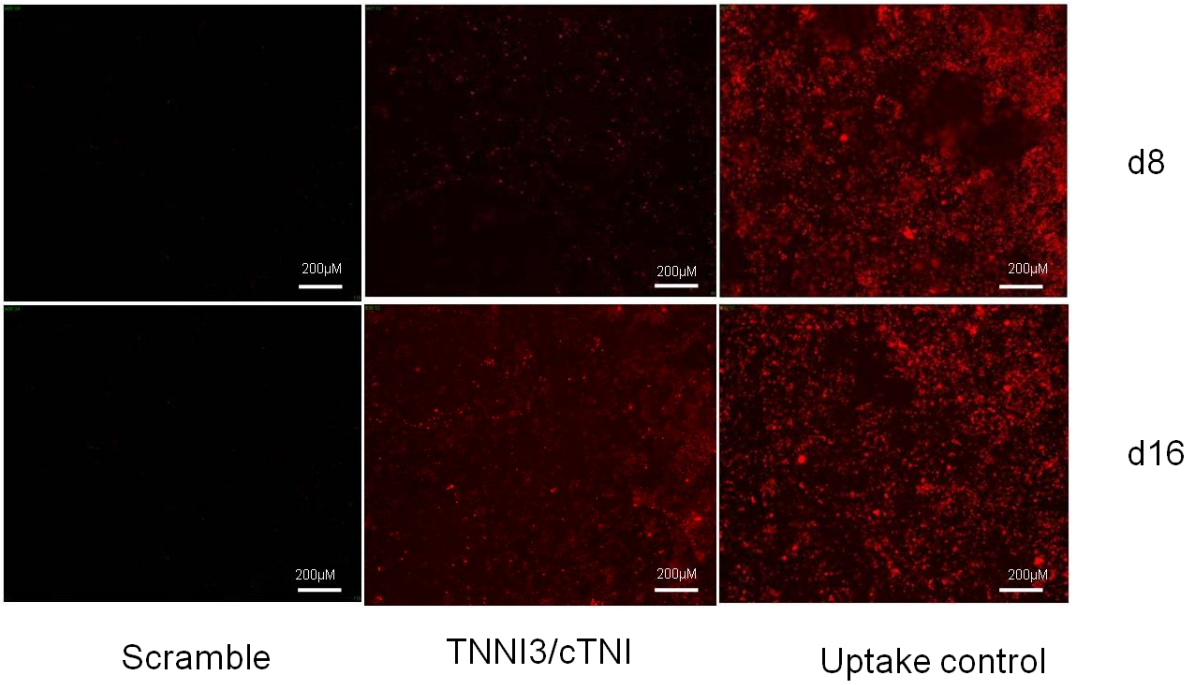

Figure 20. Differentiation of iPSCs to cardiomyocytes stained with SmartFlares™. D8 and d16 of differentiation evaluated using SmartFlares™ probes for cardiac troponin I (TNNI3). Scramble probes are included to get a measurement of background fluorescence and uptake control to see that the cells are able to take up the probes. Photos are captured using a 10x objective.

A prerequisite for use of SmartFlare in live cells are that they are non-toxic. Cell viability was observed based on appearance and contraction of the cells, and it was concluded that no difference could be observed between normal cells and cells incubated with SFs (data not shown).

A new differentiation was thereafter initiated and the cells were incubated with 100pM and 200pM SFs for 24h d3-4 of differentiation. It has been shown that cardiomyocytes differentiated from iPSCs with the Lian protocol have a small number cTNT positive cells d4 of differentiation (Pernevik, 2015). It is desired to see if the SF probes can be used with the FACS sorting unit to isolate a positive population for the probe used. The cells were sorted using FACS into RLT for further RT-qPCR analysis. The results from the PCR are shown in Figure 21 . Labels on x-axis from left; the whole d4 cell population, cells incubated with 100pM cTNI SF and sorted on the negative cTNI population, cells incubated with 100pM cTNI SF and sorted on the positive cTNI population, cells incubated with 200pM cTNI SF and sorted on the negative cTNI population and cells incubated with 200pM cTNI SF and sorted on the positive cTNI population. GATA4 and Nkx2.5 represent cardiac progenitors, cTNT and cTNI cardiomyocytes and CD31 endothelial cells.

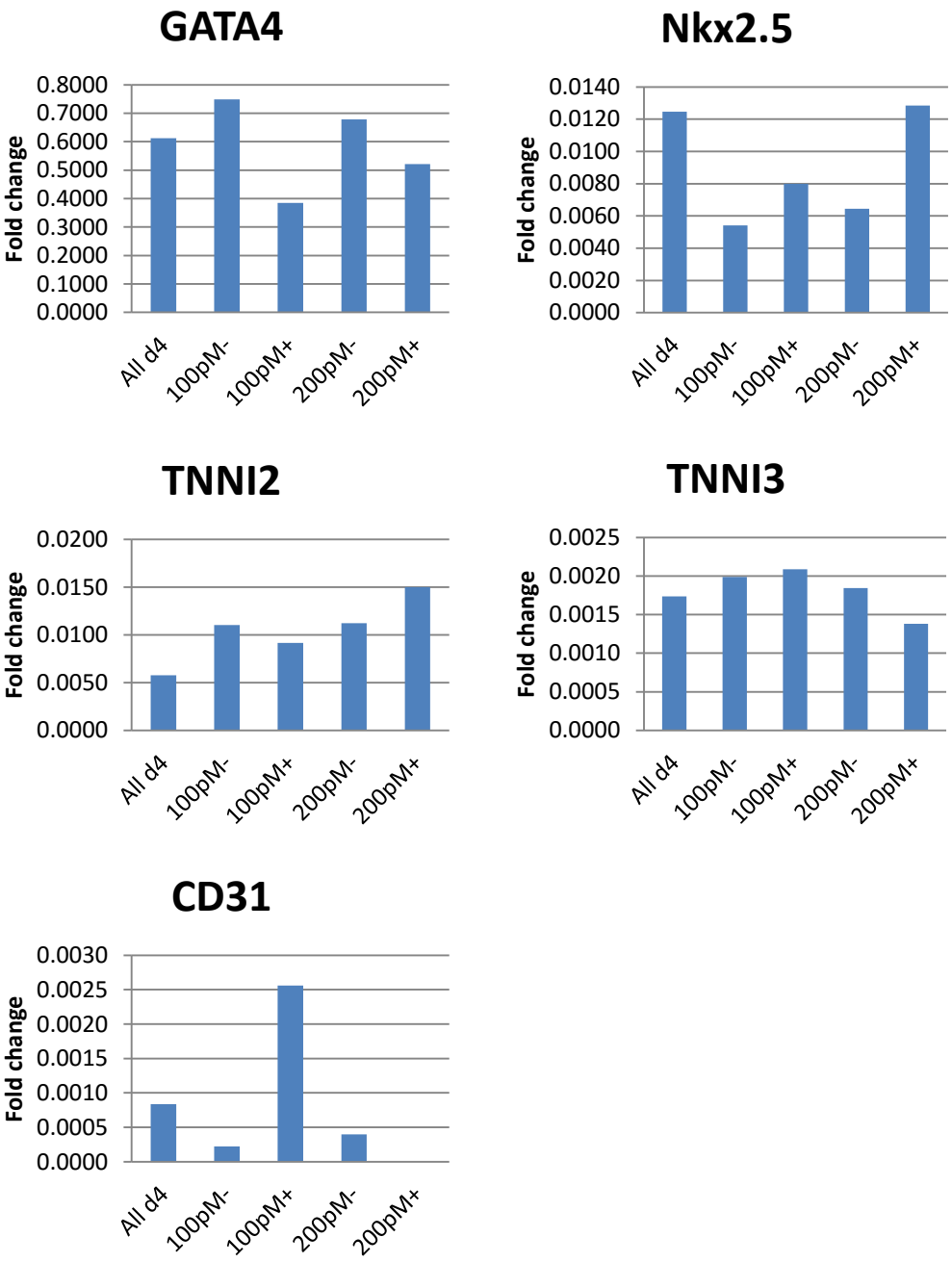

Figure 21. RT-qPCR results from iPSCs differentiated towards cardiomyocytes at day 4 of differentiation sorted using TNNI3 SF probe. Labels on x-axis from left; the entire cell population, cells incubated with 100pM TNNI3 SF and sorted on the negative population, cells incubated with 100pM TNNI3 SF and sorted on the positive population, cells incubated with 200pM TNNI3 SF and sorted on the negative population and cells incubated with 200pM TNNI3 SF and sorted on the positive population. GATA4 and Nkx2.5 represent cardiac progenitors, TNNI2 (aka TNT) and TNNI3 (aka cTNI) cardiomyocytes and CD31 endothelial cells. Two replicates were used for each sample and gene.

mRNA levels between genes cannot be compared, so only conclusions about relative expression within one gene can be drawn. The result from cTNI shows that the 200pM+ population contain lower mRNA expression relative the 200pM- population. The 100pM- and 100pM+ populations have very similar expression for cTNI. More difference can be seen for cTNT, in which the 200pM+ population contain a higher relative amount of mRNA than the 200pM- population. The 100pM- and 100pM+ populations are quite similar also in this case. The endothelial marker

CD31 has relatively high mRNA expression in the 100pM+ population and low in all the others. The GATA4 mRNA expression is relatively high and very similar to each other for 100pM- and 200pM- and a decrease can be seen for both 100pM+ and 200pM+. The opposite is seen for Nkx2.5, namely relative high mRNA expression for the positive populations and a lower, similar to each other, expression for the negative ones.

5. Discussion

The laboratory work in this project includes three main sections. They consisted of an antibody evaluation for large formats, culture of biopsy cells in different conditions and a SmartFlare™ evaluation for both iPSCs, cardiomyocytes differentiated from iPSCs and sorting of differentiated cells.

ICC is usually performed in 96w- or 384w-formats to decrease the amount of antibody used and minimize the time of imaging and image analysis. The C-kit+CD45- subpopulation constitute less than 1% of the cell population in a biopsy from the right atrium (Sandstedt et al, 2014), and culture in small formats will most likely get none or very few of the correct cell type in each well. A 6-well format increases the chance of getting as many C-kit+CD45- cells as possible in each well and still being able to use an image based analysis method. The main issue is cost since primary antibodies are expensive. It is therefore not realistic to use the same dilution used for a 96-well (surface area 0.3cm^2) with 100% positive cells for a 6-well (surface area 9.6cm^2) were the expected number of positive cells are only a few percentages. The antibody evaluation was therefore performed using model systems with small percentages of positive cells to evaluate if the same amount of antibody used for a 96-well could stain the same number of positive cells, but in a much larger volume. As can be seen in Figure 9, Figure 10 and Figure 12, the small amount antibodies used were sufficient to locate and mark positive cells in all of the three co-culture model systems used. This indicates that it is possible to use small antibody amounts in large volumes and still be able to mark a small amount of positive cells. It can be seen in Figure 12 that the HUVECs most probably grows in clusters and maybe also have gained a more stretched out morphology due to the co-culturing if comparing the scale bars between HUVECs alone and the co-culture system. The co-culture system was only developed to have model systems for biopsies, and no further optimization of them were performed since the result was satisfying. It was observed that Nkx2.5 expression was lower than cTNT expression in all iPSC derived cardiomyocytes. However, this can be expected since Nkx2.5 is an early marker for cardiac differentiation and that the cardiomyocytes had been in culture for a couple of weeks and might have lost most of the Nkx2.5 expression due to maturity.

Heart tissue from a maze operation was obtained and the large amount of tissue enabled a larger pilot study were several different conditions and differentiation protocols could be evaluated. Culture conditions for the 8 plates were obtained based on experiences from both Sahlgrenska University Hospital and from AstraZeneca. The differentiation protocols from Lian *et al* and Kattman *et* al were chosen based on success from using them on iPSCs. The protocol from the van Berlo group was found during literature studies and was chosen based on its simplicity. As can be seen in table 15, most of the conditions resulted in zero or some sporadic positive regions of interest regarding C-kit, cTNT and Nkx2.5 positive cells. However, only 1/10 of the well areas were imaged due to practical reasons and more positive cells might therefore exist. The lack of C-kit positive cells might be that they have already differentiated into other cell types. However, most conditions induced formation of vascular structures and endothelial commitment. The only condition resulting in more than one ROI was plate 2 (expanded in NutriStem medium) in combination with the van Berlo protocol. NutriStem is a medium used for maintenance of pluripotency in iPSC culture and it might be that NutriStem as an expansion medium maintain the pluripotency of the cardiac stem cells as well. NutriStem resulted in fewer cells that attached to the surface after dissociation, and for the two other protocols the cell growth never got

started again. But when using the van Berlo protocol for differentiation, the cells proliferated in quick pace and gained 100% confluency as all the other conditions when time for fixation. The cell population targeted in this thesis is the C-kit+CD45- population, but the positive cells might also come from other progenitor populations in the heart as well. The origin of the positive cells was not evaluated in this project, but is an interesting question for further studies. The cells stained positive for cTNT should not be remaining cardiomyocytes from the biopsy, since those cells were excluded during the dissociation to avoid false positives.

The culture system used for this project included growing a heterogeneous cell population consisting of all cells from a heart biopsy except cardiomyocytes since they are destroyed during dissociation and also excluded by use of a cell strainer. The heterogeneous population makes it crucial to identify an effective method that can be used for live-cell isolation of the cells of interest. One such method is the new SmartFlare™ (SF) technology. This technology has not been used before in either of the groups this project was carried out within, but it has been shown to be effective for detection of pluripotency markers in embryonic cells and IPSCs (Lahm *et al*, 2015). SmartFlares has also been used for detection of cancer stem cells by targeting mRNA that is known for stemness in pluripotent cells (McClellan *et al*, 2015). The first step was therefore evaluation of the method in iPSCs to gain more knowledge about the procedure, since the probes have been successful in that cell type in previous studies. The following factors need to be considered according to the manufacturer when evaluating SmartFlares for a new cell type: 1) do the cells take up SmartFlares (endocytosis) 2) SF concentration 3) SF exposure/incubation time and 4) background noise.

OCT4 is known to be highly expressed in the iPSC line used, and is continuously monitored by FACS as a quality control. OCT4 expression was therefore used as positive control and compared to expression of the uptake control. 200pM and 400pM final concentrations of SF probes were incubated with iPSCs for 24h. As can be seen in Figure 16 and Figure 17, both concentrations show similar signal when comparing uptake control probe and OCT4 probe. It is very promising that the OCT4 expression and uptake control signal are so similar due to the fact that this is the expected outcome since OCT4 should be highly expressed. iPSCs are thus able to take up SF probes and OCT4 can be used as a highly expressed positive control. When comparing the peak intensities for the 200pM and 400pM samples, it can be seen that they are relatively similar. However, the 400pM concentration resulted in a larger overlap of the scramble and OCT4/uptake control expressions (larger area under the curves), indicating that the background fluorescence increases when using the higher SF concentration. This might result in higher number of false positives in events such as sorting. 400pM was therefore excluded from the following experiments to reduce that possibility and also to reduce cost.

A 24h incubation time is convenient, but Merck Millipore recommends that the incubation time should be between 16-24h and should be optimized to best suit the cell type used. This evaluation can be seen in Figure 18 and shows that 24h incubation results in higher expression intensity and also lower background signal (smaller area under the curves) than incubation for 16h. It was therefore concluded that 24h incubation is most optimal for use of SF in iPSCs.

Thereafter, iPSCs was differentiated to cardiomyocytes using the Lian protocol to evaluate the cardiomyocyte marker cTNI/TNNI3. However, there were problems with the protocol and several attempts were carried out before contracting cardiomyocytes were obtained. The

problem was that the cells detached from the wells during media change second week of differentiation. The problem was identified as low confluency of the iPSC prior to seeding. The iPSCs were seeded at a high concentration (312k/cm^2) when preparing for differentiation, but the iPSC starting material was not 100% confluent. High confluency probably halts proliferation of the cells and the lower confluence most likely resulted in the cells continuing to proliferate and therefore getting a too high confluency after one week of differentiation, making them very sensitive. The movement from the fluid when changing medium was therefore strong enough to detach the cells. The differentiation was successful when using highly confluent iPSCs from the start. There were some problems with robustness and yield of contracting cardiomyocytes after that, but the problem with detachment of cells was never experienced again. SF probe was added to the cells at two different time points of differentiation to see if a difference in cTNI expression could be detected. It is expected that the cTNI expression increases as differentiation proceeds. Both 100pM and 200pM concentrations were tested. Data from 100pM incubation is not shown since it gave similar results as 200pM, but in a much lower intensity. The results from 200pM incubation are shown in Figure 20 and the difference in cTNI expression can clearly be seen. The images were obtained in the middle of the wells, but the cells mainly aggregate and contract in the edges of the wells. A higher cTNI expression would therefore most likely be observed if the images would have been obtained from the edges, but the relative intensity between the two different time points should still be the same, just a bit higher. The uptake controls are similar for both days indicating that differentiated iPSCs are able to take up SF probes. Different wells were used for the two days to avoid background signaling from remaining SF probes.

The last experiment was sorting of a small population of cTNI-positive cells to imitate the conditions that will be when sorting out cardiac committed cells from a heart tissue biopsy. It has been shown that cells differentiated with the Lian protocol show a low expression of cTNT positive cells at d3-4 of differentiation (Pernevik, 2015) and such cells were therefore used for this purpose. The sorted cells were analyzed using RT-qPCR (Figure 21). It would be desired that the 100pM+ and 200pM+ samples show mRNA expression of cTNI, while the 100pM- and 200pM- samples contain low levels of cTNI mRNA. However, that is not the case. cTNT and cTNI mRNA expression is relatively similar between the samples. However, a quite narrow gating was used to make sure that the positive populations should not include false positives. It is therefore reasonable that a relatively high number of positive cells were included in the negative populations. Optimization of gating should therefore be conducted. GATA4 and Nkx2.5 are both markers for cardiac progenitors cells and have high relative expression in the entire population, which is expected. However, the two positive populations have a lower expression relatively the negative populations for GATA4 mRNA expression and vice versa for Nkx2.5 which is peculiar. All populations except the 200pM+ one also express endothelial mRNA (CD31). This is not desired when trying to differentiate cells to cardiomyocytes, but it is possible that some of the iPSCs were spontaneously differentiated to other origins, such as an endothelial fate. This might be due to the high initial seeding used, which might put stress on the cells and thus driving them towards other fates instead of the cardiac fate that is desired. It has been problems reaching high yields of contracting cardiomyocytes using the Lian protocol, and this might be one of the explanations. It is difficult to draw conclusions from the results, more than that the sorting steps need to be further optimized. It might be better to use a more homogenous population such as fibroblasts with a low percentage of cardiomyocytes for this purpose to get a better controlled testing platform.

5. Conclusion

The main goal of this project was to optimize the conditions for culture of heart biopsy cells to expand the small resident progenitor population within. ICC was used as analysis technique and it was shown that small percentages of positive cells can be detected using small amounts of antibodies. Expansion in NutriStem and differentiation using the van Berlo protocol was most successful in generating cardiac committed cells.

The SmartFlare™ method was evaluated in iPSCs and it was shown that iPSCs are able to take up the probes and that OCT4 can be used as a highly expressed positive control for these cells. A concentration of 200pM and 24h incubation was most optimal. Cardiomyocytes differentiated from iPSCs could also take up the SF probes and could detect different mRNA expression of cTNI during differentiation. It was also concluded that SFs can be used for sorting, but that the sorting step needs to be optimized.

This work shows that it is possible to expand and detect small progenitor populations using different culture conditions and that SmartFlares™ is a promising method for isolation and characterization of such cells.

6. Future work

The work done in this project has only scratched the surface in the subject and there is much that can be done to continue developing this approach. It is important to optimize the SmartFlare isolation step to being able to isolate as many of the rare cells as possible. The culture system used for culturing of heart biopsy cells in this study consist of a heterogeneous population with a low percentage of cardiac committed cells and it is therefore crucial to have an effective method for sorting out these cells to be able to further expand and analyse them. Steps that can be further optimized regarding isolation with SmartFlares include incubation time and concentration of probes. A longer incubation time with SmartFlares can increase the amount of cardiac cells detected but might also result in a higher number of false positive cells. A shorter incubation time can decrease the number of false positive cells but might lead to that some of the positive cells are not detected at all. The same goes for higher or lower concentration of SmartFlares; a high concentration can detect more cells but increases false positives, while a low concentration reduces false positives but might not detect all of the positive cells. It would also be interesting to do similar experiments as with the antibody evaluation, namely to mix a negative population (such as fibroblasts) with a known percentage of positive cells (such as cardiomyocytes differentiated from iPSCs) and then try to optimize sorting based on those cells. This could show if all of the positive cells can be isolated since their number is known. This would also show if it is possible to isolate and further cultivate rare positive cells, which could later be utilized on cells from human heart biopsies. The next step following optimized sorting would be to culture biopsy cells in the condition found most optimal in this work, sort them and then characterize that population with RT-qPCR to increase knowledge about the cell population. It would also be possible to further optimize growth conditions for cardiac biopsies using factorial experimental design (FED) to be able to test a larger number of conditions on the limited material available from each heart biopsy. A FED model with more conditions might contribute to identification of factors that are the most important for successful expansion of resident cardiac stem cells. A long-term goal could be to expand the cells after isolation and then transfer them to a scaffold for 3D-culturing, but that requires a large amount of cells. For this to be possible, a more optimal culture system is required as well as more effective isolation- and expansion steps to reach high enough cell numbers.

There were some problems with the protocol used for differentiation of iPSCs into cardiomyocytes. Since these cells are valuable as model systems for cardiac progenitors in the heart and can be used for most of the optimization steps, it would be great to further optimize the protocol to have a reliable and stable system that generates cardiomyocytes each time. The importance of confluence was evaluated in this project, but further optimization such as source of iPSCs, passage number of iPSCs and time points for addition of small molecules (CHIR99021 and IWP2) could be further optimized to identify which factors that is the most important for reliable differentiation of iPSCs into cardiomyocytes. It would also be of interest to compare cardiomyocytes differentiated from iPSCs with the isolated cardiac stem cells from heart biopsies, using for example RT-qPCR to compare similarities in gene expression. This could determine the biological relevance of iPSC differentiated cardiomyocytes since they are of great interest for AstraZeneca to use as a model system for testing of medical compounds.

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Appendix I. Materials and Reagents

Materials and reagents used for this project are listed in table 16. Included are catalogue numbers and suppliers as well as the full name of the products used.

Table 16. List of reagents and materials used in this work. Catalog number and supplier are included.

Reagent/Material	Cat. No	Supplier
B27	080085SA	Life Technologies
B27 minus insulin	A1895601	Life Technologies
Bovine Serum ALbumine	A1470-100G	Sigma-Aldrich
CHIR99021	13122	Cayman Chemicals
Collagen I , Rat tail	A10483-01	Life Technologies
Corning® 96 Well Clear Flat Bottom Polystyrene TC-Treated Microplates	3997	Corning
Corning® Primaria™ 6 Well Cell Clear Flat Bottom Surface-Modified Multiwell Culture Plate	353846	Corning
Cover Glass	631-1585	VWR
Corning® Costar® cell culture flasks 162 cm2	CLS3151-25EA	Sigma-Aldrich
Corning® cell culture flasks 75 cm2	CLS430641-20EA	Sigma-Aldrich
DAPI	D1306	Life Technologies
Dexamethasone	D4902	Sigma-Aldrich
DMEM:F12, no gluatmine	21331-020	Life Technologies
DNase I	10104159001	Roche
EDTA	155575-038	Life Technologies
EGM®-2 MV SingleQuots®	CC-4147	Lonza
EMB®-2	CC-3156	Lonza
Ethylenediaminetetreaacetic acid	E6758-100G	Sigma
Falcon® 96 Well Black with Clear Flat Bottom TC-Treated Imaging Plate	353219	Corning
Formaldehyde 4%	02176	HistoLab®
GlutaMax	35050038	Life Technologies
HBSS	14175-053	LifeTechnologies
IWP2	681671	CalbioChem
KO-DMEM	829018	Life Technologies
Liberase TH	05401151001	Roche
L-glutamine 200mM	G7513	Sigma
LN-211	Laminin 211 cell culture	Biolamina
LN-221	Laminin 221 cell culture	Biolamina
LN-521	human rLaminin-521™	Biolamina
Matrigel NutriStem™ XF/FF Culture Medium	356230 lotnr 4174004 01-0005	Corning Stemgent

PBS -/-	14190094	Life technologies
PBS +/+	14190091	Life technologies
PEST 100x	SV30010	Thermo Scientific
Phospho-L-Ascorbic acid	49752	Sigma-Aldrich
ProLong® Gold antifade reagent with DAPI	P36935	Life Technologies
RPMI 1640X GlutaMax	61870010	Life Technologies
SB431542	S4317	Sigma-Aldrich
Selenium	S5261	Sigma-Aldrich
Transferrin	T3309	Sigma-Aldrich
Triton®-X 100	107K0065	Sigma-Aldrich
Trypsin-EDTA (0.05%)	25300-054	Life Technologies
Y27632 (Rock inhibitor)	68800	CalbioChem