# THE POTENTIAL OF DIRECT CARDIAC REPROGRAMMING IN CARDIAC REGENERATION

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Tiivistelmä/Referat – Abstract

Heart failure is a major public health problem and a leading cause of mortality worldwide. The most common cause of heart failure is myocardial infarction. Following a myocardial infarction, a large number of cardiomyocytes die and cardiac muscle is replaced by fibrotic scar tissue. Since the adult heart has inadequate endogenous regenerative capacity, loss of muscle tissue often causes a progressive decrease in cardiac function eventually leading to heart failure. At the moment heart transplantation is the only curative treatment for heart failure, but the low number of donor hearts is limiting the use of this treatment option. As current drugs only slow down the progression of the disease, there is a great need for new regenerative treatments.

Direct cardiac reprogramming is a new approach for generating cardiomyocytes for cardiac regeneration. Unlike pluripotent stem cell-based strategies, direct reprogramming enables conversion of a terminally differentiated cell type directly into another cell type without first producing a pluripotent intermediate. Due to their abundancy and role in the repair of myocardial injury, fibroblasts represent an attractive starting cell type for direct cardiac reprogramming. Fibroblasts have been directly reprogrammed to induced cardiomyocytes (iCMs) by overexpression of key cardiac transcription factors, microRNAs (miRNA) or by modulating specific signal transduction pathways with small-molecule compounds. Despite successful reports of direct reprogramming both *in vitro* and *in vivo*, the efficiency of direct reprogramming remains, however, too low for potential clinical applications.

The aim of this M.Sc. thesis work was to establish direct reprogramming of mouse embryonic fibroblasts (MEFs) to iCMs by viral overexpression of cardiac transcription factors Hand2 (H), Nkx2.5 (N) Gata4 (G), Mef2c (M) and Tbx5 (T) and a small-molecule compound screening platform for identifying small-molecule compounds that could enhance the reprogramming efficiency and potentially replace cardiac transcription factors in direct cardiac reprogramming. In accordance with previous publications MEFs were successfully directly reprogrammed to iCMs using both HGMT and HNGMT cardiac transcription factor combinations. The screening platform was tested using the TGF-β inhibitor SB431542, which has recently been reported to increase the cardiac reprogramming efficiency. In line with previous publications, the reprogramming efficiency was significantly increased by treatment with SB431542. Initial tests with other small-molecule compounds did not have a positive effect on the reprogramming efficiency.

The results of this M.Sc. thesis work verify previous publications and demonstrate a method for *in vitro* small-molecule compound screening, which can be used to identify compounds that increase the reprogramming efficiency in direct cardiac reprogramming. However, the results shown here are only preliminary and more replicates are needed in order to confirm the current results. Nonetheless, the results of this thesis work set a foundation for finding small-molecule compounds that in the future might be used to target direct cardiac reprogramming as a regenerative therapy for myocardial infarction and heart failure.



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Tiivistelmä/Referat – Abstract

Sydämen vajaatoiminta on merkittävä yhteiskuntaa kuormittava terveysongelma ja yksi yleisimpiä kuolinsyitä maailmassa. Sen taustalla on yleensä sydäninfarkti. Infarktin seurauksena osa sydämestä joutuu hapenpuutteen vuoksi kuolioon ja tuhoutunut sydänlihas korvautuu arpikudoksella. Aikuisen ihmisen sydämen uusiutumiskyky on rajallinen, minkä vuoksi sydänlihasvaurio yleensä johtaa etenevään sydämen pumppaustehon heikkenemiseen ja lopulta vajaatoimintaan. Sydämensiirto on toistaiseksi ainoa parantava hoitovaihtoehto sydämen vajaatoimintaa sairastaville, mutta elinluovuttajien vähäinen määrä rajoittaa sen käyttöä. Koska nykyiset käytössä olevat lääkkeet vain hidastavat taudin etenemistä, tarve uusille parantaville hoitomuodoille on suuri.

Solujen suora uudelleenohjelmointi sydänlihassoluiksi (engl. direct cardiac reprogramming) on uusi menetelmä, jolla pystytään tuottamaan sydänlihassoluja muista solutyypeistä sydämen regeneraatiota varten. Toisin kuin monikykyisiin kantasoluihin perustuvat menetelmät, solujen suora uudelleenohjelmointi mahdollistaa jo erilaistuneen solutyypin muuntamisen suoraan toiseksi erilaistuneeksi solutyypiksi ilman, että se ensin muodostaa monikykyisen kantasoluvälituotteen. Fibroblastit ovat houkutteleva solutyyppi solujen suorassa uudelleenohjelmoinnissa sydänsoluiksi, johtuen niiden runsaasta määrästä sydämessä sekä niiden roolista sydänlihasvaurion korjautumisessa. Fibroblasteja on onnistuttu uudelleenohjelmoimaan suoraan sydänsoluiksi yli-ilmentämällä sydänsoluille tyypillisiä transkriptiotekijöitä tai mikro-RNA:ita (miRNA) tai vaikuttamalla tiettyihin signalointireitteihin pienmolekyyleillä. Siitä huolimatta, että solujen suora uudelleenohjelmointi sydänsoluiksi on onnistunut sekä *in vitro* että *in vivo*, menetelmän tehokkuus on edelleen liian heikko kliinisiä sovelluksia varten.

Tämän pro gradututkimuksen ensimmäisenä tavoitteena oli onnistua hiiren alkion fibroblastien (MEF-solujen, mouse embryonic fibroblasts) suorassa uudelleenohjelmoinnissa indusoiduiksi sydänsoluiksi (iCMs) yli-ilmentämällä transkriptiotekijöitä (Hand2 (H), Nkx2.5 (N) Gata4 (G), Mef2c (M) ja Tbx5 (T)) virusvektoreilla. Toisena tavoitteena oli kehittää pienmolekyylien seulontamenetelmä, jonka avulla voitaisiin löytää pienmolekyylejä, jotka lisäävät suoran uudelleenohjelmoinnin tehokkuutta ja jotka voisivat mahdollisesti korvata transkriptiotekijöiden käytön suorassa solujen uudelleenohjelmoinnissa sydänsoluiksi. MEF-solujen uudelleenohjelmointi suoraan indusoiduksi sydänsoluiksi onnistui sekä HGMT- ja HNGMT- transkriptiotekijöiden yhdistelmillä. Nämä tulokset ovat linjassa aikaisempien tutkimustulosten kanssa. Pienmolekyylien seulontamenetelmää testattiin käyttämällä transformoivaa kasvutekijä-β (engl. tranforming growth factor beta, TGF-β)-estäjää SB431542:ta, jonka on hiljattain raportoitu lisäävän tehokkuutta solujen suorassa uudelleenohjelmoinnissa sydänsoluiksi. Aikaisempien tutkimustulosten kaltaisesti SB431542 lisäsi merkittävästi suoran uudelleenohjelmoinnin tehokkuutta. Alustavissa seulontakokeissa muut tutkittavat pienmolekyylit eivät lisänneet uudelleenohjelmoinnin tehokkuutta.

Tämän tutkimuksen tulokset vahvistavat aikaisempia tutkimustuloksia. Työssä kehitettiin *in vitro* pienmolekyylien seulontamenetelmä, jonka avulla voidaan tunnistaa pienmolekyylejä, jotka lisäävät tehokkuutta suorassa solujen uudeleenohjelmoinnissa sydänsoluiksi. On kuitenkin huomioitava, että tässä työssä esitetyt tulokset ovat alustavia ja lisää tutkimuksia tarvitaan, jotta ne voidaan vahvistaa. Tutkimuksen tulokset luovat kuitenkin pohjaa uusien pienmolekyylien löytämiselle, joita tulvaisuudessa mahdollisesti voidaan käyttää solujen suorassa uudelleenohjelmoinnissa sydänsoluiksi sydäninfarktin tai sydämen vajaatoiminnan hoidossa.



Avainsanat – Nyckelord – Keywords
suora solujen uudelleenohjelmointi, sydämen regeneraatio, fibroblasti, sydänlihassolu, lääkekehitys, lääkeseulonta

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ATTACHMENTS

ABBREVIATIONS

| | |
|---|---|
| AC | Adenylyl cyclase |
| AGM | Astrocyte growth medium |
| ALK | Activin receptor-like kinase |
| BMP | Bone morphogenetic protein |
| CF | Cardiac fibroblast |
| CFP | Cyan fluorescent protein |
| cTnT | Cardiac Troponin T |
| DF | Derman fibroblast |
| DMSO | Dimethyl sulfoxide |
| E | Embryonic day |
| EB | Embryonic body |
| EC | Endothelial cell |
| EF | Ejection fraction |
| ERK1 | Extracellular signal-regulated kinase 1 |
| FF | Foreskin fibroblast |
| FGF | Fibroblast growth factor |
| GATA | GATA binding protein |
| GFP | Green fluorescent protein |
| GMT | Gata4, Mef2c andTbx5 |
| GSK3 | Glycogen synthase kinase 3 |
| Hand | Heart- and neural crest derivatives-expressed protein |
| hESC | Human emryonic stem cell |
| HF | Heart failure |
| HGMT | Hand2, Gata4, Mef2c and Tbx5 |
| hiPSC | Human induced pluripotent stem cell |
| HMTase | Histone-lysine methyltransferase |
| HNGMT | Hand2, Nkx2-5, Gata4, Mef2c and Tbx5 |
| iCM | Induced cardiomyocyte |
| IGF1 | Insulin-like growth factor 1 |

| | |
|---|---|
| iPSC | Induced pluripotent stem cell |
| JAK | Janus kinase |
| LSD1 | Lysine specific histone demethylase 1 |
| MAPK | Mitogen-activated protein kinase |
| MEF | Mouse embryonic fibroblast |
| Mef | Myocyte-specific enhancer factor |
| MI | Myocardial infarction |
| miRNA | Micro-RNA |
| Myocd | Myocardin |
| mTORC1 | Mitochondrial target of rapamycin complex 1 |
| Nkx | NK homeobox protein |
| PBS | Bhosphate buffered saline |
| PDGFR | Platelet-derived growth factor receptor |
| PI3K | Phosphoinositol 3-kinase |
| PSC | Pluripotent stem cell |
| Ras-GAP | Ras GTPase-activating protein |
| ROCK | Rho-associated coiled coil forming protein serine/threonine kinase |
| SB | SB431542 |
| SMC | Smooth muscle cell |
| Tbx | T-box transcription factor |
| TF | Transcription factor |
| TGF- β | Transforming growth factor- β |
| TTF | Tail tip fibroblast |
| VEGF | Vascular endothelial growth factor |
| α-MHC | α-myosin heavy chain |

# 1 INTRODUCTION

Heart failure (HF) is a major public health problem and a leading cause of mortality worldwide (Bui et al. 2011). It affects over 23 million people in the world and the prevalence is increasing due to the aging population, causing considerable health care costs. At the moment heart transplantation is the only curative treatment for HF, but the low number of donor hearts is limiting the use of this treatment option (Doppler et al. 2015). As current drugs only slow down the progression of the disease, the mortality risk 5 years after diagnosis of HF is still 45–60%. Thus, there is a great need for new regenerative treatment options (Bui et al. 2011).

The most common cause of HF is a myocardial infarction (MI) (Bui et al. 2011). After an acute myocardial infarction, a large number of cardiomyocytes die due to ischemia, which causes an inflammatory reaction (Travers et al. 2016). As a response, cardiac fibroblasts (CFs) are activated and start proliferating and transdifferentiate to myofibroblasts that secrete increased levels of collagen and other extracellular matrix proteins. This ultimately leads to the formation of fibrotic scar tissue and pathological remodelling of the myocardium, which includes hypertrophic thickening of the left ventricular wall and fibrosis. The purpose of this CF-mediated response is to maintain the pressure-generating capacity of the heart, but since the adult heart has inadequate endogenous regenerative capacity, it often causes a progressive decrease in cardiac function eventually leading to HF.

Replacing lost cardiomyocytes with new ones is a major objective in the field of regenerative medicine and a potential strategy for developing new treatments for MI and HF (Doppler et al. 2015). Direct cardiac reprogramming is new a strategy for cardiac regenerative therapy that targets CFs. As CFs play a major role in the pathogenesis of HF, they are an attractive target for future HF therapies (Travers et al. 2016). This M.Sc. thesis work discusses the recent progress in cardiac regeneration and the potential of direct reprogramming of fibroblasts to cardiomyocytes as a future cardiac regenerative therapy.

# 2 LITERATURE REVIEW

## 2.1 Cardiac development as the basis for cardiac regeneration

The human body has a limited capacity to repair tissue and organs following injury and the heart is one of the least regenerative organs in the body (Laflamme and Murry 2011). The field of regenerative medicine aims to restore function of damaged tissue by replacing lost cells. Direct cardiac reprogramming aims to generate new cardiomyocytes as a potential treatment of MI or HF (Sahara et al. 2015). Understanding the major processes driving the development of cardiomyocytes and formation of the heart is central in developing future cardiac regenerative therapies such as direct reprogramming.

The mammalian heart is a complex and highly specialised organ and the formation of the heart, cardiogenesis, is a highly controlled process that requires generation of a diverse set of both muscle and non-muscle cells (Brade et al. 2013). During cardiogenesis embryonic stem cells commit to the cardiac cell fate and undergo cardiac differentiation, ultimately forming the heart. Cardiac cells are derived from a common mesodermal progenitor cell population that during gastrulation divides into two different cardiac progenitor populations called the first heart field and the second heart field (figure 1) (Meilhac et al. 2004, Buckingham et al. 2005). Also progenitor cells from the proepicardial organ and cardiac neural crest cells contribute to the formation of the heart.

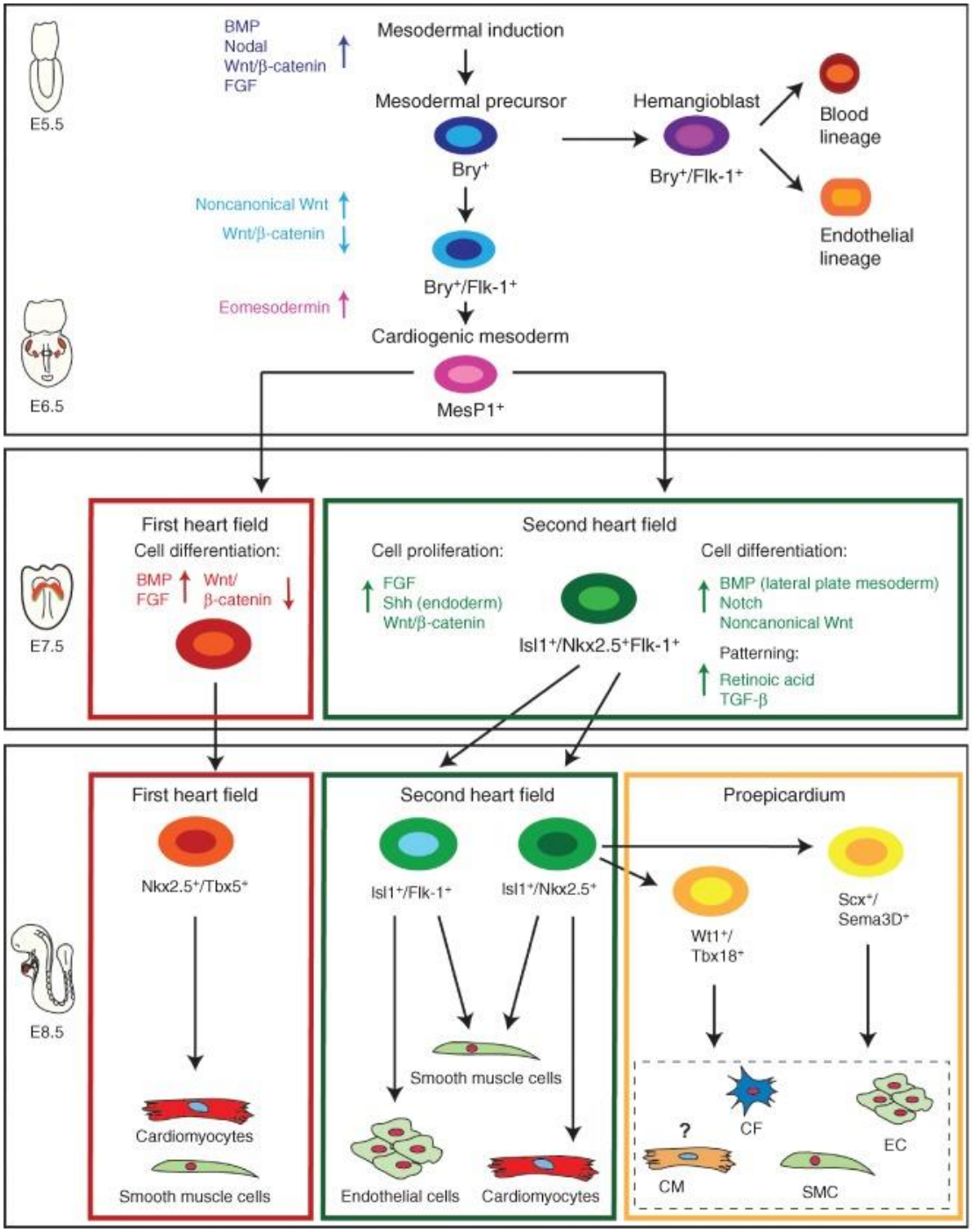


Figure 1. Schematic of cardiac progenitor cells, lineage specification and the major signalling pathways governing cardiac development in mouse embryonic development. Cardiac cells are derived from a common mesodermal progenitor cell that during gastrulation divides into two different cardiac progenitor populations, the first and the second heart field. These progenitors give rise to cardiomyocyes (CM) that contribute to distinct parts of the heart. In addition to the two heart fields also cells derived from the proepicardium contribute to the formation of the heart including cardiac fibroblasts (CF), smooth muscle cells (SMCs) and endothelial cells (EC) (Brade et al. 2013).

Much is already known about the processes governing cardiac development, but there are still many aspects that remain unclear. Further uncovering the basic biology underlying cardiac development, cell lineage commitment and cardiac differentiation will be crucial in developing direct reprogramming into a future cardiac regenerative therapy. The following chapter discusses fundamentals of cardiac development, focusing on aspects that are important for understanding direct cardiac reprogramming. Insight into the development of the heart has been gained mostly through animal studies of mouse and chicken embryos.

### 2.1.1 Cell types in the heart

A diverse array of cell types, both muscle and non-muscle, are needed to make up the functional heart (Xin et al. 2013). The muscle wall, myocardium, is formed by atrial and ventricular cardiomyocytes and the cardiac conduction system is formed by highly specialised pacemaker cardiomyocytes, including cells of the sinoatrial node, atrioventricular node and Purkinje fibers. However, less than a third of all the cells in the heart are cardiomyocytes. The inner layer of tissue that lines the chambers, the endocardium, and the interior of blood vessels is formed by endothelial cells, while vascular smooth muscle cells are found in the coronary arteries and veins. Fibroblasts, which are connective tissue cells, were previously thought to be the most abundant cell type in the heart. Recent studies however suggest that fibroblasts only account for about 20% of the non-myocyte cells in the heart and endothelial cells are in fact the most abundant cell type (Pinto et al. 2016). All cell types interact with each other through physical contact and paracrine and endocrine factors and contribute to the function and properties of the heart (Tirziu et al. 2010, Xin et al. 2013).

### 2.1.2 Essential signalling pathways during cardiac development

Cardiogenesis is a precise and complex process regulated by a number of signalling pathways and transcriptional regulators (Brade et al. 2013). Cardiac lineage commitment and cardiac differentiation is induced by spatiotemporal intrinsic and extrinsic signals and cross talk between neighbouring tissues. Central signalling pathways that regulate embryonic cardiac development are shown in figure 2. These signals direct cardiac differentiation by inducing the expression of cardiac transcription factor (TF) genes.

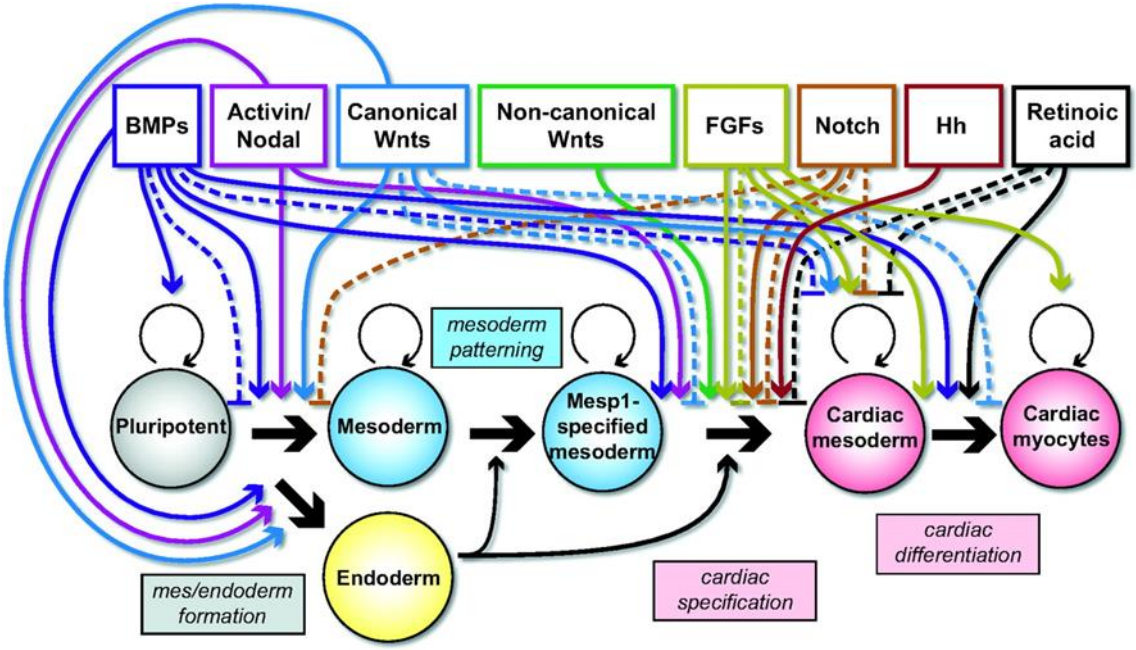


Figure 2. Essential signalling pathways that regulate embryonic cardiac development. Cardiac cell fate determination requires coordination and cooperation of multiple signals, both positive and negative. These signals direct cardiac differentiation by inducing the expression of cardiac transcription factor genes. Solid lines indicate inducing signals, dotted lines indicate inhibiting signals and circular arrows indicate self-renewal (Noseda et al. 2011).

Bone morphogenetic protein (BMP) signalling is an essential driver of cardiac development (van Wijk et al. 2007). BMPs are a subfamily of multifunctional proteins

that belong to the transforming growth factor- β (TGF- β) superfamily. BMP signalling is critical during gastrulation and in early mesoderm formation (Mishina et al. 1995). BMP signalling has also been shown to be essential in cardiac differentiation and maturation of second heart field progenitors (Yang et al. 2006). The role of BMPs in cardiogenesis is conserved in all studied species, indicating their importance for cardiac development (Noseda et al. 2011).

The Activin/Nodal signalling pathway is critical in early vertebrate embryonic development during gastrulation and in the induction of most endodermal and mesodermal cell types, including cardiac cells (Conlon et al. 1994, Schier 2003). Like BMPs, Activin and Nodal are members of the TGF-β superfamily. Activin A, together with BMPs, has been shown to increase the efficiency of cardiac differentiation in human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) (Laflamme et al. 2007, Kattman et al. 2011).

The Wnt signalling pathway plays multiple roles in cardiac development (Gessert and Kuhl 2010). Wnt ligands can be divided into the so called canonical Wnts (Wnt1, -2a, -3a, and -8) that require β-catenin for signalling and to the non-canonical Wnts (Wnt5a and Wnt11). The canonical Wnt/ β-catenin pathway has been suggested to have a biphasic role during cardiogenesis: activation of the pathway is needed for gastrulation and mesoderm formation, while inhibition of the pathway seems to be required for cardiac specification (Ueno et al. 2007, Paige et al. 2010, Sahara et al. 2015). Activation of non-canonical Wnt signalling seems to be required for cardiac specification and differentiation (Sahara et al. 2015).

The fibroblast growth factor (FGFs) pathway regulates a number of biological processes during embryonic development including cell proliferation, differentiation, and migration (Turner and Grose 2010). The mammalian FGF family consists of approximately 20 ligands that bind to 4 transmembrane receptor tyrosine kinases (Fgfr1, Fgfr2, Fgfr3 and Fgfr4). Fgf8 has been shown to be important for the

development of the second heart field in mice (Ilagan et al. 2006). Studies on cultured mesoderm cells also indicate that cooperative signalling of certain BMPs and FGFs are required to induce cardiogenesis in non-precardiac mesoderm (Barron et al. 2000). Furthermore, expression of *Fgf9, Fgf16*, and *Fgf20* in the mouse epicardium and endocardium during embryonic development has been associated with myocardial proliferation and differentiation (Lavine et al. 2005).

Several other signalling pathways in addition to those described above are involved in cardiac development, such as hedgehog signalling, Notch signalling and retinoic acid signalling (Noseda et al. 2011). Cardiac cell fate determination requires coordination and cooperation of multiple signals, both positive and negative. Unveiling signalling pathways that drive cardiac development has been crucial in the development of potential cardiac regenerative therapies (Sahara et al. 2015). Increased understanding of the molecular pathways that regulate cardiac differentiation has led to the development of several strategies to generate cardiomyocytes for cardiac replacement therapies, including direct cardiac reprogramming. These central signalling pathways are also potential drug targets when screening for small-molecule compounds that could increase the efficiency of direct cardiac reprogramming or even replace cardiac TFs altogether.

### 2.1.3 Transcriptional regulation of cardiogenesis

Transcription factors are proteins that control gene expression by binding to specific sequences in DNA and regulating the transcription of target genes to messenger RNA (mRNA) (Kohli et al. 2011). They regulate gene transcription in response to intrinsic or extrinsic signals. Furthermore, certain micro-RNAs (miRNAs) and epigenetic mechanisms, such as chromatin remodelling and histone modification participate in regulating gene expression.

Cardiac TFs are downstream targets of cardiac signalling pathways and they regulate cardiac development by controlling the spatiotemporal expression patterns of cardiac genes (Kohli et al. 2011). The evolutionarily conserved cardiac TF network is essential for cardiac cell fate determination, differentiation and morphogenesis. The core transcriptional network involved in gene regulation during cardiac development includes NK2 homeobox protein 5 (Nkx2.5), Myocyte-specific enhancer factor 2C (Mef2c), Heart- and neural crest derivatives-expressed protein 1 and 2 (Hand1 and Hand2), T-box transcription factor 5 (Tbx5) and GATA binding protein 4 (Gata4). These cardiac TFs have also proven to be essential tools in direct cardiac reprogramming.

Among the earliest cardiac TFs expressed in embryonic cardiac progenitor cells are Nkx2.5, a member of the NK homeobox gene family and Gata4, a member of the double zinc-finger TF family Gata (Lints et al. 1993, Akazawa and Komuro 2005, Kohli et al. 2011). Despite its early expression, Nkx2.5 has been shown to be important in later stages of cardiac development, rather than earlier stages of cardiogenesis (Lyons et al. 1995). The importance of Gata4 in cardiogenesis has been evidenced by loss of function studies: mice embryos lacking normal Gata4 had severe morphogenic defects and were unable to form the linear heart tube, which resulted in lethality between E7.0 and E9.5 (Molkentin et al. 1997). Gata4 has been shown to also physically interact with several other cardiac TFs, including Gata6, Nkx2.5, Hand2, Tbx5 and Mef2c, to cooperatively or synergistically activate cardiac genes (Charron et al. 1999, Morin et al. 2000, Dai et al. 2002, Garg et al. 2003, Zhang et al. 2007, Kinnunen et al. 2015, Luna-Zurita et al. 2016).

Another key cardiac TF is Mef2c, which belongs to the myocyte-specific enhancer factor 2 (Mef2) family (Morin et al. 2000). Mef2 TFs participate in the regulation of skeletal and cardiac muscle cell differentiation and several important myogenic genes have promoters that bind Mef2 TFs (Black and Olson 1998). Loss-of-function studies have shown that Mef2c is essential for cardiac myogenesis and right ventricular development (Lin et al. 1997). Loss of Mef2c function has also been associated with

down-regulation of the gene encoding for the basic helix-loop-helix TF Hand2. Hand2 has together with Hand1 been shown to be essential for the development of the ventricles (Srivastava et al. 1997, Firulli et al. 1998).

Furthermore, the TF Tbx5 is an essential regulator of cardiac development (Plageman and Yutzey 2005). It belongs to the family of T-box containing TFs that are especially important for cardiac lineage determination and the development of the chambers and the conductive system. The importance of Tbx5 in heart development was first discovered when it was shown that a mutation in the *Tbx5* gene is the cause of Holt-Oram syndrome, a genetic disorder characterised by structural defects of the heart and limbs (Basson et al. 1997). In mice lacking both *Tbx5* alleles the expression of several cardiac genes, including natriuretic peptide A (Nppa) and gap junction protein alpha 5 *(Gja5)*, was decreased and severe underdevelopment and defects were observed in the developing heart (Bruneau et al. 2001).

Several other TFs, in addition to the core cardiac TF network, are involved in the regulation of cardiac development. Even though many of the key regulators are well known, the interactions and the way these TFs work together to regulate the development of the heart remains poorly understood. Also, the importance of epigenetic regulation of gene expression is becoming more and more apparent.

## 2.2 Strategies for generating new cardiomyocytes

Replacing lost cardiomyocytes with new ones is a major goal in regenerative medicine and a potential strategy for developing new treatments for HF. There have already been some initial clinical cell transplantation trials with autologous cardiac progenitor cells, but the results from these studies are inconclusive (Yacoub and Terrovitis 2013). The growing field of stem cell research has, however, opened new potential and interesting strategies for cardiac regeneration. The following chapter reviews some of

the current promising strategies for generating new cardiomyocytes for cardiac regeneration (figure 3).

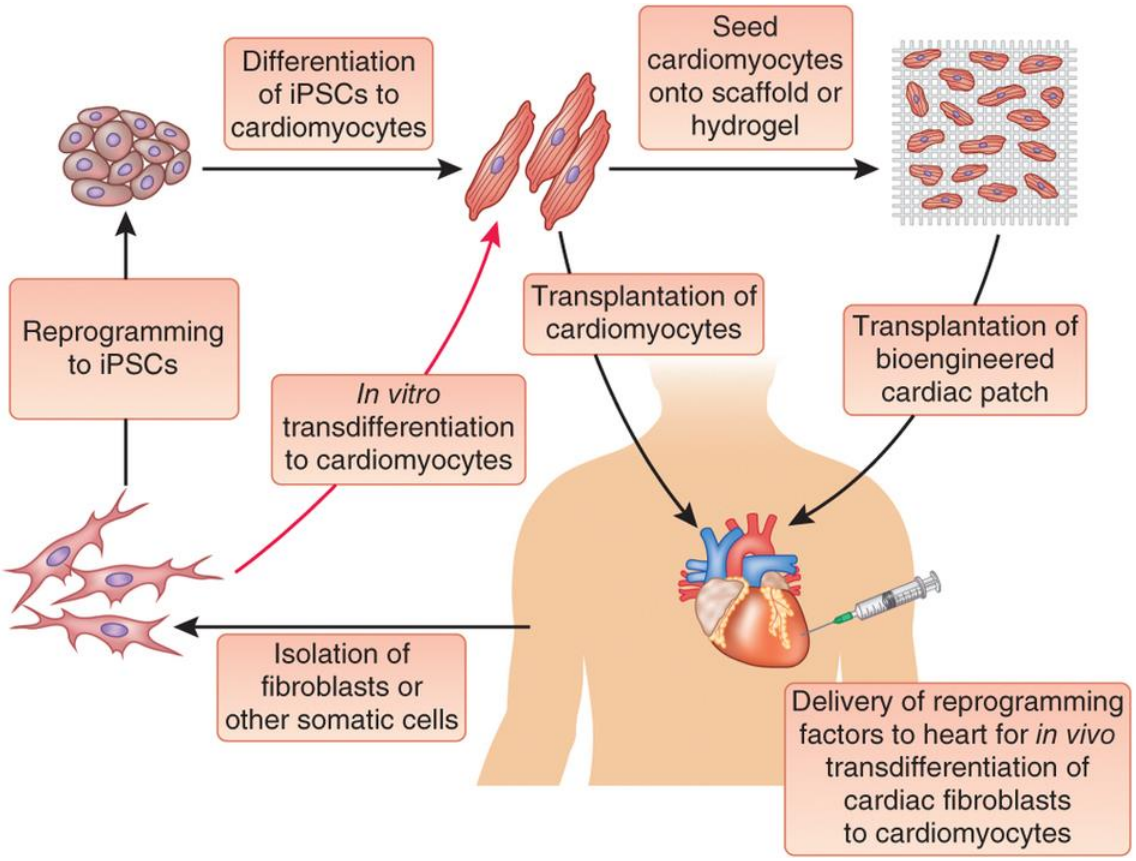


Figure 3. A schematic illustration of different strategies currently being investigated for cardiac regeneration. Following a myocardial infarction, a large number of cardiomyocytes die and cardiac muscle is replaced by fibrotic scar tissue. Since the adult heart has inadequate endogenous regenerative capacity, loss of muscle tissue often causes a progressive decrease in cardiac function eventually leading to heart failure. Therefore, replacing lost cardiomyocytes with new ones is a major goal in regenerative medicine and a potential strategy for developing new treatments for myocardial infarction and heart failure (Addis and Epstein 2013).

### 2.2.1 Stimulating cardiomyocyte proliferation

Adult mammalian cardiomyocytes have a limited regenerative capacity and it has long been thought that cardiomyocytes exit the cell cycle after birth and are unable to proliferate (Sahara et al. 2015). Recently this paradigm has been questioned, as an increasing amount of evidence indicate that mammalian cardiomyocytes are actually

able to renew themselves, but the turnover rate is very slow and inefficient (Bergmann et al. 2009, Porrello et al. 2011). This has provoked great interest in finding ways to stimulate this innate regenerative capacity, as it is a potential strategy for replacing lost cardiomyocytes following cardiac injury.

Regeneration of cardiac tissue and cardiomyocytes has traditionally been studied in lower vertebrates. In contrast to mammals, many amphibians and fish efficiently regenerate cardiac tissue following injury (Sahara et al. 2015). Research in zebrafish has shown that the adult zebrafish heart can regenerate fully after amputation of up to 20% of the ventricle and the regenerative capacity does not decrease with age (Poss et al. 2002, Raya et al. 2003, Itou et al. 2012). Recent lineage-tracing studies demonstrate that these new cardiomyocytes are generated through proliferation of pre-existing mature cardiomyocytes (Jopling et al. 2010, Kikuchi et al. 2010). These studies have also showed that prior to re-entering the cell cycle and proliferating, zebrafish cardiomyocytes undergo partial dedifferentiation by disassembling their sarcomeric structure and start expressing cardiac TF Gata4 and cell-cycle regulators. In most mammals, a large percentage of adult cardiomyocytes are binucleated with very stable sarcomeric structures, which might prevent them from re-entering the cell cycle (Paradis et al. 2014).

Also mammalian cardiomyocytes seem to have some regenerative capacity. Neonatal mouse hearts have been shown to regenerate after partial surgical resection during the first days following birth, but this capacity is lost by the age of 1 week (Porrello et al. 2011). This suggests that mammalian postnatal hearts possess a regenerative capacity, but it is inhibited or turned off in adult cardiomyocytes. Also adult mammalian cardiomyocytes have been shown to have some capacity for cardiomyocyte renewal, but the turnover rate is very low (Bergmann et al. 2009, Mollova et al. 2013, Senyo et al. 2013). Even though the regenerative capacity seems to be slightly increased following cardiac injury, it decreases with age and is too inefficient to regenerate the heart following myocardial infarction. There is also some

uncertainty to the source of these new cardiomyocytes. According to recent studies, pre-existing cardiomyocytes that undergo dedifferentiation and then proliferation are the main source of new cardiomyocytes, but cardiac progenitor cells have also been shown to contribute to cardiomyocyte renewal especially after cardiac injury (Hsieh et al. 2007, Malliaras et al. 2013, Senyo et al. 2013).

These findings suggest that adult mammalian cardiomyocytes also have the capacity to proliferate, but there are some mechanisms that inhibit proliferation in adult cells. Ongoing research is now focusing on finding central signalling pathways that control or inhibit post-natal cardiomyocyte proliferation as they are potential therapeutic targets for cardiac regeneration. One such pathway is the p38 MAPK signalling pathway. Inhibition of the p38 MAP kinase has been shown to be important for cardiomyocyte proliferation in both zebrafish and rats (Engel et al. 2005, Jopling et al. 2012). Inhibition of the homeodomain TF Meis1 has also been shown to stimulate cardiomyocyte mitosis in adult mice (Mahmoud et al. 2013). Certain microRNAs (miRNAs) have also been investigated for their therapeutic potential, including miR-590 and miR-199a (Eulalio et al. 2012).

Although knowledge about the mechanisms involved in cardiac regeneration in zebrafish and neonatal mice has opened new possibilities for stimulating cardiomyocyte proliferation in humans, much is still unknown and research is in early stages. A study by Andersen et al. (2014) was unable to repeat the findings about cardiac regeneration in neonatal mice by Porello et al. (2011), questioning the regenerative capacity of the neonatal mammalian heart. Even though a number of other investigators have been able to confirm the results by Porello et al. (2011) there is still some question to what extent the mammalian heart is capable of regeneration (Sadek et al. 2014).

### 2.2.2 Cardiac differentiation of pluripotent stem cells

Cardiomyocytes can be generated from pluripotent stem cells *in vitro* for transplantation purposes by directed differentiation of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) (Sahara et al. 2015). Embryonic stem cells are cells derived from the inner cell mass of a blastocyst at an early stage of embryogenesis (Dixit and Katare 2015). They are pluripotent, meaning that they have the potential to differentiate to cells of any of the three germ layers (mesoderm, ectoderm, and endoderm) and the capacity to proliferate indefinitely. Human embryonic stem cells (hESCs) were first isolated by Thomson et al. (1998) and have since been used as a source for *in vitro* cardiomyocytes. There are, however, many issues associated with the use of hESCs, such as teratoma formation and potential immune rejection of transplanted allogenic cells (Nussbaum et al. 2007). The use of hESCs has also raised ethical concerns as they are isolated from human embryos (Garbern and Lee 2013).

In 2006 Takahashi and Yamanaka made the discovery that fully differentiated mouse fibroblasts could be reprogrammed to an embryonic pluripotent stem cell-like state by retroviral overexpression of the TFs Oct3/4, Sox2, c-Myc and Klf4. These cells, termed induced pluripotent stem cells (iPSCs), had similar characteristics as ESCs including the ability to differentiate to any cell of the three germ layers. The following year human fibroblasts were also successfully reprogrammed to iPSCs (hiPSCs), opening new possibilities to generate patient-specific cells for disease modelling and regenerative therapies and overcoming many of the issues associated with hESCs (Takahashi et al. 2007, Yu et al. 2007). Induced pluripotent stem cell technology introduced a new way of generating cardiomyocytes for cardiac regeneration and in 2009 two independent groups were the first to differentiate iPSCs to cardiomyocytes (Zhang et al. 2009, Zwi et al. 2009).

A number of cardiac differentiation protocols have been developed for both hESCs and hiPSCs over the years and the efficiency of cardiac differentiation has been improved

by optimisation of the differentiation protocol and the addition of different agents acting on specific cardiogenic signalling pathways (Sahara et al. 2015). Generally two different techniques have been employed: embryonic body (EB) formation, where EBs spontaneously differentiate to derivatives of all three primary germ layers, and monolayer differentiation. In one of the earliest attempts to differentiate hESCs to cardiomyocytes, Kehat et al. (2001) generated cardiomyocytes utilizing the EB formation technique, but the method was very inefficient and spontaneously contracting areas were seen in only 8,1% of the EBs. Also early studies with iPSCs utilizing the serum-dependent spontaneous EB formation technique resulted in poor cardiomyocyte yields (Zhang et al. 2009, Zwi et al. 2009).

Laflamme et al. (2007) showed that the efficiency of cardiac hESC differentiation can be increased to over 30% by using a serum-free monolayer differentiation protocol with timed addition of activin A and BMP-4. Several protocols mimicking the signalling environment of embryonic cardiac development have also been developed for iPSCs resulting in increased cardiomyocyte yields and purity (Mummery et al. 2012). Recently a robust cardiac differentiation technique using small-molecule compounds to temporally modulate the canonical Wnt signalling pathway was shown to generate up to 98% cardiomyocytes (Lian et al. 2012).

*In vivo* transplantation studies in rodents have shown that cardiomyocytes derived from hESCs can integrate with the host myocardium and improve cardiac function after injury (Caspi et al. 2007, van Laake et al. 2007, Shiba et al. 2012). Promising results have also been obtained in non-human primates, as a recent study showed that transplanted hESC-derived cardiomyocytes generated remuscularisation of the infarcted monkey heart (Chong et al. 2014). This study however also highlighted some of the risks associated with PSC derived cardiomyocyte transplantation, as most of the transplanted cardiomyocytes were only partially mature and diverse in phenotype, causing non-lethal ventricular arrhythmias in the treated monkeys. These risks and

others, such as potential tumour formation, need to be addressed before either hESC or iPSC derived cardiomyocytes can be used for clinical applications.

### 2.2.3 Direct cardiac reprogramming of somatic cells

An upcoming and alternative approach for generating cardiomyocytes is direct cardiac reprogramming, also known as direct lineage reprogramming or transdifferentiation (Addis and Epstein 2013). Unlike pluripotent reprogramming, direct reprogramming enables conversion of a fully differentiated somatic cell directly into another cell type without first producing a pluripotent intermediate. It involves a change of the gene expression pattern from one cell type into another, which is achieved by overexpression of key lineage-specific TFs, with microRNAs (miRNA) or by modulating specific signal transduction pathways with small-molecule compounds.

Direct reprogramming is a promising new approach for cardiac regeneration as it is faster compared to iPSC generation and could potentially be achieved *in situ* without the need for cell transplantation (Sahara et al. 2015). The research is however in early stages and challenges including low efficiency and use of viral vectors need to be overcome before potential clinical applications. Direct reprogramming and the generation of cardiomyocytes using the direct reprogramming method will be discussed in more detail in the following chapter.

## 2.3 Direct reprogramming of fibroblasts to cardiomyocytes

Direct reprogramming is defined as the conversion of a fully differentiated cell type into another without going through a pluripotent intermediate. Direct reprogramming can be dated back to the 1980's, when it was first discovered that ectopic expression of the muscle-specific master regulator TF MyoD, was sufficient to convert fibroblasts to cells with myogenic features (Davis et al. 1987). Following this discovery, there was great interest in finding other single TFs that could directly convert somatic cells into

other cell types. However, no similar master regulators were found for other cell lineages, including cardiomyocytes, and for years it seemed that direct reprogramming was only possible within closely related cell lineages.

The research of direct reprogramming took a new direction, after the discovery that somatic cells could be reprogrammed to iPSCs by viral overexpression of a combination of TFs (Takahashi and Yamanaka 2006). This discovery inspired researchers to look for TF combinations that would allow for direct reprogramming to specific cell types across germ layers. As a result, terminally differentiated somatic cells have since been directly reprogrammed into a variety of cell types, including neurons, β-cells, hepatocytes, and cardiomyocytes, using viral overexpression of lineage specific TF combinations (Zhou et al. 2008, Ieda et al. 2010, Vierbuchen et al. 2010, Huang et al. 2011).

Direct reprogramming offers an alternative for generating cardiomyocytes for cardiac regeneration with several advantages compared to iPSC methods. By bypassing the pluripotent state, cardiomyocyte generation by direct reprogramming is both faster and easier than iPSC based cardiac reprogramming and the risk for teratoma formation is smaller (Addis and Epstein 2013). Direct reprogramming also offers a potential solution to the problems related to cell transplantation, as *in vivo* direct reprogramming would allow for conversion of endogenous fibroblasts in the heart to cardiomyocytes and would thus not involve cell transplantation. However, there are still many challenges associated with direct reprogramming, including low efficiency and varying results between different groups. Also the mechanisms behind direct cell conversion remain unclear, which might prevent the development of efficient direct reprogramming protocols. The progress of direct reprogramming in the field of cardiac regeneration will be discussed in the following. A summary of published reports of *in vitro* and *in vivo* direct cardiac reprogramming are presented in table 1 and table 2.

### 2.3.1 The starting cell population

Functioning cardiomyocytes are vital for a healthy heart, but they only account for less than a third of the total number of cells in the heart (Xin et al. 2013). The adult heart is composed of a wide array of different cell types including vascular cells and CFs. Cardiac fibroblasts are connective tissue cells that support the structure of the heart and play a key role in the formation of scar tissue and fibrosis following cardiac injury. Due to their abundancy in the heart and their role in the pathogenesis of congestive heart disease, fibroblasts have been an attractive starting cell type for cardiac reprogramming.

Several different types of fibroblasts have been employed for direct cardiac reprogramming, including CFs, mouse embryonic fibroblasts (MEFs), tail tip fibroblasts (TTFs), dermal fibroblasts (DFs) and human foreskin fibroblasts (FFs) (Doppler et al. 2015). The choice of fibroblast type can however have a great effect on the reprogramming efficiency as the different fibroblast types have different properties and some fibroblast types are more amenable to reprogramming than others (Srivastava and Ieda 2012). Embryonic fibroblasts are generally considered easier to convert as they are still immature and have higher plasticity (Doppler et al. 2015). Cardiac fibroblasts are also considered easier to convert to cardiomyocytes, as they and cardiomyocytes are derived from a common progenitor cell type and therefore might have fewer epigenetic barriers preventing reprogramming. Cardiac fibroblasts have also been shown to express higher levels of a number of cardiac genes, including *Gata4, Tbx20, Tbx5, Nkx2-5, Hand2* and *Mef2c*, when compared with tail fibroblasts and might thus be more amenable to direct cardiac reprogramming (Furtado et al. 2014). However, with the use of CFs there is also a risk for cardiomyocyte contamination when isolating the fibroblasts, which might lead to false positives (Srivastava and Ieda 2012).

Table 1. Published reports of *in vitro* direct cardiac reprogramming. CF = cardiac fibroblast, TTF = tail tip fibroblast, MEF = mouse embryonic fibroblast, FF = foreskin fibroblast, DF = dermal fibroblast, ESC = embryonic stem cell, cTnT = cardiac troponin T, α-MHC = alpha myosin heavy chain, MI = myocardial infarction, iCM = induced cardiomyocyte.

| Reference | Species | Starting cell type | Reprogramming factors | Outcome measure |
|---|---|---|---|---|
| Ieda et al. 2010 | Mouse | Post-natal CFs and TTFs | Gata4, Mef2c, Tbx5 | α-MHC reporter/cTnT |
| Efe et al. 2011 | Mouse | MEFs | Oct4, Sox2, Klf4, c-My + JAK-inhibitor | cTnT |
| Chen et al. 2012 | Mouse | CFs and TTFs | Gata4, Mef2c, Tbx5 | α-MHC Nkx2.5/cTnT reporters |
| Jayawardena et al. 2012 | Mouse | Neonatal CF, adult CF and TTFs | miR-1, -133, -208, -499 + JAK-inhibitor | α-MHC-CFP reporter |
| Protze et al. 2012 | Mouse | MEFs and neonatal CFs | Tbx5, Mef2c, Myocd | α-MHC reporter/cTnT |
| Song et al. 2012 | Mouse | Adult CFs and TTFs | Gata4, Hand2, Mef2c, Tbx5 | α-MHC reporter/cTnT |
| Inagawa et al. 2012 | Mouse | Adult CFs | Gata4, Mef2c, Tbx5 | α-MHC reporter |
| Addis et al. 2013 | Mouse | MEFs and adult CFs | Hand2, Nkx2-5, Gata4, Mef2c, Tbx5 | functional cTnT-GCaMP calcium reporter |
| Christoforou et al. 2013 | Mouse | MEFs | Gata4, tbx5, Mef2c, Srf, Myocd, Smarcd3, Mesp1 | MHC-GFP reporter |
| Nam et al. 2013 | Human | Neonatal FFs, adult CFs and neonatal DFs | GATA4, HAND2, MYOCD, TBX5, miR-1, miR-133 | cTnT |
| Fu et al. 2013 | Human | ESC-derived fibroblasts, fetal CFs and neonatal DFs | GATA4, MEF2C, TBX5, ESRRG, MESP1, MYOCD, ZFPM2 | αMHC-reporter + cTnT |
| Wada et al. 2013 | Human | CFs and DFs | GATA4, MEF2C, TBX5, MESP1, MYOCD | α-actinin and cTnT |
| Ifkovits et al. 2014 | Mouse | MEFs and adult CFs | Hand2, Nkx2-5, Gata4, Mef2c, Tbx5 + SB431542 | functional cTnT-GCaMP calcium reporter |
| Wang et al. 2014 | Mouse | MEFs and TTFs | Oct4 + small molecule cocktail | beating iCMs |
| Wang et al. 2015 | Mouse | CFs | polycistronic Mef2c-Gata4-Tbx5 | cTnT |
| Fu et al. 2015 | Mouse | MEFs | small molecule cocktail | α-actinin |
| Zhao et al. 2015 | Mouse | MEFs | Gata4, Hand2, Mef2c, Tbx5 + TGF-β inhibitor A83–01 | cTnT |
| Zhou et al. | Mouse | MEFs | Gata4, Hand2, Mef2c, | cTnT |

| | | | | |
|---|---|---|---|---|
| 2015 | | | Tbx5, Akt/PKB | |
| Cao et al. 2016 | Human | FFs | small molecule cocktail | beating iCMs |

Table 2. Published reports of *in vivo* cardiac reprogramming. MI = myocardial infarction, cTnT = cardiac troponin T, α-MHC = alpha myosin heavy chain, CFP = cyanide fluorescent protein, EF = ejection fraction

| **Reference** | **Species** | ***in vivo* model** | **Reprogramming factors** | **Outcome measure** |
|---|---|---|---|---|
| Jayawardena et al. 2012 | Mouse | MI-model | miR-1, -133, -208, -499 | lineage tracing and α-MHC-CFP reporter |
| Qian et al. 2012 | Mouse | MI-model | Gata4, Mef2c, Tbx5 | lineage tracing, α-actinin |
| Song et al. 2012 | Mouse | MI-model | Gata4, Hand2, Mef2c, Tbx5 | lineage tracing, cTnT |
| Inagawa et al. 2012 | Mouse | MI-model | Gata4, Mef2c, Tbx5 | α-MHC reporter, α-actinin |
| Mathison et al. 2012 | Mouse | MI-model | polycistronic Gata4-Mef2c-Tbx5 | Fibrosis ↓, EF ↑ |
| Jayawardena et al. 2015 | Mouse | MI-model | miR-1, -133, -208, -499 | Lineage-tracing, cTnT |
| Ma et al. 2015 | Mouse | MI-model | polycistronic Mef2c-Gata4-Tbx5 | Lineage-tracing, α-actinin |

### 2.3.2 Transcription factor-mediated direct cardiac reprogramming of murine fibroblasts to induced cardiomyocytes

Successful direct reprogramming of terminally differentiated somatic cells to cardiomyocytes was first reported by Ieda et al. (2010). They identified a combination of three cardiac TFs, Gata4, Mef2c, and Tbx5 (GMT), which were sufficient for converting mouse cardiac and DFs into cardiomyocyte-like cells *in vitro*. Using a transgenic reporter system where green fluorescent protein (GFP) was driven by an *α-myosin heavy chain (α-MHC)* promoter, which is activated in mature cardiomyocytes, and flow cytometry for quantification, they showed that lentiviral expression of GMT resulted in 20% α-MHC–GFP+ cells. These cells were termed induced cardiomyocytes (iCMs). The reprogramming was demonstrated to be direct using genetic lineage tracing, which showed that iCMs did not express cardiac progenitor markers (Mesp1 and Isl1) during the reprogramming process.

These results were the first to confirm that direct reprogramming to cardiomyocytes was possible. However, when examining these results, it is important to keep in mind that only a third of these cells expressed sarcomere marker cardiac troponin T (cTnT) and only few cells started beating spontaneously after 4–5 weeks in culture (Ieda et al. 2010). It is therefore likely that the majority of iCMs were only partially reprogrammed and the actual reprogramming efficiency was much lower than reported.

The effectiveness of the GMT TF combination was later questioned by Chen et al. (2012), who reported that lentiviral expression of GMT factors in mouse TTFs and CFs only resulted in partial and insufficient cardiac reprogramming. They reported that despite significant overexpression of GMT, no α-MHC or Nkx2.5 expression was detected. Expression of cTnT was however detected in 35% of the cells, but no spontaneously beating cells were seen.

The reason why Chen et al. (2012) were unable to reproduce the results by Ieda et al. (2010) is unclear, but might be explained with differences in the reprogramming protocols. Recently Wang et al. (2015) demonstrated that the optimal stoichiometry of G, M, T TF expression greatly influences the reprogramming efficiency *in vitro*. Using a polycistronic vector that expressed different levels of TFs depending on the splicing order, they found that the vector expressing a higher level of Mef2c and lower levels of Gata4 and Tbx5 resulted in a significantly higher reprogramming efficiency than separate transduction of GMT. These results suggest that also non-optimal expression levels of GMT might be the reason for unsuccessful reprogramming experiments.

Since the initial report by Ieda et al. (2010), several other TF combinations have been examined and additional TFs have been reported to improve direct reprogramming of fibroblasts to iCMs. Song et al. (2012) showed that the addition of Hand2 to the GMT combination resulted in a higher reprogramming efficiency compared with just GMT. Like Ieda et al. (2010), they used an α-MHC-GFP reporter to screen for the optimal TF combination in adult mouse TTFs. The combination of GMT + Hand2 (GHMT)

generated iCMs with an average efficiency of 9.2%, while GMT only converted an average of 2.9% of the transduced cells.

Addis et al. (2013) used a functional reporter system, in which a GCaMP calcium indicator is driven by a cTnT promoter, to evaluate the optimal TF combination for direct cardiac reprogramming in MEFs. They identified the combination of Hand2, Nkx2.5, Gata4, Mef2c, and Tbx5 (HNGMT) as the most effective TF combination. Lentiviral transduction of the HNGMT combination was reported to be 52-fold more efficient compared with GMT.

In addition to the above described TF combinations several other TFs, including myocardin (Myocd), Mesp1, Smarcd3 and SRF have been reported to enhance the reprogramming efficiency in addition to GMT (Sahara et al. 2015). Also different screening methods have been employed to identify the most optimal factor combination. Protze et al. (2012) used an alternative method for identifying reprogramming factors. Instead of using a screening system relying on only one reporter gene, they used a system that assessed the ability of different TF combinations to activate the expression of five cardiac genes (*Myh5, Myl2, Actc1, Nkx2.5* and *Scn5a*) in mouse fibroblasts. As a result, they found that the combination of Tbx5, Mef2c and Myocd upregulated a broader spectrum of cardiac genes than the GMT combination.

### 2.3.3 MicroRNA-mediated direct reprogramming

Micro-RNAs (miRNAs) are a class of small, single-stranded, non-coding RNA that act as negative regulators of gene expression (Katz et al. 2016). miRNAs have been shown to play an important role in cardiac development and are important regulators of cell fate determination and differentiation. They have also been recognised as potential mediators of direct cardiac reprogramming.

Jayawardena et al. (2012) identified a combination of miRNAs (miR-1, -133, -208, -499) that were able to directly convert neonatal mouse fibroblasts into iCMs both *in vitro* and *in vivo*. Using a transgenic αMHC-CFP (cyan fluorescent protein) reporter cell line to assess reprogramming efficiency, they found that transfection with the miRNA combination resulted in αMHC activation in approximately 1.5–7.7% of transfected fibroblasts. Direct cardiac reprogramming with miRNAs has also been examined by Muraoka et al. (2014), who found the addition of miR-133 to the GMT TF combination to result in up to 7-fold more beating cells compared with GMT alone in MEFs. They were however unable to convert MEFs into iCMs using the same miRNA combination as previous reported by Jayawardena et al. (2012). Overall, these studies suggest that miRNAs could potentially be used for cardiac regenerative medicine to replace lost cardiomyocytes. In contrast to viral delivery of cardiac TFs, miRNAs do not have a risk of genomic integration, making them more feasible for clinical use.

### 2.3.4 Direct reprogramming of human fibroblasts to induced cardiomyocytes

One of the ultimate goals for direct cardiac reprogramming is to be able to utilise the technique for cardiac regenerative medicine (Addis and Epstein 2013). A necessary step toward potential clinical applications is to transfer the direct reprogramming technique from murine fibroblasts to human fibroblasts. This has however proven to be challenging, as the gene regulatory networks in human cells are different and more complex than those in mouse cells (Sahara et al. 2015). The same reprogramming factor combinations that have converted mouse fibroblasts to iCMs seem to be insufficient for reprogramming human fibroblasts to iCMs and additional TFs are necessary (Fu et al. 2013, Nam et al. 2013).

Nam et al. (2013) were the first to achieve successful direct reprogramming of human fibroblasts to iCMs. They found that a combination of GATA4, TBX5, HAND2, MYOCD, miR-1 and miR-133 converted neonatal and adult human FFs to iCMs that expressed cardiac markers and had sarcomere-like structures. The reprogrammed cells also

displayed calcium oscillations and rare cells started beating spontaneously. Direct reprogramming of human fibroblasts into iCMs has also been reported by Wada et al. (2013), who found the combination of GMT together with MESP1 and MYOCD to convert human cardiac and DFs to immature iCMs, and Fu et al. (2013), who achieved direct cardiac reprogramming using a combination of GMT together with ESRRG (estrogen-related receptor gamma), MESP1, MYOCD and ZFPM2 (zinc finger protein, FOG family member 2).

Despite successful reprogramming of human fibroblasts to iCMs, all studies resulted in relatively immature iCMs and only rare spontaneously contracting cells were observed (Fu et al. 2013, Nam et al. 2013, Wada et al. 2013). Both Nam et al. (2013) and Wada et al. (2013) reported that the generated iCMs were morphologically immature and the cells only displayed slow calcium oscillations. Reprogramming of human fibroblasts into iCMs also seems to result in heterogeneous populations of iCMs and varying reprogramming efficiencies (Nam et al. 2013). However, these studies show that human fibroblast can also be directly reprogrammed to cardiomyocyte-like cells. Despite the initial success, the research is still in early stages and further research is needed in order to improve the efficiency and to generate mature and functional cardiomyocytes for clinical use.

### 2.3.5 Direct cardiac reprogramming in vivo

One of the advantages of direct cardiac reprogramming compared to iPSC-based methods is the possibility of converting endogenous CFs to iCMs *in vivo* by delivering reprogramming factors directly to the heart and thus avoiding cell transplantation. Following the first successful conversions of fibroblasts to iCMs *in vitro,* several groups began to investigate whether the same results could be achieved *in vivo*.

Qian et al. (2012) and Song et al. (2012) were the first groups to report *in vivo* cardiac reprogramming. Qian and colleagues used genetic fate mapping to demonstrate that non-myocytes in the infarcted mouse heart could be reprogrammed into iCMs by

directly injecting retroviruses expressing GMT TFs into the mouse heart (Qian et al. 2012). Using a Cre recombinase driven by a fibroblast marker promoter (*periostin* or *Fsp1*) together with an R26RlacZ reporter line, in which β-galactosidase is activated only in periostin-Cre-expressing cells, to label endogenous CFs and immunofluorescent staining with β-galactosidase and α-actinin to determine reprogramming efficiency, they showed that new iCMs derived from non-myocytes were generated with a reprogramming efficiency of 10–15%. Functional studies performed three months after the coronary ligation also showed a decreased degree of fibrosis and modest but statistically significant improvement in cardiac function.

Song and colleagues used a similar lineage tracing approach (Fsp1-Cre and inducible Tcf21-iCre) (Song et al. 2012). They reported a reprogramming efficiency of 1–8% after injecting retroviral vectors expressing GHMT factors into infarcted mouse hearts *in vivo*. Even though they reported a lower reprogramming efficiency than Qian and colleagues, Song et al. demonstrated a greater improvement in cardiac function. In functional studies three months after the left coronary artery ligation, the scar size was reduced by 50% and the ejection fraction (EF) was increased by 2-fold compared with controls.

Both groups reported a statistically significant improvement in cardiac function, even though the reprogramming efficiency remained relatively low. This suggests that there are other factors besides generation of iCMs contributing to the improved cardiac function. Song et al. (2012) speculated that mechanisms, such as decreased activation of CFs, enhanced survival of endogenous cardiomyocytes, enhanced differentiation of cardiac progenitors into cardiomyocytes or increased angiogenesis could contribute to the improved cardiac function. Gata4 has previously been shown to act as survival factor for cardiomyocytes by enhancing myocardial angiogenesis, decreasing apoptosis and increasing generation of cardiac stem like cells (Rysä et al. 2010). The reported improvement in cardiac function might therefore be partially due to overexpression of Gata4.

Inagawa and colleagues used an alternative approach to determine *in vivo* direct reprogramming by co-transducing retroviral GMT factors and a reporter gene into the infarcted hearts of immunosuppressed mice (Inagawa et al. 2012). Even though only 1% of the transduced fibroblasts in the infarcted mice hearts were reprogrammed to iCMs, Inagawa and colleagues showed that using a polycistronic viral delivery system resulted in morphologically more mature iCMs compared to iCMs generated by separately delivered GMT factors. As a conclusion they proposed that delivery of reprogramming factors via a polycistronic vector might be beneficial for *in vivo* cardiac reprogramming.

In a more recent study Ma et al. (2015) used a polycistronic MGT vector that expresses a higher level of M and lower levels of G and T for *in vivo* direct reprogramming in mice. In contrast to the results by Inagawa et al. (2012), Ma et al. (2015) showed that injecting mice with the MGT polycistronic vector resulted in a higher reprogramming efficiency compared to using separate TF vectors. These results are in line with previous results by the same group of the effect of TF stoichiometry on direct reprogramming *in vitro* (Wang et al. 2015). However, Ma and colleagues did not find a significant difference in level of iCM maturity when comparing reprogramming with the polycistronic MGT and traditional GMT TF delivery with separate vectors.

In addition to traditional TF-mediated direct reprogramming, several groups have attempted alternative methods also for *in vivo* cardiac reprogramming. Mathison et al. (2012) were able to improve cardiac function by injecting vascular endothelial growth factor (VEGF) to infarcted rat harts 3 weeks prior to GMT TF gene delivery. They reported that treatment with GMT and VEGF increased the ejection fraction by 4-fold compared to treatment with GMT alone. *In vivo* cardiac reprogramming has also been achieved using miRNAs. Jayawardena et al. (2012) demonstrated *in vivo* reprogramming using lentiviral vectors to deliver a combination of miR-1, -133, -208, and -499 into infarcted *Fsp1*-Cre mice hearts. However cardiac function was not

assessed and the study did not take into consideration that some of the generated iCMs could be the result of cell fusion of fibroblasts with endogenous cardiomyocytes. Recently, also significant improvement of cardiac function, as measured by serial echocardiography, was demonstrated by the same group using the same miRNA combination (Jayawardena et al. 2015).

Overall *in vivo* direct cardiac reprogramming seems to result in more mature iCMs and higher reprogramming efficiencies compared with *in vitro* direct reprogramming. It has been suggested that factors in the cardiac microenvironment such as the extracellular matrix and paracrine factors might be beneficial for the reprogramming process (Doppler et al. 2015). The higher reprogramming efficiencies achieved in vivo might also at least partially be explained with CFs in the heart being easier to convert to iCMs as they and cardiomyocytes are derived from a common progenitor cell. However, there have been great variations in the results reported by different groups. Like with *in vitro* reprogramming studies, some of these variations might be due to variations in the experimental procedures. It is none the less clear that both *in vitro* and *in vivo* cardiac reprogramming is still in its infancy and more comparable studies are needed.

### 2.3.6 Underlying mechanisms of direct cardiac reprogramming

Understanding the mechanism of action for direct reprogramming is of great importance in overcoming barriers that limit the reprogramming efficiency and potential clinical use. Fundamentally, converting one cell type into another involves epigenetic silencing of the starting cell type's master genes and activation of the target cell specific gene expression (Xu et al. 2015). In direct cardiac reprogramming this means shifting from expression of fibroblast genes to expression of cardiomyocyte specific genes. Even though direct reprogramming has been applied for several different cell types, the mechanisms underlying these changes remain poorly understood. Some studies suggest that inhibition of TGF-β might be critical for direct reprogramming. Ifkovits et al. (2014) showed that the TGF-β inhibitor SB431542 increased the reprogramming efficiency five-fold when used together with the NHGMT

TF combination. Muraoka et al. (2014) showed that direct reprogramming with GMT + miR-133 was mediated through suppression of TF gene *Snail1*, which is a downstream target of TGF-β. However, there are also some results contradicting this, as Fu et al. (2013) reported that activation of TGF-β promoted direct cardiac reprogramming of human fibroblasts. None the less TGF-β signalling seems to play a role in direct cardiac reprogramming.

### 2.3.7 Evaluation of direct cardiac reprogramming and characterisation of induced cardiomyocytes

To date, multiple outcome measures have been employed by different groups to evaluate the efficiency of direct cardiac reprogramming, causing great variations in reported reprogramming efficiencies and making the results of different groups difficult to compare with each other (Doppler et al. 2015). In order to get reliable and comparable results, it is important to first clearly define what features are characteristic for cardiomyocytes and to use unambiguous and relevant outcome measures (Addis and Epstein 2013). Not all reported outcome measures are equally reliable and less stringent outcome measures, such as the expression of single genes, often result in higher reprogramming efficiencies compared to more stringent outcome measures (Figure 4).

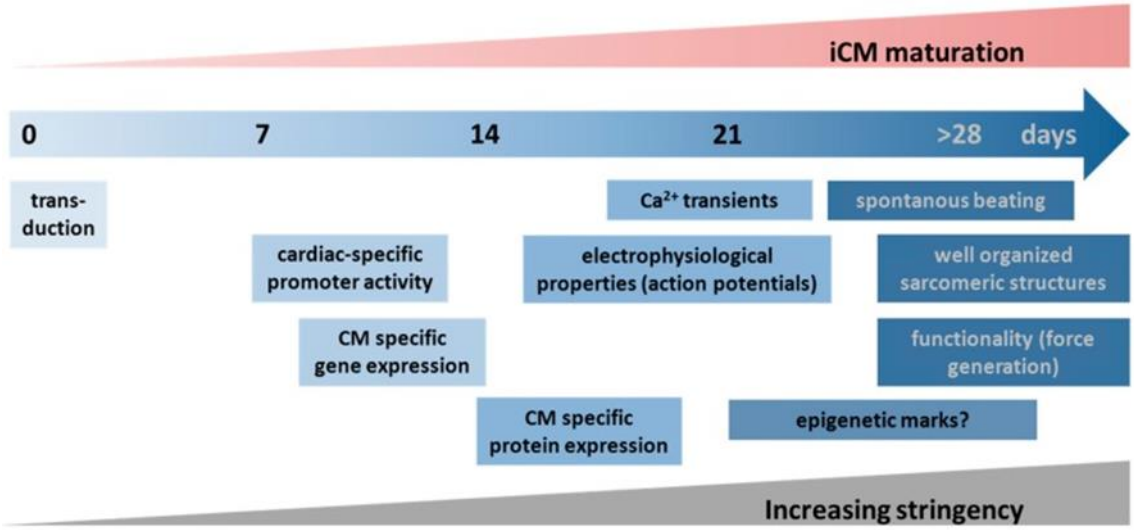


Figure 4. Reliability and stringency of different outcome measures used in direct cardiac reprogramming. Non-functional outcome measures, such as immunostaining of cardiac proteins, are easy to quantify, but usually have lower stringency compared to functional measurements. Functional outcome measures, such as detection of calcium oscillation, action potentials and spontaneous contraction, require the activation and coordination of several cardiac genes, making them more stringent and reliable. A combination of several outcome measures is generally required in order to get a reliable result (Doppler et al. 2015).

Both non-functional- and functional outcome measures have been used to evaluate direct cardiac reprogramming. Non-functional outcome measures include detection of cardiomyocyte-specific gene activation, immunostaining of cardiac proteins and activation of reporter transgenes driven by cardiomyocyte-specific gene promoters (Doppler et al. 2015). These readouts are easy to quantify and can be detected at an early stage post-induction, making them feasible for large scale screening. However, they usually also have lower stringency compared to functional measurements and can result in false positives that can be hard to rule out (Addis and Epstein 2013). Functional outcome measures, such as detection of calcium oscillation, action potentials and spontaneous contraction, require the activation and coordination of several cardiac genes, making these outcome measures more stringent and reliable.

There are no universal standards for defining an iCM (Addis and Epstein 2013). However, a wide variety of attributes can be assessed in order to determine if a cell is a cardiomyocyte, including gene expression patterns, epigenetic marks and cell

morphology. One key feature of mature functioning cardiomyocytes is a well-organised sarcomere structure, which can be visualised with immunostaining for cardiac sarcomere proteins, such as α-actinin and cTnT (Nam et al. 2014). Sarcomere protein expression and well-organised sarcomere structure do, however, not always correlate and thus evaluating reprogramming efficiency only based on sarcomere protein expression might lead to overestimation of the reprogramming efficiency (Doppler et al. 2015).

The ability to generate action potentials and to contract is generally considered the most characteristic feature of a cardiomyocyte (Addis and Epstein 2013, Doppler et al. 2015). Identification of spontaneously contracting cells has therefore been considered the most stringent outcome measure and the standard measure of success in direct cardiac reprogramming (Addis and Epstein 2013). The prevalence of spontaneously beating cells can, however, be difficult to quantify. Addis et al. (2013) described a method to visualise calcium oscillations and contracting cells by using a transgenic calcium reporter driven by the cardiac troponin T promoter, making it easier to identify and quantify beating cells. It should be noted, that spontaneous contraction is not a typical property of all mature cardiomyocytes and spontaneously beating iCMs identified in direct reprogramming experiments might in fact be immature cardiomyocytes (Addis and Epstein 2013) .

A combination of several outcome measures is generally required in order to get a reliable result and assessing just a single feature can lead to overestimation of the reprogramming efficiency (Addis and Epstein 2013). In addition to choosing valid outcome measures, it is important to pay attention to how the reprogramming efficiency is reported. In contrast to the non-reprogrammed cells, iCMs usually stop proliferating (Ieda et al. 2010). This can cause underestimation of the real reprogramming efficiency, if the efficiency is reported as the percentage of iCMs per total number of cells at the time of quantification (Addis and Epstein 2013). Another

possible way to present the reprogramming efficiency is by dividing the number of iCMs with the number of initially transduced cells.

## 2.4 Direct cardiac reprogramming with small-molecule compounds

### 2.4.1 Small-molecule compounds as regulators of gene expression

Direct reprogramming is emerging as a promising alternative for generating cardiomyocytes for cardiac regenerative medicine, disease modelling and drug discovery. The use of viral vectors for delivery of TFs might however limit the clinical applications, as it involves a risk for genomic integration of exogenous genes into the host genome (Li et al. 2013). Developing methods that would avoid genetic manipulation is one of the major challenges facing regenerative medicine. A potential solution would be to use small-molecule compounds to replace viral-mediated TF gene transfer. Small molecules are chemical compounds with low molecular weight that have the ability to modulate specific protein targets rapidly and often reversibly (Yu et al. 2014). They are an attractive alternative to the conventional use of viral vectors, as they are cost-effective, easy to manipulate and standardise and concentrations can easily be controlled, improving both efficacy and safety, and making the approach more feasible for clinical applications.

Small-molecule compounds have been studied extensively in iPSC reprogramming to reduce the number of viral vectors and to improve efficiency. In 2013 Hou et al. were the first to report iPSC generation using only small-molecule compounds. The use of small-molecule compounds in direct reprogramming is a relatively new topic and the published reports of the use of small-molecule compounds in direct reprogramming are limited. Chemically-mediated direct reprogramming is however an emerging trend and more and more research is focusing on finding small-molecule compounds that could either enhance reprogramming efficiency in combination with TFs or completely replace viral delivery of TFs.

Small-molecule compounds can work as regulators of gene expression and thus have an impact on cell fate (Yu et al. 2014). Generally small-molecule compounds that regulate gene expression can be divided into four classes based on their mechanism of action: signalling pathway modulators, modulators of epigenetic proteins, metabolic regulators and nuclear receptor agonists and antagonists (Figure 5). Signalling pathway modulators either activate or inhibit a component of a signalling cascade to indirectly regulate the expression of downstream genes. Modulators of epigenetic proteins can reactivate or silence genes by acting on enzymes that are involved in epigenetic gene regulation such as histone-modifying enzymes or enzymes that regulate DNA methylation. Metabolic regulators modulate metabolic pathways and nuclear receptor agonists or antagonists regulate gene expression directly by binding to nuclear receptors.

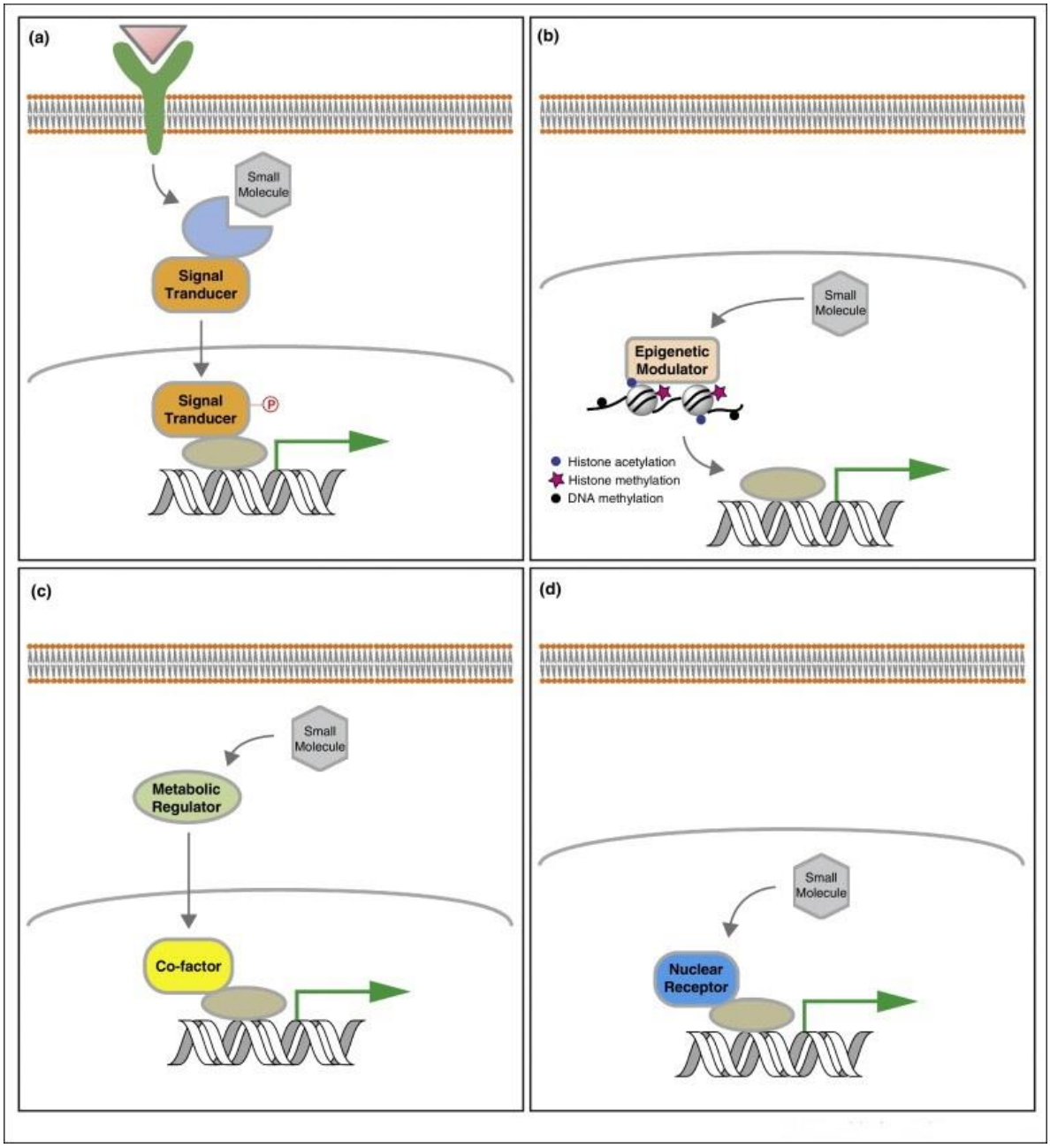


Figure 5. Small-molecule compounds can regulate gene expression through four different mechanisms: a) by activating or inhibiting signalling pathways that regulate transcriptional activity, b) by modulating epigenetic proteins, c) by acting as metabolic regulators and d) by directly activating or inhibiting nuclear receptors (Yu et al. 2014).

### 2.4.2 Advances in small-molecule compound-mediated direct reprogramming

Small-molecule compounds might prove to be important tools in overcoming some of the major challenges involved in direct reprogramming, including low reprogramming efficiency, slow reprogramming process and safety issues related to the use of viral vectors (Li et al. 2013). Small-molecule compounds have been identified to enhance direct reprogramming in addition to lineage specific TFs for a number of cell types. The ultimate goal is to identify small-molecule compounds that could replace the use of TFs completely, but this has proven to be challenging.

The use of small-molecule compounds in direct reprogramming has been most extensively investigated in the generation of neurons. There are several reports where small-molecule compounds have been used in direct neural reprogramming to either enhance the reprogramming efficiency in addition to reprogramming factors or to reduce the number of reprogramming factors (Ladewig et al. 2012, Liu et al. 2013 Kim et al. 2014). The first reports of chemical-only direct reprogramming of fibroblasts to neural cells without passing through a progenitor stage were published by Hu et al. (2015) and Li et al. (2015). These studies showed that small-molecule compounds are able to convert cell fate, taking direct reprogramming a huge step toward future clinical applications. Since these initial studies a number of additional reports of small-molecule compound-mediated direct reprogramming to neurons have been published (Cheng et al. 2015, Zhang et al. 2015, Zhang et al. 2016). Additionally there have also been single reports of chemical-only direct reprogramming toward pancreatic cells, endothelial cells and cardiomyocytes (Pennarossa et al. 2013, Sayed et al. 2015, Cao et al. 2016). The use of small-molecule compounds in direct cardiac reprogramming will be reviewed in the following section.

### 2.4.3 Potential pharmacological targets in direct cardiac reprogramming

Targeting direct reprogramming by pharmacological intervention is an intriguing strategy for cardiac regeneration. However, the underlying mechanisms of direct cardiac reprogramming remain mostly unknown, which has hindered specific targeting of direct reprogramming with small-molecule compounds. Still, understanding of the major signalling pathways and transcriptional regulation controlling cardiac differentiation during embryonic development offers potential drug targets for cardiac regeneration. Also recent studies have revealed a number of potential targets that might be pharmacologically modulated to facilitate direct reprogramming.

One such potential target is the pro-fibrotic TGF- β signalling pathway. Members of the TGF- β superfamily bind to the type I and type II TGF- β serine-threonine kinase receptors that activate target gene expression by phosphorylating TFs Smad2 and Smad3 (Inman et al. 2002). Zhao et al. (2015) recently demonstrated that activation of TGF- β signalling works as a barrier to direct cardiac reprogramming by activating pro-fibrotic signalling. They showed that treatment with the TGF- β ligand TGF- β1 significantly reduced the reprogramming efficiency in HGMT-transduced fibroblasts. Further, inhibition of the pathway with a small-molecule compound TGF-β-inhibitor significantly increased the reprogramming efficiency and generated iCMs with a remarkable reprogramming efficiency of up to 67%. Inhibition of TGF- β signalling has also previously been reported to increase the reprogramming efficiency (Ifkovits et al. 2014). Furthermore, TGF- β signalling is also considered as a potential drug target for inhibiting fibrotic remodelling of the heart (Talman and Ruskoaho 2016). TGF-β therefore serves as a promising potential drug target for cardiac regeneration.

In addition to inhibition of TGF-β, Zhao and colleagues also showed that inhibition of Rho-associated kinase signalling, which is another pro-fibrotic signalling pathway, resulted in an increased reprogramming efficiency (Zhao et al. 2015). The fundamental mechanism behind direct cardiac reprogramming involves a change in cell fate by switching from expression of fibroblast genes to expression of cardiomyocyte specific

genes (Xu et al. 2015). Therefore, activating signalling pathways that inhibit the expression of fibroblast genes or inhibiting pro-fibrotic signalling with small-molecule compounds is a potential strategy for pharmacological intervention in direct cardiac reprogramming. Further characterisation and a more detailed understanding of the signalling pathways that are involved in regulating the expression of fibroblast or cardiac genes might lead to the discovery of new potential drug targets.

Another signalling pathway that has been identified as a potential pharmacological target in direct cardiac reprogramming is the Akt1/Protein kinase B signalling pathway (Zhou et al. 2015). Zhou et al. (2015) showed that GHMT-treatment combined with overexpression of Akt1 significantly increased the generation of iCMs *in vitro* compared with GHMT treatment alone. They hypothesized that Akt1 likely increases reprogramming via a pathway activated by insulin-like growth factor 1 (IGF1) that goes via phosphoinositol 3-kinase (PI3K) to Akt1 and then activates downstream signals including the mitochondrial target of rapamycin complex 1 (mTORC1) and forkhead box o3 (Foxo3a). Interestingly this same pathway has previously been identified as a mediator of cardiac hypertrophy (Aoyagi and Matsui 2011). Zhou et al. (2015) reported that the reprogramming efficiency was enhanced by the addition of IGF1 to GHMT TFs, indicating that the increase in direct reprogramming efficiency is caused by activation of the IGF1 receptor. However, the reprogramming efficiency achieved with this combination was lower than the combination of Akt1 plus HGMT. Thus, there might be other mediators of Akt1 signalling that might serve as more efficient drug targets.

Recent direct cardiac reprogramming research has focused on identifying epigenetic barriers that hinder direct cardiac reprogramming. Epigenetic gene regulation plays a critical role in cardiac lineage differentiation and specification by regulating the expression of cardiac genes (Oyama et al. 2014). Epigenetic proteins, including histone-modifying enzymes such as histone deacetylase, histone methyltransferase and histone demethylase or enzymes that regulate DNA methylation, such as DNA methyltransferase can be modulated by small-molecule compounds (Christ et al.

2013). As efficient direct reprogramming requires a remodelling of the epigenetic landscape of the reprogrammed cell, epigenetic proteins represent attractive drug targets in direct cardiac reprogramming.

As an example of a potential epigenetic drug target, Zhou et al. (2016) recently identified the polycomb complex protein Bmi1 to be a major epigenetic barrier for direct cardiac reprogramming. The inhibitory effect of Bmi1 on direct cardiac reprogramming was revealed to be partially caused by Bmi1 directly binding to the regulatory regions of a number of cardiogenic genes and suppressing them. Identifying other epigenetic barriers of direct cardiac reprogramming is an important strategy for increasing the reprogramming efficiency and will most likely lead to the identification on novel drug targets in direct cardiac reprogramming.

### 2.4.4 Small-molecule compounds in direct cardiac reprogramming

Small-molecule compounds have initially been used in direct cardiac reprogramming to increase reprogramming efficiency. Among the first to report the use of a small-molecule compound to facilitate cardiac reprogramming were Efe et al. (2011), who showed that a small molecule Janus kinase (JAK) inhibitor in addition to overexpression of Oct4, Sox2, Klf4 and c-Myc in cardiomyocyte-favourable culture conditions directed reprogramming of MEFs directly to cardiomyocytes instead of iPSCs (Efe et al. 2011). Even though these results were an encouraging first step toward small-molecule compound-mediated direct cardiac reprogramming, Efe and colleagues could not altogether rule out the possibility that the cardiomyocytes were in fact derived from contaminating cardiac precursors or iPSC intermediates and not directly reprogrammed.

The same group later identified a small-molecule compound cocktail consisting of TGF-β inhibitor SB431542, glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021, lysine specific histone demethylase 1 (LSD1) inhibitor parnate and adenylyl cyclase (AC) activator forskolin that enabled direct cardiac reprogramming with only the TF Oct4

(Wang et al. 2014). They reported that during the reprogramming process the cells did pass through a cardiac precursor stage, but not through a pluripotent state. However, lineage tracing was not used confirm this.

Ifkovits et al. (2014) set out to find small-molecule compounds that could increase reprogramming efficiency in addition to the HNGMT TF combination in MEFs. They selected small-molecule compounds that have previously been used to facilitate iPSC generation and cardiac differentiation and found that the TGF-β inhibitor SB431542 increased the reprogramming efficiency up to 5 fold compared to only HNGMT. They further showed that inhibition of TGF-β by SB431542 increased the reprogramming efficiency in a dose-dependent matter.

Recently there have been two different reports of small-molecule compound only-mediated direct cardiac reprogramming. The first group to report direct cardiac reprogramming using only small-molecule compounds were Fu et al. (2015). They set out to repeat the generation of chemically induced iPSC from MEFs reported previously by Hou et al. (2013) but found instead that treatment with the combination of 6 small molecules resulted in beating clusters of cardiomyocytes (table 3) (Fu et al. 2015). After optimisation of their protocol they reported that the generated iCMs expressed cardiomyocyte markers, had sarcomere structures and showed calcium oscillation. The cells were shown to originate from fibroblasts by lineage tracing and were determined to pass through a cardiac progenitor stage rather than a pluripotent intermediate by quantitative RT-PCR and time-lapse imaging. Even though these results seem promising and the implications of these results can be great, no lineage tracing with pluripotency factors was done to rule out the possibility that the cells passed through a pluripotent intermediate.

Recently Cao et al. (2016) also demonstrated small-molecule compound direct cardiac reprogramming of human fibroblasts. They screened for small-molecule compounds using an α-MHC-GFP reporter to identify iCMs and found that a combination 9

different small-molecule compounds resulted in the highest reprogramming (table 3). After 30 days 6.6 ± 0.4% of the treated cells expressed cardiac marker cTnT and of these over 97% were reported to beat spontaneously. Similarly to the results by Fu et al. (2015) also Cao et al. (2016) reported that the cells went through a cardiac precursor stage before becoming iCMs. However, they did not either direct lineage tracing to rule out the possibility of the cells going through a pluripotent stage.

Table 3. Small-molecule compounds used by Fu et al. (2015) and Cao et al. (2016) to achieve chemically induced direct cardiac reprogramming.

| Small-molecule compound | Mechanism of action | Study |
|---|---|---|
| **RepSox** | Transforming growthfactor (TGF)-β kinase/activin receptor-like kinase (ALK 5) inhibitor | Fu et al. 2015 |
| **Forskolin** | adenylyl cyclase (AC) activator | Fu et al. 2015 |
| **Valporic acid (VPA)** | histone deacetylase (HDAC) inhibitorF | Fu et al. 2015 |
| **Parnate** | lysine specific histone demethylase 1 (LSD1) inhibitor | Fu et al. 2015 |
| **TTNPB** | retinoic acid analog | Fu et al. 2015 |
| **CHIR99021** | glycogen synthase kinase 3 (GSK3) inhibitor | Fu et al. 2015, Cao et al. 2016 |
| **A83-01** | TGF-β kinase/ ALK 5 inhibitor | Cao et al. 2016 |
| **BIX01294** | histone-lysine methyl-transferase (HMTase) inhibitor | Cao et al. 2016 |
| **AS8351** | histone demethylase inhibitor | Cao et al. 2016 |
| **SC1** | extracellular signal-regulated kinase 1 (ERK1) and Ras GTPase-activating protein (Ras-GAP) inhibitor | Cao et al. 2016 |
| **Y27632** | Rho-associated coiled coil formingprotein serine/threonine kinase (ROCK) inhibitor | Cao et al. 2016 |
| **OAC2** | epigenetic modulator | Cao et al. 2016 |
| **SU16F** | Platelet-derived growth factor receptor (PDGFR)-β inhibitor | Cao et al. 2016 |
| **JNJ10198409** | PDGFR-α and PDGFR-β inhibitor | Cao et al. 2016 |

These initial reports with small-molecule compound direct reprogramming seem promising; however, more research is needed before any conclusions can be made

about therapeutic potential. Finding ways to replace cardiac TF with small-molecule compounds in direct cardiac reprogramming has huge implications for future cardiac regenerative therapy as it brings direct reprogramming closer to actual therapeutic applications. Even though small-molecule compounds decrease the risk of genome alteration compared to the use of viral vectors there might be other safety concerns associated with the use of small-molecule compounds that need to be thoroughly investigated. Furthermore, generation of mature iCMs is also a challenge for chemically induced direct cardiac reprogramming (Cao et al. 2016). Unveiling the molecular mechanisms underlying direct reprogramming will be important in the search for new small-molecule compounds that could induce high efficiency direct cardiac reprogramming.

# 3 AIM OF THE STUDY

Even though the reprogramming efficiency of direct cardiac reprogramming has been improved by inclusion of additional TFs and optimised reprogramming protocols, the efficiency still remains too low for any potential clinical application (Addis and Epstein 2013). The use of small-molecule compounds in direct cardiac reprogramming has previously been shown to increase the reprogramming efficiency (Ifkovits et al. 2014). The aim of this M.Sc. thesis work was to establish a small-molecule compound screening platform for direct reprogramming of MEFs to iCMs in order to identify new small-molecule compounds that together with overexpression of TFs would enhance direct cardiac reprogramming.

In order to set up a small-molecule compound screening platform the first goal was to be able to successfully directly reprogram MEFs to iCMs and to optimise the reprogramming protocol. After being able to successfully reprogram MEFs to iCMs the second goal was to set up the small-molecule compound screening platform. The aim with the screening platform is to be able to use it to screen for possible candidate molecules that could either increase the reprogramming efficiency in addition to TFs or

identify small-molecule compounds that could replace the use of TFs altogether. Initial tests of the platform were carried out with small-molecule compounds that were selected from the literature based on their reported abilities to facilitate direct cardiac reprogramming, directed differentiation from stem cells to cardiomyocytes or reprogramming of fibroblasts to iPSCs. Also preliminary tests of new molecular entities were carried out.

# 4 MATERIALS AND METHODS

## 4.1 Primary mouse embryonic fibroblasts

Primary MEFs used in this study were derived from E13.5 (embryonic day 13.5) embryos of wild type C57BL/6J mice. Mouse embryonic fibroblasts were chosen as the source of somatic cells, because of their wide use as a starting cell population for direct reprogramming to different targets, including cardiomyocytes. Mouse embryonic fibroblasts are easy to maintain and they proliferate rapidly, which also makes them an attractive cell model for drug screening experiments. The MEFs used in this study were a kind gift from Dr. Maxim Bespalov (University of Helsinki).

## 4.2 Lentiviral vectors

Lentiviruses are a type of retrovirus that are highly suitable for gene transfer, as they can infect both dividing and non dividing cells (Tolmachov et al. 2011). Lentiviruses have often been used as gene vectors for direct reprogramming, because of their ability to deliver genes with a high efficiency to the nucleus of the target cell, enabeling stable transgene expression. The lentiviral vectors used in this study were second generation lentiviruses, generated by the Biomedicum Functional Genomics Unit (Helsinki, Finland) (Table 4). In second generation lentiviral vectors the lentivirus genome is edited to include only genes necessary for virus production and the genes are split into three plasmids (a packaging plasmid, an envelope plasmid and a transfer

plasmid) in order to increase bio-safety by reducing the risk of generating replication competent lentivirus.

The lentiviral vectors had been packaged in human embryonic kidney (HEK) 293T cells (Lenti-X 293T cells, Clontech) by transfecting the cells with the backbone plasmid, psPAX2 packaging plasmid (Addgene plasmid 12260) and the pMD2.G envelope plasmid (Addgene plasmid 12259). The plasmids had been sequenced prior to viral production. The viral titer was determined by measuring the p24 capsid protein concentration (pg/ml) from the cell culture supernatant with a p24 specific enzyme-linked immunosorbent assay (ELISA). Lentiviruses used for all experiments had a minimum titer of $10^5$ pg/ml.

Viral vectors consisting of the desired TF open reading frame cloned into a doxycycline-inducible destination vector (FU-tetO-Gateway) with a Tet-ON promoter were used in order to regulate the expression of the transgenes. When combined with the reverse tetracycline transactivator (rtTA) –lentiviral vector (FUdeltaGW-rtTA), the expression of the TFs could be induced at a chosen time with addition of doxycycline. Each used lentivirus contained a single TF. The used TF lentiviral vectors also contained a V5 epitope tag at the C-terminus for identification with immunostaining.

Table 4. Lentiviral vectors used for direct reprogramming and the titers of the virus stock determined by p24 capsid protein specific enzyme-linked immunosorbent assay. FuGu refers to Biomedicum Functional Genomics unit at the University of Helsinki.

| Construct | Addgene catalog numbers | Virus stock viral titer (pg/ml) | Estimated infectious units (IFU/ml) | Producer |
|---|---|---|---|---|
| FUdeltaGW-rtTA | 19780 | $5.91x10^5$ | $5.91x10^6$ – $5.91x10^7$ | FuGu |
| tetO-Hand2 | 46028 | $5.87x10^5$ | $5.87x10^6$ – $5.87x10^7$ | FuGu |
| tetO-NKX2-5 | 46029 | $2.72x10^5$ | $2.72x10^6$ – $2.72x10^7$ | FuGu |
| tetO-GATA4 | 46030 | $7.93x10^5$ | $7.93x10^6$ – $7.93x10^7$ | FuGu |
| tetO-MEF2C | 46031 | $1.58x10^6$ | $1.58x10^7$ – $1.58x10^8$ | FuGu |
| tetO-TBX5 | 46032 | $4.49x10^5$ | $4.49x10^6$ – $4.49x10^7$ | FuGu |
| TroponinTGCaMP5-Zeo | 46027 | $1.57x10^5$ | $1.57x10^6$– $1.57x10^7$ | FuGu |

4.3 Candidate small-molecule compounds

Small-molecule compounds were tested for their ability to increase reprogramming efficiency or induce Gata4 protein expression. Commercially available small-molecule compounds (Sigma-Aldrich, Steinheim, Germany), KY02111 (Xcess Biosciences Inc, CA, USA), IWP-4 (Stemgent Inc, MA, USA) and Sodium butyrate (Sigma-Aldrich) were selected from the literature based on their reported abilities to facilitate directed differentiation from stem cells to cardiomyocytes or reprogramming to iPSCs. In addition to commercially available compounds also compounds synthesised at the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki were tested. These molecules are referred to as 3i-1047, 3i-1103 and 3i-1070. Molecule I-666 and I-595 were purchased from Pharmatory Ltd (Oulu, Finland). All used small-molecule compounds were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

## 4.4 Culturing of primary cells

Primary MEFs were maintained on gelatin-coated culture dishes at 37 °C with 5% carbon dioxide (CO2) in a humidified incubator. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (MEF medium). Medium was changed every 2-3 days. Primary MEFs (passage 1 or 2) were stored frozen at - 150 °C and thawed and grown for 2-3 days prior to passaging. When the cells reached approximately 80-90% confluency, they were passaged using trypsin TrypLE™ Select Enzyme (Gibco Life Technologies, Paisley, UK) and plated. Cells were used at passage 2 or 3 for all reprogramming experiments.

## 4.5 Optimisation of lentiviral vectors

The FUdeltaGW-rtTA lentiviral vector transduction efficiency was optimised using a tetO-GFP reporter lentiviral vector that expresses GFP when combined with the FUdeltaGW-rtTA vector. Mouse embryonic fibroblasts were plated on a gelatin-coated 24-well plate (Corning® Costar®, Corning Inc., Corning, NY, USA) at 15 000 cells / well and transduced with different amounts of FUdeltaGW-rtTA and tetO-GFP approximately 30 min after plating. Expression of the tetO-GFP vector was induced the following day with 2 µg/ml doxycycline (Sigma-Aldrich). The cells were examined by fluorescence microscopy (EVOS® FL Cell Imaging System, Thermo Fisher Scientific Inc., Rockford, IL, USA) for GFP expression 48 hours after induction.

For optimisation of transduction of TF lentivirus, MEFs were plated on a gelatin-coated 24-well plate (Corning® Costar®) at 15 000 cells / well. The cells were transduced with FUdeltaGW-rtTA (50 µl / well) 24 hours after plating and after another 24 hours the cells were transduced with 25 µl or 100 µl of one of the TF lentiviruses. Each of the 5 TF vectors (tetO-*Hand2*, tetO-*NKX2.5*, tetO-*GATA4*, tetO-*MEF2C*, and tetO-*TBX5*) were tested. Expression of the tetO-TF lentiviral vectors were induced the following day with 2 µg/ml doxycycline. The cells were stained for V5 expression two days after induction.

To optimise the transduction efficiency of the TroponinT-GCaMP5-Zeo reporter lentivirus, mouse atrial embryonic cardiomyocytes (harvested from E14.5 embryos) on a 12-well plate (cell dencity could not be counted due to poor dissociation), were transduced with 250 µl of virus. The cells were observed with fluorescence microscopy (EVOS® FL Cell Imaging System) 2-3 days after transduction for detection of GCaMP activity. The cells were kindly provided by Robert Leigh (University of Helsinki).

### 4.6 Direct reprogramming of mouse embryonic fibroblasts to induced cardiomyocytes

Direct reprogramming of MEFs to cardiomyocytes was performed by overexpressing cardiac TFs Hand2, NKX2.5, GATA4, MEF2c, and TBX5 (HNGMT). The experiments were carried out using an adaptation of a protocol originally published by Ifkovits et al. 2014. The protocol was optimised by modifying various conditions, such as culture medium, plating substrate, viral vector concentrations, time of transduction and cell density. An outline of the final protocol is shown in figure 5.

The first reprogramming experiments were carried out using a Troponin T-GCaMP reporter system, identical to the one used by Ifkovits et al. (2014), as a functional outcome measure. In the Troponin T-GCaMP reporter system the expression of the calcium indicator GCaMP5 is driven by a cardiomyocyte-specific Troponin T promoter. The GCaMP5 protein contains a calcium-binding calmodulin domain, which is fused to GFP. When the intracellular calcium level is high and calcium is bound to the calmodulin domain, GFP is expressed resulting in bright fluorescence. In our experiments, however, the GFP flashing generated by calcium oscillation in cardiomyocytes was not detectable with the available microscope (EVOS® FL Cell Imaging System). Due to this, immunocytochemical analysis with cardiac Troponin T (cTnT) was used as readout instead.

The final reprogramming experiments were carried out either in a flat-bottomed 24-well plate (Corning® Costar®) or black-bottomed 96-well ViewPlate® microplate

(PerkinElmer Inc., MA, USA). At Day -2 the plates were coated with poly-L-Lysine solution (Sigma-Aldrich,) and incubated in room temperature for one hour, after which they were washed with phosphate-buffered saline (PBS) and left to dry for a minimum of 2 hours. Mouse embryonic fibroblasts (passage 3) were then plated at 15 000 cells/well for the 24-well plate or 2500 cells/well for the 96-well plate together with FUdeltaGW-rtTA lentiviral vector (50 µl/well or 8,4µl/well) and MEF medium so that the total volume of each well was 750 µl (24-well plate) or 120 µl (96-well plate). The MEF medium was supplemented with 8 µg/ml of Polybrene® (hexadimethrine bromide, Sigma-Aldrich, Steinheim, Germany) to increase the transduction efficiency. On Day -1 cell culture medium was changed to fresh MEF medium with Polybrene® and cells were transduced with each tetO-TF lentivirus (25 µl/well in 24-well plate or 4,2 µl/well in 96-well plate of each TF expression virus). In experiments testing small-molecule compounds, also compounds and vehicle control (DMSO) were added on day -1. Expression of the doxycycline-inducible tetO-TF lentiviral vectors were induced on Day 0 by changing the medium to reprogramming medium consisting of astrocyte growth media without epidermal growth factor (AGM, CC-3186, Lonza, Walkersville, MA, USA) and supplemented with 2 µg/ml doxycycline. Reprogramming medium was changed every 2 days. When testing candidate small-molecule compounds, fresh small-molecule compounds and control vehicles were added with every medium change. The cells were kept in culture for 2-4 weeks after induction.

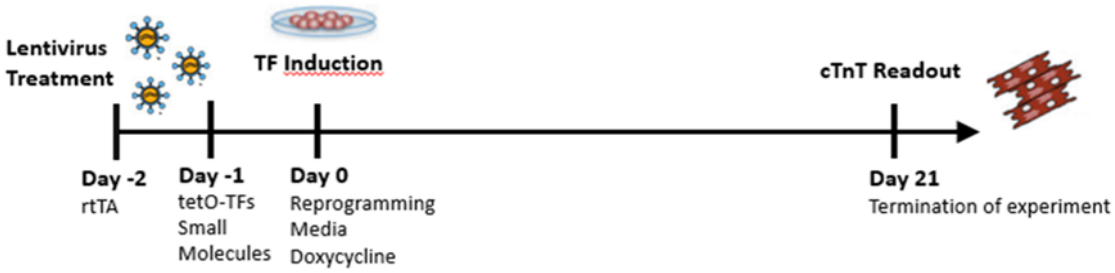


Figure 5. A schematic illustration of the final direct reprogramming protocol. Mouse embryonic fibroblasts (MEFs) were transduced with a reverse tetracycline transactivator (rtTA) -lentiviral vector at Day -2. After 24 h (Day -1) the cells were transduced with transcription factor (TF) lentiviral vectors with a Tet-ON promoter (tetO-TFs). Expression of transcription factors was induced with doxycycline the following day (Day 0). Medium was changed every 2-3 days. When testing candidate small molecules, cells were first treated with small molecules at Day -1 and fresh small molecules were added with every medium change. The cells were kept growing until Day 14 or 21 post-induction, when they were fixed analysed with immunostaining for cardiac Troponin T (cTnT).

4.7 Immunocytochemistry

For immunocytochemical staining, cells from reprogramming experiments were fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature and rinsed with PBS. The plates were then either stored at + 4 °C or used immediately for immunocytochemistry.

The fixed cells were permeabilised with 0,5% Triton X-100 diluted in PBS for 15 minutes at room temperature. To reduce nonspecific antibody binding, the cells were then incubated in Lab Vision™ Ultra V Block- protein block solution (Thermo Fisher Scientific) for 10 minutes before adding the primary antibody (table 5). The cells were incubated in 250 µl primary antibody (1:200 or 1:500, diluted in 3% bovine serum albumin, BSA in PBS) overnight, rocking at + 4 °C. The following day the cells were washed 6 times for 10 minutes with PBS and then incubated in secondary antibody dilution (1:500, diluted in 3% BSA in PBS) for 1 hour in the dark at room temperature. After incubation in secondary antibody, the cells were washed 3 times for 5 minutes

with PBS and finally mounted in Vectashield™ mounting media (Vector Laboratories Inc., Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining and examined by fluorescence microscopy (EVOS® FL Cell Imaging System) and CellInsight™ high content imaging system (Thermo Scientific). The used secondary antibodies are listed in table 6.

Table 5. Primary Antibodies used for immunocytochemistry. Ig = immunoglobulin.

| **Antibody** | **Manufacturer** | **Code** | **Description** | **Dilution** |
|---|---|---|---|---|
| anti-V5 | Life technologies | R960-25 | mouse monoclonal IgG2ak | 1:200 |
| anti-cardiac Troponin T | Thermo Scientific | MS-295-P | mouse monoclonal IgG1 | 1:200 |
| anti-GATA4 | Santa Cruz biotechnology | SC-1237 | goat polyclonal IgG | 1:200 |
| anti-Myl2 | Santa Cruz biotechnology | SC-34490 | goat polyclonal IgG | 1:150 |
| anti-Alpha smooth muscle actin | Sigma-Aldrich | A2547 | mouse monoclonal IgG2a | 1:200 |
| anti-Thy-1 | Santa Cruz Biotechnology | SC-9163 | rabbit polyclonal IgG | 1:200 |

Table 6. Alexa Fluor® Secondary Antibodies used for immunocytochemistry. Ig = immunoglobulin.

| **Antibody** | **Manufacturer** | **Code** | **Description** | **Dilution** |
|---|---|---|---|---|
| Alexa Fluor® 488 | Life Technologies | A11055 | Donkey Anti-goat IgG, green | 1:500 |
| Alexa Fluor® 488 | Life Technologies | A21202 | Donkey Anti-mouse IgG, green | 1:500 |
| Alexa Fluor® 594 | Life Technologies | A21207 | Donkey Anti-rabbit IgG | 1:500 |
| Alexa Fluor® 488 | Life Technologies | A21206 | Donkey Anti-rabbit IgG | 1:500 |
| Bdm 647 | Santa Cruz Biotechnology | SC-362286 | Bovine anti-mouse IgG-CFL 647 | 1:200 |

### 4.8 Cell imaging and quantification of induced cardiomyocytes

Cells were observed every 1-2 days during the whole direct reprogramming process using light microscopy (Leica DM IL LED, Leica Microsystems, Wetzlar, Germany) for changes in cell morphology, signs of cell toxicity and for spontaneously beating cells. For quantification of iCMs after immunostaining for cTnT, cells were imaged with fluorescence microscopy (EVOS® FL Cell Imaging System,). Pictures were taken of 4 unique fields of view (4 x magnification) in a single well and cells were counted with the cell counter plugin in ImageJ software. Reprogramming efficiency was assessed by comparing the number of cTnT+ cells to the total number of cells stained with DAPI. A minimum of 500 total cells were counted per condition for reprogramming experiments carried out with the optimised protocol. Values are expressed as mean ± standard deviation (SD). Statistical analysis was not preformed due to lack of replicates in different experiments.

## 5 RESULTS

### 5.1 Preliminary experiments to evaluate lentiviral vectors

The first goal towards direct reprogramming of MEFs to iCMs was to optimise the transduction efficiency of the lentiviral vectors in order to ensure overexpression of the desired TFs. The p24 capsid protein concentration (pg/ml) of the virus stock can only be used to estimate the amount of functional virus in the stock (IFU/ml). In order to ensure high enough levels of expression of the TFs, the viral vectors were tested and the transduction efficiency determined. Also the TroponinT-GCaMP5-Zeo lentiviral vector was tested to ensure that the readout of the experiments was valid and reliable.

### 5.1.1 Optimisation of transduction efficiency of the FUdeltaGW-rtTA vector

Since the FUdeltaGW-rtTA vector is required for Tet-on regulated activation of the transgene expression, it works as a limiting factor for transduction efficiency. Using enough FUdeltaGW-rtTA vector was therefore critical for the reprogramming experiments. The optimal amount of FUdeltaGW-rtTA was determined by transducing MEFs with 20 µl, 50 µl and 100 µl of the FUdeltaGW-rtTA and with a tetO-GFP lentiviral vector. The tetO-GFP lentiviral vector was also used to test the FUdeltaGW-rtTA construct, as seeing GFP expression from the tetO promoter when FUdeltaGW-rtTA virus is included suggests that the construct is working as it should.

The transduction efficiency was assessed two days post-induction by comparing the number of cells expressing GFP to the total number of cells stained with DAPI (Figure 6). The highest transduction efficiency was achieved with 100 µl of the rtTA viral vector, which resulted in a transduction efficiency of 97%, while 50 µl viral vector resulted in an efficiency of 94% and 20 µl in 81%. However, the difference between the transduction efficiency gained with 50 µl and 100 µl was so small that based on these results it was decided to use 50 µl FUdeltaGW-rtTA virus for the reprogramming experiments.

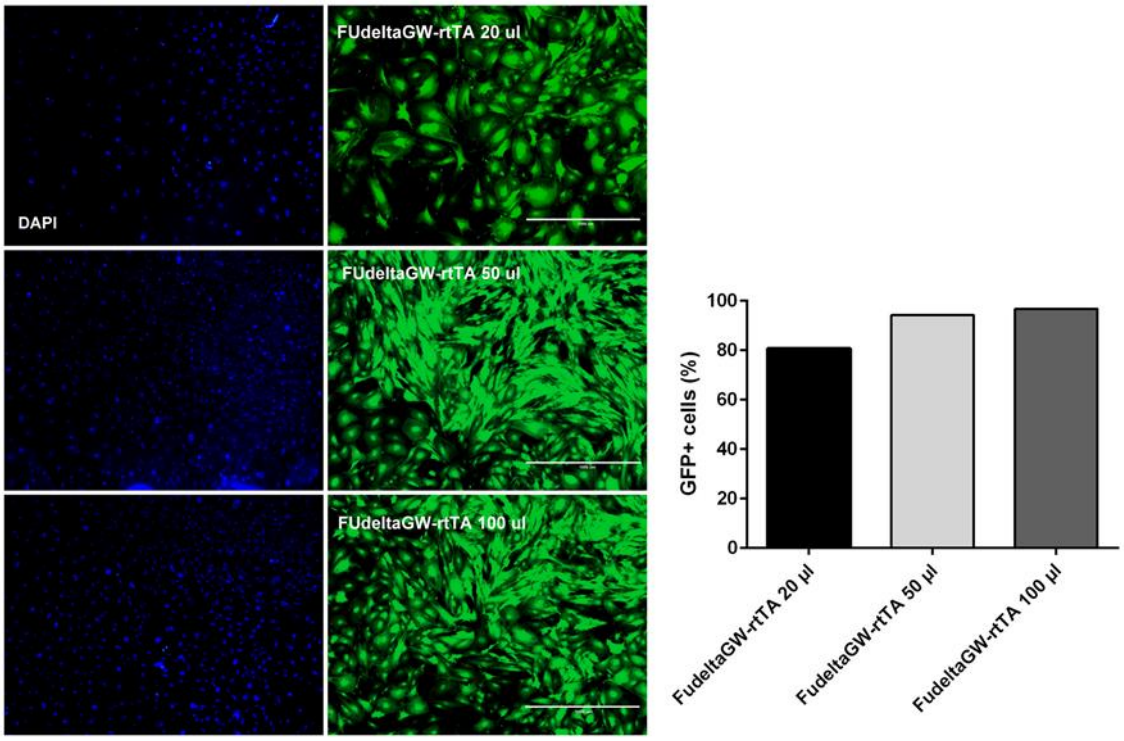


Figure 6. Optimisation of transduction efficiency of FUdeltaGW-rtTA viral vector. Transduction efficiency was evaluated at Day 2 post induction using a green fluorescent protein (GFP) marker vector with a with a Tet-ON promoter that expresses GFP when combined with the FUdeltaGW-rtTA vector (n=1). The transduction efficiency was quantified the using cell counter plugin in ImageJ software. Scale bar is 1000 µm. DAPI = 4',6-diamidino-2-phenylindole.

### 5.1.2 Optimisation of transduction efficiency of transcription factor viral vectors

Viral transduction efficiency and overexpression of TFs was evaluated for MEFs transduced with TF viral vectors at Day 2 post-induction, using immunostaining for the V5 epitope tag at the C-terminus of the vectors containing the TFs. The viral transduction efficiency was evaluated for each TF separately and for all 5 TFs (HNGMT) together (Figure 7). All TF vectors had a transduction efficiency over 70%. The joint viral transduction efficiency for all 5 TF vectors was determined to be 84%. It is however to be noted that the joint viral transduction efficiency only means that that the cells got at least one TF and the percentage of cells that received all five TFs is not

known. The transduction efficiency of the tetO-Hand2 viral vector could not be determined due to a stop codon located in the vector backbone before the C-terminus.

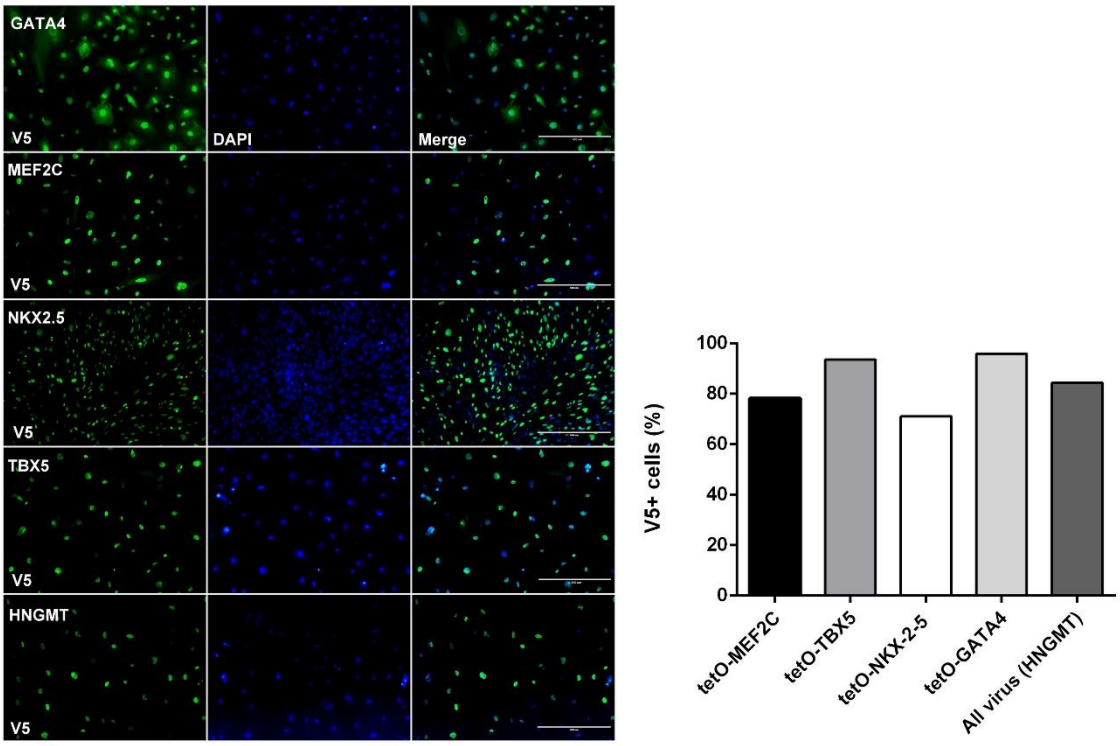


Figure 7. Determination of viral transduction efficiency. Transduction efficiency was evaluated at Day 2 post induction using immunostaining for the V5 epitope tag at the C-terminus of the transcription factor viral vectors. The joint viral transduction efficiency for all 5 transcription factor vectors was determined to be 84% (n=1). The transduction efficiency is expressed as the percentage of V5+ quantified using cell counter plugin in ImageJ software. Scale bar is 400 µm. DAPI = 4',6-diamidino-2-phenylindole.

### 5.1.3 Optimisation of transduction efficiency of the TroponinT-GCaMP5-Zeo reporter lentivirus

The transduction efficiency of the TroponinT-GCaMP5-Zeo reporter lentivirus was tested and optimised by transducing mouse atrial embryonic cardiomyocytes with the reporter vector (TroponinT-GCaMP5-Zeo). TheCaMP5 activity in the cells was observed with fluorescence microscopy 2-3 days after transduction. As previously described,

calcium oscillations in beating cardiomyocytes transfected with the reporter vector should result in green fluorescent flashing (Addis et al. 2013). When observing the cells with fluorescence microscopy, clusters of robust beating cells were clearly visible and they expressed GFP (figure 8). However, no GFP flashing was seen, even though the cells were beating. Due to these observations, the conclusion was made that GFP flashing could not be seen with the available microscope. Based on this, the decision was made to change the readout to immunocytochemical analysis for cTnT, in order to get a quantifiable readout.

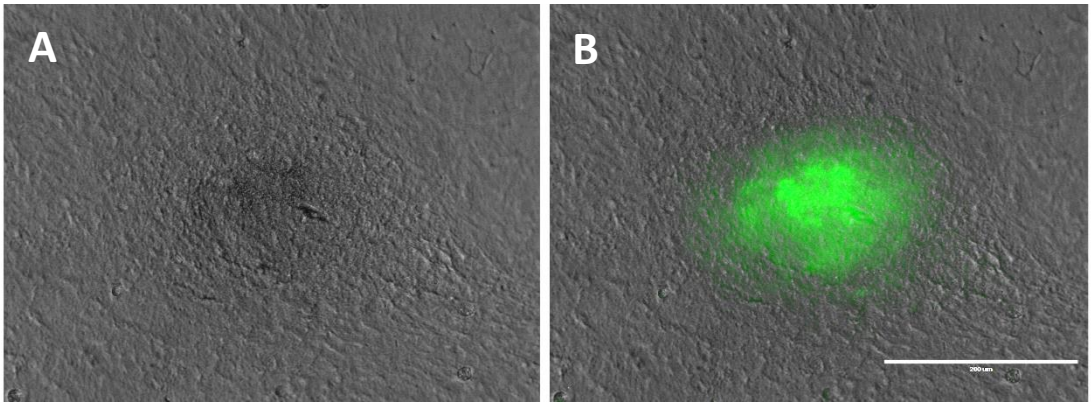


Figure 8. Validation of the TroponinT-GCaMP5-Zeo reporter lentiviral vector in mouse atrial embryonic cardiomyocytes. Robustly beating clusters of cardiomyocytes expressed green fluorescent protein (GFP) at Day 3 post transduction, but no GFP flashing was observed. A.) Beating cluster of mouse atrial embryonic cardiomyocytes. B.) GFP is expressed in beating cardiomyocytes. Scale bar is 200 µm.

## 5.2 Optimisation of the reprogramming protocol

It has been suggested that seemingly small differences in the direct reprogramming experimental procedures, such as culture medium, plating substrate, cell density and viral titers, may be behind the varying and inconsistent reprogramming efficiencies reported by different groups (Addis and Eppstein, 2013). In order to find the optimal conditions for the direct reprogramming experiments, different conditions in the protocol were changed to observe which conditions resulted in the highest reprogramming efficiency. Also different lengths of cell culture times were tested

during the optimisation process. This was done to find out if longer culture time would have a positive effect on the reprogramming.

### 5.2.1 Determining optimal amount of of transcription factor vectors

As described earlier, the p24 capsid protein concentration only gives an estimate of the functional virus in the viral vector stock. Therefore, specific concentrations of virus stock could not be used for the experiments, as the exact concentration of infectious units in the virus stocks could not be calculated. Instead MEFs were transduced with different amounts of the HNGMT viral vectors (25 µl, 50 µl, 75 µl and 100 µl of each TF viral vector) to see which amount resulted in the highest reprogramming efficiency. Upon quantification at Day 32, the highest reprogramming efficiency was achieved with 25 µl of each HNGMT virus, which generated 5,20% iCMs (Figure 9). The reprogramming efficiency as well as the total cell number decreased with an increasing amount of virus. Treatment with 50 µl of each HNGMT viral vectors generated 4,56% iCMs, while 75 µl only generated iCMs with an efficiency of 1,47%. With 100 µl of each HNGMT viral vectors the cell death was so extensive that no quantification could be done.

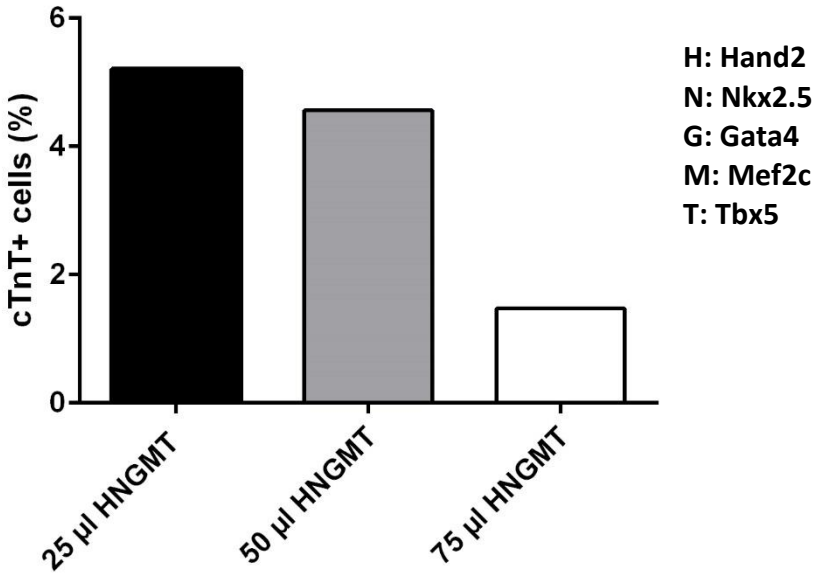


Figure 9. Determination of the optimal amount of transcription factor viral vector for direct reprogramming. Mouse embryonic fibroblasts (MEFs) were transduced with different amounts of viral vectors (25 µl, 50 µl and 75 µl of each transcription factor vector) and the transduction efficiency was assessed 32 days after induction using immunostaining for cardiac troponin T. The highest transduction efficiency was achieved with 25 µl of each virus (n=1).

Next the effect of TF stoichiometry on reprogramming efficiency was examined. Based on observations during previous experiments, NKX2.5, TBX5 and Hand2 seemed to be more toxic to the cells than MEF2C and GATA4. Since the previous experiments also showed that higher viral toxicity resulted in lower reprogramming efficiency, it was examined if lower expression levels of NKX2.5, TBX5 and Hand2 had a positive effect on reprogramming efficiency. Also, according to a recent publication by Wang et al. (2015), optimal stoichiometry of Gata4, Mef2c and Tbx5 has a great influence on reprogramming efficiency and on the expression of mature cardiac genes in iCMs. Their study showed that vectors that expressed higher levels of Mef2c and lower levels of Gata4 and Tbx5 resulted in a higher reprogramming efficiency compared to the other vectors.

Based on these results 6 different conditions were selected and their effect on the reprogramming efficiency was compared: 10 µl of each TF viral vector (HNGMT), 10 µl

of each HNGT viral vector + 100 µl of M, 100 µl of G and M + 10 µl of each HNT viral vector, 100 µl of each HNT viral vector + 10 µl of G and M, 25 µl of each HNGMT viral vector and 50 µl of each HNGMT viral vector. The results are shown in figure 10. The combination consisting of 10 µl of each HNGT + 100 µl of M (0,18%) did not result in a higher reprogramming efficiency compared to the other conditions. An increase in the reprogramming efficiency was, however, observed with higher levels of G and M (10 µl of each HNT + 100 µl of G and M, 1,03%) when compared with 10 µl of each HNGT + 100 µl of M (0,18%) and 100 µl of each HNT + 10 µl of G and M (0,04%). The highest reprogramming efficiency was still achieved with 25 µl of each HNGMT TF (1,90%). Based on these results it was decided to use 25 µl of each HNGMT TF for the reprogramming experiments. It is to be noted that these results are based on single experiments and therefore no conclusions on the effect of HNGMT stoichiometry on reprogramming efficiency can be drawn from on these results.

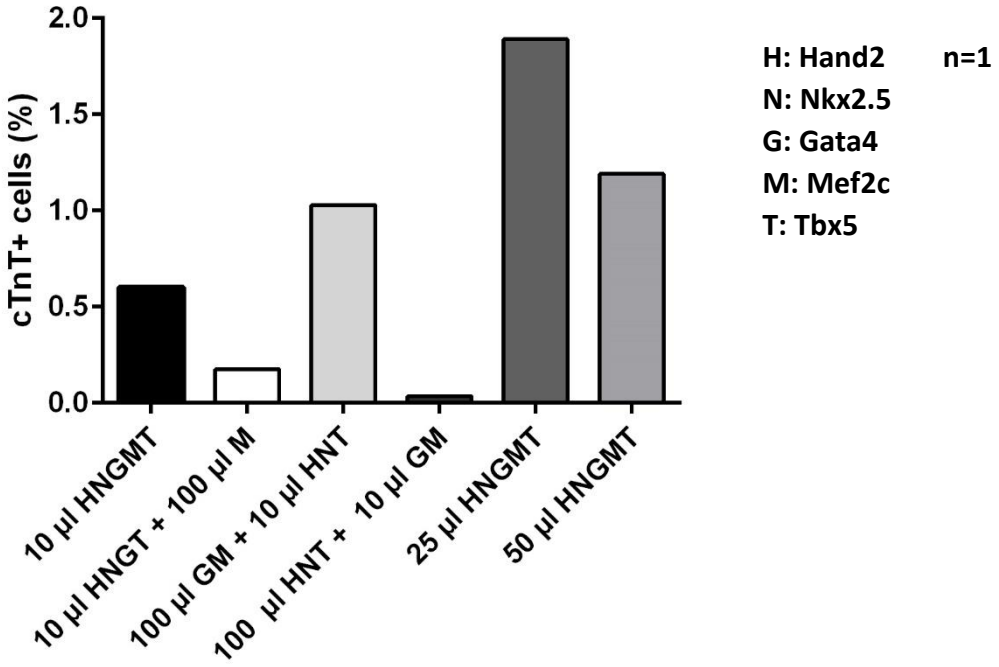


Figure 10. The effect of transcription factor stoichiometry on reprogramming efficiency. Mouse embryonic fibroblasts (MEFs) were transduced with different amounts of HNGMT viral vectors and the transduction efficiency was assessed 26 days after induction with immunostaining for cardiac troponin T (cTnT) (n=1). The reprogramming efficiency is expressed as the percentage of cTnT+ cells analysed with immunostaining and quantified using cell counter plugin in ImageJ software.

### 5.2.2 Determining optimal culture conditions

During the optimisation of the protocol, culture conditions were changed systematically in order to find the optimal conditions for the direct reprogramming experiments. The parameters to optimise were cell culture medium, plating substrate, cell density and culturing times.

In the direct reprogramming protocol developed by Ifkovits et al. (2014) they used AGM without epidermal growth factor for their reprogramming experiments. In order to see if the same results could be achieved using MEF medium, the effects of these two culture mediums on reprogramming efficiency were compared. The reprogramming efficiency gained using AGM (3,8 %) was notably higher than the efficiency in MEF medium (<0,01%). Therefore, AGM was selected for the future reprogramming experiments.

The choice of plating substrate affects the attachment, growth pattern and number of the cells and can thus have an indirect effect on direct reprogramming. In order to get clear and quantifiable data, effect of gelatin and poly-L-lysine as plating substrates on reprogramming efficiency was compared. Mouse embryonic fibroblasts plated on either gelatin- or poly-L-lysine coated wells were transduced with the TF GATA4 and the transduction efficiency was determined with anti-Gata4 immunostaining at Day 4 post-induction. Both transduction efficiency and total cell number were higher with poly-L-lysine than with gelatin (data not shown). Even though the difference between the two plating substrates was not great, it was decided to use poly-L-lysine for future experiments, as the cells were higher in number and looked more viable.

The initial experiments showed that treatment with the viral vectors was toxic for the cells and the number of surviving cells after 2-4 weeks in culture was low. Therefore, it was examined if a higher cell density (25 000 cells/well) at plating would have a positive effect on the cell survival and the reprogramming efficiency. The experiments

however showed that even though higher initial cell density at plating resulted in a higher number of surviving cells, the reprogramming efficiency was not greatly influenced by this (data not shown).

Finally, it was examined if a longer culture time resulted in a higher reprogramming efficiency. The culturing times for the different experiments were varied between 2-4 weeks. Longer culturing times were found to seemingly result in higher reprogramming efficiencies and more mature cardiomyocytes, as indicated by higher number of beating cells and cells with visible sarcomere structure (data not shown). Longer cell culturing times did however also result in fewer total number of cells at the time of quantification (data not shown). Individual experiments, even though executed following the same protocol, resulted in very varying reprogramming efficiencies.

## 5.3 Immunocytochemical analysis of induced cardiomyocytes

Immunocytochemical analysis of HNGMT-transduced MEFs revealed expression of the cardiac specific marker cTnT. The percentage of cells expressing cTnT varied with each experiment. Representative images of iCMs stained for cTnT are shown in figure 11. While some iCMs had clearly organised sarcomeres and a cardiomyocyte-like rod shaped morphology, the majority of cells expressing cTnT did not have organised sarcomeres and the morphologies of these cells were varying and irregular. Non-transfected cells and cells transfected with only FUdeltaGW-rtTA lentivirus were used as controls to rule out the possibility that cTnT protein expression would be caused by components of the medium or cytokines. No cTnT expression was detected in cells that were not transduced with TF lentiviruses (figure 12). Some cell batches transduced with HNGMT did not express cTnT at all. These experiments were defined as failed and the results are not included in analyses.

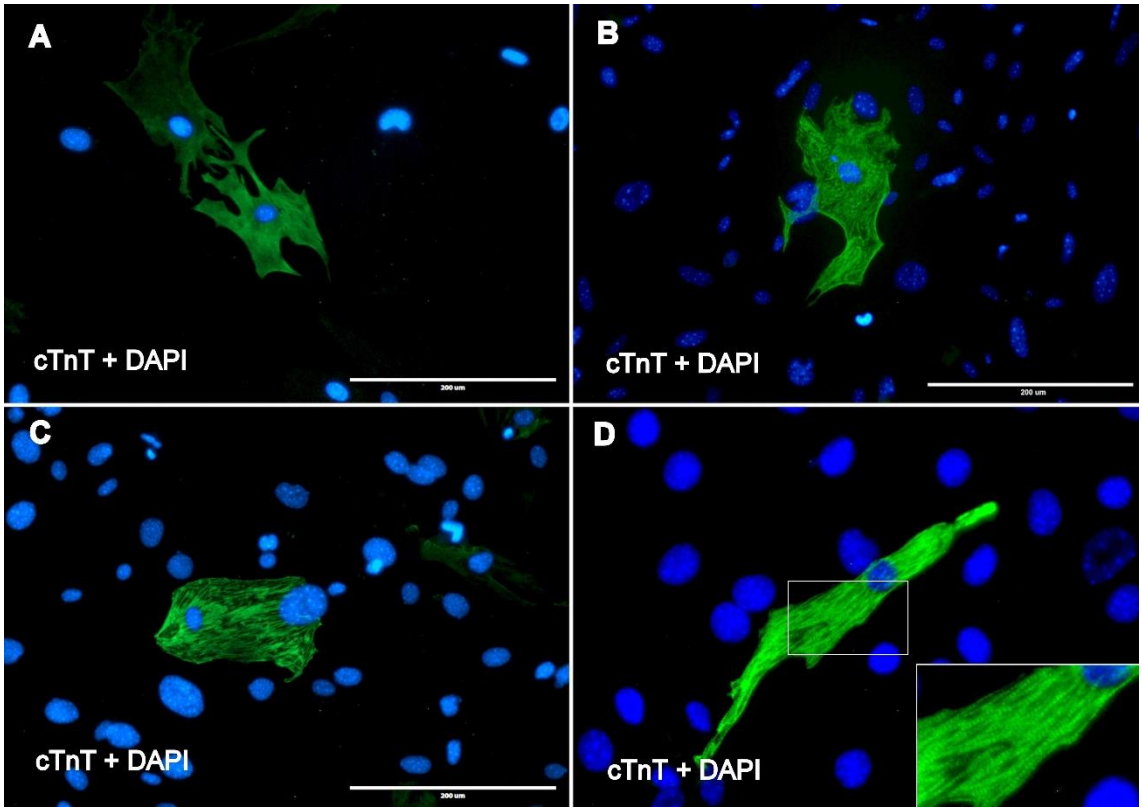


Figure 11. Representative immunofluorescence images of Day 14 HNGMT (Hand2, NKX2.5, GATA4, MEF2C and TBX5) -induced cardiomyocytes (iCMs) stained for cardiac troponin T (cTnT) (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue). Images A-D show iCMs with different levels of sarcomere assembly indicating different levels of maturation of iCMs. In A-B no sarcomere formation is seen and the cell morphology is highly irregular. In C sarcomere structure is starting to organise and D represents a more mature iCM with a rod-shaped morphology and well organised sarcomeres. The region in the white box is enlarged to show sarcomere structure. All types were commonly observed in the reprogramming experiments. Scale bars are 200 µm.

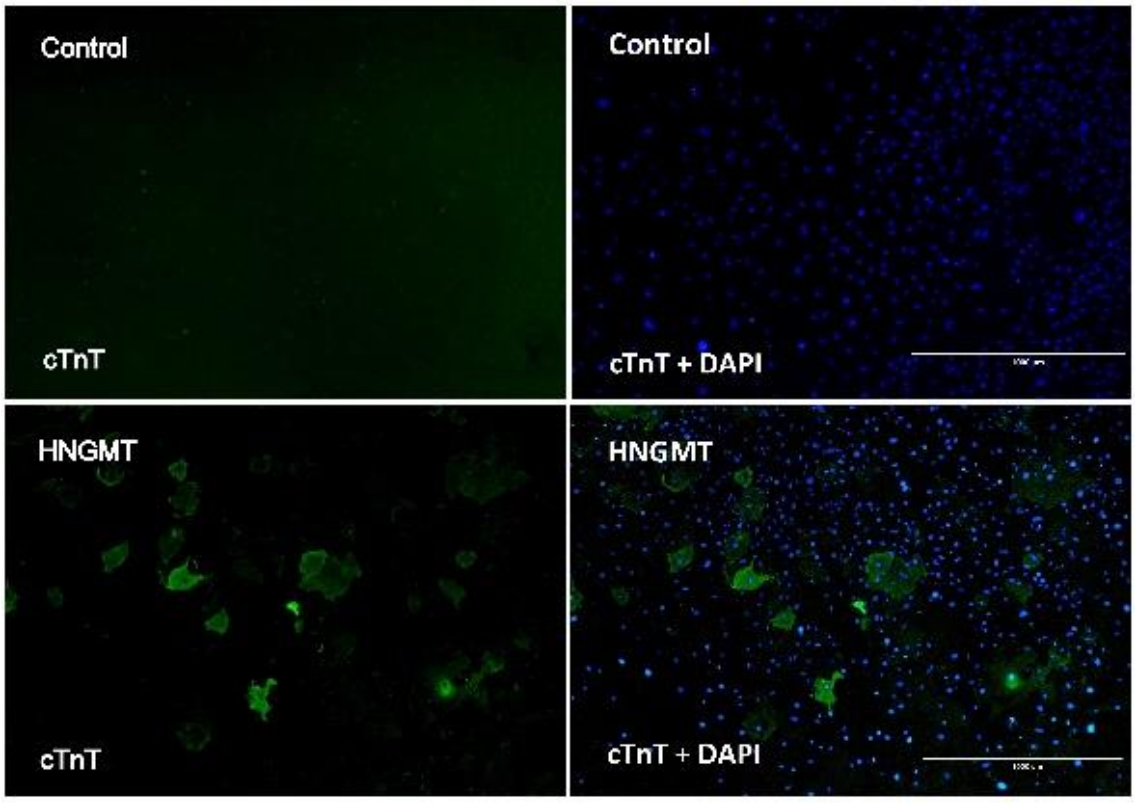


Figure 12. Representative immunofluorescence images of reverse tetracycline transactivator (rtTA) –lentiviral vector only control compared with HNGMT-transduced mouse embryonic fibroblasts (MEFs) at Day 14 post-induction stained for cardiac troponin T (cTnT) (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue). MEFs that were not transduced with transcription factor lentiviruses did not stain positive for cTnT. Scale bar is 1000 µm. H = Hand2, N= NKX2.5, G = GATA4, M = MEF2C, T = TBX5.

The cells were also analysed for expression of the smooth-muscle cell marker α-smooth muscle actin (α-SMA). The expression of α-SMA in the cells was analysed, as it is a marker of immature cardiomyocytes and normally expressed in early stages of cardiomyocyte differentiation. α-SMA is however not a specific cardiomyocyte marker and it is also expressed in other smooth muscle cells and in myofibroblasts. Immunocytochemical analysis reveald α-SMA expression in both HNGMT-transduced MEFs and in the control with only FUdeltaGW-rtTA-lentivirus (Figure 13). Upon quantification at Day 21 post-induction approximately 41,2% of the HNGMT-transduced cells and 21,9% of the control without TF lentiviruses expressed α-SMA (N = 1, data not shown). Fibroblasts are known to start expressing α-SMA in cell culture as

they transdifferentiate into myofibroblasts, which is most likely the cause of α-SMA expression the control cells and in most HNGMT-transduced cells.

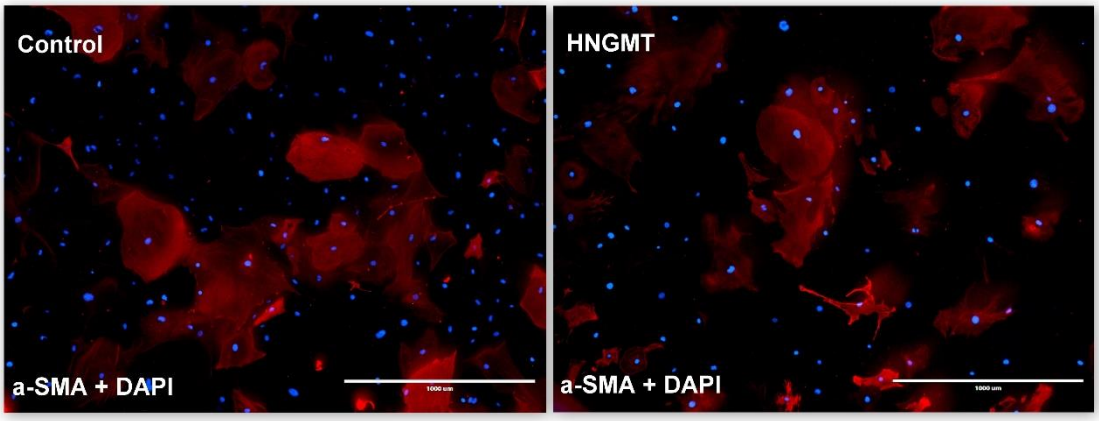


Figure 13. Representative immunofluorescence images of Day 21 HNGMT (Hand2, NKX2.5, GATA4, MEF2C and TBX5)-transduced mouse embryonic fibroblasts and control stained for α-smooth muscle actin (α-SMA) (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bar is 1000 µm.

## 5.4 Beating induced cardiomyocytes

All reprogramming experiments were qualitatively observed with light microscope every 1-2 days during the whole direct reprogramming process in order to find spontaneously beating cardiomyocytes. Sporadic single beating cells were seen in HNGMT-transduced iCMs as early as Day 13 post-induction (shown in supplemental movie 1). Beating cells were however not observed in all experiments. The occurrence of beating cells during the direct reprogramming process also seemed to correlate with a higher reprogramming efficiency when the cells were analysed with immunocytostaining. This was however not quantified.

## 5.5 Candidate small-molecule compound testing

### 5.5.1 Small-molecule compound testing in direct cardiac reprogramming

After being able to successfully convert MEFs to iCMs the next step was to test out the screening platform. For initial testing of the screening platform the TGF-β inhibitor SB431542 (SB) and histone deacetylase inhibitor sodium butyrate were selected. SB has previously been shown to increase the reprogramming efficiency of HNGMT-transduced MEFs up to 5-fold compared with baseline condition of HNGMT+DMSO (Ifkovits et al. 2014). Sodium byturate was selected based on its ability to increase the efficiency of iPSC derivation from fibroblasts (Mali et al. 2010). Upon quantification at Day 26, the baseline condition (HNGMT+DMSO) generated iCMs with an efficiency of 0,50% (figure 14). Treatment with 0,5 µM SB increased the reprogramming efficiency to 3,21% and treatment with 2 µM SB to 5,86%. Sodium butyrate on the other hand decreased the reprogramming efficiency with both 0,25 µM (0,01%) and 1 µM (0,04%). Also a decrease in the total cell number was observed with sodium butyrate. Even though the reprogramming efficiencies were low in this experiment, SB increased the reprogramming efficiency compared with baseline. This is in line with previous publications (Ifkovits e. 2014).

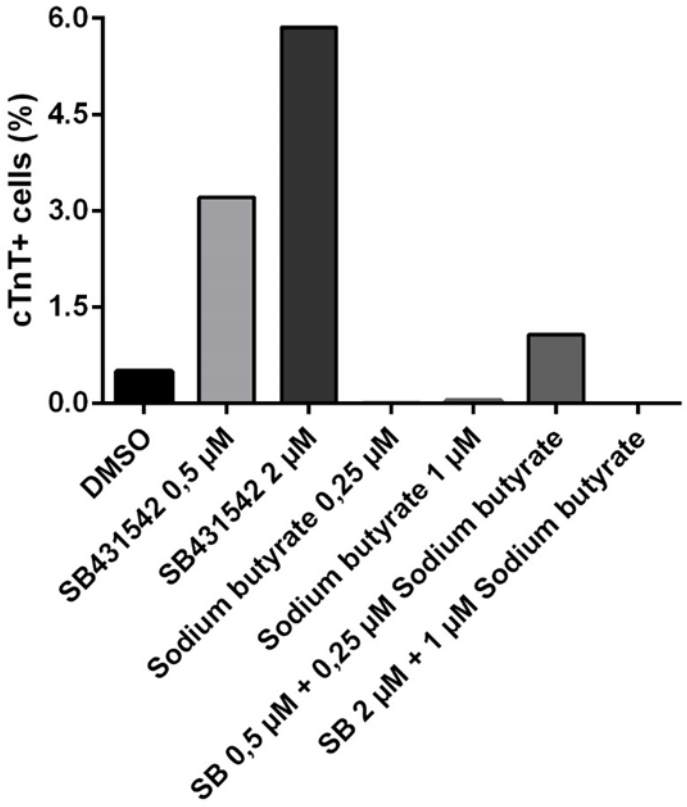


Figure 14. Initial testing of the small-molecule compound screening platform with small-molecule compound treatment. Mouse embryonic fibroblasts (MEFs) were transduced with HNGMT (Hand2, NKX2.5, GATA4, MEF2C, and TBX5) transcription factor combination and SB431542 (SB) (0,5 µM and 2 µM) and/or Sodium butyrate (0,25 µM and 1 µM) The small-molecule compounds were added 1 day before inducing the transcription factor expression and maintained in culture during the whole experiment. The cells were fixed at Day 26 and analysed with immunostaining for cardiac troponin T (cTnT) (n=1). The reprogramming efficiency is expressed as the percentage of cTnT+ cells analysed with immunostaining and quantified using cell counter plugin in ImageJ software.

Next the effect of small-molecule compounds on the reprogramming efficiency was evaluated using two different TF combinations: HNGMT and HGMT. Addison et al. (2013) found that the addition of Nkx2.5 to the HGMT TF combination increases the reprogramming efficiency. However, previous publications have reported a decrease in the cardiac reprogramming efficiency with the addition of Nkx2.5 in screens for TF combinations (Ieda et al. 2010, Song et al. 2012).

For this experiment four small-molecule compounds were selected. SB was chosen as the baseline in order to obtain enough iCMs, as previous experiments had shown that iCMs were not generated consistently without SB. The Wnt signalling pathway is one of the major signalling cascades involved in cardiac muscle development and it has been shown to have a biphasic role in cardiac development (Sahara et al. 2015). Therefore both a Wnt signalling agonist CHIR99021 and a Wnt inhibitor IWP-4 were selected for testing. Also two small-molecule compounds, 3i-666 and 3i-595 that are targeted to modulate the activity of cardiac TFs were selected.

Upon quantification on Day 21, the control conditions HNGMT + DMSO produced 6,4 ± 2,9% (n=2, mean ± SD) iCMs and HGMT + DMSO produced 5,3 ± 1,3% (n=2) iCMs (figure 15, Figure 16). Consistent with the previous results, an increase in the reprogramming efficiency was observed with 2 µM SB treatment in both HNGMT (14,o ± 6,1%, n=3) and HGMT-transduced cells (13,1 ± 1,3, n=3). Also an increase in spontaneously beating iCMs was qualitatively observed with SB treatment in both HNGMT and HGMT conditions (data not shown). None of the other small-molecule compounds had a positive effect on the reprogramming efficiency. Treatment with 5 µM I-666 together with 2 µM SB decreased the iCM yield for both HNGMT-transduced cells (8,5 ± 1,8%, n=3) and HGMT-transduced cells (8,8 ± 1,7%, n=3). A decrease in the reprogramming efficiency and in the total number of cells was also observed with 1,2 µM IWP-4 treatment in both HNGMT-transduced cells (2,1 %, n=1) and HGMT-transduced cells (1,5 ± 0,1%, n=2). Addition of 2 µM SB to the IWP-4 treatment increased the reprogramming efficiency for both HNGMT (5,3 ± 2,3%, n=2) and HGMT (6,6 ± 2,5%, n=2), but the yield was still lower than for SB alone. Treatment with CHIR99021 and I-595 was so toxic to the cells that no results could be obtained for these compounds. A minimum of 500 total cells were analysed for each condition / experiment.

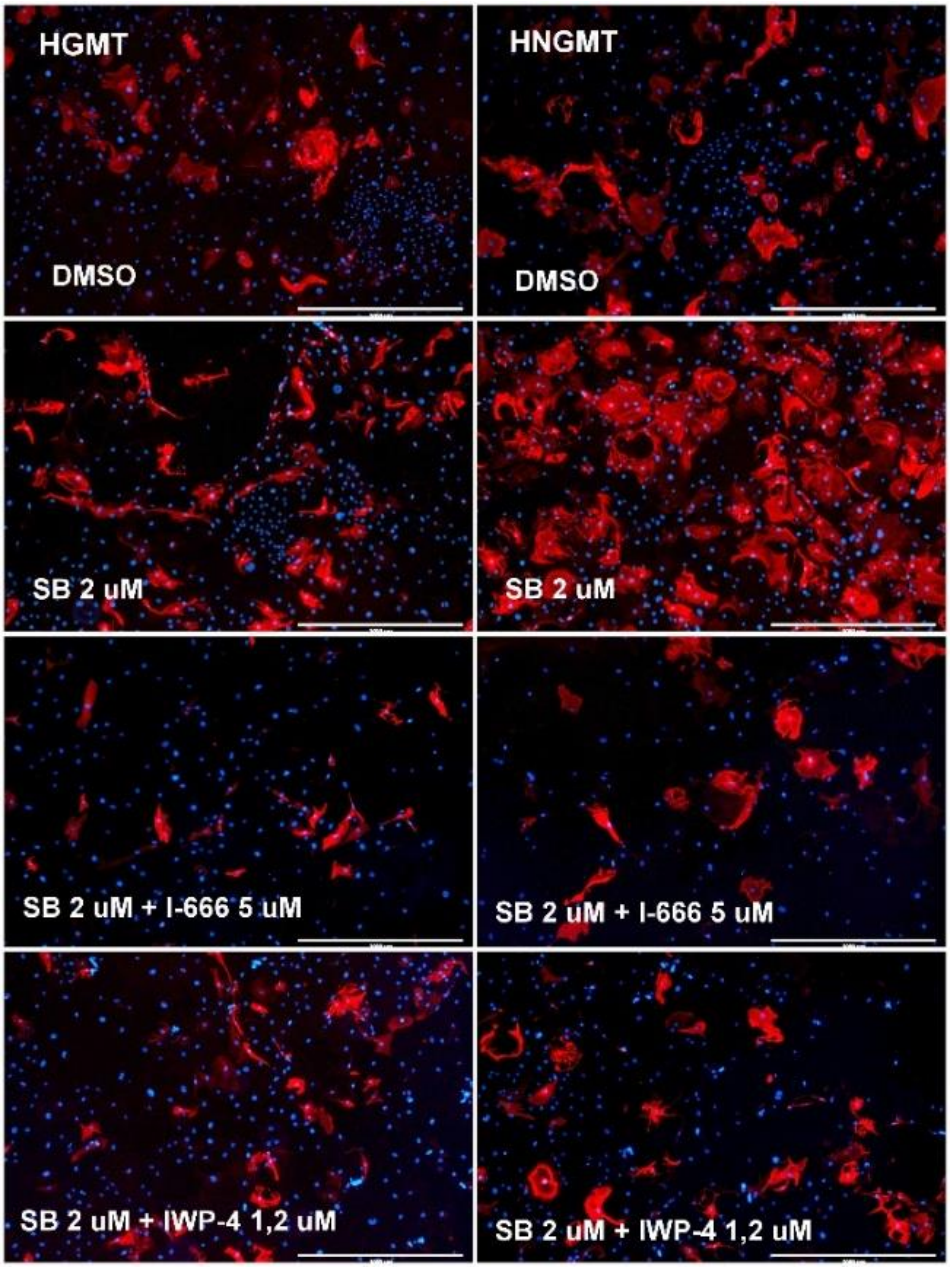


Figure 15. Representative immunocytochemistry images for HGMT- and HNGMT-induced cardiomyocytes (iCMS) treated with small-molecule compounds. Small-molecule compounds were added one day before inducing the transcription factor expression and maintained in culture during the whole experiment. The cells were fixed and stained for cardiac troponin T (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue) at Day 21 post-induction. Scale bar is 1000 µm. SB = SB431542, H = Hand2, N= NKX2.5, G = GATA4, M = MEF2C, T = TBX5.

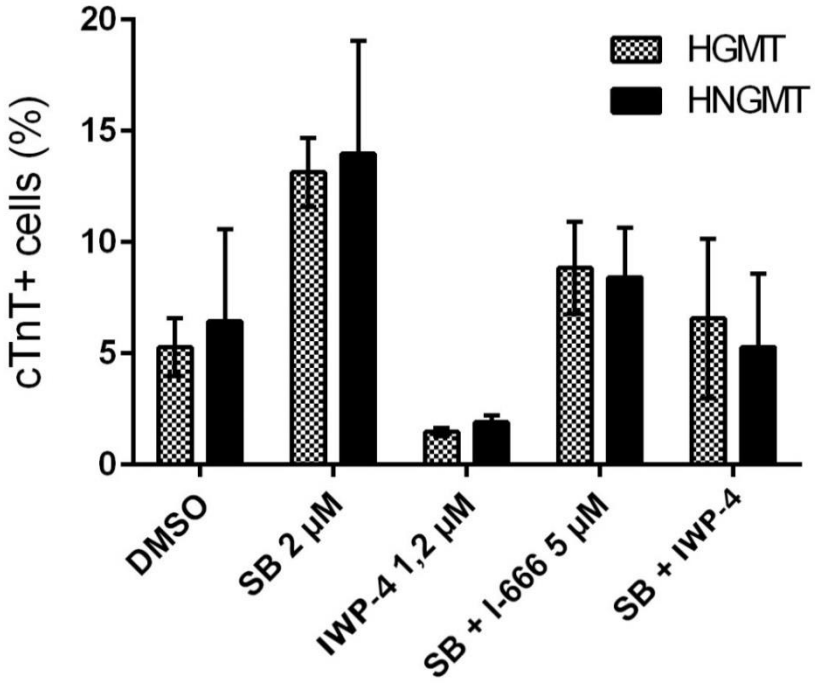


Figure 16. Comparison of the effect of small-molecule compounds on reprogramming efficiency for HGMT- and HNGMT-transduced mouse embryonic fibroblasts. Mouse embryonic fibroblasts were transduced with either HGMT or HNGMT transcription factor combination and treated with DMSO (n=3), SB431542 (SB) (n=3), IWP-4 (HGMT n=2, HNGMT n=1), SB + I-666 (n=3) or SB + IWP-4 (n=2). The reprogramming efficiency was assessed at Day 21. The reprogramming efficiency is expressed as the percentage of cTnT+ cells analysed with immunostaining and quantified using cell counter plugin in ImageJ software. Values are expressed as mean ± standard deviation (SD). H = Hand2, N= NKX2.5, G = GATA4, M = MEF2C, T = TBX5.

### 5.5.2 Gata4 protein induction with small-molecule compounds

In addition to screening for small-molecule compounds that would enhance direct cardiac reprogramming, compounds were also tested for their ability to induce Gata4 protein expression in MEFs. For this four small-molecule compounds were selected: I-666, 3i-1047, 3i-1103 and 3i-1070. These molecules are targeted to modulate the activity of cardiac TFs. Mouse embryonic fibroblasts were exposed to 1 µM, 3 µM, 5µM and 10 µM of I-666, 3i-1047, 3i-1103 or 3i-1070 24 hours after plating and the small-molecule compounds were maintained in culture during the whole experiment. Mouse embryonic fibroblasts transduced with tetO-GATA4 lentivirus were used as a positive

control and medium only and vehicle only (MEF medium containing 0.1% DMSO) were also were used as a negative control. After 14 days in culture, the cells were fixed and analysed with immunostaining for Gata4. The stained cells were imaged for Gata4 protein expression, but no expression of Gata4 protein was observed for any of the four small-molecule compounds (data not shown).

# 6 DISCUSSION

Small-molecule compounds have been suggested to be a potential solution in overcoming some of the major challenges involved in direct reprogramming. The results of this M.Sc. thesis work demonstrate a method for *in vitro* small-molecule compound screening that can be used to identify compounds that increase the reprogramming efficiency in direct cardiac reprogramming. The aim is to in the future be able to use small-molecule compounds to achieve direct cardiac reprogramming as a regenerative therapy for MI and HF.

A great part of setting up the screening platform consisted of finding the optimal conditions for direct reprogramming of MEFs to iCMs. Previous research has shown great variation in the reported results of direct cardiac reprogramming. A possible explanation can be found in different experimental procedures and protocols, as seemingly small differences in protocols might influence the reprogramming efficiency (Addis and Epstein 2013). Several different parameters were optimised, including cell density, medium, plating substrate and culturing times. None of the parameters alone resulted in notable increases of reprogramming efficacy, but in total the optimisation of protocol produced a reprogramming efficiency of 6,44 ± 2,92% (n=2) (HNGMT + DMSO).

The reprogramming efficiency achieved after optimisation of the protocol is in line with previous research by Ifkovits et al. (2014), who reported a reprogramming efficiency of 4,96 ± 1,80% using the same TF combination. When comparing these

results to other publications it is however important to note that the differences in starting cell population, used TF combinations, outcome measures and evaluated time points affect the reprogramming efficiency and the results might not be comparable with each other. In this study immunocytochemical analysis of cTnT expression was used as a read out measure. Compared to the functional Troponin T-GCaMP reporter used by Ifkovits et al., measuring cTnT protein expression is a less stringent method as it only measures the expression of a single protein, which usually gives a higher reprogramming efficiency. The achieved reprogramming efficiencies in this study could therefore have been expected to be higher, as Ifkovits et al. (2014) achieved a similar reprogramming efficiency using the same viral constructs, but with a more stringent outcome measure.

High enough expression levels of cardiac TFs is crucial for successful direct reprogramming. Chen et al. (2012) were unable to directly reprogram mouse TTFs and CFs by lentiviral overexpression of the GMT TF combination. It has been suggested that the lentiviral vectors used by Chen et al. could not express GMT at high enough levels (Addis and Epstein 2013). Prior to starting the reprogramming experiments the transduction efficiencies of the lentiviral vectors used in this study were optimised and the transduction efficiency was determined. Even though high enough transduction efficiencies were achieved for NKX2.5, GATA4, MEF2C and TBX5 the transduction efficiency for Hand2 could not be determined and it can therefore not be confirmed that the cells expressed high enough levels of Hand2. As the TFs were delivered with separate vectors for each TF the amount of cells that expressed all five TFs could also not be confirmed. It is possible that the total transduction efficiency remained too low, which might explain the low reprogramming efficiencies. However, the constructs used in this study were identical to the ones used by Ifkowits et al. (2014) , who achieved a reprogramming efficiency of 5% at baseline using the same separate vector delivery strategy.

Optimal TF stoichiometry has also been reported to influence the efficiency and quality of direct cardiac reprogramming. Wang et al. (2015) showed that higher expression levels of Mef2c and lower levels of Gata4 and Tbx5 resulted in a higher reprogramming efficiency. Unlike what was expected, higher levels of MEF2C did not result in a higher reprogramming efficiency compared to the other conditions. Instead the highest reprogramming efficiency was achieved with equal volumes of all five TFs (HNGMT). However, when examining these results, it needs to be noted that the viral vector stocks used in this study all had different viral titers and all TFs were delivered on separate vectors leading to heterogenous and uncontrolled ratios of TF expression. The MEF2C viral vector stock had the highest viral titer and therefore when giving cells equal volumes of TF lentiviral vectors the achieved expression level of MEF2C was probably higher than the expression levels of the other factors. However, capsid-based titering is very unreliable and can only be used to estimate the viral titer so the the real infectious units are unknown. Also, a possible explanation to the difference in our results is that Wang et al. (2015) used the GMT TF combination and the effect of Nkx2.5 and Hand2 stoichiometry on direct cardiac reprogramming has not been studied before.

The second phase of this thesis consisted of examining the effects of a set of small-molecule compounds on the reprogramming efficiency. An initial set of compounds were selected based on their reported abilities to facilitate direct cardiac reprogramming, directed differentiation from stem cells to cardiomyocytes or reprogramming of fibroblasts to iPSCs. Of the tested molecules only the TGF-β inhibitor SB induced a clear increase on reprogramming efficiency The reprogramming efficiency increased over 2-fold when compared with control. SB has previously been reported to increase the reprogramming efficiency over 5-fold (Ifkovits et al. 2014). Even though the results here were in line with previous publications, they also demonstrate the great variability in results by different groups.

The TGF-β superfamily consists of a large number of growth factors that regulate several cellular functions, including proliferation and differentiation (Inman et al. 2002). SB is a specific inhibitor of the TGF-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7 that phosphorylate Smad2/Smad3 and thus activate downstream signalling pathways. In an attempt to find the mechanism of action behind the effect of SB on the cardiac reprogramming efficiency, Ifkovits el al. (2014) reported that specifically the inhibition of the TGF-β signalling component of the superfamily led to the increased reprogramming efficiency. A recent study by Zhao et al. (2015) confirmed these results, by achieving the highest yet reported cardiac reprogramming efficiency, 67%, by overexpressing HGMT cardiac TFs, miR-1 and miR-133 combined with a small-molecule compound TGF-β inhibitor. It has previously been shown that direct reprogramming with GMT + miR-133 was mediated through suppression of TF gene *Snail1*, which is a downstream target of TGF-β (Muraoka et al. 2014). It is therefore possible that the effect of TGF-β inhibition on direct cardiac reprogramming is mediated through downregulation of *Snail1.* Together these results suggest that inhibition of TGF-β signalling serves as a potential target for drug development in direct cardiac reprogramming.

In addition to testing the effect of the small-molecule compounds in HNGMT-mediated cardiac reprogramming the effect of the compounds was also evaluated with HGMT-transduced MEFs. There has been conflicting results regarding the effect of Nkx2.5 on the reprogramming efficiency as both an increase and decrease in the efficiency has been reported with the addition of Nkx2.5 (Ieda et al. 2010, Song et al. 2012, Addis et al. 2013). Here no difference in the reprogramming efficiency was found when comparing the two TF combinations (Ieda et al. 2010). Also, no difference in the effect of the small-molecule compounds was found between the two combinations.

The small molecule screening platform was established succesfully. However, setting up a reprogramming thechnique is challenging and time-consuming and several questions were left unanswered and need to be confirmed in further studies. There

were also several limitations to the study that might affect the reliability of the results and need to be taken into account when interpreting the results. First of all, most of the data are preliminary results and more replicates are needed in order to confirm the current results. Also, great variability was seen in the results of the individual reprogramming experiments and more consistent results are needed before the platform can be used for actual screening.

Further characterisation of the generated iCMs is necessary in order to validate the platform. Immunocytochemical staining for cTnT expression showed clearly organised sarcomeres in only a part of the iCMs while no sarcomere structure was observed in most of the generated iCMs. This indicates that most of the cells were immature and only partially reprogrammed. Also, only sporadic beating cells were observed. The generation of immature iCMs might become a problem in clinical applications, as they might trigger arrhythmias (Sadahiro et al. 2015). In order to further characterise the iCMs and assure complete direct reprogramming the gene expression pattern of the iCMs should be determined by RT-PCR, microarray or RNA sequencing. Further, action potential or calcium oscillations should be determined using a functional outcome measure. Also further optimisation of the reprogramming protocol is needed in order to achieve higher reprogramming efficiencies.

Many questions and concerns remain regarding the potential of direct cardiac reprogramming as a future regenerative therapy for MI and HF. In order to achieve high enough reprogramming efficiencies for therapeutic application the underlying mechanism and barriers of direct reprogramming need to be determined. Determining the effect of small-molecule compounds on the reprogramming efficiency may help determine the relevant signalling pathways and targets for future drug development targeting direct cardiac reprogramming. Also, increased understanding of epigenetic regulation of cell fate and epigenetic barriers inhibiting cardiac reprogramming is needed in order to achieve the full potential of direct reprogramming.

# 7 CONCLUSIONS

Direct cardiac reprogramming holds great potential as a regenerative therapy. As CFs play a major role in the pathogenesis of HF, direct reprogramming of fibroblasts to cardiomyocytes represents an attractive target for future HF therapies. Direct reprogramming has successfully been achieved both *in vitro* and *in vivo*. However, several issues, including low reprogramming efficiency, generation of immature cardiomyocytes and potential risk of genomic integration associated with the use of viral vectors, still remain to be resolved before the technique can be applied to clinical practice.

The results of this M. Sc. thesis work demonstrate a method for *in vitro* small-molecule compound screening, which can be used to identify compounds that increase the efficiency of direct cardiac reprogramming or even replace cardiac TFs altogether. The results also verify previous publications that MEFs can be directly reprogrammed to iCMs by overexpression of both HGMT and HNGMT TF combinations. In accordance with recent research it was found that the reprogramming efficiency is significantly increased by treatment with the TGF-β inhibitor SB. None of the other tested small-molecule compounds were found to have an effect on the reprogramming efficiency.

As no statistical analysis was performed due to lack of replicates the results are, however, only directional and no real conclusions can be made based on these results. Further optimisation of the method and additional reprogramming experiments are needed in order to validate the established small-molecule compound screening platform. Nonetheless, these results set a foundation for finding small-molecules that in the future might be used to target direct cardiac reprogramming as a regenerative therapy for MI or HF. However, future research will show the true value of direct cardiac reprogramming in cardiac regeneration.

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