Universidade de Aveiro

2019/2020

Departamento de Biologia

Paula Magda Teixeira Correia

Exploiting the role of long non-coding RNAs in the direct conversion of fibroblasts into functional cardiomyocytes

Estudo da importância dos RNAs não codificantes longos na conversão direta de fibroblastos em cardiomiócitos funcionais

Universidade de Aveiro

2019/2020

Departamento de Biologia

Paula Magda Teixeira Correia

Exploiting the role of long non-coding RNAs in the direct conversion of fibroblasts into functional cardiomyocytes

Estudo da importância dos RNAs não codificantes longos na conversão direta de fibroblastos em cardiomiócitos funcionais

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Bruno Bernardes de Jesus, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação da Professora Doutora Sandrina Nóbrega Pereira, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro.

universidade de aveiro
FCT Fundação para a Ciência e a Tecnologia
Cofinanciado por: COMPETE 2020
PORTUGAL 2020
UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento
POPH PROGRAMA OPERACIONAL POTENCIAL HUMANO

Dedico esta dissertação aos meus pais e irmã pelo constante apoio.

Dedicatória Póstuma:

Dedico esta dissertação a Maria Augusta Brandão, a minha avó.

"Accept the difficulty of what you cannot yet change. But do not accept the impossibility of ever changing it."

Aubrey de Grey, PhD, Co-founder of SENS Research Foundation

O júri

Presidente- Professor Doutor António José de Brito Fonseca Mendes Calado, Professor Auxiliar, Universidade de Aveiro

Vogal- Arguente Principal: Doutor Simão Teixeira da Rocha, Investigador, Instituto de Medicina Molecular João Lobo Antunes

Vogal- Orientador: Doutor Bruno Miguel Bernardes de Jesus, Professor Auxiliar em Regime Laboral, Universidade de Aveiro

Agradecimentos

Em primeiro lugar, agradeço ao Departamento de Biologia da Universidade de Aveiro por me ter acolhido no mestrado de Biologia Molecular e Celular, pelas suas iniciativas inovadoras e pelo corpo docente exemplar.

Ao Professor Bruno Bernardes de Jesus, Professor Auxiliar no Departamento de Ciências Médicas da Universidade de Aveiro, Investigador Principal no Instituto de Biomedicina da Universidade de Aveiro e orientador desta dissertação, por me ter acolhido e dado oportunidade de trabalhar na sua equipa, pela disponibilidade, apoio, ajuda no laboratório, ensinamentos e ajuda na construção deste manuscrito.

À Professora Sandrina Nóbrega Pereira, Professora Auxiliar no Departamento de Ciências Médicas da Universidade de Aveiro, Investigadora no Instituto de Biomedicina da Universidade de Aveiro e coorientadora desta dissertação, pelo apoio no laboratório principalmente em citometria, pela ajuda na escrita do artigo de revisão, pela ajuda na análise estatística e apoio na construção deste manuscrito.

Ao Professor Mário Pacheco ex-diretor do Mestrado de Biologia Molecular e Celular pelo esclarecimento de dúvidas e ajuda na documentação relativa à elaboração desta dissertação de mestrado.

Ao Professor António Calado atual diretor do Mestrado de Biologia Molecular e Celular pela resposta a todas as questões relativas à elaboração deste manuscrito e ajuda no tratamento de documentação necessária para a entrega da dissertação.

Ao Francisco por toda a disponibilidade e ajuda no laboratório.

Aos colegas de mestrado de Biologia Molecular e Celular pelo companheirismo em especial às pessoas com quem trabalhei em diversas disciplinas, a Ana Martinho, o José Franco, o Tiago Bonacho, a Rita Gouveia, a Sofia Baptista e a Natália Félix.

Aos meus pais, Victor e Paula, que sempre estiveram presentes e me motivaram sempre para alcançar esta etapa.

Á minha irmã Clara pelo seu sentido de humor apurado e sempre me fazer rir mesmo nos momentos mais difíceis.

Á minha restante família pela união e pela motivação.

Aos meus amigos pela atenção que sempre me deram e a ajuda em todos os momentos. Em especial queria agradecer à Márcia, minha amiga de infância desde os 5 anos de idade, sempre esteve ao meu lado em todos os momentos. Também um agradecimento especial à Vanessa, minha amiga desde a licenciatura em Coimbra, por toda a compreensão e carinho.

Palavras-Chave: Insuficiência cardíaca, Reprogramação direta, Cardiomiócitos, lncRNAs, Metabolismo

Resumo:

As doenças cardíacas são uma das principais causas de mortalidade nos países desenvolvidos. A patologia associada é tipicamente caracterizada pela perda de cardiomiócitos que leva, eventualmente, à insuficiência cardíaca. Atualmente, existem muitas estratégias promissoras para a regeneração cardíaca. A reprogramação cardíaca direta tem se tornado conhecida como uma nova abordagem terapêutica para regeneração cardíaca depois de uma lesão. A reprogramação cardíaca direta é um processo simples e rápido, no entanto os seus mecanismos moleculares e de maturação celular continuam maioritariamente desconhecidos.

A reprogramação cardíaca direta é uma abordagem terapêutica com grande potencial para se tornar uma das principais estratégias da medicina regenerativa no combate à insuficiência cardíaca, uma vez que os fibroblastos estão facilmente disponíveis no coração e dividem-se facilmente ao contrário dos cardiomiócitos. Os fibroblastos cardíacos são uma população alargada no coração que, após uma lesão, tornam-se em miofibroblastos ativos contribuindo para a fibrose. Atualmente, sabe-se que uma combinação específica de três fatores de transcrição, *Mef2c, Gata4* e *Tbx5* (MGT), é suficiente para reprogramar fibroblastos cardíacos de ratinho em cardiomiócitos induzidos. Por outro lado, quando fibroblastos humanos são infetados com MGT apresentam uma pequena percentagem de conversão.

Com o retrovírus MGT transfectamos com sucesso: fibroblastos adultos de ratinho (MAFs), Feeders e Gm 03348 (fibroblastos humanos com 10 anos de idade). Através da análise de qPCR, avaliamos a expressão dos lncRNAs: *Gm 15856, Mir22hg, Gm 027028* e *Gm 28592*. O nosso objetivo foi estudar quais os lncRNAs são os melhores candidatos para knockdown, e assim melhorar a eficiência da reprogramação cardíaca direta. Para além disso, estudamos como a manipulação de nutrientes nos meios de cultura pode influenciar a reprogramação cardíaca direta. Verificou-se que meios com níveis mais altos de glucose e glutamina apresentaram maiores taxas de sobrevivência e proliferação celular.

Keywords: Heart failure, Direct reprogramming, Cardiomyocytes, lncRNAs, Metabolism

Abstract:

Heart disease is one of the leading causes of mortality in developed countries. The associated pathology is typically characterized by the loss of cardiomyocytes that leads, eventually, to heart failure. Presently, there are many promising strategies for cardiac regeneration. Direct cardiac reprogramming is becoming known as a novel therapeutic approach to regenerate injured hearts. Direct cardiac reprogramming is a simple and quick process however, the molecular mechanisms of cardiac reprogramming and cardiomyocyte-like cells functional maturation remain to be understood.

Direct cardiac reprogramming has great potential to become one of the main strategies for regenerative medicine in heart failure since fibroblasts, contrary to cardiomyocytes which do not divide, are easily available in the heart, they are a large population of cells in the heart, which become activated and turn to myofibroblasts, contributing to fibrosis after cardiac injury. Currently it is known that a specific combination of three transcription factors, *Mef2c, Gata4* and *Tbx5* (MGT), are enough to reprogram non-myocyte mouse heart cells into induced cardiomyocyte-like cells. Nevertheless, human fibroblasts when infected with MGT appeared to have a small percentage of conversion.

With MGT retrovirus we successfully transfected: mouse adult fibroblasts (MAFs), Feeders and Gm 03348 (human fibroblasts with 10 years old). Through qPCR analysis we evaluated the expression of lncRNAs: *Gm 15856, Mir22hg, Gm 027028* and *Gm 28592*. Our goal was to understand which lncRNAs are the best candidates to knockdown in order no enhance direct cardiac reprogramming. In addition, we studied how nutrient manipulation in cell culture media can influence direct cardiac reprogramming. It was found that media with higher levels of glucose and glutamine had larger rates of cellular survival and proliferation.

Contents

List of figures

List of tables

Abbreviations

2D- Two-dimensional

3D- Three-dimensional

α-MHC- α-cardiac myosin heavy chain

Acetyl-coA- Acetyl-coenzyme A

ACTC1- Actin alpha cardiac muscle 1

AuNPs- Cationic gold nanoparticles

BSA- Bovine serum albumin

Bvht- Braveheart

CCK-8- Cell Counting Kit-8

CFs- Cardiac fibroblasts

CMs- Cardiomyocytes

CREB- cAMP-response element-binding

CRIP2- Cysteine Rich Protein 2

cTnT- cardiac Troponin

DMEM- Dulbecco′s Modified Eagle′s Medium

DMSO- Dimethylsulfoxide

DNA- Deoxyribonucleic Acid

dNTP- Deoxyribonucleotide triphosphate

DTT- Dithiothreitol

FACS- Fluorescence-activated cell sorting

FADH2- Flavin adenine dinucleotide hydrogen

FAs- Fatty acids

FBS- Fetal bovine serum

iPSCs- Induced pluripotent stem cells

iCMs- Induced cardiomyocytes

I/R- Ischemia-reperfusion

Gata4- Gata Binding Protein 4

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GFP- Green fluorescent protein

GMT- Gata4, Mef2c, and Tbx5

GHMT- Gata4, Hand2, Mef2c, and Tbx5

H_2O- Water

H3K27me3- Trimethylation of the lysine 27 of histone H3

Hand2- Heart and Neural crest derivatives expressed 2

HGP- Human Genome Project

HIF- Hypoxia inducible factor

lncRNAs- Long noncoding RNAs

MAFs- Mouse embryonic fibroblasts

Mef2c- Myocyte enhancer factor 2C

MESP 1- mesoderm posterior 1

MHC- Myosin heavy chain

MI- Myocardial infarction

miR combo- miR-1, miR-133a, miR-208a, and miR-499

miRNAs- MicroRNA

MAFs- Mouse adult fibroblasts

MEFs- Mouse embryonic fibroblasts

MIAT- Myocardial infarction-associated transcript

MGT- Mef2c, Gata4, and Tbx5

mRNA- Messenger RNA

MyoD- Myocardin

NADH- Nicotinamide adenine dinucleotide hydrogen

ncRNAs- Noncoding RNAs

NRF2- Nuclear factor erythroid 2-related factor

OXPHOS- Oxidative phosphorylation

PBS- Phosphate-buffered saline

PCR- Polymerase Chain Reaction

PDH- Pyruvate dehydrogenase

PDK1, 2, 4- Phosphoinositide-dependent kinase-1, 2, 4

PDP1, 2- Pyruvate dehydrogenase phosphatase-1, 2

PFA- Paraformaldehyde

PRC1- Polycomb repressive complex 1

PRC2- Polycomb repressive complex 2

qPCR- Quantitative polymerase chain reaction

RNA- Ribonucleic acid

ROS- Reactive oxygen species

RT- Room temperature

Sarrah- SCOT1-antisense RNA regulated during aging in the heart

shRNA- Short hairpin RNA

SD- Standard deviation

Tbx5- T-Box Transcription Factor 5

TCA- Tricarboxylic acid cycle

TFs- Transcription factors

WHO- World health organization

CHAPTER 1 - INTRODUCTION

1. Heart disease

The World Health Organization (WHO) has been reporting every year that cardiovascular diseases are the leading causes of death in the world (Chang *et al.*, 2019). Remarkably, aging and cardiovascular diseases lead to a progressive loss of cardiomyocytes, yet the adult mammalian heart has limited regenerative capacity (Klattenhoff *et al.*, 2013). Heart attack or necrosis of the myocardium leads to the loss of cardiomyocytes. This loss has linked to an impaired heart function and hampered regenerative capability (Chang *et al.*, 2019). When a large amount of cardiomyocytes are lost, the human heart is incapable to replace them due to its very low rate of turnover (Hudson and Porrello, 2013). The heart is not considered a post-mitotic organ however, the turnover of cardiomyocytes in the adult heart is around 1% per year which is insufficient to replace the cardiomyocytes loss caused by myocardial infarction (MI) (Malandraki-Miller *et al.*, 2018).

There are multiple causes of heart failure, coronary artery disease is the most common one, and it can lead to MI. Presently there are available a large range of pharmaceutical drugs and surgical techniques to prevent further deterioration or restore function to the failing heart. Some of the most prominent treatments currently include beta-blockers, angiotensin converting enzyme or receptor inhibitors, surgical valve replacement/reconstruction and reperfusion/revascularisation, and all these strategies have contributed to a substantial decrease in patient mortality rates. However, for end-stage heart failure the only long-term option is heart transplantation which has considerable disadvantages as reduced availability of matched donor hearts and life-long immune-suppression (Hudson and Porrello, 2013).

Subsequently after cardiac injury, cardiomyocytes undergo necrotic and apoptotic cell death and cardiac fibroblasts are activated to produce collagen and other extracellular matrix components, leading to fibrosis and harmed cardiac function. Following injury, the capacity for regeneration of adult mouse heart is limited however, the neonatal mouse heart can regenerate. The main goal of regenerative cardiovascular medicine is to repair injured hearts by replacing cardiomyocytes and diminishing fibrosis. Transplantation of cardiac stem cells or stem cell-derived cardiomyocytes to improve cardiac function has clinical potential, however these techniques present low efficiency (Song *et al.*, 2012). In addition, although embryonic stem cells have a clear cardiogenic potential, its efficiency in cardiac differentiation brings risk of tumour formation, and issues of cellular rejection (Ieda, J. Fu, *et al.*, 2010; Hashimoto *et al.*, 2019).

During the last years it has been explored direct reprogramming of resident cardiac fibroblasts (CFs) by cardiogenic transcription factors (TFs) into induced cardiac-like myocytes,

bypassing the pluripotent state. The human heart is composed of cardiomyocytes, vascular cells, and cardiac fibroblasts. Indeed, 50% of the cells in the heart are cardiac fibroblasts. Cardiac fibroblasts (CFs) are somatic cells completely differentiated that offer structure support, secrete signals, and contribute to scar formation after cardiac injury. Most population of endogenous CFs are a potential source of cardiomyocytes for regenerative therapy, in the case directly reprogram the resident fibroblasts into beating cardiomyocytes (Ieda, J. Fu, *et al.*, 2010; Hashimoto *et al.*, 2019).

2. Noncoding RNAs in the heart

Since the Human Genome Project (HGP) was concluded, it is known that about 20,000–25,000 protein-coding genes exists in human species, however just about 2-3% of the transcriptome codes for proteins (Laks, 1982; Trembinski *et al.*, 2020). HGP generated interest in the scientific community to investigate the functions of noncoding sequences, specifically lncRNAs (Laks, 1982). More than 98% of the genome is actively transcribed to produce thousands of noncoding RNAs (ncRNAs). Various classes of noncoding RNAs have been described over the years, including ribosomal RNAs, transfer RNAs, microRNAs, long non-coding RNAs (lncRNAs), etc. (Figure 1) (Trembinski *et al.*, 2020).

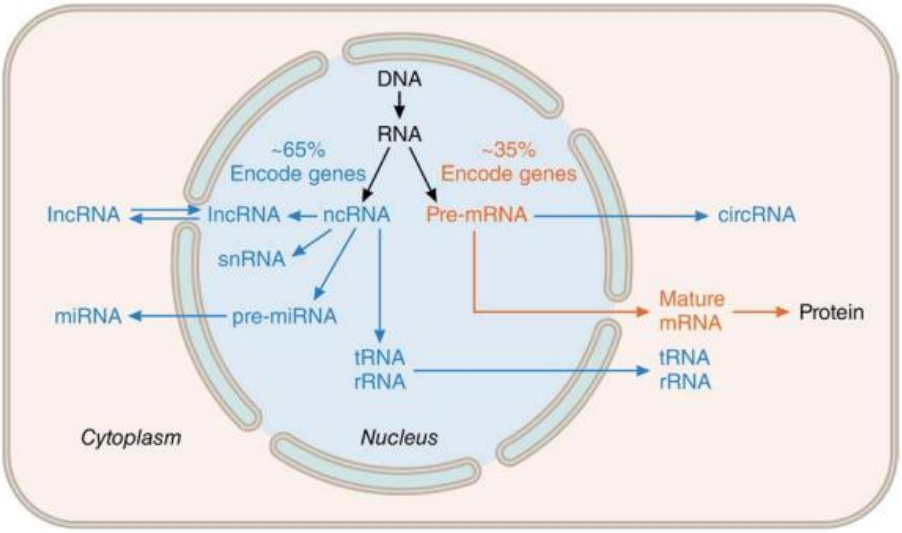


Figure 1. Transmission of genetic information: primary transcripts give origin to protein coding mRNAs and noncoding RNAs. A small portion of mRNAs are translated into protein but, the majority are noncoding RNA molecules subdivided into: lncRNA, circular RNA, miRNA, transfer RNA, ribosomal RNA, small nuclear RNA, etc. Adapted from (Bär, Chatterjee and Thum, 2016).

MicroRNAs (miRNAs) are a class of small noncoding RNAs (~22 nucleotides) and more than 2,000 miRNAs have been found in human genome, several were conserved through evolution. miRNAs repress gene expression by degrading target mRNAs and/or inhibiting their translation every time that happens an imperfect base pairing with mRNAs in a sequence-dependent manner. Also was discovered that miRNAs have an important role in the regulation of a broad range of biological activities and diseases (Laks, 1982).

More recently researchers found that lncRNAs (~200 nucleotides) have several implications in a variety of biological processes. Investigating the role of miRNAs and lncRNAs in gene expression regulation during cardiovascular development and function will greatly facilitate the development of new therapeutics of treating cardiovascular disease (Laks, 1982; Hobuß, Bär and Thum, 2019). Given the emerging regulatory potential of non-coding RNAs for controlling diverse cellular processes, these molecules may offer potential solutions in this pursuit of cardiac regeneration (Hudson and Porrello, 2013).

The subclass of ncRNAs better studied is miRNAs, they have a crucial role in development and stress adaptation in the heart. miRNAs initiate biological pathways by targeting numerous mRNAs implicated in cell growth, differentiation, and apoptosis by suppressing the translation of central protein effectors. LncRNAs are characterized by a variety of molecular functions due to their ability to fold into complex structures and act as scaffolds for protein-protein interactions and/or chaperones that direct protein complexes to specific RNA or DNA sequences. Essential roles for some lncRNAs in heart development have been discovered over the last years (Matkovich et al., 2014). lncRNAs display multifaceted biological functions and interact with a range of other RNAs or proteins. Differing on their subcellular localization in the nucleus or cytoplasm, lncRNAs can interact with transcriptional and post-transcriptional gene regulation, as well as mRNA translation, respectively (Hobuß, Bär and Thum, 2019).

2.1. MiRNAs in the heart

Total inhibition of miRNAs expression in the heart is the first step to understanding the function of miRNAs in cardiogenesis. For miRNA maturation is necessary a RNase III endoribonuclease named Dicer. In case of Dicer’s deletion there is an early embryonic lethality in mice due to dilated cardiomyopathy and heart failure. Many miRNAs have indispensable roles in cardiac development (Laks, 1982).

Deregulated cardiac growth is a characteristic feature of heart disease (Bischof and Krishnan, 2016). miR-1 is tissue-specifically expressed in the heart and skeletal muscle, and its genetic deletion compromises cardiogenesis and the expression of many cardiac contractile proteins (Laks, 1982). In addition, miR-1 is downregulated in heart disease, it is capable to induce the expression of genes necessary for deregulated cardiac growth through repression of calmodulin and myocyte enhancer factor 2 A (*Mef2a*) expression (Bischof and Krishnan, 2016).

Another miRNA is miR-133, it is transcribed from the same bicistronic transcription unit as miR-1 and is also expressed specifically in cardiac and skeletal muscle. Inhibition of miR-133 *in vitro* and *in vivo* causes hypertrophic growth, whereas ectopic miR-133 expression showed to inhibit cardiac hypertrophy *in vitro*. Conversely, cardiac specific ectopic miR-133 expression reduced cardiomyocyte apoptosis, attenuated fibrosis and helped with the maintenance of normal cardiac function (Bischof and Krishnan, 2016).

Recently were identified about 40 miRNAs that greatly increased cell proliferation in neonatal mouse and rat cardiomyocytes. The miRNAs, miR-590 and miR-199a demonstrated to induce cardiomyocyte proliferation *in vitro* and *in vivo*. All these evidences together suggest that miRNAs have an important role in the regulation of cardiomyocytes proliferation and heart regeneration, suggesting their significant therapeutic potential to treat heart failure (Laks, 1982).

Ischemia is an independent risk factor of cardiovascular incidents, which leads to MI and ischemia-reperfusion (I/R) injury. Numerous miRNAs have a role in the regulation of these pathologic processes, particularly cardiomyocyte apoptosis after MI and I/R injury. miR-92a is a member of the miR-17-92 cluster implicated in cardiomyocytes proliferation and survival. Studies reported that inhibition of miR-92a improved cardiac function and reduced cardiomyocyte apoptosis after MI in mice. On otherwise miR-320 is downregulated after I/R injury, it was demonstrated that miR-320 promotes cardiomyocyte apoptosis. Collectively, these studies reveal that miRNAs are key regulators of cardiomyocyte survival and cardiac remodelling in response to pathophysiological stresses (Laks, 1982).

2.2. LncRNAs in the heart

Although thousands of lncRNAs were discovered in eukaryotes, many of them are species specific also, seem to be less conserved than protein-coding genes. Importantly, lncRNAs are differentially expressed in tissues, suggesting that they regulate lineage commitment (Klattenhoff *et al.*, 2013; Bischof and Krishnan, 2016). Presently, more than 100,000 lncRNAs have been described in humans and numerous lncRNAs have been identified

to play important roles in homeostasis and disease (Goldman and Poss, 2020; Trembinski *et al.*, 2020).

Braveheart (Bvht) is a novel lncRNA, the first to be found in mice, which was discovered to be a critical regulator of cardiovascular commitment from embryonic stem cells (Laks, 1982). *Bvht* is cardiac-enriched approximately threefold compared with other tissues, but it is expressed in the heart is at similar levels in embryos and adults (Matkovich et al., 2014). It is necessary for activation of a core gene regulatory network that included key cardiac transcription factors (e.g., *MesP1, Gata4, Hand1, Hand2, Nkx2.5*, and *Tbx5*) and EMT genes (e.g., Snail1 and Twist). Further analysis revealed a significant overlap between the genes regulated by *Bvht* and *MESP1*, a master regulator of cardiovascular potential. Moreover, forced expression of *MESP1* rescued the *Bvht* depletion phenotype, indicating that these two factors function in a similar genetic pathway. *Bvht* interacts with *SUZ12*, suggesting that this interaction may be critical for epigenetic regulation of network genes. Also, *Bvht* is crucial for maintenance of cardiac fate in *ex vivo* neonatal cardiomyocytes. Constitutive cardiac expression of *Bvht* indicates that it may have "housekeeping" roles in adult hearts in addition to its canonical role upstream of *MESP1* to stimulate and preserve cardiomyocyte fate (Matkovich et al., 2014). The findings about *Bvht* suggest that lncRNAs play a role as molecular modulators that can regulate directly cell fate (Klattenhoff *et al.*, 2013).

Fendrr is another important lncRNA that has been identified as an essential regulator of heart and body wall development. *Fendrr* is expressed in the mouse lateral plate mesoderm, and the heart and body wall precursors are derived from it, also the knockout of *Fendrr* resulted in heart development malformations (Laks, 1982).

A study of *MALAT1* loss-of-function in a genetic model suggested that *MALAT1* is not crucial for mouse prenatal and postnatal development. Moreover, it was proved that depletion of *MALAT1* is not involved in global gene expression, splicing factor level and phosphorylation status, or alternative pre-mRNA splicing. Nevertheless, a few genes were deregulated in adult *MALAT1* knockout mice, many neighboured *MALAT1*, indicating a potential cis-regulatory role of *MALAT1* gene transcription. Interestingly, inhibition of *MALAT1 in vivo* by oligonucleotides decreased vascularization, indicating that *MALAT1* can be interesting target to manipulate angiogenic processes (Devaux *et al.*, 2015). This finding leads to suspect that probably *MALAT1* plays a role in cardiovascular diseases.

Over the last years others lncRNAs were linked to heart disease. *ANRIL* is a lncRNA identified as a risk factor for coronary disease. However still not clear how *ANRIL* functions, evidences suggest that it may have a role in the regulation of histone methylation. *MIAT*

(myocardial infarction-associated transcript) was identified as a determinant lncRNA associated with patients with MI, though how *MIAT* controls MI status continues unknown (Laks, 1982).

Mechanistically, lncRNAs use various molecular regulatory mechanisms to control gene expression, one of the strategies are being antisense transcripts that directly bind mRNA or acting as chaperones that engage to macromolecular protein complexes at specific sequence specified locations in the genome. Also, lncRNAs are involved in chromatin remodelling that evokes long-term changes in transcriptional activity (Matkovich et al., 2014).

3. Direct Reprogramming of Fibroblasts into Cardiomyocytes

3.1. Defined factors

A specific combination of three different TFs, *Gata4, Mef2c,* and *Tbx5* (GMT) can create functional beating cardiomyocytes directly from mouse postnatal cardiac or dermal fibroblasts, assuming the fully reprogrammed induced cardiomyocytes (iCMs) a cardiomyocyte-like gene expression profile. The three TFs interact with one another, activating cardiac gene expression, and promoting cardiomyocyte differentiation. *Gata4* opens the chromatin structure in cardiac *loci*, thus allows the binding of *Mef2c* and *Tbx5* to their specific target sites and leading to full activation of the cardiac program (Ieda, J. Fu, *et al.*, 2010; Qian *et al.*, 2012).

The addition of *Hand2* into GMT combination (GHMT) enhanced direct cardiac reprogramming. Notably, it was demonstrated direct reprogramming of resident cardiac fibroblasts in the heart to iCMs following cardiac injury by forced expression of GMT or GHMT *in vivo* (Song *et al.*, 2012; Zhang *et al.*, 2019). The overexpression of cardiac TFs GMT or GHMT direct reprogram fibroblasts into iCMs, without passing through a stem cell state (Liu *et al.*, 2017; Muraoka *et al.*, 2019).

Direct cardiac reprogramming of fibroblasts to into iCMs has emerged as an attractive strategy. Since the first attempt based on retroviral delivery of the pivotal cardiac TFs GMT, alternative sets of reprogramming factors based on different TFs combinations, microRNAs, chemical compounds capable to inhibit specific signalling pathways, enzymes involved in epigenetic modifications, defined culture conditions, and small molecules (including TGFβ and Wnt inhibitors), were studied in order to promote cardiac reprogramming (Figure 1) (Hashimoto *et al.*, 2019; Muraoka *et al.*, 2019; Testa *et al.*, 2020).

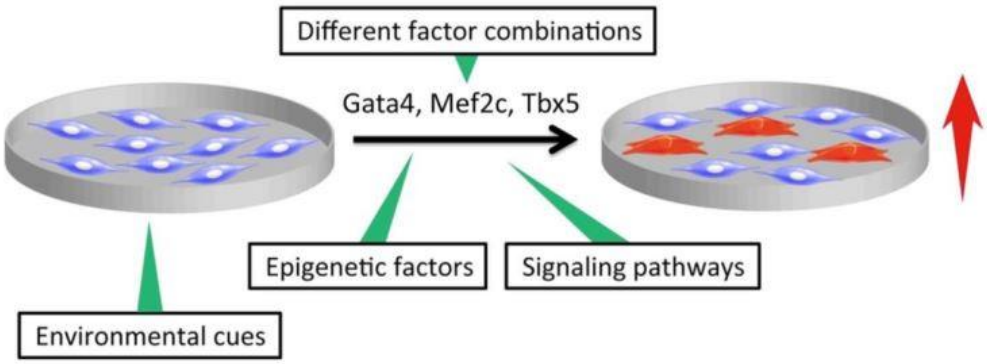


Figure 2. Optimization of TFs, culture conditions, and epigenetic factors to enhance the efficiency of direct cardiac reprogramming. Adapted from (Tani, 2018).

Recently comparative gene expression analyses reported that iCMs induced *in vitro* exhibited more adult cardiomyocyte-like features, such as fatty-acids (FAs) oxidation and cell-cycle exit, than exhibited by induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CMs) (Muraoka et al., 2019).

The silencing of fibroblast program is a prerequisite for direct cardiac reprogramming, however the molecular mechanism underlying this process continues not to be understood. Furthermore, improvements in reprogramming efficiency in mouse embryonic fibroblasts (MEFs) were demonstrated however, direct cardiac reprogramming from more differentiated fibroblasts, such as mouse postnatal and adult tail-tip fibroblasts, persists inefficient. For clinical relevance, it is desirable to generate iCMs efficiently from postnatal and adult fibroblasts; but, the barriers to cardiac reprogramming associated with aging remain undefined (Muraoka *et al.*, 2019). Even though fully direct reprogramming into beating cardiomyocytes is not complete *in vitro*, gene transfer of GMT or GHMT into mouse hearts produced new cardiomyocytes from endogenous cardiac fibroblasts and enhanced cardiac function after MI (Wada, Muraoka, Inagawa, Yamakawa, Miyamoto and Sadahiro, 2013).

Importantly, experiments in mice demonstrated that four miRNAs (miR-1, miR-133a, miR-208a, and miR-499) named miR combo, could direct reprogram fibroblasts into cardiomyocyte-like cells. Moreover miR-133a appears to enhance cardiac reprogramming mediated by *Gata4*, *Mef2c*, and *Tbx5* through direct repression of *Snail1* which ends up silencing the fibroblasts signature (Dal-Pra *et al.*, 2017; Zhang *et al.*, 2019).

The identification and modulation of target molecules engaged in lineage conversion still a major challenge. Through screening for epigenetic regulators with a significant role in iCM generation was discovered that reprogramming efficiency of GMT was substantially

improved by the knockdown of the essential component of the polycomb repressive complex 1 (PRC1), *Bmi1*. The silencing of *Bmi1* by shRNAs suppressed the activity of *Gata4* during the reprogramming process substituting the need of exogenous *Gata4* during the process. Importantly, the positive effect provoked by *Bmi1* knockdown was confirmed at early stage after viral transduction (Testa *et al.*, 2020).

Several approaches have been implemented to improve cardiac reprogramming efficiency thus, a precise comparison of the reprogramming efficiency between the different protocols should be performed. Another strategy documented reprogram fibroblasts into cardiomyocytes has been the partial reprogramming of the cells into cardiac progenitor cells, bypassing a pluripotent state. Forced expression of a combination of five genes encoding early cardiac factors *Mesp1, Gata4, Tbx5, Nkx2-5,* and *Baf60c* reprogrammed murine fibroblasts into an expandable multipotent cardiac progenitor cell population. These induced cardiac progenitor-like cells were transplanted into murine hearts after MI, and enhanced cellular survival (Hashimoto, Olson and Bassel-Duby, 2018).

Despite successful lineage conversion of mouse fibroblasts into a range of relevant cell types, only neuronal direct reprogramming has been shown in human cells. Because human cells are more resistant to the reprogramming process, it is reasonable to speculate that additional regulatory events are required to propel human cells toward alternative cell fates (J. Fu *et al.*, 2013; Nam *et al.*, 2013).

GHMT factors alone for direct cardiac reprogramming of human fibroblasts were ineffective in activating cardiac gene expression. Two muscle-specific miRNAs, miR-1 and miR-133, further improved myocardial conversion of human fibroblasts and eliminated the requirement of *Mef2c*. It was demonstrated that miR-1 and miR-133 are regulated by *Mef2c*, which likely contributes to their ability to replace this transcription factor in the reprogramming mixture (Nam *et al.*, 2013; Wada, Muraoka, Inagawa, Yamakawa, Miyamoto, Sadahiro, *et al.*, 2013).

Also studies have shown that expression of a combination of the transcription factors protein c-ETS2 (*ETS2*) and *MESP1* converted human dermal fibroblasts into cardiac progenitors that expressed early cardiac markers such as *ISL1* and *Nkx2-5*, which are not found with direct reprogramming of mouse fibroblasts. Remarkably, for direct cardiac reprogramming of human cells, GMT or GHMT reprogramming cocktails need additional factors such as myocardin, *MESP1*, oestrogen-related receptor-γ (*ESRRγ*), and zinc-finger protein *ZFPM2*, or even miR-1 and/or miR-133, to successfully induce the conversion of human fibroblasts towards a cardiac fate (Ghiroldi *et al.*, 2017; Hashimoto, Olson and Bassel-Duby, 2018).

Direct cardiac reprogramming in human cells is substantially challenging compared to mouse cells, given the low reprogramming efficiency and the longer time needed for human cells to exhibit cardiomyocyte features. The difficulty in reprogramming human cells could be attributed to the difference in the epigenetic landscape between mouse and human fibroblasts, suggesting additional epigenetic barriers for reprogramming human cells. Another feature to consider is that humans have a longer developmental time than mice, which may contribute to the species differences in cell reprogramming. Intriguingly, in spite of the requirement of different factors for direct cardiac reprogramming between species, crucial endogenous signalling pathways such as the TGFβ1 and WNT signalling pathways contribute similarly to cardiac reprogramming in mice and human cells (Hashimoto, Olson and Bassel-Duby, 2018). Taken together, cardiac reprogramming may offer a potential platform to develop therapeutic strategies for heart diseases and drug screening (Zhang *et al.*, 2019).

3.2. Stoichiometry of the factors

As mentioned before, direct cardiac reprogramming uses forced overexpression of specific TFs. On the first studies it was used the standard *Gata4, Mef2c* and *Tbx5* cocktail utilizing retroviral delivery of the three factors packaged as separate viruses. In order to reprogram, starting cells have to take up each three individual viruses, this leads to low cell fate conversion rate since only a small percentage of cells receive all three TFs. Stochastically, only a minor fraction of cells receive the ideal ratio and dose of the three TFs for cell reprogramming (Vaseghi, Liu and Qian, 2017).

Cardiac remodelling through generation of iCM from fibroblasts holds a great promise however, its efficiency is still very challenging. Fibroblasts appears to have a low reprogramming rate to iCMs suggesting the existence of major rate-limiting barrier(s) requiring a balanced expression of *Gata4, Mef2c,* and *Tbx5* to promote effective and complete reprogramming. Currently, iCM generation requires transducing fibroblasts with pooled viruses encoding the three individual reprogramming factors. Studies were made with different ratios of *Gata4, Mef2c,* and *Tbx5* expression through the use of a polycistronic vector that encoded *Gata4, Mef2c,* and *Tbx5* in a single transgene (Wang *et al.*, 2014; Hashimoto, Olson and Bassel-Duby, 2018).

Different sets of polycistronic constructs containing *Gata4, Mef2c,* and *Tbx5* in all possible mRNA splicing orders were tested. Each splicing order of *Gata4, Mef2c,* and *Tbx5* gave rise to distinct ratios of *Gata4, Mef2c,* and *Tbx5* protein expression and consequently substantially different reprogramming efficiencies. The difference in protein stoichiometry of

Gata4, *Mef2c*, and *Tbx5* alone is sufficient to confer a considerably different effect on cardiac reprogramming outcomes (Wang *et al.*, 2014).

At the molecular level, the more optimal *Gata4*, *Mef2c*, and *Tbx5* stoichiometry is defined by higher protein expression level of *Mef2c* and lower levels of *Gata4* and *Tbx5* (MGT) (Figure 3) (Wang, Liu, Yin, Zhou, *et al.*, 2015). This stoichiometry is correlated with higher expression of mature cardiac myocyte markers. Currently it is known that the stoichiometry of *Gata4*, *Mef2c*, and *Tbx5* influences both efficiency and quality of iCM induction (Wang *et al.*, 2014; Vaseghi, Liu and Qian, 2017).

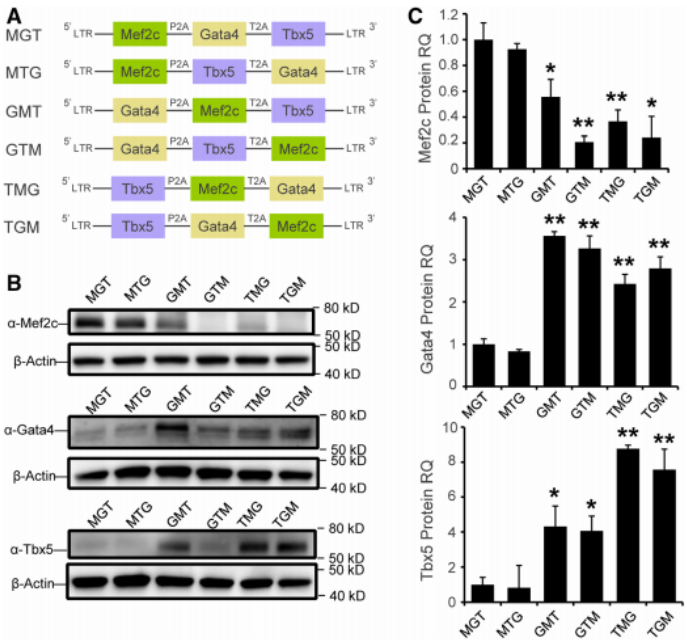


Figure 3. Complete set of polycistronic vectors that result in different *Gata4*, *Mef2c*, and *Tbx5* (G, M, T) protein levels. A) Diagram of the 6 polycistronic vectors with G, M, T in different splicing orders; B) Western blot analysis of cardiac fibroblasts expressing each of the 6 different polycistronic vectors; C) Quantification of G, M, T protein expression levels. Adapted from (Wang, Liu, Yin, Asfour, *et al.*, 2015).

A study comparing GHMT reprogramming ability with GMT demonstrated similar sarcomere protein induction efficiency however, GHMT increased drastically the number of fibroblasts adopting contractile structures and functions. It was concluded that GHMT would further enhance the cardiomyocyte reprogramming process (Zhang, Zhang and Nam, 2019).

Additionally, a study *in vivo* demonstrated reprogramming efficiency in mouse model using the polycistronic MGT vector compared to reprogramming with separate *Gata4*, *Mef2c* and *Tbx5* viruses (Ma *et al.*, 2015).

3.3. Long non-coding RNAs as targets to regenerative therapeutics

As mentioned before, over the latest years several lncRNA *loci* have been identified as critical components of the gene regulatory network that controls cardiovascular development (Ritter *et al.*, 2019).

In addiction some lncRNAs were suggested to have important roles in development of the heart and also in heart failure. Nevertheless, these early findings have uncertain interpretation about how lncRNAs are regulated in different cardiac developmental and disease states and whether regulated lncRNAs differ between these states (Matkovich et al., 2014).

Many times, cardiovascular diseases are a consequence of aging. In the heart, aging has been characterized by an increase in stiffness, fibrosis and cardiomyocyte apoptosis and consequently increased heart failure. A few strategies have been suggested to be potential therapy to counteract cardiac disfunction caused by aging. For instance, inhibition of miR-34a directly regulates *PNUTS*, and consequently reduces cardiomyocyte apoptosis and fibrosis after acute MI. However, the role of *PNUTS* and its relation with aging is not documented yet (Trembinski *et al.*, 2020).

Another lncRNA that was identified and demonstrated to be involved with the aging of the heart is *Sarrah* (SCOT1-antisense RNA regulated during aging in the heart). Apparently, *Sarrah* when silenced induces apoptosis and delays cardiac contractile force development in human engineered heart tissue. Mechanistically, *Sarrah* creates a DNA-DNA-RNA triplex with promoters of cardiac survival genes to recruit Cysteine Rich Protein 2 (*CRIP2)* and activate gene expression. One of these target genes conferring the antiapoptotic function is nuclear factor erythroid 2-related factor (*NRF2)* (Trembinski *et al.*, 2020).

4. Epigenetics Barriers of Reprogramming

During direct reprogramming, cardiac fibroblasts must overcome epigenetic barriers to become cardiomyocytes. To achieve efficient reprogramming, the TFs must be able to engage genes that are developmentally silenced and repress the expression of the genes responsible for the starting cell population (Tani, 2018).

For direct cardiac reprogramming it is necessary the activation of the cardiogenic transcriptional program in concert with the repression of the fibroblastic transcriptional program. Epigenetic modifications have a huge impact in cell fate decision during embryonic

development and cell differentiation by modulating chromatin accessibility and transcriptional activity. Trimethylation of the lysine 27 of histone H3 (H3K27me3) is a hallmark of transcriptional repression (Wang *et al.*, 2016). This repression is the resulted of the activity of methyltransferases Ezh1 and Ezh2 which associate with *Eed* and *SUZ12* to form the polycomb Repressive Complex 2 (*PRC2)*. The regulation of H3K27 methylation is essential for cardiac development and homeostasis. The conditional knockout of the enzyme Ezh2 in cardiac progenitors and cardiomyocytes leads to lethal congenital heart defects. Also, the loss of Ezh2 in cardiomyocytes of the anterior heart field results in hypertrophy. To conclude, global gene analysis of conditional Ezh2 mutants shown that Ezh2 promoted the cardiogenic transcriptional program (Dal-Pra *et al.*, 2017).

During embryonic stem cell differentiation to cardiomyocytes, cardiac gene determinants progressively lose H3K27me3. Also, GMT-induced cardiomyocytes appear to have low levels of H3K27me3 at the promoter of cardiac markers. The elimination of H3K27me3 is achieved by the activity of the demethylases Kdm6A and Kdm6B. The knockout of Kdm6A in female mice induces severe congenital heart defects. Similarly, cardiovascular differentiation of embryonic stem cell is compromised when Kdm6A and Kdm6B are absent, supporting the idea that regulation of H3K27 methylation is essential for cardiac fate. Curiously, *PRC2* and H3K27 methylation also play an important role during somatic reprogramming to pluripotency, but their role during direct reprogramming to cardiomyocyte is unknown (Dal-Pra *et al.*, 2017; Testa *et al.*, 2020).

As mentioned before, direct cardiac reprogramming of fibroblasts can be induced by miR-combo by modulating the epigenetic landscape. miR combo has the capacity to repress the enzyme Ezh2, whereas it upregulates the Kdm6A and Kdm6B expression. Therefore, H3K27me3 levels are decreased in miR-combo transfected fibroblasts, which leads to a global depression of transcription in these cells (Dal-Pra *et al.*, 2017).

5. *In vivo* direct cardiac reprogramming

Evidences suggested that it is possible to direct cardiac reprogram *in vivo* through an injection of GMT-encoding retrovirus into the mouse heart reprogramming this way endogenous nonmyocytes (largely activated fibroblasts) into functional CMs after coronary artery ligation. The iCMs completely reprogrammed, demonstrating synchronous contractions with endogenous CMs and other iCMs. GMT induction *in vivo* can decrease scar size and increase cardiac function. The addiction of *Hand2* to GMT leads to a greater reprogramming result both *in vivo* and also *in vitro* as mentioned before (J. D. Fu *et al.*, 2013). It was also

studied the administration of miR combo into the ischemic myocardium, 10% of the cardiomyocytes in the infarct border zone were from a fibroblast origin two months after MI, decreasing fibrosis (Dal-Pra *et al.*, 2017). Therefore, numerous factors might reinforce the molecular network and reprogram cardiac fibroblasts into iCMs *in vivo*, including environmental cues and/or mechanical forces that enhance reprogramming (J. Fu *et al.*, 2013).

After infection with GMT virus vectors *in vivo*, about 5% of the mice fibroblasts expresses α-MHC-GFP and cardiac Troponin T (cTnT) after one week. Additionally, local viral infection of GMT encoding retrovirus into mouse heart following coronary ligation leads to approximately 10% of α-Actinin^{+} iCMs from cardiac fibroblasts in the infarcted region. Unfortunately, presently mouse direct cardiac reprogramming *in vivo* is still quite inefficient. In order to address this difficulty, studies suggested that the addition of other transcription factors (e.g. *MESP1, MyoD, Baf60c* and *Hand2*), combination of miRNAs (e.g. miR-1, miR-133, miR-208, and miR-499) and chemical inhibitors (e.g. SB431542, and XAV939) along with GMT transduction improve cardiac reprogramming (Qian *et al.*, 2012; Chang *et al.*, 2019).

Although infection with GMT virus vectors *in vivo* has only been tested in the left ventricle, it is most likely translatable to the right ventricle as GMT has induced cardiomyocyte reprogramming in mouse fibroblasts of diverse origin including left ventricular neonatal and adult cardiac, dermal and tail tip fibroblasts (Di Salvo, 2015).

GMT injection with the addition of *Hand2* can convert cardiac fibroblasts into iCMs up to about 28% after three weeks in mouse MI models. However, it is important to mention that the safety issues associated with the use of lentivirus and retrovirus namely genomic integrations are a barrier for human potential application of this *in vivo* therapeutic. Thus, it started to be studied non-viral reprogramming systems to allow the clinical application of *in vivo* cardiac reprogramming in patients. In recent decades, cationic gold nanoparticles (AuNPs) have emerged as a favourable platform for gene and drug delivery due to their easy preparation, large surface area, simplicity of surface functionalization, and inertness (Chang *et al.*, 2019). AuNPs could be a possibility to *in vivo* human direct cardiac reprogramming.

6. Cardiac metabolism

The mammalian heart has to contract constantly thus, the need for an optimal energy fuel is huge. Mitochondria is the organelle that coordinates the energy transduction function and it is responsible to produce more than 95% of ATP (Doenst, Nguyen and Abel, 2013) utilized

by the heart. Additionally, mitochondria regulates intracellular calcium homeostasis, signalling and apoptosis (Kolwicz, Purohit and Tian, 2013).

The effects of metabolism on growth, proliferation, and survival pathways have been documented over the last years. Even though a large fraction of the metabolic fluxes in the heart is devoted to oxidative metabolism for ATP synthesis, substrate metabolism has significant impact on multiple aspects of cardiac biology (Kolwicz, Purohit and Tian, 2013).

The heart has a low capacity for energy storage, consequently it has different metabolic networks to ATP production. The heart is capable of utilizing all classes of energy substrates as glucose, pyruvate, triglycerides, glycogen, lactate, ketone bodies, FAs and amino acids for ATP production in the mitochondria (Kolwicz, Purohit and Tian, 2013; Malandraki-Miller *et al.*, 2018).

ATP can be produced in the cytosol via glycolysis, the end-product of glycolysis is pyruvate, which can be further reduced into lactate. In case of carbohydrate deficiency, gluconeogenesis of pyruvate, reoxygenation of lactate or glycerol metabolism, can be utilized as sources of glucose synthesis. Otherwise, pyruvate can enter the mitochondria in the form of acetyl-coenzyme A (acetyl-coA) and be oxidized in the Krebs cycle (TCA cycle), in a process called oxidative phosphorylation. The reducing equivalents of this chained reaction act as hydrogen carriers: nicotinamide adenine dinucleotide hydrogen (NADH) and flavin adenine dinucleotide hydrogen (FADH2) and move into the electron transport chain. There the coupled transfer of electrons and H^+ generates an electrochemical proton gradient that leads to the production of ATP (Figure 4) (Malandraki-Miller *et al.*, 2018).

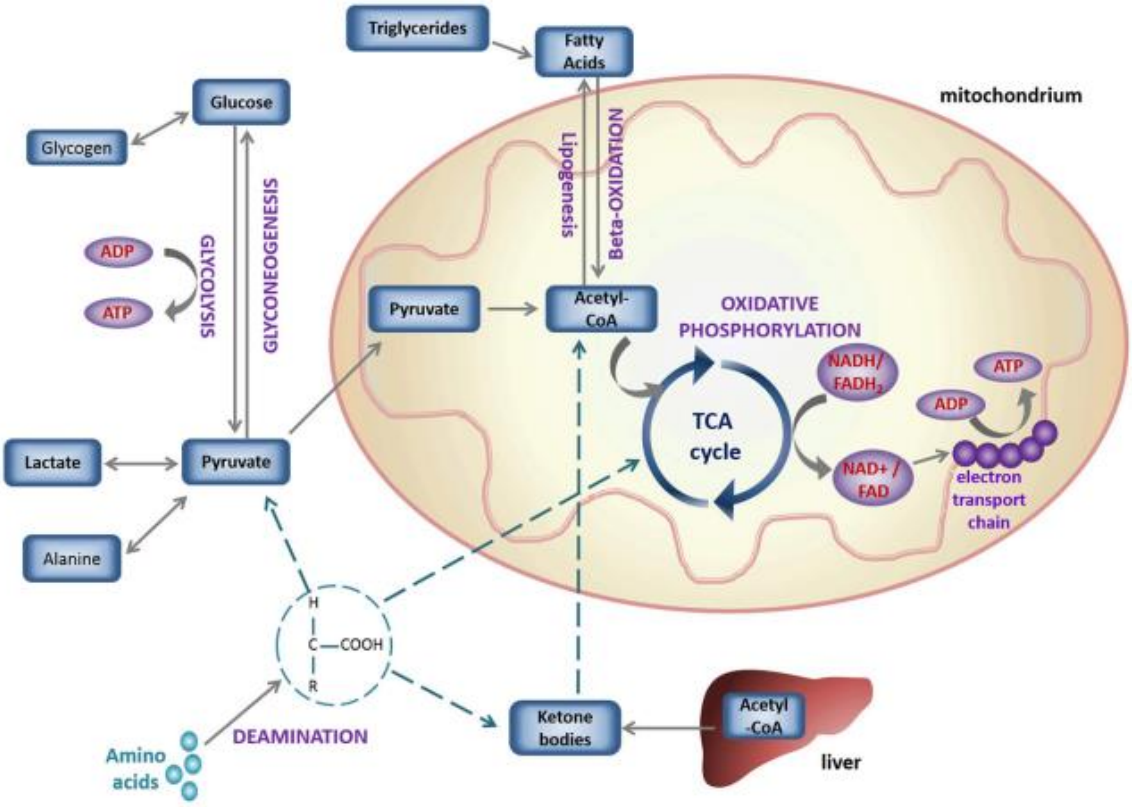


Figure 4. Cell metabolic pathways for energy production. Adapted from (Malandraki-Miller et al., 2018).

Substrates as lactate, ketone bodies and amino acids, can go into mitochondria directly for oxidation. Metabolism of ketone bodies yields acetyl-CoA while amino acid catabolism yields keto-acids are metabolized to go through the TCA cycle. The contribution of ketone bodies and amino acids to overall cardiac oxidative metabolism is minimal due to the low availability of these substrates in normal physiological conditions (Kolwicz, Purohit and Tian, 2013).

Nearly 70% to 90% of cardiac ATP is produced by the oxidation of FAs. The remaining 10% to 30% comes from the oxidation of glucose and lactate, as well as small amounts of ketone bodies and certain amino acids (Doenst, Nguyen and Abel, 2013).

Cardiomyocytes are the cell type with the highest mitochondria content, it occupies one third of the cell volume. Mitochondria in healthy hearts are largely fuelled by fatty acyl-CoA and pyruvate, which are the primary metabolites of FAs and carbohydrates, respectively (Kolwicz, Purohit and Tian, 2013). ATP can as well be produced by the degradation of lipids (including triglycerides) into FAs, which are metabolized in the mitochondria via beta-oxidation

(Figure 4), which transforms Fatty acyl-CoA to acetyl-CoA for flux into the TCA cycle and ATP synthesis (Kolwicz, Purohit and Tian, 2013; Malandraki-Miller *et al.*, 2018).

As already mentioned, more than 95% of ATP production comes from oxidative phosphorylation and in the healthy heart 50–80% of the energy is produced via beta-oxidation, under aerobic conditions. Oxidative phosphorylation yields 36 ATP/glucose molecule, being more efficient than glycolysis which only yields 2 ATP/glucose. Due to lipids reduced state, they are more oxygen-demanding than glucose, but also they have higher yield of ATP/carbon (Malandraki-Miller *et al.*, 2018).

The rest 5% of ATP comes from glycolysis and to a lesser extent from the TCA cycle. The heart consumes about 60-70% of its generated ATP to fuel contraction and the remaining 30-40% for various ion pumps, especially the Ca^{2+}-ATPase in the sarcoplasmic reticulum (Doenst, Nguyen and Abel, 2013).

The heart has an incredible ability of adaptation to changes in its physiological state by selecting the most efficient substrate, depending on its environment conditions. Hypoxia is one of the examples of cardiac metabolism adaptation. It is a upregulation of the hypoxia inducible factor (HIF) that increases glycolysis and suppress mitochondrial oxidative metabolism, in low oxygen conditions due to the fact that FAs require more oxygen than glucose to generate ATP (Malandraki-Miller *et al.*, 2018).

There is a network of interrelated signalling pathways that control the flux of glucose and FAs metabolism to allow the heart to switch substrates rapidly. This was for the first time described by Randle in 1963 as the glucose-fatty acid cycle, however the complexity of this network is yet to be fully explored (Malandraki-Miller *et al.*, 2018). In sum, it is known that FAs are the predominant substrate utilized in the adult myocardium. Importantly, the cardiac metabolic network is highly flexible in using other substrates when they are highly available in the heart (Kolwicz, Purohit and Tian, 2013).

6.1. Metabolism switch between postnatal cardiomyocytes to adult cardiomyocytes

Mammalian cardiomyocytes undergo extensive metabolic remodelling after birth in order to adapt to high-energy demands of the postnatal life. In mice, neonatal cardiomyocytes use glycolysis as their major source of ATP later, during the neonatal period, rodent cardiomyocytes suffer a metabolic switch and adult cardiomyocytes produce their energy via mitochondrial oxidative phosphorylation, a more efficient process than glycolysis (Vivien, Hudson and Porrello, 2016). The mouse heart loses the ability to regenerate after seven days of

birth, it is intriguing to think that the metabolic shift would have a role in suppressing the ability to repair (Martik, 2020). The fetal heart's environment is low in oxygen and FAs, thus fetal cardiomyocytes are highly dependent on glycolysis for ATP production. During development, the heart suffers a major metabolic alteration; the main physiological changes during the transition to the postnatal stage are the enhanced workload, and the demand for growth, that cannot be supported by glucose and lactate metabolisms (Malandraki-Miller *et al.*, 2018).

Through mitochondrial oxidative phosphorylation, electron leak produces reactive oxygen species (ROS). Increased production of ROS in postnatal cardiomyocytes leads to cardiomyocyte cell-cycle arrest through the activation of DNA damage response pathway. Cardiomyocyte cell-cycle arrest are dependent of the FAs oxidation however, it is important to mention that FAs utilization is directly linked with ROS increase. Importantly, cardiomyocytes from highly regenerative species such as zebrafish have a preference for glycolysis (Vivien, Hudson and Porrello, 2016; Fukuda *et al.*, 2019). Therefore, the "fetal switch" to oxidative metabolism of glucose and FAs has been associated to the loss of the regenerative capacity (Malandraki-Miller *et al.*, 2018). Recently, it was demonstrated that the HIF1 signalling localisation pattern controls the embryonic switch toward oxidative metabolism, disruption of which influences cardiac maturation (Menendez-Montes *et al.*, 2016). Furthermore, postnatal cardiomyocytes also revealed a shift in the energetic substrate utilization from pyruvate to FAs that are energetically more favourable (Cardoso *et al.*, 2020).

Adult heart can not regenerate lost or damaged myocardium although it does have a limited myocyte turnover. This cell capacity turnover is insufficient for restoration of contractile dysfunction. However, the neonatal mammalian heart is capable of substantial regeneration following injury, but its regenerative capacity is lost by postnatal day 7, which corresponds with cardiomyocyte binucleation and cell-cycle arrest. As a result, numerous pathways that regulate cardiomyocyte cell-cycle arrest postnatally have been identified. Even though many postnatal regulators of cardiomyocyte cell-cycle arrest have been already studied, the upstream signals that cause permanent cell-cycle arrest of most cardiomyocytes continue unidentified. The brief window of regenerative response following injury of the mammalian neonatal heart is mediated by proliferation of pre-existing cardiomyocytes (Cardoso *et al.*, 2020).

The constant use of FAs and its oxidation provokes a FAs utilization dependency by inhibiting glucose oxidation via the TCA cycle, in which acetyl-coA produced from fatty-acid oxidation inhibits the mitochondrial enzyme pyruvate dehydrogenase (PDH). The regulation of cardiac PDH activity is made by various isoforms of pyruvate dehydrogenase kinases (PDK1, PDK2 and PDK4) and phosphatases (PDP1 and PDP2), with phosphorylation resulting in enzyme inhibition. PDK4 is mainly responsible for inhibiting PDH when FAs are present and

enhance the heart commitment on fatty-acid oxidation for energy production. Mitochondria produce an elevated rate of H_2O_2 using FAs compared with pyruvate usage as a respiratory substrate (Cardoso *et al.*, 2020).

Current studies are focusing to clarify whether modulating substrate utilization would affect DNA damage and promote cell-cycle re-entry in cardiomyocytes. Diet manipulation with FAs deficiency results in a prolongation of the postnatal window of cardiomyocyte proliferation however, it is associated with a marked hepatomegaly and steatosis as a result of amplified FAs synthesis. Cessation of cardiomyocyte proliferation coincided with enhanced FAs synthesis by the liver. In sum, impaired dietary supply of FAs can delay, but not prevent, postnatal cardiomyocyte cell-cycle arrest due to a compensatory increase in hepatic fatty-acid biosynthesis (Cardoso *et al.*, 2020).

Studies of PDK4 knock-out were used to understand the role of inhibiting FAs utilization by cardiomyocyte mitochondria on cell cycle progression. PDK4 deletion in adult cardiomyocytes results in a marked shift in myocardial substrate utilization decreasing FAs and enhancing glucose-derived pyruvate utilization resulting in a significant decrease in DNA damage and marked increase in cardiomyocyte mitosis and cytokinesis. There is a possibility that PDK4 exerts effects not solely dependent on interaction with the PDH complex. Evidence indicates that PDK4 binds to and stabilizes the cAMP-response element-binding (CREB) protein, resulting in mTORC1 activation. In addition, the activation of PDH through administration of dichloroacetate or loss of PDK4 expression in mice improves glucose utilization and is cardioprotective with regards to infarct size and contractile dysfunction following I/R injury (Cardoso *et al.*, 2020).

6.2. Influence of metabolism on heart regeneration

Zebrafish are able to regenerate its heart after injury: heart muscle cells close to the wound divide to generate new cells that gradually replace the scar tissue and restore its normal function (Vivien, Hudson and Porrello, 2016; Honkoop *et al.*, 2019). This repair process has a lot in common with the heart developmental process in zebrafish embryos. In the human heart, cardiac injury leads to scarring and ultimately heart failure, thus the understanding of the links between heart development and regeneration in zebrafish could help with the improvement of heart regeneration efficiency in humans (Martik, 2020). In this section, it is explored the role of metabolism in cardiac regeneration, particularly how metabolism can possibly enhance direct cardiac reprogramming efficiency.

Heart regeneration in zebrafish is incredibly effective and relies on the proliferation of pre-existing cardiomyocytes. But not only cardiomyocytes contribute to regeneration, the process also involves other cell types such as epicardial and endocardial cells respond to the heart injury by the upregulation of injury-induced genes. Additionally, the injured heart is infiltrated by immune cells and fibroblasts. All these processes after the heart injury complicate the detection of cardiomyocyte specific gene responses. However, research by the use of single cell transcriptomics overcame these limitations and allowed to identify and characterize the different cardiomyocyte populations in the regenerating zebrafish heart. Additionally, it was found that increased mitochondrial oxidative phosphorylation (OXPHOS) activity promotes cardiomyocyte maturation and reduces the proliferative capacity of cardiomyocytes. This correlates well with the loss of regenerative capacity of the murine heart in the first week after birth at which time the metabolism in cardiomyocytes changes from predominantly glycolysis to mitochondrial OXPHOS (Honkoop *et al.*, 2019).

Research with iPSC-CM shown its structural and functional integration in healthy host cardiac tissue *in vivo*. However, even after the initiation of cardiac beating in these cells, they did not have the metabolic features of mature cardiomyocytes. Despite the mitochondrial remodelling and upregulation of oxidative metabolism, newly differentiated iPSC-CM in culture shown to preserve predominantly glycolytic metabolism (Malandraki-Miller *et al.*, 2018). Based on this data came to us the assumption that metabolism has an important role in cell-arrest cycle and in cellular maturation and possibly in regenerative therapies.

Progenitor cells in the developing of mice embryo as well as iPSC depend on glycolysis to preserve its proliferation capacity. It was discovered that glycolytic enzymes such as PKM2 and PFKFB4 can also directly interact with cell cycle regulators to boost proliferation. Concluding, the exact role for glycolysis in driving the cellular reprogramming during heart regeneration requires to be further investigated using genetic loss- and gain-of-function experiments combined with metabolomics (Honkoop *et al.*, 2019).

Overall, still not fully understand why the injured heart shifts to glycolysis in order to proliferate. It is important to understand if there is because proliferation is a high-energy demanding process, or it is glycolysis necessary for other critical processes during the heart regeneration process (Martik, 2020).

CHAPTER 2 - OBJECTIVES

Cardiac reprogramming has been a technique highly explored over the last years. Several TFs combinations were tried in both mice and human fibroblasts in order to differentiate these cells in iCMs. It was shown that MGT retroviruses were the most efficient viruses with better rate of cells conversion after the transduction process in mice. With all of these findings in consideration, one of the main objectives of the experimental work of this dissertation was to transdifferentiate mice and human fibroblasts to iCMs through the use of MGT retrovirus.

After that, the biggest innovation of our experimental approach was the understanding of how lncRNAs, specifically its silencing, influence direct cardiac reprogramming and if lncRNAs knockdowns can possibly enhance reprogramming efficiency.

On the other hand, as also mentioned during this chapter, zebrafish heart uses always glucose to energy production, and it is capable to regenerate after injury. Additionally, postnatal mammal hearts also have the capacity to regenerate and have proliferative capacity until the cell-cycle arrest that overlaps the switch between glucoses to FAs as the main source to ATP production. Based on this data we decided, through media nutrient manipulation, to study the influence of cellular metabolism in direct cardiac reprogramming.

CHAPTER 3 - METHODS

1. Cell Culture

Five cell lines were used during the experiments: Feeders, MAFs, Gm 03348 (10 years old human fibroblasts), HL-1 and Wi-38. All cell lines were previously used at Institute of Biomedicine of University of Aveiro. MAFs and Feeders were obtained from C57Bl6 mice. MAFs were prepared form adult (10–30-weeks old) and old (70–100-weeks old) fibroblasts obtained from mouse ears as previosly described (Li *et al.*, 2007). Feeders were prepared from embryonic fibroblasts primary cultures (MEFs) with Mitomycin-C from *Streptomyces caespitosus* (Sigma-Aldrich) or γ-irradiation, those MEFs were prepared from total mouse embryos as previously described (Palmero and Serrano, 2001). HL-1 are a cardiac muscle cell line from mice (Sigma- Aldrich) and were cultured with Claycomb medium (Sigma- Aldrich), supplemented with 10% fetal bovine serum (FBS), 1mM of L-glutamine and 1% penicillin/streptomycin (100 U/mL:100 μg/mL) at 37ºC with 5% CO_2. Wi-38 is a human cell line composed of fibroblasts derived from lung tissue of a 3-month-gestation aborted female fetus (Sigma- Aldrich), and were cultured in Dulbecco′s Modified Eagle′s Medium (DMEM) 41966 (Thermo Fisher Scientific), supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/mL:100 μg/mL), at 37ºC with 5% CO_2. Both cell lines were platted in gelatine coated plates.

The experiments with the remaining three cells lines (Feeders, MAFs and Gm 03348) were used in culture media DMEM 41966 (Thermo Fisher Scientific), supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/mL:100 μg/mL), at 37ºC with 5% CO_2. However, other growth media were used in cell culture named: All high, No FBS, No Glutamine and Low Glucose. Table 1 shows media composition, of all of them was used DMEM 11880 (Thermo Fisher Scientific).

Table 1. Different growth media used in cell culture.

All high	**No FBS**	**No Glutamine**	**Low Glucose**
-DMEM 11880; -1% Penicillin/Streptomycin (100 U/mL:100 μg/mL); -15% of FBS; -Glutamine at 4mM (from 400mM stock); -Glucose at a 4.5 g/L (from 20 g/L stock).	-DMEM 11880; -1% Penicillin/Streptomycin (100 U/mL:100 μg/mL); -Glutamine at 4mM (from 400mM stock); -Glucose at a 4.5 g/L (from 20 g/L stock).	-DMEM 11880; -1% Penicillin/Streptomycin (100 U/mL:100 μg/mL); -15% of FBS; -Glucose at a 4,5 g/L (from 20 g/L stock).	-DMEM 11880; -1% Penicillin/Streptomycin (100 U/mL:100 μg/mL); -15% of FBS; -Glutamine at 4mM (from 400mM stock);

2. Retroviral infection

At day 1 the factors were transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific). For each transfection it was mixed 30 µl of lipofectamine in 576 µl Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific), then were incubated 5 minutes at room temperature (RT) (Iacovides *et al.*, 2016).

In 4 separate tubes, 4 µg of each of pMXsMGT and pBabeGFP vectors were mixed with 4 µg pCL-Ampho (packaging plasmid). Next, Lipofectamine/Opti-MEM the was mixed with the plasmid mix and, next incubated at room temperature (RT) for 45min. In the meantime, $4.5x10^6$ HEK-293T cells in each 10 cm plate (1 for each factor) were platted. At the end of 45min the pMXs retroviral vectors and retroviral packaging vectors were added to the HEK-293T cells to generate viruses with $3x10^6$ IFU/ml (Kitamura *et al.*, 2003; J. D. Fu *et al.*, 2013; Iacovides *et al.*, 2016).

At day 2 HEK-293T cells medium was replaced by fresh growth medium and $3x10^5$ fibroblasts were platted in each 10 cm gelatine-coated plates. After this process during days 3 and 4 during mornings and afternoons, 4 viral infections were made. For that supernatants were collected from HEK-293T cells and re-feeded. The supernatants were filtered with 0.45 µm filters to remove debris and cells. Next it was added polybrene at a dilution of 1:1000 to the final volume of viral supernatants making the cocktails needed (equal volume of each factor). After the viral supernatants were added on each fibroblast's plates drop by drop. At day 5, after all viral infections, the growth medium was changed to DMEM 41966 + 10% of FBS for about 5 days, then GFP expression was evaluated on Fluorescence-activated cell sorting (FACS) (Takahashi and Yamanaka, 2006; Iacovides *et al.*, 2016). The viruses yielded a transduction efficiency indicated by the GFP retroviral infection (J. D. Fu *et al.*, 2013). During all process cells were incubated at 37ºC with 5% CO_2.

3. RNA isolation

Total RNA was isolated from cells using TRIzol™ Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells medium was removed and after it was added TRIzol™ Reagent directly to the culture dish to lyse the cells. Then, the lysates were pipetted up and down several times to homogenize. Next, the lysates were centrifuged and after transferred the clear supernatant to new tubes. Following, there was a period of incubation to allow complete dissociation of the nucleoprotein complexes. Next it was added chloroform, mix

was incubated and centrifuged. The aqueous phase of the mixtures was transferred to new 1.5ml tubes.

To precipitate the RNA isopropanol was added to the aqueous phase and incubated. After incubation it was centrifuged, total RNA was precipitated in a white gel-like pellet at the bottom of the tube. The supernatant was discarded with a micropipettor.

Next step RNA was washed by resuspending the pellet with 75% ethanol. The samples were vortexed briefly and then centrifuged. Lastly, it was discarded the supernatant with a micropipette and the RNA pellet was dried at RT for 10 minutes.

To solubilize the RNA, the pellet was resuspended in RNase-free water by pipetting up and down. After it was incubated in a heat block at 55ºC for 15 minutes and kept at -20ºC until further use.

RNA concentration was quantified using a NanoDrop™ Spectophotometer and the absorbance was measure at 260 nm and 280 nm. RNA concentration was calculated using the formula:

$$A_{260} \times \text{dilution} \times 40 = \mu g \text{ RNA/mL} \quad \textbf{Equation 1}$$

Lastly was calculated the A_{260}/A_{280} ratio. All RNAs extracted from different cell lines had a A_{260}/A_{280} ratio ≈ 2 which is considered pure RNA.

4. Reverse Transcriptase (RT) reaction for cDNA synthesis

The RNAs extracted were reverse-transcribed into cDNA using the SuperScript™ II Reverse Transcriptase (Invitrogen) (Chang *et al.*, 2019).

The manufacturer protocols was followed briefly, it was added random primers, RNA quantity necessary for the final concentration of 1 ng/µl, dNTPs and sterile and distilled water.

Next the mixture was heated to 65°C and then quick chill on ice. Following it was collected the contents of the tube by brief centrifugation and added: 5X First-Strand Buffer, 0.1 M DTT and RNaseOUT™ (40 units/µL).

The contents were mixed on the tube gently and after incubated at 25ºC. Next it was added SuperScript™ II RT and mixed by pipetting gently up and down. The tubes were incubated at 25°C, next were incubate at 42°C and lastly to inactivate the reaction, the tubes were heated at 70°C.

5. Quantitative PCR (qPCR)

qPCR was performed on an Applied Biosystems 7500 Real-Time PCR System using SYBR Green PCR Master Mix (Thermo Fisher Scientific). The final volume of each reaction was 15 µl: 7.5 µl of SYBR Green PCR Master Mix, 0.4 µl of each pair of primers (forward and reverse at 10 µM), 5 µl of cDNA and 1.7 µl of H_2O. Quantifications were made applying the ΔΔCt method (Ct of nuclear DNA gene – Ct of mitochondrial DNA gene), followed 2^(ΔΔCt). The mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and actin alpha cardiac muscle (ACTC1) mRNA (J. D. Fu *et al.*, 2013; Patel *et al.*, 2018). All primer sequences are listed below on Table 2:

Table 2. Amplified genes and primers used for qPCR.

Primers	**Sequence** (F- Forward primer; R- Reverse primer)
ACTC1	F: TGCCCCCGGCTGCTC R: GTTCTGTAGGCGTGCTAGGG
GAPDH	F: TTCACCACCATGGAGAAGGC R: CCCTTTTGGCTCCACCCT
Gata4	F: TGTGCCAACTGCCAGACTAC R: TGGGCTTCCGTTTTCTGGTT
Mef2c	F: GAGCCGGACAAACTCAGACA R: TCAAAGCTGGGAGGTGGAAC
Tbx5	F: CTTCTATCGCTCGGGCTACC R: GCTATAGGAGGGCATGCTGG
Gm28592	F: GCGAGTGAAGAGGCTGGT R: TCAAGCTGAAGGAATTGCAC
Phlda1	F: TCATCACAGTTGCAGGAAGC R: GGAGGTGGCCTACATTCAGA
Mir22hg	F: GAAGAACTGTTGCCCTCTGC R: ACTGTCTTGTGCCTGCCTCT
Gm27028	F: GCATCCTGCAGCCTTCTTAC R: AACAGCCTTCTCCCAGCTTT
Gm15856	F: AAATACCCCTGGGGAGAATG R: GGAGAGTGGGGAGATGAACA
Bvht	F: TGGGCCTAAGGAAAGCCG R: ATCTCCGTTGGATTTGGAGGG

6. Cell Proliferation / Cytotoxicity Assay

Initially Gm 03348 cells grow in adherent monolayer in 6-well plaques with the four media previously described. After 12 days of culture, it was removed the culture growth medium and cells were washed with PBS (1x). TrypleTM Express (Gibco, 12605-028) was added and cells were incubated 5 minutes followed by inactivation with complete medium. After obtaining cell suspension, the cell density was determined by trypan blue exclusion method.

Gm 03348 cells were then inoculated on a 96-well plate at a density of 3000 cells/well and 5000 cells/well (n=6 well/each). To study the optimal culture condition for these cells, the four different culture media already mentioned were tested: Low glucose, All high, No FBS and No glutamine. Following 5 days of platting, the proliferation of Gm 03348 cells in each culture condition were examined after four hours of incubation (37ºC under 5% CO_2) with the Cell Counting Kit-8 (CCK-8) (Dojindo, Europe) recorded at an absorbance of 450 nm using a Microplate reader (Tecan 200).

7. Intracellular staining and fluorescence-activated cell sorting (FACS)

To prepare the cells to flow cytometry, cells were trypsinized and the pellet was resuspended in 200 µl of PBS (1x), then cells were fixed adding 200 µl of paraformaldehyde (PFA) (8%) for 15 minutes on ice. Following it was centrifuged 3000 x g for 5 minutes.

The first wash was made with the stain buffer: PBS (1x) + 0.2% Bovine serum albumin (BSA) following centrifugation of 3000 x g for 5 minutes. Next step was permeabilization, it was used 0.05% tween 20 + 0.5% Dimethylsulfoxide DNA(DMSO) followed by a spin equal to the ones before (Patel *et al.*, 2018).

For blocking it was used PBS (1x) + 0.5% BSA and then incubated at RT for 10 minutes, in the end of this step it was added mouse monoclonal anti-tropomyosin antibody (Sigma) at a dilution of 1:100. For 1 hour in rotation. Following incubation cells were washed following the exact process of the first wash.

Next cells pellet was resuspended in 300 µl of PBS (1x) + 0.5% BSA and added the secondary antibody, in the case Alexa Fluor 488 Dye (Thermo Fisher Scientific) at a dilution of 1:600 for, 30 minutes at RT. After cells were washed one last time being this wash equal to the previously washes. Last step of the process was to resuspend the pellet in 200 µl PBS (1x) +

0.2% BSA following acquisition in a Accuri™ C6 Flow Cytometer (BD Biosciences) and subsequent analysis. Using the FlowJo software (Nam *et al.*, 2013).

8. Statistical Analysis

The results are presented as mean values and their standard deviations (mean ± SD) for each experimental group. Differences between samples comparing with the control conditions were estimated by T student test to evaluate samples i.e. parametric analysis. A P value < 0.05 was considered statistically significant. Error bars indicate standard deviation (Sperandei, 2014; Vaseghi *et al.*, 2016). Statistical analysis was performed using Microsoft Excel software.

CHAPTER 4 - RESULTS

1. Gene expression in MGT transfected cardiac cells

1.1. MGT mRNA expression

In vitro cardiac differentiation recapitulates the embryonic development of the heart *in vivo* which is a progressive process. Therefore, the elaborate regulation of cardiac differentiation involves stepwise integration of transcription factors and signalling pathways (Li *et al.*, 2019).

Gata4 plays an important role in general cardiac development both in human and mice, its genetic knockout causing embryonic lethality due to a block of cardiogenesis (Chen *et al.*, 2012).

Mef2c is essential for cardiovascular development, its activity is modulated by post-translational modifications in response to cytoplasmic signals including calcium (Hao *et al.*, 2011). Moreover, *Mef2c* knockout mice are embryonically lethal due to prominent heart defects and importantly also demonstrate a vascular phenotype characterized by a failure of organization of endothelial cells (Sturtzel *et al.*, 2014; Laszlo *et al.*, 2015).

During the embryonic stage of cell proliferation, the cardiac cells express the first molecular markers of cardiac development, including *Tbx5*. This TF has an important role in heart development, mutations in *Tbx5* display many cardiac abnormalities. *Tbx5* is indispensable to control embryonic cardiac cell proliferation and cell number by regulating the length of the embryonic cardiac cell cycle (Goetz, Brown and Conlon, 2006).

qPCR was performed using two different housekeeping genes in order to guarantee authenticity to the results, i.e. to verify if mRNA expression of the different TFs and lncRNAs had the same tendency using different normalizations.

First it was maintained in culture two murine cellular lines, Feeders and MAFs, following fibroblasts retroviral transduction protocol. After 3 days of the retroviral transduction process the cells were observed through the microscope and, it was visible GFP^+ in the control cells (Figure 5).

a) **b)**

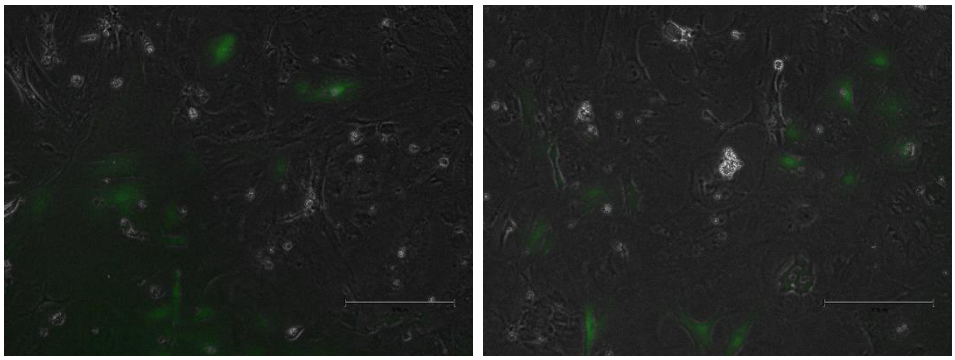


Figure 5. Feeders [a)] and MAFs [b)] expressing GFP^+, used as positive control for the transduction process, observed under the phase-contrast imaging 3 days after the retroviral infections. Scale bars represent 300 μm in both a) and b). The magnification used was 10x in both a) and b).

Next RNA was isolated and retrotranscribed into cDNA and MGT expression was analysed by qPCR. TFs mRNA expression in MAFs infected with MGT were compared with GFP infected MAFs and with Feeders infected with MGT, the results can be seen in Figure 6.

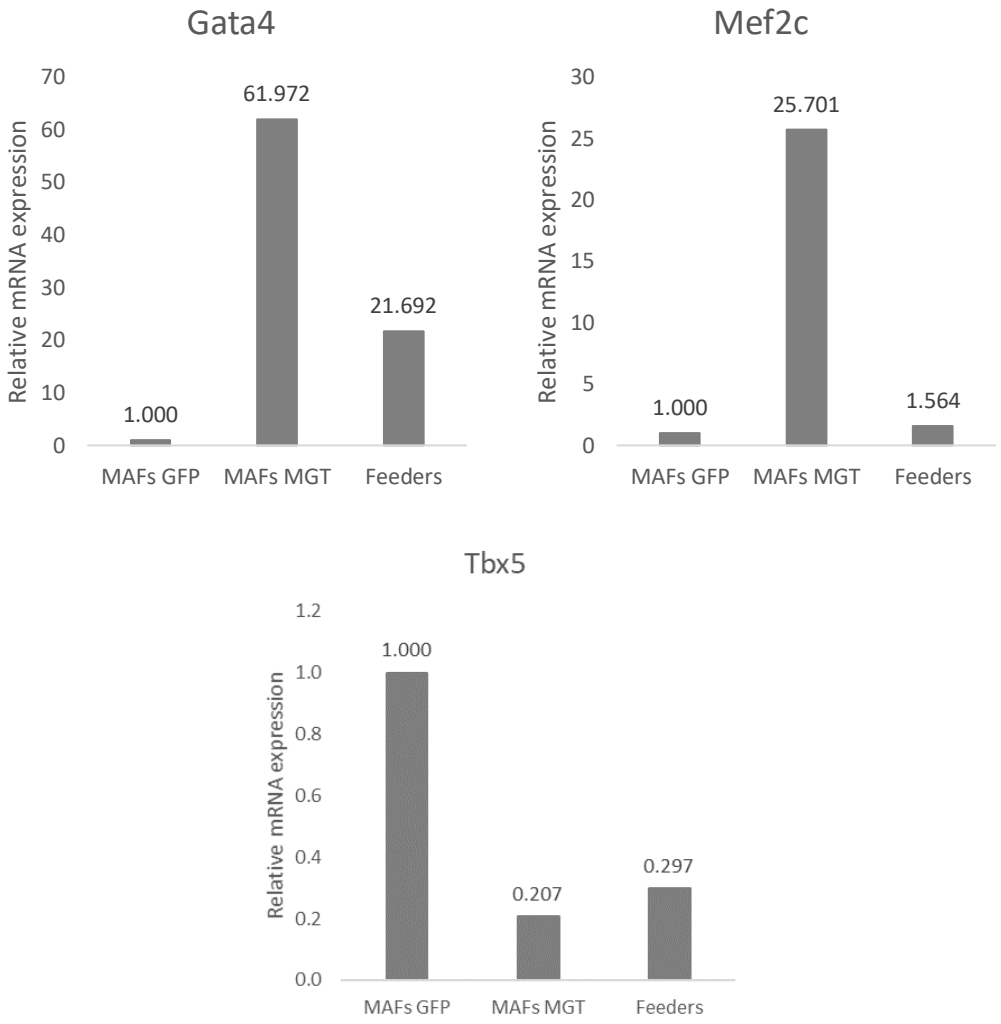


Figure 6. MGT expression in MAFs infected with GFP, MAFs infected with MGT and Feeders infected with MGT using the *GAPDH* as housekeeping gene. The calculation was made based in *GAPDH* expression. Data refers to n=2 from 1 independent experiment.

Figure 6 represents the expression of the three TFs responsible for direct cardiac reprogramming. *Gata4* expression was increased in MAFs infected with MGT compared with MAFs infected with GFP. Feeders infected with MGT also showed a higher mRNA expression comparing with MAFs infected with GFP. *Mef2c* followed the exact same tendency that *Gata4*. *Tbx5* was mostly expressed in MAFs infected with GFP comparing with MAFs and Feeders infected with MGT.

The process was repeated using *ACTC1* as housekeeping gene. Figure 13 (annex 1) represents the expression of the three TFs responsible for direct cardiac reprogramming. Comparing *Gata4* expression between MAFs infected with GFP and MAFs infected with MGT, it is observed an increased expression of this cardiac TF in MAFs infected with MGT. In

addition, *Mef2c* also showed a clear expression difference among cells infected with GFP and MGT, being its expression larger in MAFs infected with MGT. *Tbx5* was the TF with dissimilar results in MAFs infected with MGT relatively with MAFs infected with GFP. MAFs infected with GFP presented higher expression for this TF. These results can be possibly justified by the fact that the chosen pairs of primers were not the most efficient. In conclusion, *ACTC1* results are in accordance with the *GAPDH* results.

1.2. lncRNAs expression

lncRNAs have been demonstrated to modulate biological processes, in particular the regulation of gene expression networks. Several lncRNAs were identified as being expressed in the heart, however there is a long way to assess the potential of modulating lncRNAs for cardiac regeneration. The inhibition of lncRNAs has been performed using for example antisense oligonucleotides. Knockdown using antisense oligonucleotides may be a very promising strategy for therapeutic applications targeting nuclear-localised lncRNAs, as they are effective in reducing expression levels via RNAse H to mediate destruction of the lncRNA (Hudson and Porrello, 2013).

The mRNA expression of some lncRNAs was evaluated using qPCR. lncRNAs candidates were chosen based in preliminary data from RNAseq results of the day 1 and day 7 in mice neonatal cardiomyocytes kindly provided by our collaborator Christian Bär from Institute of Molecular and Translational Therapeutic Strategies, Hannover Medical School, Germany. The lncRNAs selected were: *Mir22hg*, *Gm15856*, *Gm27028* and *Gm28592*. The intention of this analysis was to understand the best lncRNA candidates to knockdown and how the lncRNAs knockdown could influence the efficiency of direct cardiac reprogramming of fibroblast in cardiomyocytes.

Additionally, it was studied *Phlda 1* expression to guarantee the veracity of *Gm28592* analysis since *Gm28592* is an antisense lncRNA to *Phlda1* gene. *Bvht* was used as a positive control given the fact that this lncRNA is strongly expressed in the heart (see chapter one). The expression of lncRNAs in MAFs infected with MGT and MAFs infected with GFP can be seen at Figure 7.

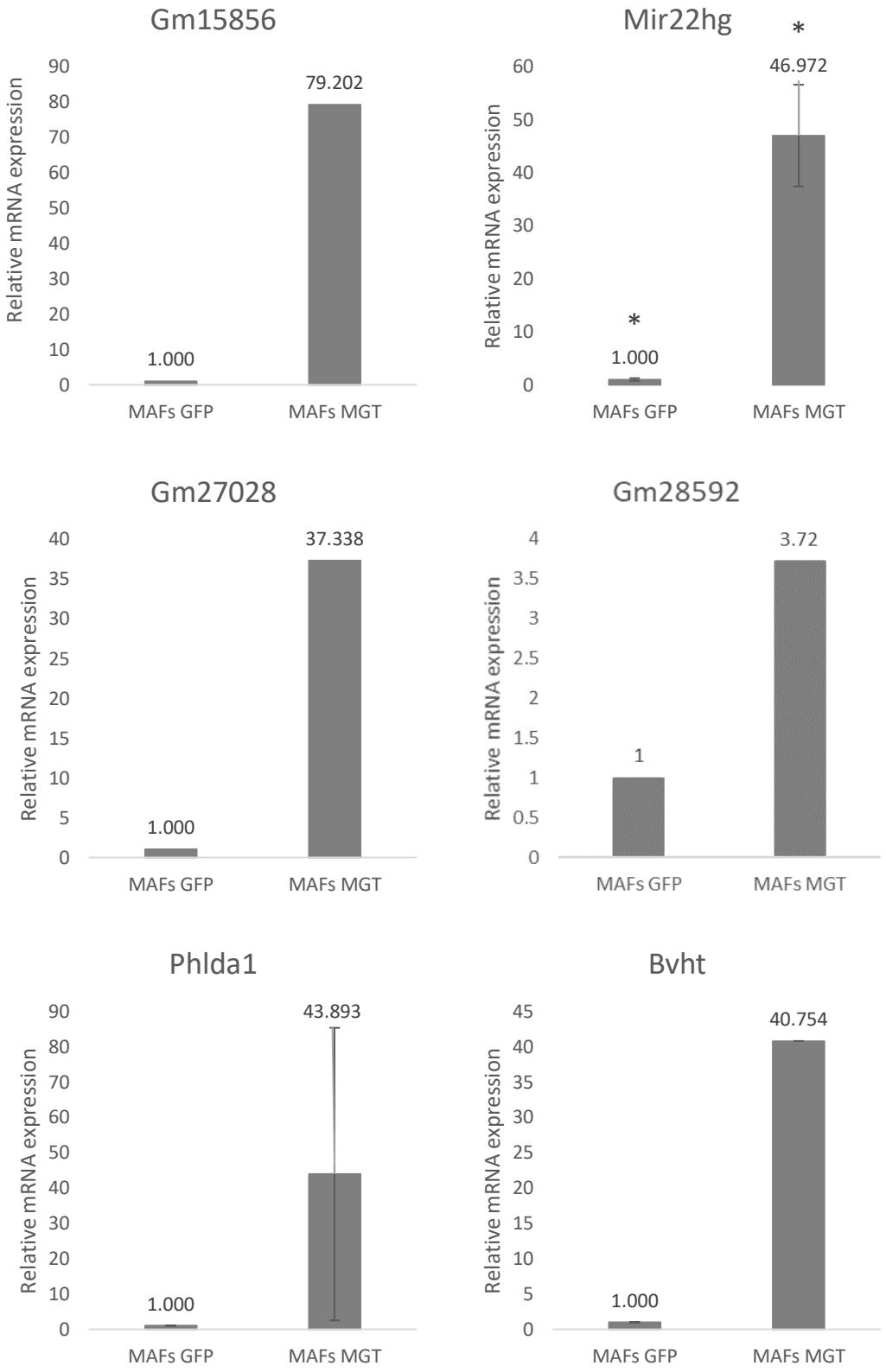


Figure 7. lncRNAs expression in MAFs infected with MGT comparing with MAFs infected with GFP using *ACTC1* as housekeeping gene. Data refers to n=2 from 1 independent experiment. Data are presented as mean ± SD. Statistical significance was determined by two-tailed Student t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Of all lncRNAs *Gm15856* was the one with the most significant expression in MAFs infected with MGT. *Mir22hg* were the second lncRNA candidate that showed significant expression in MGT infected cells. Relatively to *Gm27028*, this lncRNA also presented increased expression in MAFs infected with MGT comparing with MAFs infected with GFP. *Gm28592* had lower expression comparing with the expression of other lncRNAs, the difference between MAFs infected with GFP and MAFs infected with MGT was minor. In the end, all lncRNAs had a higher expression in cells infected with MGT as it was expected.

The same selected lncRNAs were analysed in comparison to *GAPDH* expression and also using Feeders infected with MGT to understand how aging affects the reprograming and mRNA expression of these lncRNAs. The results are presented at Figure 14 (annex 2).

Gm15856 had considerable expression in MAFs infected with MGT and next in Feeders infected with MGT comparing with MAFs infected with GFP. *Mir22hg* demonstrated higher expression in MGT infected cells however, its larger expression was observed in Feeders. Also, the *Mir22hg* mRNA expression between MAFs infected with GFP and MAFs infected with MGT was very small. *Gm28592* had lower expression in MAFs (both infected with GFP and MGT) comparing with Feeders infected with MGT. *Phlda 1* and *Bvht* mRNA expression was significant higher in MAFs infected with MGT comparing with MAFs infected with GFP. In conclusion, the *GAPDH* results are in accordance with previous ones with *ACTC1* which brings authenticity to the analysis.

2. Flow cytometry assays of Gm 03348

2.1. Monitoring transfection efficiency by GFP expression

First it was maintained in culture Gm 03348 cells following our fibroblasts retroviral transduction protocol. After three days of the retroviral transduction process, GFP^+ were observed under the microscope (Figure 8).

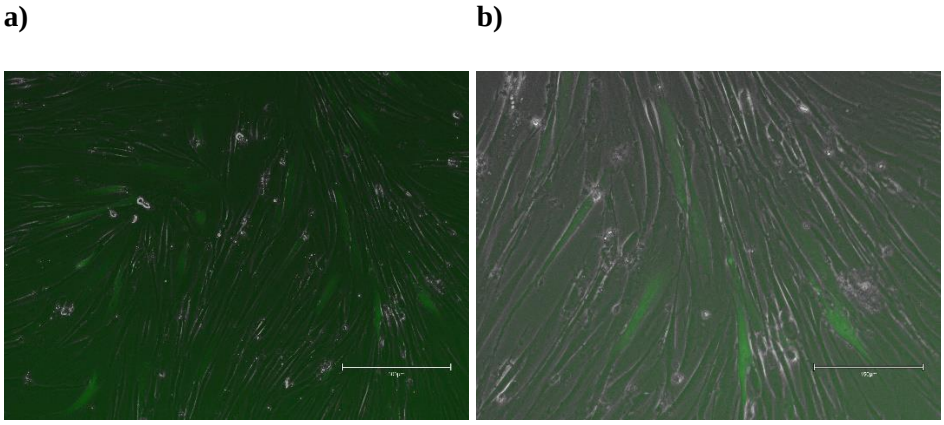


Figure 8. Gm 03348 cells expressing GFP$^+$, used as positive control for the transduction process, observed under the phase-contrast imaging 3 days after the retroviral infections. Scale bars represent 300 µm in a) and in 150 µm b). The magnification used was 10x in a) and 20x in b).

Flow cytometry has been used for the analysis of surface and intracellular proteins of entire non-muscle tissues or organs. Flow cytometry analysis has the ability to simultaneously detect several proteins of interest using samples labelled with numerous antibodies. In addition, flow cytometry analysis is less time consuming and even more sensitive than other methods, such as western blotting and gel electrophoresis for example (Jackaman *et al.*, 2007). Figure 9 shows the results obtained in the control group and in the cells infected with GFP as a readout for the viral infection efficiency.

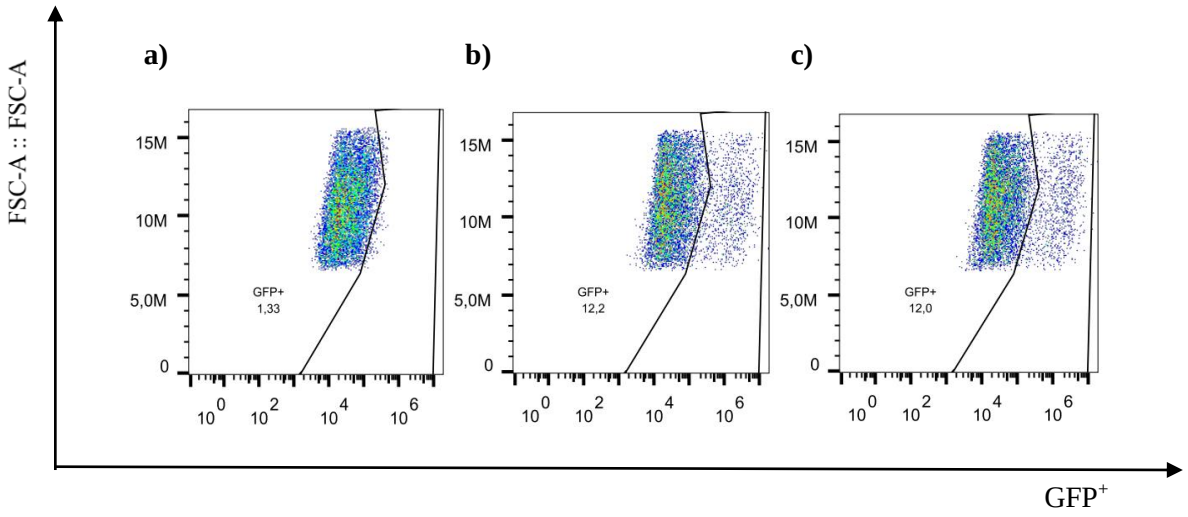


Figure 9. FACS were ran in Accuri™ C6 Flow Cytometer (BD Biosciences) and analysed using Flow Jo Software. Displaying the 1.33% of GFP positive cells in the live-singlet population; a) Gm 03348 cells not infected (negative control); b) and c) Gm 03348 cells infected with GFP (positive controls); with n=2 each.

It is observed that Gm 03348 cells have an autofluorescence of 1.33%. At Figure 9 in b) and c) GFP had 12.2% and 12.0% respectively of successfully viral transfection, concluding that, the transfection rate in Gm 03348 cells were on average 10.77% [((12.2+12.0)/2)-1.33].

2.2. HL-1 and Wi-38 comparison

Tropomyosin is one of the proteins that regulates cardiac physiology. Additionally, it plays a key role in controlling calcium regulated sarcomeric contraction through its interactions with actin and the troponin complex. Tropomyosin regulates the rates of cardiac contraction and relaxation, along with conferring differences in myofilament calcium sensitivity and sarcomere tension development (Jagatheesan, Rajan and Wieczorek, 2010). The flow cytometry studies of certain proteins as tropomyosin is useful to understand the effectiveness of an experiment in case the direct cardiac reprogramming (Jackaman *et al.*, 2007).

HL-1 cells are mice cardiomyocytes that in this context will help us to understand what levels of tropomyosin are expected in cardiomyocytes. Comparing HL-1 tropomyosin results with Gm 03348 cells infected with MGT it is possible to understand the efficiency of the retroviral transduction process and whether Gm 03348 cells are manifesting cardiac features.

Wi-38 is a diploid human cell line composed of fibroblasts derived from lung tissue of a 3-month-gestation aborted female fetus that were used to understand the efficiency of tropomyosin staining by the anti-human tropomyosin antibody used. In order to define the best stanning condition for detecting tropomyosin expression in our experimental setting, we also tested the use of the conjugated anti-human. The tropomyosin primary antibody alone or combined with a secondary antibody (anti-goat Alexa 488) results for FACS analysis can be seen at Figure 10.

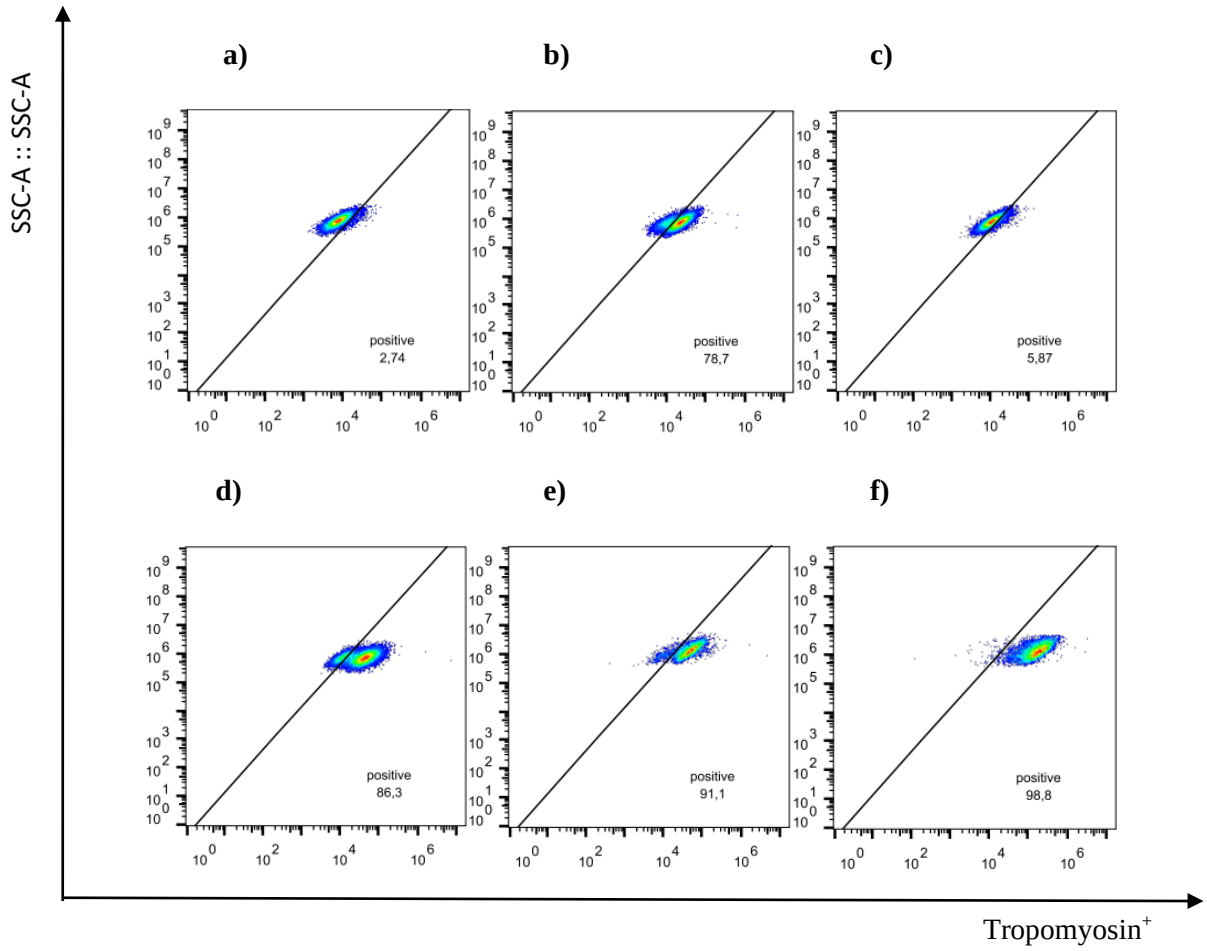


Figure 10. FACS were ran in Accuri™ C6 Flow Cytometer (BD Biosciences) and analysed using Flow Jo Software. a) HL-1 cells unstained (negative control); b) HL-1 cells with primary antibody; c) HL-1 cells with secondary antibody; d) HL-1 cells with primary and secondary antibodies; e) Wi-38 cells with primary antibody; f) Wi-38 with primary and secondary antibodies.

The results obtained in HL-1 cells with primary antibody only and with primary and secondary antibodies have similar result which means that the primary antibody tropomyosin conjugated to anti-goat Alexa 488 is totally functional being the percentage of alive HL-1 cells expressing tropomyosin 78.7%, with both antibodies the percentage was 86.3%. The difference between the b) and d) is probably only due to the secondary anti-goat Alexa 488 antibody autofluorescence. In summary, and as expected, HL-1 cells have a significant expression of tropomyosin and the use of anti-goat Alexa 488 conjugated tropomyosin primary antibody alone gives satisfactory results for flow cytometry analysis.

Wi-38 FACS analysis shown a percentage of 91.1% of alive cells expressing tropomyosin when was used the primary antibody only. Wi-38 cells, being embryonic cells, this high expression of tropomyosin was not expected. Then, it was necessary a negative control, i.e. Wi-38 with no antibody to verify the level of autofluorescence from unstained cells. Using both

antibodies the result was 96.8% and as for the HL-1 cells. This difference may be due to the anti-goat Alexa 488 secondary antibody autofluorescence.

2.3. The impact of cell culture media composition in the expression of tropomyosin by Gm 03348 MGT infected cells

Gm 03348 cells were transduced with MGT and after the fourth viral infection were cultured with different media: All high, No FBS, No glutamine and Low glucose. After culturing for 15 days, cells were stained with tropomyosin antibody and the percentage of cells expressing tropomyosin was calculated through flow cytometry. The results can be seen at Figure 11.

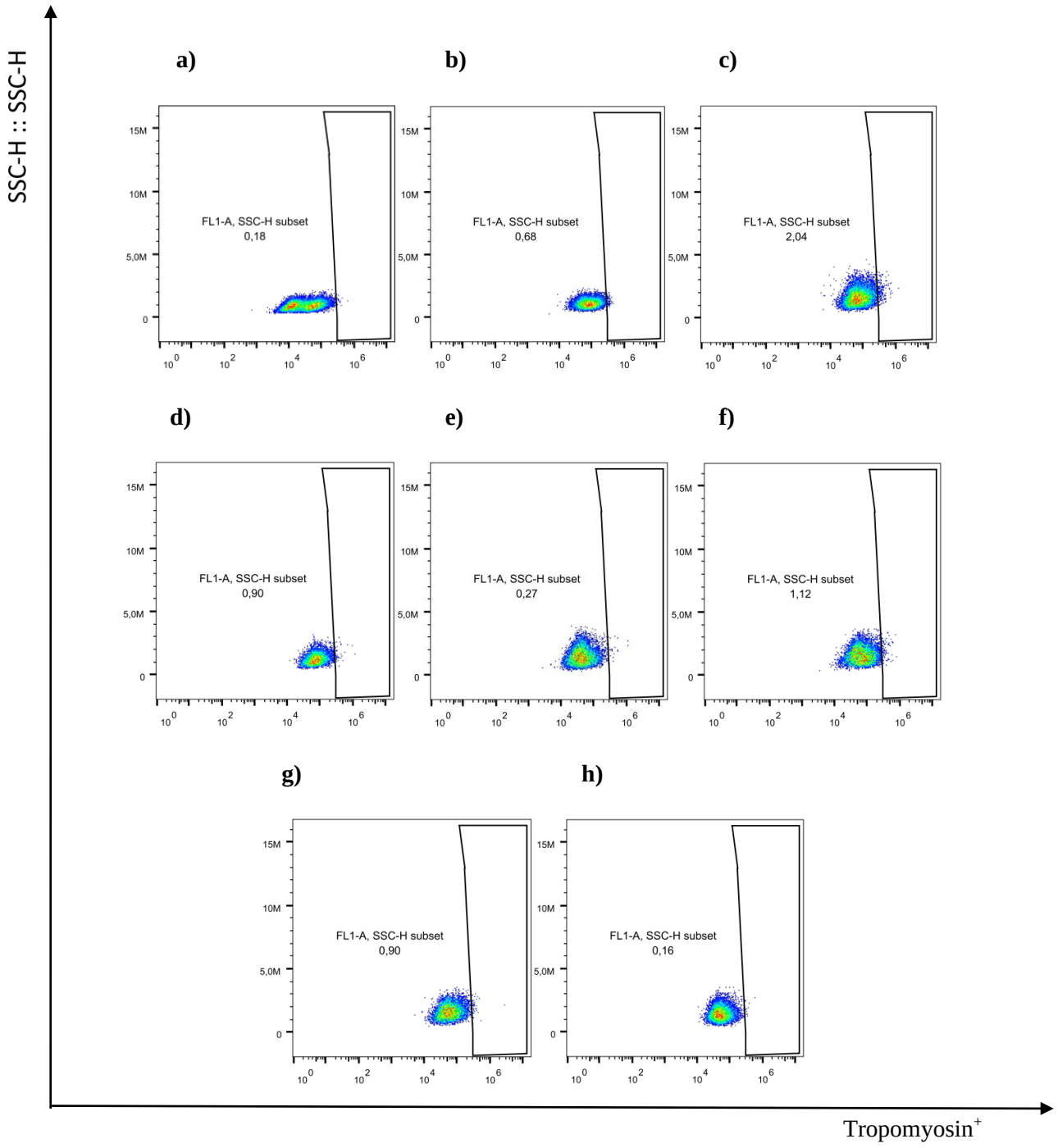


Figure 11. FACS were ran in Accuri™ C6 Flow Cytometer (BD Biosciences) and analysed using Flow Jo Software. a) Gm 03348 cells infected (positive control); b) Gm 03348 cells not infected (negative control); c) Gm 03348 cells infected cultured in "All high" medium; d) Gm 03348 cells infected cultured in "No FBS" medium; e) Gm 03348 cells infected cultured in "No Glutamine" medium; f) Gm 03348 cells infected cultured in "Low glucose" medium; g) Gm 03348 cells infected cultured in "All high medium with 10% of Claycomb" medium; h) Gm 03348 cells infected cultured in "No FBS medium with 10% of Claycomb" medium; with n=1 each.

Analysing the FACS results it can be concluded that All high medium was the one with best results with a 2.04% of alive cells expressing tropomyosin. Low glucose was the second medium that demonstrated better performance with 1.12% of Gm 03348 alive cells expressing

tropomyosin whereas No FBS and No glutamine media revealed lowest efficiency with 0.3% and 0.9% respectively.

The fact that All high and Low glucose media had the highest score of alive cells expressing tropomyosin is mainly because their composition are very similar being the glucose concentration the only difference. This data suggests that glucose can possibly be one of the main energy sources for these cells. Moreover, results of cells cultured with No glutamine medium indicates that glutamine is crucial to cell survival and metabolism.

After the retroviral transduction process, cells were culture with the media All high and No FBS supplemented with 10% of Claycomb medium from HL-1 cultured cells and analysed for tropomyosin expression by FACS. The results can be seen in Figure 11 g) and h).

In both cases it was observed diminished expression of tropomyosin when Claycomb medium was added. Cells culture in All high medium + 10% of Claycomb medium showed a regression of 1.14% comparing with the cells cultured with just All high medium. Equally, cells cultured with No FBS medium + 10% of Claycomb medium showed a decrease of 0.74% comparing with the cells cultured with just No FBS medium. These results are quite surprising, it was expected an enhancement in the percentage of alive cells expressing tropomyosin.

3. Cellular viability

In order to evaluate the impact of the different growth media used in the proliferation and viability of Gm 03348 cells, we assayed cellular viability using the Cell Counting Kit-8 (CCK-8), a kit that provides information about cell proliferation and cytotoxicity. In a 96-well Gm 03348 cells were platted at different cells densities, 3000 cells and 5000 cells per well, and cells were cultured with different media: All high, No FBS, No glutamine and Low glucose as before. The cells were maintained in culture for 15 days, in which it was studied the cellular viability using the CCK-8 kit. The results can be seen at Figure 12.

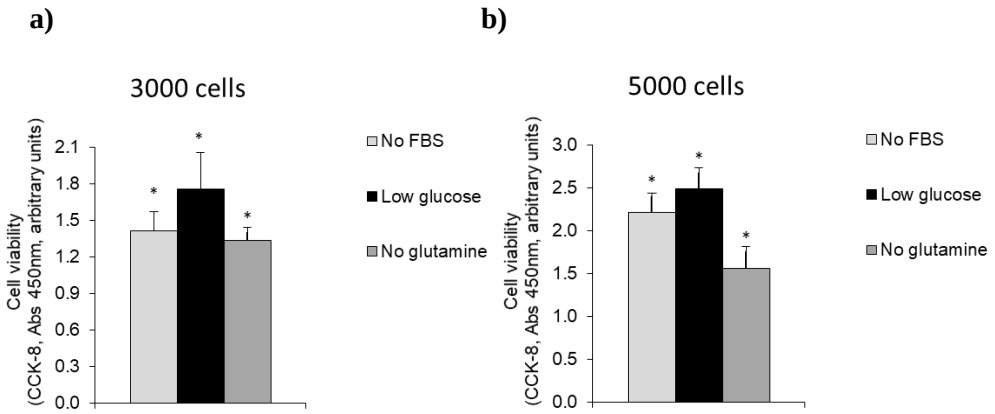


Figure 12. Cellular viability of Gm 03348 plated [a) 3000 cells and b) 5000 cells per well] measured by CCK-8 absorbance at 450 nm detected in a Microplate Tecan 2000 reader, with n=6 for each medium. Data are presented as mean ± SD. Statistical significance was determined by two-tailed Student t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Cells plated whit Low glucose medium demonstrated the best results, next was with No FBS medium, followed by No glutamine medium. Similar results were obtained when 5000 cells were plated.

As mentioned before Low glucose and All high media are identical just differing at glucose concentration, thus similar results were expected. However, we obtained significant different results between these media which can be an artefact, these results can be seen at Figure 15 (annex 3).

CHAPTER 5 - DISCUSSION

Heart diseases affect more than 30 million individuals worldwide and they are the most significant cause of morbidity and mortality in the world, representing 30% of all global deaths. The mammalian heart lacks an adequate capacity to generate new cardiomyocytes and re-establish its normal function after injury (Talkhabi, Razavi and Salari, 2017; Shah, 2020). For these reasons the prevention of disease and death owing to heart failure needs to be made a global health priority (Ponikowski *et al.*, 2014).

After injury, cardiac fibroblasts generate fibrotic scars that cause remodelling and hypertrophy which compromises heart function (Rastegar-Pouyani *et al.*, 2017). Numerous medical interventions including drug therapy, organ transplantation and cell therapy have been developed to treat patients. It seems that cell therapy has more advantages in repairing the cardiac normal functions after injury. In the last years, different types of human cells have been studied clinically and experimentally to enhance the cardiac regeneration as for example iPSCs (Talkhabi, Razavi and Salari, 2017).

iPSCs technology opened a new avenue for generating different cell types from differentiated somatic cells, only by overexpressing specific TFs or miRNAs (Talkhabi, Razavi and Salari, 2017). Nevertheless, iPSC-derived cardiomyocytes present several concerns about the maturity and functional heterogeneity of these cells, their low survival when delivered to the injured myocardium and their potential tumorigenicity (Jayawardena, Mirotsou and Dzau, 2014; Miyamoto *et al.*, 2018).

In this regard, transdifferentiation or direct reprogramming, in which the identity of one type of somatic cells is transformed to other adult cell types without intermediate reversion to a pluripotent state (Talkhabi, Razavi and Salari, 2017) holds great promise for regenerative medicine as it overcomes several problems of the iPSC-based therapy (Jayawardena, Mirotsou and Dzau, 2014; Miyamoto *et al.*, 2018).

Others advantages of the direct cardiac reprogramming are that the overexpression of cardiac reprogramming factors in fibroblasts not only induced the cardiac program but also repressed fibroblast signatures such as cell proliferation, synthesis of the extracellular matrix, and expression of cytokines (Miyamoto *et al.*, 2018).

In our study, to evaluate the transdifferentiation efficiency, MGT expression analysis was evaluated by qPCR. *Gata4* and *Mef2c* had a clear increase in expression in MAFs infected with MGT comparing with MAFs infected with GFP, these findings lead to the conclusion that the retroviral infection was effective. However, for *Tbx5* the results were different, in fact *Tbx5*

had lower mRNA expression in MAFs infected with MGT, as already mentioned probably due to the use of less efficient primers (Figures 13).

When retroviral infection were tested in cells with different ages, Feeders and MAFs, *Gata4* and *Mef2c* present increase expression in MAFs infected with MGT comparing with Feeders infected with MGT (Figure 6), this results are expected since retroviral infection is only effective in mitotic cells. Feeders were inactivated during the mitomycin-C process which means that they lost their proliferative capacity, thus it is expected that MAFs have higher mRNA expression of the TFs. However, it is important to mentioned that Feeders had senescent aspect.

Cellular senescence is an irreversible cell cycle arrest process that occurs in response to different stresses. Besides cell cycle exit, senescent cells undergo phenotypic changes including increased cellular volume, increased reactive oxygen species (ROS) level, persistent DNA damage response, loss of proliferative potential, resistance to apoptosis and increased metabolic activity (Khosla *et al.*, 2020; Lin *et al.*, 2020; Yu *et al.*, 2020).

Tbx5 had lower mRNA expression in both cells (MAFs and Feeders) infected with MGT comparing with MAFs infected GFP for the reasons already discussed in chapter 4 (Figure 6).

In 2010, for the first time it was directly reprogrammed mouse cardiac fibroblasts and tail tip fibroblasts into beating CMs by overexpressing *Gata4*, *Mef2c*, and *Tbx5*. It seems that GMT are the "master regulators" for direct cardiac reprogramming. However, in this first study, the efficiency of reprogramming was low, which increased the necessity of other strategies to enhance the efficiency of iCMs generation (Talkhabi, Razavi and Salari, 2017).

One of the main goals of this dissertation/experiments was to understand which of the previously selected lncRNAs are the best candidates for modulation/knocking down in order to increase direct reprogramming efficiency. qPCR analysis shown that MGT infected cells (MAFs and Feeders) had larger expression of the lncRNAs that the MAFs infected with GFP. *Mir22hg* it was elected as the best candidate to the knockdown since it was the lncRNA candidate that shown the most solid results using both *ACTC1* and *GAPDH* as housekeeping references (Figures 7 and 14).

Comparing both infected MGT- MAFs and Feeders, it was observed that *Gm 15856* and *Gm 28592* had a large expression difference amongst cells infected with MGT which lead to the suspicion that are not the best candidates to the knockdown. *Gm 27028* expression analysis was inconclusive using *GAPDH* as the housekeeping gene, making it is necessary to repeat in the near future. We used *Phlda1* and *Bvht* worked as positive controls, and its results allowed to

guarantee that *Gm 28592* amplification was not being mistaken by its antisense gene *Phlda1*, and *Bvht* being a known lncRNA that is highly expressed in the heart demonstrated what the level of mRNA expression detected was the expected for lncRNAs (Figures 7 and 14).

Human cells are more resistant to the reprogramming process therefore, it is reasonable to speculate that additional regulatory events are necessary to propel human cells toward alternative cell fates. Others have shown infection of human fibroblasts with GMT only present of 3.45% of tropomyosin positive cells. (Nam *et al.*, 2013). In our experiments Gm 03348 cells infected with MGT presented a percentage of 10.77% GFP positive cells by FACS (Figure 9).

Over the last years studies reported that modification of reprogramming factors, manipulating signalling pathways, or the use of defined culture conditions promoted cardiac reprogramming in mouse and human fibroblasts (Miyamoto *et al.*, 2018). It is known that GMT that reprogrammed iCMs from mouse fibroblasts, are able to reprogram human fibroblasts into iCMs *in vitro*; nevertheless, inclusion of additional reprogramming factors resulted in increased successful reprogramming rates (Fu and Srivastava, 2015). These studies opened the possibility to modulate other variables in cardiac environment, including nutrient alterations, as a strategy to increase transdifferentiation efficiency.

In this regard, we decided to modulate the levels of energy supplying nutrients (including glucose, glutamine, and lipids) in the Gm 03348 cells growth medium. Flow cytometry analysis revealed that following MGT infection higher tropomyosin percentages were obtained with media rich in glucose instead of media with “No FBS” and “No glutamine”. From that, we can conclude that through the transdifferentiation process from fibroblasts to cardiomyocytes, cells preferably choose metabolism pathways involving glucose instead of FAs or glutamine (Figure 11). The GFP^+ with different media for Gm 03348 cells were: 2.04 % to “All high” medium, 1.12% to “Low glucose” medium, 0.9 % to “No FBS” medium and 0.27 % to “No glutamine” medium.

Proliferating cells often display enhanced uptake of glucose, providing an important source of carbon to support lipid production and the biosynthesis of nucleotides and non-essential amino acids, which ensues via redirection of metabolites of glycolysis and the TCA cycle. To replace TCA cycle intermediates that are used to produce biomass, cells use anaplerosis, and an important anaplerotic substrate is glutamine (Zhu and Thompson, 2019).

Additionally, as observed in tumours, cells obtain glucose from glycogenolysis of stored glycogen, but also from gluconeogenic mechanisms, using lactate and glutamine as alternative fuels to favour cell proliferation. Glutamine is the most abundant amino acid in the organism and is a pillar fuel for cancer cells that, similar to glucose, provides the energy generation, biomass and redox control (Afonso *et al.*, 2020). Based on these facts, it is expected that cells

grown in culture medium rich in glucose and glutamine had better proliferation/viability and therefore, increased retroviral infections rates.

Since both glucose and glutamine fuel pathways that are vital for cellular proliferation and survival, several enzymes that control these pathways can be considered attractive for therapeutic targets (Méndez-Lucas *et al.*, 2020), including for our experiments of direct cardiac reprogramming.

Fatty-acid utilization is energetically favourable for post-mitotic adult cardiomyocytes (Cardoso *et al.*, 2020) however, increased fatty-acid oxidation perpetuates dependence on fatty-acid utilization by inhibiting glucose oxidation via the TCA cycle, in which acetyl-coA generated from fatty-acid oxidation inhibits the mitochondrial enzyme PDH (Figure 4). Shortly after birth, cardiomyocytes exhibit a shift in energetic substrate utilization, from pyruvate to FAs. This postnatal metabolic shift coincides with increased DNA damage, expression of DNA-damage markers, and cell-cycle arrest of cardiomyocytes. The inhibition of fatty-acid utilization by cardiomyocyte mitochondria results in a marked shift in myocardial substrate utilization to glucose-derived pyruvate utilization and increased cardiomyocytes proliferation. This was accompanied by a significant decrease in DNA damage, both base oxidation and double-strand breaks (Cardoso *et al.*, 2020). In our experiments, cells cultured in "No FBS" medium had the second lower survival and transdifferentiation rates (Figure 11). FAs depletion is important for promoting glucose utilization and cardiomyocytes proliferation, this is in accordance with our data since glucose richer media are more beneficial for direct reprogramming from cardiac fibroblasts.

During cellular viability test using the CCK-8 it was visible in both cells densities (3000 cells and 5000 cells) (Figures 12 and 15) plated that the survival rate was bigger in cells plated in "Low glucose" medium. On the other hand, "All high" medium had the lower survival rate during both platting. Since "All high" had similar composition with the "Low glucose" medium, it was expected similar results. Surprisingly, "No FBS" medium had the second-best survival rate, which leads to the conclusion already made that high levels of glucose enhance survival and proliferation in cells. "No glutamine" medium shown poorest cell viability results suggesting that glutamine as well as glucose is crucial for cellular proliferation and survival (Afonso *et al.*, 2020; Méndez-Lucas *et al.*, 2020).

To induce further cardiac maturation in direct cardiac reprogramming of human fibroblasts, conditioned media from murine cardiomyocytes was used according to Wada *et. al.* In Wada *et. al*. work, after one week of transduction, cells were re-plated onto neonatal rat cardiomyocytes and expression of cardiac markers, such as α-actinin and cardiac troponin was observed in the transdifferentiate cells. After seven days of co-culture, 5% of the transduced

cells contracted synchronously with surrounding cardiomyocytes; nevertheless, conditioned media from rat cardiomyocytes did not induce spontaneous contraction in the transduced cells. Compared with mouse iCMs, human iCMs require coculture with murine cardiomyocytes to differentiate into beating cardiomyocytes (Wada, Muraoka, Inagawa, Yamakawa, Miyamoto, Sadahiro, *et al.*, 2013).

In our experiments, 10% of conditioned media from HL-1 cells (Claycomb medium) was used as stimulus to increase the transdifferentiation rate instead of coculture with murine cardiomyocytes. However, different results were obtained, in fact the percentage number of cells expressing tropomyosin after this process was lower comparing to the cells just infected with MGT [Figure 11 g) and h)]. These surprising results could be due to the fact that Claycomb is recommended for mice cardiomyocytes culture and here it was used in human cells or some specific factor presenting the medium composition that decreased the transdifferentiation rate in Gm 03348 cells.

To have a perception, which is the level of tropomyosin expressed by mice cardiomyocytes, we analysed its expression in HL-1 cells by FACS (Figure 6). As expected, the percentage of HL-1 cells expressing tropomyosin was considerable high (Jackaman *et al.*, 2007; Jagatheesan, Rajan and Wieczorek, 2010). Although relevant, it is important to mention that in these experiments these cells were not the perfect positive control since we are using human Gm 03348 fibroblasts and it would have been important to use human cardiomyocytes as positive control.

For the Wi-38 cells flow cytometry analysis, revealed even higher tropomyosin expression compared to HL-1 (Figure 10), such results were not expected since Wi-38 cells are embryonic cells and HL-1 are cardiomyocytes. In the future, it will be necessary to perform a negative control of these cells, i.e. Wi-38 with no antibody, to understand if some tropomyosin expression is due from autofluorescence.

The major challenge in the field is to achieve direct cardiac reprogramming *in vivo*, studies shown that gene transfer of retroviral GMT or GMT plus *Hand2*, or lentiviral microRNAs into mouse infarct hearts reprogrammed resident CFs into iCMs *in vivo*, improved cardiac function and reduced fibrosis after MI (Miyamoto *et al.*, 2018). Nevertheless, all these iCMs were generated using integrating retroviruses or lentiviruses, which could disrupt endogenous gene expression and are associated with the risk of insertional mutagenesis. Moreover, the process of the induction of functional iCMs *in vitro* with these vectors is slow and of low efficiency, which is an obstacle to the research of the mechanisms of cardiac reprogramming and progress to its clinical applications (Miyamoto *et al.*, 2018).

Thus, development of an integration-free cardiac reprogramming method that is appropriate for both *in vitro* and *in vivo* applications with high efficiency would be ideal to the benefit of clinical translation and basic biology (Miyamoto *et al.*, 2018). The fact that fibroblasts are derived from different origins, even in the same organ such as heart is an obstacle to the efficiency of direct cardiac reprogramming (Talkhabi, Razavi and Salari, 2017).

Direct cardiac reprogramming is currently far from cardiac cell-based therapy. Improving the efficiency and maturity is not enough, and for producing a large number of CMs lost after a MI, 2D culture systems must become 3D culture systems. Therefore, finding novel transcriptional activators that increase the efficiency and maturity of iCMs in 2D culture systems could pave the way for producing a large number of iCMs required in cell-based therapy (Talkhabi, Razavi and Salari, 2017). In sum, despite using different strategies and protocols and the considerable effort spent in identifying new TFs for increasing the efficiency and functionality of generated iCMs, the efficiency and functional properties of the generated cells are still not adequate to be used in clinical trials (Rastegar-Pouyani *et al.*, 2017). LncRNAs and nutrient experiments are relevant for the reason that they can bring us new clues to the development of this therapeutic strategy.

In sum, our study provides considerable information for a current challenge in the field, in which the efficiency of the direct conversion of mouse and human fibroblasts into iCMs. It is important to mention that cardiac fibroblasts are heterogeneous, which raises the question of whether we can find a subpopulation of cardiac fibroblasts that is favourable for direct cardiac reprogramming. In addition, we still do not have an appropriate culture method to facilitate or maintain the maturation of adult CMs *in vitro*. Therefore, we need to identify the optimal conditions for cell culture to enhance *in vitro* cardiac reprogramming (Fu and Srivastava, 2015; Miyamoto *et al.*, 2018).

CHAPTER 6 - CONCLUSIONS AND FUTURE PERPECTIVES

The heart has a limited self-renewing capacity, including after injury, establishment of a strategy for heart regeneration has been desired. Direct cardiac reprogramming has great potential to become one of the main therapies of regenerative medicine in heart failure. During the experimental work of this dissertation, some advances were made in mouse and human systems indicating that cardiac reprogramming efficiency can be improved by various strategies, as lncRNAs silencing or metabolism manipulation, and might eventually become powerful enough for clinical application.

MGT seems to be the master regulator of rodent cardiac reprogramming *in vitro*. However, as documented in the first chapter, other TF combinations can also direct cardiac reprogram successfully, both in mice and human cells. Thus, it is important to consider in the future that other factors such as growth factors and epigenetic regulating factors can assist MGT in this regard. Nevertheless, it seems that MGT must be included in the final combination for cardiac reprogramming in different species. With our experimental work we shown that is possibly to transdifferentiate with MGT retrovirus murine fibroblasts of different ages and human fibroblasts. In addition, it was observed that the chosen lncRNAs had an increased expression in MAFs and Feeders after the transduction process.

Another fascinating feature of direct cellular reprogramming in most cell types is the progressive, yet rapid, alteration of cellular phenotype, genome-wide epigenetic and transcriptional changes occur to establish the necessary landscape for a new cell type without progression through a progenitor state, this is a huge advantage against iPSCs, due to the fact that this last ones have a bigger probability of tumour development.

Our studies of cellullar metabolism, through the nutrient manipulation in media, elucidate us that cells are more viable in glucose rich media. This finding leads us to the conclusion that metabolism manipulation can be a strategy to enhance direct cardiac reprogramming efficiency.

Mice exhibit significant cardiovascular differences compared to humans. Besides the differences such as small size and short lifespan, mouse differ from humans in various of anatomical, physiological, energetic, electrophysical, and mechanical features that include heart rate, coronary artery structure, and contraction/relaxation kinetics. Although significant progress has been achieved in direct cardiac reprogramming in mice, research in reprogramming human cells lags far behind, reprogramming human fibroblasts requires the addition of extra factors. Spontaneously beating cells are rare, suggesting that is necessary more work to translate findings from the mouse to human and uncover undiscovered molecular barriers in human reprogramming.

In the future additional research is necessary to translate direct cardiac reprogramming into a clinical therapy. Required steps include continued basic research, research in large animal models, improvement in human reprogramming, and bioengineering of delivery mechanisms. Also, it is required a better understanding of the mechanism of late stage reprogramming events and iCMs maturation.

One intriguing area worth describing is iCM maturation. As mentioned before, experiments of direct cardiac reprogramming were made using co-culture of mice cardiomyocytes with the intention of potential beneficial effects of environmental cues like extra-cellular matrix, signalling pathways and mechanical or electrical stimulation. In our case we tried to give an extra stimulus with Claycomb medium used previously in HL-1, in order to enhance reprogramming. Our results were not satisfactory, therefore it is important to explore ways of cellular stimuli.

The continuous findings of the regulatory functions of lncRNAs in diverse cellular processes will lead to improvements on the understanding of cardiac homeostasis and disease and will possibly provide us with additional therapeutic targets and a more sophisticated tools for cellular transdifferentiation approaches in heart regeneration. Compared to other classes of ncRNAs, lncRNAs demonstrate a surprisingly wide range of sizes, shapes, and functions. These characteristics have endowed them with previously underappreciated functional potentials. LncRNAs have various roles in all aspects of gene expression by different mechanisms of action. These versatile functions of lncRNAs are dependent of their subcellular localization and the adoption of specific structural modules with interacting partners, a process that may undergo dynamic changes in response to local environments in cells, based on these facts it is important to keep exploring the role of lncRNAs during the transdifferentiation process in order to improve direct cardiac reprogram efficiency.

CAHPTER 7 - ANNEXES

Annex 1

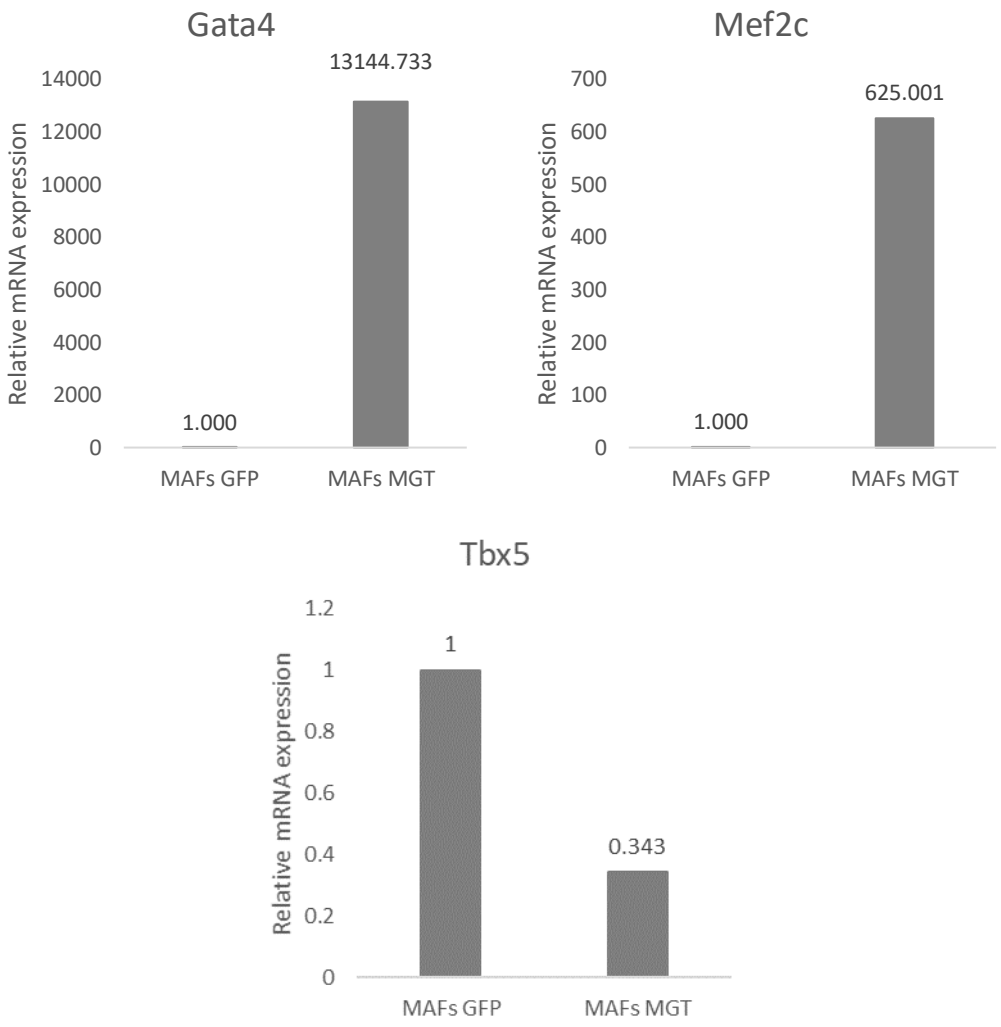


Figure 13. MGT expression in MAFs infected with MGT comparing with MAFs infected with GFP using the *ACTC1* as housekeeping gene. The calculus was made based in *ACTC1* expression. Data refers to n=2 from 1 independent experiment.

Annex 2

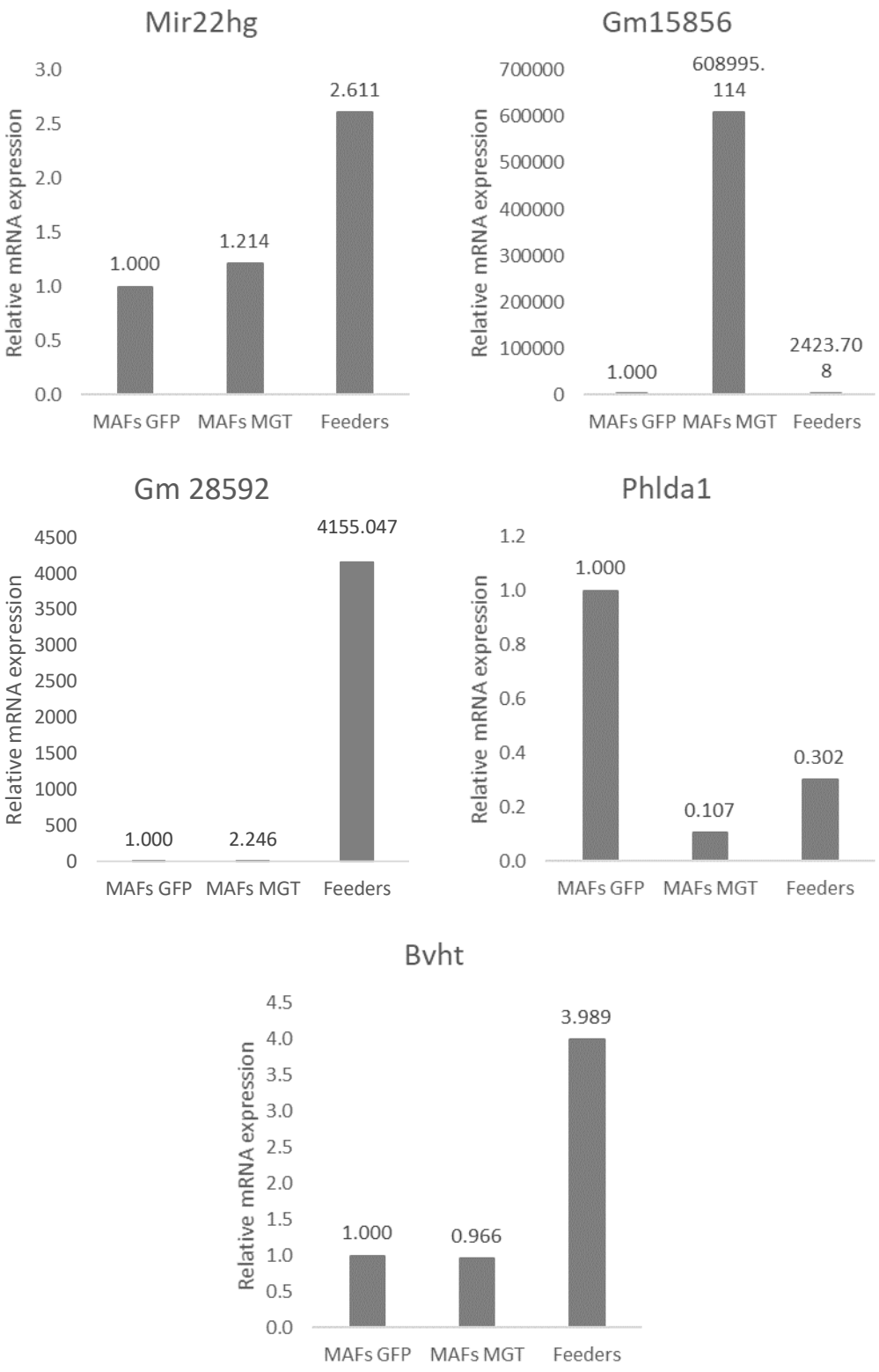


Figure 14. lncRNAs expression in MAFs infected with GFP, MAFs infected with MGT and Feeders infected with MGT using the *GAPDH* as housekeeping gene. Data refers to n=2 from 1 independent experiment.

Annex 3

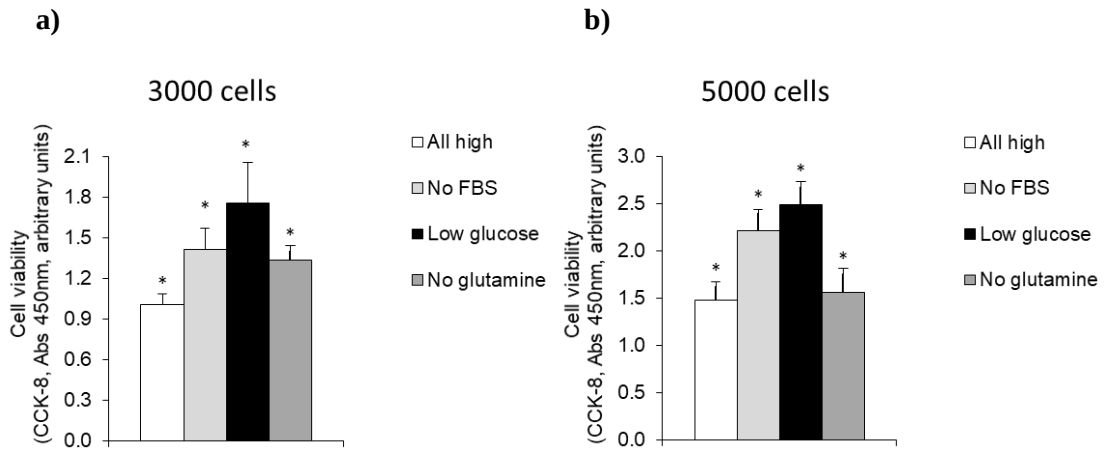


Figure 15. Cellular viability of Gm 03348 cells [a) 3000 cells per well and b) 5000 cells per well] measured by CCK-8 absorbance at 450 nm detected in a Microplate Tecan 2000 reader, with n=6 for each medium. Data are presented as mean ± SD. Statistical significance was determined by two-tailed Student t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Annex 4

To Be Published in: Article collection *Non-coding RNA as Therapeutic Target: A Game Changer in Cardiac Regenerative Strategies?* Frontiers in Physiology - Clinical and Translational Physiology.

Age-related pathways in cardiac regeneration: a role for lncRNAs?

Francisco Santos [1*], Magda Correia [1*], Sandrina Nóbrega-Pereira [1,2#], and Bruno Bernardes de Jesus [1#]

[1] Department of Medical Sciences and Institute of Biomedicine - iBiMED, University of Aveiro, 3810-193 Aveiro, Portugal

[2] Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Av. Professor Egas Moniz, 1649-028 Lisboa, Portugal

Corresponding authors: Department of Medical Sciences and Institute of Biomedicine - iBiMED, University of Aveiro, 3810-193 Aveiro , Portugal, Tel: +(351) 234 370 213
E-mail addresses: sandrina.pereira@ua.pt (S. NóbregaPereira) and brunob.jesus@ua.pt (B. Bernardes de Jesus)

Running title: Understanding the characteristics of neonatal heart regenerative potential

Abstract

Aging imposes a barrier for tissue regeneration. In the heart, aging leads to a severe rearrangement of the cardiac structure and function and to a subsequent increased risk of heart failure. An intricate network of distinct pathways contributes to age-related alterations during healthy heart aging and account for the higher susceptibility of heart disease. Our understanding of the systemic aging process has already leaded to the design of anti-aging strategies or to the adoption of protective interventions such has the cardioprotective role of exercise. Still, our understanding of the molecular determinants operating during cardiac aging or repair remains limited. Here, we will recapitulate the molecular and physiological alterations operating during heart aging, highlighting the potential role for long non- coding RNAs (lncRNAs) as novel and valuable targets in cardiac regeneration/repair.

Introduction

Heart failure is a devastating disease leading to millions of deaths worldwide (Yusuf et al., 2001; Lloyd-Jones et al., 2009; Lloyd-Jones et al., 2010). Aging is probably the highest risk factor for heart failure (Li et al., 2020a). Indeed, heart failure is still the major cause of death in the elderly in industrialized countries. Contrary to the neonatal heart, adult mammalian hearts lost the capacity to fully regenerate after an exogenous or endogenous harm (Lam and Sadek, 2018). This may be mediated through several interconnected processes including cellular senescence and secreted factors, telomere attrition, mitochondrial damage, cell death or inflammation (for a comprehensive review on age-related pathways affecting heart see (Li et al., 2020a)). Although a partial myocyte turnover has been observed in adult heart after damage (e.g. myocardial infarct), it only partially and slightly restores heart function. Understand what distinguishes neonatal to adult hearts or, in other words, understand the functional alterations occurring during the early days of neonatal heart development to adult will permit to potentially design novel strategies targeted to the adult heart. For instance, it has been recently demonstrated that manipulation of telomere length through the expression of telomerase, which expression is silenced in the mice heart from day 5 to 7 (Blasco et al., 1995;Borges and Liew, 1997;Richardson et al., 2012), may prove beneficial in heart healing and healthspan (Bernardes de Jesus and Blasco, 2011;Bar et al., 2014). Here, we will challenge the cardiac regeneration properties of neonatal versus adult hearts in the light of their intrinsic and distinct properties. Namely, we will discourse their different expression profiles paying particular attention to the role of non-coding transcripts in particular long non-coding RNAs (lncRNAs). LncRNAs have been associated with several biological processes, including chromosome dosage compensation, genomic imprinting, epigenetic regulation, aging, and cell differentiation (Mercer et al., 2009; Rinn and Chang, 2012; Sousa-Franco et al., 2019). Additionally, we will summarize different approaches aimed at converting the identity of heart cells and their effectiveness in healing a damaged heart.

Cardiac Regeneration in Neonatal and Adult Hearts

There is a general consensus on the capacity of neonatal hearts to regenerate, after distinct types of damage (**Figure 1** - (Porrello et al., 2011;Haubner et al., 2012;Jesty et al., 2012;Mahmoud et al., 2013;Porrello et al., 2013;Rubin et al., 2013;Andersen et al., 2014;Aurora et al., 2014;Mahmoud et al., 2014;Sadek et al., 2014;Bryant et al., 2015;Darehzereshki et al., 2015;Han et al., 2015;Jiang et al., 2015;Konfino et al., 2015;Mahmoud et al., 2015;Aix et al., 2016;Andersen et al., 2016;Blom et al., 2016;Haubner et al., 2016;Kang et al., 2016;Tao et al., 2016;Valiente-Alandi et al., 2016;Xiong and Hou, 2016;Yu et al., 2016;Ai et al., 2017;Bassat et al., 2017;Malek Mohammadi et al., 2017;Zebrowski et al., 2017;Ahmed et al., 2018;Ingason et al., 2018;Sampaio-Pinto et al., 2018;Sereti et al., 2018;Cai et al., 2019;Elhelaly et al., 2019;Wang et al., 2019b;Fan et al., 2020;Li et al., 2020b;Li et al., 2020c;Pei et al., 2020)). Interestingly, the repair of some types of injuries (e.g. apical resection) may be addressed differentially from other lesions (cryoinjury or ischemia). A comprehensive overview of neonatal heart regeneration studies has been previously and elegantly detailed by Nicholas Lam et al. (Lam and Sadek, 2018). Neonatal heart regeneration seems to be mediated through a concerted action of the proliferation of the cardiomyocytes, something lost during heart development (Eschenhagen et al., 2017), and the extension and characteristics of the injury. This occurs in an extremely short time window (less than 10 days), where the cardiomyocytes loss their dividing properties, and rewire several of their characteristics including their metabolic needs. This implies huge alterations at the level of transcription networks, including coding and non-coding genes. It is accepted that the division of pre-existing cardiomyocytes is fuelling cardiac regeneration, rather than mobilization of circulation or resident stem and progenitor cells (Valiente-Alandi et al., 2016;Lam and Sadek, 2018). Indeed, several novel strategies designed for adult heart healing address the death of mouse tissues through forcing the pre-existing cardiomyocytes to re-enter cell cycle, to convert other cell types of the heart (e.g. cardiac fibroblasts – addressed in the next chapter) in dividing cardiomyocytes, or to cell replacement therapies through the expansion of cardiomyocytes in vitro (Qian et al., 2012;Addis and Epstein, 2013;Nam et al., 2013;Wada et al., 2013;Ghiroldi et al., 2017;Amin et al., 2018;Engel and Ardehali, 2018).

Reprogramming the Heart

Cellular reprogramming has upsurge as a novel strategy allowing the conversion of fully determined cells in cells with the potency to be differentiated in novel tissues,

including tissues from different development lineages (Takahashi and Yamanaka, 2006;Yamanaka, 2009;Abad et al., 2013). Variables of the process have been adapted to different needs, overpassing limitations and safety concerns of the original protocol. Namely, direct conversion between two distinct cell types has been widely explored and advocated has a safer and applicable strategy for adult tissue dysfunction. Additionally, many of the reprogramming barriers such as the obstacles imposed by aging have been, for instance, addressed through the direct manipulation of the tumour suppressor genes, p53 or Ink4a/ARF (Li et al., 2009;Marion et al., 2009) or the EMT factor ZEB2 (De Jesus et al., 2018;Santos et al., 2019). Here, the lncRNA Zeb2-NAT which controls the levels of Zeb2 (7) and facilitate cellular reprogramming of aged tissues, may have a potential role in cardiac remodelling. Cardiac fibroblast to myofibroblast phenoconversion is a critical step during cardiac fibrosis (Czubryt, 2019). Zeb2 increases with aging and plays a crucial role by repressing Meox2 leading to the upregulation of key myofibroblast markers (Cunnington et al., 2014). Targeting of Zeb2-NAT may prove beneficial as an anti-fibrotic target.

As previously stated, several strategies have been adapted to the heart. Here, for the sake of clarity, we will singly discuss the reprogramming into functional cardiomyocytes. Mouse postnatal cardiac or dermal fibroblasts can be transdifferentiated into functional beating cardiomyocytes through the combined expression of three different transcription factors, *Gata4, Mef2c,* and *Tbx5* (GMT). GMT activates a cardiac-like gene expression program and promotes cardiomyocyte differentiation (Ieda et al., 2010;Qian et al., 2012). Compensation of the GMT cocktail with *Hand2* showed to enhance direct cardiac reprogramming of mouse cells, being however inefficient for human samples (Sahara et al., 2015).

Remarkably, this approach has been adapted *in vivo* where cardiac fibroblasts have been transdifferentiated into induced cardiomyocytes (iCMs) (Song et al., 2012;Zhang et al., 2019a;Zhang et al., 2019b) bypassing the need to dedifferentiate to a stem cell state (Liu et al., 2017;Muraoka et al., 2019).

Direct cardiac reprogramming of fibroblasts into iCMs has emerged as an attractive strategy. Alternative sets of reprogramming factors based on different TFs, microRNAs, chemical compounds, epigenetic modifications, defined culture conditions, and small molecules, have been studied in order to promote cardiac reprogramming (Hashimoto et al., 2018;Hashimoto et al., 2019;Muraoka et al., 2019;Testa et al., 2020). Comparative gene expression analyses reported that iCMs induced *in vitro* exhibited bona-fide adult cardiomyocyte-like features, (e.g. such as fatty-acids (FAs) oxidation or cell-cycle exit), surpassing the characteristics of induced pluripotent stem cell (iPSC)-derived cardiomyocytes (Muraoka et al., 2019). Although silencing the

fibroblast program is a prerequisite for cardiac reprogramming, the molecular mechanisms underlying this process remain unknown. For instance, the desirable conversion of aged adult fibroblasts into different cell lineages is still limited through defined and undefined age-related barriers (Price et al., 2014;Muraoka et al., 2019). Even considering the low efficiency of the transdifferentiation process, new cardiomyocytes reprogrammed from endogenous cardiac fibroblasts enhanced cardiac function after MI (Wada et al., 2013) fully demonstrating their promise for adult-heart repair. Numerous approaches have been applied to improve cardiac reprogramming efficiency. A strategy passes through the partial reprogramming of the original cells into cardiac progenitors, bypassing pluripotency. Forced expression of a combination of five TFs: *Mesp1, Gata4, Tbx5, Nkx2-5,* and *Baf60c* reprogrammed murine fibroblasts into an expandable multipotent cardiac progenitor cell population, with potential to be transplanted into murine hearts after MI, and enhancing survival (Hashimoto et al., 2018). One week after GMT expression *in vivo*, about 5% of mice fibroblasts expressed α-MHC and cardiac Troponin T (cTnT). Additionally, GMT expression following coronary ligation lead to approximately 10% of α-Actinin^{+} iCMs from cardiac fibroblasts in the infarcted region. In order to improve cardiac reprogramming efficiency other TFs (e.g. *MESP1, MyoD, Baf60c* and *Hand2*), combination of miRNAs (e.g. miR-1, miR-133, miR-208, and miR-499) and chemical inhibitors (e.g. SB431542, and XAV939) have been placed along with GMT transduction (Qian et al., 2012;Chang et al., 2019). Addition of *Hand2* can increase the efficiency about 28% after three weeks in mouse MI models. It is important to mention, however, that the safety issues associated with the use of lentivirus and/or retrovirus is a barrier for the potential translational aspect of this application. Non-viral reprogramming systems such as the use of cationic gold nanoparticles (AuNPs) have emerged as a promising platform for gene and drug delivery due to their easy preparation and inertness (Chang et al., 2019).

Because human cells are more resistant to the reprogramming process, it is reasonable to speculate that additional regulatory events are required to propel human cells toward alternative cell fates (Fu et al., 2013;Nam et al., 2013). GHM or GHMT factors alone seem insufficient for the reprogramming of human fibroblasts. Additional factors such as myocardin, *MESP1*, oestrogen-related receptor-γ (*ESRRγ*), zinc-finger protein *ZFPM2*, or miR-1 / miR-133 are needed to successfully induce the conversion of human fibroblasts towards a cardiac fate
(Ghiroldi et al., 2017;Hashimoto et al., 2018).

A role for lncRNAs in heart regeneration

The importance of lncRNAs in heart regeneration has shed some light recently (Bar et al., 2016). LncRNAs are a vast category of non-coding, poorly conserved and tissue- and developmental stage-specific transcripts with distinct functions in several biological processes, including epigenetic, transcriptional and posttranscriptional regulation. Regarding the role of lncRNAs in heart regeneration we will discuss some recent studies describing lncRNAs directly acting (facilitating or inhibiting) on heart regeneration. In 2018, Cai and colleagues explored the role of lncRNAs during heart regeneration after ischemic injury, in both neonatal and adult mice (Cai et al., 2018). *CAREL*, a lncRNA whose expression gradually increased in the neonatal hearts from P1 to P10 mice, with P7 corresponding to the timepoint at which the heart regenerative capacity is lost in mice (Cai et al., 2018). Cardiac-specific overexpression of *CAREL* led to a decrease of cardiomyocyte proliferation and reduced heart regeneration in neonatal mice after injury. On the contrary, silencing *CAREL* promoted cardiac regeneration and improved heart functional parameters after myocardial infarction in neonatal and adult mice (Cai et al., 2018). *CAREL* was found to be an endogenously competing RNA (ceRNA), sequestering miR-296. It was suggested that the *CAREL-miR-296* interaction led to the activation of Trp53inp1 and Itm2a, leading to a decrease in cardiomyocyte proliferation, thus resulting in a reduction of regeneration. Intramyocardial administration of *CAREL* to p1 neonatal mice inhibited cardiomyocyte mitosis and increased the formation of cardiac scar and, on the other hand, overexpression of miR-256 promoted cardiomyocyte proliferation and cardiac regeneration after injury. Similarly to CAREL, the lncRNA CPR (cardiomyocyte proliferation regulator) was shown to be a negative regulator of cardiomyocyte proliferation and cardiac repair. Ponnusamy and colleagues observed that higher levels of CPR hampered cardiomyocyte proliferation, whilst silencing CPR resulted in cardiomyocyte proliferation in postnatal and adult hearts (Ponnusamy et al., 2019). CPR expression levels were found to be higher in the adult heart, which is consistent with their lack of regeneration. The authors reported that CPR recruits DNMT3A to several locus leading, in particular, to increased levels of methylation in the MCM3 promoter (Ponnusamy et al., 2019). In dividing tissues, MCM3 promotes the initiation of DNA replication and cell cycle progression (Lin et al., 2008), something halted by CPR in the heart and leading to the inhibition of cardiomyocytes proliferation. Another lncRNA that appears to be involved in cardiac regeneration is NR_045363, whose expression correlates with the regenerative capacity of mice. P7 mice subjected to LAD ligation and injected with adenovirus containing NR_045363 exhibited improved left ventricular ejection fraction and reduced infarct size compared to the control-

injected group (Wang et al., 2019a). Mice overexpressing NR_045363 showed higher expression of cardiomyocyte mitotic markers, such as Ki67 and phosphorylated histone H3 (pH3), suggesting that improved heart function after MI was due to cardiomyocyte proliferation. The authors reported that NR_045363 acted as a ceRNA, binding to miR-216a (Wang et al., 2019a). miR-216a is known to repress JAK2, leading to decreased levels of phosphorylation of STAT3 (Hou et al., 2015). Furthermore, deletion of STAT3 was shown to impair cardiomyocyte proliferation after apical resection (Kurdi et al., 2018), suggesting that NR_045363 promoted cardiomyocyte proliferation by modulating the JAK2-STAT3 pathway. So, the absence of NR_045363 (which results in an upregulation of miR216a) led to reduced activity of the JAK2-STAT3, whilst NR_045363 overexpression (which leads to a downregulation of miR-216a) resulted in an increase of the phosphorylation levels of JAK2 and STAT3, thus promoting cardiomyocyte proliferation (Wang et al., 2019a). More recently, NR_045363 was associated with cardiomyocyte apoptosis. Chen and colleagues reported that loss of NR_045363 led to activation of the p53 signaling pathway, promoting apoptosis. On the other hand, overexpressing NR_045363 inhibited apoptosis and improved cardiac function after MI (Chen et al., 2020), thus potentially mediating the cardiac functions observed after NR_045363 modulation. Another lncRNA with a possible role in modulation cardiac regeneration is LncDACH1. This lncRNA was found to be gradually upregulated in postnatal hearts, which is in accordance with the loss of myocardial regenerative capacity soon after birth (Cai et al., 2020). Cardiac-specific overexpression of LncDACH1 resulted in the suppression of neonatal heart regeneration and aggravation of cardiac function after apical resection. These phenotypes were accompanied by a decrease in the number of cardiac-cells expressing proliferative markers (Cai et al., 2020). LncRNA ECRAR (endogenous cardiac regeneration-associated regulator) was found to be upregulated in the fetal heart, and its expression gradually decreased in postnatal hearts. Overexpression of ECRAR in postnatal rat cardiomyocytes, both *in vitro* and *in vivo*, resulted in an increase of DNA synthesis, and an increase of cytokinesis (pH3 and aurora B kinase), suggesting a direct involvement in cardiomyocytes proliferation (Chen et al., 2019). Overexpression of ECRAR resulted in the phosphorylation of ERK1/2, their subsequent translocation to the nucleus and the transcription of cell proliferation and cell cycle-related genes (Chen et al., 2019). Li and colleagues identified Sirt1 antisense lncRNA (Sirt1-as), whose expression was high during heart development. Overexpression of this lncRNA resulted in an increase of Ki67- and pH3-positive cardiomyocytes. On the other hand, silencing of Sirt1-as, both *in vitro* and *in vivo*, led to a decrease of Ki67- and pH3-positive cardiomyocytes, indicating a potential decline

in cell division (Li et al., 2018a). Furthermore, overexpression of Sirt1-as after MI in adult mice resulted in an increased expression of cell-cycle specific factors Ki67 and pH3, thus suggesting a potential implication in cardiac health (Li et al., 2018a). Cardiomyocyte regeneration-related lncRNA (CRRL) was also found to play a role in heart regeneration. CRRL silencing was associated with an increased expression of EdU, Ki67 and pH3 in P1 and P7 rat cardiomyocytes (Chen et al., 2018). Similar results were obtained in neonatal rats post-MI, concomitantly with better prognosis such as reduction of the fibrotic length of the infarct wall and fibrosis area in the non-infarct zone. Instead, overexpression of CRRL leads to a decrease in pH3-positive cardiomyocytes and inhibition of functional recovery postMI. Similarly to other lncRNAs, CRRL function seemed to be mediated through the binding to miR-199a-3p resulting in an increased expression of Hopx, which is a negative regulator of cardiomyocyte proliferation (Trivedi et al., 2010). LncRNA AZIN2-sv, a splice variant of the AZIN2 gene, was found to be upregulated in human adult hearts. AZIN2-sv was reported to negatively regulate cardiomyocyte proliferation, both *in vitro* and *in vivo (Li et al., 2018b)*. Overexpression of AZIN2-sv led to an anti-proliferative phenotype, marked by decreased levels of EdU-, Ki67-, pH3- and Aurora-B. On the other hand, silencing AZIN2-sv promoted cardiomyocyte proliferation and improved cardiac function after MI. AZIN2-sv sequesters miR-214, leading to the release of its target PTEN, resulting in a decrease in the phosphorylation of Akt and Cyclin-D, therefore inhibiting cardiomyocyte proliferation. Reduced levels of AZIN2-sv allow miR-214 to repress PTEN, leading to increased levels of phosphorylated Akt and Cyclin-D, thus promoting cardiomyocyte proliferation. More recently, Trembinski and colleagues identified lncRNA *SARRAH* (SCOT1-antisense RNA regulated during aging in the heart), whose expression declines during aging. Inhibition of *Sarrah* induces caspase activity in mouse and human cardiomyocytes, promoting apoptosis. Gene set enrichment analysis after *SARRAH* silencing, showed an enrichment of apoptosis related pathways, corroborating previous observations (Trembinski et al., 2020). *SARRAH* was also found to directly bind to the promoters through RNADNA triplex helix structures, suggesting that its binding may activate gene expression. Indeed, it was reported that *SARRAH* interacted with CRIP2 (cardiac transcription factor cysteine-rich protein 2) and p300, which acetylates histone H3 lysine 27 to activate transcription (Trembinski et al., 2020). On the contrary, overexpression of SARRAH led to a decrease in caspase activity. In adult mice a decline in apoptosis was observed after overexpressing SARRAH, suggesting that reduced expression levels of this lncRNA in aged mice might contribute to cardiomyocyte cell death *in vivo*. Furthermore, reduced levels of Sarrah were observed in the infarcted and border

regions after acute MI (Trembinski et al., 2020). Other lncRNAs involved with aged heart include the lncRNA H19 (downregulated in aged or ischemic heart (Hofmann et al., 2019)); MALAT1 a lncRNA which is, himself, regulated by an antisense lncRNA transcript (TALAM1) (Zong et al., 2016), was also shown to be decreased in aged hearts (Bink et al., 2019;Gomes et al., 2019), and this decrease was shown to be involved in cardiac dysfunction (Zhu et al., 2019;Li et al., 2020a).

Conclusions

As previously discussed several lncRNAs are deregulated during the development of the heart or during heart pathologies. LncRNA targeting may be a novel strategy against heart diseases (Bar et al., 2016). Technically, the development of specific and deliverable antisense transcripts (e.g. LNA-GapmeRs) has been proved powerful and efficient carriers for in vivo targeting and RNase H-mediated degradation of specific targets (Bernardes de Jesus et al., 2018). Similar approaches may be designed for expression of selected lncRNAs, down-regulated in cardiac diseases. In conclusion, lncRNAs are critical regulators of heart health and disease. Understand their specific profiles in dividing versus non-dividing cardiomyocytes may allow the detection of potentially druggable targets for adult heart repair.

Funding: This work was supported by Fundação para a Ciência e Tecnologia (FCT) (ERA-CVD 2018 / 3599-PPCDT - ERA-CVD/0001/2018 - INNOVATION)

Conflicts of Interest: The authors declare no conflict of interest.

References

Abad, M., Mosteiro, L., Pantoja, C., Canamero, M., Rayon, T., Ors, I., Grana, O., Megias, D., Dominguez, O., Martinez, D., Manzanares, M., Ortega, S., and Serrano, M. (2013). Reprogramming in vivo produces teratomas and iPS cells with totipotency features. *Nature* 502, 340-345.

Addis, R.C., and Epstein, J.A. (2013). Induced regeneration--the progress and promise of direct reprogramming for heart repair. *Nat Med* 19, 829-836.

Ahmed, A., Wang, T., and Delgado-Olguin, P. (2018). Ezh2 is not required for cardiac regeneration in neonatal mice. *PLoS One* 13, e0192238.

Ai, S., Yu, X., Li, Y., Peng, Y., Li, C., Yue, Y., Tao, G., Pu, W.T., and He, A. (2017). Divergent Requirements for EZH1 in Heart Development Versus Regeneration. *Circ Res* 121, 106-112.

Aix, E., Gutierrez-Gutierrez, O., Sanchez-Ferrer, C., Aguado, T., and Flores, I. (2016). Postnatal telomere dysfunction induces cardiomyocyte cell-cycle arrest through p21 activation. *J Cell Biol* 213, 571-583.

Amin, M., Kushida, Y., Wakao, S., Kitada, M., Tatsumi, K., and Dezawa, M. (2018). Cardiotrophic Growth Factor-Driven Induction of Human Muse Cells Into Cardiomyocyte-Like Phenotype. *Cell Transplant* 27, 285-298.

Andersen, D.C., Ganesalingam, S., Jensen, C.H., and Sheikh, S.P. (2014). Do neonatal mouse hearts regenerate following heart apex resection? *Stem Cell Reports* 2, 406-413.

Andersen, D.C., Jensen, C.H., Baun, C., Hvidsten, S., Zebrowski, D.C., Engel, F.B., and Sheikh, S.P. (2016). Persistent scarring and dilated cardiomyopathy suggest incomplete regeneration of the apex resected neonatal mouse myocardium--A 180 days follow up study. *J Mol Cell Cardiol* 90, 47-52.

Aurora, A.B., Porrello, E.R., Tan, W., Mahmoud, A.I., Hill, J.A., Bassel-Duby, R., Sadek, H.A., and Olson, E.N. (2014). Macrophages are required for neonatal heart regeneration. *J Clin Invest* 124, 1382-1392.

Bar, C., Bernardes De Jesus, B., Serrano, R., Tejera, A., Ayuso, E., Jimenez, V., Formentini, I., Bobadilla, M., Mizrahi, J., De Martino, A., Gomez, G., Pisano, D., Mulero, F., Wollert, K.C., Bosch, F., and Blasco, M.A. (2014). Telomerase expression confers cardioprotection in the adult mouse heart after acute myocardial infarction. *Nat Commun* 5, 5863.

Bar, C., Chatterjee, S., and Thum, T. (2016). Long Noncoding RNAs in Cardiovascular Pathology, Diagnosis, and Therapy. *Circulation* 134, 14841499.

Bassat, E., Mutlak, Y.E., Genzelinakh, A., Shadrin, I.Y., Baruch Umansky, K., Yifa, O., Kain, D., Rajchman, D., Leach, J., Riabov Bassat, D., Udi, Y., Sarig, R., Sagi, I., Martin, J.F., Bursac, N., Cohen, S., and Tzahor, E. (2017). The extracellular matrix protein agrin promotes heart regeneration in mice. *Nature* 547, 179-184.

Bernardes De Jesus, B., and Blasco, M.A. (2011). Aging by telomere loss can be reversed. *Cell Stem Cell* 8, 3-4.

Bernardes De Jesus, B., Marinho, S.P., Barros, S., Sousa-Franco, A., Alves-Vale, C., Carvalho, T., and Carmo-Fonseca, M. (2018). Silencing of the lncRNA Zeb2-NAT facilitates reprogramming of aged fibroblasts and safeguards stem cell pluripotency. *Nat Commun* 9, 94.

Bink, D.I., Lozano-Vidal, N., and Boon, R.A. (2019). Long Non-Coding RNA in Vascular Disease and Aging. *Noncoding RNA* 5.

Blasco, M.A., Funk, W., Villeponteau, B., and Greider, C.W. (1995). Functional characterization and developmental regulation of mouse telomerase RNA. *Science* 269, 1267-1270.

Blom, J.N., Lu, X., Arnold, P., and Feng, Q. (2016). Myocardial Infarction in Neonatal Mice, A Model of Cardiac Regeneration. *J Vis Exp*.

Borges, A., and Liew, C.C. (1997). Telomerase activity during cardiac development. *J Mol Cell Cardiol* 29, 2717-2724.

Bryant, D.M., O'meara, C.C., Ho, N.N., Gannon, J., Cai, L., and Lee, R.T. (2015). A systematic analysis of neonatal mouse heart regeneration after apical resection. *J Mol Cell Cardiol* 79, 315-318.

Cai, B., Ma, W., Ding, F., Zhang, L., Huang, Q., Wang, X., Hua, B., Xu, J., Li, J., Bi, C., Guo, S., Yang, F., Han, Z., Li, Y., Yan, G., Yu, Y., Bao, Z., Yu, M., Li, F.,

Tian, Y., Pan, Z., and Yang, B. (2018). The Long Noncoding RNA CAREL Controls Cardiac Regeneration. *Journal of the American College of Cardiology* 72, 534-550.

Cai, B., Ma, W., Wang, X., Sukhareva, N., Hua, B., Zhang, L., Xu, J., Li, X., Li, S., Liu, S., Yu, M., Xu, Y., Song, R., Xu, B., Yang, F., Han, Z., Ding, F., Huang, Q., Yu, Y., Zhao, Y., Wang, J., Bamba, D., Zagidullin, N., Li, F., Tian, Y., Pan, Z., and Yang, B. (2020). Targeting LncDACH1 promotes cardiac repair and regeneration after myocardium infarction. *Cell Death and Differentiation*.

Cai, W., Tan, J., Yan, J., Zhang, L., Cai, X., Wang, H., Liu, F., Ye, M., and Cai, C.L. (2019). Limited Regeneration Potential with Minimal Epicardial Progenitor Conversions in the Neonatal Mouse Heart after Injury. *Cell Rep* 28, 190-201 e193.

Cunnington, R.H., Northcott, J.M., Ghavami, S., Filomeno, K.L., Jahan, F., Kavosh, M.S., Davies, J.J., Wigle, J.T., and Dixon, I.M. (2014). The Ski-Zeb2-Meox2 pathway provides a novel mechanism for regulation of the cardiac myofibroblast phenotype. *J Cell Sci* 127, 40-49.

Czubryt, M.P. (2019). Cardiac Fibroblast to Myofibroblast Phenotype Conversion-An Unexploited Therapeutic Target. *J Cardiovasc Dev Dis* 6.

Chang, Y., Lee, E., Kim, J., Kwon, Y.W., and Kwon, Y. (2019). Efficient in vivo direct conversion of fibroblasts into cardiomyocytes using a nanoparticle-based gene carrier. *Biomaterials* 192, 500-509.

Chen, G., Li, H., Li, X., Li, B., Zhong, L., Huang, S., Zheng, H., Li, M., Jin, G., Liao, W., Liao, Y., Chen, Y., and Bin, J. (2018). Loss of long non-coding RNA CRRL promotes cardiomyocyte regeneration and improves cardiac repair by functioning as a competing endogenous RNA. *Journal of Molecular and Cellular Cardiology* 122, 152-164.

Chen, X., Wang, J., Nie, Y., and Chu, M. (2020). The long noncoding RNA NR_045363 involves cardiomyocyte apoptosis and cardiac repair via p53 signal pathway. *Cell Biology International*.

Chen, Y., Li, X., Li, B., Wang, H., Li, M.S., Huang, S., Sun, Y., Chen, G., Si, X., Huang, C., Liao, W., Liao, Y., and Bin, J. (2019). Long Non-coding RNA ECRAR Triggers Postnatal Myocardial Regeneration by Activating ERK1/2 Signaling. *Molecular Therapy* 27, 29-45.

Darehzereshki, A., Rubin, N., Gamba, L., Kim, J., Fraser, J., Huang, Y., Billings, J., Mohammadzadeh, R., Wood, J., Warburton, D., Kaartinen, V., and Lien, C.L. (2015). Differential regenerative capacity of neonatal mouse hearts after cryoinjury. *Dev Biol* 399, 91-99.

De Jesus, B.B., Marinho, S.P., Barros, S., Sousa-Franco, A., Alves-Vale, C., Carvalho, T., and Carmo-Fonseca, M. (2018). Silencing of the lncRNA Zeb2-NAT facilitates reprogramming of aged fibroblasts and safeguards stem cell pluripotency. *Nature Communications* 9, 1-11.

Elhelaly, W.M., Cardoso, A.C., Pereira, A.H.M., Elnawasany, A., Ebrahimi, S., Nakada, Y., and Sadek, H.A. (2019). C-Kit Cells Do Not Significantly Contribute to Cardiomyogenesis During Neonatal Heart Regeneration. *Circulation* 139, 559-561.

Engel, J.L., and Ardehali, R. (2018). Direct Cardiac Reprogramming: Progress and Promise. *Stem Cells Int* 2018, 1435746.

Eschenhagen, T., Bolli, R., Braun, T., Field, L.J., Fleischmann, B.K., Frisen, J., Giacca, M., Hare, J.M., Houser, S., Lee, R.T., Marban, E., Martin, J.F., Molkentin, J.D., Murry, C.E., Riley, P.R., Ruiz-Lozano, P., Sadek, H.A., Sussman, M.A., and Hill, J.A. (2017). Cardiomyocyte Regeneration: A Consensus Statement. *Circulation* 136, 680-686.

Fan, Y., Cheng, Y., Li, Y., Chen, B., Wang, Z., Wei, T., Zhang, H., Guo, Y., Wang, Q., Wei, Y., Chen, F., Sha, J., Guo, X., and Wang, L. (2020). Phosphoproteomic Analysis of Neonatal Regenerative Myocardium Revealed Important Roles of Checkpoint Kinase 1 via Activating Mammalian Target of Rapamycin C1/Ribosomal Protein S6 Kinase b-1 Pathway. *Circulation* 141, 1554-1569.

Fu, J.D., Stone, N.R., Liu, L., Spencer, C.I., Qian, L., Hayashi, Y., Delgado-Olguin, P., Ding, S., Bruneau, B.G., and Srivastava, D. (2013). Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state. *Stem Cell Reports* 1, 235247.

Ghiroldi, A., Piccoli, M., Ciconte, G., Pappone, C., and Anastasia, L. (2017). Regenerating the human heart: direct reprogramming strategies and their current limitations. *Basic Res Cardiol* 112, 68.

Gomes, C.P., Nobrega-Pereira, S., Domingues-Silva, B., Rebelo, K., Alves-Vale, C., Marinho, S.P., Carvalho, T., Dias, S., and Bernardes De Jesus, B. (2019). An antisense transcript mediates MALAT1 response in human breast cancer. *BMC Cancer* 19, 771.

Han, C., Nie, Y., Lian, H., Liu, R., He, F., Huang, H., and Hu, S. (2015). Acute inflammation stimulates a regenerative response in the neonatal mouse heart. *Cell Res* 25, 1137-1151.

Hashimoto, H., Olson, E.N., and Bassel-Duby, R. (2018). Therapeutic approaches for cardiac regeneration and repair. *Nat Rev Cardiol* 15, 585-600.

Hashimoto, H., Wang, Z., Garry, G.A., Malladi, V.S., Botten, G.A., Ye, W., Zhou, H., Osterwalder, M., Dickel, D.E., Visel, A., Liu, N., Bassel-Duby, R., and Olson, E.N. (2019). Cardiac Reprogramming Factors Synergistically Activate Genome-wide Cardiogenic Stage-Specific Enhancers. *Cell Stem Cell* 25, 69-86 e65.

Haubner, B.J., Adamowicz-Brice, M., Khadayate, S., Tiefenthaler, V., Metzler, B., Aitman, T., and Penninger, J.M. (2012). Complete cardiac regeneration in a mouse model of myocardial infarction. *Aging (Albany NY)* 4, 966-977.

Haubner, B.J., Schuetz, T., and Penninger, J.M. (2016). A reproducible protocol for neonatal ischemic injury and cardiac regeneration in neonatal mice. *Basic Res Cardiol* 111, 64.

Hofmann, P., Sommer, J., Theodorou, K., Kirchhof, L., Fischer, A., Li, Y., Perisic, L., Hedin, U., Maegdefessel, L., Dimmeler, S., and Boon, R.A. (2019). Long noncoding RNA H19 regulates endothelial cell aging via inhibition of STAT3 signalling. *Cardiovasc Res* 115, 230-242.

Hou, B.H., Jian, Z.X., Cui, P., Li, S.J., Tian, R.Q., and Ou, J.R. (2015). miR-216a may inhibit pancreatic tumor growth by targeting JAK2. *FEBS Lett* 589, 2224-2232.

Ieda, M., Fu, J.D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G., and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142, 375-386.

Ingason, A.B., Goldstone, A.B., Paulsen, M.J., Thakore, A.D., Truong, V.N., Edwards, B.B., Eskandari, A., Bollig, T., Steele, A.N., and Woo, Y.J. (2018). Angiogenesis precedes cardiomyocyte migration in regenerating mammalian hearts. *J Thorac Cardiovasc Surg* 155, 1118-1127 e1111.

Jesty, S.A., Steffey, M.A., Lee, F.K., Breitbach, M., Hesse, M., Reining, S., Lee, J.C., Doran, R.M., Nikitin, A.Y., Fleischmann, B.K., and Kotlikoff, M.I. (2012). ckit+ precursors support postinfarction myogenesis in the neonatal, but not adult, heart. *Proc Natl Acad Sci U S A* 109, 13380-13385.

Jiang, J., Burgon, P.G., Wakimoto, H., Onoue, K., Gorham, J.M., O'meara, C.C., Fomovsky, G., Mcconnell, B.K., Lee, R.T., Seidman, J.G., and Seidman, C.E. (2015). Cardiac myosin binding protein C regulates postnatal myocyte cytokinesis. *Proc Natl Acad Sci U S A* 112, 9046-9051.

Kang, J., Hu, J., Karra, R., Dickson, A.L., Tornini, V.A., Nachtrab, G., Gemberling, M., Goldman, J.A., Black, B.L., and Poss, K.D. (2016). Modulation of tissue repair by regeneration enhancer elements. *Nature* 532, 201-206.

Konfino, T., Landa, N., Ben-Mordechai, T., and Leor, J. (2015). The type of injury dictates the mode of repair in neonatal and adult heart. *J Am Heart Assoc* 4, e001320.

Kurdi, M., Zgheib, C., and Booz, G.W. (2018). Recent Developments on the Crosstalk Between STAT3 and Inflammation in Heart Function and Disease. *Front Immunol* 9, 3029.

Lam, N.T., and Sadek, H.A. (2018). Neonatal Heart Regeneration: Comprehensive Literature Review. *Circulation* 138, 412-423.

Li, B., Hu, Y., Li, X., Jin, G., Chen, X., Chen, G., Chen, Y., Huang, S., Liao, W., Liao, Y., Teng, Z., and Bin, J. (2018a). Sirt1 Antisense Long Noncoding RNA Promotes Cardiomyocyte Proliferation by Enhancing the Stability of Sirt1. *Journal of the American Heart Association* 7.

Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Canamero, M., Blasco, M.A., and Serrano, M. (2009). The Ink4/Arf locus is a barrier for iPS cell reprogramming. *Nature* 460, 1136-1139.

Li, H., Hastings, M.H., Rhee, J., Trager, L.E., Roh, J.D., and Rosenzweig, A. (2020a). Targeting Age-Related Pathways in Heart Failure. *Circ Res* 126, 533-551.

Li, X., He, X., Wang, H., Li, M., Huang, S., Chen, G., Jing, Y., Wang, S., Chen, Y., Liao, W., Liao, Y., and Bin, J. (2018b). Loss of AZIN2 splice variant facilitates endogenous cardiac regeneration. *Cardiovascular Research* 114, 1642-1655.

Li, Y., Feng, J., Hu, S., and Nie, Y. (2020b). Achieving stable myocardial regeneration after apical resection in neonatal mice. *J Cell Mol Med* 24, 6500-6504.

Li, Y., Feng, J., Song, S., Li, H., Yang, H., Zhou, B., Yue, Z., Lian, H., Liu, L., Hu, S., and Nie, Y. (2020c). Gp130 Controls Cardiomyocyte Proliferation and Heart Regeneration. *Circulation*.

Lin, D.I., Aggarwal, P., and Diehl, J.A. (2008). Phosphorylation of MCM3 on Ser-112 regulates its incorporation into the MCM2-7 complex. *Proc Natl Acad Sci U S A* 105, 8079-8084.

Liu, Z., Wang, L., Welch, J.D., Ma, H., Zhou, Y., Vaseghi, H.R., Yu, S., Wall, J.B., Alimohamadi, S., Zheng, M., Yin, C., Shen, W., Prins, J.F., Liu, J., and Qian, L. (2017). Single-cell transcriptomics reconstructs fate conversion from fibroblast to cardiomyocyte. *Nature* 551, 100-104.

Lloyd-Jones, D., Adams, R., Carnethon, M., De Simone, G., Ferguson, T.B., Flegal, K., Ford, E., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., Mcdermott, M., Meigs, J., Mozaffarian, D., Nichol, G., O'donnell, C., Roger, V., Rosamond, W., Sacco, R., Sorlie, P., Stafford, R., Steinberger, J., Thom, T., Wasserthiel-Smoller, S., Wong, N., Wylie-Rosett, J., and Hong, Y. (2009). Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 119, e21-181.

Lloyd-Jones, D., Adams, R.J., Brown, T.M., Carnethon, M., Dai, S., De Simone, G., Ferguson, T.B., Ford, E., Furie, K., Gillespie, C., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, P.M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., Mcdermott, M.M., Meigs, J., Mozaffarian, D., Mussolino, M., Nichol, G., Roger, V.L., Rosamond, W., Sacco, R., Sorlie, P., Thom, T., Wasserthiel-Smoller, S., Wong, N.D., and Wylie-Rosett, J. (2010). Heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation* 121, e46-e215.

Mahmoud, A.I., Kocabas, F., Muralidhar, S.A., Kimura, W., Koura, A.S., Thet, S., Porrello, E.R., and Sadek, H.A. (2013). Meis1 regulates postnatal cardiomyocyte cell cycle arrest. *Nature* 497, 249-253.

Mahmoud, A.I., O'meara, C.C., Gemberling, M., Zhao, L., Bryant, D.M., Zheng, R., Gannon, J.B., Cai, L., Choi, W.Y., Egnaczyk, G.F., Burns, C.E., Burns, C.G., Macrae, C.A., Poss, K.D., and Lee, R.T. (2015). Nerves Regulate Cardiomyocyte Proliferation and Heart Regeneration. *Dev Cell* 34, 387-399.

Mahmoud, A.I., Porrello, E.R., Kimura, W., Olson, E.N., and Sadek, H.A. (2014). Surgical models for cardiac regeneration in neonatal mice. *Nat Protoc* 9, 305311.

Malek Mohammadi, M., Kattih, B., Grund, A., Froese, N., Korf-Klingebiel, M., Gigina, A., Schrameck, U., Rudat, C., Liang, Q., Kispert, A., Wollert, K.C., Bauersachs, J., and Heineke, J. (2017). The transcription factor GATA4 promotes myocardial regeneration in neonatal mice. *EMBO Mol Med* 9, 265279.

Marion, R.M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M., and Blasco, M.A. (2009). A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. *Nature* 460, 1149-1153.

Mercer, T.R., Dinger, M.E., and Mattick, J.S. (2009). Long non-coding RNAs: insights into functions. *Nat Rev Genet* 10, 155-159.

Muraoka, N., Nara, K., Tamura, F., Kojima, H., Yamakawa, H., Sadahiro, T., Miyamoto, K., Isomi, M., Haginiwa, S., Tani, H., Kurotsu, S., Osakabe, R., Torii, S., Shimizu, S., Okano, H., Sugimoto, Y., Fukuda, K., and Ieda, M. (2019). Role of cyclooxygenase-2-mediated prostaglandin E2-prostaglandin E receptor 4 signaling in cardiac reprogramming. *Nat Commun* 10, 674.

Nam, Y.J., Song, K., Luo, X., Daniel, E., Lambeth, K., West, K., Hill, J.A., Dimaio, J.M., Baker, L.A., Bassel-Duby, R., and Olson, E.N. (2013). Reprogramming of human fibroblasts toward a cardiac fate. *Proc Natl Acad Sci U S A* 110, 55885593.

Pei, J., Wang, F., Pei, S., Bai, R., Cong, X., Nie, Y., and Chen, X. (2020). Hydrogen Sulfide Promotes Cardiomyocyte Proliferation and Heart Regeneration via ROS Scavenging. *Oxid Med Cell Longev* 2020, 1412696.

Ponnusamy, M., Liu, F., Zhang, Y.H., Li, R.B., Zhai, M., Liu, F., Zhou, L.Y., Liu, C.Y., Yan, K.W., Dong, Y.H., Wang, M., Qian, L.L., Shan, C., Xu, S., Wang, Q., Zhang, Y.H., Li, P.F., Zhang, J., and Wang, K. (2019). Long Noncoding RNA CPR (Cardiomyocyte Proliferation Regulator) Regulates Cardiomyocyte Proliferation and Cardiac Repair. *Circulation* 139, 2668-2684.

Porrello, E.R., Mahmoud, A.I., Simpson, E., Hill, J.A., Richardson, J.A., Olson, E.N., and Sadek, H.A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* 331, 1078-1080.

Porrello, E.R., Mahmoud, A.I., Simpson, E., Johnson, B.A., Grinsfelder, D., Canseco, D., Mammen, P.P., Rothermel, B.A., Olson, E.N., and Sadek, H.A. (2013). Regulation of neonatal and adult mammalian heart regeneration by the miR15 family. *Proc Natl Acad Sci U S A* 110, 187-192.

Price, J.D., Park, K.Y., Chen, J., Salinas, R.D., Cho, M.J., Kriegstein, A.R., and Lim, D.A. (2014). The Ink4a/Arf locus is a barrier to direct neuronal transdifferentiation. *J Neurosci* 34, 12560-12567.

Qian, L., Huang, Y., Spencer, C.I., Foley, A., Vedantham, V., Liu, L., Conway, S.J., Fu, J.D., and Srivastava, D. (2012). In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* 485, 593-598.

Richardson, G.D., Breault, D., Horrocks, G., Cormack, S., Hole, N., and Owens, W.A. (2012). Telomerase expression in the mammalian heart. *FASEB J* 26, 48324840.

Rinn, J.L., and Chang, H.Y. (2012). Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 81, 145-166.

Rubin, N., Darehzereshki, A., Bellusci, S., Kaartinen, V., and Ling Lien, C. (2013). FGF10 Signaling Enhances Epicardial Cell Expansion during Neonatal Mouse Heart Repair. *J Cardiovasc Dis Diagn* 1.

Sadek, H.A., Martin, J.F., Takeuchi, J.K., Leor, J., Nie, Y., Giacca, M., and Lee, R.T. (2014). Multi-investigator letter on reproducibility of neonatal heart regeneration following apical resection. *Stem Cell Reports* 3, 1.

Sahara, M., Santoro, F., and Chien, K.R. (2015). Programming and reprogramming a human heart cell. *EMBO J* 34, 710-738.

Sampaio-Pinto, V., Rodrigues, S.C., Laundos, T.L., Silva, E.D., Vasques-Novoa, F., Silva, A.C., Cerqueira, R.J., Resende, T.P., Pianca, N., Leite-Moreira, A., D'uva, G., Thorsteinsdottir, S., Pinto-Do, O.P., and Nascimento, D.S. (2018). Neonatal Apex Resection Triggers Cardiomyocyte Proliferation, Neovascularization and Functional Recovery Despite Local Fibrosis. *Stem Cell Reports* 10, 860-874.

Santos, F., Moreira, C., Nobrega-Pereira, S., and Bernardes De Jesus, B. (2019). New Insights into the Role of Epithelial(-)Mesenchymal Transition during Aging. *Int J Mol Sci* 20.

Sereti, K.I., Nguyen, N.B., Kamran, P., Zhao, P., Ranjbarvaziri, S., Park, S., Sabri, S., Engel, J.L., Sung, K., Kulkarni, R.P., Ding, Y., Hsiai, T.K., Plath, K., Ernst, J., Sahoo, D., Mikkola, H.K.A., Iruela-Arispe, M.L., and Ardehali, R. (2018). Analysis of cardiomyocyte clonal expansion during mouse heart development and injury. *Nat Commun* 9, 754.

Song, K., Nam, Y.J., Luo, X., Qi, X., Tan, W., Huang, G.N., Acharya, A., Smith, C.L., Tallquist, M.D., Neilson, E.G., Hill, J.A., Bassel-Duby, R., and Olson, E.N. (2012). Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* 485, 599-604.

Sousa-Franco, A., Rebelo, K., Da Rocha, S.T., and Bernardes De Jesus, B. (2019). LncRNAs regulating stemness in aging. *Aging Cell* 18, e12870.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663-676.

Tao, G., Kahr, P.C., Morikawa, Y., Zhang, M., Rahmani, M., Heallen, T.R., Li, L., Sun, Z., Olson, E.N., Amendt, B.A., and Martin, J.F. (2016). Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. *Nature* 534, 119-123.

Testa, G., Russo, M., Di Benedetto, G., Barbato, M., Parisi, S., Pirozzi, F., Tocchetti, C.G., Abete, P., Bonaduce, D., Russo, T., and Passaro, F. (2020). Bmi1 inhibitor PTC-209 promotes Chemically-induced Direct Cardiac Reprogramming of cardiac fibroblasts into cardiomyocytes. *Sci Rep* 10, 7129.

Trembinski, D.J., Bink, D.I., Theodorou, K., Sommer, J., Fischer, A., Van Bergen, A., Kuo, C.C., Costa, I.G., Schürmann, C., Leisegang, M.S., Brandes, R.P., Alekseeva, T., Brill, B., Wietelmann, A., Johnson, C.N., Spring-Connell, A., Kaulich, M., Werfel, S., Engelhardt, S., Hirt, M.N., Yorgan, K., Eschenhagen, T., Kirchhof, L., Hofmann, P., Jaé, N., Wittig, I., Hamdani, N., Bischof, C., Krishnan, J., Houtkooper, R.H., Dimmeler, S., and Boon, R.A. (2020). Aging-regulated anti-apoptotic long non-coding RNA Sarrah augments recovery from acute myocardial infarction. *Nature Communications* 11.

Trivedi, C.M., Zhu, W., Wang, Q., Jia, C., Kee, H.J., Li, L., Hannenhalli, S., and Epstein, J.A. (2010). Hopx and Hdac2 interact to modulate Gata4 acetylation and embryonic cardiac myocyte proliferation. *Dev Cell* 19, 450-459.

Valiente-Alandi, I., Albo-Castellanos, C., Herrero, D., Sanchez, I., and Bernad, A. (2016). Bmi1 (+) cardiac progenitor cells contribute to myocardial repair following acute injury. *Stem Cell Res Ther* 7, 100.

Wada, R., Muraoka, N., Inagawa, K., Yamakawa, H., Miyamoto, K., Sadahiro, T., Umei, T., Kaneda, R., Suzuki, T., Kamiya, K., Tohyama, S., Yuasa, S., Kokaji, K., Aeba, R., Yozu, R., Yamagishi, H., Kitamura, T., Fukuda, K., and Ieda, M. (2013). Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. *Proc Natl Acad Sci U S A* 110, 12667-12672.

Wang, J., Chen, X., Shen, D., Ge, D., Chen, J., Pei, J., Li, Y., Yue, Z., Feng, J., Chu, M., and Nie, Y. (2019a). A long noncoding RNA NR_045363 controls cardiomyocyte proliferation and cardiac repair. *Journal of Molecular and Cellular Cardiology* 127, 105-114.

Wang, Z., Cui, M., Shah, A.M., Ye, W., Tan, W., Min, Y.L., Botten, G.A., Shelton, J.M., Liu, N., Bassel-Duby, R., and Olson, E.N. (2019b). Mechanistic basis of neonatal heart regeneration revealed by transcriptome and histone modification profiling. *Proc Natl Acad Sci U S A* 116, 18455-18465.

Xiong, J., and Hou, J. (2016). Apical Resection Mouse Model to Study Early Mammalian Heart Regeneration. *J Vis Exp*, e53488. Yamanaka, S. (2009). A fresh look at iPS cells. *Cell* 137, 13-17.

Yu, W., Huang, X., Tian, X., Zhang, H., He, L., Wang, Y., Nie, Y., Hu, S., Lin, Z., Zhou, B., Pu, W., and Lui, K.O. (2016). GATA4 regulates Fgf16 to promote heart repair after injury. *Development* 143, 936-949.

Yusuf, S., Reddy, S., Ounpuu, S., and Anand, S. (2001). Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. *Circulation* 104, 2746-2753. Zebrowski, D.C., Jensen, C.H., Becker, R., Ferrazzi, F., Baun, C., Hvidsten, S., Sheikh, S.P., Polizzotti, B.D., Andersen, D.C., and Engel, F.B. (2017). Cardiac injury of the new-born mammalian heart accelerates cardiomyocyte terminal differentiation. *Sci Rep* 7, 8362.

Zhang, Z., Zhang, A.D., Kim, L.J., and Nam, Y.J. (2019a). Ensuring expression of four core cardiogenic transcription factors enhances cardiac reprogramming. *Sci Rep* 9, 6362.

Zhang, Z., Zhang, W., and Nam, Y.J. (2019b). Stoichiometric optimization of Gata4, Hand2, Mef2c, and Tbx5 expression for contractile cardiomyocyte reprogramming. *Sci Rep* 9, 14970.

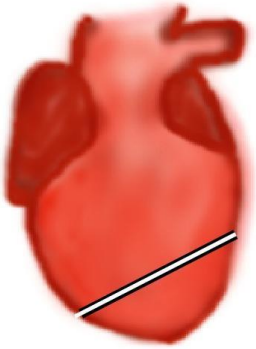
Zhu, B., Zhang, L., Liang, C., Liu, B., Pan, X., Wang, Y., Zhang, Y., Xie, W., Yan, B., Liu, F., Yip, H.K., Yu, X.Y., and Li, Y. (2019). Stem Cell-Derived Exosomes Prevent Aging-Induced Cardiac Dysfunction through a Novel Exosome/lncRNA MALAT1/NF-kappaB/TNF-alpha Signaling Pathway. *Oxid Med Cell Longev* 2019, 9739258.

Zong, X., Nakagawa, S., Freier, S.M., Fei, J., Ha, T., Prasanth, S.G., and Prasanth, K.V. (2016). Natural antisense RNA promotes 3' end processing and maturation of MALAT1 lncRNA. *Nucleic Acids Res* 44, 2898-2908.

Figure 1 – Studies on neonatal heart regeneration

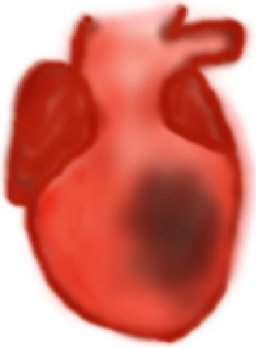
Figure 1 - Santos *et al.*

APICAL RESECTION



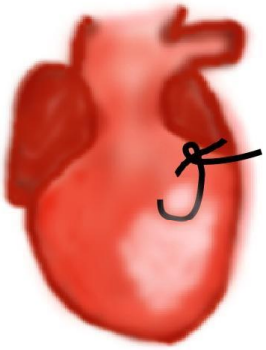
Study	Strain Background	Year
Porrello, E. R.,*et al*	ICR (CD-1)	2011
Mahmoud, A. I.,*et al*	ICR (CD-1)	2014
Sadek, H. A.,*et al*	C57BL/6	2014
Bryant, D. M.,*et al*	ICR (CD-1)	2015
Konfino, T.,*et al*	ICR (CD-1)	2015
Jiang, J.,*et al*	129SvEv	2015
Mahmoud, A. I.,*et al*	ICR (CD-1)	2015
Han, C.,*et al*	ICR (CD-1), C57BL/6J and others	2015
Yu, W.,*et al*	mixed background	2016
Kang, J.,*et al*	ICR (CD-1)	2016
Xiong, J.,*et al*	C57BL/6	2016
Valiente-Alandi, I.,*et al*	mixed background (C57BL/6x129)	2016
Tao, G.,*et al*	mixed background	2016
Bassat, E.,*et al*	ICR (CD-1)	2017
Sampaio-Pinto, V.,*et al*	C57BL/6	2018
Ingason, A. B.,*et al*	ICR (CD-1)	2018
Ahmed, A.,*et al*	mixed background	2018
Elhelaly, W. M.,*et al*	mixed background	2019
Li, Y.,*et al* (b)	several backgrounds	2020
Li, Y.,*et al* (c)	C57BL/6 and B6.129S2	2020
Fan, Y.,*et al*	ICR (CD-1)	2020
Andersen, D. C.,*et al*	C57BL/6	2014
Andersen, D. C.,*et al*	C57BL/6	2016
Zebrowski, D. C.,*et al*	ICR (CD-1)	2017
Cai, W.,*et al*	C57BL/6J	2019

CRYO-INJURY



Study	Strain Background	Year
Jesty, S. J.,*et al*	B6.DBA2(F2) or C57BL/6J	2012
Aix, E.,*et al*	C57BL/6	2016
Mohammadi, M. M.,*et al*	SV129/CD-1	2017
Rubin, N.,*et al*	FVB/n	2013
Darehzereshki, A.,*et al*	ICR (CD-1)	2015

LAD LIGATION



Study	Strain Background	Year
Haubner, B. J.,*et al*	C57BL/6J	2012
Porrello, E. R.,*et al*	ICR (CD-1)	2013
Mahmoud, A. I.,*et al*	C57BL/6xSV129/CD1	2013
Mahmoud, A. I.,*et al*	ICR (CD-1)	2014
Aurora, A. B.,*et al*	ICR (CD-1)	2014
Mahmoud, A. I.,*et al*	ICR (CD-1)	2015
Blom, J. N.,*et al*	C57BL/6x CD-1 IG-S	2016
Haubner, B. J.,*et al*	C57BL/6J, ICR and C57BL/6JxSv129	2016
Ai, S.,*et al*	C57BL/6xSV129	2017
Sereti K-I.,*et al*	mixed background, C57BL/6	2018
Ahmed, A.,*et al*	mixed background	2018
Wang, Z.,*et al*	ICR (CD-1)	2019
Pei, J.,*et al*	C57BL/6	2020
Konfina, T.,*et al*	ICR (CD-1)	2015

Annex 5

To Be Published in: Article collection *Non-coding RNA as Therapeutic Target: A Game Changer in Cardiac Regenerative Strategies?* Frontiers in Physiology - Clinical and Translational Physiology.

Interplay between lncRNAs and metabolic signaling in cardiac regeneration

Magda Correia [1], Bruno Bernardes de Jesus [1#], and
Sandrina Nóbrega-Pereira [1,2#]

[1] Department of Medical Sciences and Institute of Biomedicine - iBiMED, University of Aveiro, 3810-193 Aveiro, Portugal.
[2] Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Av. Professor Egas Moniz, 1649-028 Lisboa, Portugal;

Corresponding authors: Department of Medical Sciences and Institute of Biomedicine - iBiMED, University of Aveiro, 3810-193 Aveiro, Portugal, Tel: +(351) 234 370 213
E-mail addresses: sandrina.pereira@ua.pt (S. Nóbrega-Pereira) and brunob.jesus@ua.pt (B. Bernardes de Jesus)

Running title: lncRNAs and metabolic signaling in cardiac regeneration

ABSTRACT

Heart disease is the leading cause of mortality in developed countries. The associated pathology is typically characterized by the loss of cardiomyocytes that leads, eventually, to heart failure. Although conventional treatments exist, novel regenerative procedures are warranted for improving cardiac regeneration and patients well fare. Whereas following injury the capacity for regeneration of adult mammalian heart is limited, the neonatal heart is capable of substantial regeneration but this capacity is lost at postnatal stages. Interestingly, this is accompanied by a shift in the metabolic pathways and energetic fuels preferentially used by cardiomyocytes from embryonic glucose-driven anaerobic glycolysis to adult oxidation of substrates in the mitochondria. Apart from energetic sources, metabolites are emerging as key regulators of gene expression and epigenetic programs which could impact cardiac regeneration. Long non-coding RNAs (lncRNAs) are known master regulators of cellular and organismal carbohydrate and lipid metabolism and play multifaceted functions in the cardiovascular system. Still, our understanding of the metabolic determinants and pathways that can promote cardiac regeneration in the injured hearth remains limited. Here, we will discuss the molecular interplay between lncRNAs and metabolic signaling in the regenerative heart and whether their manipulation could provide ground for the development of innovative treatments.

INTRODUCTION

The World Health Organization (WHO) has been reporting every year that cardiovascular diseases (CVD) are the leading cause of death in the world. Although currently there are large range of pharmaceutical drugs and surgical options that prevent further deterioration or restore function to the failing heart, for end-stage heart failure, the only long-term selection is heart transplantation which presents several limitations (Hudson and Porrello, 2013). Therefore, the development of improved cardiac regenerative strategies is an area of growing interest.

Subsequent to cardiac injury, cardiomyocytes undergo necrotic and apoptotic cell death and cardiac fibroblasts are activated to produce collagen and other extracellular matrix components, leading to fibrosis and harmed cardiac function (Song et al., 2012; Hashimoto et al., 2018). The main goal of regenerative cardiovascular medicine is to repair injured hearts by replacing cardiomyocytes and diminishing fibrosis. In order to suppress the outcomes of heart failure several regenerative strategies have been proposed, including post-injury activation of cardiomyocyte proliferation, recruitment of stem cells or progenitor cells, delivery of *de novo* cardiomyocytes from iPSCs, and direct reprogramming of resident cardiac fibroblasts (CFs) into induced cardiac-like myocytes (iCLMs) (Ieda *et al.*, 2010; Song *et al.*, 2012; Ghiroldi *et al.*, 2018; Hashimoto, Olson and Bassel-Duby, 2018)

Besides holding great promise, most cardiac regenerative strategies still lack effective clinical outcomes (Ghiroldi et al., 2017; Hashimoto et al., 2018). Therefore understanding the molecular mechanisms and players governing cardiac regeneration in the injured hearth is warranted for improving the efficiency of cardiac regenerative strategies. In this context, long non-coding RNAs (lncRNAs), a class of >200 nucleotides-long ribonucleic acid sequences, are abundantly expressed in the cardiovascular system and are part of a complex regulatory network governing cardiovascular function in health and disease (Bär et al., 2016; Das et al., 2018; Hobuß et al., 2019). Essential roles for few lncRNAs in heart development have been described (Matkovich et al., 2014; Bär et al., 2016; Haemmig et al., 2017) and exploring the role of lncRNAs in cardiovascular function may facilitate the development of new therapeutics for treating cardiovascular disease (Bär et al., 2016; Hobuß et al., 2019).

Although the adult mammalian heart has limited regenerative capacity, with estimation of only ≈1% *de novo* cardiomyogenesis per year (Neidig et al., 2018), the neonatal heart is capable of substantial regeneration but this capacity is lost by

postnatal day (P) 7 (Soonpaa et al., 1996). Interestingly, this lost in proliferative potential is accompanied by a shift in the main energetic metabolic pathway and fuels preferentially used by cardiomyocytes from embryonic glucose-driven anerobic glycolysis to adult oxygen-dependent oxidative phosphorylation (OXPHOS) nof pyruvate and fatty acids (FAs) in the mitochondria (Lopaschuk et al., 1992; Lehman and Kelly, 2002). Apart from energetic sources, metabolites have emerged as key regulators of gene expression programs acting as essential substrates or cofactors for epigenetic enzymes (Intlekofer and Finley, 2019) opening the possibility for a two-way communication between metabolism and lncRNAs in cardiac heart regeneration. Interestingly, lncRNAs are emerging as master regulators of cellular and systemic carbohydrate and lipid metabolism with clear implications for cardiovascular function (Zhao, 2015; van Soligen, 2018; Mongelli et al., 2019).

Here, we will discuss the molecular interplay between lncRNAs and metabolic signaling in the heart highlighting recent evidence in lncRNA modulation that improves cardiac regeneration. Also, particular emphasis will be given to those lncRNAs regulating metabolic targets in the cardiovascular system which manipulation could provide ground for the development of innovative cardiovascular treatments.

CAN AN EMBRYONIC-LIKE METABOLIC PROGRAM PROMOTE HEART REGENERATION?

The fetal heart's environment is low in oxygen and FAs, thus fetal cardiomyocytes are highly dependent on glycolysis for ATP production (Lopaschuk et al., 1992). The heart suffers a major metabolic alteration driven by the physiological changes at postnatal stages, as enhanced workload and the demand for growth, that cannot be supported by glucose and lactate metabolism (Malandraki-Miller et al., 2018). The mammalian heart has to contract constantly thus, the need for an optimal energy fuel is imperative. Mitochondria is the organelle that coordinates the energy transduction function and it is responsible to produce more than 95% of ATP utilized by the heart (Doenst et al., 2013). Additionally, mitochondrion regulates intracellular calcium homeostasis, signaling and apoptosis (Kolwicz et al., 2013). As a result, mammalian cardiomyocytes undergo extensive metabolic remodeling after birth. In order to adapt to high-energy demands of the postnatal life, cardiomyocytes suffer a metabolic switch and produce their energy via mitochondrial OXPHOS, a more efficient process than glycolysis (Lehman and Kelly, 2002; Vivien et al., 2016). Postnatal cardiomyocytes also revealed a shift in the energetic substrate utilization from pyruvate

to FAs that are energetically more favorable (Lopaschuk et al., 1992; Lehman and Kelly, 2002). As the neonatal mammalian heart regenerative capacity is lost by P7, which corresponds with cardiomyocyte binucleation and cell-cycle arrest (Soonpaa et al., 1996; Porrello et al., 2011), it is intriguing to think that the “fetal metabolic shift” would have a role in suppressing cardiomyocyte proliferation and heart repair (Martik, 2020). Currently many studies are focusing in understanding the role of mitochondrial metabolism in regulating cell-cycle arrest postnatally and new regenerative strategies could arise.

Heart regeneration in zebrafish is incredibly effective and relies on the proliferation of pre-existing cardiomyocytes. Apart from cardiomyocytes, other cell types (such as epicardial, endocardial, immune cells and fibroblasts) respond to the heart injury and contribute for the healing process (Vivien et al., 2016; Honkoop et al., 2019). Cardiomyocytes from highly regenerative species such as zebrafish have a preference for glycolysis and increased OXPHOS activity promotes cardiomyocyte maturation and reduces the proliferative capacity (Vivien et al., 2016; Honkoop et al., 2018; Fukuda et al., 2019). Although the “fetal switch” to mitochondrial respiration has been associated to loss of the regenerative capacity (Malandraki-Miller et al., 2018), the role of bioenergetics in regulating cardiogenesis remains unclear. Recent evidence suggest that hypoxia inducible factor 1 (HIF1) signaling, an important inducer of aerobic glycolysis and the Warburg effect in cancer cells (Kroemer and Pouyssegur, 2008), controls the embryonic switch toward oxidative metabolism in developing mouse heart (Menendez-Montes et al., 2016).

The adult mammalian heart cannot regenerate lost or damaged myocardium although it does present a limited myocyte turnover that reveals insufficient for restoration of contractile dysfunction. The brief window of regenerative response following injury seems to be also driven by proliferation of pre-existing cardiomyocytes (Porrello et al., 2011; Elhelaly et al., 2019). Strikingly, increased production of mitochondrial-derived reactive oxygen species (ROS) and DNA oxidation leads to cell-cycle arrest in mouse postnatal cardiomyocytes through the activation of DNA damage response pathways (Puente et al., 2014). FAs oxidation is directly linked with increased production of ROS and cardiomyocyte cell-cycle arrest (Cardoso et al., 2020). Moreover, the constant use of FAs as an energetic fuel provokes a dependency on this substrate as the acetyl-coA produced from FAs oxidation inhibits the mitochondrial enzyme pyruvate dehydrogenase (PDH) (Rindler et al., 2013). Currently the focus is to clarify whether modulating substrate utilization would affect DNA damage and promote cell-cycle re-entry in cardiomyocytes. Supplementation of FAs depleted diets in mice

prolongs the postnatal window for cardiomyocyte proliferation however, it is associated with a marked hepatomegaly and steatosis due to a compensatory increase in hepatic de novo fatty-acid synthesis (Cardoso et al., 2020). Moreover, deletion of the dehydrogenase kinases isoform 4 (PDK4), main responsible for PDH inhibition and FAs usage, in adult cardiomyocytes results in a marked shift in myocardial substrate utilization with decrease FAs and enhanced pyruvate-driven glucose oxidation, leading to a decrease in DNA damage and increase in cardiomyocyte proliferation (Cardoso et al., 2020). Activation of PDH through administration of dichloroacetate in mice also resulted improved glucose utilization and resulted cardioprotective (Cardoso et al., 2020).

In sum, the intricate relationship between the “fetal metabolic switch” and loss of cardiomyocyte proliferative potential in the mammalian heart is beginning to shed light into important regulatory axis, including HIF signaling, mitochondrial-dependent ROS formation and bioenergetic fuels (FAs, glucose, pyruvate). Several important questions and opportunities are still open in the field. Can other cardiac regenerative strategies, as generation of induced cardiac-like myocytes (iCLMs) from iPSCs or resident cardiac fibroblasts (CFs), be potentiated by induction of the “fetal metabolic switch”? And, is there evidence for systemic metabolic shifts, as nutritional stages and diets, favoring cardiac regeneration pós-injury in the mammalian heart?

LncRNAs CONTROLING METABOLIC PATHWAYS IN THE HEART

LncRNAs represent one of the most prominent but least understood transcriptome in the heart. Thousands of lncRNAs have been identified to be dynamically transcribed during the development, differentiation, and maturation of cardiac myocytes (Devaux et al., 2015; He et al., 2016; Li et al., 2017; Beermann et al., 2018). Due to their unique regulatory action and tissue-specific expression, lncRNAs are attractive candidates for modulation and diagnosis of cardiac pathophysiological conditions (Bär et al., 2016; Hobuß et al., 2019). lncRNAs execute their functions by forming RNA-DNA, RNA-protein, and RNA-RNA interactions that regulate gene expression through diverse mechanisms, including epigenetic remodeling, transcriptional activation or repression, posttranscriptional regulation, and modulation of protein activity (Schonrock et al., 2012; Kornfeld and Brüning, 2014; Devaux et al., 2015; Thum and Condorelli, 2015; Muret et al., 2019).

Interestingly, lncRNAs are emerging as master regulators of cellular and organismal carbohydrate and lipid metabolism in adipose tissue and liver (Kornfeld and Brüning,

2014; Zhao, 2015; van Soligen, 2018; Mongelli et al., 2019; Muret et al., 2019). Alteration in blood lipids levels is one of the most relevant risk factor for CVD. In the recent years, several studies have highlighted the complex contribution of lncRNAs in controlling systemic and cell-type-specific cholesterol, FAs and triglyceride metabolism, with important implications for CVD. For instance, several lncRNAs, including *H19*, lncRNA HCV regulated 1 (*lncHR1*), *MALAT-1* and *lncARSR*, have been shown to regulate the expression of the sterol regulatory element binding protein 1c (*SREBP-1c*), a transcription factor that regulates lipid synthesis and uptake in the liver (Liu et al., 2018; Li D. et al., 2018; Yan et al., 2016; Zhang M. et al., 2018). Other examples are the liver-specific triglyceride regulator lncRNA Lancaster (*lncLSTR*) that regulates triglyceride plasma levels and energy metabolism (Li P. et al., 2015) or *AT102202* that inhibits cholesterol synthesis in the liver by targeting the rate- limiting enzyme HMGCR (Liu et al., 2015). Whether lncRNAs-mediated control of systemic lipid metabolism directly impacts cardiac regeneration remains unknown.

As previously discussed, of particular interest are the lncRNAs controlling the "fetal metabolic switch" from embryonic glycolysis to adult mitochondrial respiration and the preferred usage of FAs as energetic fuel in differentiated cardiomyocytes. Although most of our knowledge in lncRNAs control of metabolism comes from studies in lipogenic tissues and/or cancer energetics (Gomes et al., 2019), some mechanistic insights in cardiac muscle development and function, particularly concerning mitochondrial metabolism, are beginning to arise (Table 1). Due to the implication of mitochondrial-dependent FAs oxidation and ROS production in cardiomyocyte loss of proliferation (Puente et al., 2014; Cardoso et al., 2020), lncRNAs that regulate these pathways are particularly attractive for cardiac regeneration.

In heart and skeletal muscle, the lncRNA *LINC00116* is among the most significantly downregulated genes in aging muscles (GEO: GSE362 and GSE674). Interestingly, a small region of the most predominant isoform is actively translated in human and mouse and has been found to encode a highly conserved transmembrane microprotein, named mitoregulin (Mtln), that localizes to the inner mitochondrial membrane, enhancing mitochondrial membrane potential while decreasing ROS formation (Stein et al., 2018). The impact of Mtln expression in cardiovascular disease and regeneration is still unclear but GTEx portal annotates the existence of common genetic variants that strongly associate with *LINC00116* expression in human heart (Stein et al., 2018). *NEAT1* (nuclear enriched abundant transcript 1) is another lncRNAs with increased expression in non-regenerative cardiomyocytes (Table 1). In skeletal muscle, NEAT1 modulates myogenesis by accelerating myoblast proliferation

and suppressing myoblast differentiation and fusion (Wang et al., 2019). NEAT1 act by recruiting EZH2 to target gene promoters, decreasing the expression of the cyclin-dependent kinase inhibitor *p21* and suppressing the myoblast differentiation program. Strikingly, several mitochondrial regulators have been identified to associate to *NEAT1* in paraspeckles, a type of nuclear body with multiple roles in gene expression (Wang et al., 2018). Specifically, *NEAT1* depletion lead to profound effects on mitochondrial dynamics and function by altering the paraspeckles-specific sequestration of essential mito-mRNAs, including *CYCS* (cytochrome c), *NDUFA13* (NADH:Ubiquinone Oxidoreductase Subunit A13) and *CPT1A (*Carnitine Palmitoyltransferase 1A) (Wang et al., 2018) and *NEAT1*-depleted HeLa cells showed a reduction in mitochondrial DNA, ATP production and proliferation rate (Wang et al., 2018).

Cardiac muscle is extremely metabolically active and undergoes significant changes in its energy metabolism during disease. In mouse cardiomyocytes, cardiac apoptosis-related lncRNA (*CARL*) bound to and sequesters miR-539, a microRNA found to target the mRNA of the PHB2 sub-unit of prohibitin, a protein localized to the inner mitochondrial membrane that regulates mitochondrial homeostasis (Wang et al., 2014). Downregulation of PHB2 during pathological insults was found to be dependent on upregulation of miR-539. *CARL* act as the endogenous sponge for this microRNA, suppressing mitochondrial fission and cardiomyocyte apoptosis (Wang et al., 2014), highlighting the therapeutic potential of lncRNAs during myocardial infarction. The lncRNA *CDKN2B-AS1* (also known as *ANRIL*) has been described as a genetic risk factor for coronary artery disease (CAD) (Deloukas et al., 2013). *ANRIL* expression level is associated with left ventricular dysfunction after myocardial infarction (Vausort et al., 2014). Experimental manipulation in several human cell lines (including HEK and HeLa), showed that knock-down of *ANRIL* decreases the expression of *ADIPOR1* (adiponectin receptor 1), *TMEM258* (also known as *C11ORF10* for chromosome 11 open reading frame 10) and *VAMP3* (vesicle associated membrane protein 3), both at the transcript and protein level, which are important genes in the regulation of glucose and fatty-acid metabolism (Bochenek et al., 2013). The mechanistic action of *ANRIL* and the existence of *ANRIL*-mediated metabolic regulation in cardiomyocytes remains to be explored. Conversely, in patients with myocardial infarction the levels of the lncRNA hypoxia inducible factor 1A antisense RNA 2 *(HIF1A-AS2)* was found to be upregulated (Vausort et al., 2014). In humans, the HIF pathway is induced early in acute myocardial and remains activated in chronic human heart failure (Zolk et al., 2008). Due to the role of the HIF signaling in controlling myocardial metabolism and differentiation in the neonatal heart (Menendez-Montes et al., 2016) and the implication

of the lncRNA *lincRNA-p21* in hypoxia-enhanced glycolysis (Yang et al., 2014), manipulation of the lncRNAS/HIF regulatory network might modulate metabolism and potentiate regeneration in the failing heart.

Type 2 diabetes (T2D) is a multifactorial disorder and diabetic cardiomyopathy (DCM) is a critical complication (Jia et al., 2018). Studies suggest that lncRNAs that regulate metabolic targets are aberrantly regulated in DCM, thus targeting lncRNAs could have potential implications for DCM diagnosis or therapy. The mitochondrial long intergenic noncoding RNA predicting cardiac remodeling (*MT-LIPCAR*) is a lncRNA possibly transcribed from mitochondrial DNA that cross the membrane barrier being released into the circulation (Dorn, 2014). Plasma levels of *MT-LIPCAR* were positively associated with left ventricular diastolic dysfunction in T2D patients with DCM showing prognostic value as an indicator of future heart failure and patient mortality. *MT-LIPCAR* was the first proof that plasma lncRNAs might be used for cardiovascular disease prognostic (Kumarswamy *et al.*, 2014). Despite the invaluable potential as a cardiac biomarker, *MT-LIPCAR* targets and metabolic impact remains unclear. Evidence suggested that the complete *MT-LIPCAR* sequence could map to the mitochondrial genes *CYTB* (Mitochondrially Encoded Cytochrome B) and *COX2* (Mitochondrially Encoded Cytochrome C Oxidase II) (Dorn, 2014), raising further questions regarding MT-LIPCAR biogenesis as a mitochondrial or nuclear pseudogene transcript.

H19 is a lncRNA transcribed from H19/ insulin-like growth factor-II (IGF2) genomic imprinted cluster which accumulates in cardiomyocytes of the mature myocardium in humans and rodents (Pant et al., 2018; Viereck et al., 2020). Decreased expression of cardiac *H19* was reported in a rat model of DCM (Li et al., 2016; Zhuo et al., 2017). Overexpression of *H19* in myocardial tissues was able to suppress oxidative stress, inflammation and improved left ventricular function leading to DCM amelioration. Mechanistically, *H19* serves as template for microRNA-675 expression from its first exon (Zhang et al., 2017; Pant et al., 2018). Since microRNA-675 has multiple biological targets, H19 is able to regulate a number of mitochondrial functions including suppression of apoptosis by targeting voltage-dependent anion channel 1 (*VDAC1*) (Li et al., 2016), or inhibiting autophagy activation in cardiomyocytes exposed to high glucose through the down-regulation of the GTP- binding protein Di-Ras-3 (*DIRAS3*) (Zhuo et al., 2017).

In sum, recent work on lncRNAs has started to shed light on their regulatory potential on heart metabolic homeostasis during health and disease. A question to be

exploited is whether lncRNAs-mediated control of metabolic targets may be applied for cardiac regeneration.

lncRNAs AND METABOLISM ARE CENTRAL EPIGENETIC PLAYERS IN CARDIAC REGENERATION?

A hallmark function of lncRNAs is their ability to mediate epigenetic regulation and lncRNAs have crucial roles in regulating cardiac chromatin structure during heart development and pathological remodeling (Schonrock et al., 2012). lncRNAs exhibit tissue-specific and regulated expression patterns which are frequently lost during disease (Cabili et al., 2011). How lncRNAs are regulated in different cardiac developmental and disease states is still unclear. Strikingly, inhibition of epigenetic modifications alters the expression pattern of lncRNAs (Schonrock et al., 2012). Metabolites are emerging as key regulators of gene expression programs and epigenetic modifications, acting as essential substrates or cofactors for enzymes that deposit or remove chemical modifications in DNA and/or histones (Intlekofer and Finley, 2019). FAs and cholesterol have been show to regulate lncRNAs expression in lipogenic tissues. For instance, the lncRNA *CHROME* which is upregulated in nonhuman primates with atherosclerotic vascular disease, regulates cellular and systemic cholesterol homeostasis and conversely, *CHROME* expression is influenced by dietary and cellular cholesterol (Hennessy et al., 2019). Also, the expression of the lncRNAS *H19* and *MALAT1* is upregulated by FAs exposure, coinciding with an increase in (SREBP)-1c in hepatic cells (van Soligen, 2018) and *HULC* is induced by cholesterol in hepatoma cells via the retinoic receptor RXRA, leading to lipogenesis (Cui et al., 2015). Evidence for the direct implication of nutritional signals in the epigenetic alterations that govern lncRNAs expression is still lacking but it seems clear that lncRNAs and metabolites engage in a two-way communication road in the control of systemic metabolism. Recently the nutritional microenvironment has also been show to control the specification of skeletal cell fate, highlighting the possibility for a similar network to potentiate cardiac regeneration. When lipids are scarce, skeletal progenitors activate forkhead box O (FOXO) transcription factors leading to a Sox9-dependent suppression of FAs oxidation and chondrogenic commitment (van Gastel et al., 2020). Moreover, glucose metabolism is crucial for muscle stem cells (MuSCs) commitment.

In proliferating MuSCs, glucose is dispensable for mitochondrial respiration and becomes available for maintaining high histone acetylation via acetyl-CoA whereas differentiating MuSCs increase glucose oxidation and have consequently reduced

acetylation (Yucel et al., 2019). Pyruvate dehydrogenase (PDH) is pivotal for this switch and determines the differentiation potential of myogenic progenitors during muscle regeneration (Yucel et al., 2019). Whether metabolic choices also directly impinge cardiomyocyte cell fate decisions and if dietary cues can impact cardiac regeneration by the control of lncRNAs expression remains to be explored.

CONCLUDING REMARKS

Given the emerging regulatory potential of lncRNAs, it is undoubted that these molecules offer potential solutions in the pursuit for cardiac regeneration (Hudson and Porrello, 2013). But can we boost cardiac regeneration by modulating the lncRNAs-metabolism node? Accumulating evidence suggests that exploring the two-way communication road between lncRNAs and (cardiac or systemic) metabolism may offer new perspectives for increasing the regenerative potential of the injured heart.

AUTHOR CONTRIBUTIONS

MC, BBJ and SN-P planned, wrote and discussed the paper. BBJ and SN-P revised the paper. All authors contributed to the article and approved the submitted version.

Funding: This work was supported by LISBOA-01-0145-FEDER-028534, projeto cofinanciado pelo FEDER através POR Lisboa 2020 - Programa Operacional Regional de Lisboa, do PORTUGAL 2020 e pela Fundação para a Ciência e a Tecnologia (FCT).

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Bär, C., Chatterjee, S., and Thum, T. (2016). Long Noncoding RNAs in Cardiovascular Pathology, Diagnosis, and Therapy. *Circulation* 134, 1484–1499. doi:10.1161/CIRCULATIONAHA.116.023686.

Beermann, J., Kirste, D., Iwanov, K., Lu, D., Kleemiß, F., Kumarswamy, R., et al. (2018). A large shRNA library approach identifies lncRNA Ntep as an essential regulator of cell proliferation. *Cell Death Differ.* 25, 307–318. doi:10.1038/cdd.2017.158.

Bochenek, G., Häsler, R., Mokhtari, N. E. El, König, I. R., Loos, B. G., Jepsen, S., et al. (2013). The large non-coding RNA ANRIL, which is associated with atherosclerosis, periodontitis and several forms of cancer, regulates ADIPOR1, VAMP3 and C11ORF10. *Hum. Mol. Genet.* 22, 4516–4527. doi:10.1093/hmg/ddt299.

Cabili, M., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., et al. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* 25, 1915–1927. doi:10.1101/gad.17446611.

Cardoso, A. C., Lam, N. T., Savla, J. J., Nakada, Y., Pereira, A. H. M., Elnwasany, A., et al. (2020). Mitochondrial substrate utilization regulates cardiomyocyte cell-cycle progression. *Nat. Metab.* 2, 167–178. doi:10.1038/s42255-020-0169-x.

Cui, M., Xiao, Z., Wang, Y., Zheng, M., Song, T., Cai, X., et al. (2015). Long noncoding RNA HULC modulates abnormal lipid metabolism in hepatoma cells through an miR-9-mediated RXRA signaling pathway. *Cancer Res.* 75, 846–857. doi:10.1158/0008-5472.CAN-14-1192.

Das, A., Samidurai, A., and Salloum, F. N. (2018). Deciphering Non-coding RNAs in Cardiovascular Health and Disease. *Front. Cardiovasc. Med.* 5, 1–31. doi:10.3389/fcvm.2018.00073.

De Gonzalo-Calvo, D., Kenneweg, F., Bang, C., Toro, R., van der Meer, R. W., Rijzewijk, L. J., et al. (2016). Circulating long-non coding RNAs as biomarkers of left ventricular diastolic function and remodelling in patients with well-controlled type 2 diabetes. *Sci. Rep.* 6, 37354. doi:10.1038/srep37354.

Deloukas, P., Kanoni, S., Willenborg, C., Farrall, M., Assimes, T. L., Thompson, J. R., et al. (2013). Large-scale association analysis identifies new risk loci for coronary artery disease. *Nat. Genet.* 45, 25–33. doi:10.1038/ng.2480.

Devaux, Y., Zangrando, J., Schroen, B., Creemers, E. E., Pedrazzini, T., Chang, C. P., et al. (2015). Long noncoding RNAs in cardiac development and ageing. *Nat. Rev. Cardiol.* 12, 415–425. doi:10.1038/nrcardio.2015.55.

Doenst, T., Nguyen, T. D., and Abel, E. D. (2013). Cardiac Metabolism in Heart Failure. *Circ. Res.* 113, 709–724. doi:10.1161/circresaha.113.300376.

Dorn, G. W. 2nd (2014). LIPCAR: a mitochondrial lnc in the noncoding RNA chain? *Circ. Res.* 114, 1548–1550. doi:10.1161/CIRCRESAHA.114.304028.

Elhelaly, W. M., Cardoso, A. C., Pereira, A. H. M., Elnawasany, A., Ebrahimi, S., Nakada, Y., et al. (2019). C-Kit Cells Do Not Significantly Contribute to Cardiomyogenesis During Neonatal Heart Regeneration. *Circulation* 139, 559–561. doi:10.1161/CIRCULATIONAHA.117.033150.

Fukuda, R., Aharonov, A., Ong, Y. T., Stone, O. A., El-Brolosy, M. A., Maischein, H. M., et al. (2019). Metabolic modulation regulates cardiac wall morphogenesis in zebrafish. *Elife* 8, 1–14. doi:10.7554/eLife.50161.

Ghiroldi, A., Piccoli, M., Ciconte, G., Pappone, C., and Anastasia, L. (2017). Regenerating the human heart: direct reprogramming strategies and their current limitations. *Basic Res. Cardiol.* 112, 1–14. doi:10.1007/s00395-017-0655-9.

Ghiroldi, A., Piccoli, M., Cirillo, F., Monasky, M. M., Ciconte, G., Pappone, C., et al. (2018). Cell-Based Therapies for Cardiac Regeneration: A Comprehensive Review of Past and Ongoing Strategies. *Int. J. Mol. Sci.* 19. doi:10.3390/ijms19103194.

Gomes, C. P., Nóbrega-Pereira, S., Domingues-Silva, B., Rebelo, K., Alves-Vale, C., Marinho, S. P., et al. (2019). An antisense transcript mediates MALAT1 response in human breast cancer. *BMC Cancer* 19. doi:10.1186/s12885-019-5962-0.

Haemmig, S., Simion, V., Yang, D., Deng, Y., and Feinberg, M. W. (2017). Long noncoding RNAs in cardiovascular disease, diagnosis, and therapy. *Curr. Opin. Cardiol.* 32, 776–783. doi:10.1097/HCO.0000000000000454.

Hashimoto, H., Olson, E. N., and Bassel-Duby, R. (2018). Therapeutic approaches for cardiac regeneration and repair. *Nat. Rev. Cardiol.* 15, 585–600. doi:10.1038/s41569-018-0036-6.

Hashimoto, H., Wang, Z., Garry, G. A., Malladi, V. S., Botten, G. A., Ye, W., et al. (2019). Cardiac Reprogramming Factors Synergistically Activate Genome-wide Cardiogenic Stage-Specific Enhancers. *Cell Stem Cell* 25, 69-86.e5. doi:10.1016/j.stem.2019.03.022.

He, C., Hu, H., Wilson, K. D., Wu, H., Feng, J., Xia, S., et al. (2016). Systematic Characterization of Long Noncoding RNAs Reveals the Contrasting Coordination of Cis- and Trans-Molecular Regulation in Human Fetal and Adult Hearts. *Circ. Cardiovasc. Genet.* 9, 110–118. doi:10.1161/CIRCGENETICS.115.001264.

Hennessy, E. J., van Solingen, C., Scacalossi, K. R., Ouimet, M., Afonso, M. S., Prins, J., et al. (2019). The long noncoding RNA CHROME regulates cholesterol homeostasis in primates. *Nat. Metab.* 1, 98–110. doi:10.1038/s42255-018-0004-9.

Hobuß, L., Bär, C., and Thum, T. (2019). Long non-coding RNAs: At the heart of cardiac dysfunction? *Front. Physiol.* 10. doi:10.3389/fphys.2019.00030.

Honkoop, H., Bakker, D. E. M. De, Aharonov, A., Kruse, F., Tessadori, F., Peterson, J. C., et al. (2018). A metabolic switch from OXPHOS to glycolysis is essential for cardiomyocyte proliferation in the regenerating heart Summary The capacity to regenerate damaged tissues , such as the heart , various enormously amongst species . While heart regeneration is g. 1–39.

Honkoop, H., de Bakker, D. E. M., Aharonov, A., Kruse, F., Shakked, A., Nguyen, P. D., et al. (2019). Single-cell analysis uncovers that metabolic reprogramming by ErbB2 signaling is essential for cardiomyocyte proliferation in the regenerating heart. *Elife* 8, 1–27. doi:10.7554/eLife.50163.

Hudson, J. E., and Porrello, E. R. (2013). The Non-coding Road Towards Cardiac Regeneration. 909–923. doi:10.1007/s12265-013-9486-8.

Ieda, M., Fu, J., Delgado-olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., et al. (2010). Direct Reprogramming of Fibroblasts into Functional Cardiomyocytes by Defined Factors. *Cell* 142, 375–386. doi:10.1016/j.cell.2010.07.002.

Intlekofer, A. M., and Finley, L. W. S. (2019). Metabolic signatures of cancer cells and stem cells. *Nat. Metab.* 1, 177–188. doi:10.1038/s42255-019-0032-0.

Jia, G., Hill, M. A., and Sowers, J. R. (2018). Diabetic Cardiomyopathy: An Update of Mechanisms Contributing to This Clinical Entity. *Circ. Res.* 122, 624–638. doi:10.1161/CIRCRESAHA.117.311586.

Kolwicz, S. C., Purohit, S., and Tian, R. (2013). Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. *Circ. Res.* 113, 603–616. doi:10.1161/CIRCRESAHA.113.302095.

Kornfeld, J. W., and Brüning, J. C. (2014). Regulation of metabolism by long, non-coding RNAs. *Front. Genet.* 5, 1–8. doi:10.3389/fgene.2014.00057.

Kroemer, G., and Pouyssegur, J. (2008). Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell* 13, 472–482. doi:10.1016/j.ccr.2008.05.005.

Kumarswamy, R., Bauters, C., Volkmann, I., Maury, F., Fetisch, J., Holzmann, A., et al. (2014). Circulating long noncoding RNA, LIPCAR, predicts survival in patients with heart failure. *Circ. Res.* 114, 1569–1575. doi:10.1161/CIRCRESAHA.114.303915.

Lehman, J. J., and Kelly, D. P. (2002). Transcriptional activation of energy metabolic switches in the developing and hypertrophied heart. *Clin. Exp. Pharmacol. Physiol.* 29, 339–345. doi:10.1046/j.1440-1681.2002.03655.x.

Li, X., Wang, H., Yao, B., Xu, W., Chen, J., and Zhou, X. (2016). lncRNA H19/miR-675 axis regulates cardiomyocyte apoptosis by targeting VDAC1 in diabetic cardiomyopathy. *Sci. Rep.* 6, 36340. doi:10.1038/srep36340.

Li, Y., Zhang, J., Huo, C., Ding, N., Li, J., Xiao, J., et al. (2017). Dynamic Organization of lncRNA and Circular RNA Regulators Collectively Controlled Cardiac Differentiation in Humans. *EBioMedicine* 24, 137–146. doi:10.1016/j.ebiom.2017.09.015.

Lopaschuk, G. D., Collins-Nakai, R. L., and Itoi, T. (1992). Developmental changes in energy substrate use by the heart. *Cardiovasc. Res.* 26, 1172–1180. doi:10.1093/cvr/26.12.1172.

Malandraki-Miller, S., Lopez, C. A., Al-Siddiqi, H., and Carr, C. A. (2018). Changing Metabolism in Differentiating Cardiac Progenitor Cells—Can Stem Cells Become Metabolically Flexible Cardiomyocytes? *Front. Cardiovasc. Med.* 5, 1–21. doi:10.3389/fcvm.2018.00119.

Martik, M. L. (2020). Metabolism makes and mends the heart. *Elife* 9, 9–11. doi:10.7554/elife.54665.

Matkovich, S. J., Edwards, J. R., Grossenheider, T. C., De Guzman Strong, C., and Dorn, G. W. (2014). Epigenetic coordination of embryonic heart transcription by dynamically regulated long noncoding RNAs. *Proc. Natl. Acad. Sci. U. S. A.* 111, 12264–12269. doi:10.1073/pnas.1410622111.

Menendez-Montes, I., Escobar, B., Palacios, B., Gómez, M. J., Izquierdo-Garcia, J. L., Flores, L., et al. (2016). Myocardial VHL-HIF Signaling Controls an Embryonic Metabolic Switch Essential for Cardiac Maturation. *Dev. Cell* 39, 724–739. doi:10.1016/j.devcel.2016.11.012.

Mongelli, A., Martelli, F., Farsetti, A., and Gaetano, C. (2019). The dark that matters: Long noncoding RNAs as master regulators of cellular metabolism in noncommunicable diseases. *Front. Physiol.* 10, 1–13. doi:10.3389/fphys.2019.00369.

Muret, K., Désert, C., Lagoutte, L., Boutin, M., Gondret, F., Zerjal, T., et al. (2019). Long noncoding RNAs in lipid metabolism: literature review and conservation analysis across species. *BMC Genomics* 20, 882. doi:10.1186/s12864-019-6093-3.

Neidig, L. E., Weinberger, F., Palpant, N. J., Mignone, J., Martinson, A. M., Sorensen, D. W., et al. (2018). Evidence for minimal cardiogenic potential of stem cell antigen 1-positive cells in the adult mouse heart. *Circulation* 138, 2960–2962. doi:10.1161/CIRCULATIONAHA.118.035273.

Pant, T., Dhanasekaran, A., Fang, J., Bai, X., Bosnjak, Z. J., Liang, M., et al. (2018). Current status and strategies of long noncoding RNA research for diabetic cardiomyopathy. *BMC Cardiovasc. Disord.* 18, 1–10. doi:10.1186/s12872-018-0939-5.

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., et al. (2011). Transient regenerative potential of the neonatal mouse heart. *Science (80-.).* 331, 1078–1080. doi:10.1126/science.1200708.

Puente, B. N., Kimura, W., Muralidhar, S. A., Moon, J., Amatruda, J. F., Phelps, K. L., et al. (2014). The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* 157, 565–579. doi:10.1016/j.cell.2014.03.032.

Rindler, P. M., Crewe, C. L., Fernandes, J., Kinter, M., and Szweda, L. I. (2013). Redox regulation of insulin sensitivity due to enhanced fatty acid utilization in the mitochondria. *Am. J. Physiol. Circ. Physiol.* 305, H634–H643. doi:10.1152/ajpheart.00799.2012.

Schonrock, N., Harvey, R. P., and Mattick, J. S. (2012). Long noncoding RNAs in cardiac development and pathophysiology. *Circ. Res.* 111, 1349–1362. doi:10.1161/CIRCRESAHA.112.268953.

Song, K., Nam, Y. J., Luo, X., Qi, X., Tan, W., Huang, G. N., et al. (2012). Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* 485, 599–604. doi:10.1038/nature11139.

Soonpaa, M. H., Kim, K. K., Pajak, L., Franklin, M., and Field, L. J. (1996). Cardiomyocyte DNA synthesis and binucleation during murine development. *Am. J. Physiol.* 271, H2183-9. doi:10.1152/ajpheart.1996.271.5.H2183.

Stein, C. S., Jadiya, P., Zhang, X., McLendon, J. M., Abouassaly, G. M., Witmer, N. H., et al. (2018). Mitoregulin: A lncRNA-Encoded Microprotein that Supports Mitochondrial Supercomplexes and Respiratory Efficiency. *Cell Rep.* 23, 3710-3720.e8. doi:10.1016/j.celrep.2018.06.002.

Thum, T., and Condorelli, G. (2015). Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. *Circ. Res.* 116, 751–762. doi:10.1161/CIRCRESAHA.116.303549.

Van Gastel, N., Stegen, S., Eelen, G., Schoors, S., Carlier, A., Daniëls, V. W., et al. (2020). Lipid availability determines fate of skeletal progenitor cells via SOX9. *Nature* 579, 111–117. doi:10.1038/s41586-020-2050-1.

Van Soligen, C. (2018). Long Noncoding RNAs in Lipid Metabolism. *Curr Opin Lipidol.* 29, 224–232.

Vausort, M., Wagner, D. R., and Devaux, Y. (2014). Long noncoding RNAs in patients with acute myocardial infarction. *Circ. Res.* 115, 668–677. doi:10.1161/CIRCRESAHA.115.303836.

Viereck, J., Bührke, A., Foinquinos, A., Chatterjee, S., Kleeberger, J. A., Xiao, K., et al. (2020). Targeting muscle-enriched long non-coding RNA H19 reverses pathological cardiac hypertrophy. *Eur. Heart J.* doi:10.1093/eurheartj/ehaa519.

Vivien, C. J., Hudson, J. E., and Porrello, E. R. (2016). Evolution, comparative biology and ontogeny of vertebrate heart regeneration. *npj Regen. Med.* 1. doi:10.1038/npjregenmed.2016.12.

Wang, K., Long, B., Zhou, L.-Y., Liu, F., Zhou, Q.-Y., Liu, C.-Y., et al. (2014). CARL lncRNA inhibits anoxia-induced mitochondrial fission and apoptosis in cardiomyocytes by impairing miR-539-dependent PHB2 downregulation. *Nat. Commun.* 5, 3596. doi:10.1038/ncomms4596.

Wang, S., Zuo, H., Jin, J., Lv, W., Xu, Z., Fan, Y., et al. (2019). Long noncoding RNA Neat1 modulates myogenesis by recruiting Ezh2. *Cell Death Dis.* 10. doi:10.1038/s41419-019-1742-7.

Wang, Y., Hu, S. Bin, Wang, M. R., Yao, R. W., Wu, D., Yang, L., et al. (2018). Genome-wide screening of NEAT1 regulators reveals cross-regulation between paraspeckles and mitochondria. *Nat. Cell Biol.* 20, 1145–1158. doi:10.1038/s41556-018-0204-2.

Yang, F., Zhang, H., Mei, Y., and Wu, M. (2014). Frontiers. *Mol. Cell* 53, 88–100. doi:https://doi.org/10.1016/j.molcel.2013.11.004.

Yucel, N., Wang, Y. X., Mai, T., Porpiglia, E., Lund, P. J., Markov, G., et al. (2019). Glucose Metabolism Drives Histone Acetylation Landscape Transitions that Dictate Muscle Stem Cell Function. *Cell Rep.* 27, 3939-3955.e6. doi:10.1016/j.celrep.2019.05.092.

Zhang, L., Zhou, Y., Huang, T., Cheng, A. S. L., Yu, J., Kang, W., et al. (2017). The Interplay of LncRNA-H19 and Its Binding Partners in Physiological Process and Gastric Carcinogenesis. *Int. J. Mol. Sci.* 18. doi:10.3390/ijms18020450.

Zhao, X.-Y. (2015). Long Noncoding RNAs: a New Regulatory Code in Metabolic Control. *Trends Biochem. Sci.* 40, 586–596. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4584418/.

Zhuo, C., Jiang, R., Lin, X., and Shao, M. (2017). LncRNA H19 inhibits autophagy by epigenetically silencing of DIRAS3 in diabetic cardiomyopathy. *Oncotarget* 8, 1429–1437. doi:10.18632/oncotarget.13637.

Zolk, O., Solbach, T. F., Eschenhagen, T., Weidemann, A., and Fromm, M. F. (2008). Activation of negative regulators of the hypoxia-inducible factor (HIF) pathway in human end-stage heart failure. *Biochem. Biophys. Res. Commun.* 376, 315–320. doi:10.1016/j.bbrc.2008.08.152.

CHAPTER 8 - REFERENCES

Afonso, J. *et al.* (2020) 'Competitive glucose metabolism as a target to boost bladder cancer immunotherapy', *Nature Reviews Urology*, 17(2), pp. 77–106. doi: 10.1038/s41585-019-0263-6.

Bär, C., Chatterjee, S. and Thum, T. (2016) 'Long Noncoding RNAs in Cardiovascular Pathology, Diagnosis, and Therapy', *Circulation*, 134(19), pp. 1484–1499. doi: 10.1161/CIRCULATIONAHA.116.023686.

Bischof, C. and Krishnan, J. (2016) 'Exploiting the hypoxia sensitive non-coding genome for organ-specific physiologic reprogramming', *Biochimica et Biophysica Acta - Molecular Cell Research*. Elsevier B.V., 1863(7), pp. 1782–1790. doi: 10.1016/j.bbamcr.2016.01.024.

Cardoso, A. C. *et al.* (2020) 'Mitochondrial substrate utilization regulates cardiomyocyte cell-cycle progression', *Nature Metabolism*. Springer US, 2(2), pp. 167–178. doi: 10.1038/s42255-020-0169-x.

Chang, Y. *et al.* (2019) 'Efficient in vivo direct conversion of fibroblasts into cardiomyocytes using a nanoparticle-based gene carrier', *Biomaterials*. Elsevier, 192(November 2018), pp. 500–509. doi: 10.1016/j.biomaterials.2018.11.034.

Chen, D. *et al.* (2012) 'Dual function of the UNC-45b chaperone with myosin and GATA4 in cardiac development'. doi: 10.1242/jcs.106435.

Dal-Pra, S. *et al.* (2017) 'Demethylation of H3K27 Is Essential for the Induction of Direct Cardiac Reprogramming by MIR Combo', *Circulation Research*, 120(9), pp. 1403–1413. doi: 10.1161/CIRCRESAHA.116.308741.

Devaux, Y. *et al.* (2015) 'Long noncoding RNAs in cardiac development and ageing', *Nature Reviews Cardiology*. Nature Publishing Group, 12(7), pp. 415–425. doi: 10.1038/nrcardio.2015.55.

Doenst, T., Nguyen, T. D. and Abel, E. D. (2013) 'Cardiac Metabolism in Heart Failure', *Circulation Research*, 113(6), pp. 709–724. doi: 10.1161/circresaha.113.300376.

Fu, J. *et al.* (2013) 'Stem Cell Reports', *Stem Cell Reports*. The Authors, 1(3), pp. 235–247. doi: 10.1016/j.stemcr.2013.07.005.

Fu, J. D. *et al.* (2013) 'Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state', *Stem Cell Reports*. The Authors, 1(3), pp. 235–247. doi: 10.1016/j.stemcr.2013.07.005.

Fu, J. D. and Srivastava, D. (2015) ‘Direct reprogramming of fibroblasts into cardiomyocytes for cardiac regenerative medicine’, *Circulation Journal*, 79(2), pp. 245–254. doi: 10.1253/circj.CJ-14-1372.

Fukuda, R. *et al.* (2019) ‘Metabolic modulation regulates cardiac wall morphogenesis in zebrafish’, *eLife*, 8, pp. 1–14. doi: 10.7554/eLife.50161.

Ghiroldi, A. *et al.* (2017) ‘Regenerating the human heart : direct reprogramming strategies and their current limitations’, *Basic Research in Cardiology*. Springer Berlin Heidelberg, 112(6), pp. 1–14. doi: 10.1007/s00395-017-0655-9.

Goetz, S. C., Brown, D. D. and Conlon, F. L. (2006) ‘TBX5 is required for embryonic cardiac cell cycle progression’, 2584, pp. 2575–2584. doi: 10.1242/dev.02420.

Goldman, J. A. and Poss, K. D. (2020) ‘Gene regulatory programmes of tissue regeneration’, *Nature Reviews Genetics*. Springer US. doi: 10.1038/s41576-020-0239-7.

Hao, H. *et al.* (2011) ‘The Transcription Factor Neural Retina Leucine Zipper (NRL) Controls Photoreceptor-specific Expression of Myocyte Enhancer Factor Mef2c from an Alternative Promoter *’, 286(40), pp. 34893–34902. doi: 10.1074/jbc.M111.271072.

Hashimoto, H. *et al.* (2019) ‘Cardiac Reprogramming Factors Synergistically Activate Genome-wide Cardiogenic Stage-Specific Cardiac Reprogramming Factors Synergistically Activate Genome-wide’, *Stem Cell*. Elsevier Inc., 25(1), pp. 69-86.e5. doi: 10.1016/j.stem.2019.03.022.

Hashimoto, H., Olson, E. N. and Bassel-Duby, R. (2018) ‘Therapeutic approaches for cardiac regeneration and repair’, *Nature Reviews Cardiology*. Springer US, 15(10), pp. 585–600. doi: 10.1038/s41569-018-0036-6.

Hobuß, L., Bär, C. and Thum, T. (2019) ‘Long non-coding RNAs: At the heart of cardiac dysfunction?’, *Frontiers in Physiology*, 10(JAN), pp. 1–9. doi: 10.3389/fphys.2019.00030.

Honkoop, H. *et al.* (2019) ‘Single-cell analysis uncovers that metabolic reprogramming by ErbB2 signaling is essential for cardiomyocyte proliferation in the regenerating heart’, *eLife*, 8, pp. 1–27. doi: 10.7554/eLife.50163.

Hudson, J. E. and Porrello, E. R. (2013) ‘The Non-coding Road Towards Cardiac Regeneration’, (May), pp. 909–923. doi: 10.1007/s12265-013-9486-8.

Iacovides, D. *et al.* (2016) 'Direct conversion of mouse embryonic fibroblasts into functional keratinocytes through transient expression of pluripotency-related genes', *Stem Cell Research and Therapy*. Stem Cell Research & Therapy, 7(1), pp. 1–12. doi: 10.1186/s13287-016-0357-5.

Ieda, M., Fu, J. D., *et al.* (2010) 'Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors', *Cell*. Elsevier Ltd, 142(3), pp. 375–386. doi: 10.1016/j.cell.2010.07.002.

Ieda, M., Fu, J., *et al.* (2010) 'Direct Reprogramming of Fibroblasts into Functional Cardiomyocytes by Defined Factors', *Cell*. Elsevier Ltd, 142(3), pp. 375–386. doi: 10.1016/j.cell.2010.07.002.

Isomi, M., Sadahiro, T. and Ieda, M. (2019) 'Progress and Challenge of Cardiac Regeneration to Treat Heart Failure', *Journal of Cardiology*. Japanese College of Cardiology, 73(2), pp. 97–101. doi: 10.1016/j.jjcc.2018.10.002.

Jackaman, C. *et al.* (2007) 'Novel application of flow cytometry: Determination of muscle fiber types and protein levels in whole murine skeletal muscles and heart', *Cell Motility and the Cytoskeleton*, 64(12), pp. 914–925. doi: 10.1002/cm.20239.

Jagatheesan, G., Rajan, S. and Wieczorek, D. F. (2010) 'Investigations into tropomyosin function using mouse models', *Journal of Molecular and Cellular Cardiology*. Elsevier Ltd, 48(5), pp. 893–898. doi: 10.1016/j.yjmcc.2009.10.003.

Jayawardena, T., Mirotsou, M. and Dzau, V. J. (2014) 'Direct Reprogramming of Cardiac Fibroblasts to Cardiomyocytes Using MicroRNAs', *Methods Mol Biol*, 1150, pp. 263–272. doi: 10.1007/978-1-4939-0512-6.

Khosla, S. *et al.* (2020) 'The role of cellular senescence in ageing and endocrine disease', *Nature Reviews Endocrinology*. Springer US, 16(5), pp. 263–275. doi: 10.1038/s41574-020-0335-y.

Kitamura, T. *et al.* (2003) 'Retrovirus-mediated gene transfer and expression cloning: Powerful tools in functional genomics', *Experimental Hematology*, 31(11), pp. 1007–1014. doi: 10.1016/S0301-472X(03)00260-1.

Klattenhoff, C. A. *et al.* (2013) 'Braveheart, a long noncoding RNA required for cardiovascular lineage commitment', *Cell*. Elsevier Inc., 152(3), pp. 570–583. doi: 10.1016/j.cell.2013.01.003.

Kolwicz, S. C., Purohit, S. and Tian, R. (2013) 'Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes', *Circulation Research*, 113(5), pp. 603–616. doi: 10.1161/CIRCRESAHA.113.302095.

Laks, H. (1982) *Etiology and Morphogenesis of Congenital Heart Disease, The Annals of Thoracic Surgery*. doi: 10.1016/s0003-4975(10)61422-1.

Laszlo, G. S. *et al.* (2015) 'High expression of myocyte enhancer factor 2C (MEF2C) is associated with adverse-risk features and poor outcome in pediatric acute myeloid leukemia : a report from the Children ' s Oncology Group', *Journal of Hematology & Oncology*. Journal of Hematology & Oncology, pp. 1–10. doi: 10.1186/s13045-015-0215-4.

Li, H. *et al.* (2007) 'Deletion of Ku70 , Ku80 , or Both Causes Early Aging without Substantially Increased Cancer', 27(23), pp. 8205–8214. doi: 10.1128/MCB.00785-07.

Li, T. *et al.* (2019) 'The status of MAPK cascades contributes to the induction and activation of Gata4 and Nkx2 . 5 during the stepwise process of cardiac di ff erentiation', *Cellular Signalling*. Elsevier, 54(October 2018), pp. 17–26. doi: 10.1016/j.cellsig.2018.11.019.

Lin, C. *et al.* (2020) 'Arginine hypomethylation-mediated proteasomal degradation of histone H4—an early biomarker of cellular senescence', *Cell Death and Differentiation*. Springer US. doi: 10.1038/s41418-020-0562-8.

Liu, Z. *et al.* (2017) 'Single-cell transcriptomics reconstructs fate conversion from fibroblast to cardiomyocyte', *Nature*. Nature Publishing Group, 551(7678), pp. 100–104. doi: 10.1038/nature24454.

Ma, H. *et al.* (2015) 'In vivo cardiac reprogramming using an optimal single polycistronic construct', *Cardiovascular Research*, 108(2), pp. 217–219. doi: 10.1093/cvr/cvv223.

Malandraki-Miller, S. *et al.* (2018) 'Changing Metabolism in Differentiating Cardiac Progenitor Cells—Can Stem Cells Become Metabolically Flexible Cardiomyocytes?', *Frontiers in Cardiovascular Medicine*, 5(September), pp. 1–21. doi: 10.3389/fcvm.2018.00119.

Martik, M. L. (2020) 'Metabolism makes and mends the heart', *eLife*, 9, pp. 9–11. doi: 10.7554/elife.54665.

Matkovich, Scot J. *et al.* (2014) 'Epigenetic coordination of embryonic heart transcription by dynamically regulated long noncoding RNAs', *Proceedings of the National Academy of Sciences of the United States of America*, 111(33), pp. 12264–12269. doi: 10.1073/pnas.1410622111.

Méndez-Lucas, A. *et al.* (2020) 'Identifying strategies to target the metabolic flexibility of tumours', *Nature Metabolism*. Springer US, 2(4), pp. 335–350. doi: 10.1038/s42255-020-0195-8.

Menendez-Montes, I. *et al.* (2016) 'Myocardial VHL-HIF Signaling Controls an Embryonic Metabolic Switch Essential for Cardiac Maturation', *Developmental Cell*, 39(6), pp. 724–739. doi: 10.1016/j.devcel.2016.11.012.

Miyamoto, K. *et al.* (2018) 'Direct In Vivo Reprogramming with Sendai Virus Vectors Improves Cardiac Function after Myocardial Infarction', *Cell Stem Cell*. Elsevier Inc., 22(1), pp. 91-103.e5. doi: 10.1016/j.stem.2017.11.010.

Muraoka, N. *et al.* (2019) 'Role of cyclooxygenase-2-mediated prostaglandin E2-prostaglandin E receptor 4 signaling in cardiac reprogramming', *Nature Communications*. Springer US, 10(1), pp. 1–5. doi: 10.1038/s41467-019-08626-y.

Nam, Y. J. *et al.* (2013) 'Reprogramming of human fibroblasts toward a cardiac fate', *Proceedings of the National Academy of Sciences of the United States of America*, 110(14), pp. 5588–5593. doi: 10.1073/pnas.1301019110.

Palmero, I. and Serrano, M. (2001) 'Induction of Senescence by Oncogenic Ras', 138(1993), pp. 247–256.

Patel, V. *et al.* (2018) 'p63 Silencing induces reprogramming of cardiac fibroblasts into cardiomyocyte-like cells', *Journal of Thoracic and Cardiovascular Surgery*. Elsevier Inc., 156(2), pp. 556-565.e1. doi: 10.1016/j.jtcvs.2018.03.162.

Ponikowski, P. *et al.* (2014) 'Heart failure : preventing disease and death worldwide', (August), pp. 4–25. doi: 10.1002/ehf2.12005.

Qian, L. *et al.* (2012) 'In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes', *Nature*. Nature Publishing Group, 485(7400), pp. 593–598. doi: 10.1038/nature11044.

Rastegar-Pouyani, S. *et al.* (2017) 'Meta-Analysis of Transcriptome Regulation During Induction to Cardiac Myocyte Fate From Mouse and Human Fibroblasts', *Journal of Cellular Physiology*, 232(8), pp. 2053–2062. doi: 10.1002/jcp.25580.

Ritter, N. *et al.* (2019) 'The lncRNA Locus Handsdown Regulates Cardiac Gene Programs and Is Essential for Early Mouse Development', *Developmental Cell*, 50(5), pp. 644-657.e8. doi: 10.1016/j.devcel.2019.07.013.

Sadahiro, T. (2019) ‘Cardiac regeneration with pluripotent stem cell-derived cardiomyocytes and direct cardiac reprogramming’, *Regenerative Therapy*. Elsevier Ltd, 11, pp. 95–100. doi: 10.1016/j.reth.2019.06.004.

Di Salvo, T. G. (2015) ‘Evolving targeted therapies for right ventricular failure’, *Expert Opinion on Biological Therapy*, 15(9), pp. 1263–1283. doi: 10.1517/14712598.2015.1054277.

Shah, S. (2020) ‘Genome-wide association and Mendelian randomisation analysis provide insights into the pathogenesis of heart failure’, (2020), pp. 1–12. doi: 10.1038/s41467-019-13690-5.

Song, K. *et al.* (2012) ‘Heart repair by reprogramming non-myocytes with cardiac transcription factors’, *Nature*. Nature Publishing Group, 485(7400), pp. 599–604. doi: 10.1038/nature11139.

Sperandei, S. (2014) ‘Special Lessons issue: in biostatistics Responsible writing in science’, 24(3), pp. 311–320.

Sturtzel, C. *et al.* (2014) ‘The Transcription Factor MEF2C Negatively Controls Angiogenic Sprouting of Endothelial Cells Depending on Oxygen’, 9(7). doi: 10.1371/journal.pone.0101521.

Takahashi, K. and Yamanaka, S. (2006) ‘Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors’, *Cell*, 126(4), pp. 663–676. doi: 10.1016/j.cell.2006.07.024.

Talkhabi, M., Razavi, S. M. and Salari, A. (2017) ‘Global transcriptomic analysis of induced cardiomyocytes predicts novel regulators for direct cardiac reprogramming’, *Journal of Cell Communication and Signaling*. Journal of Cell Communication and Signaling, 11(2), pp. 193–204. doi: 10.1007/s12079-017-0387-5.

Tani, H. (2018) ‘Direct Cardiac Reprogramming : A Novel Approach for Heart Regeneration’, pp. 1–13. doi: 10.3390/ijms19092629.

Tani, H., Sadahiro, T. and Ieda, M. (2018) ‘Direct cardiac reprogramming: A novel approach for heart regeneration’, *International Journal of Molecular Sciences*, 19(9), pp. 1–13. doi: 10.3390/ijms19092629.

Testa, G. *et al.* (2020) ‘Bmi1 inhibitor PTC-209 promotes Chemically-induced Direct Cardiac Reprogramming of cardiac fibroblasts into cardiomyocytes’, *Scientific Reports*, 10(1), pp. 1–16. doi: 10.1038/s41598-020-63992-8.

Trembinski, D. J. *et al.* (2020) 'Aging-regulated anti-apoptotic long non-coding RNA Sarrah augments recovery from acute myocardial infarction', *Nature Communications*. Springer US, 11(1). doi: 10.1038/s41467-020-15995-2.

Vaseghi, H., Liu, J. and Qian, L. (2017) 'Molecular barriers to direct cardiac reprogramming', *Protein and Cell*. Higher Education Press, 8(10), pp. 724–734. doi: 10.1007/s13238-017-0402-x.

Vaseghi, H. R. *et al.* (2016) 'Generation of an inducible fibroblast cell line for studying direct cardiac reprogramming', *Genesis*, 54(7), pp. 398–406. doi: 10.1002/dvg.22947.

Vivien, C. J., Hudson, J. E. and Porrello, E. R. (2016) 'Evolution, comparative biology and ontogeny of vertebrate heart regeneration', *npj Regenerative Medicine*. The Author(s), 1(1), pp. 1–14. doi: 10.1038/npjregenmed.2016.12.

Wada, R., Muraoka, N., Inagawa, K., Yamakawa, H., Miyamoto, K. and Sadahiro, T. (2013) 'Induction of human cardiomyocyte-like cells from fi broblasts by de fi ned factors', 110(31). doi: 10.1073/pnas.1304053110.

Wada, R., Muraoka, N., Inagawa, K., Yamakawa, H., Miyamoto, K., Sadahiro, T., *et al.* (2013) 'Induction of human cardiomyocyte-like cells from fibroblasts by defined factors', *Proceedings of the National Academy of Sciences of the United States of America*, 110(31), pp. 12667–12672. doi: 10.1073/pnas.1304053110.

Wang, L. *et al.* (2014) 'Efficiency and Quality of Induced Cardiac Myocyte Reprogramming', pp. 237–244. doi: 10.1161/CIRCRESAHA.116.305547.

Wang, L., Liu, Z., Yin, C., Zhou, Y., *et al.* (2015) 'Improved generation of induced cardiomyocytes using a polycistronic construct expressing optimal ratio of Gata4, Mef2c And Tbx5', *Journal of Visualized Experiments*, 2015(105), pp. 1–7. doi: 10.3791/53426.

Wang, L., Liu, Z., Yin, C., Asfour, H., *et al.* (2015) 'Stoichiometry of Gata4, Mef2c, and Tbx5 influences the efficiency and quality of induced cardiac myocyte reprogramming', *Circulation Research*, 116(2), pp. 237–244. doi: 10.1161/CIRCRESAHA.116.305547.

Wang, Z. *et al.* (2016) 'The long noncoding RNA Chaer defines an epigenetic checkpoint in cardiac hypertrophy', *Nature Medicine*. Nature Publishing Group, 22(10), pp. 1131–1139. doi: 10.1038/nm.4179.

Yu, B. *et al.* (2020) 'Mitochondrial phosphatase PGAM5 modulates cellular senescence by regulating mitochondrial dynamics', *Nature Communications*, 11(1), p. 2549. doi: 10.1038/s41467-020-16312-7.

Zhang, Z. *et al.* (2019) 'Ensuring expression of four core cardiogenic transcription factors enhances cardiac reprogramming', *Scientific Reports*. Springer US, 9(1), pp. 1–10. doi: 10.1038/s41598-019-42945-w.

Zhang, Z., Zhang, W. and Nam, Y. J. (2019) 'Stoichiometric optimization of Gata4, Hand2, Mef2c, and Tbx5 expression for contractile cardiomyocyte reprogramming', *Scientific Reports*. Springer US, 9(1), pp. 1–10. doi: 10.1038/s41598-019-51536-8.

Zhu, J. and Thompson, C. B. (2019) 'Metabolic regulation of cell growth and proliferation', *Nature Reviews Molecular Cell Biology*. Springer US, 20(7), pp. 436–450. doi: 10.1038/s41580-019-0123-5.