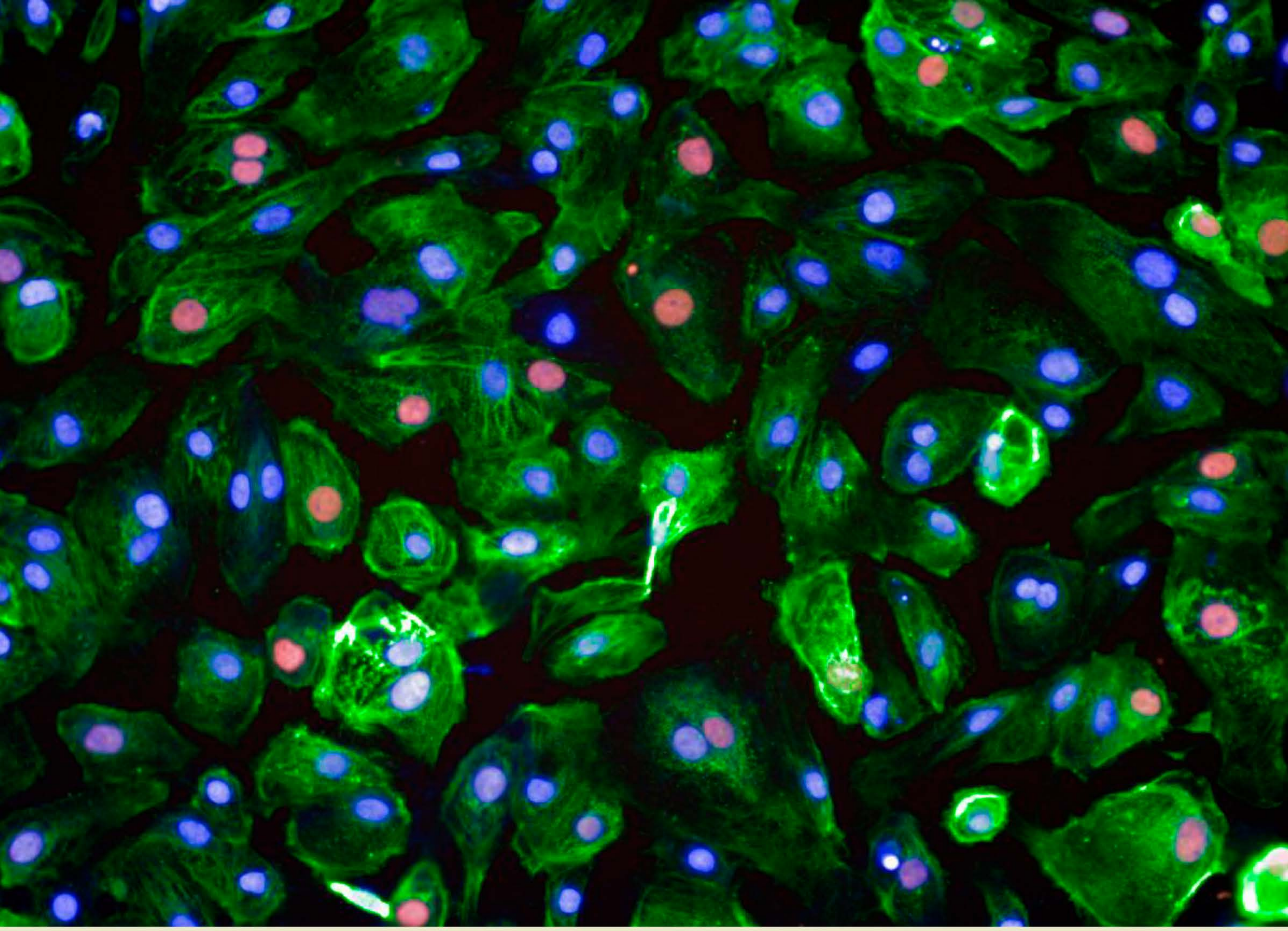

Raquel Figuinha Videira

Cardiac ischemia-reperfusion injury: *in vitro* models and regulation by microRNAs

Dissertação de Mestrado em Biologia Celular e Molecular,
orientada por Doutor Miguel Mano e Professora Doutora Ana Luísa Carvalho
e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra.

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UNIVERSIDADE DE COIMBRA

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Miguel Mano e da Professora Doutora Ana Luísa Carvalho, Centro de Neurociências e Biologia Celular, Universidade de Coimbra.

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“The important thing is to never stop questioning”

Albert Einstein

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Abbreviations

Abbreviations

2’-F – 2’-Fluor

2’-OMe – 2′-O-Methyl

2’-MOE – 2’-Methyoxyethyl

3’ UTR – 3’ Untranslated region

α- SMA – Alpha smooth muscle actin

A

AAVs – Adeno-associated virus

Ago2 – Argonaute 2

Akt – Protein kinase B

Arl2 – ADP-Ribosylation factor-like 2

ASOs – Antisense oligonucleotides

ATP – Adenosine triphosphate

B

Bcl2 – B-cell lymphoma 2

BSA – Bovine serum albumin

C

CAD – Coronary artery diseases

Cdc42 – Cell division control protein 42 homolog

CMs – Cardiomyocytes

CRISPR – Clustered regularly interspaced short palindromic repeats

CTGF – Connective tissue growth factor

D

DAMPs – Damage-associated molecular pattern molecules

DDX – DEAD box proteins

DGCR8 – DiGeorge syndrome critical region gene 8

DMEM – Dulbecco modified Eagle’s medium

DNA – Deoxyribonucleic acid

ds – Double strand

E

ECM – Extracellular matrix

EdU – 5-Ethynyl-2'-deoxyuridine

EDTA – Ethylenediaminetetraacetic acid

eIF4E – Eukaryotic translation initiation factor 4E

ET1 – Endothelin 1

F

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

Fsp1 – Fibroblast-specific protein 1

H

HCFs – Human cardiac fibroblasts

HCV – Hepatitis C virus

hiPSCs-CMs – Human induced pluripotent stem cell derived cardiomyocytes

HIF-1α – Hypoxia-inducible factor 1 alpha

HSP60 – Heat shock protein 60

I

iCell-CMs – Human induced pluripotent stem cell derived cardiomyocytes from Cellular Dynamics International

iCMs – human induced pluripotent stem cell derived cardiomyocytes from iBET

IL – Interleukins

IMS – Ischemia mimetic solution

IMS -Lac – Ischemia mimetic solution without lactate

IMS +Lac – Ischemia mimetic solution with lactate

L

LNA – Locked nuclei acid

M

MAPK – Mitogen activated protein kinases

MASP – Mannan-binding lectin serine protease

Mcl-1 – Myeloid cell leukemia 1

MI – Myocardial infarction

miRs – MicroRNAs

MMPs – Matrix metalloproteinases

mRNA – Messenger RNA

MRTF – Myocardin-related transcription factors

N

NFAT - Nuclear factor of activated T-cells

NF-Kb – Nuclear factor of kappa light polypeptide gene enhancer in B-cells

NHE-1 – Sodium-hydrogen antiporter 1

NO – Nitric oxide

P

p16 – Cyclin-dependent kinase-4 inhibitor

p38 – Mitogen-activated protein kinase

p85a – Inhibitory subunit of PI3K

P-bodies – Processing bodies

P-selectin – Platelet selectin

PBS – Phosphate buffer saline

PFA – Paraformaldehyde

PI3K – Phosphoinositide-3 kinase

PKC – Protein kinase C

PNA – Peptide nucleic acid

PS – Phosphorothioate

PTEN – Phosphatase and tensin homolog

R

RISC – RNA-induced silencing complex

RNA – Ribonucleic acid

ROCK – Rho-associated protein kinase

ROCKi – ROCK inhibitor

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute medium

RT – Room Temperature

S

SAFE – Survivor activating factor enhancement

SD – Standard deviation

siRNA – Small interfering RNA

SIRT1 – Sirtuin 1

SMAD – Mothers against decapentaplegic homolog

SRF – Serum response factor

STAT3 – Signal transducer and activator of transcription 3

T

TAR – RNA regulatory element

TCA cycle – Tricarboxylic acid cycle

Tcf 21 – Transcription factor 21

TGF-β – Transforming growth factor beta

TIMPs – Tissue inhibitor of metalloproteinases

TLR – Toll-like receptors

TNF-α – Tumor necrosis factor alpha

tPA – Tissue plasminogen activator

TRBP – RNA-binding protein TAR

tRNA – Transfer RNA

TRP – Transient receptor potential channels

X

XPO5 – Exportin 5

1.1 Abstract

Cardiovascular diseases, including myocardial infarction, are a leading cause of morbidity and mortality worldwide. Ischemia is a major event during myocardial infarction and results from the deprivation of blood to the heart, caused by the obstruction of a coronary artery. Reperfusion, i.e. the restoration of blood flow following an ischemic event, is the routine clinical procedure. Although reperfusion is essential to preserve the hypoxic myocardium, the sudden reoxygenation of the hypoxic tissues has important adverse effects, including initiation of cell death programs. This phenomenon, known as ischemia-reperfusion injury, is responsible for a fraction of the myocyte death observed following myocardial infarction. Since the human heart has limited regenerative capacity, dead cardiomyocytes are not renewed and, instead, a reparative scarring mechanism occurs. Cardiac fibrosis promoted by cardiac fibroblasts is the principal event underlying scar formation.

Upon ischemia-reperfusion injury, cardiac fibroblasts increase their proliferative rate and transform into a contractile and active form, called myofibroblasts. Myofibroblasts are hyper stimulated cells, that produce large amounts of ECM proteins such as collagens, contributing to ECM deposition. Although fibrosis is essential to mending a damaged heart, excessive scarring and persistence of myofibroblasts in the heart is a maladaptive event that leads to ventricle wall stiffness and impairment of heart function.

To minimize the effects caused by myocardial infarction, two main therapeutic endpoints could be pursued: i) increase of cardiomyocyte proliferation, to promote replacement of cells lost upon injury, and ii) blocking of excessive scarring process and fibrosis, which contributes to heart dysfunction.

MicroRNAs are endogenous small non-coding RNAs that play a major role in the post-transcriptional regulation of gene expression. Currently, 2,588 mature microRNAs are annotated in the human genome (miRBase Release 21, June 2014) and it is estimated that microRNAs might control the expression of ca. 60% of the human genes. MicroRNAs have been shown to be involved in diverse cellular functions such as proliferation, differentiation and apoptosis. The observation that each microRNA can regulate multiple target transcripts, together with the fact that microRNA expression can be modulated by synthetic molecules that mimic or prevent microRNA function (microRNA mimics and inhibitors, respectively), position microRNAs as attractive therapeutic tools.

This project is focused on the two main pathological events of myocardial infarction, ischemia-reperfusion and fibrosis, and the potential role of microRNAs in regulating these processes.

In the first stage of the project, we optimized experimental conditions to model cardiac ischemia-reperfusion injury *in vitro* and assessed the impact of injury to cardiomyocyte survival. For this purpose, we used two sources of human cardiomyocytes, both derived from induced pluripotent stem cells. Cells were first exposed to ischemia, achieved by incubation in a hypoxic environment for 24 or 48 hours in the absence of nutrients (except lactate in selected conditions) and subsequently returned to normal culturing conditions, mimicking the reperfusion step. We observed a strong decrease of cardiomyocyte number following ischemia-reperfusion injury, accompanied by changes in cell

morphology. In addition, we also optimized a reverse transfection protocol that can be used for transfection of microRNAs in large-scale screenings aimed at clarifying the role of microRNAs in ischemia-reperfusion injury. From these experiments, we were able to demonstrate that hsa-miR-302d-3p strongly induces proliferation of human cardiomyocytes.

The second stage of the project was focused on human cardiac fibroblasts. Cardiac fibroblasts were exposed to ischemia-reperfusion injury *in vitro* using a protocol similar to that applied for cardiomyocytes. Interestingly, our results suggested that ischemia-reperfusion leads to a slight increase of cardiac fibroblast proliferation, which is triggered by reperfusion. A reverse transfection protocol for microRNA transfection was also optimized and we performed a "proof-of-principle" pilot screening with 36 microRNAs, aiming at identifying microRNAs controlling myofibroblast activation. Among those, we identified microRNAs that have a strong activity in promoting (hsa-miR-26b-5p, hsa-miR-19b-2-5p, hsa-miR-2052, hsa-miR-875-5p, hsa-miR-210-3p and hsa-miR-19a-5p) or preventing (hsa-miR-1281, hsa-miR-130a-5p and hsa-miR-143-5p) myofibroblast activation. Additionally, it was also possible to establish a preliminary correlation between cardiac fibroblast activation and proliferation after microRNA treatment. Our findings indicate that cardiac fibroblasts transformation into myofibroblasts results in a significant decrease of their proliferation rate.

Overall, this study has established the experimental conditions for performing large-scale screening studies in human cardiomyocytes and cardiac fibroblasts, which will be important to identify novel microRNAs involved in ischemia-reperfusion injury.

Keywords: Ischemia-reperfusion injury, Cardiac fibrosis, Myofibroblasts, MicroRNAs, Cardiac regeneration

1.2 Resumo

As doenças cardiovasculares, nomeadamente o enfarte do miocárdio, são uma das principais causas de morbilidade e mortalidade a nível mundial. A isquémia é o principal acontecimento de um enfarte do miocárdio, e resulta da obstrução de uma artéria coronária, reduzindo o fluxo sanguíneo no coração. A reperfusão é uma intervenção médica na qual o fluxo sanguíneo é restabelecido após isquémia. Apesar de essencial à preservação do miocárdio isquémico, a rápida reoxigenação provocada pela reperfusão tem efeitos devastadores no tecido cardíaco, que incluem a ativação de mecanismos de morte celular. Este fenómeno, conhecido como lesão de isquémia-reperfusão, é em parte responsável pela morte de cardiomiócitos observada após o enfarte do miocárdio. Uma vez que o coração humano é incapaz de se regenerar, os cardiomiócitos danificados não são repostos e, em vez disso, é ativado um mecanismo reparador, conhecido como cicatrização. A fibrose cardíaca promovida por fibroblastos cardíacos é o principal evento subjacente à formação da cicatriz.

Após exposição a isquémia-reperfusão, os fibroblastos cardíacos aumentam a sua taxa proliferativa e transformam-se numa forma ativa e contrátil denominada de miofibroblastos. Os miofibroblastos são células híper estimuladas, que produzem grandes quantidades de proteínas de matriz extracelular (MEC), nomeadamente colagénio, contribuindo assim para a deposição de MEC. Embora a fibrose seja essencial para reparar danos cardíacos, a excessiva cicatrização e a permanência de miofibroblastos no coração é um processo patológico que pode conduzir à rigidez da parede do ventrículo, deteriorando a função cardíaca.

Para diminuir os efeitos provocados pelo enfarte do miocárdio, duas abordagens terapêuticas podem ser implementadas: i) aumentar a proliferação de cardiomiócitos para promover a substituição das células danificadas durante o enfarte e ii) bloquear a cicatrização excessiva e fibrose cardíaca, que contribuem para a disfunção cardíaca.

MicroARNs são pequenos ARN endógenos não-codificantes, que desempenham um papel chave na regulação pós-transcricional da expressão de genes. Atualmente, estão descritos 2588 microARNs maduros no genoma humano (miRBase Release em 21, Junho 2014) e é estimado que estes microARNs possam controlar até 60% da expressão de genes humanos. Foi demonstrado que os microARNs estão envolvidos em diversas funções celulares como a proliferação, diferenciação celular e apoptose. A observação de que cada microARN é capaz de regular vários transcritos alvo, conjuntamente com o facto de que a sua expressão pode ser modulada por moléculas sintéticas que mimetizam ou antagonizam a função de cada microARN (mímicos ou inibidores de microARNs, respetivamente), posicionam os microARNs como atrativas ferramentas terapêuticas.

Este projeto foca-se nos dois eventos patológicos associados ao enfarte do miocárdio, nomeadamente, a isquémia-reperfusão e a fibrose cardíaca, e no potencial papel dos microARNs na regulação destes processos.

A primeira parte deste projeto visou a otimização das condições experimentais para mimetizar os danos provocados pela isquémia-reperfusão cardíaca, e posteriormente, a avaliação do impacto desses mesmos danos na sobrevivência dos cardiomiócitos. Para tal, foram usados cardiomiócitos humanos de duas origens diferentes, mas ambos derivados de células estaminais pluripotentes induzidas. As células foram expostas a isquémia, obtida

através de incubação das células num ambiente hipóxico, durante 24 ou 48 horas na ausência de nutrientes (exceto lactato, em condições específicas), e subsequentemente, devolvidas às condições normais de cultura, simulando o passo da reperfusão. Após a lesão de isquémia-reperfusão observou-se um forte decréscimo no número de cardiomiócitos, acompanhado por alterações na morfologia celular. Adicionalmente, foi também possível otimizar o protocolo de transfeção reversa de microARNs que poderá ser usado para a realização de um screening de larga escala com o objetivo de clarificar o papel dos microARNs na lesão de isquémia-reperfusão. Através destas experiências foi possível demonstrar que o hsa-miR-302d-3p induz um forte aumento da capacidade proliferativa de cardiomiócitos humanos.

A segunda fase do projeto focou-se em fibroblastos cardíacos humanos. Os fibroblastos cardíacos foram expostos a lesão de isquémia-reperfusão *in vitro* usando um protocolo semelhante ao utilizado em cardiomiócitos. Curiosamente, os resultados obtidos sugerem que a isquémia-reperfusão leva a um ligeiro aumento da proliferação de fibroblastos cardíacos, que é estimulado pela reperfusão. O protocolo de transfeção reversa para microARNs foi também otimizado, e foi realizado ainda um screen piloto com 36 microRNAs, que serviu de "prova de princípio" para a validação da tecnologia e identificação de microARNs que controlam a ativação de miofibroblastos. De entre os microRNAs testados, identificámos microARNs com um efeito marcante na estimulação (hsa-miR-26b-5p, hsa-miR-19b-2-5p, hsa-miR-2052, hsa-miR-875-5p, hsa-miR-210-3p e hsa-miR-19a-5p) ou na repressão (hsa-miR-1281, hsa-miR-130a-5p e hsa-miR-143-5p) da ativação de miofibroblastos. Adicionalmente, foi também possível estabelecer uma correlação preliminar entre a ativação e a proliferação dos fibroblastos cardíacos após tratamento com microARNs. Os nossos resultados indicam que a transformação de fibroblastos cardíacos em miofibroblastos resulta num significativo decréscimo da sua taxa de proliferação.

Em geral, este estudo estabeleceu as condições experimentais necessárias à realização de um screen em larga escala em cardiomiócitos e fibroblastos cardíacos humanos, que será importante para a identificação de novos microARNs envolvidos na lesão de isquémia-reperfusão.

Palavras-chave: Lesão de isquémia-reperfusão, Fibrose cardíaca, Miofibroblastos, microARNs, Regeneração Cardíaca

2. Introduction

2.1 Heart Regeneration

From early embryogenesis, human life depends on a healthy heart to survive, the organ responsible for pumping blood to all parts of the body. Heart dysfunction caused by multiple insults has deleterious consequences that can ultimately result in death.

The human heart is a heterogeneous organ composed by different cells types, including epicardium cells that form the outer layer of the heart and cardiomyocytes that constitute the myocardium, the middle muscular layer of the heart. Notably, cardiomyocytes are supported by other types of cells, most importantly cardiac fibroblasts, which are responsible for supporting cardiomyocyte function and extracellular matrix (ECM) remodeling. The heart is also composed of pacemaker cells and purkinje fibers responsible for the generation and transmission of electrical impulses. In addition, endothelial cells and smooth muscle cells constitute the endocardium (heart inner layer), blood vessels and cardiac valves (Fig. 1). Despite their distinct functions, the different cardiac cell types interact, influence and respond to each other stimuli and the proper function of all cell types is required for normal heart function [1] .

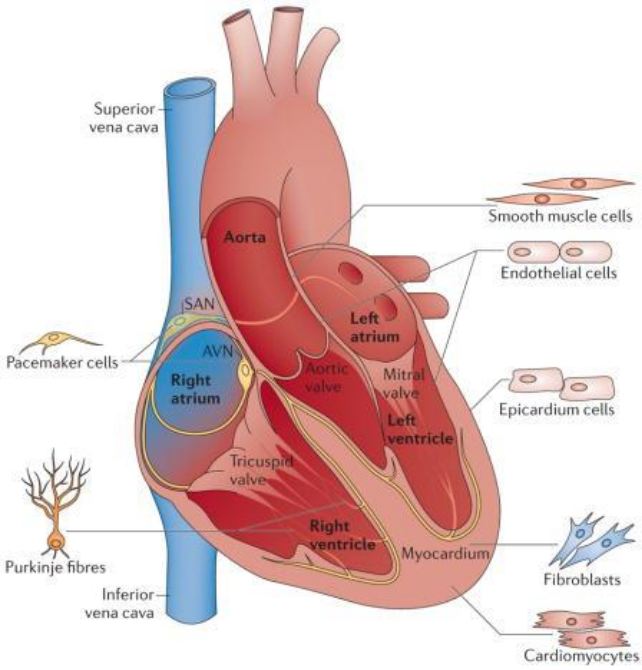


Figure 1. Human heart cell types. The heart is composed by several different cell types, including cardiomyocytes and cardiac fibroblasts. Adapted from: Xin, M., E.N. Olson, and R. Bassel-Duby, Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol, 2013. 14(8): p. 529-41.

Cardiovascular diseases, including myocardial infarction (MI), are a leading cause of morbidity and mortality worldwide. In the United States, 400,000 new cases of MI are reported every year [2]. Unfortunately, only 25 to 30% of these individuals survive in the following five years [3]. During MI, the myocardium is subjected to ischemia for a long period of time, and consequently, cardiomyocytes are lost and a scar is formed mainly via fibrosis [4]. Ideally, this loss should be overcome by cardiomyocyte regeneration, a process where damaged cardiomyocytes are replaced by new functional cardiomyocytes, but this process is highly inefficient in higher mammals.

Heart regeneration, achieved via proliferation of existing cardiomyocytes, has been shown to occur in adult animals, in particular lower vertebrates such as zebrafish, but not in higher mammals [5]. Following apical resection of the heart, zebrafish cardiomyocytes are able to proliferate and recover their quantitative, morphologic and contractile properties with a new ventricular wall being formed at the injured area. In this scenario, regeneration occurs mainly through proliferation of preexisting cardiomyocytes [6]. Unlike zebrafish, when mammals undergo heart injury, such as that induced by prolonged ischemia, cardiomyocyte proliferation is very limited and the necrotic myocardium is not renewed [7] and, instead, an adaptive mechanism involving scarring occurs. It is important to note that, although cardiac fibrosis is a necessary reparative system in the first phase to avoid cardiac rupture, fibrosis can become maladaptive and compromise heart function.

Similar to humans, the adult mouse heart also demonstrates limited regenerative capacity; however, in the first days after birth the regenerative potential is still high and sufficient to counteract injury. Indeed, the heart of neonatal mouse is able to restore cardiac function after partial surgical resection (ventricular apex) through cardiomyocyte proliferation, showing that the neonatal mouse heart has regenerative capacity similar to what is observed in zebrafish, where after 20% ventricle resection, heart completely regenerates without forming a fibrotic tissue [8].

In 2009, Bergmann *et al.* [9] reported that 55% of human cardiac cells persist from birth to adult life while 45% is renewed over the human lifespan, suggesting a very low renewal rate – at the age of 20, only 1% of cardiomyocytes is renewed per year. Moreover, it was also observed a decrease of human cardiomyocyte renewal throughout human life; in fact, at age of 75 only 0.4% cells are regenerated per year. Using hearts from rats with 4, 12, 16 and 27 months of age (representing young adult, fully mature adult, aged and senescence stages, respectively) Kajustura *et al.* [10] showed that, at 4 and 12 months of age, cardiomyocytes with short telomeres represent 6% of the total population, while at the age of 27 months this number rises to 16%, indicating that the number of cardiomyocytes with proliferative capacity has decreased. Moreover, differences in p16 expression (a senescence marker) were even more dramatic: after birth p16 is present in only 10% of cardiomyocyte, where in hearts from old animals this number increases to 80%. This study showed that although cardiomyocytes have proliferative capacity, it is very limited, and substantially decreases with aging.

Under normal circumstances, this low regenerative capacity does not compromise heart function, but in the event of an insult such as MI, it becomes problematic since the low rate of cardiomyocyte proliferation cannot compensate deleterious events and recover heart function. Several strategies have been tested in an attempt to overcome the lack of cardiomyocyte proliferation after injury.

One strategy consists in the usage of cardiomyocytes derived from embryonic stem cells or induced pluripotent stem cells (iPSCs), a type of pluripotent cells that can be obtained from human fibroblasts through forced expression of four transcription factors [11]. These pluripotent cells can then be induced to differentiate in cells of the cardiac lineage, in particular cardiomyocytes [12]. Importantly, the newly differentiated cardiomyocytes have been shown to exhibit similar properties and function of regular cardiomyocytes [12].

Other approaches are based on the stimulation of cardiomyocyte proliferation by modulating molecules such as cytokines [13] and microRNAs [14] (discussed below). These approaches can either function individually or be combined with other therapies, aiming at enhancing treatment efficacy. Furthermore, they could also be employed in models to study tissue injury caused by ischemia, e.g. by promoting cardiomyocyte differentiation from iPSCs.

2.2 Ischemia-Reperfusion

Ischemia is a pathological event described as the sudden lack of blood supply and subsequent restriction of oxygen and nutrients, essential for proper cell function, survival and homeostasis. Ischemia typically occurs due to occlusion of an artery, e.g. caused by a clot [4].

The majority of ischemia studies are focused on brain, heart and kidney, because of their severity; however, ischemia can also occur in the limbs, or other tissues and organs.

In the case of ischemia due to occlusion of an artery, the standard clinical intervention is the urgent recovery of blood flow (revascularization) with subsequent restauration of oxygen and nutrients to the affected tissues. This can be achieved through percutaneous coronary intervention (PCI, also known as angioplasty with stent) which is a catheter-based procedure to insert a stent to open an obstructed artery (Fig. 2) or by the administration of drugs such as tPA (tissue plasminogen activator) to break the clots. Although revascularization is essential to prevent the irreversible damage caused by ischemia, tissue reperfusion has also consequences and can aggravate injury caused by ischemia.

A number of pathological conditions are directly related with ischemia-reperfusion tissue injury including brain stroke, acute kidney injury, sleep apnea, solid organ transplantation, cardiac arrest and coronary artery diseases (CAD) [15]. Myocardial infarction, included in the CAD group of disease, results from prolonged exposure to ischemia caused by obstruction of a coronary artery that irrigates the heart. As a consequence, the heart does not receive oxygen and nutrients and myocardial cells become ischemic. This process is frequently related with other conditions such as arteriosclerosis, where accumulation of cholesterol forms a plaque diminishing blood flow [16]. Myocardial infarction can be divided in two main events, the first is ischemia-reperfusion that results in heart damage which, in turn, induces cardiac fibrosis, the second focal event [15].

Cardiac ischemia-reperfusion can lead to myocardial stunning (decrease of heart contraction), microvascular dysfunction, alterations of tissue barrier function and endothelial injury. Eventually, a severe and continued ischemia-reperfusion injury could lead to multiple organ dysfunction and heart failure [16].

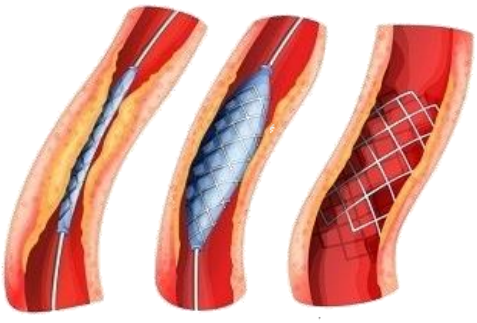


Figure 2. Overview of the percutaneous coronary intervention (PCI). Briefly, a deflated balloon catheter and a closed stent are inserted into the obstructed artery. The stent is then expanded by inflation of the balloon. Finally, catheter is removed and blood flow is restored. Adapted from: *www.healthwork.com.br*, "Um novo stent começa a ser utilizado" published by Adin in 9/12/2014 visited in 20/6/2016

Several changes also occur both at the metabolic and structural levels. Ischemia-reperfusion injury is a complex phenomenon that involves the immune system, transcriptional reprogramming, cell death programs, and ultimately, irreversible damage that can culminate in cell death - lethal ischemia-reperfusion injury [17]**.**

2.2.1 Molecular Findings

The initial phase of myocardial infarction is associated with inflammation, however, this is a sterile process since rarely bacteria or other microorganisms are involved. Damage-associated molecular patterns (DAMPs) are ligands able to induce an immune response in sterile conditions. They can derive from endogenous or exogenous sources, namely necrotic cells, or result from ECM degradation. Upon a damage stimulus, DAMPs can act as neoepitopes, triggering innate immune response receptors (Fig. 3) such those mediated by the Toll-like receptor family (TLRs) [18]. Among the elements of this family, TLR4 appears to be the most important during inflammation response, and its activation induces the release of cytokines and triggers an immune response that includes neutrophil and monocyte recruitment, complement system activation and platelet aggregation [15].

The complement system is part of the innate immune system and it is constituted by multiple cytoplasm proteins including C5a and C5b9, responsible for inflammation and cellular lysis, respectively (Fig. 3). In fact, C5a activates the release of cytokines as IL-1, IL- 6 and TNF-α, recruiting monocytes and neutrophils to the risk area, whereas, C5b9 affects cell membrane pore formation which facilitates leucocyte adhesion via NF-Kb activation [16].

Additionally, different stimuli such as antibodies (also known as the classical pathway), MASP (mannose binding lectin-associated serine proteases) pathway or alternative pathways are also prone to activate the complement system.

As outlined, with the onset of cardiac ischemia-reperfusion injury, TLRs are activated and neutrophils are recruited. Neutrophils are phagocytic leucocytes able to activate platelets that release inorganic phosphate, which acts as a pro-inflammatory molecule. In addition, neutrophils are recruited by inflammatory molecules, such as IL-6, TNF-α and IL-8, to the damaged areas of the myocardium. Reactive oxygen species (ROS) can also recruit

neutrophils, however, the reverse process can also happen, and neutrophils can lead to an increase in ROS and to an amplification of the immune response [18].

During ischemia, hypoxanthine accumulates inside cells and when myocardial cells are reoxygenated, xanthine oxidase generates ROS from hypoxanthine. In addition to xanthine oxidase, other enzymes can contribute to ROS production, such as cyclooxygenase and cytochrome c oxidase. Indeed, several potent oxidants are produced in a few minutes following ischemia [16].

An increment of ROS levels can be particularly dangerous to the cells, since ROS damage DNA and inactivate specific enzymes. Moreover, ROS can also lead to peroxidation of polyunsaturated lipids present on the cell membrane, damaging the sarcolemma and affecting the function of membrane-bound proteins. At the same time, high levels of ROS accumulate inside mitochondria, activating pro-apoptotic molecules and pathways [19]. Vessel vasoconstriction can also be induced by conjoint action of ROS and endothelin-1 (ET1) [16]. Additionally, ROS can also promote leucocyte activation, which is enhanced due to a raise in arachnoid acid via phospholipase A2 increase [19]. ROS can also prone activation of TLR4 resulting in neutrophil recruitment [15].

ROS increment is closely related with hypoxic pathways. Under hypoxia, hypoxia-inducible factor 1 alpha (HIF-1α) is no longer targeted to ubiquitination and degradation. Thus, HIF-1α is able to migrate into the nucleus where it acts as a transcription factor, enhancing the expression of glycolytic enzyme genes and inhibiting oxidative phosphorylation related-enzymes, particularly pyruvate dehydrogenase [20]. In summary, HIF-1α stabilization promotes transcriptional reprogramming and a metabolic shift from oxidative phosphorylation to glycolysis.

As consequence of glycolysis activation, lactate accumulates in the cytoplasm leading to a decrease of the intracellular pH, which disrupts membrane potential and impairs ATP production. Without ATP, the Na^+/K^+ pump becomes dysfunctional, stimulating the Na^+/H^+ exchange type 1 (NHE-1) system, and subsequently sodium accumulates inside the cell triggering the reversion of the Na^+/Ca^{2+} exchanger. As a consequence of the increase of intracellular calcium levels, calcium accumulates in mitochondria and the mitochondrial permeability transition pore opens promoting mitochondrial swelling that, conjointly with other events, leads to cellular death [21].

Hypoxia also activates NF-KB, a transcription factor involved in apoptosis and inflammation. It should be noted that the metabolic switch from oxidative phosphorylation to glycolysis allows cells to be viable for a longer period of time, allowing generation of energy (ATP) in the absence of oxygen [20].

As mentioned previously, reperfusion, although necessary, has also adverse effects, leading to activation of cell death programs including apoptosis, autophagy and necrosis (Fig. 3).

Apoptosis is a controlled form of cell death, characterized by nuclear fragmentation, cell shrinkage and blebbing. Apoptosis can be triggered by cytokines such as TNF-α, leading to caspase activation, namely caspase 3, and cytochrome c release. Although myocardial apoptosis is rare, under ischemic conditions, cell death increases. As aforementioned, during ischemia-reperfusion a transcriptional reprogramming occurs and the expression of pro-apoptotic genes is upregulated, while that of pro-survival genes is repressed [22].

Necrosis is triggered by high levels of intracellular calcium, which impair the respiratory chain and increase inner mitochondrial membrane permeability. Consequently, the electrical gradient is lost and the mitochondrial pore opens. Water enter mitochondria provoking mitochondria swelling, matrix deformation and ATP production stops. Eventually, the cell membrane ruptures and the cell content is released without any functional organelles (in contrast to apoptosis) [22].

Autophagy is responsible for degradation of unnecessary/abnormal proteins or organelles via fusion with the lysosome. Although autophagy is a process that operates continuously, it can also be triggered by injury. Ischemia-reperfusion injury damages myocardial cells, resulting in cell starvation caused by the lack of nutrients and oxygen, and consequently decreases the levels of ATP. To cope with those lower energy levels, autophagy is activated, providing an additional source for energy (e.g. by releasing amino acids); however, when physiological limits are exceeded, massive cellular destruction by autophagy leads to cell dysfunction and consequently death. Autophagy distinguishes from other cell death programs by the involvement of lysosomes and autophagosomes [22].

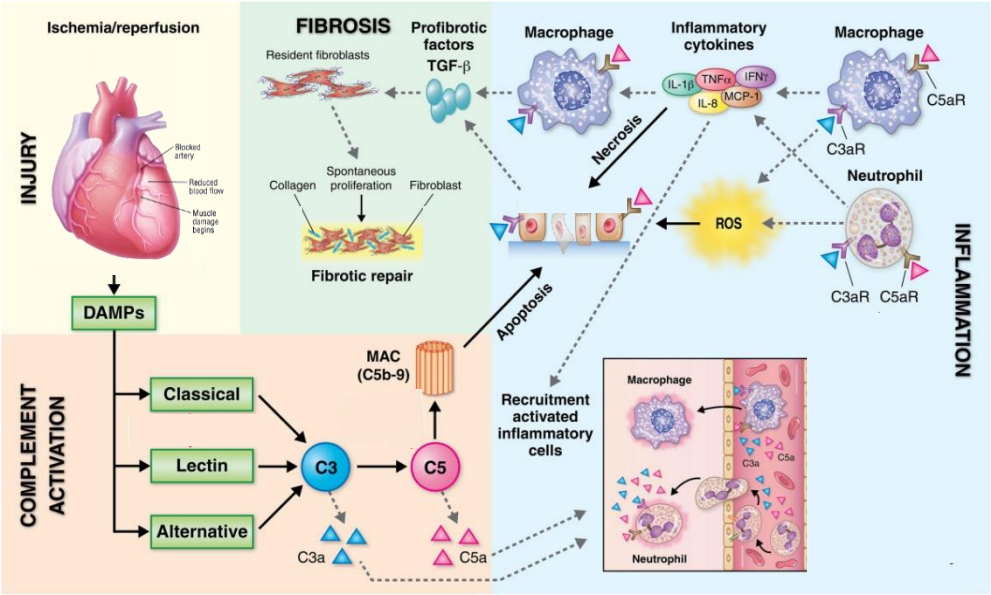


Figure 3. Cardiac ischemia-reperfusion injury pathways. During ischemia-reperfusion injury, DAMPs induce inflammation in sterile conditions, triggering complement system activation including proteins C3 and C5 which recruit neutrophils and macrophages to the affected area. In turn, increased ROS and interleukins activate cell death programs, which leads to release of pro fibrotic factors as TGF-β and consequently cardiac fibrosis. Cardiac fibrosis is a reparative response mediated by fibroblasts proliferation and ECM protein deposition, in particular collagen. Adapted from: www.drugs.com "Heart Attack (Myocardial Infarction)" published by Harvard Health Publications, consulted in 20/6/2016 and Danobeitia, Juan S., Arjang Djamali, and Luis A. Fernandez. "The role of complement in the pathogenesis of renal ischemia-reperfusion injury and fibrosis." Fibrogenesis & tissue repair, (2014) 7(1) 1.

2.3 Cardiac Fibrosis

2.3.1 Cardiac Fibroblasts and Scar Formation

During myocardial infarction, ischemia is the primordial event responsible for activation of pro-fibrotic pathways. Briefly, cardiomyocyte death stimulates an inflammatory response, probably through TGF-β signaling pathway (Fig. 3), leading to fibrosis and myofibroblast phenotype activation. Reversion of myocardium damage requires that cardiomyocyte regeneration occurs without scar formation [23].

Cardiac fibrosis is manifested by fibroblast proliferation and migration to the affected area and an accumulation of large amounts of extracellular matrix (ECM). The ECM network plays an important role in contractile force transmission and, since it serves as a scaffold, it also confers mechanical support to the heart. As such, cardiac contraction and relaxation depends strictly on the ECM.

The heart matrix is comprised primarily of elastins, fibronectins, basement membranes and fibrillar collagen, namely, collagen I and III. Collagen I associated with thick fibers maintains tensile strength and collagen III is related with thin fibers and it is thus responsible for muscle elasticity.

In basal conditions, ECM undergoes turnover with sustained degradation and synthesis of collagens and other components. This process is balanced by matrix metalloproteases (MMPs) that degrade the matrix, and MMP inhibitors, also known as TIMPs, blocking MMP action. In turn, synthesis of ECM is stimulated by cytokines and growth factors such as TNF-α, IL-6, TGF-β and CTGF. Cardiac fibroblasts, spindle-shape cells, are responsible for ECM regulation [24].

Cardiac fibroblasts are the most numerous cell type in the heart, and play an important role during development, physiology and injury. In the healthy adult mammalian heart, fibroblasts remain in a quiescent state and their proliferation rate is low. Fibroblasts support cardiomyocytes by producing and remodeling the ECM scaffold, which results in a more efficient distribution of the mechanical stress, homeostasis maintenance, and heart function [25]. Indeed, loss of cardiac fibroblasts, or at least, a decrease of their activity, creates a lack of mechanical strength, required for the heart to function properly. Upon an ischemia-reperfusion insult, homeostatic interactions are disrupted and heart requires reparative mechanisms for healing. This process is intimately related with cardiac fibroblasts and it is composed by three main phases: inflammation, proliferation and maturation.

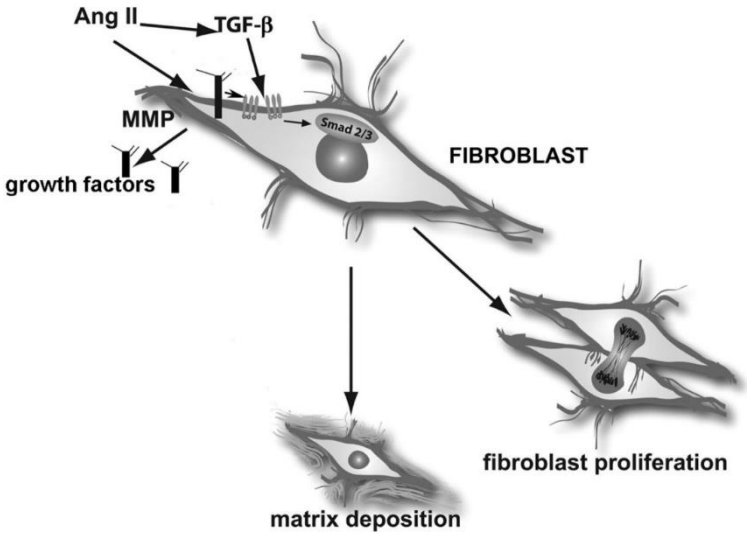


Figure 4. Cardiac fibrosis. Fibrosis, triggered by pro-fibrotic molecules such as AngII and TGF-β, is manifested by matrix deposition and fibroblast proliferation. Adapted from: Frangogiannis NG. Syndecan-1: a critical mediator in cardiac fibrosis. Hypertension. 2010;55 p. 233–235.

During inflammation, the first phase of the healing process, inflammatory molecules released by injured cells lead to the production of MMPs, which actively degrade the matrix and modify the ECM composition. Inflammatory molecules also trigger cardiac fibroblast proliferation (second phase) and activation into myofibroblasts. Subsequently, synthesis of collagen I and non-fibrillar collagen (collagen type VI) is upregulated and the matrix is reestablished, forming the scar tissue (maturation phase) [25]. However, at least two thirds of cardiac fibroblasts remain into the activated form, myofibroblasts, and continue to release pro-fibrotic signals as TGF-β (Fig. 4). As a result, the adaptive response becomes maladaptive and contractile function of the heart its compromised [26].

2.3.2 Myofibroblasts

Myofibroblasts are cells extremely sensitive to growth factors and pro-inflammatory molecules. Interestingly, myofibroblasts share characteristics of smooth muscle cells, due to expression of contractile stress fibers, and of fibroblasts, since they also exhibit an extensive endoplasmic reticulum that allows secretion of large amounts of ECM proteins. Indeed, myofibroblasts are able to contract and proliferate (Fig. 5).

The major source of myofibroblasts are endogenous cardiac fibroblasts, although they can also derive from other cells, including bone-marrow, blood and endothelial cells. Therefore, different forms of fibrosis may be due to transformation of cells from different sources into myofibroblasts [25].

Although specific and sensitive gene markers for cardiac fibroblasts have not been described, the most commons include Fibroblast-specific protein 1 (Fsp1) and Tcf21 (essential to cardiac fibroblast cell fate determination). Myofibroblasts major markers include periostin, which is expressed only by few cells in the normal heart, but it is clearly increased in cardiac fibroblasts following injury, and alpha smooth muscle actin (α-SMA) which is typically expressed in mature myofibroblasts. Alpha SMA is an actin isoform that provides mechanical

support and enables cell movement and, along with myosin and ATP, it aids cell contraction [25].

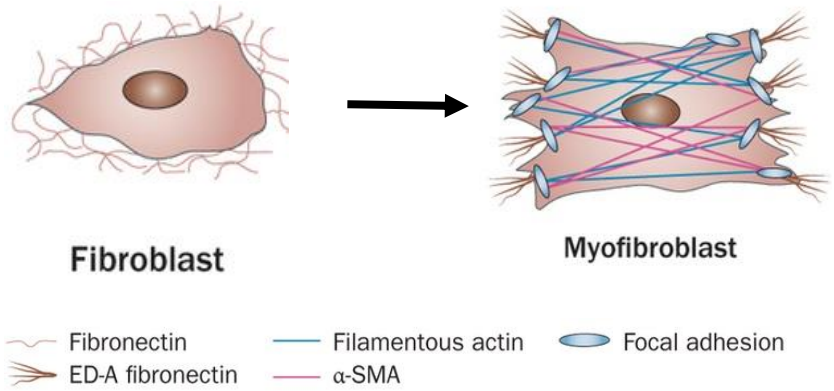


Figure 5. Myofibroblast phenotype activation. Transformation of cardiac fibroblasts in myofibroblasts involves expression of filamentous actin and α-SMA. Adapted from: Falke, Lucas L., et al. "Diverse origins of the myofibroblast - implications for kidney fibrosis." Nature Reviews Nephrology, 2015, 11(4) p. 233-244.

2.3.3 Regulation of Myofibroblast Activation

Myofibroblast transformation mediated by cardiac injury is supported by an environment rich in biochemical and mechanical stimuli provided by activation of different signaling pathways.

At an early stage of cardiac injury, fibrotic remodeling and myofibroblast activation is promoted by high levels of TGF-β that can further instigate canonical (SMAD) and non-canonical (MAPK-p38) pathways [26]. In the first case, binding of TGF-β to its receptor allows SMAD4 activation and translocation into the nucleus where it increases the rate of pro-myofibroblast gene transcription while in the non-canonical pathway, MAPK and its signaling branches, namely JNK and p38, are activated, also leading to the expression of myofibroblast-related genes. Similar phenotypes are observed when canonical or non-canonical TGF-β pathways are repressed, indicated that, potentially, both of them are key regulators of the fibrotic response, including of myofibroblast activation [26].

Angiotensin II (AngII), a pro-myofibroblast factor upregulated upon cardiac injury, is an upstream inducer of TGF-β signaling. AngII is able to activate the expression of pro-fibrotic genes like collagen and fibronectin both by SMAD and MAPK-SRF signaling. Indeed, AngII inhibitors such as losartan, impair the fibrotic response in the heart. Moreover, it has been reported that AngII increases ET1, a peptide which expression is triggered after cardiac injury, stimulating the wound healing process. This suggests that AngII, ET1 and TGF-β act synergistically to promote and maintain a myofibroblast phenotype [26].

In a later phase, establishment of the contractile and migratory phenotype of myofibroblasts is supported by the Rho-MRTF-SRF pathway. Promoter regions of genes responsible for myofibroblast differentiation namely the α-SMA gene, are enriched in serum response factor (SRF) binding sites. SRF is a transcriptional factor that has as cofactor the myocardin-related transcription factor family (MRTF) and therefore SRF activity is positively regulated by MRTF. In fact, repression of SRF function via short hairpin RNA administration represses myofibroblast activation [27].

Another relevant activator of myofibroblast phenotype is the transient receptor potential (TRP) channel family. TRP channels are permeable to calcium and sodium and are activated by mechanical stress that for example can be observed under cardiac injury, among other stimuli. TRP channels are able to modulate calcium signaling pathways such as the calcineurin/nuclear factor of activated T-cells (NFAT), which can be initiated by mechanical stress [26]. It was also reported that constitutive expression of calcineurin or TRPC6 (a member of the canonical family of TRP) is able to overcome the loss of SRF, inducing conversion of cardiac fibroblasts into myofibroblasts [27].

Moreover, when matrix tension increases in the area exposed to MI, fibroblasts and other precursor fibrotic cells, highly sensitive to their mechanical microenvironment, differentiate into myofibroblasts. In line with this observation, fibroblast quiescent state is achieved when cells are cultured in a soft matrix. When ECM rigidity increases, enough tension is provided to activate TGF-β pathway, and as a result more myofibroblasts are formed and a positive feedback is established. Remarkably, α-SMA stress fibers can be formed in the absence of TGF-β stimulus. Matrix rigidity sensed by actin cytoskeleton is able to activate Rho-MRFT-SRF pathway, triggering α-SMA expression [26].

In fibroblasts, a family of GTPases is able to activate Rho associated kinase (ROCK) influencing actin dynamics and cytoskeletal reorganization which translates in cellular migration and contraction. In support of this mechanism, administration of pharmacologic ROCK inhibitor in cardiac fibroblasts is able to prevent α-SMA expression, which underlies myofibroblasts differentiation.

Although, myofibroblasts are essential for myocardium repair, continued presence of myofibroblasts due to persistence of inflammatory signals may result in excessive scarring and impairment of systolic and diastolic function. The initial naïve adaptive fibrosis created to maintain tensile strength and pressure becomes a maladaptive response, defined by cardiomyocyte hypertrophy and potentially leading to heart failure [26].

In summary, cardiac fibrosis is a dichotomous event, and a better knowledge of the role of myofibroblasts and of when cardiac fibrosis becomes maladaptive is required in order to target myofibroblast phenotype at a “critical turning point”, and completely restore heart function.

2.4 Conditioning

Heart injury leads to activation of defense mechanisms known as cardioprotection mechanisms, aimed at minimizing those harmful effects. Conditioning consists in short periods of non-lethal ischemia-reperfusion and confers resistance to subsequent ischemic insults. Conditioning can occur before, pre-conditioning, or after, post-conditioning, an ischemic insult and at the same organ or in an organ remote from the affected one – remote conditioning [28].

Pre-conditioning acts as an endogenous adaptation improving the resistance against hypoxia. The benefits of conditioning were first shown by Murry *et al.* [29] in a canine model, showing that 4 cycles of ischemia (5 minutes each), followed by 40 minutes of occlusion, results in a reduction of the lesion by 75%. Endogenous stimuli, such as adenosine, triggered by non-lethal ischemia, sensitizes different signaling pathways that converge on protein

kinase C (PKC) activation. PKC, in particular PKCα, plays a major role in cardioprotection since it stimulates transcription factors such as NF-KB, STAT3, heat shock transcription factor 1 and other survival pathways, namely, the SAFE pathway [30]. Although the mechanisms underlying pre-conditioning are not fully understood, evidences indicate that NO, TNF-α, K^+ ATP channels, ROS and mitochondrial transition pore opening are involved [30].

Despite limited clinical application in the context of myocardial infarction, pre-conditioning can be useful in other contexts such as surgical interventions (e.g. coronary artery bypass, transplants) [31].

Post-conditioning has also been shown to reduce infarct size and improve the efficiency of heart transplant surgery. Post-conditioning was first used in dog models by *Zhao* and collaborators [32] and, unlike pre-conditioning, it acts rapidly after ischemia-reperfusion phenomenon, preventing undesirable effects.

Studies have suggested that post-conditioning may limit inflammation by restricting P-selectin expression and thereby attenuating endothelial dysfunction and neutrophil accumulation. Moreover, it also reduces the no-reflow phenomenon that can occur when blood flow is not fully restored due to platelet aggregation and leucocyte accumulation into blood vessels, ROS generation and apoptosis. However, post-conditioning depends on intrinsic and extrinsic factors such as age, gender, drug regimen and reflow method. In addition, it is also difficult to find the exact period of time in which post-conditioning is relevant [33].

Remote conditioning can be applied before (pre), after (post) or during (per) the ischemic insult and its cardioprotective effects include improvement of heart contractile function, ATP recovery and reduction of infarct size. Usually, the limb is selected for this procedure because it requires less invasive methods; for example, the usage of a standard blood pressure cuff on the arm or leg is enough to trigger a response [34].

2.5 Therapies

Myocardial infarction and ischemia-reperfusion injury results in cardiomyocyte loss and cardiac fibrosis, which together lead to organ dysfunction. Therefore, control of ischemia-reperfusion damage and, concomitantly limiting of excessive scaring, is critical to reverse damage and prevent heart failure. Current therapies include pharmacological treatments, genetic approaches, mechanical interventions and, as last resource, organ replacement (transplantation).

Currently, the most used pharmacological agents are adenosine receptor agonists, bradykinin, anisomycin, diazoxide (an activator of potassium channels), acetylsalicylic acid, thrombolysis mediators and ionotropic stimulators (which increase contractility of a stunned heart). Furthermore, leucocyte-endothelial interaction-involved molecules such as aspirin and TFN-α antagonists are usually administered [4].

The usage of therapeutic gases is also common, for example, administration of nitric oxide (NO) controls regular blood oxygenation, vascular tone and endothelial function, attenuating damage associated with ischemia-reperfusion. Mechanical interventions such as hypothermia and gradual reperfusion have also been shown to have cardioprotective effects [4].

Drugs based on cyclosporine A are in clinical trials. This powerful immunosuppressive compound is a mitochondrial membrane pore blocker. Since during cardiac ischemia-reperfusion injury there is activation of apoptosis via mitochondrial membrane pore formation, prevention of pore opening by cyclosporine A can potentially increase cell viability [35].

Heart transplantation is usually used as last resort given the scarcity of organs, the complexity of the procedure and the high rate of rejection. Furthermore, this procedure also involves ischemia-reperfusion insult and consequent cardiac injury, so other solutions are usually preferred.

Concerning cardiac fibrosis, therapy is based on decreasing fibroblast proliferation and reduction of myofibroblast activation The current approaches contemplate inhibition of the receptors of the two main players in cardiac fibrosis, TGF-β and AngII, with GW 788388 (inhibitor TGF-β receptor type I) and losartan, respectively [36]. Moreover, the usage of inflammation suppressors is also effective in reducing cardiac fibrosis (e.g. IL-10) [13]. Hydrogels combined with antifibrotic molecules provide mechanical support, diminish myofibroblast-related stress and improve cardiac function [36] .

Recently, a new approach based on reprogramming cardiac fibroblasts into cardiomyocytes has been explored. Briefly, mouse embryonic fibroblasts were treated with a cocktail of small molecules, without requiring transcription factor expression, which promoted cardiomyocyte-like phenotype and function [37]. A similar strategy was applied by administering a cocktail of selected microRNAs (miR-1, -133, -208 and -499) in mouse cardiac fibroblasts, forcing their reprograming into the cardiomyocyte lineage [38]. The obtained cells resembled cardiomyocytes, but it should be mentioned that these procedures are still experimental, highly inefficient and further studies are needed before these approaches can be eventually translated into clinical procedure.

2.6 MicroRNAs

Molecular strategies based on the modulation of non-coding RNAs have emerged in the past years as promising therapeutic approaches against heart disorders [39].

MicroRNAs are a class of conserved single-stranded small noncoding RNAs (21-23 nucleotides) that play a major role in the regulation of gene expression. It is estimated that microRNAs can modulate up to 60% of mammalian gene expression at the post-transcriptional level, through mRNA silencing [40].

MicroRNAs were first described in 1993, during a lin-4 gene study in *Caenorhabditis elegans*. The product of this non-coding gene was able to block translation of the lin-14 gene [41]**;** since then, microRNAs have gained great interest within the scientific community.

Currently, 2,588 microRNAs are annotated in the human genome (miRBase, release 21, Jun 2014) and have been implicated in different biological process including cellular proliferation, differentiation, migration and apoptosis. MicroRNA dysfunction or dysregulation has also been associated with several pathologies including cancer, HIV, atherosclerosis and heart failure, among others [40].

Even though microRNAs are present predominantly inside cells, they can also be found in plasma and secretions such as urine and saliva. Several studies have reported the presence of microRNAs in circulating exosomes, microvesicles or apoptotic bodies, associated with proteins or lipoprotein complexes. The levels of circulating microRNAs can potentially be used for the diagnosis and prognosis of different pathologies, myocardial infarction and cardiac ischemia [42].

Of note, microRNAs can tolerate chemical modifications to improve their pharmacokinetic and pharmacodynamic profiles. This, together with the fact microRNAs can simultaneously target multiple genes, make them excellent candidates for the development of novel therapeutics.

2.6.1 MicroRNA Biogenesis

MicroRNA biogenesis can result from canonical or non-canonical pathways, both of them consisting of two main steps, which occur sequentially in the nucleus and in the cytoplasm.

In the nucleus, RNA polymerase II transcribes intergenic, intronic or polycistronic loci forming primary microRNAs (pri-microRNAs or pri-miRs) [40], with some exceptions where microRNAs can be derived from a tRNA precursor [43]. After transcription, those long sequences of RNA (pri-miRs) are guided to Drosha, a member of RNAse III family that digests pri-miRs to produce precursor microRNAs (pre-microRNAs or pre-miRs) which are approximately 70 nucleotides in length and have a hairpin structure. Drosha is regulated by glycogen synthase kinase 3β, allowing nuclear localization, and ribonucleoprotein binding partner DiGeorge syndrome critical region 8 (DGCR8), which stabilizes Drosha activity through Drosha-DGCR8 complex assembly (Fig. 6) [40]. DGCR8 recognizes pri-miRs and acts like an anchor, positioning Drosha at the correct site for the cleavage. Pri-miR expression is also controlled by DDX5 and DDX17 which interact with Drosha-DGCR8 complex creating a super microprocessor, essential to process pri-miRs in pre-miRs and to establish a crosstalk between pathways.

After Drosha-DGCR8 processing, pre-miRs are exported to the cytoplasm through

exportin-5. This step is promoted by Ran-GTP which is responsible for XPO5/pre-microRNA binding (Fig. 6). Indeed, when XPO5 is mutated pre-microRNAs remain trapped inside the nucleus [40].

Once in the cytoplasm, the terminal loop of the pre-microRNA hairpin is cleaved by Dicer, a RNase III enzyme that is positively regulated by TAR RNA-binding protein (TRBP) phosphorylation. This step leads to the formation of the mature microRNA [40]. Next, the double stranded microRNA is loaded into the RNA-induced silencing (RISC or miRISC) complex which is composed by multiple protein factors and Argonaute (Ago) protein family, namely, Ago2. Ago proteins are a set of catalytic enzymes that bind to microRNAs mediating post-transcriptional regulation by silencing mRNAs [40].

Finally, the double stranded microRNA is unwound (via RISC-associated helicase) based on its thermodynamic properties and tissue-specific factors and eventually, one strand is discarded. Typically, the less stable strand is elected to be the guide, surviving to degradation, but if both strands are equally stable, they both could act as guides. When the guide strand derives from the 5' end of the precursor, microRNAs are named -5p while if the guide strand derives from the 3' end, microRNAs are named -3p [44].

Alternatively, microRNA biogenesis can occur via a non-canonical pathway, where short hairpin introns, called mirtrons, avoid Drosha cleavage, and are alternatively processed by spliceosomes that splice and debranch mirtrons [40]. This, however, occurs only for a minor fraction of the miRNome.

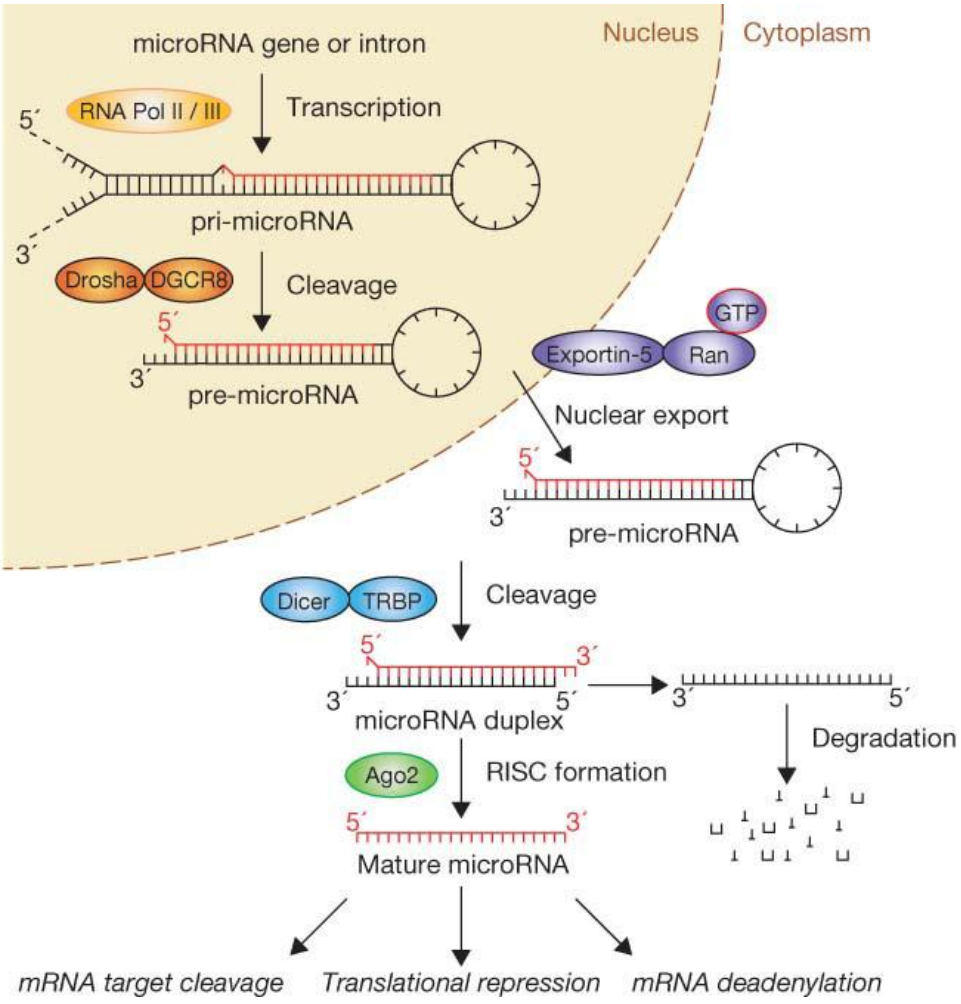


Figure 6. MicroRNA biogenesis and mechanisms of action. MicroRNAs are transcribed from introns or gene regions by RNA polymerase II, forming a pri-miR. This long ds-RNA is cleaved by Drosha resulting in a shorter hairpin called pre-miR. Next, pre-miR is exported from the nucleus to the cytoplasm by exportin 5. In the cytoplasm, pre-miR is exposed to Dicer cleavage to originate the form a microRNA duplex. Finally, the double strand is processed by the RISC complex, leading to the formation of the mature guide strand and degradation of the passenger strand. Mature microRNAs exercise their function by mRNA target cleavage, translational repression or mRNA deadenylation and degradation. Adapted from: Winter, Julia, et al. "Many roads to maturity: microRNA biogenesis pathways and their regulation." Nature cell biology, 2009, 11(3) p.228-234.

2.6.2 Mechanisms of MicroRNA-Mediated Gene Silencing

As mentioned, the most common mechanism of action of microRNAs is the repression of gene expression at a post-transcriptional level. The major determinant of microRNA binding to mRNA is the seed region located at the 5' end of microRNAs, which recognizes target mRNAs through Watson-Crick base complementary usually at 3' UTR [45].

The seed sequence of microRNAs corresponds to nucleotides 2-7 at the 5' end of microRNAs. There are three types of seed sequences, depending on the complementarity with their targets: 5'-dominant canonical, 5'-dominant seed and 3'-compensatory. Regarding 5' canonical, both 3' and 5' ends are well paired with their targets, while 5'-dominant seed sites complement perfectly from positions 2–8 of microRNAs. Additionally, 3'-compensatory

seed sites are characterized by high 3' end complementary; nonetheless, minimal 5' end complementary is always required [46].

MicroRNAs can silence mRNAs and downregulate gene expression through three different mechanisms: i) repression of translation initiation, ii) inhibition of translation elongation or iii) destabilization of mRNA (deadenylation). Among other factors, the complementarity degree will determine the mechanism that will prevail, less complementary levels leading to translation repression whereas high complementary degree leads to mRNA degradation through destabilization [47].

Although the exact mechanism of inhibition of translation initiation by microRNAs is still unknown, some models have been proposed. One is based on the fact that GW182 competes with translation initiation factor 4F, namely the eIF4E subunit, for the binding to mRNA 5' cap. Other models suggest that miRISC can inhibit the assembly of 80S ribosomal complex, or alternatively, mRNAs can accumulate in processing bodies (P-bodies) [48].

Inhibition of translation elongation can also occur, whereby target mRNA is bound to miRISC promoting release of mRNA and consequently its premature separation from ribosomes.

Finally, it has also been proposed to occur that mRNA destabilization by RISC may involve deadenylating enzymes that remove poly (A) tail, exposing mRNA to degradation and Ago protein slicer activity. This system has been suggested to be the main mechanism of action for microRNA-mediated gene silencing in mammalian cells [40].

2.6.3 Modulation of MicroRNA Activity

The main two approaches to modulate microRNA activity are restoring or inhibiting microRNA function The increase of the levels of a downregulated microRNA can be achieved by administering a molecule that mimics its activity, either synthetic or expressed from a plasmid or viral vector. Synthetic double-stranded microRNA mimics are composed by a guide strand, that mimics the microRNA of interest, and the passenger strand that is complementary to the guide strand. In physiological conditions, passenger strand is in general eliminated, but in this context it can also be used to enhance delivery and cellular uptake. For that purpose, RNA duplexes can harbor chemical modifications (discussed below) that cannot only confer resistance to enzymes and stability, but also enrich tissue specific delivery and cellular intake [45]; such modification are more tolerated in the passenger strand.

In a different scenario, a decrease of microRNA activity can be reached by administration of antisense oligonucleotide (ASOs) or microRNA inhibitors, and microRNAs sponges. ASOs, anti-miRs or microRNA inhibitors, are single-stranded oligonucleotides that target directly microRNAs via perfect complementarity; usually these oligonucleotides are chemically modified to increase affinity of binding and intracellular delivery since unmodified RNA oligonucleotides have limited cell membrane penetration [45].

MicroRNAs sponges are able to bind to the target microRNA due to the presence of multiple tandem binding sites in their sequence. As such, they selectively sequester microRNAs by an inhibitory competition mechanism, thus allowing the expression of otherwise targeted mRNAs. The efficiency of microRNA sponges was reported by *Ebert et al.* [49] which has shown lower levels of microRNA after utilization of sponges. In this study, the authors used a modified ASO with multiple binding sites against a microRNA. This method

increased the efficacy of repression when compared to other microRNA inhibitory strategies (including anti-miRs).

An alternative strategy to block microRNA action, specific for a particular target, involves the use of target site blockers or masks. These molecules inhibit microRNAs by binding directly to the mRNA target at a region that overlap the microRNA binding site, thus preventing microRNA/mRNA association [50].

Recently, approaches based on the CRISPR/Cas9 technology have also emerged as new strategies to block microRNA by knocking out microRNA codifying sequences [51].

The therapeutic potential of microRNAs has been demonstrated by miravirsen, a drug against Hepatitis C virus (HCV) that is based on an anti-miR that targets miR-122. This liver-specific microRNA has a main role in the life cycle of HCV. Miravirsen blocks miR-122, avoiding the binding of the microRNA to the 5' region the HCV genome, which is required for its replication [52].

2.6.4 MicroRNA Modifications and Delivery

MicroRNA modifications are used to improve affinity, biostability and pharmacokinetic profile of microRNA mimics and anti-miRs. However, in microRNA mimics, modifications are limited since the seed sequence and consequently microRNA activity has to be preserved, whereas in anti-miRs modifications are more tolerated. The most common modification of microRNA mimics consists of the addition of a cholesterol molecule to the 3' end of the passenger strand. MicroRNA sugar modifications include 2'-O-methyl (2'-OMe), 2'-methyoxyethyl (2'-MOE), 2'-fluoro (2'-F) and locked nucleic acid (LNA). Additional, well described modifications comprise backbone linkage modification to phosphorothioate (PS), peptide nucleic acid (PNA) or morpholino oligomers [45]. It should also be mentioned that there is an important molecular asymmetry between how modifications are tolerated at strand ends. For example, a study by *Chiu* and collaborators [53] showed that microRNA guide strands tolerate well chemical modifications at 3' end but not at the 5' end indicating that 5´ end must have a crucial role in loading and silencing.

Of all microRNA modifications, the most efficient and extensively used is the addition of LNA bases, bicyclic RNA analogues in which a methylene bridge between the 2'-O to the 4'-C is introduced, locking the sugar moiety into N-type (3' endo) sugar ring conformation. This allows a stronger duplex formation enhancing Tm and consequently binding affinity and specificity [45]. Among all modifications, LNA modified anti-miRs exhibit the greatest potency due to their reduced size, which enables an efficient cellular uptake.

Another important step in the development of microRNA based therapies concerns microRNA delivery. Aside from determining the optimal dose, target and tissue specificity, finding a vehicle with perfect balance between efficient treatment and absence of deleterious effects is an obstacle for microRNA therapy that still needs to be overcome. These obstacles are more notorious *in vivo* than *in vitro*, where cells can be easily transfected with desirable microRNAs. Briefly, *in vivo* delivery strategies include the use of viral vectors, liposomes, nanoparticles, antibodies and scaffold matrices [40]. Among these, adeno-associated virus and liposome-based systems are the most commonly used for *in vitro* and *in vivo* experiments.

Adeno-associated virus (AAV) is a small non-enveloped single-strand DNA parvovirus. AAVs are not able to replicate, requiring a helper virus like adenovirus or herpes virus. They are non-pathogenic in humans and are able to transduce several tissues, particularly post-mitotic tissues, at high efficiencies, making them promising therapeutic vectors. Although these vectors are in clinical trials, there are still issues regarding potential immunogenicity that need to be overcome before their broad application can be achieved. For the heart, the AAV serotype 9 (rAAV9) appears to be the most efficient serotype and therefore the most promising for heart-related microRNA modulation [54].

Concerning the use of liposomes for heart diseases, *Higashi et al.* [55] demonstrated recently that liposome delivery of miR-145 induced repair of the infarcted myocardium, showing a protective effect *in vivo* using a rabbit model of myocardial infarction (MI) and *in vitro* H9c2 rat cardiomyoblasts. Briefly, miR-145 reduces MI size by targeting FRS2 and simultaneously activating PI3K/Akt survival signaling. Despite promising results, liposome-based *in vivo* delivery of siRNA, microRNA mimics and ASOs needs to be optimized to reduce toxicity and nonspecific uptake.

2.7 MicroRNAs and Cardiovascular Diseases

MicroRNAs play an important role in several cardiac functions and homeostasis, and have also been associated to different pathologies, namely, cardiac ischemia-reperfusion injury and scar formation. The most important microRNAs associated with myocardial infarction are described below (Fig. 7).

As outlined above, microRNA expression depends on the cell type and tissue. The microRNAs miR-1 and miR-133 are particularly important in the heart, being involved in myocardial cell differentiation and heart development. Furthermore, these microRNAs also control cardiac contraction by modulating connexin 43 expression in gap junctions and by regulating calcium homeostasis. Several studies have reported miR-1 upregulation in areas exposed to ischemia-reperfusion, which appears to be related with apoptosis, possibly through interaction with Bcl-2. Accordingly, administration of LNA-based anti-miR-1 has been shown to increase cell viability and reduce infarct size. Among other suggested miR-1 targets are heat shock protein (HSP60), caspase-9, GJA1 and KCNJ2 [56].

After ischemia-reperfusion insult, miR-133 is downregulated. Indeed, miR-133 overexpression is considered a pro-survival approach since its upregulation is correlated with an increase in cell viability, probably by repression of caspase 9, which is indicated as a miR-133 target.

Similar to miR-133, other cardioprotective microRNAs include miR-17 and miR-92 that are part of a microRNA cluster that induces cardiomyocyte proliferation, although they also control angiogenesis and neovascularization. Recently, *Hinkel et al.* [57] reported that the usage of LNA-based miR-92a inhibitor at the onset of cardiac reperfusion in pigs decreases infarct size and apoptosis, improves heart function and reduces inflammation. Those processes probably occur due to targeting of PTEN (a negative regulator of survival and proliferation) and SIRT1 by the miR-17-92 cluster.

After ischemia-reperfusion, the levels of miR-208 decrease dramatically; usually, miR-208 is highly expressed in the heart and participates in stress-induced induction of myosin

heavy chain response. In mammals, miR-208b is the most common member of the miR-208 family and is beneficial during cardiac remodeling [58].

Under basal conditions, members of the miR-15 family, namely miR-195, participate in cardiomyocyte post-natal switch from a proliferative to a differentiated state. This microRNA family is upregulated after myocardial infarction and their predicted targets include cell cycle related genes such as ADP-ribosylation factor-like 2 (Arl2) and pro-apoptotic molecules. *Porrelo et al.* have demonstrated that inhibition of microRNAs of the miR-15 family leads to a reduction in infarct size by increasing cardiomyocyte proliferation, resulting in improved left ventricular systolic function [59].

Recently, *Eulalio et al.* [14] have identified other microRNAs able to induce reentry of cardiomyocytes in the cell cycle and proliferation, most importantly hsa-miR-199a-3p and hsa-miR-590-3p. Overexpression of these microRNAs following myocardial infarction led to a reduction of infarct size and ameliorated cardiac function. Among other relevant targets, miR-199 has been shown to target HIF-1α and prevent both SIRT1 and p53 expression, thus inhibiting apoptosis [60].

MiR-21 is probably the most studied cardiac fibrosis-related microRNA being ubiquitously expressed in the heart, though at higher levels within fibroblasts and at lower levels in cardiomyocytes. During cardiac fibrosis, miR-21 levels increase, repressing Sprouty homologue 1 action, and consequently activating MAPK signaling and enhancing fibroblast survival and growth factor secretion. Additionally, miR-21 also controls expression of matrixmetalloproteinase-2 (MMP-2), through the PTEN pathway, which is involved in tissue injury, particularly, in inflammation [24, 61].

MiR-29 has also been strongly associated with fibrosis-related genes, namely, collagens. This microRNA is highly expressed in the heart and its downregulation has an anti-apoptotic effect. Mcl-1, a member of the anti-apoptotic Bcl-2 family, p85a (the regulatory subunit of PI3K), and cell division cycle 42 (Cdc42) are indicated as potential targets of miR-29 [24, 61].

MiR-101 is downregulated after MI, and indeed, treatment with miR-101 microRNA mimics prevents AngII-induced proliferation and inhibits the expression of ECM proteins as collagens, MMPs and fibronectins, among others [24].

Recently, a group of microRNAs upregulated in hypoxia have been denoted as hypoxamiRs. Among these, miR-210 is considered the master hypoxamiR since it is directly regulated by HIF-1α. MiR-210 targets fibroblast growth factor receptor like 1 thus inhibiting cell proliferation and TCA cycle proteins, preventing mitochondrial metabolism and leading to lactate accumulation. Interestingly, even after reperfusion, miR-210 levels remain increased, indicating that it has a long lasting effect; moreover, miR-210 also supports angiogenesis and cell differentiation, but prevents DNA repair system function [62].

Given their important role in control of gene expression, modulation of microRNAs can be exploited to inhibit cell death and cardiac fibrosis or increment cardiomyocyte proliferation to potentially improve the consequences of ischemia-reperfusion injury. Nonetheless, more studies must be performed to understand which microRNA may better serve this purpose, before any therapy can be developed.

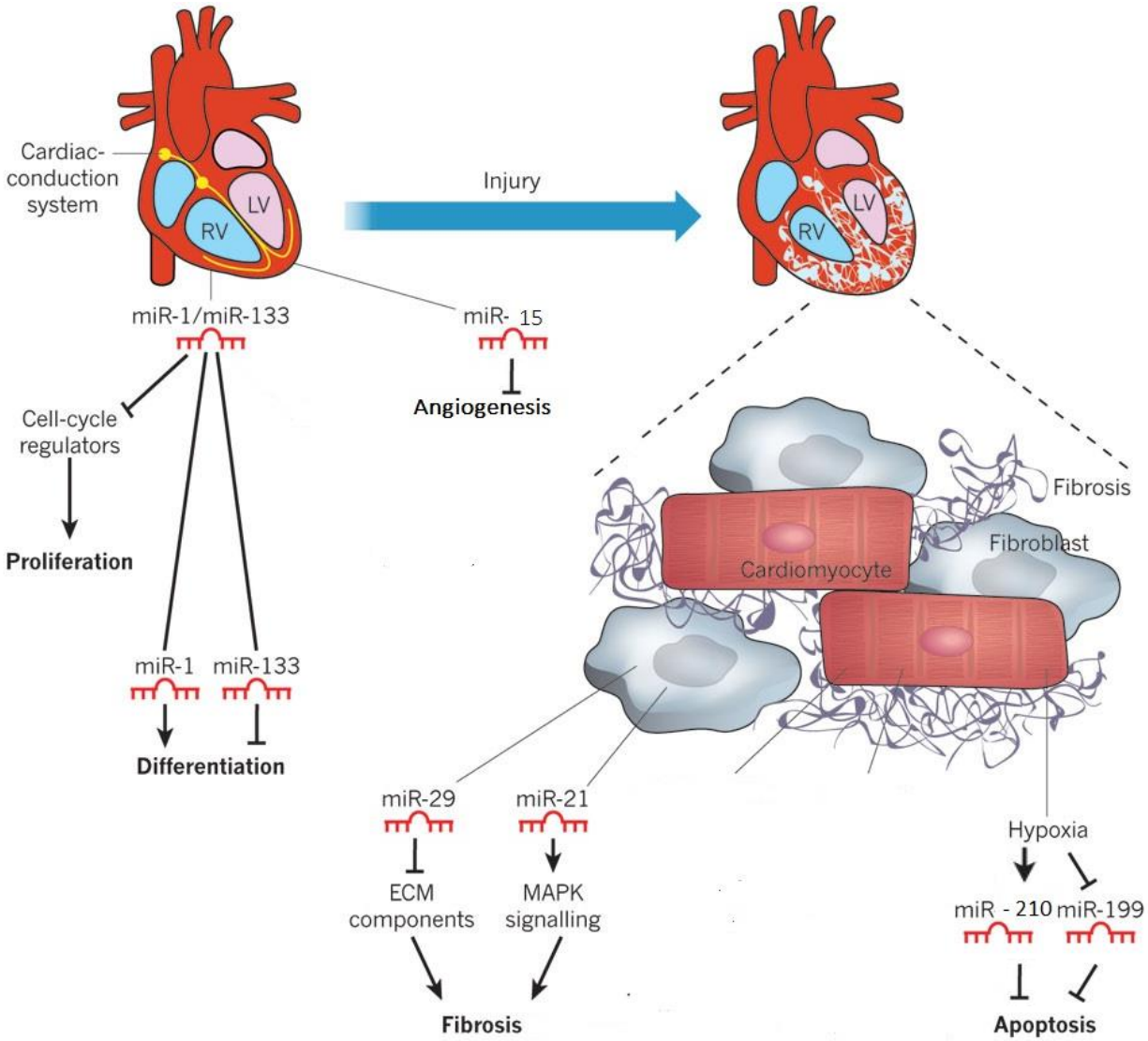


Figure 7. MicroRNAs and cardiovascular diseases. Several microRNAs are activated after injury, namely, miR-21 leading to activation of pro-fibrotic pathways such as MAPK signaling, miR-210 which promotes apoptosis. MicroRNAs are also involved in the development and normal function of the heart, such as miR-1, that promotes cardiomyocyte differentiation and miR-133 that blocks cardiomyocyte differentiation**.** Adapted from: Small, Eric M., and Eric N. Olson. "Pervasive roles of microRNAs in cardiovascular biology." Nature, 2011, 469.7330 p. 336-342.

3.Objectives

Main Goal and Objectives

The main goal of this work was to optimize the experimental conditions to perform large-scale screenings in human cardiomyocytes and cardiac fibroblasts, using genome-wide libraries of microRNA mimics and inhibitors, aimed at identifying microRNAs relevant to cardiac diseases, in particular myocardial infarction.

Specific objectives:

- Optimization of the experimental conditions mimicking myocardial infarction *in vitro*, namely, cardiac ischemia-reperfusion.
- Evaluation of the impact of ischemia-reperfusion injury to cardiomyocytes and cardiac fibroblasts, by assessing cell proliferation and cell number.
- Optimization of procedures for efficient transfection of microRNAs into cardiomyocytes and cardiac fibroblast, amenable to large-scale screenings using libraries of microRNA mimics and inhibitors.
- "Proof-of-principle" investigation of the relevance of selected microRNAs on cardiac fibrosis, specifically in myofibroblast activation.

4.Materials and Methods

4.1 Cell Culture

a) Human iPSC-Derived Cardiomyocytes – iCMs

Human iPSC-derived cardiomyocytes were obtained from a collaboration with Prof. Paula Alves, Animal Cell Technology Unit, iBET. These cells were derived from a commercially available iPSC cell line – human iPS DF19-9-11T.H (Cell Research Institute, Inc.) through the addition of growth factors and small-molecule Wnt modulation, as described by *Burridge et al.* [63] and *Lian et al.* [64], with minor modifications. In this study, these cells were designated as iCMs.

The cells were used at day 15 of the differentiation process, when cells have acquired a cardiomyocyte phenotype and function, showing cardiomyocyte-specific markers particularly, cardiac troponin T, sarcomeric α-actinin, myosin heavy chain and NKx2, and spontaneous beating. At this stage, cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640, #21875-091, Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS, #10270160, Life Technologies) and B27 supplement minus insulin (#A1895601, Life Technologies).

One hour before plating iCMs for the experiments, ROCK inhibitor (ROCKi) (Y-27632 dihydrochloride, #1254, Tocris) was added to iCMs plated in 12- or 24-well plates (1:1000), to improve survival of dissociated cardiomyocytes and facilitating cell detachment. Then, cells were washed once with phosphate buffered saline (PBS) 1x and TripLE Select Enzyme 1x (#12563-029, Life Technologies) was added to the cells for 5 minutes. TripLE Select was then neutralized with culture medium containing ROCKi and the monolayer was dissociated mechanically by repeated pipetting. Cells were then transferred dropwise to a 50 ml falcon tube with culture medium containing ROCKi and centrifuged at 300 x g for 5 min, at room temperature (RT). Next, the supernatant was removed and cells were resuspended in culture medium containing ROCKi. Cells were then counted and plated at the desired density in plates previously coated with Matrigel (Matrigel® hESC-Qualified Matrix, #354277, Corning), which was removed immediately before plating the cells.

Unless specified, cells were incubated and maintained in culture at 37ºC, 5% CO_2 in a humidified atmosphere; one day after plating media was replaced by culture medium without ROCKi and media was change every two days.

Coating of multiwell plates was started at least 1 h before cell plating, by adding Matrigel diluted in DMEM/F12 (#11320-074, Life Technologies); 100 µl was used per well in 96-well plates.

b) Human iPSC-Derived Cardiomyocytes – iCell-CMs

iCell® Cardiomyocytes (iCell-CMs) were purchased from Cellular Dynamics International (Madison, Wisconsin) as cryopreserved cells. In brief, these cells were obtained through differentiation of human iPSCs. iCell Cardiomyocytes are composed of a homogeneous population of cells - 95% pure population of ventricular, atrial, and nodal cells. Cells were thawed, seeded and subcultivated according to the manufacturer's instructions using RPMI supplemented with 10% FBS and B27 minus insulin as basal growth medium.

For the experiments, 1 h before plating, multiwells were coated with Matrigel diluted in DMEM/F-12 as described above for iCMs, and cells were thawed by immersing the cryovial for 4 min in a 37ºC water bath. Cells were then transferred dropwise to a 50 ml falcon tube containing culture medium. Cell suspension was homogenized and cells were then counted and plated at the desired density. Matrigel was aspirated immediately before plating the cells and, unless specified cells were maintained at 37ºC, 5% CO_2 in a humidified atmosphere. Culture medium was replaced 24 h after plating and then every two days.

c) Human Cardiac Fibroblasts (HCF)

Human cardiac fibroblasts were purchased from Promocell GmbH (Heidelberg, Germany). Briefly, primary human cardiac fibroblasts were isolated from the ventricles of a healthy female adult and then cryopreserved at passage 2 using a serum-free freezing medium. Flow cytometry analysis of these cells was negative for α-smooth muscle actin (α-SMA), a marker of myofibroblasts. Cells were thawed, seeded and subcultivated according to the manufacturer's instructions. Human cardiac fibroblasts were thawed in one of the following media:

i) Fibroblast Growth Medium 3 (#c-23025, Promocell GmbH) supplemented with Fibroblast Growth Medium 3 SupplementMix (#C-39345, Promocell GmbH) – final composition 10% (v/v) Fetal Calf Serum, 1 ng/ml Basic Fibroblast Growth Factor (recombinant human), 5 µg/ml Insulin (recombinant human);

ii) Dulbecco modified Eagle's medium (DMEM) - high glucose (#31966-047, Life Technologies), supplemented with 10% (v/v) FBS, 1 ng/ml Fibroblast Growth Factor (#PHG0026, Life Technologies), 5 µg/ml Insulin (#I9278-5ML, Sigma)

and maintained in culture as a monolayer in DMEM (media ii) at 37ºC, 5% CO_2 in a humified atmosphere.

For the experiments, subconfluent cultures (70-90% confluence) were washed with PBS 1x and detached with Trypsin/EDTA (0.05% porcine trypsin in HBSS, 0.2% EDTA, without calcium, magnesium; #11581861, GE Healthcare Hyclone). After 5 min incubation at RT, trypsin was neutralized by adding culture medium. Cell suspension was then centrifuged at 220 x g for 3 min at RT and supernatant was discarded. Cells were then resuspended, counted, plated at the desired density and incubated as previously described.

4.2 Ischemia-Reperfusion Insult

For the ischemia-reperfusion experiments, cardiomyocytes were plated at a density of 15 x 10^3 cells per well in 100 µl, in 96-well microplates. Twenty-four hours after plating, cardiomyocytes were exposed to an ischemia-reperfusion insult to simulate myocardial infarction injury.

In order to mimic the ischemia insult, cells were incubated in a hypoxic incubator environment composed of 0.1% O_2 and 5% CO_2 in ischemia mimetic solution (IMS) consisting of 125 mM NaCl, 8 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 6.25 mM $NaHCO_3$, 20 mM HEPES, 5 mM sodium lactate (IMS +Lac), or alternatively, without lactate (IMS -Lac) (Table 1). IMS media with or without lactate were adjusted to pH 6.6 in order to resemble the ischemia microenvironment characterized by lactate increment and a decrease of pH. To mimic reperfusion, 24 or 48 h after ischemia, cell medium was replaced by fresh culture medium (RPMI without ROCKi) and cells were moved to a normoxia incubator for 24 h.

As a control for these experiments, cells were not exposed to hypoxia neither to IMS, remaining in culture medium and in a normoxic chamber for the whole duration of the experiment.

Table 1- Composition of Ischemia Mimetic Solution (IMS). All values are in mM, pH 6.6

Reference	NaCl	KCl	KH_2PO_4	$CaCl_2$	$MgSO_4$	$NaHCO_3$	HEPES	Sodium Lactate	Others
Xie et al., 2014 [65]	125	8	1.2	1.2	1.25	6.25	20	5	20 deoxyglucose
Vila-Petroff et al., 2007 [66]	123	8	-	2.5	0.5	-	20	20	0.9 NaH_2PO_4 pH 6.2
Brady et al., 2006 [67]	125	8	1.2	1.2	1.25	6.25	20	5	-
Hamacher-Brady et al., 2006 [68]	125	8	1.2	1.2	1.25	6.25	20	5	-
Arstall et al., 1998 [69]	119	5.36	1.2	2.5	0.95	25	-	-	11 deoxyglucose
This study	125	8	1.2	1.2	1.25	6.25	20	5	-

For human cardiac fibroblasts, a similar procedure was adopted to mimic ischemia-reperfusion injury, with minor modifications. Briefly, cells were plated in DMEM supplemented with FGF and insulin (media ii) at 4 x 10^3 per well in 100 µl, in 96-well microplates for 24 h prior to the ischemic insult. Cells were then incubated for 24 h with IMS -Lac or IMS +Lac into in a hypoxic incubator as described above for cardiomyocytes (0.1% O_2, 5% CO_2). In the case of fibroblasts, literature described IMS as a more alkaline media at pH 7.4 [70]. For this reason, we prepared IMS at pH 6.6 (as used in cardiomyocytes) and pH 7.4,

so that we could also evaluate the impact of pH decrement. Following ischemia, reperfusion was achieved as described for cardiomyocytes; briefly, IMS was replaced by normal culture media and cells were returned to a normoxic incubator. To test the impact of reperfusion and determine the effect of ischemia *per se*, a subset of cardiac fibroblasts was exposed only to ischemia. Control cells were not subjected to hypoxia or IMS.

4.3 MicroRNA Reverse Transfection

Reverse transfection was used to examine the role of microRNAs in cardiomyocyte or cardiac fibroblast proliferation and myofibroblast phenotype activation. Reverse transfection differs from forward transfection in the sequence by which the components are added to the multiwell. In the reverse transfection protocol, the genetic material is firstly added to the empty wells, then the transfection reagent is added and finally the cells. In the forward transfection protocol, cells are seeded before transfection (typically one day before transfection) and complexes of the genetic material and transfection reagent are then added to cells. The reverse transfection protocol allows to save time, since cells do not need to be seeded before the beginning of the experiments, and it is also easier to automate using robotic systems.

The microRNA mimics (miRIDIAN microRNA mimics) used in this study were purchased from Dharmacon (GE Healthcare).

a) MicroRNA Reverse Transfection – iPSC-Derived Cardiomyocytes

Selected microRNA mimics known to increase rat and mouse cardiomyocyte proliferation (hsa-miR-199-3p, hsa-miR-302d-3p, hsa-miR-590-3p), cel-miR-67 (a C. elegans microRNA without homology to human microRNAs) and siRNA UBC (a siRNA that targets the essential Ubiquitin C gene) were used to test the transfection conditions for human cardiomyocytes.

Briefly, the microRNAs and siRNAs were diluted in siRNA buffer 1x (B-002000-UB-100, Dharmacon, GE Healthcare) at a concentration of 500 nM and transferred to 96-well microplates (15 µl/well). The transfection reagent, Lipofectamine RNAiMax (0.4 µl/well; #13778-150, Life Technologies) was diluted in Opti-MEM Reduced Serum Medium (#21875-034, Life Technologies 35 µl/well), incubated for 5 minutes at RT and then added to the microplates.

After 30 min incubation, cardiomyocytes were added to the wells in culture medium (15×10^3/well, 100 µl/well) and 48 h after transfection the medium was replaced by fresh medium. Finally, cells were fixed 72 h after transfection and cardiomyocyte proliferation was assessed as described below.

Negative controls were included in all experiments (siRNA buffer, transfection reagent and Opti-MEM) and non-transfected cells. All conditions were plated in triplicates.

b) MicroRNA Reverse Transfection – HCF Pilot Screen

Transfection of the microRNA mimics for the pilot screen, aimed at identifying microRNAs with a potential role in cardiac fibroblast activation, was performed using a reverse transfection protocol at a final concentration of 50 nM. The microRNA mimics used in this pilot screening corresponded to microRNA mimics known to increase rat and mouse cardiomyocyte proliferation (Table 2).

Briefly, microRNA mimics diluted in siRNA buffer 1x at a concentration of 500 nM were transferred to 96-well microplates (15 µl/well) using an automated robotic system. A total of 36 microRNA mimics were tested, arrayed in columns 2 to 6 of 96-well microplates; columns 1 and 7 were occupied with controls as follows: buffer, cel-miR-67 (a C. elegans microRNA that has no homology with human microRNAs), non-targeting siRNA #5 (NT #5; a siRNA that has no targets in the human genome), siRNA UBC (a siRNA that targets the essential Ubiquitin C gene) and non-transfected cells. The transfection reagent, Lipofectamine RNAiMax (0.3 µl/well) was diluted in Opti-MEM Reduced Serum Medium (35 µl/well) and incubated for 5 min at RT; this mix was added to the wells (35 µl/well) containing the microRNA mimics or controls and incubated for 30 minutes at RT.

Human cardiac fibroblasts were then added to the microplate at a density of 5 x 10^3 well (100 µl) in DMEM supplemented with 10% (v/v) FBS and incubated at 37°C, 5% CO2 in a humidified atmosphere. Forty-eight hours after transfection, media was replaced by fresh media. HCFs were fixed at 96 h after reverse transfection and immunostaining against α-SMA was performed to evaluate myofibroblast phenotype.

Concerning transfection controls, siRNA UBC was used as positive control, since it targets an essential gene, Ubiquitin C – successful transfection of this siRNA results in efficient cell death. Moreover, cel-miR-67 and NT#5 were used as negative controls.

Table 2 - MicroRNAs selected for the HCF pilot screen

Name	miRBase Accession #	Sequence
hsa-miR-18a-3p	MIMAT0002891	ACUGCCCUAAGUGCUCCUUCUGG
hsa-miR-18b-3p	MIMAT0004751	UGCCCUAAAUGCCCCUUCUGGC
hsa-miR-19a-5p	MIMAT0004490	AGUUUUGCAUAGUUGCACUACA
hsa-miR-19b-2-5p	MIMAT0004492	AGUUUUGCAGGUUUGCAUUUCA
hsa-miR-23a-3p	MIMAT0000078	AUCACAUUGCCAGGGAUUUCC
hsa-miR-23b-3p	MIMAT0000418	AUCACAUUGCCAGGGAUUACC
hsa-miR-26b-5p	MIMAT0000083	UUCAAGUAAUUCAGGAUAGGU
hsa-miR-30a-3p	MIMAT0000088	CUUUCAGUCGGAUGUUUGCAGC
hsa-miR-33b-3p	MIMAT0004811	CAGUGCCUCGGCAGUGCAGCCC
hsa-miR-130a-5p	MIMAT0004593	UUCACAUUGUGCUACUGUCUGC
hsa-miR-143-5p	MIMAT0004599	GGUGCAGUGCUGCAUCUCUGGU
hsa-miR-152-3p	MIMAT0000438	UCAGUGCAUGACAGAACUUGG
hsa-miR-181a-5p	MIMAT0000256	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-199a-3p	MIMAT0000232	ACAGUAGUCUGCACAUUGGUUA
hsa-miR-210-3p	MIMAT0000267	CUGUGCGUGUGACAGCGGCUGA
hsa-miR-219-5p	MIMAT0000276	UGAUUGUCCAAACGCAAUUCU
hsa-miR-302b-3p	MIMAT0000715	UAAGUGCUUCCAUGUUUUAGUAG
hsa-miR-302c-5p	MIMAT0000716	UUUAACAUGGGGGUACCUGCUG
hsa-miR-302d-3p	MIMAT0000718	UAAGUGCUUCCAUGUUUGAGUGU
hsa-miR-302e	MIMAT0005931	UAAGUGCUUCCAUGCUU
hsa-miR-335-3p	MIMAT0004703	UUUUUCAUUAUUGCUCCUGACC
hsa-miR-372-3p	MIMAT0000724	AAAGUGCUGCGACAUUUGAGCGU
hsa-miR-373-3p	MIMAT0000726	GAAGUGCUUCGAUUUUGGGGUGU
hsa-miR-455-5p	MIMAT0003150	UAUGUGCCUUUGGACUACAUCG
hsa-miR-511-5p	MIMAT0002808	GUGUCUUUUGCUCUGCAGUCA
hsa-miR-520a-3p	MIMAT0002834	AAAGUGCUUCCCUUUGGACUGU
hsa-miR-520b	MIMAT0002843	AAAGUGCUUCCUUUUAGAGGG
hsa-miR-520c-3p	MIMAT0002846	AAAGUGCUUCCUUUUAGAGGGU
hsa-miR-590-3p	MIMAT0004801	UAAUUUUAUGUAUAAGCUAGU
hsa-miR-875-5p	MIMAT0004922	UAUACCUCAGUUUUAUCAGGUG
hsa-miR-885-5p	MIMAT0004947	UCCAUUACACUACCCUGCCUCU
hsa-miR-1244	MIMAT0005896	AAGUAGUUGGUUUGUAUGAGAUGGUU
hsa-miR-1248	MIMAT0005900	ACCUUCUUGUAUAAGCACUGUGCUAAA
hsa-miR-1281	MIMAT0005939	UCGCCUCCUCCUCUCCC
hsa-miR-2052	MIMAT0009977	UGUUUUGAUAACAGUAAUGU

4.4 Analysis of Cell Proliferation

Cardiomyocyte and cardiac fibroblast proliferation was evaluated by scoring the number of 5-ethynyl-2'-deoxyuridine (EdU) positive cells. EdU Assay (Click-iT® EdU Alexa Fluor®647 or 594 Imaging Kit, #C10340 or C10339, Life technologies) is based on a modified thymidine analogue (EdU) that is added to cells and is incorporated in DNA during DNA synthesis in the S phase of the cell cycle. The presence of this nucleotide is revealed subsequently by a reaction with a fluorescently labelled azide.

Briefly, EdU was diluted in culture media to a final concentration of 5 µM and incubated with cells for 24 h (cardiomyocytes) or 4 h (fibroblasts) prior to the fixation step. The EdU labelling Reaction was performed after cell fixation and immunostaining, according to the manufacturer's instructions. In summary, a solution composed by Alexa fluor azide 594 or 647, $CuSO_4$, 1X Click-iT® reaction buffer and reaction buffer additive (35 µl/well) was added to cells and incubated for 30 min in the dark, then Hoechst 33342 was used to counterstain the nuclei. Image acquisition was performed in an Operetta automated fluorescence microscopy (Perkin Elmer).

4.5 Immunostaining

We used immunostaining to identify α-actinin and α-SMA proteins, allowing the recognition of cardiomyocytes and myofibroblasts, respectively.

Cells were fixed with 4% of paraformaldehyde (PFA) for 15 min, washed 3x with PBS 1x for 5 min each, and permeabilized with 0.5% Triton X-100 in PBS 1x for 10 min at RT. Following a 5 min PBS 1x wash, cells were blocked with 1% Bovine Serum Albumin Fraction V (#8076.3, Carl Roth GmbH) and incubated with the primary antibody diluted in blocking solution. After incubation with the primary antibody, cells were washed 3x with PBS 1x for 5 min each and incubated with Alexa-488 donkey anti-mouse secondary antibody (1:400) (#A-21202, Life Technologies), for 1 h at RT. After 3x washes with PBS 1x for 5 min each, cells were processed for EdU Reaction, if indicated, and after 3 additional washes cells were incubated with Hoechst 33342 (#H3570, Life Technologies) diluted in PBS 1x (1:2000), to counterstain cell nuclei.

Concerning cardiomyocytes, cells were blocked for 30 min at RT and incubated with a monoclonal anti α-actinin antibody (1:200) (#ab9465, abcam), a cardiomyocyte marker, overnight at 4ºC.

For staining of myofibroblasts, cells were blocked for 1 h at RT and incubated with a monoclonal antibody against α-SMA (1:2000) (#A2547, Sigma) for 2 h at RT.

4.6 Image Acquisition and Analysis

Image acquisition was performed using an Operetta automated high-content screening fluorescence microscope (Perkin Elmer) at 10x magnification. A total of 9 fields were acquired per wavelength, well and replicate.

Columbus HCS Analysis, version 2.5.0.120577, was used to analyse the acquired images. Segmentation of the cell nuclei and cytoplasm was based on morphological and intensity properties, which also allowed to exclude cellular residues, apoptotic cells or other background particles (Fig. 8). To identify cardiomyocytes, a threshold intensity for Alexa 488 (α-actinin) was applied while to recognize proliferative cardiomyocytes, the first population was then submitted to a threshold intensity for Alexa 647 or 594 (EdU). For the identification of myofibroblasts, cells were automatically categorized based on texture, intensity and morphology properties of cardiac fibroblasts, following manual training of the software with sample images.

4.7 Statistical Analysis

All data are presented as mean ± SD of at least three independent experiments. Results were statistically analysed by one-way analysis of variance (ANOVA) combined with the Tuckey posthoc test for multiple comparisons or Dunnet posthoc for comparisons with control and considered significant when p value <0.05. Statistical analysis was performed on Prism Software (GraphPad) or STATISTIC 7 software. Probability levels of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) presented statistical differences.

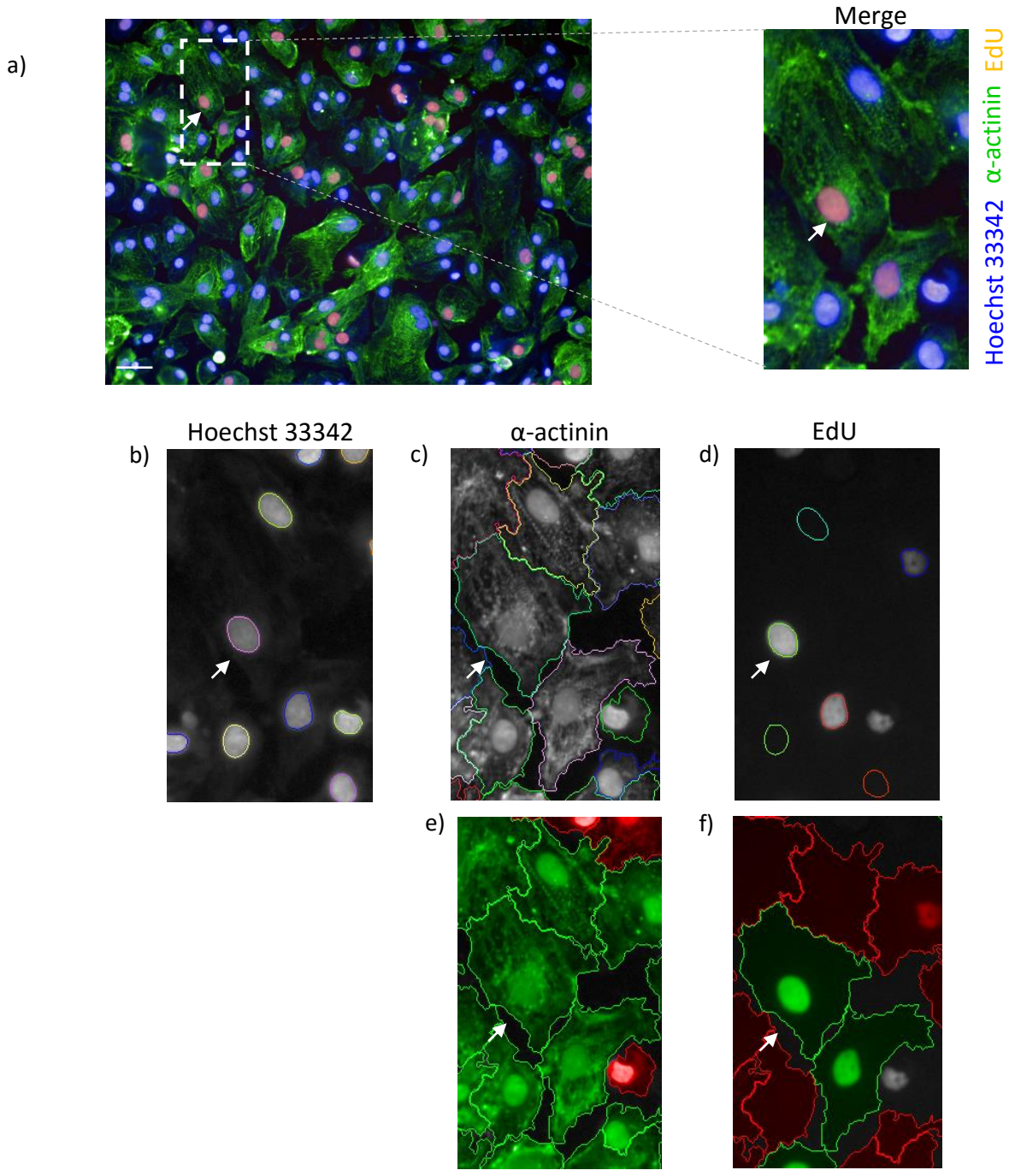


Figure 8. Illustration of the workflow used for image analysis. An image of iCMs treated with hsa-miR-302d-3p is used as an example. Cells were stained for α-actinin (Alexa 488, green), proliferative nuclei were stained with Alexa 594 (EdU assay, red) and nuclei were counterstained in Hoechst 33342 (blue). Scale bar at 70 µm. **(a)** Original imaging (expanded) acquired after treatment with hsa-miR-302d-3p; **(b)** First step of image analysis – "Find nuclei" using Hoechst 33342 channel; **(c)** Second step of imaging analysis – "Find cytoplasm" using Alexa 488 channel; **(d)** Analysis of Alexa 594 channel (EdU) of α-actinin positive cells; **(e)** Population Selection of α-actinin positive cells (cardiomyocytes); **(f)** Population Selection of double positive α-actinin and EdU (proliferative cardiomyocytes).

5. Results and Discussion

5.1 Cardiac Ischemia-Reperfusion Injury

5.1.1 Modelling of Cardiomyocyte Ischemia-Reperfusion *In Vitro*

Myocardial infarction is a leading cause of mortality in developed countries and typically occurs after prolonged exposure to ischemia. Cardiac ischemia results from restriction of blood flow to the heart, which deprives the myocardium of essential nutrients and oxygen. The recovery of blood flow, named reperfusion, is achieved through medical intervention. Together, ischemia and reperfusion induce a metabolic shift of the heart, characterized by the activation of glycolysis and lactate accumulation inside the cells, which contributes to cardiomyocyte death.

The development of reliable *in vitro* models is critical to a better understanding of human disease, as well as to the testing and development of novel therapeutic approaches. In order to establish experimental conditions that closely resemble the events of myocardial infarction and revascularization *in vitro*, we subjected human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) to hypoxia and nutrient deprivation, followed by reoxygenation and nutrient restoration.

For these experiments, cardiomyocytes from two different sources were used: cardiomyocytes derived from hiPSCs, provided by the group of Prof. Paula Alves (iBET), which will be mentioned as iCMs, and iCell cardiomyocytes available commercially from Cellular Dynamics International (see Methods section), which will be cited as (iCell-CMs).

To simulate the nutrient deprivation that occurs during ischemia, two saline solutions, designated ischemia mimetic solution (IMS - see Methods section) with or without lactate (referred to as IMS +Lac or IMS -Lac, respectively) were used. To better mimic the ischemia microenvironment characterized by lactate increment and a decrease of the intercellular pH, the pH of IMS was adjusted to pH 6.6 (see Methods section). As a control, we used the normal culture medium of hiPSC-CMs, composed of RPMI supplemented with B27 minus insulin.

Concerning hypoxia, cells were incubated for 24 or 48 hours into a hypoxic incubator with 0.1% O_2, 5% CO_2, with each of the three different media. Reperfusion was reproduced by placing cells into normoxic conditions (18% O_2, 5% CO_2) and replacing the medium by normal culture medium. As a control for these experiments, cardiomyocytes were maintained in normal culture medium and normoxic conditions (Fig. 9a and Fig. 10a for iCMs and iCell-CMs, respectively).

Representative images of iCMs and iCell-CMs showed differences in cell number (Fig. 9b and Fig. 10b) and morphology (Fig. 9c and Fig. 10c) between the different conditions; given the greater heterogeneity between the different batches of iCMs, results for these cells were normalized to the normoxia control (Fig. 9b). The results obtained from both iCMs and iCell-CMs revealed a decrease in the number of cardiomyocytes in samples treated with ischemia mimetic solution (IMS) when compared to the control medium. This effect was slightly more pronounced for cells treated with ischemia mimetic solution (IMS), when compared to the

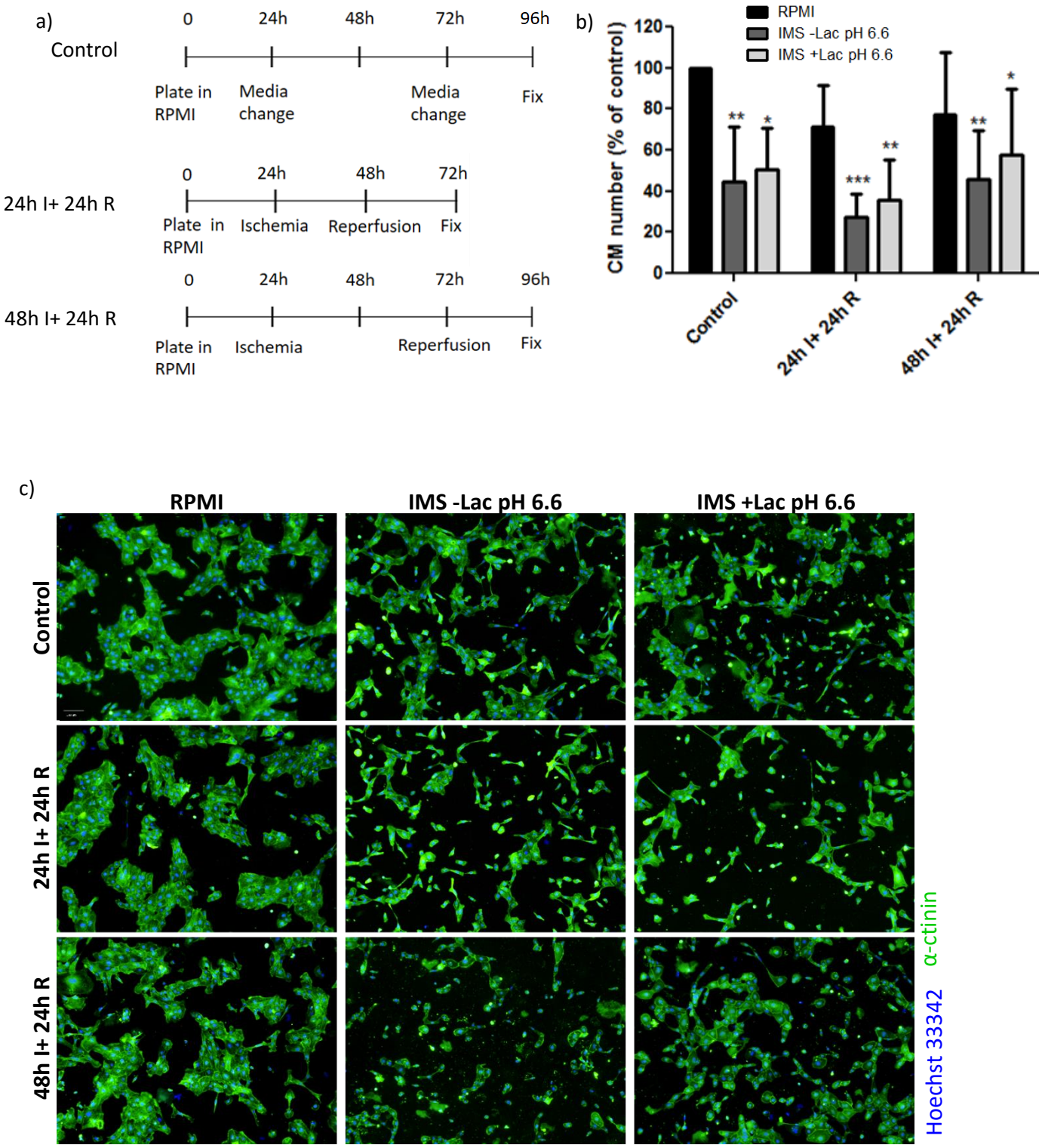


Figure 9. Modelling of cardiomyocyte ischemia-reperfusion *in vitro* in iCMs (a) Workflow for ischemia-reperfusion insult of control condition (Control, top), 24h Ischemia + 24h Reperfusion (24h I+ 24h R; middle) and 48h Ischemia + 24h Reperfusion (48h I+ 24h R; bottom); to note that when cells were maintained in complete culture medium, Ischemia (I) corresponds to hypoxia **(b)** Number of cardiomyocytes (iCMs), shown as percentage of the control condition; **(c)** Fluorescence microscopy of iCMs stained for α-actinin (green). Nuclei were counterstained using Hoechst 33342 (blue). Scale bar 100 µm. Results in (b) are shown as mean±S.D.; *p<0.5, **p< 0.01, ***p< 0.001 relative to control.

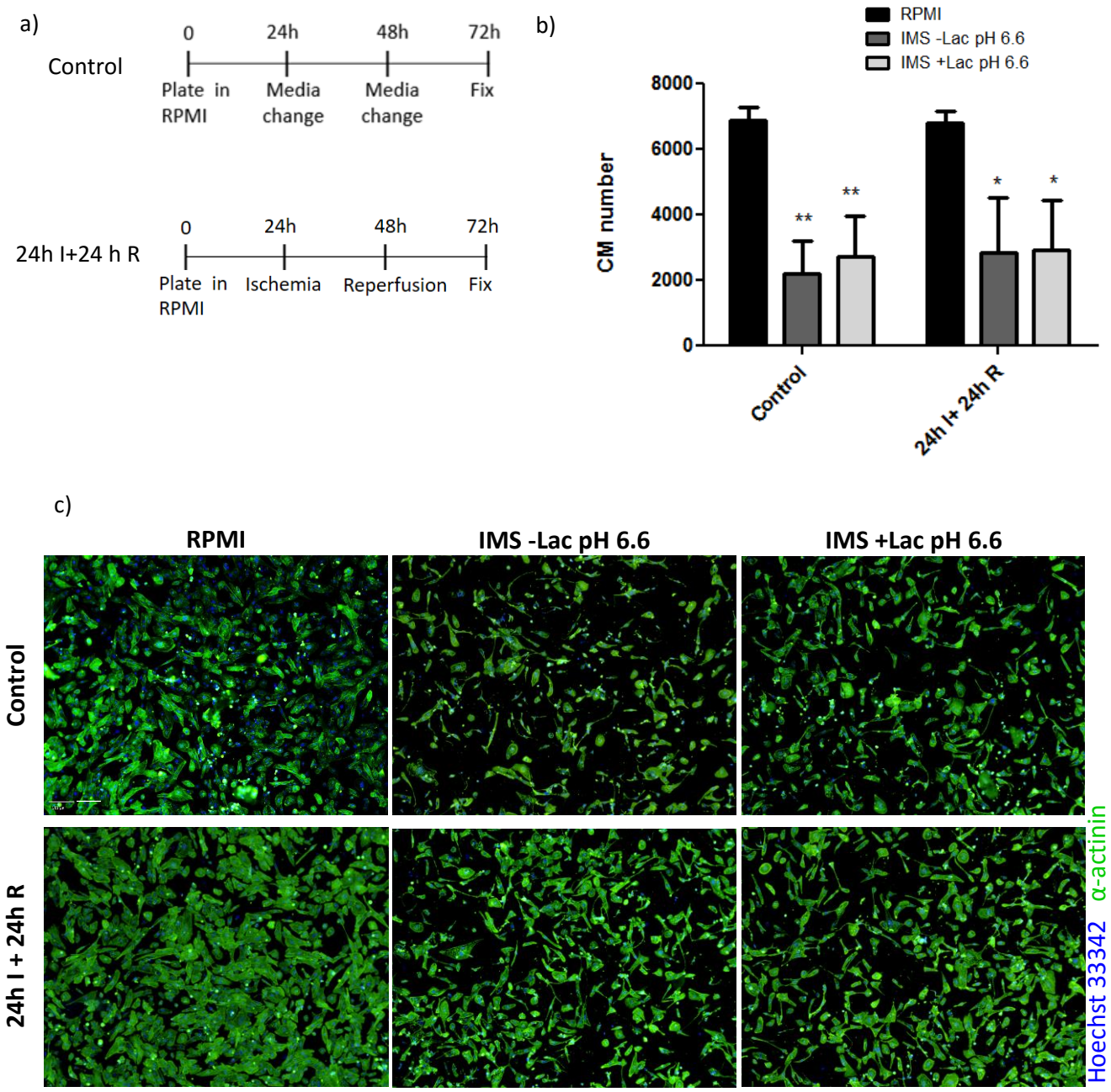


Figure 10. Modelling of cardiomyocyte ischemia-reperfusion *in vitro* in iCell-CMs (a) Workflow for ischemia-reperfusion insult in control condition (Control, top) and 24h Ischemia + 24h Reperfusion (24h I+ 24h R, bottom). To note that When cells were maintained in complete culture medium, Ischemia (I) corresponds to hypoxia **(b)** Number of cardiomyocytes; **(c)** Fluorescence microscopy of α-actinin stained (green) iCell-CMs. Nuclei were counterstained using Hoechst 33342 (blue). Scale bar 100 µm. Results in (b) are shown as mean± S.D.*p<0.5, **p< 0.01 relative to control.

control medium. This effect was slightly more pronounced for cells treated with ischemia mimetic solution without lactate (IMS -Lac) (Fig. 9b, c and Fig. 10b, c).

Treatment of cells with ischemia mimetic solution (with and without lactate) also induced a significant change of cell morphology into a shrink and shrivel appearance.

Interestingly, cells exposed to 24 or 48 hours of hypoxia did not show significant changes when compared to the corresponding controls in normoxia, for the three media

tested. These results suggest that, in contrast to nutrient deprivation, restriction of oxygen *per se* does not result in a visible phenotype. Although no differences in the cardiomyocyte number or morphology could be detected, it is strongly expected that molecular changes do indeed occur.

Taken together, these results showed that media composition has a stronger phenotypical impact on cardiomyocyte number and morphology, when compared with oxygen deprivation. We hypothesize that such changes occur due to a metabolic shift from oxidative phosphorylation to glycolysis, which allows cells to survive in hypoxic conditions. Regarding the impact of the different media, we speculate that a nutrient deprived environment could activate cell death programs, as apoptosis, that ultimately lead to cell death, therefore reducing the number of live cells.

Finally, despite the fact that both iCMs and iCell-CMs showed similar responses to the *in vitro* conditions mimicking ischemia-reperfusion injury, and although both cells are derived from human induced pluripotent stem cells, these two types of cells have a slightly different phenotype, iCell-CMs being more homogenous than iCMs. This observation reinforces the importance of the source and homogeneity of the cells for the outcome of these experiments.

Collectively, the experiments using hiPSC-CMs are an important step in translational research since allow a better study of the human response to an insult, namely, myocardial infarction, and possibly more predictive of the efficacy of novel therapeutic agents. Moreover, the use of hiPSC-CMs can potentially address patient-specific genetic differences, eventually allowing the development of more efficient, personalized medicine approaches.

Nonetheless, there are important limitations in using hiPSC-CMs to model disease, most importantly the fact that these cultures are heterogeneous, containing other cells in addition to cardiomyocytes, and that the obtained cardiomyocytes are not mature cells, which can influence the response to certain stimuli. Moreover, as any other induced pluripotent stem cell derived cell, hiPSC-CMs retain some degree of epigenetic memory, possibly interfering with cellular behavior.

5.1.2 Optimization of MicroRNA Transfection through a Reverse Transfection Protocol

MicroRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level, and have been implicated in multiple cellular functions. Previous studies have shown that microRNAs are dysregulated in several pathologies, including myocardial infarction. Modulation of microRNA levels has also been shown to constitute a potential therapeutic approach. Nonetheless, the identification of the microRNAs relevant for different pathologies, including cardiac diseases, and the consequences of their modulation, is still very preliminary.

Large-scale screenings using libraries of microRNA mimics and inhibitors constitute a valid approach to identify microRNAs relevant to different biological processes, including ischemia-reperfusion injury. To apply these approaches for the identification of microRNAs able to promote a regenerative phenotype, for example by inducing proliferation of cardiomyocytes, the optimal conditions for microRNA mimic transfection must first be optimized. In particular, a reverse transfection protocol would be preferred, when compared to a forward transfection protocol, since it is faster and it is more amenable to automation.

To optimize the conditions for reverse transfection of microRNAs into cardiomyocytes, mimics of selected microRNAs known to increase cardiomyocyte proliferation of both rat and mice were used. In particular, mimics of hsa-miR-199-3p, hsa-miR-302d-3p and hsa-miR-590-3p, were tested in both iCMs and iCell-CMs. As negative controls, cel-miR-67, a *C. elegans* microRNA that has no homology with human microRNAs was used; cells treated with the transfection mix only served also as control to measure the overall transfection toxicity.

As a positive control for transfection, a siRNA targeting UBC was administered. The UBC gene encodes for ubiquitin C, which is essential to cell survival. Cell treatment with siRNA UBC results in a toxic effect, leading to cell death. As such, the use of this siRNA allows an easy assessment of the transfection efficiency.

After treatment with the different siRNAs and microRNAs, cardiomyocyte number and cardiomyocyte proliferation (measured by EdU incorporation) were evaluated. Briefly, 48 hours after transfection and plating (reverse transfection), cells were incubated with EdU for 24 hours. The cells were then fixed and stained for α-actinin and the EdU labeling reaction was performed. The optimization of microRNA transfection was performed in both iCMs and iCell-CMs (Fig. 11a and Fig. 12a).

Concerning iCMs, from the three tested microRNA mimics, only hsa-miR-302d-3p increased cardiomyocyte proliferation, up to 15% of EdU positive cardiomyocytes (EdU labels cells in S phase, for more details see Methods section) (Fig. 11b and Fig. 11d). Hsa-miR-590-3p increased proliferation rate slightly, however, this difference was not significant (Fig. 11b and Fig. 11d). A small increase of cardiomyocyte proliferation was also observed for cel-miR-67 (Fig. 11b and Fig. 11d), suggesting that this microRNA is interfering with transcripts in these cells and therefore a better negative control should be used. Proliferation after treatment with hsa-miR-199-3p was similar to control, with a proliferation rate close to 2-3% (Fig. 11b

and Fig. 11d). Regarding the number of cells, results were normalized as described previously to correct for the heterogeneity between the different cell batches. Significant differences were observed after treatment with hsa-miR-302d-3p and siRNA UBC (Fig. 11c). Samples transfected with hsa-miR-302d-3p increased the number of cardiomyocytes, which could be explained taking into account the higher proliferative rate; in samples treated with siRNA UBC only few cells were observed when compared to other conditions, confirming that transfection was successful. No toxicity was observed with the transfection mix alone, indicating that the transfection is not toxic *per se*.

Regarding iCell-CMs, the results were essentially similar to those obtained with the iCMs (compare Fig. 11 and Fig. 12). Treatment of these cells with the different microRNAs resulted in unchanged proliferative rates (Fig. 12b) and number of cardiomyocytes (Fig. 12c), except for hsa-miR-302d-3p that augmented the proliferation rate up to 5%. The basal level of EdU positive cardiomyocytes is slightly lower (1%) when compared to iCMs (compare Fig. 11b and Fig. 12b). Samples exposed to siRNA UBC presented a lower number of cardiomyocytes (Fig. 12d), although a more dramatic cell death was expected. Therefore, we can speculate that iCell-CMs are more resistant to transfection, which could be overcome by increasing RNA concentration or use a different transfection reagent. Interestingly, these results could also indicate that UBC is not an essential gene to iCell-CMs, and therefore a siRNA targeting an alternative essential gene should be tested.

Overall, transfection conditions were appropriate for microRNA reverse transfection since the expected phenotypes were observed. Nevertheless, transfection appeared more efficient when handling iCMs compared to iCell-CMs. For instance, the role of hsa-miR-302d-3p, which significant increased cardiomyocyte proliferation, was significantly higher in iCMs, showing an increase to 15%, against only 5% in iCell-CMs.

It is interesting to note that hsa-miR-302d-3p increases cardiomyocyte proliferation in rat, mouse and human species, whereas the effect of other microRNAs (e.g. hsa-miR-199-3p and hsa-miR-590-3p) is evident in rat and mouse cardiomyocytes, but not in human cells. Most likely, the different effects observed for these microRNAs in rat and mouse cardiomyocytes, compared to human cells, reflect differences in target transcripts that preclude, or favor, critical microRNA/mRNA interactions.

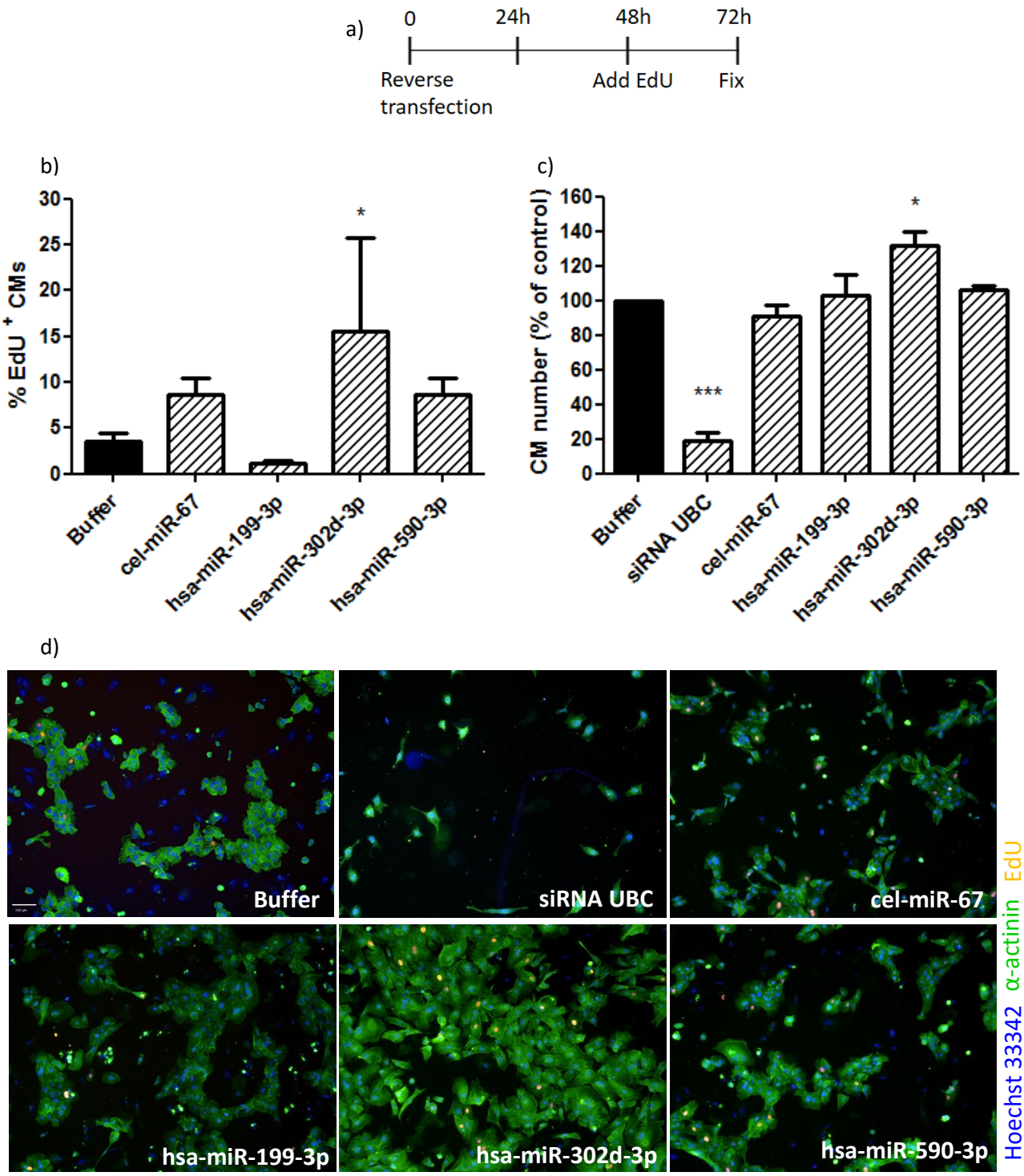


Figure 11. MicroRNA reverse transfection in iCMs (a) Schematic of workflow to assess the effects of microRNA transfection on iCMs; **(b)** Percentage of proliferative (EdU+) cardiomyocytes; **(c)** Number of cardiomyocytes (iCMs), expressed as percentage of the control; **(d)** Fluorescence microscopy of iCMs treated with selected microRNA mimics or controls. CMs were immunostained against α-actinin (green). Proliferative nuclei were stained using EdU (orange) and nuclei were counterstained using Hoechst 33342 (blue). Scale bar, 100 µm. Data in panels (b) and (c) are shown as mean±S.D.; *p<0.5, ***p< 0.001 relative to buffer condition.

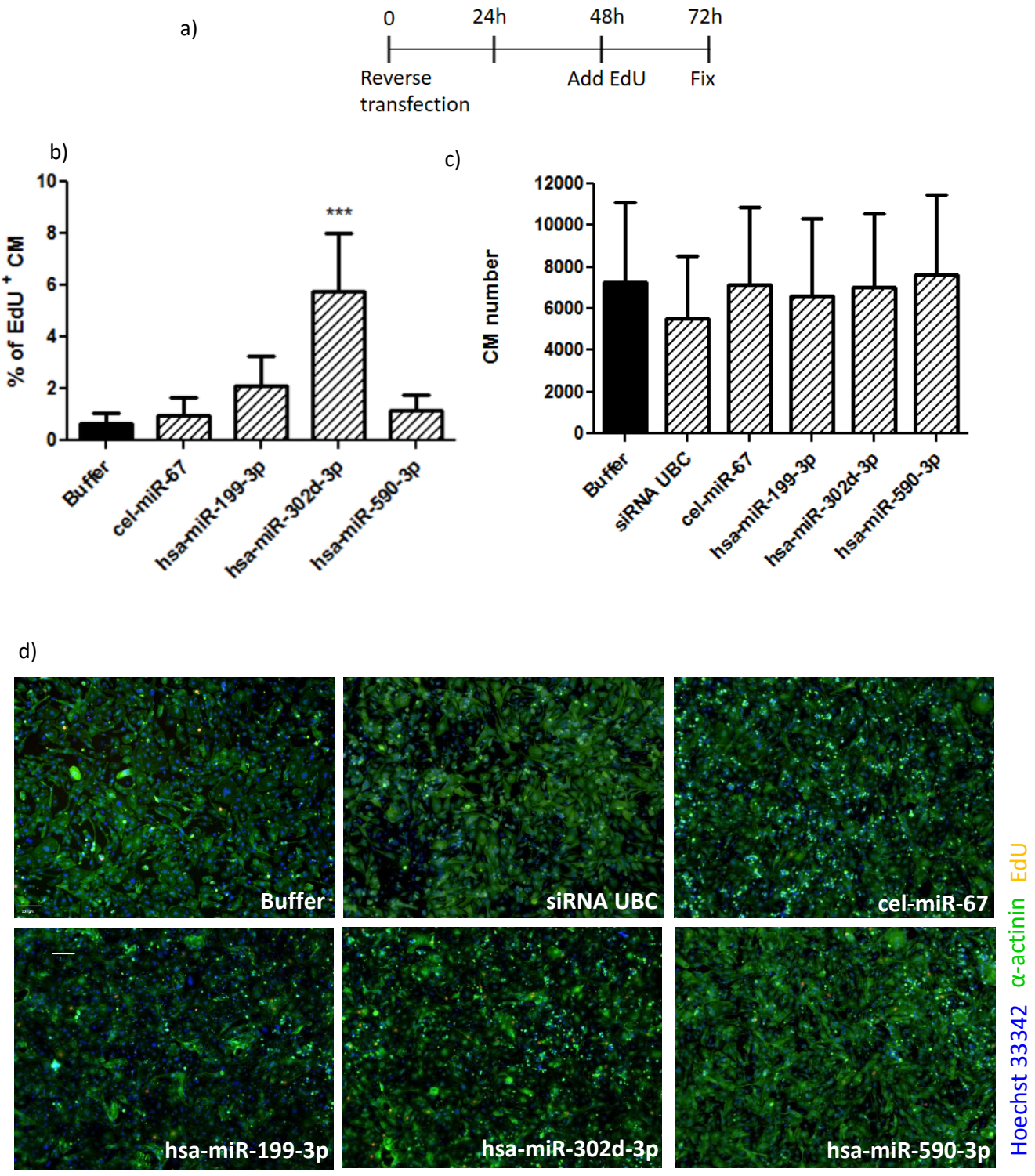


Figure 12. MicroRNA reverse transfection in iCell-CMs (a) Schematic of workflow to assess the effects of microRNA transfection on iCell-CMs; **(b)** Percentage of proliferative cardiomyocytes; **(c)** Number of cardiomyocytes (iCell-CMs); **(d)** EdU staining of iCell-CMs treated with selected mimics microRNAs or controls. CMs were immunostained against α-actinin (green). Nuclei were counterstained using Hoechst 33342 (blue) and proliferative nuclei were stained using EdU (orange). Scale bar, 100 µM. Results in panels (b) and (c) are shown as mean±S.D.; ***p< 0.001 relative to basal condition.

5.2 Cardiac Fibrosis

5.2.1 Modelling of Cardiac Fibroblast Ischemia-Reperfusion *In Vitro*

The heart has limited regenerative capacity and thus, as a consequence of myocardial infarction, dead cardiomyocytes are not replaced and reparative mechanisms are activated, mainly fibrosis. Cardiac fibrosis, the second main event of MI, occurs as consequence of ischemia-reperfusion injury and it is characterized by proliferation of cardiac fibroblasts and ECM deposition. Cardiac fibroblasts are mainly responsible for ECM remodeling, heart support and leakage block. However, after injury, their excessive proliferation and ECM secretion increases the stiffness of the ventricle wall, compromising heart function, and potentially leading to heart failure.

Nonetheless, the rate at which human cardiac fibroblasts proliferate after cardiac ischemia-reperfusion injury and the impact of ischemia-reperfusion on these cells is still not well characterized. In this context, we assessed cardiac fibroblast proliferation after ischemia-reperfusion injury. In addition, we also assessed the effect of ischemia *per se* in cardiac fibroblasts.

To evaluate the proliferative rate of human cardiac fibroblasts, cells were exposed to a protocol mimicking ischemia-reperfusion insult, similar to that described previously for cardiomyocytes. The use of human cells in these experiments increases the relevance of this study and facilitates the extrapolation of the results to human disease.

Nutrient deprivation typical of ischemia was imposed by changing the medium to IMS -Lac and IMS +Lac, as previously performed in cardiomyocytes. Notably, reports in the literature regarding cardiac fibroblasts described IMS as a more alkaline media with pH 7.4. For this reason, in these experiments we used IMS at both pH 6.6 and pH 7.4. Along with media exchange, cardiac fibroblasts were incubated for 24 hours in a hypoxic incubator composed by 0.1% O_2, 5% CO_2. When indicated, reperfusion was reproduced by replacing IMS for normal culture medium, i.e. DMEM high glucose supplemented with FBS, FGF and insulin (see Methods section), and placing cells into a normoxic incubator. Alternatively, in control condition, cardiac fibroblasts were maintained in normal culture medium and normoxic conditions (Fig. 13a).

As previously observed in cardiomyocytes, after treatment with IMS the number of cardiac fibroblasts drastically decreased (Fig. 13b). In contrast, hypoxia *per se* (cells exposed to hypoxic environment in normal culture medium), did not aggravate the phenotype (Fig. 13b and Fig. 13d).

Proliferation of cardiac fibroblasts was examined by analyzing EdU incorporation. Briefly, at 20 hours of reperfusion, or alternatively, ischemia, cells were incubated with EdU and, after 4 hours, cardiac fibroblasts were fixed and EdU labeling reaction was performed (Fig. 13a). Of note, this is a purified primary culture and therefore all cells were considered cardiac fibroblasts.

Regarding cardiac fibroblast proliferation, a basal level of 15% of proliferation was observed, clearly higher than that observed for cardiomyocytes, as expected. Of note, the proliferation capacity of HCFs was not lost after ischemia-reperfusion. In fact, EdU incorporation after treatment with IMS pH 7.4 reached 25% (Fig. 13c and Fig. 13d), a slight increment when compared to the control condition. However, this effect was not statistically significant. In contrast, samples exposed to hypoxia and treated with IMS (ischemia only), showed a dramatic decrease in proliferation, to nearly 1% when contrasted with 15% of control condition (Fig. 13c and Fig. 13d).

Taken together, these results suggest that ischemia-reperfusion injury contributes to cardiac fibroblast death, but simultaneously promotes their proliferation, operating as a pro-fibrotic stimulus. Since this stimulus is only noticed after reperfusion, and not ischemia alone, it is likely that reperfusion is accountable for the activation of reparative mechanisms as pro-fibrotic pathways, ultimately leading to scar formation.

Finally, results from both cardiomyocytes and cardiac fibroblasts suggest that the ischemia-reperfusion protocol used in this study adequately models ischemia-reperfusion injury *in vitro*.

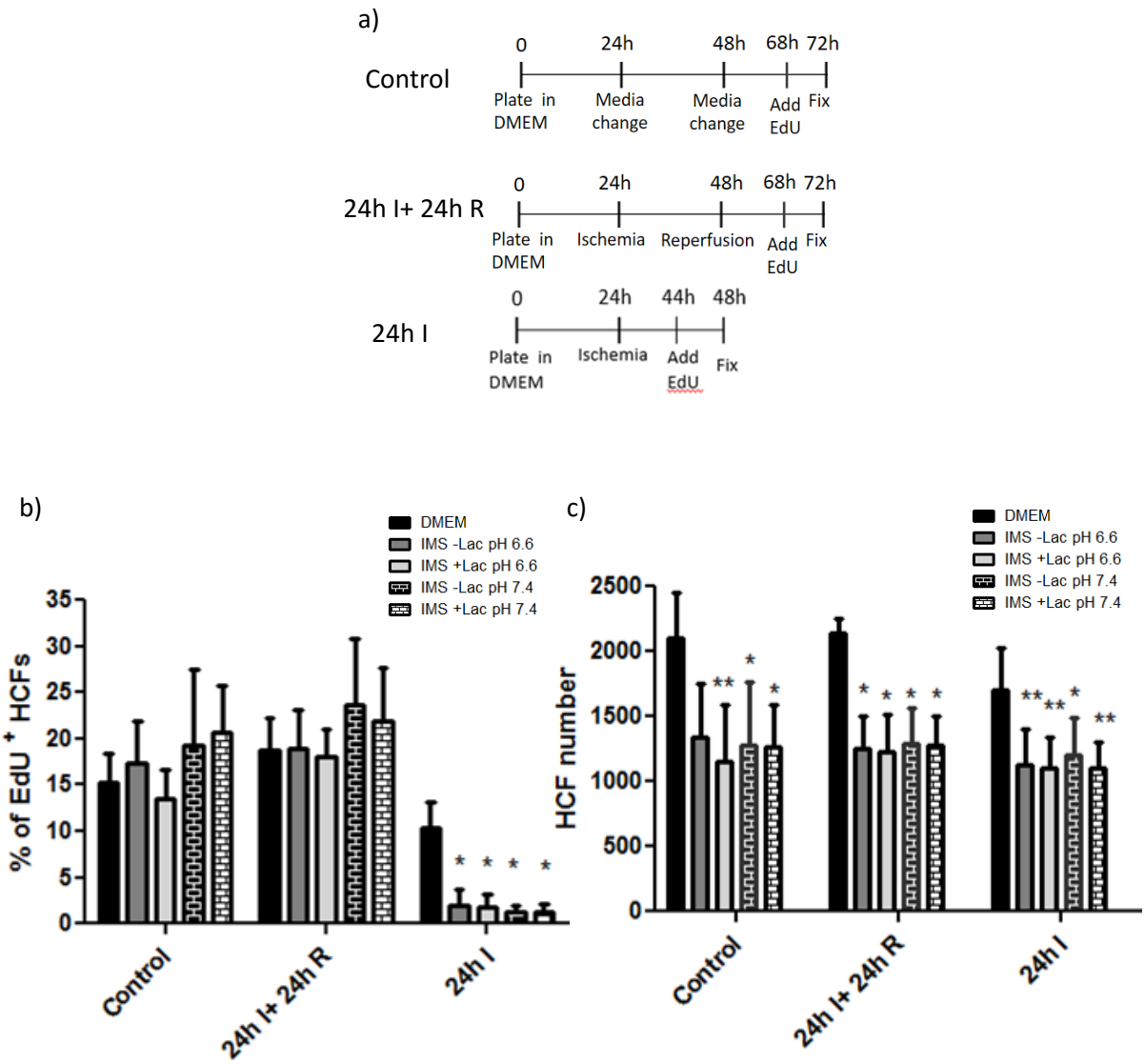
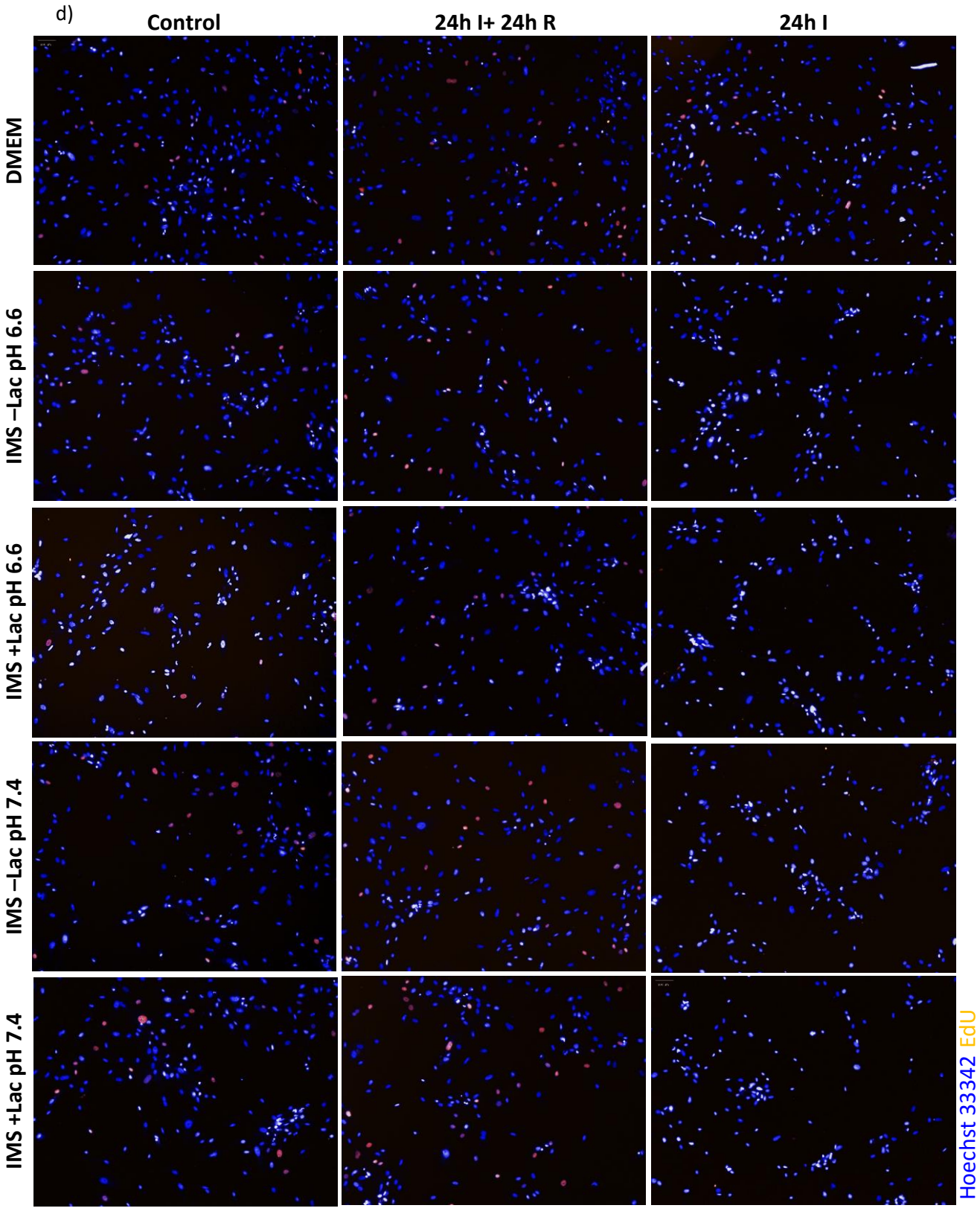


Figure 13. Modelling of cardiac fibroblast ischemia-reperfusion *in vitro.* **(a)** Workflow for ischemia-reperfusion insult and evaluation of cell proliferation in control (top), 24h ischemia+ 24h reperfusion (24h I+24h H, middle) and 24h ischemia condition (24h I, bottom); to note that when cells were maintained in complete culture medium, Ischemia (I) corresponds to hypoxia. **(b)** Percentage of proliferative human cardiac fibroblasts; **(c)** Number of human cardiac fibroblasts; **(d)** Representative fluorescence microscopy images of human cardiac fibroblasts. Cardiac fibroblasts nuclei were stained with Hoechst 33342 (blue) and proliferative nuclei were labeled with EdU (red). Scale bar 100 µm. Data in panels (b) and (c) are shown as mean±S.D.; *p<0.5, **p< 0.01 relative to control condition.


d)
Control
24h I+ 24h R
24h I
DMEM
IMS –Lac pH 6.6
IMS +Lac pH 6.6
IMS –Lac pH 7.4
IMS +Lac pH 7.4
Hoechst 33342 EdU

5.2.2 Optimization of a Large-Scale Screen to Identify MicroRNAs Regulating Myofibroblast Activation

In the human heart, cardiomyocytes and cardiac fibroblasts are in close contact, sending and receiving reciprocal stimuli.

Myofibroblasts are an active and contractile form of fibroblasts characterized for being highly responsive migratory cells. During MI, myofibroblasts are the primary source of ECM proteins, inducing massive ECM synthesis and deposition, which leads to ventricle wall stiffness. These cells are also able to contract due to expression of α-smooth muscle actin (α-SMA) and stress fibers and, although myofibroblasts are extremely helpful after myocardial infarction to favor wound healing and prevent cardiac rupture, their presence at advanced stages of the reparative process leads to scar formation, which compromises heart function.

MicroRNAs have been proposed as crucial players during myofibroblast transformation, however, at the moment there is only limited information regarding microRNA regulation of cardiac myofibroblast activation, as well as concerning the involvement of microRNAs in myocardial infarction. For that reason, it is important to evaluate the role of microRNAs during cardiac fibrosis and also to elucidate which microRNAs promote or prevent myofibroblast phenotype activation, as well as other related events such as cardiomyocyte and fibroblast proliferation.

Large-scale screenings using libraries of human microRNA mimics and inhibitors have proved to be a reliable tool to identify microRNAs relevant to different biological processes.

As a preliminary approach to test the feasibility of a screening to identify microRNAs involved in myofibroblast activation, a pilot screen using microRNA mimics (36 microRNAs) known to increase cardiomyocyte proliferation in both rat and mice was performed in human cardiac fibroblasts. Briefly, HCFs were transfected (reverse transfection) and medium was changed 48 hours after reverse transfection. After an additional 48 hour period, the cells were fixed and immunofluorescence against α-SMA, a marker of myofibroblasts, was performed to assess myofibroblast activation (Fig. 14a). Previous experiments performed in our lab have demonstrated that DMEM high glucose supplemented with 10% FBS, is the most adequate media to evaluate myofibroblast activation (data not shown).

As controls for these experiments, cells were treated with cel-miR-67, a *C. elegans* microRNA with no homology to human microRNAs, and siRNA NT #5, a siRNA with no targets in the human genome. To assess toxicity of the transfection procedure, cells were treated with transfection mix only (mock). As a positive control for transfection, cells were treated with siRNA UBC.

Myofibroblast activation was evaluated by scoring the percentage of cells presenting α-SMA fibers, using automated image acquisition and analysis.

Treatment of cardiac fibroblasts with the different microRNAs led to significant differences in myofibroblast activation, as measured by α-SMA staining (Fig. 14b and Fig. 15). We identified six microRNA mimics that increase myofibroblast activation (Fig. 14b and Fig.

15), namely, hsa-miR-26b-5p, hsa-miR-19b-2-5p, hsa-miR-2052, hsa-miR-875-5p, hsa-miR-210-3p and hsa-miR-19a-5p (in descending order). The highest effect was observed after treatment with hsa-miR-26b-5p with 70% of cells showing a myofibroblast phenotype, compared to approximately 30% in the control situation.

Using this approach, we also identified three microRNA mimics that prevent myofibroblast activation, specifically, hsa-miR-1281, hsa-miR-130a-5p and hsa-miR-143-5p. The most dramatic effect was found in samples treated with hsa-miR-1281 with only 10% of cells showing a myofibroblast phenotype (Fig.14b and Fig. 15). Although a decrease in myofibroblast activation was also noticed after treatment with other microRNAs, such as hsa-miR-520b, hsa-miR-511 and hsa-miR-373, differences were not significant.

We noticed that culture of cells *per se* induced myofibroblast activation to some extent, a behavior that depended on passage number and media used for thawing. Despite some variability observed in basal conditions, the identified microRNAs exhibited a strong effect, despite the level of this basal activation (Fig. 14b).

In parallel to the measurement of myofibroblast activation, we also analyzed the cell number after treatment with the different microRNAs, to account for possible toxic effects of selected microRNAs on cardiac fibroblasts. Given the variability between different experiments in terms of overall number of cells, these results were normalized to the median of all samples (Fig. 14c). Treatment with the different microRNAs did not result into significant differences (more than two-fold) in terms of the number of cells, indicating that none of the used microRNA mimic is toxic to these cells. Despite the absence of marked effects, results denoted that some microRNAs slightly increase or decrease the number of cells, most likely due to their effects on cardiac fibroblast proliferation. As expected, treatment with siRNA UBC, resulted in a very pronounced cell death, validating transfection efficacy (Fig. 14c and Fig. 15).

Despite inducing the same phenotype, microRNAs that increase myofibroblast activation differed on seed sequence (except for hsa-miR-19a-5p and hsa-miR-19b-2-5p which are from the same family), and therefore are likely to regulate different targets. Consequently, we speculate that myofibroblast activation induced by the microRNAs could derive from different stimuli, and depending on that, different pathways can be activated.

Next, we analyzed the literature to understand the role of the microRNAs with the strongest effect in myofibroblast phenotype, namely hsa-miR-26b-5p, hsa-miR-19b-2-5p, hsa-miR-2052, hsa-miR-875-5p, hsa-miR-210-3p and hsa-miR-19a-5p, in the context of cardiac injury and regeneration.

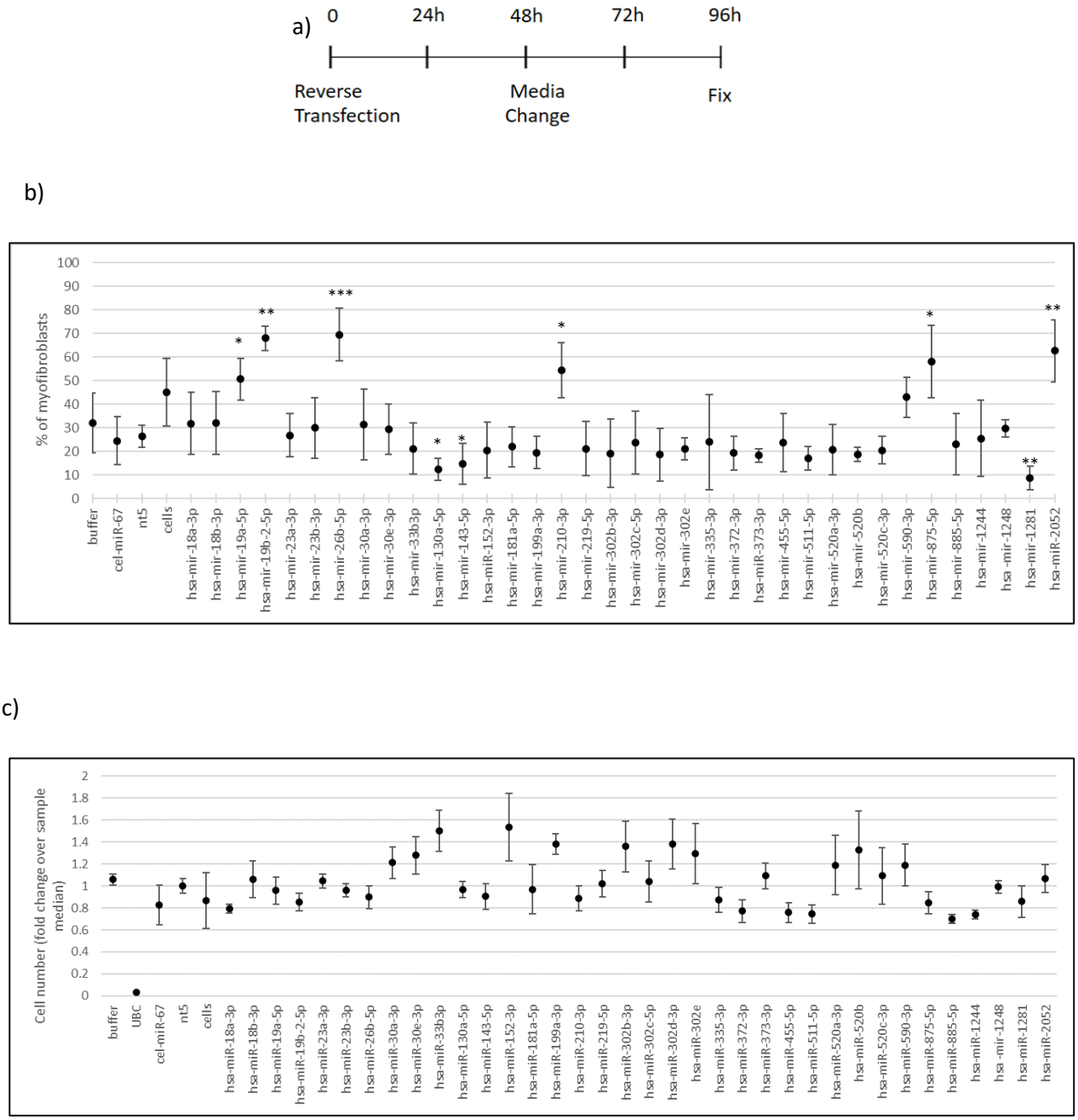


Figure 14. Pilot screen for microRNAs regulating myofibroblast activation. (a) Workflow of the protocol used for the screen; **(b)** Percentage of cells showing a myofibroblast phenotype (α-SMA positive) after reverse transfection of selected microRNAs; **(c)** Number of cells after treatment with selected microRNAs, normalized to median of all samples. Data in (b) and (c) are presented as mean±S.D.; *p<0.5, **p< 0.01, ***p<0.001 relative to control condition.

From the 6 microRNAs with the strongest effect in myofibroblast activation, the best characterized microRNA is hsa-miR-210, a master hypoxamiR that is strongly upregulated after hypoxia. The described effects of hsa-miR-210 include activation of pro-survival kinases and glycolysis switch [62, 71, 72]. Like hsa-miR-210, hsa-miR-19a-5p is a cardioprotective microRNA with an anti-apoptotic function [73]. Both of these microRNAs were found in exosomes after cardiac injury, making them also potential MI biomarkers. Of the same family

of hsa-miR-19a-5p, hsa-miR-19b-2-5p was described to promote cardiomyocyte survival and, more importantly, to control cardiac fibroblast migration and proliferation, being PTEN one of the targets [74]. PTEN was also reported to be regulated by hsa-miR-26b-5p, a microRNA upregulated in cardiomyocytes after ischemia. A previous study reported that inhibition of hsa-miR-26b-5p increases PTEN expression and consequently promotes cell survival [75]. Nothing was reported for hsa-miR-2052 and hsa-miR-875-5p in the context of myocardial infarction.

Overall, our findings suggest that the microRNA reverse transfection protocol followed in these experiments is appropriated for the transfection of cardiac fibroblasts and could be applied to a large-scale screen. Interestingly, on the one hand we observed that hsa-miR-19b-2-5p promotes myofibroblast activation in both rat and human cells [74], confirming the results from previous studies. On the other hand, the discovery of new microRNAs involved in myofibroblast activation may be useful to modulate cardiac injury, and increase our understanding of the pathology.

Despite the extensive knowledge of microRNA function, the understanding of their biological relevance is still incomplete, and therefore studying the impact of microRNAs in the context of myocardial infarction is crucial to develop an efficient microRNA-based therapy against cardiac injury.

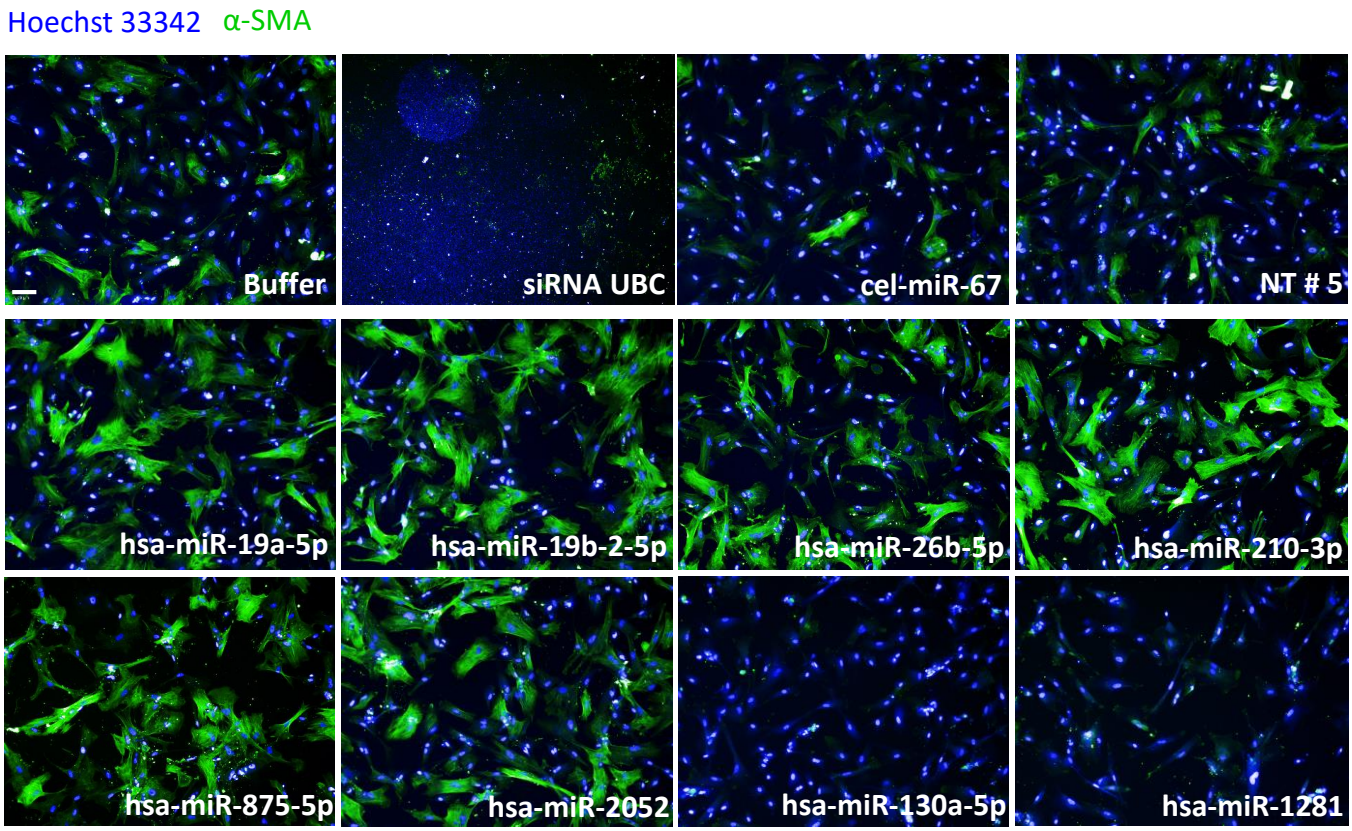


Figure 15. Representative fluorescence microscopy images of myofibroblast activation for control and top scoring microRNAs. Results for the controls, six microRNAs increasing myofibroblast activation (hsa-miR-19b-2-5p, hsa-miR-19a-5p, hsa-miR-26b-5p, hsa-miR-210-3p, hsa-miR-875-5p, and hsa-miR-2052) and 2 microRNAs decreasing myofibroblast activation (hsa-miR-130a-5p and hsa-miR-1281) are shown. Cells were immunoassayed against α-SMA (green) and nuclei were counterstained with Hoechst 33342 (blue). Scale bar 100 µm.

5.2.3 Relationship Between MicroRNAs Inducing Myofibroblast Activation and Fibroblast Proliferation

As previously stated, myofibroblasts are characterized as cells able to contract and proliferate, yet, how fibroblast proliferation and myofibroblast activation correlate and what mechanisms are activated in response to different stimuli, is poorly understood.

To gain insights into this relationship, we compared the results concerning the effect of microRNAs on myofibroblast activation (described above) with results from a previous study performed in our lab investigating proliferation of human cardiac fibroblasts and human cardiomyocytes promoted by microRNAs. A summary of the results obtained for a selected subset of microRNAs is shown in Table 3. MicroRNAs with the highest and the lowest scores regarding cardiomyocyte and fibroblast proliferation and myofibroblast activation were selected.

As outlined above, some microRNAs promoted myofibroblast activation, namely, hsa-miR-19b-2-5p, hsa-miR-26b-5p, hsa-miR-210-3p, hsa-miR-875-5p and hsa-miR-2052. Remarkably, these microRNAs do not appear to have an impact in cardiomyocyte proliferation and, interestingly, they decrease fibroblast proliferation. We also noticed that microRNAs that increased cardiac fibroblast proliferation particularly, hsa-miR-520b and hsa-miR-373-3p, decreased myofibroblast activation slightly, and also promoted the proliferation of cardiomyocyte.

In contrast, hsa-miR-33b-3p, hsa-miR-152-3p and hsa-miR-1248, microRNAs that promoted cardiomyocyte proliferation, have more heterogeneous effects in terms myofibroblast activation and fibroblast proliferation.

MicroRNAs that prevented myofibroblasts activation, hsa-miR-130a-5p, hsa-miR-143-5p and hsa-miR-1281, also presented distinct results regarding cardiomyocyte and cardiac fibroblast proliferation.

Taken together, these observations could denote that fibroblasts are in fact able to proliferate, however, results also suggested that when cardiac fibroblasts are stimulated to become active and transform into myofibroblasts they are discouraged to proliferate.

Collectively, the analysis of this small subset of microRNAs has proved that microRNAs are indeed crucial players in controlling several myocardial infarction related processes, including cell proliferation and fibrosis, acting in different cardiac cell types, particularly in cardiomyocytes and cardiac fibroblasts. This reinforces the concept that modulation of microRNAs could be used as a therapeutic strategy against cardiac ischemia-reperfusion injury. For this purpose, a microRNA should ideally promote cardiomyocyte proliferation, prevent cardiac fibroblast proliferation and do not interfere in myofibroblast activation, at least in a first stage when this process is beneficial.

We can also speculate that, although more microRNAs need to be investigated, from our results, hsa-mir-1248 appears to be the microRNA with a more promising profile, and could constitute an interesting candidate to rescue the damaged phenotype caused by cardiac ischemia-reperfusion.

Table 3. Relationship between microRNA inducing cardiomyocyte proliferation, myofibroblast activation and fibroblast proliferation.

MicroRNAs	Cardiomyocyte Proliferation	Myofibroblast Activation	Fibroblast Proliferation
hsa-miR-19a-5p	-	+	--
hsa-miR-19b-2-5p	-	+	--
hsa-miR-26b-5p	=	++	--
hsa-miR-33b-3p	+	-/=	=
hsa-miR-130a-5p	+	--	=
hsa-miR-143-5p	-/=	--	--
hsa-miR-152-3p	+	+	--
hsa-miR-210-3p	=	++	--
hsa-miR-373-3p	+	-/=	++
hsa-miR-520b	+	-	++
hsa-miR-875-5p	-	+	--
hsa-miR-1248	+	+/=	-
hsa-miR-1281	-	--	--
hsa-miR-2052	=	++	--

6. Conclusions and Future Perspectives

Cardiac Ischemia-Reperfusion

Cardiac ischemia is a major event during myocardial infarction, a common and debilitating disorder, which still lacks from efficient treatment and diagnosis. Ischemia results from the obstruction of an artery and it is typically followed by surgical restoration of blood flow and reperfusion. Conjointly, these processes lead to cardiomyocyte death. Because the adult human heart has limited regenerative capacity, dead cardiomyocytes are not replaced. Therapeutic strategies intended to minimize the effects of cardiac ischemia-reperfusion injury, for example by stimulating proliferation of preexistent cardiomyocytes, are valid approaches to promote regeneration.

MicroRNAs are small non-coding RNAs that control roughly 60% of the human genes, at a post-transcriptional level. MicroRNAs have recently urged as potential therapeutic targets and as biomarkers for a number of disorders, including ischemia. However, most of the reported studies were not performed in human cells making difficult to extrapolate the obtained findings.

In this context, this study was able to employ an in vitro model resembling cardiac ischemia-reperfusion injury, using human cardiomyocytes derived from iPSCs. Given the exploratory nature of this project, the results obtained were not sufficient to elucidate the mechanisms underlying ischemia-reperfusion injury and microRNA dysregulation. However, we were able to validate the in vitro model of ischemia-reperfusion injury showing that it alters the morphology and viability of cardiomyocytes.

Furthermore, we were also able to establish the experimental conditions required to perform large-scale experiments aimed at systematically identifying microRNAs relevant to this process. In the process of optimization, we also determined that hsa-miR-302d-3p is a strong inducer of proliferation of human cardiomyocytes.

Cardiac Fibrosis

Fibrosis arises as a reparative mechanism triggered by ischemia-reperfusion injury. Cardiac fibrosis, a dominant event during scar formation, is manifested by ECM accumulation, cardiac fibroblast proliferation and myofibroblast activation, leading to increased stiffness of the heart walls, and possibly leading to heart failure. Several studies have reported microRNAs as regulators of scar response, and indeed, their modulation via administration of microRNA mimics and inhibitors is considered a promising strategy to counteract fibrosis and rescue from heart failure.

In this study we were able to adequately model cardiac ischemia-reperfusion in human cardiac fibroblasts. Indeed, we observed that as a consequence of the insult cardiac fibroblasts increased their proliferation. Importantly, we also determined that reperfusion seems to be the pro-fibrotic stimulus during myocardial infarction.

In addition, we established the experimental conditions, in particular efficient reverse transfection protocols, required to perform large-scale experiments using libraries of microRNA mimics and inhibitors to identify microRNAs controlling proliferation of human cardiac fibroblasts and myofibroblast activation. Using this approach, we performed a “proof-

of-principle" pilot screen using 36 microRNA mimics and identified several microRNAs that have a strong activity in promoting (hsa-miR-26b-5p, hsa-miR-19b-2-5p, hsa-miR-2052, hsa-miR-875-5p, hsa-miR-210-3p and hsa-miR-19a-5p) or preventing (hsa-miR-1281, hsa-miR-130a-5p and hsa-miR-143-5p) myofibroblast activation.

In this study it was also possible to infer a biological correlation between proliferation and activation of cardiac fibroblasts following microRNA treatment. Our results determined that microRNAs involved in myofibroblasts activation prevent cardiac fibroblast proliferation, suggesting that during scar formation different stimuli are released.

Future Perspectives and Limitations

Cardiovascular diseases are a major cause of mortality in developed countries, and microRNAs have emerged as key players in myocardial infarction related events, including cardiac fibrosis and ischemia-reperfusion injury. It is expected that microRNAs will soon become of broad therapeutic use for a number of pathologies including myocardial ischemia injury and also serve as biomarkers for the diagnosis/prognosis of cardiovascular diseases.

A more challenging aspect, and probably one of main unknowns in microRNA-based therapeutics is related to the *in vivo* consequences of microRNA administration. Since microRNAs can have multiple targets with distinct functions and present in different organs and tissues, microRNA modulation can, in theory, lead to undesirable effects, affecting non-pathological pathways. Another obstacle is the specific delivery of microRNAs to a certain organ or tissue. Indeed, more studies are needed before microRNA-based therapies can become a routine application in clinics.

This study has established the experimental conditions for performing screening studies in human cells cardiomyocytes and cardiac fibroblasts, which will be explored to test libraries of microRNA mimics and inhibitors and identify novel microRNAs affecting heart-related disease phenotypes and, eventually, able to revert cardiac injury.

7.Bibliographic References

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