DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Identification of MicroRNAs to promote cell survival for the treatment of ischemic diseases

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Hugo Fernandes (CNC) e do Professor Doutor Paulo Santos (Universidade de Coimbra).

Carolina Bento dos Santos

2016

The experimental work described in this dissertation was performed at Neuroscience and Cell Biology (CNC), in Biomaterials & Stem Cell Based Therapeutics group, in the unit UC-Biotech (Biocant Park)

Todo o trabalho experimental efetuado nesta dissertação foi realizado no Centro de Neurociências e Biologia Celular (CNC), no grupo "Biomaterials & Stem Cell Based Therapeutics" UC-Biotech (Biocant Park)

This work was financially supported by *Fundação para a Ciência e Tecnologia* - FCT (PTDC/BIM-MED/1118/2012)

AGRADECIMENTOS

Este trabalho é o resultado de uma estadia bastante enriquecedora na UC-Biotech, no grupo "Biomaterials & Stem Cell Based Therapeutics" onde quero desde já agradecer a todos os colegas por me acolherem e por me apoiarem, fazendo com que a realização deste trabalho fosse possível.

Quero também agradecer ao Dr. Hugo Fernandes e Dra. Ana Barradas pelo voto de confiança, por acreditarem nas minhas capacidades, por me terem dado a oportunidade de trabalhar numa das minhas eleitas áreas da ciência e por me terem providenciado tanto os meios como a audaz orientação para a realização deste trabalho.

À Helena, Alessandra e Catarina, as minhas "coffee buddies", um enorme obrigada por toda a ajuda que me prestaram, por todo o apoio, ensinamentos e bons conselhos, e sobretudo pela amizade.

Aos bons amigos, Melissa, João, Inês e Judite pela amizade incondicional e pelos bons momentos.

Um agradecimento especial à Juliana a minha "sister from another mother",pela amizade, cumplicidade e companheirismo, por estar sempre presente na minha vida e por provar que a família não se limita a laços sanguíneos.

A todos os restantes amigos e colegas que, infelizmente, demasiados para aqui serem mencionados, mas que de uma forma ou de outra me ajudaram ou pela sua simples companhia, o meu profundo e sincero obrigada.

Ao meu namorado, Nuno, pela paciência inesgotável, pelo carinho, amor e amizade.

À minha fantástica família, a quem eu dedico este trabalho, um agradecimento muito especial por tudo. Aos meus tios Carlos e Michelle pela felicidade que demonstram pelo meu sucesso, pelo carinho e apoio que sempre me deram. Não posso também deixar de agradecer à minha super irmã, Joana, pelo companheirismo, apoio incondicional, pelos desabafos e pela compreensão. Aos meus pais pelas oportunidades que me deram, por acreditarem em mim, pela força e apoio incondicionais, pela liberdade que me ofereceram para eu seguir os meus sonhos e por serem o meu "porto" seguro. Obrigada por tudo, principalmente pelos valores e sábios conselhos.

“Nothing is too wonderful to be true, if it be consistent with the laws of nature.”

Michael Faraday

“Make up your mind to act decidedly and take the consequences. No good is ever done in this world by hesitation.”

Thomas Huxley

ABSTRACT

Cardiovascular Diseases (CVDs) are the primary cause of global mortality and morbidity. Functional recovery of ischemic tissues and organs is dependent on establishing collateral networks that supply oxygenated blood. Cell-based therapies for the prevention and treatment of cardiac dysfunction offer the potential to significantly modulate cardiac function and improve outcomes in patients with CVD.

Stem cell therapy arise as an encouraging treatment for myocardial infarction but the low survival of the transplanted cells in the infarcted myocardium is possibly the primary reason for failure of long-term improvement. The leading causes of reduced survival of stem cells *in vivo* are connected to anoikis, possible immune rejection and oxidative damage interceding apoptosis. Hence, the development of innovative pro-survival approaches to increase stem cell survival will be a major benefit to this field. The survival depends on the specific cell type, the quantity and the quality of transplanted cells, and the status of the host tissue.

In this thesis, we proposed methods (ischemic conditions) and potential molecular mechanisms for cell preconditioning *in vitro* to increase their retention after transplantation in damaged tissues *in vivo*. Approximately 1 billion cardiomyocytes are lost upon myocardial infarction, but the regeneration capacity of these cells is massively low and it is difficult to rely entirely on the heart´s naturally healing potential to overcome this.

Recent studies suggested that transplantation of endothelial cells can significantly improve the efficacy of cell based heart repair. Endothelial cells play imperative physical and functional roles in vascular homoeostasis. Destabilization in endothelial cell amount and function are strictly involved with the beginning and evolution of several CVDs.

Lately, microRNAs (miRNAs) have emerged as novel switch controllers of cardiovascular functioning and pathology. Diverse microRNAs are able to control stem

and progenitor cell functions, regulating cell survival and homing or monitoring differentiation and maturation. Moreover, experimental reports show that microRNAs can also regulate endogenous repair and possibly be valuable to increase the regeneration of the heart.

The purpose of this study was to identify microRNAs that enhance cell survival and study the role of these miRNAs in the biology of endothelial cells in order to promote cell survival after transplantation.

KEY WORDS: Cell therapies; Endothelial cells; Cell survival; Transplantation; MicroRNAs; Cardiovascular diseases; Ischemia.

RESUMO

Doenças cardiovasculares (DCVs) são a principal causa de mortalidade e morbidade a nível global. A recuperação dos órgãos e tecidos isquémicos depende do estabelecimento de redes funcionais que possam bombear o sangue para que este chegue a todos os órgãos, a fim de fornecer o oxigénio e nutrientes necessários. Terapias celulares para a prevenção e tratamento de disfunções cardíacas, oferecem a possibilidade de modular significativamente a função cardíaca e melhorar os resultados em pacientes com doenças cardiovasculares.

O uso de células estaminais em terapias celulares surgem como um possível tratamento para pós enfarte do miocárdio, porém a baixa sobrevivência das células transplantadas para o tecido isquémico é possivelmente a principal razão para o fracasso destas terapias. As principais causas de sobrevivência reduzida são a possível rejeição imunológica, "anoikis" e danos oxidativos intercedendo apoptose. Posto a isto, o desenvolvimento de abordagens inovadoras que permitam aumentar a sobrevivência das células estaminais após transplante podem vir a ser um grande benefício para estas terapêuticas. A sobrevivência celular depende do tipo específico de células a utilizar, da quantidade e da qualidade das células transplantadas e o estado do tecido hospedeiro.

Neste estudo propomos alguns mecanismos a serem usados no pré-condicionamento de células *in vitro* para aumentar a sua retenção após a injecção para os tecidos danificados *in vivo*. Aproximadamente 1 bilião de cardiomiócitos são perdidos após enfarte do miocárdio, porém a capacidade de regeneração dessas células é extremamente baixa e por isso, o coração por si só não tem um potencial regenerativo que o permita ultrapassar um insulto isquémico. Estudos recentes sugeriram que a transplantação de células endoteliais pode melhorar significativamente a eficácia da reparação de células do coração. As células endoteliais desempenham um papel fundamental na manutenção da homeostasia vascular. Qualquer desestabilização na quantidade ou na função das células endoteliais

encontra-se estritamente envolvida com o aparecimento de várias doenças cardiovasculares.

Recentemente, os microRNAs surgiram como novos controladores no funcionamento de patologias vasculares. Diferentes microRNAs são capazes de ditar as funções de células endoteliais progenitoras, regulando a sua sobrevivência, "homing" e/ou monitorizar processos de maturação/diferenciação. Alem disso, relatórios experimentais sugerem que os microRNAs podem também regular a reparação endógena a fim de aumentar a regeneração vascular e cardíaca.

O objectivo deste estudo será identificar microRNAs que aumentam a sobrevivência das células endoteliais, a fim de promover a sobrevivência celular após transplante.

Palavras-chave: Terapias celulares; Células endoteliais; Sobrevivência celular; Transplante; Doenças cardiovasculares; Isquémia.

LIST OF CONTENTS

LIST OF FIGURES AND TABLES

Figure index

Tables index

LIST OF ABBREVIATIONS

ACTB- Actin-Beta

AKT- Protein kinase B (PKB)

ANOVA- Analysis of variance

ATP- Adenosine triphosphate

CDK- Cyclin-dependent kinases

CDKN1A- Cyclin-Dependent Kinase Inhibitor 1A

cDNA- Complementary deoxyribonucleic acid

CM- Cardiomyocyte

CXCL5- C-X-C motif chemokine 5

DMEM- Dulbecco's Modified Eagle Medium

DNA- Deoxyribonucleic acid

EBM-2- Endothelial Basal Medium

EC- Endothelial cell

ECM- Extracellular matrix

EGM-2- Endothelial Cell Growth Medium

EPC- Endothelial progenitor cells

FBS- Fetal bovine serum

FGF- Fibroblast growth factor

GFR- Growth factor reduced

HIF-1α- hypoxia inducible factor-1 alpha

HTS- High-throughput screening

ICAM-1- Intercellular Adhesion Molecule 1

ITGb1- Integrin beta-1

KEGG- Kyoto Encyclopedia of Genes and Genomes

MACS- Magnetic-activated cell sorting

mRNA- Messenger Ribonucleic acid

MI- Myocardial infarction

miRNA- microRNA

nt- nucleotide

PCR- polymerase chain reaction

PDGF- Platelet-derived growth

PTEN- Phosphatase and tensin homolog

PTPRQ- Protein Tyrosine Phosphatase, Receptor Type Q

RNase- Ribonuclease

RT-PCR- Real time polymerase chain reaction

SMAD6- SMAD family member 6

SMC- Smooth muscle cells

TEVG- Tissue engineered vascular grafts

TGF-β- Transforming growth factor beta

TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling

UTR- Untranslated region

VEGF- Vascular endothelial growth factor

CHAPTER 1 - INTRODUCTION

1.1 Cardiovascular Diseases

Cardiovascular diseases (CVDs), a group of disorders that comprise diseases of the heart, vascular diseases of the brain and blood vessels, are the number one cause of death worldwide (data from World Health Organization, 2011). CVDs are responsible for over 17.3 million deaths per year and are the leading causes of death in the world, representing 30% of all global deaths (Mendis S, Puska P & Norrving B, 2011) (Figure 1). Four out of five CVD deaths are due to heart attacks and strokes. As a result, in 2030 nearly 23.6 million people will die from CVDs, mostly from heart disease and stroke (data from World Health Organization, 2011). Individuals at risk of CVD may possibly present high blood pressure, glucose, and lipids as well as overweight and obesity. The recurrent incidence of several of these risk factors, especially in developing countries, has led to a steep increase in mortality rate as a result of cardiovascular disease. In Portugal, CVDs are the most common cause of death and morbidity (Nichols et al., 2014).

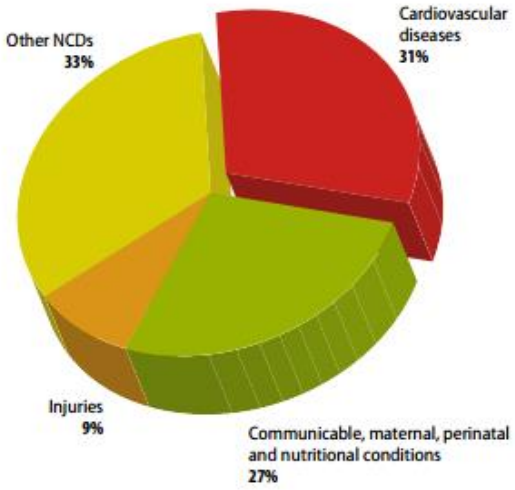


Figure 1. Distribution of major causes of death including CVDs (Mendis S, Puska P & Norrving B, 2011).

The leading cause of cardiovascular events is due to vascular obstructions that normally results in tissue ischemia. Therefore, several of the existing treatments count on the relief of ischemia, restoring vascular perfusion, with the purpose of avoid current tissue damage (Krenning et al., 2009). Nonetheless, such treatments fail to induce tissue regeneration. Consequently, the search for new therapies that induce tissue regeneration is necessary, even though these therapies do not eradicate the primary causes of cardiovascular disease (Krenning et al., 2009).

After a myocardial infarction (MI), the adult human heart has limited capacity to regenerate and consequently, the myocardial tissue lost after the insult, is usually substituted by non-contractile scar tissue (Ferreira, Pedroso, Vazão, & Gomes, 2010).

So far, there is no consistent treatment to regenerate the heart after MI , and although today's options have importantly reduced the number of patients that severely die from MI, the existing therapeutic for chronic heart failure are merely palliative. Therefore, heart transplantation is the only existing and effective therapy that addresses the problem of heart failure (Lei, Sluijter, & van Mil, 2015). Unfortunately, the number of donated hearts available is not suitable to treat all patients.

1.2 Cellular composition of the heart

The heart is mostly comprised of three types of cells: cardiomyocytes (CMs), that allows heart contractions, endothelial cells , that exist in the inner surface of heart chambers and blood vessels and finally, smooth muscle cells that make up the wall of arteries and veins (Yuan & Ma, 2014). CMs establish about 75% of the heart's volume but account for less than 40% of the heart cells (Figure 2) (Brutsaert et al., 2003).

CMs are located nearby to capillaries in a normal heart but occurs in smaller amounts compared to endothelial cells (ECs) with a ratio of 3:1 (Pinto et al., 2016). Cardiac ECs are essential for the structuration of myocardial microvasculature, controls the amount of oxygen and free fatty acids to the CMs and also release paracrine

factors in order to standardize CM metabolism, contractile function, and survival (Brutsaert et al., 2003).

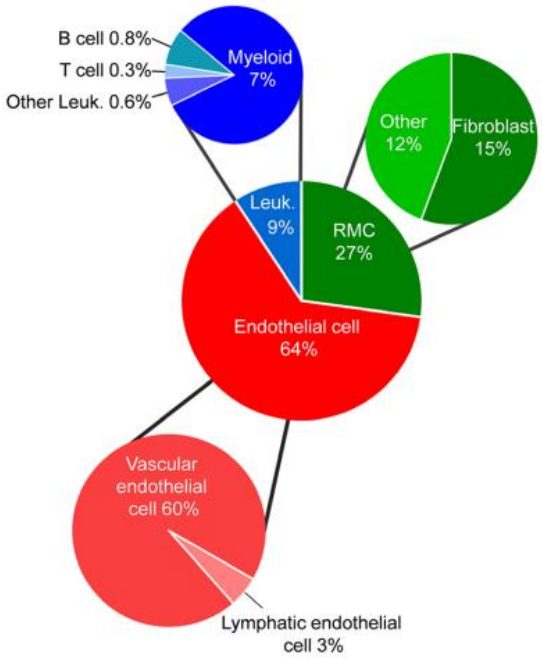


Figure 2. Distribution of cardiac cellular composition. **From** Pinto, A. R., Ilinykh, A., Ivey, M. J., Kuwabara, J. T., D'antoni, M. L., Debuque, R., … Tallquist, M. D. (2016). Revisiting cardiac cellular composition. *Circulation Research*, *118*(3), 400–409

The relation of CMs and the microvasculature is critical for successful cardiac regeneration and it is expected that a successful strategy will require the survival and engraftment of both ECs and CMs. It is known that cardiac ECs increase spatial reorganization and encourage myocyte survival (Narmoneva et al., 2004). Myocyte death significantly diminished due to the interaction of ECs and CMs. This interaction also shows that endothelium could be vital not only for the supply of blood and oxygen but also for the formation and preservation of myocardial organization (Narmoneva et al., 2004).

1.3 Cell therapy

Several strategies can be considered to compensate cell death after tissue damage due to acute ischemia: stimulate regenerative growth in damaged hearts

intervening in cell-cycle checkpoints (Pasumarthi, Nakajima, Nakajima, Soonpaa, & Field, 2005) inhibiting pro-death pathways (Foo, Mani, & Kitsis, 2005); enhancing mechanisms that happen naturally in cells (angiogenic mechanisms) using defined growth factors or vessel-forming cells (Losordo & Dimmeler, 2004) or deliver exogenous cells as a replacement or predecessor to rebuilt cardiac musculature (Rosenthal, 2003).

Among these concepts, the opportunity of tissue restoration via cell grafting in several forms has been the first approach to be translated into the clinic. Given the aging population and the crescent incidence of patients with CVDs, cell based therapies for the prevention or treatment of cardiac dysfunction have earned substantial attention (Penn & Mangi, 2008). Though, no methodology subsists for defining the finest cell population(s) to the procedure or the best treatment conditions (Doris A. Taylor & Robertson, 2009).

In the cardiovascular context, cell therapy debuted as a novel method with stage specific concerns that must be considered. On the initial stage of the disease, the crucial target is vascular damage, but once the infarction occurs, equally the injured myocardium and vasculature require treatment, and with the beginning of heart failure, it is the whole tissue or organ that needs to be restored (Doris A. Taylor & Robertson, 2009).

An equilibrium between injury and repair is necessary to maintain tissue integrity. As a result of the initial ischemic insult, the release of inflammatory chemo/cytokines into circulation and the adjacent tissue environment promote the recruitment of stem or progenitor cells to the wounded sites. If these cells allow tissue repair, inflammation declines and tissue integrity is reestablished. Still, if the amount of progenitor cells is not sufficient or if they are not functional, repair is compromised and the balance is rolled toward injury and inflammation (Figure 3). The challenge of cell therapy is to provide the suitable cells in a sufficient dose for treatment within an adequate time frame to enable the potential for repair (Doris A. Taylor & Robertson, 2009).

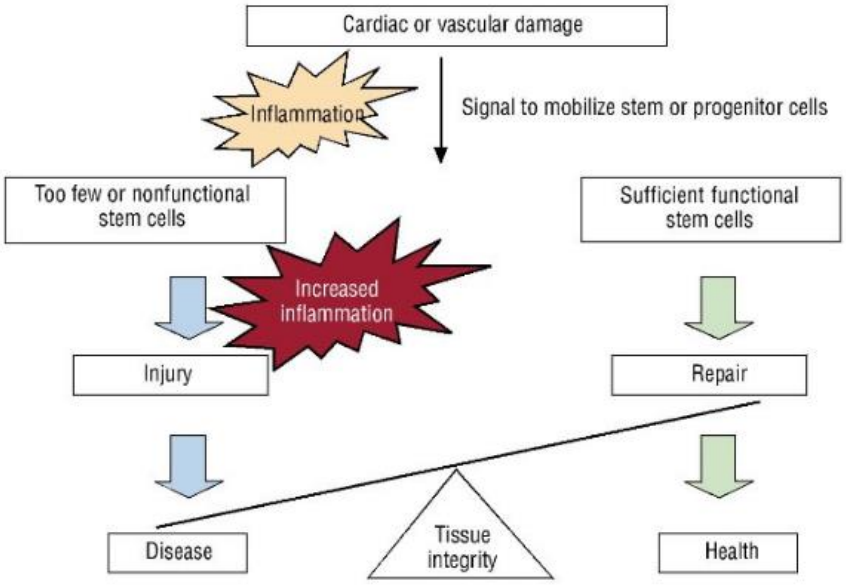


Figure 3. Tissue integrity reflects a balance between injury and repair. From Taylor, D. A., & Robertson, M. J. (2009). Cardiovascular Translational Medicine (IX) The Basics of Cell Therapy to Treat Cardiovascular Disease: One Cell Does Not Fit All. *Revista Española de Cardiología (English Edition)*, *62*(9), 1032–1044.

Tissue repair occurs throughout lifetime but appears to decrease with aging, culminating in the commencement of chronic illnesses such as cardiovascular disease (Daid A Taylor & Zenovich, 2008). At a younger age, risk factors are typically lower and there are a sufficient existing number of cells to compensate for minor insults and sustain repair. However, with aging and in the face of catastrophic events such as, for example, acute trauma and acute myocardial infarction, impairment in heart/vascular repair arises since stem/progenitor cells are no longer functional and the suitable number of cells either do not exist or cannot be mobilized in time to compensate for the injury. In these cases, cell therapy can be considered as a novel methodology aimed to relieve this impairment of endogenous repair (Doris A. Taylor & Robertson, 2009).

1.4 Stem cells in regenerative medicine

Due to their intrinsic features of self-renewal, limitless ability for proliferation and capacity adopt diverse phenotypes, stem cell transplantation has arisen as a prospective modality in cardiovascular therapeutics (Haider & Ashraf, 2008). It is already known that the delivery of adult stem cells led to the restoration of myocardial tissue as well as new vasculature, endothelium and new contractile and efficient cardiac myocytes, although more recent data propose that this procedure is not quite as simple as supposed (Penn & Mangi, 2008). Recent data suggests that the participation of stem cells in cardiac regeneration experiments cannot be explained by cell transplantation alone, and the benefits of these therapies are mostly due to paracrine effects that lead to improved capacity of native cells to resist death stimuli in the situation of ischemic insults (Gnecchi et al., 2008; Penn & Mangi, 2008).

Cell/tissue transplantation efficiency requires viable cells with good histocompatibility, proper cell proliferation, differentiation and migration along the injured tissue (Abdelwahid et al., 2015). Despite the fact of several studies have confirmed the differentiation and proliferative capability of stem cells in *in vitro*, these properties are barely perceived after transplantation *in vivo*, with less than 3% of cells persisting 1 week after transplantation (Dixit & Katare, 2015). Cell type, the number of cells injected, and the status of the host tissue is essential for an optimal survival rate (Robey et al., 2008). Substantial stem cell death occurs in all cell types, indicating the need for understanding the influence of pro-death stimuli on stem cell activity and repair mechanisms in cardiac pathological conditions (Robey et al., 2008).

There are several matters that need to be clarified before we believe that stem cell transplantation can be a new approach in the treatment of CVDs. These questions include: What is the percent of injected stem cells that survive transplantation and can contribute to a successfully engraft? Are these cells viable? What is their proliferative capacity? In what conditions is the host tissue? What is the ideal timing for transplantation – is it recommended to transplant during the acute ischemic event, or several weeks afterwards? (Penn & Mangi, 2008).

1.5 Survival of transplanted cells

One of the biggest questions in cell transplantation is how long the injected cells can survive in the host tissue. For example, human ESC-derived cardiomyocytes can maximally occur in the damage heart for 12 weeks due to the hostile micro-environment in the injured heart (Van Laake et al., 2009). Model experiments revealed that death of grafted rat neonatal cardiomyocytes restricted the formation of new myocardium after acute cryoinjury. The study shown that 30 min after grafting, the fraction of dead cells is merely 1.8(+/-0.4)% of graft cells. Still, at day 1 TUNEL-positive cells increased to 32.1(+/-3.5)% and remained high at 4 days, averaging 9.8(+/-3.8)%. By 7 days, TUNEL decreased to 1.0(+/-0.2)% (Figure 4) (M. et al. Zhang, 2001). (TUNEL is a method for detecting DNA fragmentation that results from apoptotic signaling cascades (Lozano et al., 2009)).

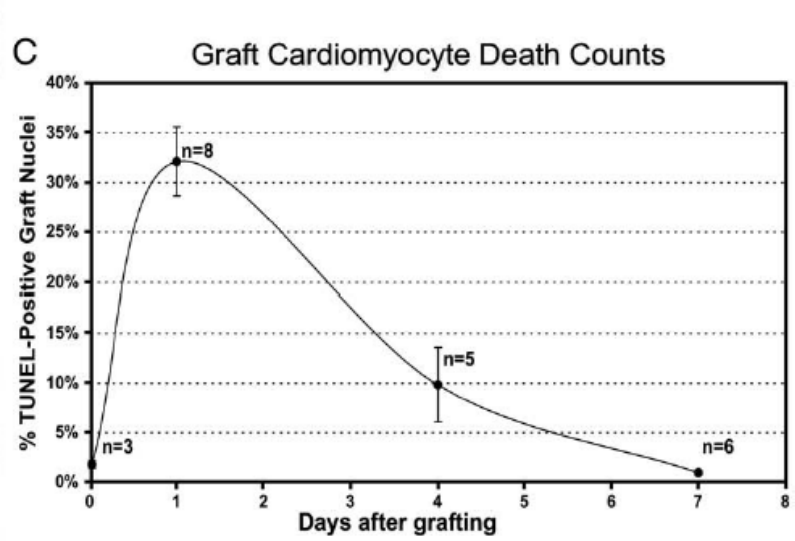


Figure 4. Cell counts indicate the rapid loss of the majority of injected cells within hours after injection. TUNEL index peaks at 24 hrs post-engraftment and falls below 1% by 7 days. From (M. et al. Zhang, 2001)

Cell death follows the first few days post-transplantation and results from a combination of ischemia, anoikis and inflammation (Figure 5) (Robey et al., 2008). During transplantation, there are three principal pathways that lead to cell death.

Right after engrafment, the first insult that cells encounter is absence of matrix support. Nonexistence of stem cell adhesion to the extracellular matrix (ECM) at the location of transplantation is the main pro apoptotic factor that have impact in the efficiency of stem cell survival (Abdelwahid et al., 2015). A pathway of cell death called “anoikis” is started when the cells that usually grow in attachment are retained in suspension (Robey et al., 2008). “Anoikis” is a greek word for homelessness and in cell biology defines a cell death pathway that is induced upon cell detachment (Reddig & Juliano, 2005). Recent studies show that ECM might have an important role enhancing cell viability providing a supportive niche for engraftment regulating stem cell maintenance and differentiation (Nie, Zhang, Liu, & Li, 2016). ECM-initiated survival signaling could be enhanced by overexpression of several growth factor genes and downstream mechanisms and constituents of survival signaling pathway, e.g. protein kinase (AKT) (Paoli, Giannoni, & Chiarugi, 2013).

The low survival rate of transplanted cells is also due to the absence of a efficient local vascular structure capable to deliver sufficient amounts of oxygen and nutrients to the injured loci (ischemia) (Yuan & Ma, 2014). Like in healthy tissue, ischemic injury in transplanted cells is accompanied by ATP reduction, triggering of anaerobic glycolysis resulting in acidosis, followed by dysregulation of calcium culminating in swelling due to an osmotic load; finally this results in membrane damage and cell death (Figure 5) (Robey et al., 2008). Disturbances of oxygen supply or cellular oxygen metabolism are common in patients with CVDs.

Finally, the third motivator of injury is inflammation. The environment that we encounter in a healing infarct is very hostile; according to the inflammatory reaction, neutrophils lead the innate cellular response in the first few days and subsequently macrophages colonize the region (Nahrendorf et al., 2007). Graft cells can be damage by oxygen-derived free radicals and inflammatory cytokines produced by leucocytes, or by caspase activation (Robey et al., 2008).

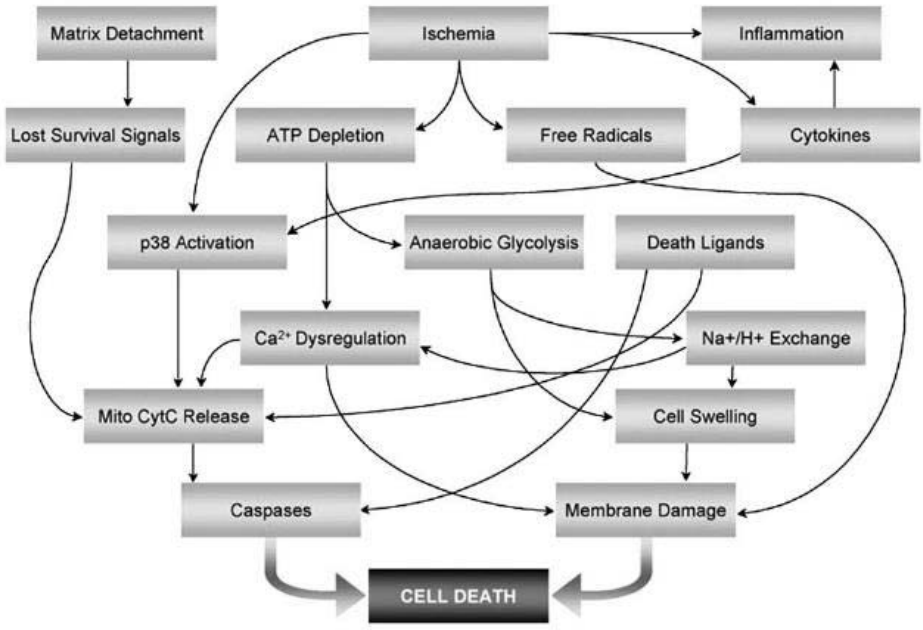


Figure 5. Potential Mechanisms of graft cell Death. From Robey, T. E., Saiget, M. K., Reinecke, H., & Murry, C. E. (2008). Systems approaches to preventing transplanted cell death in cardiac repair. *Journal of Molecular and Cellular Cardiology*, *45*(4), 567–581.

Apoptosis can be involved in both acute and chronic injury of cardiac tissues, and for that reason can be considered as a significant modulator of heart diseases. Cell death can be induced by the factors that are released by the apoptotic cells into the surrounding milieu (Yuan & Ma, 2014)

Some strategies have been proposed to enhance stem cell survival under severe conditions after implantation (Haider & Ashraf, 2008; Penn & Mangi, 2008). To improve the capacities of transplantable cells, there is a variety of pre-treatments with small molecule inhibitors, peptides, miRNAs, exosomes are performed after the implantation; preconditioning using heat shock, hypoxia or cell contact to the oxidative stress and finally genetic modification and co-transplantation with diverse cell types is also possible (Abdelwahid et al., 2015). It is already known that the combinatorial use of such interventions can significantly support cardiac repair by creating an improved environment, however the mechanisms of programmed cell death is still an unresolved issue (Robey et al., 2008).

1.6 The vascular endothelium

In the year of 1865, the anatomist Wilhelm His defined as endothelium the cells covering blood vessels and lymphatics. Nowadays, the term endothelium englobes a monocellular layer that separates all tissues from the circulating blood (Favero et al., 2014).

The effective supply of oxygen and nutrients to all tissues in the human body is an essential mechanism to maintain its complex structure. The vascular system is the important vehicle to achieve this task through a vastly diverged complex of blood vessels (Figure 6).

The human heart comprises several endothelial compartments (arteries, capillaries, and endocardium) that differ in developmental origin, function and structure showing a remarkable heterogeneity. The structure and function of the ECs in coronary arteries are very similar to ECs of other arteries in the body; whereas the structural phenotype of capillaries in the heart holds a continuous endothelium and the distance between the capillary EC and the adjacent cardiomyocyte is about 1 μm leading to an optimal diffusion of oxygen and nutrients between blood and cells (Aird, 2007).

Tissue/vascular function can suffer variations whenever endothelial hemodynamics and cell number is disturbed. In fact, deficiencies in endothelial function and can lead to alterations in the expression profile of endothelial cells (Davies et al.,1986). When the oxygen level is not in the normal parameters, endothelial cells trigger several adaptive mechanisms, such as the upregulation of vascular endothelial growth factor (VEGF) to maintain ECs integrity. Though, it is increasingly becoming apparent that endothelial cell dysfunction strictly relates to CVDs (Yuan & Ma, 2014).

The function of endothelium in maintaining the vascular homeostasis is achieved through the release of autocrine and paracrine signals in response to numerous stimuli. In fact, endothelium as several other functions such as: regulate the

vascular tone, control blood cell trafficking, switch innate and adaptive immunity and hemostasis, producing vasoactive factors, pro-coagulants and anticoagulants, inflammatory and anti-inflammatory factors, fibrinolytics and antifibrinolytics, oxidizing and antioxidizing, and other factors (Favero et al., 2014).

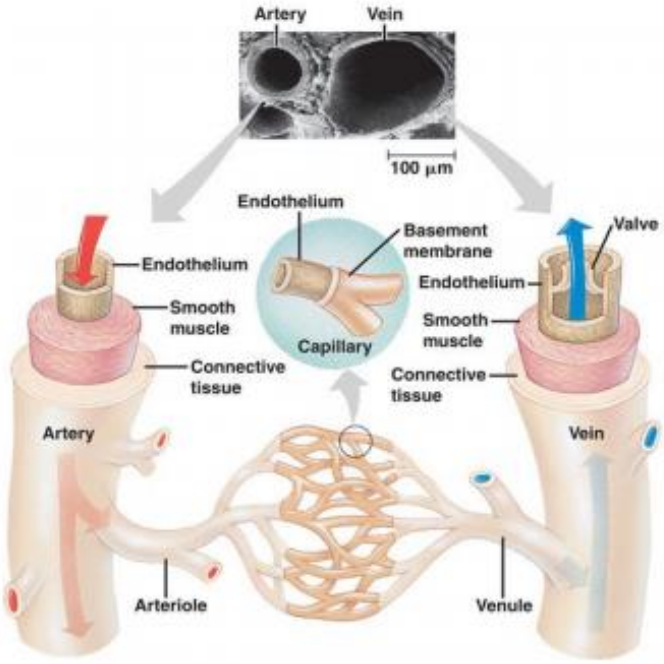


Figure 6. Diagram of different blood vessels of the cardiovascular system. Structure of different blood vessel, where endothelial cells monolayer is always present. Adapted from (http://www.baileybio.com/plogger/?level=picture&id=462)

To recover blood supply after ischemia, vessel maturation/regeneration and the communication of vascular cells with each other and with the ECM are very important mechanisms to gain and reconstitute intact vessels. Cadherins are involved in cell-to-cell communications and proteases are matrix-degrading enzymes responsible for migration of vascular cells and generation of angiogenesis-regulating molecules (Dimmeler, 2005). Integrins intercede in cell adhesion and provide anchorage for cell migration and invasion. Integrins are also critical in multiple cellular procedures, including cell adhesion, proliferation, survival, and the activation of growth factor receptors (Martin, Ye, Sanders, Lane, & Jiang, 2013).

1.7 The expansion of blood vessels

Vasculogenesis and angiogenesis are determinant mechanisms for the formation of blood vessels not only during embryonic development but also throughout adult life (Conway et al., 2001). During vasculogenesis, blood vessels can be formed *de novo* from endothelial progenitor cells or from via extension or remodeling from existing capillaries (angiogenesis) occurring in tissue regeneration for example, during wound healing (Conway et al., 2001).

1.7.1 Vasculogenesis

In premature stage of embryogenesis, vasculogenesis give rise to the first primitive vascular plexus. Angioblasts are endothelial precursor cells (EPCs) that are induced by growth factors such as fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs) (Liu, Driskell, & Engelhardt, 2006). Angioblasts differentiate into endothelial cells (ECs), which form cords, acquire a lumen, and create a network of blood vessels. During ischemia, vasculogenesis acts in concert with angiogenesis and stablishes a functional network that remodels into arteries and veins (Figure 7) (Potente, Gerhardt, & Carmeliet, 2011). Vasculogenic cells are recruited by the ischemic tissue to help in the renewal of the vascular network (Silvestre et al., 2013).

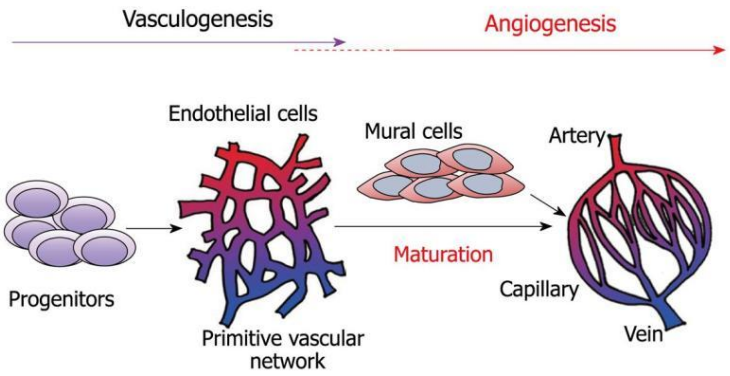


Figure 7. Schematic representation of vascular formation. The processes include vasculogenesis and angiogenesis. Adapted from: Yoh Takuwa, Wa Du, Xun Qi, Yasuo Okamoto, Noriko Takuwa, Kazuaki Yoshioka Roles of sphingosine-1-phosphate signaling in angiogenesis. World J Biol Chem: 2010, 1(10);298-306

1.7.2 Angiogenesis

The term "angiogenesis" refers to the process of vessel growth formed by sprouting and migration from existing capillaries. Decreased levels of oxygen activates hypoxia inducible factor-1 alpha (HIF-1α) which results in upregulation of pro-angiogenic factors such as VEGF, culminating in EC activation. Activated ECs proliferate and migrate leading the growth of a new sprouting vessel by degrading the basement membrane and laying down a provisional extracellular matrix. VEGF initiates the assembly of ECs, vessels are sealed by the formation of endothelial cell–cell junctions, whereas platelet derived growth factor (PDGF) recruits pericytes and SMCs to help on stabilization. The stabilization or regression of the molded vessels hinge on the variety of positive and negative signals coming from the surrounding matrix and other cells (Figure 8)(Phelps & Garcia, 2009).

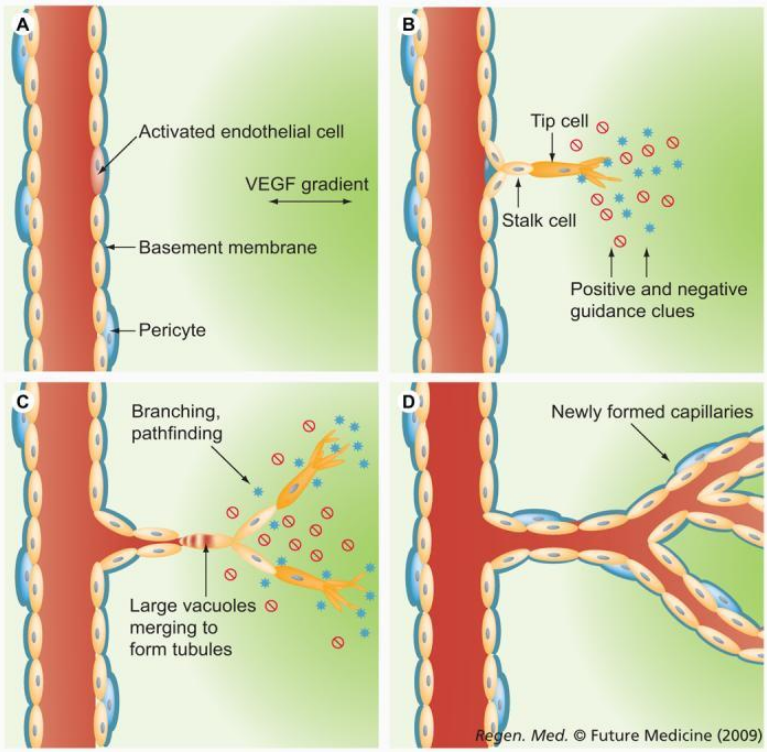


Figure 8. Angiogenesis - Sprouting and migration from existing capillaries. (A) Endothelial cell is activated in response to VEGF gradient; (B) the exposed endothelial cells respond to the proangiogenic signals; (C) process of sprouting, branching following tube formation; (D) Stabilization of new formed capillaries. From: Phelps, E. A., & Garcia, A. J. (2009). Update on therapeutic vascularization strategies. *Regenerative Medicine*, *4*(1), 65–80.

1.8 Tissue engineering and endothelial cell therapy

Recent studies indicated that ECs could be used as a source for tissue engineered vascular grafts (TEVGs) since they show very interesting features (Di Bernardini et al., 2013). For TEVGs, we should opt for a source of cells that are easy to isolate and expand in culture, and it should be able to differentiate into functional vascular cells and be non-immunogenic for tissue recipients (W. J. Zhang, Liu, Cui, & Cao, 2007). The challenge nowadays is trying to improve re-endothelialisation of vascular scaffolds recurring to autogenous cells in order to build a tissue engineered vessel with autogenous ECs.

During the past 15 years, studies revealed that ECs play a mandatory role in the endocardium regulating and sustaining cardiac function, and in the myocardial capillaries where endothelial cells are in a straight line with adjacent cardiomyocytes (Brutsaert et al., 2003). Therapeutic angiogenesis is also a new approach for the treatment of CDV since it involves ECs in the promotion of novel vessels to treat ischemic diseases (Reed et al., 2013).

ECs are more than simple coating cells, and cardiovascular structuration depends on a fully efficient endothelium. ECs must be able to react rapidly to injury to release defensive mediators, and resolve infection through innate immune pathways (Reed et al., 2013). Signals present in the extracellular environment are essential to modulate ECs responses. It is thought that microRNAs (miRs) controls the cellular response to these signals, modulating the expression of secreted growth factors and other paracrine factors (Fish & Srivastava, 2009).

It is necessary to understand the mechanisms underlying the molecular mechanisms that regulate ECs differentiation and metabolism in order to benefit the regenerative strategies for the treatment of various vascular diseases. MicroRNAs are involved in regulation of at least 30% of the mammalian genes, and for this reason they are now in the forefront of this field.

1.9 MicroRNAs

miRNAs are 20-25 nucleotide (nt) non coding RNA molecules which control gene expression typically through binding to the 3' untranslated region (UTR) of messenger RNA (mRNA) transcripts and preventing translation via RNA interference (Bartel, 2004). A single miRNA is capable of regulate numerous distinct mRNA targets, often intricate in the same cellular pathway, and are believed to modulate more than one third of the mRNA encrypted in the human genome (van Rooij et al., 2006).

miRNAs are processed by several maturation steps mediated by protein complexes, including the RNases Drosha and Dicer, and the mature miRNAs are incorporated into the RNA-inducing silencing complex to target mRNAs (Seeger et al., 2013). The expression of microRNAs can be disturbed by tissue injury, as for example myocardial infarction (van Rooij et al., 2008). The roles of distinct miRNAs in several CVDs can be determined exploiting overexpression and knockdown approaches.

1.9.1 miRNA biogenesis and mechanism of action

microRNAs are transcribed by RNA polymerase II and arise from precursor transcripts, named primary miRNAs (pri-miRNAs). Successively, these pri-mRNAs are first processed in the nucleus into an intermediate form (pre-miRNAs) by the ribonuclease (RNase) III enzyme Drosha, being later exported by Exportin 5 to the cytosol, where they are processed by the RNase III enzyme Dicer (Etheridge et al., 2011; He & Hannon, 2004). Next, this process generates an hairpin precursor with 70 to 100 nucleotides in length and finally a smaller and mature double-stranded miRNA with 18–24 nucleotides (Etheridge et al., 2011; He & Hannon, 2004). Then, a strand of the miRNA duplex is selected as a mature miRNA, while the other strand is degraded. To facilitate this process, argonaute proteins are located in the RISC complexes and bind to single-stranded miRNAs (Figure 9) (Etheridge et al., 2011). Depending on the complementary sequence with its target mRNA, the mature miRNA negatively regulates gene expression by either translational repression or mRNA degradation (Y. Lee et al., 2003).

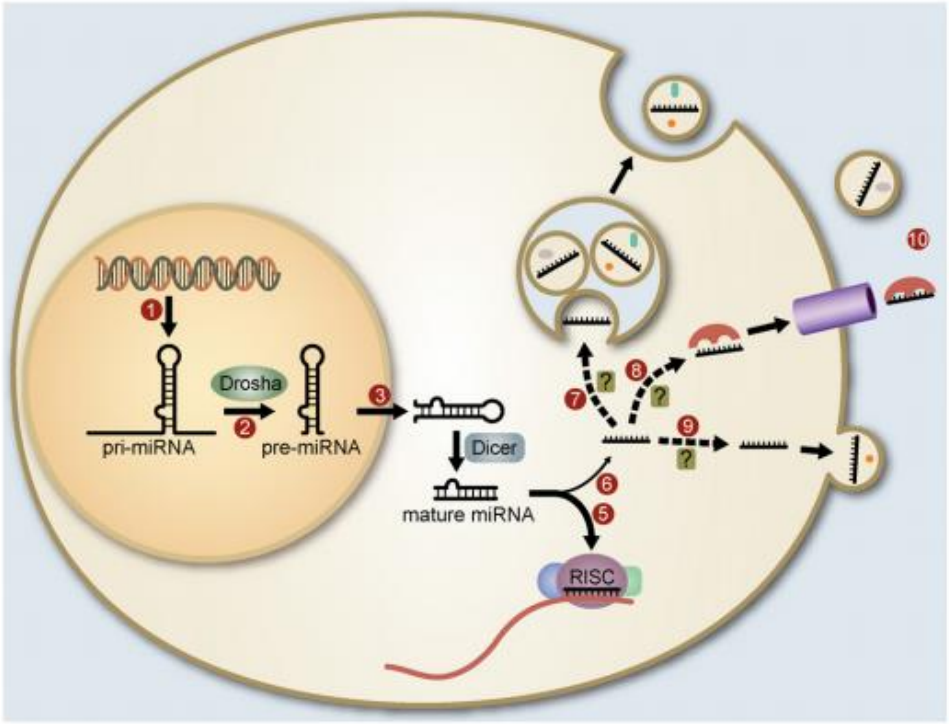


Figure 9. The biogenesis of microRNAs: transcription of pri-miRNA transcripts by RNA polymerase II or III (1); pri-miRNAs processed first in the nucleus (2) and later in the cytosol (3) by the RNase III enzymes Drosha and Dicer respectively, in order to produce pre-miRNA hairpins and eventually a mature double-stranded miRNA of 19–24 nucleotides (4); one strand of the mature miRNA duplex is incorporated into the RISC complex (5); the other strand is unwound from the guide strand and degraded (6); Some miRNAs have been found packaged in exosomes (7) or exported in the presence of RNA-binding proteins (8). Once in the extracellular space, these miRNAs could be taken up by other cells, degraded by RNases, or excreted (9). From: A. Etheridge, et al., Extracellular microRNA: A new source of biomarkers, Mutat. Res.: Fundam. Mol. Mech. Mutagen. (2011)

1.9.2 MicroRNA target identification

Interactions between miRNAs and their targets are a complex process and very often there multiple binding sites in mRNA (Lai, 2004). Most animal microRNAs are more tolerant to imperfect pairing between microRNA and its target (Thomas, Lieberman, & Lal, 2010). miRNA target identification and authentication remains a crucial step in the field. The different existing bioinformatic prediction programs recurrently predict different mRNA targets for individual miRNAs with a vast number of false positive and false negative results (Williams et al, 2009). The available bioinformatic tools end up relying on some pre-conceived information about miRNA utility, necessary to sort through hundreds of predicted targets with association to the significant biological data to identify a restricted set of significant targets to validate by functional methodologies (Fish & Srivastava, 2009). As a result, different algorithms are currently available to perform target prediction analysis (e.g., PicTar, miRanda, TargetScan, PITA).

1.9.3 Therapeutics with miRs

Several experimental studies offer substantial evidence that miRNAs might be targeted to improving cell therapy or enhancing endogenous repair processes. In spite of these promising data, the progress of miRNA therapeutics encounter several complications. First, while miRNA inhibition is very well-organized, overexpression of miRNAs is not yet established and involves the use of vectors (Stenvang, Petri, Lindow, Obad, & Kauppinen, 2012). Second, the repression of multiple targets by miRNAs requires a system biology technique to recognize the full impact on the gene expression networks. Some miRNAs target gene family members that *share* a common seed region sequence with a common biological function (eg, miR-29 targeting several matrix proteins); however, other miRNAs target genes with antagonic characteristics making more complicated to understand the biological mechanisms facing challenges with respect to unwanted side effects in other cells or tissue. Therefore, in some cases, cell specific delivery strategies may be necessary (Seeger et al., 2013)

1.9.4 MicroRNAs and cardiovascular disease

MicroRNAs have been identified as key regulators of cardiac development and response to stress functioning as master switches controlling proliferation and differentiation events (Van Rooij et al., 2008). MicroRNAs might be remarkable candidates to *ex vivo* treatment of cells before transplantation since miRNAs can change gene expression networks, interfere with cell function, homing, survival and differentiation (Chavakis & Dimmeler, 2011). Cardiovascular risk factors appear to modulate some miRs, and some deregulated miRNAs might help recover the functional aptitude in cells of patients with ischemic diseases (Figure 10).

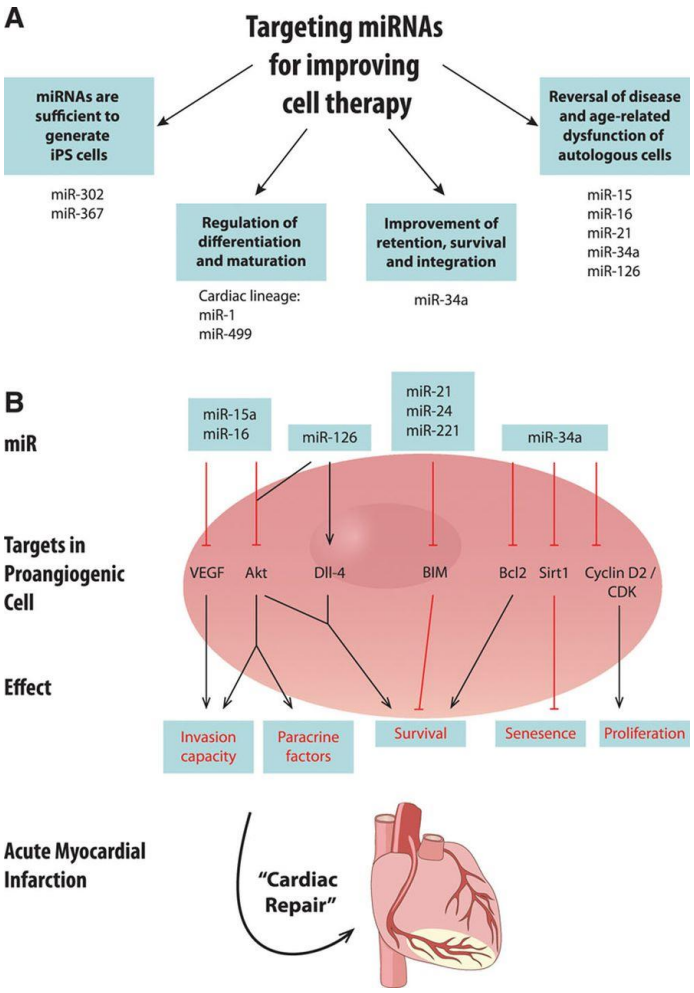


Figure 10. Effects of microRNAs (miRNAs/miRs) on cell therapy. The figure summarizes the possible use of miRNAs to improve for cell therapy (A) and shows some of the mechanisms by which miRNAs regulate the function of proangiogenic

cells for cardiovascular repair (B) VEGF and Akt indicates to be mediator of invasion capacity, release of paracrine factors amd cell survival. From: Seeger, F. H., Zeiher, A. M., & Dimmeler, S. (2013, August 1). MicroRNAs in stem cell function and regenerative therapy of the heart. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Lippincott Williams & Wilkins.

As mentioned before, a single miRNA can target multiple mRNA, and this way regulates a whole signaling cascade.

There are several microRNAs that are described to participate in several endothelial processes. The family of pro-angiogenic miRNAs includes:

- the miRNA-17-92 cluster, which is expressed in ECs and plays a role in tumour vascularization (Otsuka et al., 2008);
- the miRNA-92a, has been shown to regulate angiogenesis *in vivo* and *in vitro* and to induce efficient recovery of mice ischemic tissues (Bonauer et al., 2009);
- the miRNA-130a controls the angiogenic phenotype of ECs and is considered to promote angiogenesis in ECs (Chen & Gorski, 2008);
- The miRNA-210 is involved in EC migration and capillary-like structure formation (Fasanaro et al., 2008);
- the miRNA-378 promotes tumour angiogenesis (D. Y. Lee, Deng, Wang, & Yang, 2007);
- the miRNA-296 regulates EC migration and tube formation, and tumour angiogenesis *in vivo* (Würdinger et al., 2008);

All of the above cited pro-angiogenic miRNAs can be used as new therapeutic targets in the selective modulation of angiogenesis, for the treatment of CVDs providing that a clear understanding of their mechanism of action is available.

CHAPTER 2 - AIMS

An effective cell-based therapy depends on several components such as vascularization, immunosuppression, cellular homing and tissue growth. Consequently, novel approaches with the potential to help the translation of some therapies into the clinics are needed. For clinical efficacy it is vital that stem cells or their progenies survive and engraft into the host tissue. Though, a substantial number of cells die or are lost within hours after transplantation (usually >70 % within days). This affects stem cells from all different origins (progenies from pluripotent, fetal and adult stem cells) in different clinical settings (e.g. myocardial infarction, chronic wounds, etc…). Ischemic insults are one of the reasons for cell death and the focus of the present thesis.

In this project, it was proposed to identify microRNAs that are able to improve the survival of stem cells after transplantation, focusing on CD34+ derived endothelial cells in the cardiac context.

Specific aims:

1. Screening and identification of pro-survival microRNA's;
2. Identify molecular targets of each pro-survival microRNA using bioinformatics tools;
3. MicroRNA validation through survival, migration and angiogenesis assays using CD34+ derived endothelial cells cultured under ischemic and normal conditions;
4. Find the mechanisms of action of the pro-survival microRNAs;

CHAPTER 3 - METHODS

3.1 Isolation of $CD34^+$ cells from UCB

All human UCB samples were collected from donors, who signed an informed consent form, in compliance with Portuguese legislation. The collection was approved by the ethical committees of Dr. Daniel de Matos Maternity Hospital in Coimbra. The samples were stored in sterile bags containing 35 mL of citrate-phosphate-dextrose anticoagulant solution. CD34+ cells were isolated from mononuclear cells, obtained from UCB samples after Lymphoprep (STEMCELL technologies) density gradient separation. CD34+ cells were positively selected using the mini-MACS immunomagnetic separation system, according to the manufacturer's recommendations.

3.2 Differentiation of $CD34^+$ cells into ECs

Isolated $CD34^+$ cells were transferred onto 1% (w/v) gelatin-coated 24-well plates (2×10^5cells/well) and incubated in endothelial growth medium (EGM-2; Lonza, Gaithersburg, MD, USA) with 20% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, USA) and 50 ng/mL vascular endothelial growth factor ($VEGF_{165}$; PrepoTech Inc., Rocky Hill, USA), at 5% CO_2, 37°C. After 5 days and then every other day, half of the volume of the medium was replaced with fresh one. Expression of EC markers was evaluated by fluorescence-activated cell sorting (FACS) and immunofluorescence staining. The functionality of the cells was evaluated by incubating the cells with acetylated low-density lipoprotein (DiI-Ac-LDL; Biomedical Technologies, Stroughton, USA). At the end of the differentiation assay, EC where characterized as previously described in our group (Pedroso et al., 2011)

3.3 CD34+ Endothelial Cell Culture and Seeding

CD34 ECs were cultured in endothelial cell growth medium-2 (EGM-2), from Lonza, in flasks coated with 1% gelatin and incubated at 37ºC with 5% CO2. Cell culture medium was replaced every 2-3 days until cells were approximately 80% confluent. All

experiments were performed with cells at fourth passage. Briefly, cells media was removed and cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS®), from Invitrogen, to remove cellular waste. After that, to detach cells from the flask surface, cells were incubated with a solution of trypsin 1x at 37ºC for around 1 minute. Cells were then homogenized in Dulbecco's modified eagle medium (DMEM®), from Invitrogen, with 10% FBS, which inhibits the action of trypsin, and counted on a Neubauer hemocytometer. Cells were pelleted by centrifugation at 300 x g for 7 min before being resuspended in EGM-2 medium and plated at a cell density of 100.000 cells/mL (96 well plates: 10.000 cells/well, 6 well plates: 200.000 cells/well).

3.4 Identification of pro-survival miRNA

3.4.1 miRNA library screening

For initial screening assays 2 pools of CD34+ EC's were transfected with the miRNA mimics library (miRIDIAN miRNA mimics) corresponding to all the human mature miRNAs (2080 miRNAs, Dharmacon miRIDIAN® microRNA Library - Human Mimic (19.0)).

MiRNAs were transfected at a final miRNA concentration of 50 nM. Briefly, the transfection reagent (Lipofectamine RNAiMAX, Life Technologies) was diluted in EBM-2 (Lonza) and added to the miRNAs arrayed on 96-well plates. After 30 min, the miRNA-lipid complex was added on top of cells previously seeded overnight in 96 well plates. Forty-eight hours after transfection, culture medium was replaced by EBM-2 medium (starvation medium), and then the cells were incubated in the hypoxia chamber (0.1% O2) for another forty-eight hours (Figure 10) After this period, cells were stained with Hoechst (1µg/mL) and imaged using the In Cell analyzer 2200 (GE Healthcare Life Sciences). For each well, 8 different fields were imaged and the number of viable cells was determined using the In Cell developer software (Figure 11).

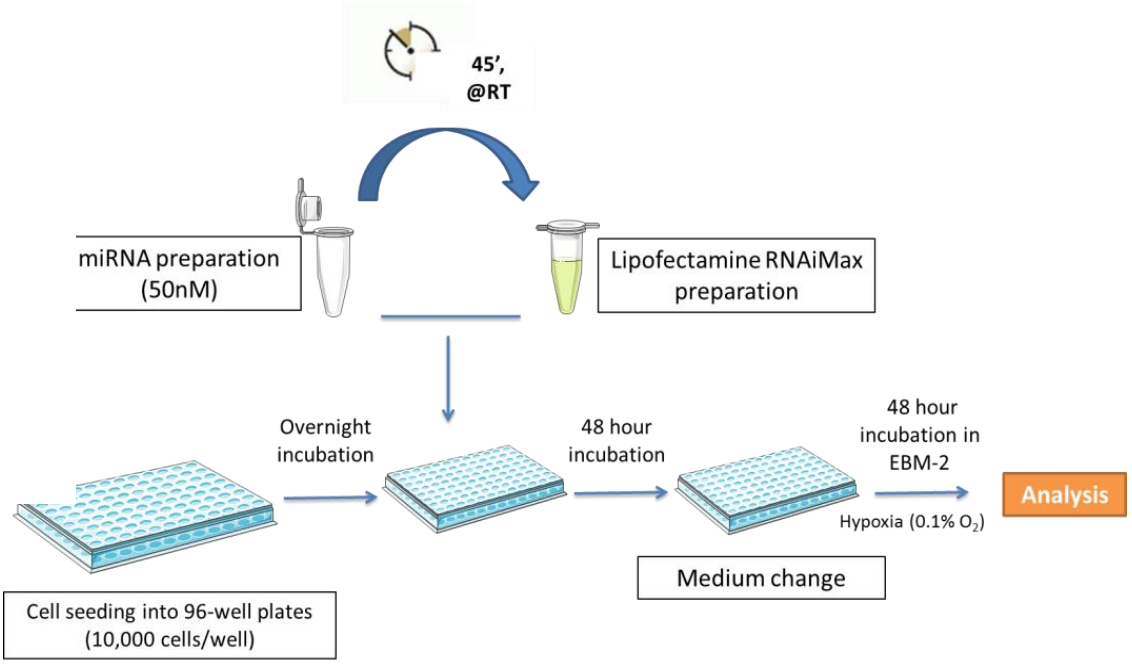


Figure 11. Experiment layout of micro RNA library screening (adapted).

3.4.2 Hit validation

miRNAs hits were identified as increasing the survival of CD34+ Endothelial cells if the total number of cells was higher than the mean + 2*SD of the control (Lipofectamine) in two different donors.

3.4.3 Gene target prediction analysis

Bioinformatic prediction of target genes and miRNA binding sites was performed using four different programs: *miRTarbase* (http://mirtarbase.mbc.nctu.edu.tw/) while computationally predicted targets were retrived from *DIANA-microT* (http://www.microrna.gr/webServer) *PITA* (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html) and *RNA22* (https://cm.jefferson.edu/rna22/) databases.

After retrieving the list of all putative gene targets for each pro-survival miRNA, a system to rank these targets was developed in order to infer which ones putatively have the highest survival potential.

First, the targets for the remaining 2055 non-survival miRNAs were obtained. Then, a score was assigned to each pro-survival miRNA gene target according to their set exclusivity, assuming that the genes with the highest survival potential would be over-represented in the pro-survival dataset relative to the non-survival one. Briefly, the absolute frequency of each gene in the pro-survival dataset was calculated and normalized to its absolute frequency in the non-survival dataset. In other words, the score for each individual gene target is the number of pro-survival miRNAs divided by the number of non-survival miRNAs that potentially target that particular gene.

This 'survival score' was then used to rank the target genes and the first 500 genes were selected for the subsequent analyses.

3.4.4 Identification of potential pathways of survival miRNAs

The hypergeometric function of the 'GOstats' package implemented in the R programming language was used to assess the degree of overlap of genes from KEGG pathways and genes targeted by each pro-survival miRNA. The estimated significance level of the overlap was determined by the Fisher's exact test and adjusted for multiple hypotheses testing using the Bonferroni correction. Pathways for which adjusted p-value is below 0.05 were identified.

3.5 Validation Survival/Migration/Angiogenesis assays

3.5.1 miRNA transfection

MiRNAs transfections into CD34+ EC's were performed 12 h after cell seeding using a typical RNAiMAX Transfection Procedure. For the survival assay, different final concentration of miRNA were evaluated: 10, 25 and 50 nM. For angiogenesis and migration assay the final miRNA concentration used was 25 nM. Briefly, the transfection reagent (Lipofectamine RNAiMAX, Life Technologies) was diluted in EBM-2 (0.3µL/well for 96 well plates or 6µL/well for 6 well plates) and added to the selected

miRNAs. After 30min, the miRNA-lipid complexes were added to the cells in EGM-2 without antibiotics and incubated at 37ºC, 5% CO_2.

3.5.2 Survival assay

For the survival assay, cell seeding and transfection was performed in 96 well plates. After 48 hours of transfection, cells were stained with Hoechst and imaged using In Cell Analyzer 2200 for counting the number of viable cells. Next, cell medium was changed to starvation medium (EBM-2) and the cells were incubated in hypoxia condition (0.1% O_2) for additionally 48hours. After removing cells from hypoxia, cell nuclei were stained with Hoechst and the number of viable cells was determined. The percentage of cell survival was obtained by the following formula: (number of viable cells after hypoxia / number of viable cells before hypoxia) *100.

3.5.3 *In vitro* tube formation assay for ECs

For the tube formation assay, cells were seeded and transfected in 6 well plates. After 48 hours of transfection, cells were harvested and 10.000 cells in 50µL of EBM-2 were seeded on 15 well chamber slides (IBIDI) previously coated with Matrigel GFR (BD Biosciences). After 4 hours, images were using the In Cell analyzer 2200.The number of tube-like structures was determined using Image J software.

3.5.44 Migration: Scratch wound healing assay

For the migration assay, cells were seeded and transfected in 96 well plates. After 48 hours of transfection, wound was created by scratching the surface with a 200µL pipette tip, and the medium was changed to EBM-2. Cells were incubated at hypoxia (0.1% O_2) or normoxia for 24 hours. Immediately after the scratch and 24 hours after incubation, images were acquired using In Cell Analyzer 2200. The wound size was measured using Axiovision software. The percentage of wound area was determined using the following formula: (wound size after 24h / wound size at 0h) + 100.

3.6 Mechanisms of action

3.6.1 Extraction of RNA from cells and tissues

For gene analysis, cells were seeded and transfected in 6 well plates. After 24hours of transfection, culture medium was removed and cells washed twice with PBS prior to the addition of 350 µl QIAzol® Lysis Reagent (Qiagen, Crawley, UK) to each well. Cells were homogenized by pipetting up and down and transferred to 1.5 ml microcentrifuge tubes for storage at -80ºC until processing.

3.6.2 RNA Isolation for validation of gene targets on CD34+ Endothelial cells

RNA isolation was performed using Qiagen RNeasy mini kit, according to the manufacturer's instructions. RNA extraction was performed using RNeasy Mini Kit for isolation of total RNA from animal cells (Qiagen), according to the manufacturer's protocol at room temperature with a centrifugal force of 8000 g unless otherwise stated. The cell pellet was resuspended in 350µl of RLT lysis buffer that disrupt the cell membrane and organelles to release total RNA contained in the sample. The lysate was then transferred into spin column and centrifuged for 2min to create a homogenous lysate by shearing high-molecular-weight genomic DNA and other cellular components. An equal volume of 70% ethanol was added to the 95 homogenized lysate to provide appropriate conditions for the RNA to bind to the RNeasy spin column membrane. At this point the mixture was applied to the RNeasy mini column placed in a 2ml collection tube and centrifuged for 30s and the flow though obtained was discarded. 350µl of RW1 washing buffer were added to the column followed by centrifugation for 15s, and the flow through was once again discarded. 350µl of RW1 washing buffer were added to the column followed by centrifugation for 15s. The flow through was discarded and 2 more washes were carried out with 500µl of RPE buffer. The washing buffers are applied to remove all contaminants. The flow through was discarded and an additional centrifugation of 1min took place to remove all remaining solution that could reduce the purity of the RNA extract. To elute the RNA, the RNeasy column was transferred to a clean 1.5ml RNase free micro centrifuge collection tube

and 30µl of RNase-free water was added to the RNeasy column membrane followed by spinning for 1min. The RNA concentration was measured using a Nanodrop Spectrophotometer at 230-260nm and 260-280nm.

3.6.3 Reverse transcription and quantitative real time polymerase chain reaction (qRT-PCR) analysis.

In all cases, cDNA was prepared using Taqman Reverse transcription reagents (Applied Biosystems)(Table 1). These reactions took place in the thermal cycle (Bio Rad) with the following PCR program: 1 cycle at 25ºC during 10 minutes, 30 seconds at 48ºC and a final cycle at 95ºC during 4 minutes.

Table 1. Taqman Reverse transcription reagents (Applied Biosystems)

Taqman RT buffer	5uL
MgCl2	11uL
dNTP	10uL
Random hexamers	2.5uL
Rnase inhibitor	1uL
Reverse transcriptase	1.25uL
Vmax. Mix	30.75uL
Vmax. RNA+water	19.25uL

Quantitative real time PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems) (Table 2) and the detection was carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative quantification was carried out through the comparative threshold cycle (Ct) method (ΔΔCt). The run method was started with the hold stage (5 minutes at 94ºC), then 40 cycles of 30 seconds at 94ºC, 30 seconds at 60ºC and 30 seconds at 72ºC were used for the PCR stage, and finally the melt curve stage with 15 seconds at 95ºC, 1 minute at 60ºC followed by a gradual increase in temperature (1°C/15 s) to 95°C. All qRT-PCR data were normalized

to beta actin (ACTB) as the reference gene. Primer sequences are given as supporting information (Table 3).

Table 2. PCR Master Mix (for each primer)

Syber Green	10uL
Rnase free water	8uL
Primer (F+R)	1uL
cDNA	1uL/well

Table 3. Primer sequences

Gene	Reverse primer sequence	Foward primer sequence
PTPRQ	CCATTTGGTTGCCGAGGTAAATA	GTGGTGAATCTCACAGTTGAGG
Akt	GCCAGCATACCATAGTGA	GTAGGGAAATGTTAAGGAC
CDK1NA (p21)	TCTCATTCAACCGCCTAG	CTCTACATCTTCTGCCTTAG
PTEN	GTTACTCCCTTTTTGTCTCTG	GGCTAAGTGAAGATGACAATC
ACTB	GATGGGGTACTTCAGGGTGA	CGTCTTCCCCTCCATCGT

3.7 Statistical analysis

Statistical analysis and graphs were achieved using GraphPad® Prism 6.0 software. For statistical comparison: One way ANOVA + Bonferroni multiple comparison test. Were considered significant when $p < 0.05$. Otherwise, p values were described as n.s. (not significant). In the figures, significant p values are indicated by asterisk (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

CHAPTER 4 - RESULTS

4.1 Identification of pro-survival microRNAs

4.1.1 Screening results

The results of these experiments indicate that 25 miRNAs play a role in enhancing endothelial cell survival under hypoxic and serum starvation conditions in two of the donors tested in the high-throughput screening (HTS) experiment. All remaining miRNAs were considered non-survival miRNAs (Figure 12).

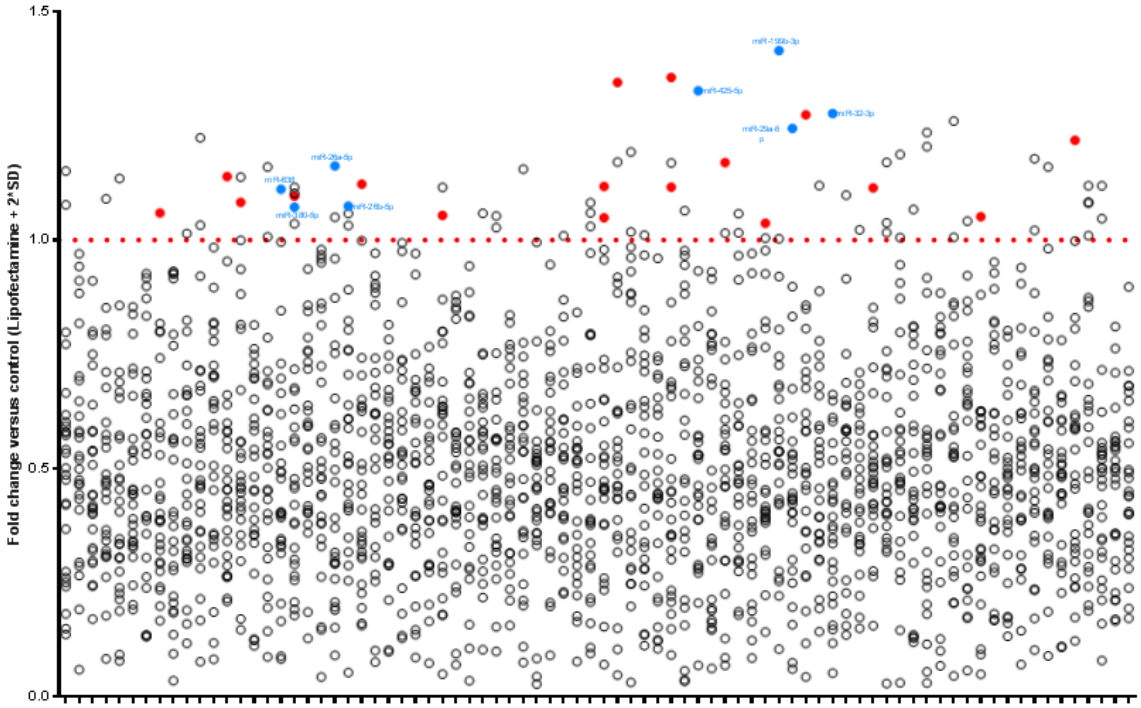


Figure 12: **MicroRNA hits were identified as increasing the survival of CD34+ Endothelial cells above 2*SD of the control (Lipofectamine) in two different donors. The red circles correspond to the microRNAs that are not described in the literature; the blue circles correspond to the micro RNAs that are already described in the literature. Fold change vs control (LF) + 2*SD.**

4.1.2 microRNA predicted targets

From the 25 miRNA that increased survival in CD34 ECs, two pro-survival-miRs were selected to perform a gene target prediction analyses (pro-survival-miR-A and pro-survival-miR-B). To provide more information regarding pro-survival-miR-A and pro-survival-miR-B associated pathways, putative gene targets were identified using target prediction programs and evaluated top gene targets in vitro.

DIANA-microT algorithm identifies targets based on conserved UTRs among human and mouse as well as free energy binding and sequence complementarity, while *PITA* and *RNA22* focus on slightly different approaches. *PITA* first predicts targets using complementarity analysis within seed regions and then compares the free energy gained from the formation of the miRNA-target duplexes and the energetic cost of un-pairing the target to make it accessible to the miRNA while *RNA22* selects mRNA 'hot-spots' (sites of intensive sequence complementarity) and then identifies miRNAs that are likely to bind to those sites based on the folding energy of the duplexes.

A 'survival score' was used to rank the target genes and the first 500 genes were selected for the subsequent analyses. The top 20 gene targets (with highest survival scores) of pro-survival-miR-A and pro-survival-miR-B are shown in Table 4.

Table 4. Top 20 gene-targets (those with highest survival scores) of the miRNAs: pro-survival-miR-A and pro-survival-miR-B

Rank	Pro-survival-miR-A	Pro-survival-miR-B
1	LIPT2	SPINK1
2	CKS1B	TEX12
3	PTPRQ	IFNG
4	PPP2CB	TCP10L2
5	YTHDF1	TRMT112
6	MANEA	PDFN4
7	USMGS	COMMD8
8	LSM6	MAP4K5
9	LRRC40	CXCL5
10	CXCL6	CFAP20
11	ANGPTL3	RSL24D1
12	CXADR	PTPRQ
13	TSTA3	YTHDF1
14	ALG2	SC5D
15	RNF11	MICU3
16	PDCD10	MANEA
17	CMTM6	RRAGB
18	C2orf47	TCTEX1D1
19	AASDHPPT	EMC7
20	FST	CAAP1

The bar graphs present the biological pathways significantly enriched (adjusted p-value < 0.05) in gene targets of the pro-survival-miR-A and pro-survival-miR-B miRNAs (Figure 13a/b). The size of each bar represents the significance of the overlap between the genes targeted by the miRNA and the genes belonging to each enriched pathway. The most targeted pathways by pro-survival-miR-A are 'P53-signaling pathway' and 'Inositol phosphate metabolism' (Figure 13a), whereas the top enriched pathways for pro-survival-miR-B are 'TGF-beta signalling pathway', 'Melanoma' and 'Cytokine-cytokine receptor interaction' (Figure 13b).

Genes from the 'P53-signaling pathway' and 'Inositol phosphate metabolism' pathways, which are among the top 500 targets (with highest survival scores) for pro-survival-miR-A, are shown in Table 5. Genes of the 'TGF-beta signalling pathway', 'Melanoma' and 'Cytokine-cytokine receptor interaction' pathways, which are among the top 500 targets (with highest survival scores) for pro-survival-miR-B, are shown in Table 6. This analysis allowed to identify predicted miR targets that are associated with relevant cellular functions, thereby defining a subset of potentially relevant genes that may be the subject of future functional studies.

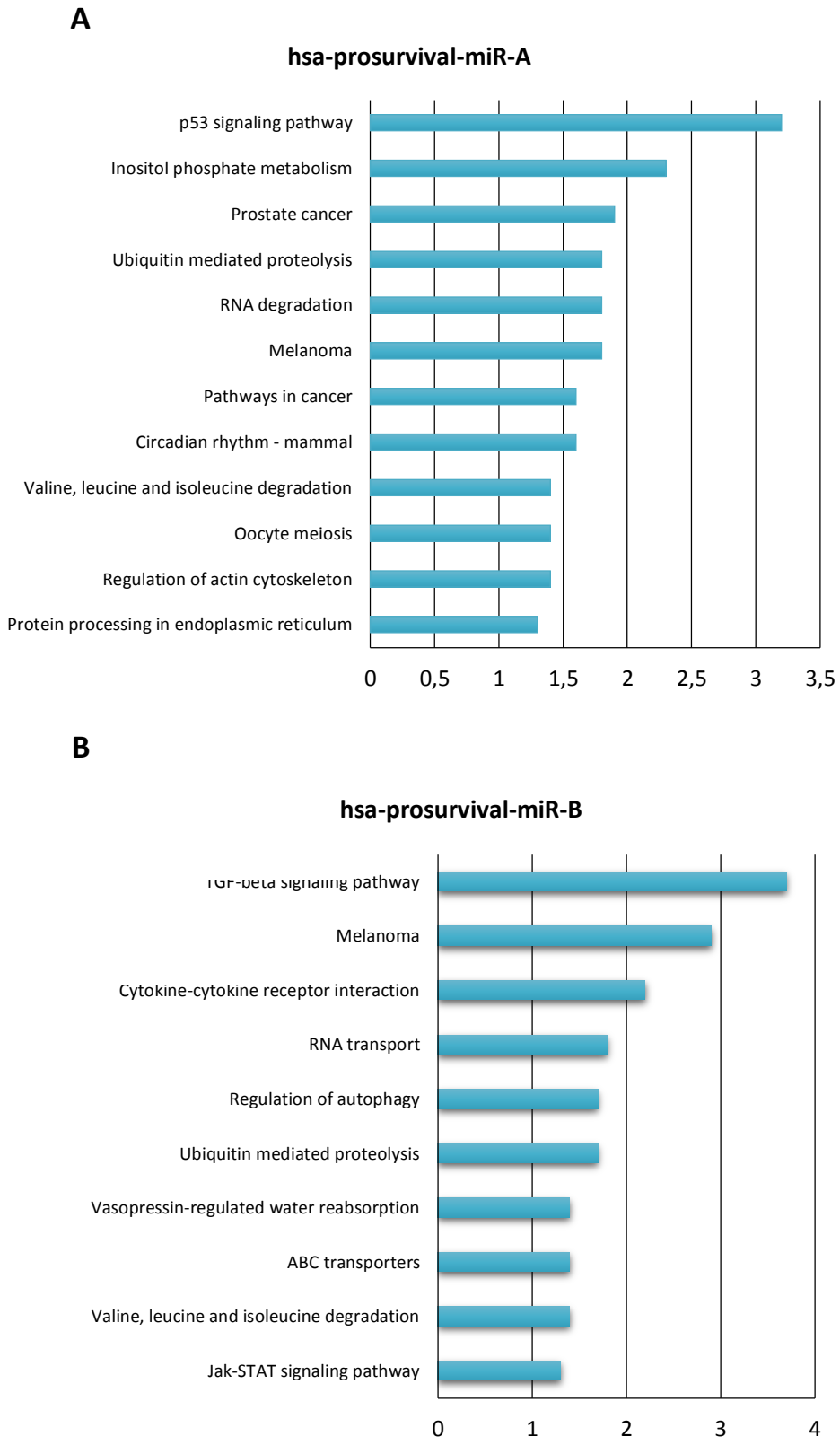


Figure 13. Biological pathways significantly enriched (adjusted p-value < 0.05) in gene targets of pro-survival-miR-A (a) and pro-survival-miR-B (b). The horizontal axis is ($-\log_{10}$(p-value)).

Table 5: 500 top genes targeted by pro-survival-miR-A present in the "p53 signalling pathway" and "Inositol phosphate metabolism" (the two most enriched pathways of pro-survival-miR-A). Rank is the ordering of the gene among the top 500 targets of pro-survival-miR-A. The total number of genes in each pathway is shown.

P53-signaling pathway (68 genes in total)	
Rank	Gene Symbol
72	PPM1D
144	PTEN
336	TP53AIP1
413	ZMAT3
458	IGF1
469	RRM2
493	MDM2
500	CASP8

Inositol phosphate metabolism (with 57 genes in total)	
Rank	Gene symbol
37	IPMK
144	PTEN
193	OCRL
328	PLCZ1
368	INPP4A
432	PIK3C3

Table 6: 500 top genes targeted by pro-survival-miR-B present in the "TGF-beta signalling pathway", "Melanoma" and "Cytokine-cytokine receptor interaction" (the three most enriched pathways of pro-survival-miR-B). Rank is the ordering of the gene among the top 500 targets of pro-survival-miR-B. The total number of genes in each pathway is shown.

Cytokine-cytokine receptor interaction (with 265 genes in total)	
Rank	**Gene symbol**
3	IFNG
9	CXCL5
44	IL22
70	HGF
78	IFNA8
101	IFNA11
130	TNFRSF11B
211	IL18RAP
297	BMPR1B
332	INHBA
341	GHR
375	ACVR1
388	ACVR2A
445	IL2
493	BMP2
494	LEPR

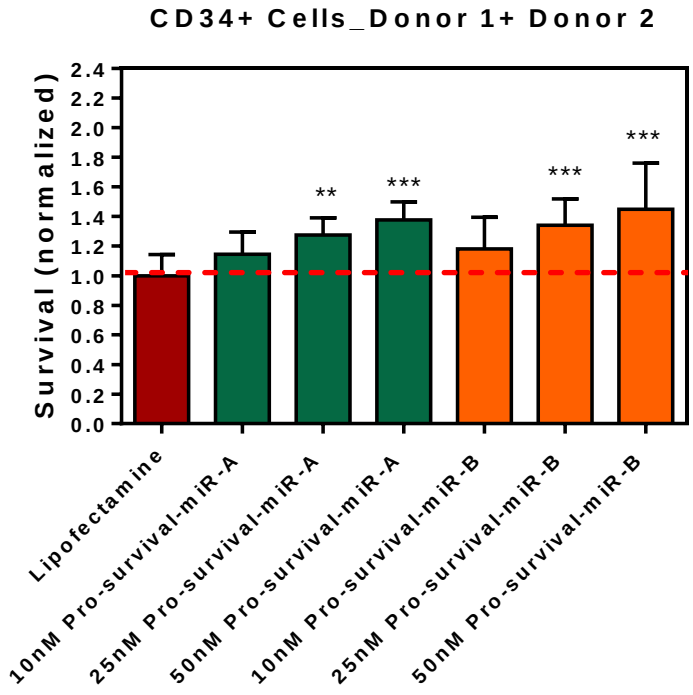
TGF-beta signaling pathway (with 84 genes in total)	
Rank	**Gene symbol**
3	IFNG
184	ID4
294	SMAD6
297	BMPR1B
332	INHBA
375	ACVR1
388	ACVR2A
410	SMAD7
493	BMP2
496	DCN

Melamona (with 71 genes in total)	
Rank	**Gene symbol**
63	FGF18
70	HGF
114	RB1
185	FGF7
192	FGF5
219	FGF9
290	PTEN
422	FGF12

4.2 Pro-survival-miR-A and Pro-survival-miR-B promote survival in CD34+Endothelial Cells

To demonstrate the pro-survival activity of different miRNAs, CD34+ ECs were incubated for 48 h after transfection, cultured in ischemic conditions (pO2 of 0.1%; media without serum) and finally counted with Hoechst. Pro-survival-miR-A and pro-survival-miR-B each significantly ($P<0.05$) increased cell survival, and this effect was dependent on the miRNA concentration (Figure 14a). This increased survival is presented in both donors comparing to the control (Lipofectamine) ($P < 0.001$; Figure 14a/b). Significant differences were seen between the different miRNAs tested ($P>0.05$). The percentage of viable cells and dead cells was also determined (Figure 14b). As we can see, pro-survival-miR's presented a higher percentage of viable cells compared to Lipofectamine.

A



B

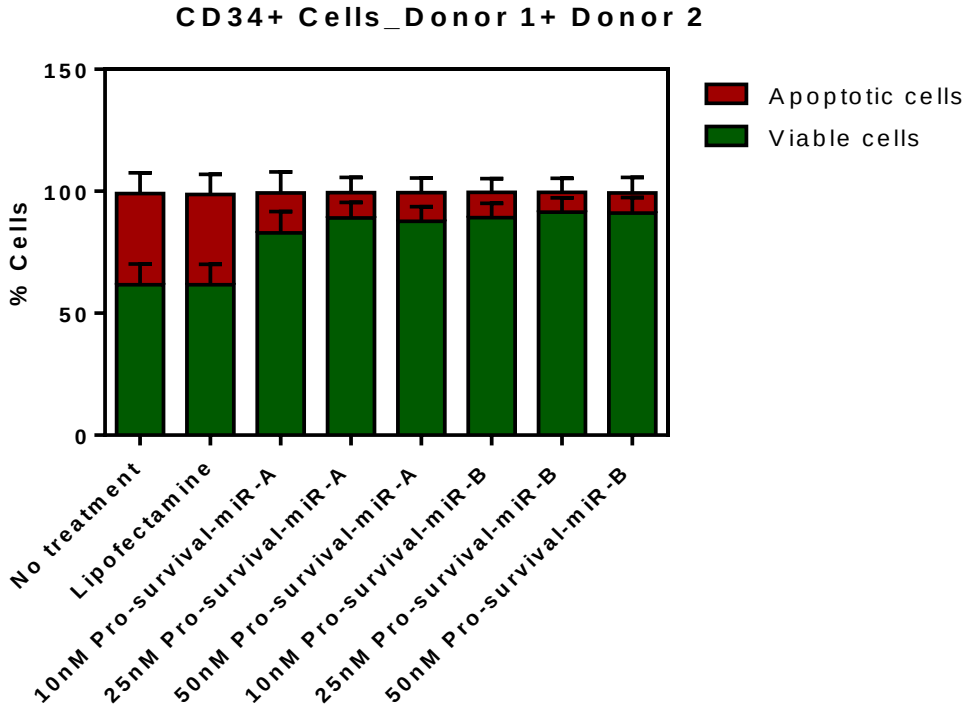


Figure 14. (a) CD34+ EC's transfected with pro-survival-miR's had a significant increase in cell survival (b) Cell survival was quantified by the % of viable cells and dead cells. * p<0.05, comparing to Lipofectamine group, one-way anova and Bonferroni post test.

4.3 Pro-survival-miR-A and pro-survival-miR-B promotes cell migration in CD34+ EC's

Cell migration was assessed through the "wound healing/scratch" assay as described in methods section. It is a simple and economical technique, in which upon creation of a gap called "scratch" on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the denuded area to close the wound until the cell-to-cell contacts are reestablished, similarly to migration of cells in vivo. The major advantage of this method is that cell migration can be monitored over time, thus estimating a rate of migratory response.

Representative images of the migration assay can be seen in Figure 15. In order to analyse migration capacity of the cells, the pictures of the wounded area were taken at 0 h and 24h. To measure the wound closure, a rectangle was set with a defined surface area and passed that on all photos of the other time points and the percentage (%) of wound area was measured (Figure 15a-q).

The results demonstrated that transfected CD34+ ECs did not migrate in the first 4h. After 24h, the cells transfected with pro-survival-miR-B migrated 50% more compared to the control (Lipofectamine), and the pro-survival-miR-A had the same percentage of wounded area than VEGF. This is suggestive of transfection itself affecting cell migration

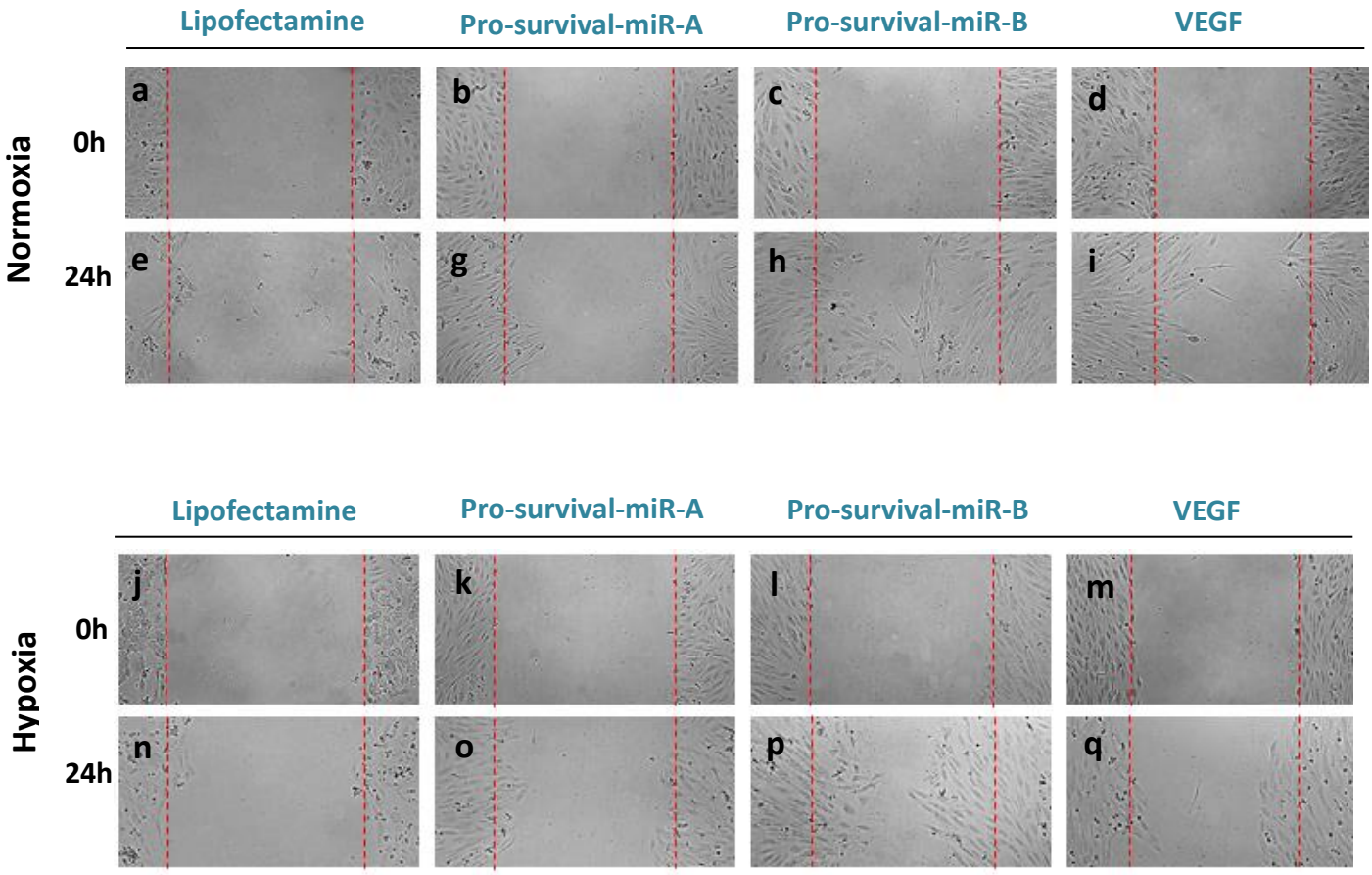


Figure 15. Images from a scratch assay experiment at different conditions (a-i) Normoxia and (j-q) Hypoxia. Transfected CD34+ EC's were plated on gelatin-coated 96-well-plates, wounded with a p20 pipette tip, and then imaged in two different time-points. The pictures show cells that have been freshly wounded (0h) and 24h

hours after wounding at which point cell migration into the area of the wound can clearly be seen. Dotted lines mark the edge of the 'wound'. Images were taken 10X magnification and area was quantified using image J.

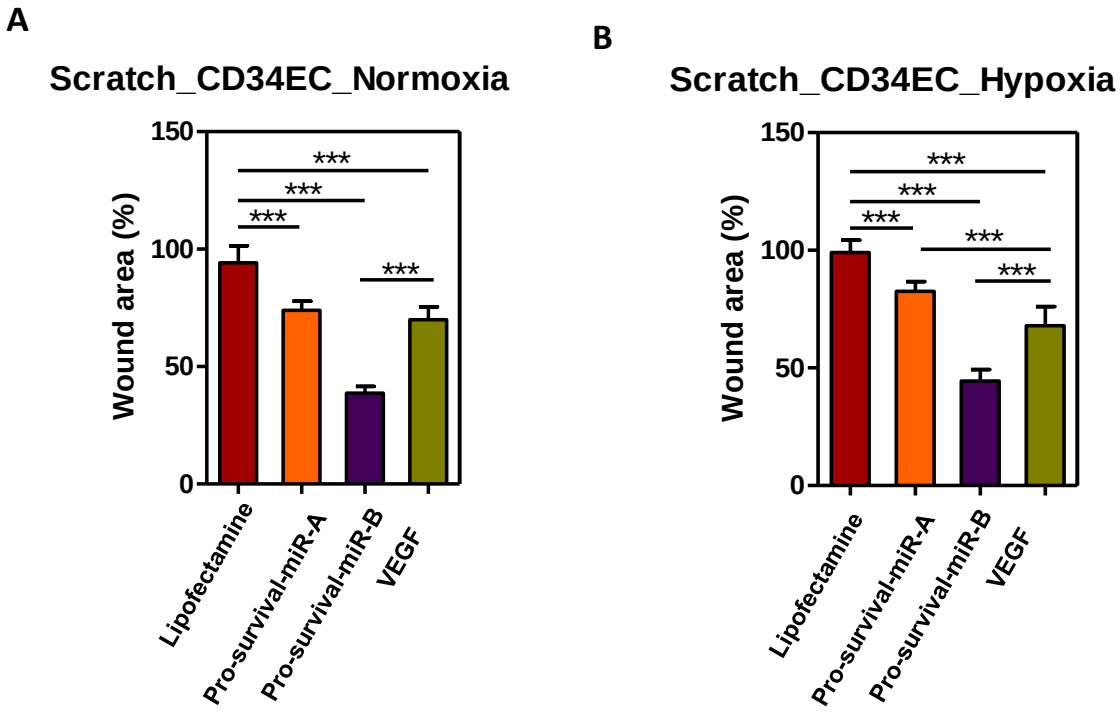


Figure 16. Quantification of CD34+ EC's migration through the percentage of wounded area over 24h in (a) Normoxia and (b) Hypoxia. Values are reported as mean + SD. *p<0.05 statistically significant difference from control group ate same time.

4.4 Angiogenesis: tube formation assay on Matrigel

The angiogenic capability of CD34+ EC's was assessed using an *in vitro* capillary formation assay on Matrigel. The *in vitro* angiogenesis assay was conducted using IBIDI micro-angiogenesis slides as described in methods section. After 4 h, tube length and number of branching points was measured and compared between conditions.

As shown in figure 17, all transfected cells presented similar efficiency in both conditions (normoxia and hypoxia). Measurement of total tube length revealed

significant but not critical differences between cells transfected with VEGF comparative to Lipofectamine. Total tube area was also similar in both pro-survival-miR's. This results suggested that this microRNA does not have significant angiogenic properties.

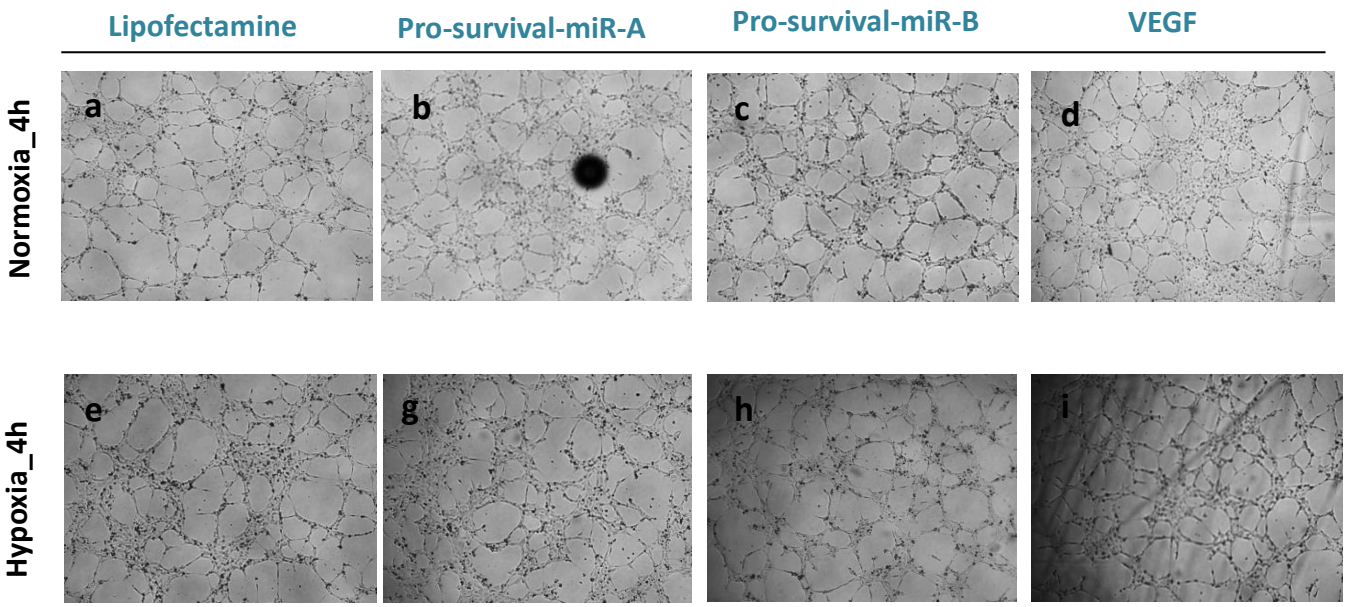


Figure 17. Vascular formation of the different groups in vitro (magnification × 100): Representative images of CD34+ EC's tubules in growth factor-reduced Matrigel after transfection and culture for 4 hours in the respective conditions. Tube formation assay was perform to verify the endothelial function and their ability to aggregate and sprout to create vessels in normoxia (a-d) and hypoxia (e-i) conditions.

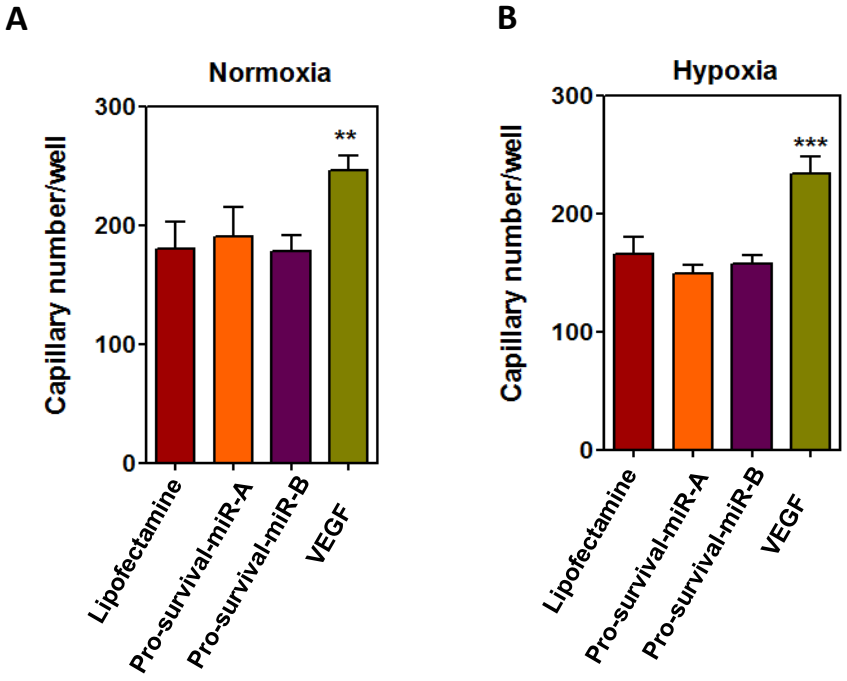


Figure 18. Transfection efficiency for the matrigel assay. Comparison of the numbers of capillary/well between groups in (a) normoxia and (b) hypoxic conditions. transfected cells with miR's, VEGF versus Lipofectamie. Error bars represent standard deviation (SD). The statistical analysis used is Newman-Keuls Multiple Comparison Test. *, **, * denote statistical significance (P,0.05, P,0.01, P,0.001, respectively).**

4.5 RT-PCR for gene expression profile of treated cells

Putative gene targets of pro-survival-miR-A and pro-survival-miR-B were identified and investigated *in vitro*. RT-PCR was performed using SYBR Green PCR Master Mix. The final results are presented using the approach $2^{-\Delta\Delta Ct}$ relative to the reference gene β-actin. The housekeeping gene is crucial for the estimation and comparison of mRNA level; B-actin is an abundant and stable housekeeping gene as it has a constant expression level across the samples.

The modulation of pro-survival-miR's levels in CD34+ endothelial cells and the gene expression analysis through real-time quantitative polymerase chain reaction (RT-qPCR) allowed the identification of downstream targets underlying pro-survival-miR's

regulation of cell survival. Bioinformatic tools and literature allowed the choice of 4 predicted targets (PTEN, PTPRQ, Akt, p21) from which phosphatase and tensin homolog (PTEN) was confirmed to be a direct target of our pro-survival-miR's.

In the first experiment (figure 19a), pro-survival-miR-A slightly upregulated the expression of Akt, while pro-survival-miR-B values for this target gene are near the control. In both miR's, there's an increase in p21 and PTPRQ levels (figure 19b), whereas the PTEN expression is downregulated.

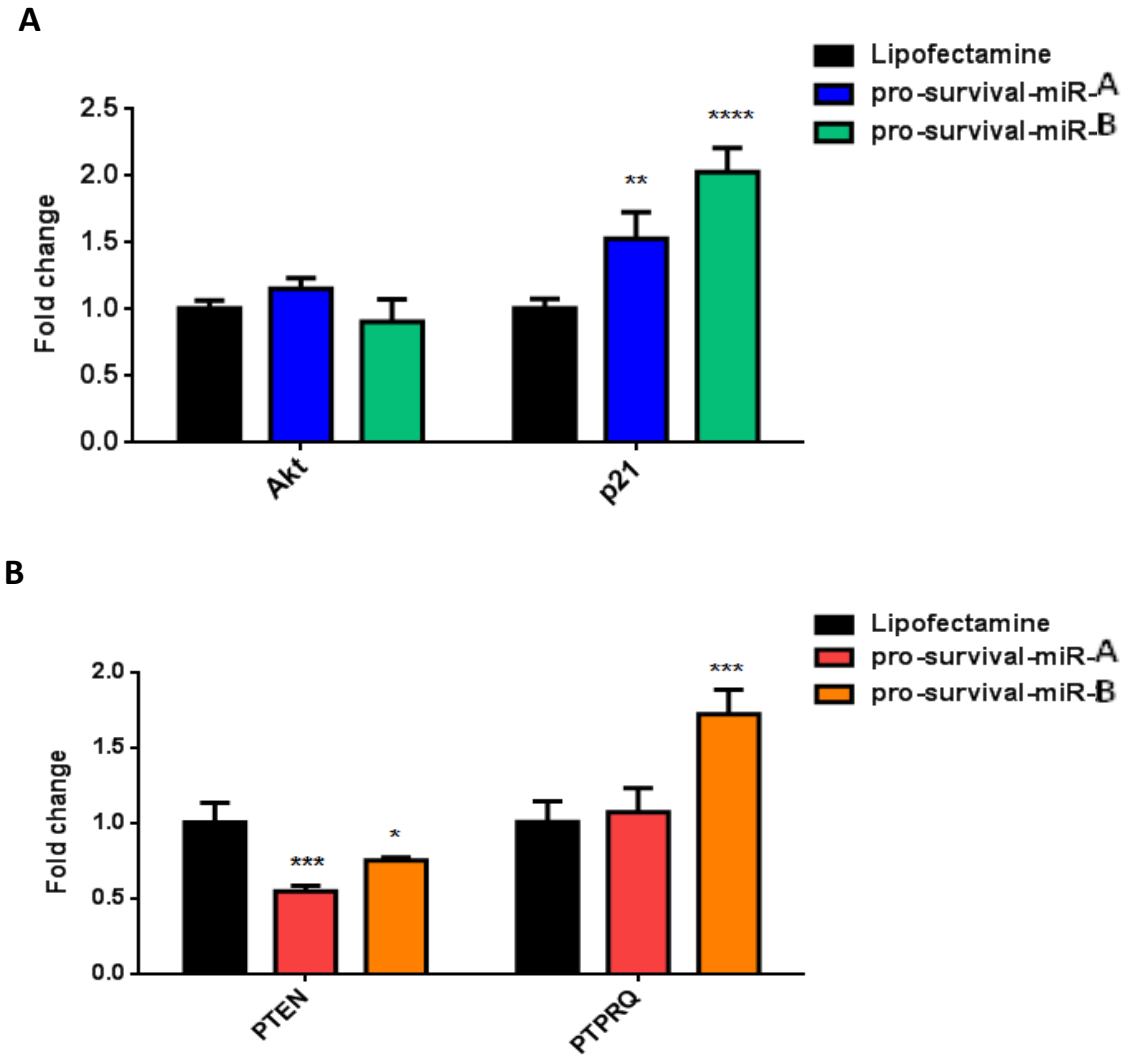


Figure 19. Gene expression modulated by pro-survival-miR-A and pro-survival-miR-B represented by $2^{-\Delta\Delta Ct}$ values. The statistical analysis used is one way ANOVA + Bonferroni multiple comparison test. p-value: ≤ 0.05 (*) ≤ 0.01 (**) ≤ 0.001(***) ≤ 0.0001(****).

The remainings of CD34+ ECs used in functional assays, were used to test more putative targets of our pro-survival-miR's **(attachment 1)**.

CHAPTER 5 - DISCUSSION

In this study, the effects of two pro-survival-microRNAs on endothelial cell survival and proliferation were tested. The experiments indicated that CD34+ ECs could be efficiently transfected with microRNAs without affecting their viability. First, we characterized our pro-survival-miRs regulation and its functional relevance in EC in response to hypoxia. Neither serum deprivation nor lack of oxygen hinder the potential of our transfected cells to survive under hypoxic conditions. These findings shown that pro-survival-miR-A and pro-survival-miR-B promotes cell survival of CD34+ ECs during starvation. Therefore we can hypothesize a broad-spectrum role of our pro-survival-miRs induction in the hypoxic response. In keeping with this explanation, recent studies found that the characteristics of the miRNAs modulated by hypoxia differs extensively according to the cell type used as much as the degree and the time of hypoxia (Hua et al., 2006). Also, pro-survival-miR-A and pro-survival-miR-B improves cell survival and inhibits apoptosis via regulation of various signaling networks.

Upon transfection with our miRNAs, cells display high migratory activity. Because ECs play an important role in cardiac repair and restoring vascularization after an ischemic insult by promoting angiogenesis and improving blood supply, these results indicate that pro-survival-miR-A and pro-survival-miR-B promote endothelial cell migration. This improvement in migration was better than VEGF on pro-survival-miR-B and, on miR-A, this enhancement was equivalent to VEGF.

One important feature of hypoxia is the increase in angiogenesis. Several characteristics of angiogenesis can be studied *in vitro*, using the capability of EC to form capillary-like structures once plated on Matrigel or other extracellular components (Darland & D'amore, 2001). The angiogenic ability of our pro-survival miR's was also explored. Transfected CD34+ EC's showed similar angiogenic capacity compared to Lipofectamine, suggesting the importance of VEGF in augmenting pro-angiogenic properties of CD34+ endothelial cells, since VEGF has been identified as a predominant mediator of angiogenesis (Yang et al., 2006). To assess whether CD34+ EC's transfection with pro-survival-miR's affected capillary-like structure formation in the absence of hypoxia, these cells were also cultured in normoxia conditions. The

results show that there is no significant difference between tube formation in hypoxia and normoxia conditions (Figure 17). It is possible that modulation of pro-survival-miR-A and B alone was insufficient to significantly alter the angiogenic potential of CD34+ EC's.

Real-time quantitative polymerase chain reaction (RT-qPCR) allowed the identification of the expression of targets underlying pro-survival-miR's regulation of cell survival. The expression of tumor suppressor related genes was downregulated activating its downstream targets, inducing cell cycle progression, survival, metabolism and migration. Based on miRNA TargetScan software analysis, other target genes may be involved in mediating the proliferation and survival effects of our pro-survival-miRs.

To study the possible molecular mechanism of our pro-survival-miR's, putative gene targets of pro-survival-miR-A and pro-survival-miR-B were identified using bioinformatic tools. To understand the observed cellular changes in our pro-survival-miR's target regulation, downstream signaling cascades were further investigated. So far, PTEN, Akt, p21 and PTPRQ were identified. However, many additional targets may be involved in the action of this pro-survival-miR's, especially in other disease conditions in which target availability may change. Although many other targets might be involved, target reconstitution experiments showed dominant roles of PTEN for the proliferation and migration effects of our microRNA's (Figure 20). The role of other potential target important for pro-survival-microRNA's biological actions remains to be elucidated. PTPRQ has very low activity against phosphotyrosine but is active against phosphatidylinositol phosphates that are involved in regulation of survival, proliferation, and subcellular architecture. PTPRQ regulates the phosphorylation state of AKT by suppressing the phosphatidylinositol 3,4,5-trisphosphate (PIP3) level (Oganesian et al., 2003). When tested in cultured cells, PTPRQ overexpression inhibited proliferation and promoted apoptosis and this activity depended on its PIPase activity rather than on its PTPase activity (Oganesian et al., 2003). Like PTEN its predominant biological activity is as a PIPase, but unlike PTEN it dephosphorylates a broader range of PIPs and depends for activity on different amino acid residues in the catalytic domain. The results demonstrate overexpression of PTPRQ, but according to

bibliography, this target should be downregulated to promote cell survival, so we presume that PTPRQ is not a direct target of pro-survival-miR's.

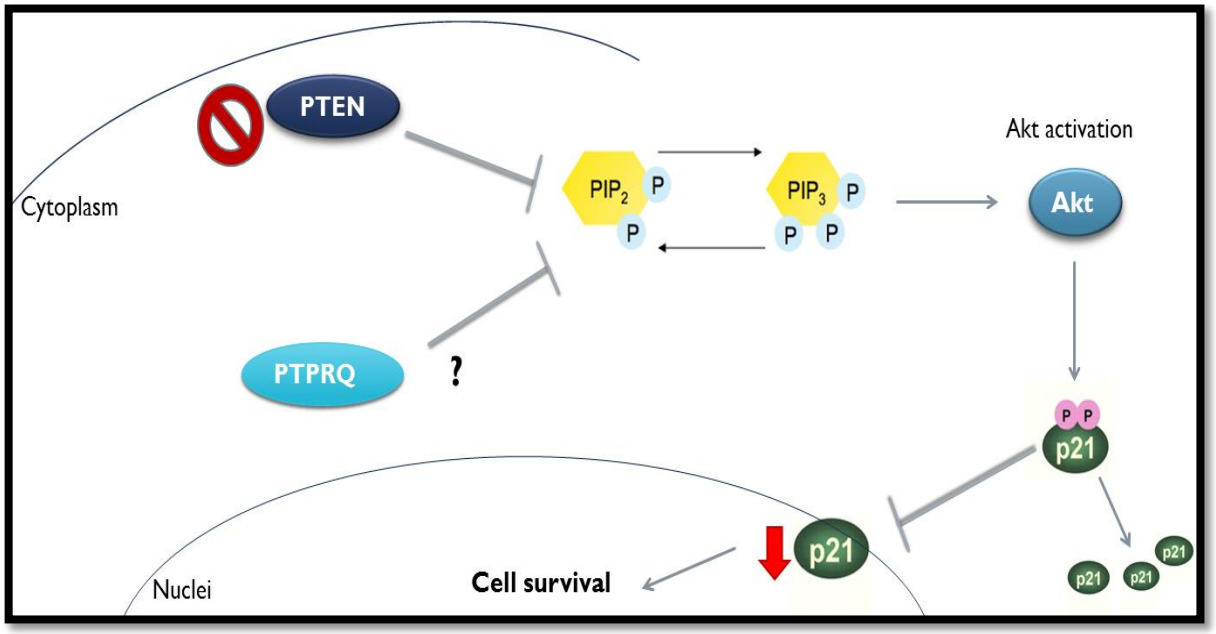


Figure 20. Potential molecular mechanisms of pro-survival-A and pro-survival-miR-B to promote cell survival. (adapted)

Although all these evidences establish that pro-survival-miR's regulates PTEN expression directly, downregulating this gene, promoting cell migration, it is still possible that indirect mechanisms may increase the potency of our pro-survival-miR's.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual lipid and protein phosphatase. Its main target is the PIP3 (Maehama & Dixon, 1998), the direct product of the phosphatidylinositol 3-kinase (PI3K). Loss of PTEN function, results in accumulation of PIP3 copying the effect of PI3K activation and triggering the activation of its downstream effectors, PDK1, AKT/PKB. Activated PDK1 phosphorylates AKT. AKT activation stimulates cell cycle progression, survival, metabolism and migration through phosphorylation of many physiological substrates (Dahia, 2000; Downward, 2004).

Cell cycle progression is regulated by the interaction between cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs) (reviewed in (Sherr & Roberts, 1999)). The role of p21 was in cyclin/cdk inhibition (Xiong et al., 1993), but more recent data also has shown that p21 is involved in positive effects on cyclin/cdk activation (Han et al., 2009). p21 is usually phosphorylated by Akt, which leads to increased p21 stability as well as improved cell survival (Li, Dowbenko, & Lasky, 2002). Cytoplasmic localization of p21 results from activation of Akt with subsequent p21 phosphorylation (Zhou et al., 2001). p21 accumulates in the cytoplasm of actively growing cells and that forced localization of p21 to the cytosolic compartment results in increased cell growth and resistance to apoptosis . Given the complex relationship between PTEN, phosphoinositide-3 kinase (PI3K), Akt, and p21, which are all signaling proteins involved in cell growth and apoptosis in cancer, we wanted to validate this targets behavior in the presence of pro-survival-miR's. RT-PCR showed that p21 is upregulated, and that can be caused by the attenuation of PTEN.

Our present findings reported here, that PTEN deficiency augments p21 stability and alters its subcellular localization so that it is situated more in the cytosolic compartment, may explain by these results (figure 19).

In summary, the role of pro-survival-miR-A and pro-survival-miR-B in cell survival and migration illustrates how miRNAs can have multiple targets in the same process and further demonstrates how miRNAs can be involved in balancing cellular responses by having both positive and negative effects on a specific biological phenomenon.

Some studies could be used to complement these results. For example, the effects of miRNAs on their target mRNA expression could examined by luciferase reporter assay. This is achieved by engineering a luciferase gene construct comprehending the predicted miRNA targeting sequence from the target gene (often located in the 3'-UTR). Also a western blot could be done in order to avaliate the impact of our pro-survival-miR's on the expression of their targets.

CHAPTER 6 - CONCLUSIONS

Strategies to biologically and genetically modify and deliver autologous endothelial cells for cardiovascular disease treatment were possible by the identification of pro-survival microRNAs and their molecular mechanisms. As therapeutic targets for vascular diseases, miRNAs could represent a major breakthrough because a single miRNA can regulate several target genes, thus influencing multiple molecular pathways.

In consideration of the above, miRNAs antagonists or mimics could become an important new class of “drugs” to regulate angiogenesis, cell survival and migration. Moreover, manipulation of miRNAs structures might increase their delivery efficiency.

In the meantime, not all targets of a particular miRNA are disease-related, so a possible drawback in the therapeutic use of miRNAs is their “off-target effects”, since any therapeutic perturbation of miRNA expression can have side effects.

In summary, this study showed that pro-survival-miR-A and pro-survival-miR-B could promote CD34+ EC’s survival and the cells ability of vascularization under hypoxic–ischemic conditions *in vitro*.

Further explorations of the implications of pro-survival-miR’s on CD34+EC’s *in vivo* will provide new approaches for the treatment of ischemic heart disease based on endothelial cell transplantation.

REFERENCES

Abdelwahid, E., Kalvelyte, A., Stulpinas, A., de Carvalho, K. A. T., Guarita-Souza, L. C., & Foldes, G. (2015). Stem cell death and survival in heart regeneration and repair. *Apoptosis*, *21*(3), 1–17. http://doi.org/10.1007/s10495-015-1203-4

Aird, W. C. (2007). Phenotypic Heterogeneity of the Endothelium: II. Representative Vascular Beds. *Circulation Research*, *100*(2), 174–190. http://doi.org/10.1161/01.RES.0000255690.03436.ae

Bartel, D. P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*. http://doi.org/10.1016/S0092-8674(04)00045-5

Bonauer, A., Carmona, G., Iwasaki, M., Mione, M., Koyanagi, M., Fischer, A., … Dimmeler, S. (2009). MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science (New York, N.Y.)*, *324*(5935), 1710–3. http://doi.org/10.1126/science.1174381

Brutsaert, D. L., Acierno, R., Agnisola, C., Venzi, R., Tota, B., Aird, W., … Solomon, S. (2003). Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. *Physiological Reviews*, *83*(1), 59–115. http://doi.org/10.1152/physrev.00017.2002

Chavakis, E., & Dimmeler, S. (2011). Homing of progenitor cells to ischemic tissues. *Antioxidants & Redox Signaling*, *15*(4), 967–980. http://doi.org/10.1089/ars.2010.3582

Chen, Y., & Gorski, D. H. (2008). Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood*, *111*(3), 1217–1226. http://doi.org/10.1182/blood-2007-07-104133

Conway, E. M., Collen, D., Carmeliet, P., Noden, D. M., Hirschi, K. K., D'Amore, P. A., … Gimbrone, M. A. (2001). Molecular mechanisms of blood vessel growth. *Cardiovascular Research*, *49*(3), 507–21. http://doi.org/10.1016/s0008-6363(00)00281-9

Dahia, P. L. (2000). PTEN, a unique tumor suppressor gene. *Endocrine-Related Cancer*, *7*(2), 115–29. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10903528

Darland, D. C., & D'amore, P. A. (2001). TGF?? is required for the formation of capillary-like structures in three-dimensional cocultures of 10T1/2 and endothelial

cells. *Angiogenesis*, *4*(1), 11–20. http://doi.org/10.1023/A:1016611824696

Davies, P. F., Remuzzi, A., Gordon, E. J., Dewey, C. F., & Gimbrone, M. A. (1986). Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(7), 2114–7. http://doi.org/10.1073/pnas.83.7.2114

Di Bernardini, E., Campagnolo, P., Margariti, A., Zampetaki, A., Mayer, M., Hu, Y., & Xu, Q. (2013). Endothelial lineage differentiation from ips cells is regulated by miRNA-21/AKT and TGF-beta2 pathways. *Heart*, *99*, A103–A104. http://doi.org/10.1136/heartjnl-2013-304019.183

Dimmeler, S. (2005). ATVB in focus: Novel mediators and mechanisms in angiogenesis and vasculogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(11), 2245. http://doi.org/10.1161/01.ATV.0000187471.06942.17

Dixit, P., & Katare, R. (2015). Challenges in identifying the best source of stem cells for cardiac regeneration therapy. *Stem Cell Research & Therapy*, *6*(1), 26. http://doi.org/10.1186/s13287-015-0010-8

Downward, J. (2004). PI 3-kinase, Akt and cell survival. *Seminars in Cell & Developmental Biology*, *15*(2), 177–182. http://doi.org/10.1016/j.semcdb.2004.01.002

Etheridge, A., Lee, I., Hood, L., Galas, D., & Wang, K. (2011). Extracellular microRNA: A new source of biomarkers. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*. http://doi.org/10.1016/j.mrfmmm.2011.03.004

Fasanaro, P., D'Alessandra, Y., Di Stefano, V., Melchionna, R., Romani, S., Pompilio, G., … Martelli, F. (2008). MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand ephrin-A3. *Journal of Biological Chemistry*, *283*(23), 15878–15883. http://doi.org/10.1074/jbc.M800731200

Ferreira, L., Pedroso, D. C. S., Vazão, H., & Gomes, R. S. M. (2010). Stem cell-based therapies for heart regeneration: what did the bench teach us? *Cardiovascular & Hematological Disorders Drug Targets*, *10*(3), 173–85. http://doi.org/BSP/CHDDT/E-Pub/00021 [pii]

Fish, J. E., & Srivastava, D. (2009). MicroRNAs: opening a new vein in angiogenesis research. *Science Signaling*, *2*(52), pe1. http://doi.org/10.1126/scisignal.252pe1

Foo, R. S. Y., Mani, K., & Kitsis, R. N. (2005, March). Death begets failure in the heart. *Journal of Clinical Investigation*. American Society for Clinical Investigation. http://doi.org/10.1172/JCI200524569

Gnecchi, M., He, H., Noiseux, N., Liang, O. D., Zhang, L., Morello, F., … Dzau, V. J. (2006). Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *20*(6), 661–669. http://doi.org/10.1096/fj.05-5211com

Haider, H. K., & Ashraf, M. (2008). Strategies to promote donor cell survival: Combining preconditioning approach with stem cell transplantation. *Journal of Molecular and Cellular Cardiology*. http://doi.org/10.1016/j.yjmcc.2008.05.004

Han, C.-T., Schoene, N. W., Lei, K. Y., Alshatwi, A., Han, C., Schoene, N., … Hung, M. (2009). Influence of zinc deficiency on Akt-Mdm2-p53 and Akt-p21 signaling axes in normal and malignant human prostate cells. *American Journal of Physiology. Cell Physiology*, *297*(5), C1188-99. http://doi.org/10.1152/ajpcell.00042.2009

He, L., & Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation TL - 5. *Nature Reviews Genetics*, *5 VN-re*(7), 522–531. http://doi.org/10.1038/nrg1379

Hua, Z., Lv, Q., Ye, W., Wong, C. K. A., Cai, G., Gu, D., … Zhang, Y. (2006). Mirna-directed regulation of VEGF and other angiogenic under hypoxia. *PLoS ONE*, *1*(1), e116. http://doi.org/10.1371/journal.pone.0000116

Krenning, G., van Luyn, M. J. A., & Harmsen, M. C. (2009). Endothelial progenitor cell-based neovascularization: implications for therapy. *Trends in Molecular Medicine*, *15*(4), 180–189. http://doi.org/10.1016/j.molmed.2009.02.001

Lai, E. C. (2004). Predicting and validating microRNA targets. *Genome Biology*, *5*(9), 115. http://doi.org/10.1186/gb-2004-5-9-115

Lee, D. Y., Deng, Z., Wang, C.-H., & Yang, B. B. (2007). MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(51), 20350–20355. http://doi.org/10.1073/pnas.0706901104

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., … Kim, V. N. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature*, *425*(6956), 415–419.

http://doi.org/10.1038/nature01957

Lei, Z., Sluijter, J. P. G., & van Mil, A. (2015). MicroRNA Therapeutics for Cardiac Regeneration. *Mini Reviews in Medicinal Chemistry*, *15*(6), 441–451.

Li, Y., Dowbenko, D., & Lasky, L. A. (2002). AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. *Journal of Biological Chemistry*, *277*(13), 11352–11361. http://doi.org/10.1074/jbc.M109062200

Liu, X., Driskell, R., & Engelhardt, J. (2006). Lung vasculogenesis and angiogenesis. *Methods in Enzymology*, *419*(6), 285–321. http://doi.org/10.1016/S0076-6879(06)19012-6

Losordo, D. W., & Dimmeler, S. (2004). Review : Clinical Cardiology : New Frontiers Therapeutic Angiogenesis and Vasculogenesis for. *Online*, *109*(21), 2487–2491. http://doi.org/10.1161/01.CIR.0000128595.79378.FA

Lozano, G. M., Bejarano, I., Espino, J., González, D., Ortiz, A., García, J. F., … Pariente, J. A. (2009). Relationship between caspase activity and apoptotic markers in human sperm in response to hydrogen peroxide and progesterone. *The Journal of Reproduction and Development*, *55*(6), 615–621. http://doi.org/10.1262/jrd.20250

Maehama, T., & Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *Journal of Biological Chemistry*, *273*(22), 13375–13378. http://doi.org/10.1074/jbc.273.22.13375

Martin, T. A., Ye, L., Sanders, A. J., Lane, J., & Jiang, W. G. (2013). Cancer Invasion and Metastasis: Molecular and Cellular Perspective. Landes Bioscience.

Mendis S, Puska P, & Norrving B. (2011). Global Atlas on cardiovascular disease prevention and control. *World Health Organization*, 156.

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J.-L., … Pittet, M. J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of Experimental Medicine*, *204*(12), 3037–47. http://doi.org/10.1084/jem.20070885

Narmoneva, D. A., Vukmirovic, R., Davis, M. E., Kamm, R. D., & Lee, R. T. (2004). Endothelial cells promote cardiac myocyte survival and spatial reorganization:

Implications for cardiac regeneration. *Circulation*, *110*(8), 962–968. http://doi.org/10.1161/01.CIR.0000140667.37070.07

Nichols, M., Townsend, N., Scarborough, P., Rayner, M., Lozano, R., Naghavi, M., … Zoghbi, W. (2014). Cardiovascular disease in Europe 2014: epidemiological update. *European Heart Journal*, *35*(42), 2950–9. http://doi.org/10.1093/eurheartj/ehu299

Nie, Y., Zhang, S., Liu, N., & Li, Z. (2016). Extracellular Matrix Enhances Therapeutic Effects of Stem Cells in Regenerative Medicine. In *Composition and Function of the Extracellular Matrix in the Human Body*. InTech. http://doi.org/10.5772/62229

Oganesian, A., Poot, M., Daum, G., Coats, S. A., Wright, M. B., Seifert, R. A., & Bowen-Pope, D. F. (2003). Protein tyrosine phosphatase RQ is a phosphatidylinositol phosphatase that can regulate cell survival and proliferation. *Proc Natl Acad Sci U S A*, *100*(13), 7563–8. http://doi.org/10.1073/pnas.1336511100

Otsuka, M., Zheng, M., Hayashi, M., Lee, J. D., Yoshino, O., Lin, S., & Han, J. (2008). Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. *Journal of Clinical Investigation*, *118*(5), 1944–1954. http://doi.org/10.1172/JCI33680

Paoli, P., Giannoni, E., & Chiarugi, P. (2013). Anoikis molecular pathways and its role in cancer progression ☆. *BBA - Molecular Cell Research*, *1833*(12), 3481–3498. http://doi.org/10.1016/j.bbamcr.2013.06.026

Pasumarthi, K. B. S., Nakajima, H., Nakajima, H. O., Soonpaa, M. H., & Field, L. J. (2005). Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. *Circulation Research*, *96*(1), 110–118. http://doi.org/10.1161/01.RES.0000152326.91223.4F

Pedroso, D. C. S., Tellechea, A., Moura, L., Fidalgo-Carvalho, I., Duarte, J., Carvalho, E., & Ferreira, L. (2011). Improved Survival, Vascular Differentiation and Wound Healing Potential of Stem Cells Co-Cultured with Endothelial Cells. *PLoS ONE*, *6*(1), e16114. http://doi.org/10.1371/journal.pone.0016114

Penn, M. S., & Mangi, A. A. (2008, June 20). Genetic enhancement of stem cell engraftment, survival, and efficacy. *Circulation Research*. http://doi.org/10.1161/CIRCRESAHA.108.175174

Phelps, E. A., & Garcia, A. J. (2009). Update on therapeutic vascularization strategies.

Regenerative Medicine, *4*(1), 65–80. http://doi.org/10.2217/17460751.4.1.65

Pinto, A. R., Ilinykh, A., Ivey, M. J., Kuwabara, J. T., D'antoni, M. L., Debuque, R., … Tallquist, M. D. (2016). Revisiting cardiac cellular composition. *Circulation Research*, *118*(3), 400–409. http://doi.org/10.1161/CIRCRESAHA.115.307778

Potente, M., Gerhardt, H., & Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell*. http://doi.org/10.1016/j.cell.2011.08.039

Reddig, P. J., & Juliano, R. L. (2005, September). Clinging to life: Cell to matrix adhesion and cell survival. *Cancer and Metastasis Reviews*. Kluwer Academic Publishers. http://doi.org/10.1007/s10555-005-5134-3

Reed, D. M., Foldes, G., Harding, S. E., & Mitchell, J. A. (2013). Stem cell-derived endothelial cells for cardiovascular disease: A therapeutic perspective. *British Journal of Clinical Pharmacology*, *75*(4), 897–906. http://doi.org/10.1111/j.1365-2125.2012.04361.x

Robey, T. E., Saiget, M. K., Reinecke, H., & Murry, C. E. (2008). Systems approaches to preventing transplanted cell death in cardiac repair. *Journal of Molecular and Cellular Cardiology*, *45*(4), 567–581. http://doi.org/10.1016/j.yjmcc.2008.03.009

Rosenthal. (2003). 030717 Prometheus's Vulture and the Stem-Cell Promise. *http://dx.doi.org/10.1056/NEJMra020849*, 1–8. Retrieved from papers3://publication/uuid/B14A4BF3-1627-42DC-AE3A-917521DA7602

Seeger, F. H., Zeiher, A. M., & Dimmeler, S. (2013, August 1). MicroRNAs in stem cell function and regenerative therapy of the heart. *Arteriosclerosis, Thrombosis, and Vascular Biology*. Lippincott Williams & Wilkins. http://doi.org/10.1161/ATVBAHA.113.300138

Sherr, C. J., & Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Development*, *13*(12), 1501–1512. http://doi.org/10.1101/gad.13.12.1501

Silvestre, J.-S., Smadja, D. M., Lévy, B. I., Abbate, A., Kontos, M., Grizzard, J., … Maini, R. (2013). Postischemic revascularization: from cellular and molecular mechanisms to clinical applications. *Physiological Reviews*, *93*(4), 1743–802. http://doi.org/10.1152/physrev.00006.2013

Stenvang, J., Petri, A., Lindow, M., Obad, S., & Kauppinen, S. (2012). Inhibition of microRNA function by antimiR oligonucleotides. *Silence*, *3*(1), 1.

http://doi.org/10.1186/1758-907X-3-1

Taylor, D. A., & Robertson, M. J. (2009). Cardiovascular Translational Medicine (IX) The Basics of Cell Therapy to Treat Cardiovascular Disease: One Cell Does Not Fit All. *Revista Española de Cardiología (English Edition)*, *62*(9), 1032–1044. http://doi.org/10.1016/S1885-5857(09)73269-4

Taylor, D. A., & Zenovich, A. G. (2008). Cardiovascular cell therapy and endogenous repair. In *Diabetes, Obesity and Metabolism* (Vol. 10, pp. 5–15). http://doi.org/10.1111/j.1463-1326.2008.00937.x

Thomas, M., Lieberman, J., & Lal, A. (2010). Desperately seeking microRNA targets. *Nature Structural & Molecular Biology*, *17*(10), 1169–1174. http://doi.org/10.1038/nsmb.1921

Van Laake, L. W., Van Donselaar, E. G., Monshouwer-Kloots, @bullet J, Schreurs, @bullet C, Passier, @bullet R, Humbel, @bullet B M, … Mummery, C. L. (2009). Extracellular matrix formation after transplantation of human embryonic stem cell-derived cardiomyocytes.

van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., … Olson, E. N. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(48), 18255–60. http://doi.org/10.1073/pnas.0608791103

van Rooij, E., Sutherland, L. B., Thatcher, J. E., DiMaio, J. M., Naseem, R. H., Marshall, W. S., … Olson, E. N. (2008). Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(35), 13027–32. http://doi.org/10.1073/pnas.0805038105

World Health Organization. (2011). WHO _ Cardiovascular diseases (CVDs). *Cardiovascular Diseases (CVDs)*. World Health Organization. http://doi.org/Fact sheet N°317

Würdinger, T., Tannous, B. A., Saydam, O., Skog, J., Grau, S., Soutschek, J., … Krichevsky, A. M. (2008). miR-296 Regulates Growth Factor Receptor Overexpression in Angiogenic Endothelial Cells. *Cancer Cell*, *14*(5), 382–393. http://doi.org/10.1016/j.ccr.2008.10.005

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993). P21 Is a Universal Inhibitor of Cyclin Kinases. *Nature*, *366*(6456), 701–704. http://doi.org/10.1038/366701a0

Yang, J., Zhou, W., Zheng, W., Ma, Y., Lin, L., Tang, T., … Hu, J. (2006). Effects of myocardial transplantation of marrow mesenchymal stem cells transfected with vascular endothelial growth factor for the improvement of heart function and angiogenesis after myocardial infarction. *Cardiology*, *107*(1), 17–29. http://doi.org/10.1159/000093609

Yuan, P., & Ma, X. (2014). Endothelial Cells Facilitate Cell-Based Cardiac Repair: Progress and Challenge. *Current Stem Cell Research & Therapy*, *9*(5), 415–423. http://doi.org/10.2174/1574888X09666140710100143

Zhang, M. et al. (2001). Cardiomyocyte Grafting for Cardiac Repair: Graft Cell Death and Anti-Death Strategies. *Journal of Molecular and Cellular Cardiology*, *33*(5), 907–921.

Zhang, W. J., Liu, W., Cui, L., & Cao, Y. (2007). Tissue engineering of blood vessel. *Journal of Cellular and Molecular Medicine*, *11*(5), 945–57. http://doi.org/10.1111/j.1582-4934.2007.00099.x

Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., & Hung, M. C. (2001). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature Cell Biology*, *3*(3), 245–252. http://doi.org/10.1038/35060032

CHAPTER 7 - ATTACHMENTS

1

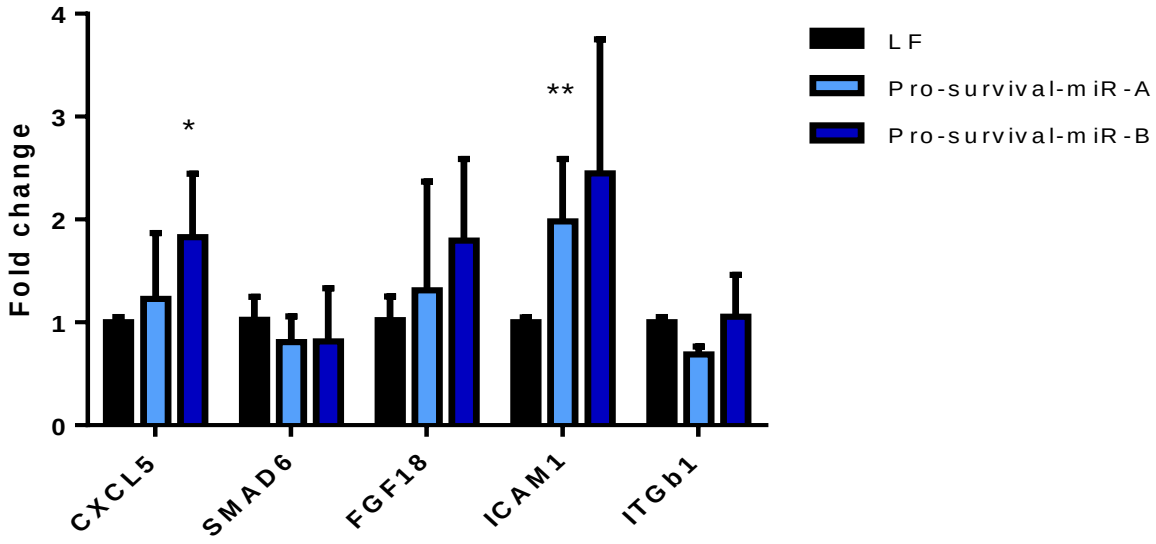


Figure 21. Gene expression modulated by pro-survival-miR-A and pro-survival-miR-B represented by $2^{-\Delta\Delta Ct}$ values. Statistically significant differences in CXCL5 and ICAM1. The statistical analysis used is one way ANOVA + Bonferroni multiple comparison test. p-value: ≤ 0.05 (*) ≤ 0.01 (**) ≤ 0.001(***) ≤ 0.0001(****).