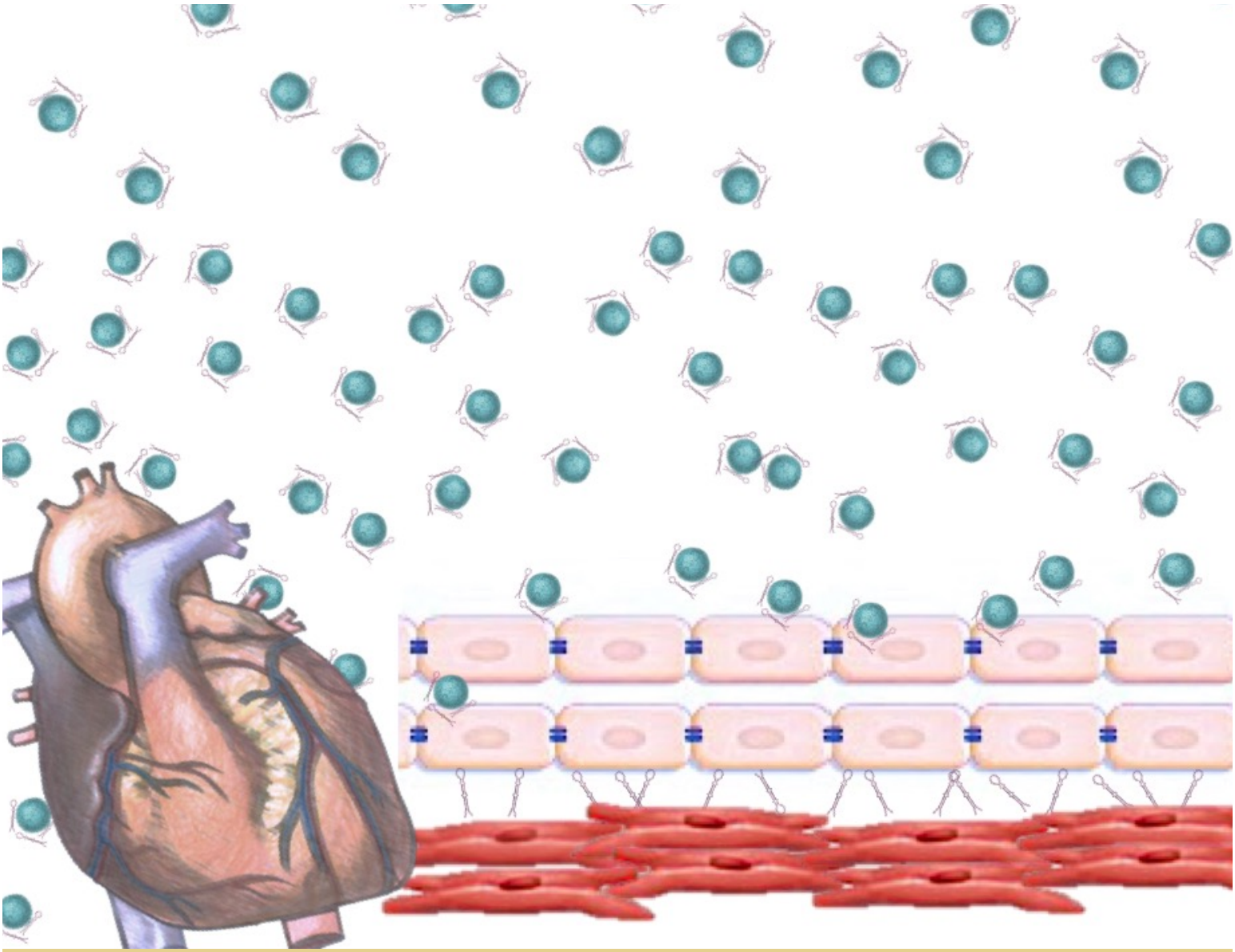

Cátia Alexandra Seabra Vieira

The role of miRNAs on cell survival

Tese de Mestrado em Investigação Biomédica

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UNIVERSIDADE DE COIMBRA

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The role of miRNAs on cell survival

Dissertação de Mestrado em Investigação Biomédica, apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre.

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*"**Brain:** You never give up, do you?*

***Heart:** No way! There's always something worth chasing!"*

From: The Awkward Yeti

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Abstract

Cardiovascular diseases are the leading cause of mortality, morbidity and health related costs in Europe and United States of America. They comprise different pathologies including myocardial infarction, cardiomyopathies and valvular heart disease all of them ultimately leading to heart failure (HF). HF is broadly characterized by and inability to efficiently fulfill the metabolic needs of the organism. The increase in life expectancy, the high prevalence of dietary and lifestyle risks factors, as well as the improved survival from cardiovascular events reinforces the need for efficient treatments to revert HF. During the last years, most of the research performed in that respect pertained to the discovery of molecules capable of enhancing cardiomyocyte proliferation. However, the results obtained with these approaches are far from ideal and a paradigm shift is necessary. Recently, several authors have demonstrated an important role of cardiac endothelium on cardiac regeneration. Moreover, miRNAs have appeared as versatile modulators of virtually all cell processes including in the cardiac context.

Our group recently performed a high throughput screening experiment to identify miRNAs capable of enhancing endothelial cell survival. In brief, we transfected endothelial cells with a library of 2080 miRNA mimics and exposed the transfected cells to ischemia (0.1% O_2 in endothelial basal medium without serum or growth factors). Fifteen miRNAs were identified as pro-survival miRNAs. In this thesis, we focused on the role of two of those miRNAs (a previously identified pro-survival miRNA and a miRNA never described in the literature). In the first part of the work we identified the mechanism of action governing the pro-survival effect of the selected miRNAs. In the second part, we explored a nanoparticle system capable of efficiently delivering the miRNAs to the cells both *in vitro* as well as *in vivo.*

Our results showed that both miRNAs increased endothelial cell survival under ischemic conditions and increased the migration of human $CD34^{+}$-derived endothelial cells. Then, we validated the direct interaction of both miRNAs with PTEN resulting in a decrease in its mRNA and protein levels. Furthermore, MAPK and PI3K/Akt pathways are activated by miR-C and miR-G both of which are well known mediators of the survival pathways. Finally, we used light-inducible polymeric nanoparticles to deliver miRNA to human derived endothelial cells with preliminary results showing their high complexation rate and strong delivery potential.

Our work presents miR-C and miR-G as strong endothelial cell survival enhancers.

Keywords: “myocardial ischemia”; miRNAs; “cell survival”; “endothelial-cardiomyocyte crosstalk”; “light-sensitive nanoparticles”.

Resumo

As doenças cardiovasculares são a causa predominante de mortalidade, morbidade e custos associados com a saúde na Europa e nos Estados Unidos da América. Estão incluídas nas doenças cardiovasculares, patologias como enfarte do miocárdio, cardiomiopatias ou doenças cardíacas valvulares, que poderão culminar em insuficiência cardíaca. Doentes com esta condição apresentam um desempenho cardíaco reduzido sendo incapazes de suprimir as necessidades metabólicas do organismo. Para além do mais, o aumento da esperança média de vida, o estilo de vida sedentário e maus hábitos alimentares, assim como a maior taxa de sobrevivência após diagnóstico de uma doença cardíaca reforçam a necessidade do desenvolvimento de tratamentos eficazes para curar a insuficiência cardíaca. Nos últimos anos, o grande foco da pesquisa nesta área tem passado pelo desenvolvimento de estratégias que estimulem a proliferação dos cardiomiócitos. Contudo, os resultados obtidos têm ficado aquém das expectativas realçando a necessidade de uma mudança de direção. Recentemente, alguns autores demonstraram o papel importante do endotélio na regeneração cardíaca. Paralelamente, os "miRNAs" surgiram como moduladores versáteis dos processos intracelulares inclusive no contexto de doença cardíaca.

O nosso grupo realizou uma experiência em larga escala para identificar "miRNAs" capazes de aumentar a sobrevivência celular endotelial. Brevemente, células endoteliais humanas foram transfectadas com 2080 "miRNA mimics" e expostas a condições de isquémia (0,1% O_2 em meio basal sem soro ou fatores de crescimento). Quinze "miRNAs" foram identificados como capazes de aumentar a sobrevivência. Nesta tese, estudámos o papel de dois desses "miRNAs" (um previamente descrito na literatura

como capaz de aumentar a sobrevivência e um nunca descrito). Na primeira parte do trabalho, desvendámos o mecanismo de acção o qual confere um efeito na sobrevivência celular. Na segunda parte, explorámos uma nanoformulação capaz de entregar os “miRNAs”.

Os nossos resultados demonstram que ambos os “miRNAs” aumentaram a sobrevivência e a capacidade de migração das células endoteliais. Em seguida, validámos a interação directa dos dois “miRNAs” com a proteína PTEN levando a uma diminuição nos seus níveis de RNA mensageiro e proteicos. Ambos os “miRNAs” levaram à ativação de duas vias intracelulares relacionados com a sobrevivência: vias “Pi3K/Akt” e “MAPK”. Por fim, usámos nanopartículas poliméricas sensíveis à luz para entregar os “miRNAs” a células endoteliais e os nossos resultados preliminares apontaram para uma alta taxa de complexação e um forte potencial de entrega.

O nosso trabalho apresenta estes “miRNAs” como fortes promotores da sobrevivência endotelial após condições isquémicas.

Palavras-chave: “isquémia do miocárdio”; “miRNAs”; “sobrevivência celular”; “interação endotélio-cardiomiócito”; “nanopartículas sensíveis à luz”.

Table of contents

INTRODUCTION.....1

1. CARDIOVASCULAR DISEASES.....3
2.HEART.....4
2.1HEART CELLULAR COMPOSITION.....4
2.2 *HEART REGENERATION*.....6
2.3 *CARDIAC ENDOTHELIUM*.....9
2.4 E*NDOTHELIUM-CARDIOMYOCYTE CROSSTALK*.....12
3. SMALL NON-CODING RNAS: MIRNAS.....14
3.1 MIRNAS BIOGENESIS AND FUNCTION.....14
3.2 MIRNAS IN THE CARDIOVASCULAR SYSTEM.....15
3.3 MIRNAS ADVANTAGES AND DISADVANTAGES: PAVING THE WAY FOR THERAPEUTICS.....17
3.3.1NANOCARRIERS.....20
3.3.1.1 *NANOCARRIERS FOR MIRNA MIMICS DELIVERY: FOCUS ON PEI POLYMERIC NPS*.....21
3.3.1.2 MIRNAS IN CLINICAL TRIALS.....22

GENERAL HYPOTHESIS.....24

MATERIALS AND METHODS.....26
CD34^{+} CELLS ISOLATION FROM UMBILICAL CORD BLOOD.....27
DIFFERENTIATION INTO CD34+-DERIVED ECS.....27
ENDOTHELIAL CELL CULTURE.....27
HIGH-CONTENT SCREENING (HCS) ASSAY.....28
IMAGE ACQUISITION AND ANALYSIS.....29
CONFIRMATION OF THE MIRNAS IDENTIFIED IN HIGH-CONTENT SCREENING ASSAY.....29
SURVIVAL ASSAY.....29
IMMUNOCYTOCHEMISTRY.....30
RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR FOR PTEN.....31
ANGIOGENESIS ASSAY.....31
MIGRATION ASSAY.....32
BIOINFORMATICS ANALYSIS.....32
LUCIFERASE REPORTER ASSAY.....35
WESTERN BLOTTING.....35
RNA SEQUENCING.....36
NANOPARTICLES PREPARATION AND CHARACTERIZATION.....37
NANOPARTICLES CONJUGATION TO MIRNA.....37
CELL TREATMENT WITH NPS CONJUGATED TO MIRNA.....38
STATISTICAL ANALYSIS.....39

RESULTS.....41
1. IDENTIFICATION OF "PRO-SURVIVAL" MIRNAS.....43
2. PRO-SURVIVAL MIRNAS AND ENDOTHELIAL CELL FUNCTION.....46
3. PRO-SURVIVAL MIRNAS TARGET PTEN AND MODULATE PI3K/AKT AND MAPK SIGNALING PATHWAYS.....50
4. DELIVERY OF MIR-G USING LIGHT-INDUCIBLE POLYMERIC NANOPARTICLES (NPS): 57
4.1 PRODUCTION AND CONJUGATION TO MIRNA.....57
4.2 CELL TREATMENT.....57

DISCUSSION......61
1. IDENTIFICATION OF "PRO-SURVIVAL" MIRNAS.....64
2. PRO-SURVIVAL MIRNAS AND ENDOTHELIAL CELL FUNCTION.....64
3. PRO-SURVIVAL MIRNAS TARGET PTEN AND MODULATE PI3K/AKT AND MAPK SIGNALING PATHWAYS.....65
4. DELIVERY OF MIR-G USING LIGHT-INDUCIBLE POLYMERIC NANOPARTICLES (NPS):
.....72

CONCLUDING REMARKS.....**75**

REFERENCES.....77

ATTACHMENTS.....97

Introduction

1. **Cardiovascular diseases**

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality in the developed countries. According to the World Health Organization, more than 17 million people die each year from CVDs - an estimated 31% of all deaths worldwide from which 80% are due to the blockage of blood supply in the heart or the brain.

During a myocardial infarction (MI), commonly named heart attack, the blood flow in a specific area of the heart is blocked and, consequently, nutrients and oxygen diffusion cannot reach the tissues downstream of the blockage leading to massive cell death. The loss of viable myocardium is compensated by increased fibrosis with non-contractile scar tissue replacing lost cardiac cells (Lister *et al.* 2016). This remodeling process is an attempt to restore function but unfortunately the newly formed tissue has reduced contractility compared to the lost native tissue.

To rescue the ischemic myocardium, reperfusion is often the first line of treatment. Despite terminating ischemia, reperfusion inflicts additional injury to the heart denominated reperfusion injury (Neri *et al.* 2017). The final infarct size will be dependent on Ischemia/Reperfusion (I/R) damage and will determine left ventricular function, remodeling and the long-term outcome of patients surviving an acute MI.

The deleterious alterations in structure and heart function can ultimately cause heart failure (HF) a condition resulting in a mismatch between the supply and metabolic needs of the body. Half of the diagnosed HF patients die within 4 years and have a 5-year survival rate even lower than breast-, lung- or colon- cancer patients (Behfar *et al.* 2014).

For several years, drugs such as angiotensin-converting-enzyme (ACE) inhibitors, beta blockers, aldosterone

antagonists or cardiac devices have relieved HF symptoms (Behfar *et al.* 2014). Still, HF has the greatest negative impact in quality of life compared to other chronic conditions, such as diabetes or arthritis. The only treatment that truly reverts HF is heart transplantation, which is by far not the best option due to shortage of donated organs and the risks and costs associated with the surgical procedure.

Regenerating the patient's myocardium and restoring proper heart function is the main goal in MI and HF research fields.

2. **Heart**

2.1 *Heart cellular composition*

The heart is a very well organized tridimensional structure composed of different cell types that work together to supply blood to the organism (Coulombe *et al.* 2014). It can be divided into three distinct layers: the epicardium, the myocardium and the endocardium (**Figure 1**).

The epicardium corresponds to the outer layer of the heart and is composed of elastic connective tissue and fat (Dueñas *et al.* 2017). It is responsible for maintaining the heart in the chest cavity and preventing the heart from over-expanding when blood volume increases.

The myocardium is the middle layer of the heart and is mainly composed, in mass and structure, by cardiomyocytes (CMs). CMs are specialized muscle cells, generally branched and multinucleated. To allow synchronous contraction and effective pump action, CMs present a dense network of mitochondria, sarcomeres composed of myosin and actin and intercalated disks connecting distinct CMs (Woodcock *et al.* 2005). Intercalated disks are CM specific

and are composed of different cell junctions: desmossomes, adherent- and gap junctions. Importantly, within CMs there is a group capable of creating autonomous electric impulses: the pacemaker cells. The rate of these impulses will determine the heart rate. Cardiac fibroblasts are also in the myocardium synthesizing extracellular matrix (ECM), which provides a scaffold for the attachment of CMs, fibroblasts themselves and endothelial cells (ECs). ECM is mainly comprised of fibrillar collagen types I and III, as well as less abundant collagen types IV, V and VI. The ECM also includes fibronectin, laminin, elastin and fibrillin, proteoglycans and glycoproteins (Fan *et al.* 2012). Cardiac ECM helps mediating mechanical connection among CMs, fibroblasts and blood vessels in the myocardium.

The endocardium is the inner layer of the heart and is composed of ECs that provide a smooth, elastic, non-adherent surface for blood collection (ref #layers). ECs in the endocardium act as a barrier between blood and the

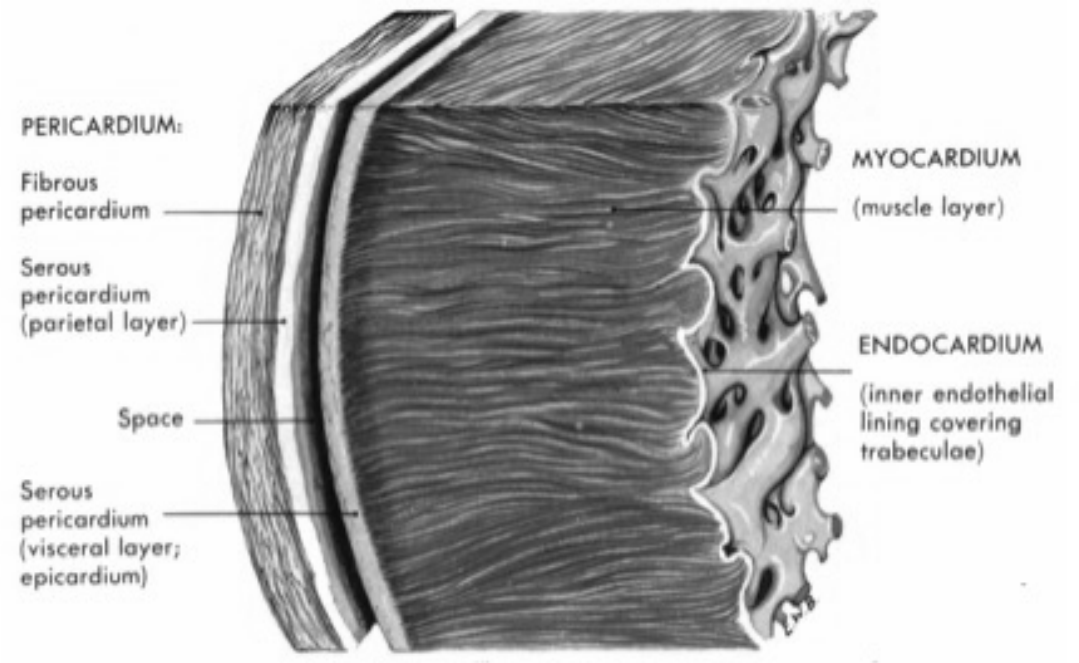


Figure 1. Transverse section of the heart wall highlighting the outer pericardium, the myocardium and the inner endocardium.
Adapted from: *Boundless, USA*: layers of the heart.

myocardium thus regulating the CMs surrounding environment (Lim *et al.* 2015).

2.2 *Heart regeneration*

The mammalian heart was for several years referred to as a post-mitotic organ. Over the past years, increasing evidence supporting the idea that heart tissue can regenerate has emerged.

Lower animals like zebrafish are the “gold standard” when it comes to heart regeneration. After CM loss due to ventricular apex resection, cryoinjury or genetic ablation, pre-existing CMs can proliferate and heart’s pumping capacity is fully restored (Poss *et al.* 2002; Wang *et al.* 2011; Chablais *et al.* 2011).

Regeneration studies in mice showed that, during embryonic development, upon injury, the myocardium can fully recover due to the proliferation of cardiac cells (Drenckhahn *et al.* 2008). Just after birth, rodents are still able to functionally recover after ventricular apex removal even though some scaring may occur (Porrello *et al.* 2011, Bryant *et al.* 2015). Porrello *et al.* (2011) was the first to report that CMs from neonatal rodents were capable of proliferating after resection of approximately 15% of the apex. The same author reported similar findings after MI in 1 day old mice (Mahmoud *et al.* 2014). Interestingly, Andersen *et al.* (2014) reached the opposite conclusion claiming that after apex removal there is only scar formation without CM proliferation. To clarify the discrepancy between these seemingly conflicting results, Bryant *et al.* (2015) systematically examined how technical considerations influenced this experimental model. By comparing and reproducing both authors removal techniques, the surgical retraction (gently fixing the ventricle just prior to excision) of the apex of the ventricle, only performed by Anderson *et al.*

(2014), was suggested as the cause for excessive fibrosis possibly hindering CM proliferation.

Using other cardiac injury models, such as MI after left coronary artery permanent ligation, Senyo *et al*. (2013) have shown a 4-fold increase in CMs turnover in the border region (**Figure 2**).

Interestingly, it seems that the heart´s regenerative capacity is maintained during 2-3 weeks following birth. This year's work by Nakada *et al.* points to the hypoxic womb state as a promoter of CM proliferation. Upon birth, the exposure to high amounts of oxygen increases reactive

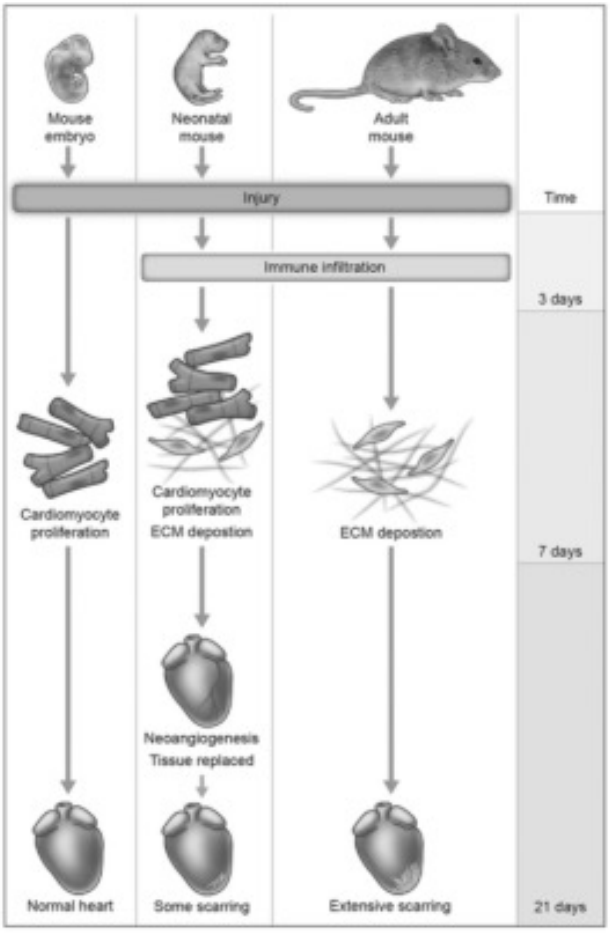


Figure 2. Regenerative capacity of the heart in mouse embryo, neonatal and adult mouse after cardiac injury. Mouse embryos after injury are capable of CM proliferation with complete heart regeneration. Neonatal mice can efficiently regenerate following injury although some scarring may occur. Adult mice lose their regenerative potential ending with scar tissue instead of functional heart tissue.
Adapted from: Uygur *et al.* 2016.

oxygen species (ROS), which induce a DNA damage response responsible for cell cycle arrest. Interestingly, when mice were gradually exposed to hypoxia, pre-existent CMs re-entered the cell cycle and proliferated corroborating the idea that oxygen levels play a key role in CM proliferation.

With respect to the human heart, Bergmann *et al.* (2009) used carbon-14 (^{14}C) uptaken by individuals during the nuclear tests of the 60s, to study heart regeneration. ^{14}C was used to date the CMs by mass spectrometry and compare it to the age of the individual. Using this technique, Bergmann reported a CM generation rate of ≈1% in individuals after 25 years of age. In contrast with the results from Bergmann, Mollova *et al.* (2013) reported a striking 3.4-fold increase in the number of CMs from 1 to 20 years of age. Clarifying the magnitude of heart regeneration is relevant for the development of therapeutic strategies but technically challenging. For instance, in the heart nucleation, both polyploidization and cell volume increase occur in parallel, potentially affecting cell turnover analyses. Moreover, the use of cell division or apoptosis markers not always proves if the process was successfully concluded (Senyo *et al.* 2014). These factors are important variables likely contributing to the different outcomes reported by several authors regarding human CM proliferation. More recently, Bergmann *et al.* (2015) have shown, using both ^{14}C dating and simultaneous detection of membrane and nuclear markers by immune-detection, that in the first decade of life, human hearts are more proliferative producing 36% of all postnatal CMs. After 20 years of age however, CM turnover decreases to less than 1% per year. On the contrary, fibroblasts, smooth muscle cells and ECs proliferate throughout life with ECs being replaced every 6 years, in the adult. Moreover, Wohlschlaeger *et al.* (2009) have studied the impact of reducing the pressure overload effects on HF patients by implanting a left ventricular assist device (LVAD). In humans, most CMs are polyploid but after LVAD, CM DNA content

decreased and the number of diploid CMs increased while polyploid CMs decreased. These results suggest the existence of a population of CMs not yet terminally differentiated and still capable of completing the cell cycle upon stimulus.

Regardless of the percentage of CM that proliferate during our life, it is a given fact that CM proliferation occurs at an extremely low rate and its stimulation, specially upon injury, is still relatively insignificant. Some strategies have been explored to promote endogenous CM proliferation. For example, the inhibition of glycogen synthase kinase-3 beta (GSK3β) or overexpression of E2F transcription factor 4 (E2F4) stimulated CM cycling *in vivo* (Kerkela *et al.* 2008; Ebelt *et al.* 2008). Other strategies like viral-delivery of miR-590-3p and miR-199a-3p have successfully induced CM proliferation in mice after MI (Eulalio *et al.* 2012).

For many years, researchers have focused on CMs proliferation culminating in exceptional advances in our understanding of the mechanisms governing CMs turnover. However, as previously mentioned, the heart is a multicellular organ comprising different cell types and, it is perhaps surprising that these other cell types have received considerable less attention. Therefore, understanding their role in CVDs and their interaction with CMs will be necessary to promote heart function and regeneration.

2.3 *Cardiac endothelium*

CMs represent 75% of the heart volume but are only 25%-30% of total cell number (Pinto *et al.* 2016). ECs represent the majority of cardiac non-myocytes and it is estimated that the ratio of ECs to CMs is 3:1 (Hsieh *et al.* 2006) (**Figure 3**).

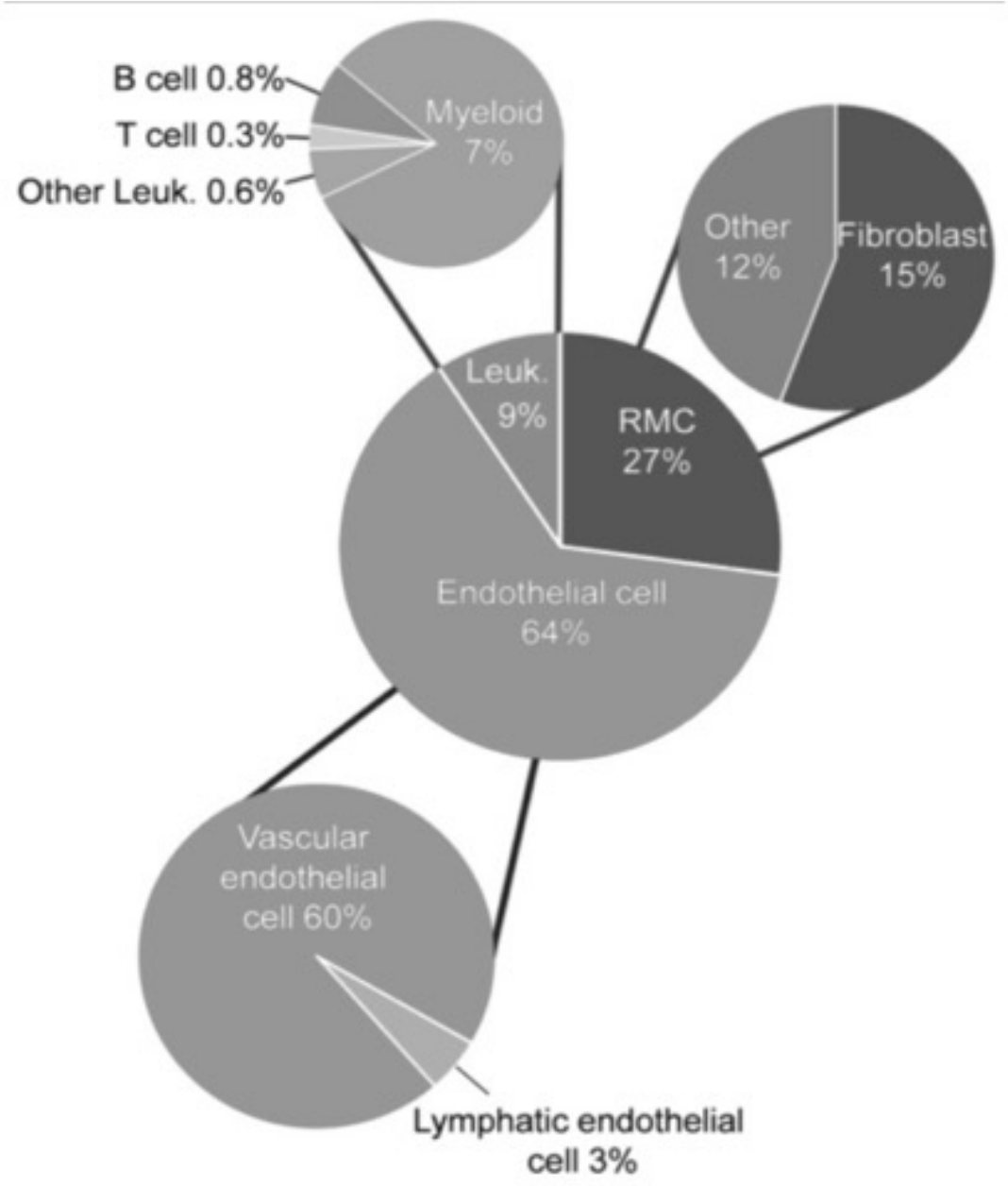


Figure 3. Cardiac non-myocyte cellular composition: ECs represent 64% from which 60% are vascular and 3% lympathic; Leukocytes represent 9% and 27% are resident stem cells (RMC) from which 15% are fibroblasts.
Adapted from: Pinto *et al.* 2016.

Cardiac vascular endothelium, similarly to the other organs of the body, is responsible for the blood flow and for preventing blood coagulation (Coulombe *et al.* 2014). ECs secrete auto- and paracrine molecules influencing growth, metabolism and contractile performance of the heart

(Brutsaert 2003). Moreover, the formation of new blood vessels from pre-existing ones, a process designated as angiogenesis, is an important adaptive mechanism in situations of increased demand. Angiogenesis involves proliferation, sprouting, and migration of ECs, followed by pruning and remodeling of the vascular network (Fish *et al.* 2009). There is a large body of literature describing how low oxygen levels induce neoangiogenesis, a process mediated by the hypoxia signaling pathway. It is noteworthy that low oxygen levels during embryonic development seem to support CM proliferation whereas, upon birth, the increase in oxygen levels seems to abolish that effect. Nakada *et al.* (2017), besides reporting the already mentioned effect of gradual exposure to hypoxia in CMs proliferation, showed a concomitant increase in EC proliferation, suggesting a relation between EC and CM proliferation. Furthermore, Porrello *et al.* (2013) showed that regeneration in neonatal rodent hearts after MI was accompanied by an increase in angiogenesis. It is well recognized that vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis and therefore clinical trials with VEGF were already performed in the cardiovascular context. However, the clinical trials for coronary or peripheral artery disease and myocardial ischemia using plasmid DNA encoding VEGF or human recombinant VEGF (Henry *et al.* 2003) have not shown a clear clinical benefit (Taimeh *et al.* 2013). Many reasons can explain these findings but recent evidence suggest that the pharmacokinetics of VEGF is a key factor regulating neoangiogenesis. Recently, Yang *et al.* (2015) have fused VEGF to a peptide that promotes its homing into the ischemic myocardium after MI. This modified VEGF improved heart function in both rat and pig MI models. In the rat heart, the modified VEGF decreased the infarct size, improved angiogenesis and increased cell survival either upon a single administration or in multi administration regimen comprising a lower dosage. Zangi *et al.* (2013)

developed a synthetic modified RNA encoding VEGF-A capable of improving heart function and enhancing long-term survival of mice after MI. This modified mRNA was more effective than the first-generation DNA based approaches. In fact, AstraZeneca is currently recruiting patients for a Phase I clinical trial using a similar modified mRNA for VEGF-A to initiate a strong, local and transient increase in VEGF-A expression (NCT02935712) which could help overcome challenges associated with previous VEGF plasmid-based approaches (Taimeh *et al.* 2013).

2.4 *Endothelium-Cardiomyocyte Crosstalk*

Endocardium and capillaries ECs are very close to CMs. In fact, capillaries ECs are within 1 μm distance from CMs, ensuring optimal diffusion of nutrients and oxygen between CMs and blood (Brusaert *et al.* 2003). This proximity also facilitates communication between these cell types. ECs and CMs are partners in the heart physio- and pathology. ECs can enhance survival and organization of nearby CMs in vitro (Narmoneva *et al.* 2004) and can also modulate CMs morphology via β1-integrin (Zhang *et al.* 2015). Furthermore, the same authors showed that in a mouse model of MI, co-transplantation of ECs and CMs enhanced cell engraftment within the ischemic border zone, diminished fibrosis and improved cardiac function when compared to transplantation of CMs alone.

Soluble molecules secreted by ECs and CMs modulate their interaction (**Figure 4**). For example, nitric oxide (NO) and neuroregulin-1 are secreted by ECs and act upon CMs (Lim *et al.* 2015). NO has a primordial role in controlling myocardial relaxation and Jaba *et al.* (2013) showed that endothelial NO induced myocardial hypertrophy by promoting PI3K/AKT/mTORC1 pathway activation. In fact,

alterations in NO are present in hypertension, atherosclerosis and HF (Lim *et al.* 2015). In turn, neuregulin-1 plays a very important role during embryonic development, particularly in the development of the cardiac muscle and cardiac conduction systems (Noireaud *et al.* 2014). In the adult heart, neuregulin-1 has a pro-survival effect on CMs via the PI3K/Akt pathway (Kuramochi *et al.* 2004). Recently, a recombinantly expressed portion of human neuregulin 1β2α, with the commercial designation of Neucardin™, has successfully completed Phase II clinical trials in China, Australia and United States of America in HF patients and entered Phase III.

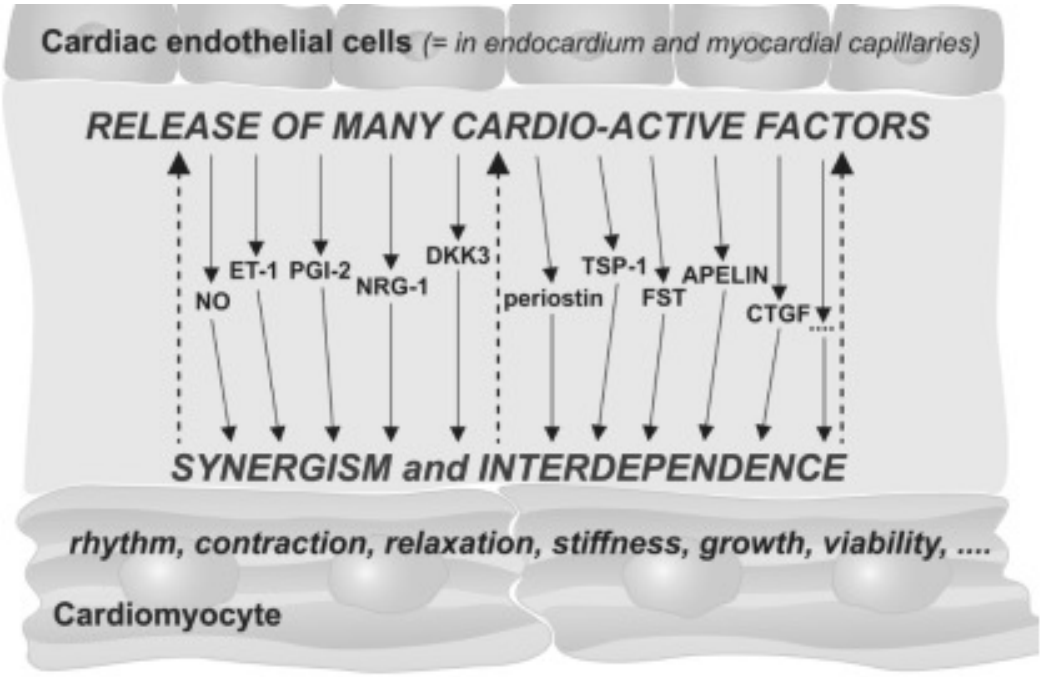


Figure 4. Soluble factors secreted by endocardium ECs and myocardial capillaries that act upon CMs modulating them. From the represented molecules the most well studied are nitric oxide (NO), endothelin-1 (ET-1), prostacyclin (PGI2), neuregulin-1 (NRG1). Other, namely Dickkopf-3 (DKK3), periostin, thrombospondin-1 (TSP-1), follistatin (FST), apelin and connective tissue growth factor (CTGF) have only emerged recently. Each roles are reviewed in (ref)
Adapted from: Lim *et al.* 2015.

3. **Small noncoding RNAs: microRNAs**

3.1 *miRNAs biogenesis and function*

Most of the human genome encodes RNAs that do not code for proteins but can modulate intracellular pathways. Noncoding RNAs include microRNAs (miRNAs), long non-coding RNAs and circular RNAs, amongst others (Matsui *et al.* 2017).

MiRNAs are composed of 18-25 nucleotides capable of inhibiting protein translation by degrading the target mRNA or inhibiting its translation.

Briefly, miRs are initially transcribed in the nucleus by RNA polymerase II in a longer hairpin precursor form capped and polyadenylated, the primary miRNA transcript (pri-miRNA) (**Figure 5**). Pri-miRNA transcript extends both 5' and 3' from the miRNA sequence therefore requiring two processing reactions to trim it into miRNA. Still in the nucleus, the pri-miRNA is processed by a protein complex, the Microprocessor complex, composed of a type III RNAse, Drosha and a protein co-factor, DGCR8. Pri-miRNA 3' and 5' strands are cleaved producing a precursor miRNA (pre-miRNA) which is translocated to the cytoplasm via Exportin 5. Now in the cytoplasm, another RNAse - Dicer - performs the second excision step removing the stem loop from the pre-miRNA thus generating a double stranded miRNA. The miRNA duplex is incorporated into the RNA-inducing silencing complex (RISC) allowing duplex unwinding and the thermodynamically stable association between one of the strands, called the guide strand, and Argonaute effector protein (Ago) (Carthew *et al.* 2009; Bernardo *et al.* 2015; Matsui *et al.* 2017; Rupaimoole *et al.* 2017). Usually the other strand is degraded but it can also go through the same process as the guide strand. The miRNA-bound Ago in association with scaffold proteins is denoted the miRISC

complex which will access the target mRNA. Most of the cases, the miRNA binding site locates in the 3 'UTR of the target mRNA (Carthew *et al.* 2009). Animal miRNAs can bind with mismatches however a perfect Watson-Crick base-pairing complementarity is needed in the seed region, represented by nucleotides 2-8 at the 5' end of miRNAs. Perfect complementarity between mRNA and miRNA will result in target degradation while mismatches will promote target repression without degradation. The mechanisms underlying miRISC regulation of translation are still not clear (Carthew *et al.* 2009). Since full complementarity between miRNA and target mRNA is not necessary, the same miRNA can target several distinct mRNAs. Also, the same mRNA can be targeted by more than one miRNA.

MiRNAs versatility implicates them in virtually all cellular processes making them master regulators of the cell fate.

3.2 *miRNAs in cardiovascular system*

miRNAs and other noncoding RNAs' have been implicated in several physiological and pathological processes, from embryogenesis to cancer to cardiac diseases.

In the cardiovascular context, miRNAs are important regulators of cardiac development and disease (Kwekkeboom *et al.* 2014). For instance, during embryonic development, depletion of cardiac miRNAs led to embryo death while in the adult heart resulted in cardiac hypertrophy, fibrosis and cardiac dysfunction (da Costa Martins *et al.* 2008). Additionally, specific knockout of Dicer in smooth muscle cells led to reduced proliferation and impaired contractility whereas in ECs its deletion impaired post-natal angiogenesis (Albinsson *et al.* 2010; Chen *et al.* 2012; Suarez *et al.* 2008).

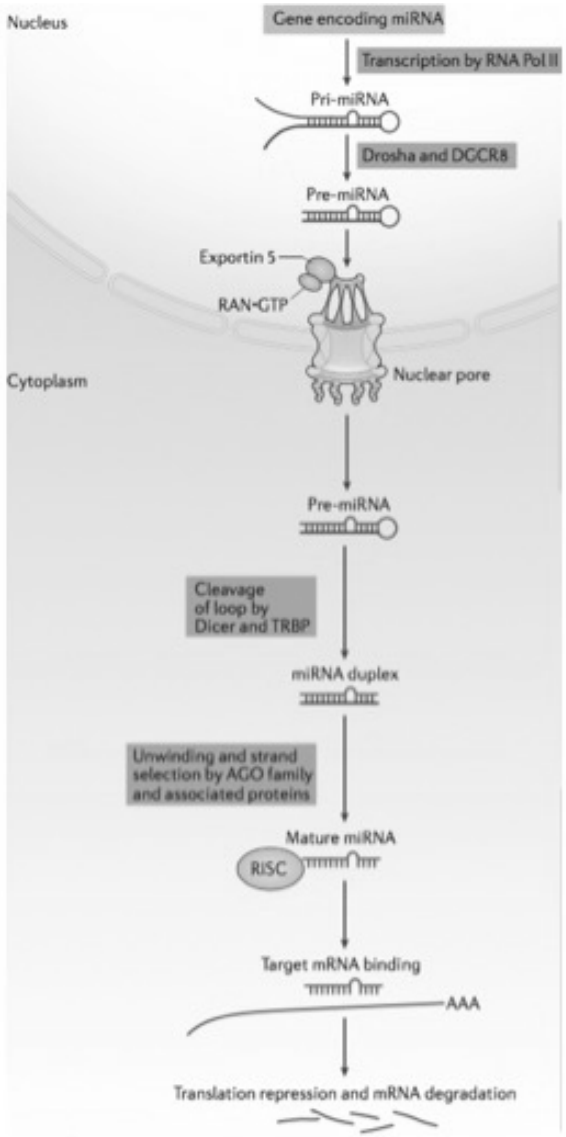


Figure 5. Schematic representation of miRNA biogenesis. In the nucleus, the gene encoding the miRNA is transcribed by RNA polymerase II generating the pri-miRNA. 3' and 5' strands of the pri-miRNA are cleaved by Drosha and cofactor protein DGCR8 resulting in the pre-miRNA. Pre-miRNA is translocated into the cytoplasm via Exportin 5. The terminal loop of the pre-mIRNA is cleaved by Dicer generating a miRNA duplex. This double stranded structure is incorporated in the RISC complex (RNA-induced silencing complex) and Ago (argonaute) family of proteins unwinds and selects the guide strand. The miRNA is now mature allowing full or partial complementarity to its target.
Adapted from: Rupaimoole *et al.* 2017.

Moreover, the involvement of miRNAs in several heart associated processes such as fibrosis or hypertrophy have been described. For example, miR-378 repressed hypertrophy and improved cardiac function in mice after thoracic aortic constriction (Ganesan *et al.* 2013); miR-208a

regulated cardiac contractility in mice (Callis *et al.* 2009); miR-15 family influenced cell cycle regulation and its knockdown in neonatal mice increased mitotic CMs (Porrelo *et al.* 2013).In MI, miR-494 was downregulated in the infarct zone and restoring its levels reduced infarct size (Wang *et al.* 2010); miR-21 was downregulated upon ischemia and has been shown to increase Akt phosphorylation in both isolated CMs and ECs (Weber *et al.* 2010; Tu *et al.* 2013). Moreover, recent findings showed that intramyocardial injection of miR-210 increased angiogenesis and reduced infarct size in mice hearts (Hu *et al.* 2010).

miRNAs are also associated to the endothelium. For instance, miR-126 inhibited vascular ECs apoptosis by targeting the PI3K/Akt pathway and its delivery to a chronic ischemic hindlimb rat model resulted in improved perfusion, vessel density and enhanced arteriolar formation (Cao *et al.* 2015). Based on these evidences, several authors believe that miRNAs will become a therapeutic option and/or important biomarkers in certain CVDs (Lim *et al.* 2015; Keulenaer *et al.* 2017).

3.3 *miRNAs advantages and disadvantages: paving the way for therapeutics*

miRNAs often target different components of the same intracellular signaling cascade. miRNAs therapeutics may perform better than therapeutics with small molecules or proteins due to their capacity of modulating more than one process at a time or by overcoming desensitization of a specific cascade component to a drug (Bernardo *et al.* 2015). However, immune response, off-target effects and specific and efficient delivery remain unresolved challenges in the field. Besides, it is necessary to understand the full miRNA “targetome” before proceeding to therapeutics because

unknown targets may cause unexpected adverse effects (Rupaimoole *et al.* 2017).

There are two therapeutic strategies for miRNA modulation: miRNA inhibition or activation.

miRNA inhibitors attenuate miRNA expression and cellular effects (Bernardo *et al.* 2015). The most commonly used inhibitors are single stranded oligonucleotides complementary to the miRNA. These antisense oligonucleotides are chemically modified to increase stability and resistance to exonucleases or to improve sensitivity. Other inhibition strategy is the use of "blockmirs" which target the miRNA-binding site in the target transcript in the cytoplasm. This strategy enables the blockage of a specific miRNA target while simultaneously allowing the miRNA to regulate its other mRNA targets. One of the limitations of miRNA inhibitors is their transient effect, with repeated administrations being necessary to prolong inhibition. To circumvent this barrier, miRNA sponges were developed. A single miRNA sponge contains multiple miRNA-binding sites which act as competitive inhibitors for miRNA binding. miRNA sponges can be designed to target multiple miRNAs families at once. Using miRNA inhibitors implies that the miRNA is endogenously transcribed in the target cell or tissue. One must keep in mind that endogenous miRNAs can be regulating important pathways, other than the pathway being targeted for inhibition (**Figure 6**).

miRNA activation can be used to restore the levels of a miRNA that has been cell depleted along the disease or to deliver a miRNA to control specific cellular functions. miRNA mimics are used for this purpose. Double stranded nucleotides are preferred over single stranded nucleotides because the duplex structure has been found to facilitate RISC loading and enhance gene silencing efficacy (Bader *et al*. 2011). MiRNA mimics face challenges when administered in vivo because they are degraded by serum nucleases, can

easily be phagocytosed and are cleared by renal excretion. In addition, their hydrophilic nature and negative charge difficult their cellular uptake (Kamps *et al.* 2016).

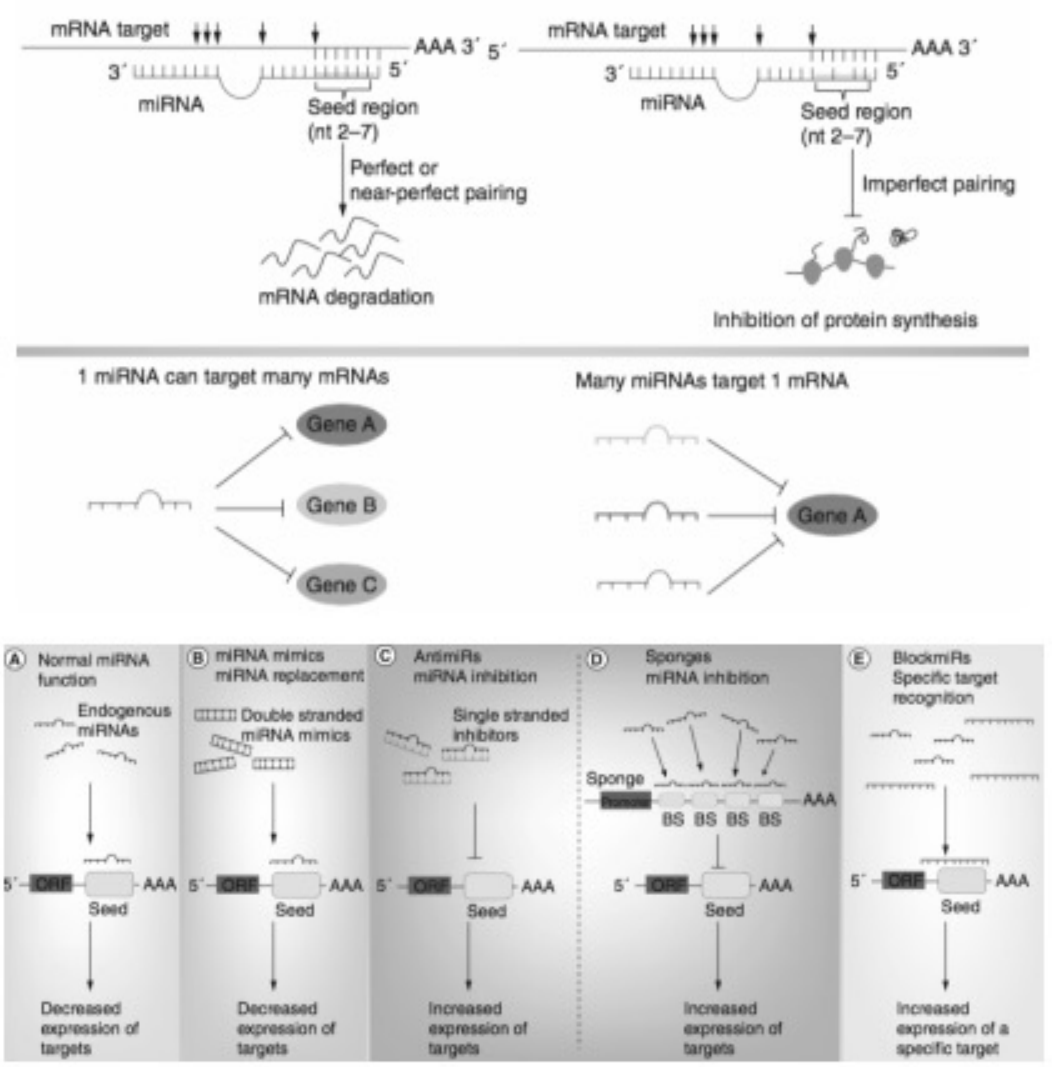


Figure 6. Schematic representation of the possible miRNA degradation mechanisms. Possible interaction between miRNAs and mRNAs. **A)** Mechanism of action between endogenous miRNA and one of its target mRNAs. **B)** miRNA mimics mode of action. **C)** Single stranded miRNA inhibitors mode of action. **D)** miRNA sponges mode of action. **E)** BlockmiRs mode of action.

Adapted from: Bernardo *et al.* 2015

One strategy to overcome this is to chemically modify the miRNA, with the substitution of ribose 2'-OH group for 2'-O-methyl, 2'-O-fluoro or 2'-methoxyethyl the most commonly used, increasing binding affinity and stability (Davis *et al.*

2006). Other possible modification is the conjugation of the miRNA to small transporter domains promoting cell entry and specificity. Rohde *et al.* (2015) used miR-126 conjugated to an aptamer with affinity for the ubiquitously expressed transferrin receptor promoting successful uptake in ECs and cancer cells. Despite improving miRNA stability, modification strategies increase toxicity and can negatively affect AGO function therefore reducing the effect of the miRNA (ref). Furthermore, miRNAs mimics are more difficult to extensively modify than miRNA inhibitors (Bernardo *et al.* 2015).

3.3.1 *Nanocarriers*

Pharmacological effectiveness requires the development of carriers for miRNAs. The ideal vehicle is non-toxic, non-immunogenic and biodegradable (Kamps *et al.* 2016). Furthermore, it needs to be specifically uptaken by the target cell or tissue and, when inside the cell, escape endosomal degradation and, in the case of miRNA delivery, interact with the RISC machinery.

Viral vectors are very efficient in targeted delivery but immunogenicity, the potential to integrate into the host genome and biological variability hinder their clinical use (Desphande *et al.* 2016). Non-viral vectors or nanocarriers, on the other hand, present low immunogenicity and high versatility (Fernandez-Piñeiro *et al.* 2017). Nanocarriers may include: polymeric and inorganic nanoparticles, dendrimers or lipid-based carriers such as liposomes and micelles. Nanoparticles (NPs) can be composed of extremely distinct materials and their high surface-to-volume ratio allows several surface modifications. Their versatility can be explored to modulate cellular uptake, bioavailability, simultaneous delivery of multiple drugs and nucleic acids (Kamps *et al.* 2016). Moreover, nanocarriers can be

developed to respond to stimuli, as pH or light, allowing spatio-temporal control of cargo release (Jhaveri *et al.* 2014).

3.3.1.1 *Nanocarriers for miRNA mimics delivery: focus on PEI polymeric NPs*

Currently, there are many nanocarriers being developed to deliver miRNAs varying in their composition and distinctive characteristics. One of the most promising studied groups of polymers used to deliver nucleic acids, including miRNAs, are cationic polymers because their positive charge allows conjugation to negative charged nucleic acids (Fernandez-Piñeiro *et al.* 2017). Polyethylenimine (PEI) is the most widely used cationic polymer since its high charge density promotes formation of polyplexes with miRNAs. Importantly, it possesses a high transfection efficacy and a strong capacity to promote endosome destabilization with release of nucleic acids into the cytoplasm (Ganju *et al.* 2016; Fernandez-Piñeiro *et al.* 2017). PEI derived particles have been successfully used for miRNA delivery: Chiou *et al.* (2012) delivered miR-145 intratumorally using modified PEI polyplexes and observed a reduction in tumor growth; Chien *et al.* (2015) developed PEI nanoparticles modified with polyurethane that delivered miRNA-122 expression plasmid and increased miR-122 levels in iPSCs enhancing their differentiation into hepatocyte-like cells; Zhang *et al.* (2015) conjugated miR-145 into PEI nanoparticles modified with polyarginine penetrating peptide and delivered intravenously by tail vein injection into a mouse prostate cancer model, inhibiting tumour growth and prolonging survival. However, this nanoformulation also increased miR-145 levels in spleen, liver and kidney, highlighting the drawback of off-target delivery. Recently, Ma *et al.* (2016) developed a delivery system for miRNAs onto ECs. miR-146a and miR-181b were packaged into PEI nanoparticles and then loaded

to microparticles that were coated with E-selectin, a known marker of EC inflammation. This formulation used PEI as a suitable miRNA vehicle and added cell specificity with E-selectin modification. When comparing the full formulation to only PEI nanoparticles with miR-146a and miR-181b, only the full formulation ameliorated endothelial inflammation and atherosclerosis in diseased mice emphasizing the importance of targeted delivery.

3.3.1.2 *miRNAs in clinical trials*

Despite miRNAs mimics based therapies not having entered the clinical trials for cardiovascular diseases, there are trials for cancer, pulmonary fibrosis or hepatitis C virus. In 2013, *Mirna Therapeutics* started a clinical trial using a miR-34 mimic for patients with liver cancer but it has been terminated due to immune related serious adverse events (Rupaimoole *et al.* 2017). A clinical trial for non-small-cell lung cancer using a mimic for miR-16 in a nanoparticle formulation that targets cancer cells with minimum toxicity is currently recruiting. Also, *miRage* is developing a phase I clinical trial with a mimic for miR-29 in cutaneous and pulmonary fibrosis.

For miRNA inhibitors, there are more ongoing clinical trials, possibly due to their potential to be chemically modified. One good example is the use of miR-122 inhibitor. Previous phase IIa clinical trials have shown safety and decrease in hepatitis C virus RNA after administration of miR-122 inhibitor (Janssen *et al.* 2013). *Regulus therapeutics* has now a clinical trial underway for hepatitis C using a modified anti-miR targeting miR-122.

General hypothesis:

Myocardial ischemia results from partial or complete blockage of coronary arteries. The consequent reduction on oxygen and nutrients availability within the area surrounding the blockage leads to massive cell death. Given the poor regenerative potential of cardiomyocytes, upon injury, the lost cardiomyocytes are replaced by non-contractile cells such as fibroblasts which contribute to a reduction in cardiac function. Moreover, a poorly vascularized area remains an important barrier for the regeneration of the heart and therefore, strategies capable of enhancing vascularization are needed. Here we propose a novel strategy consisting of miRNAs capable of enhancing endothelial cell survival in ischemic conditions.

Specific aims:

1) Validate the miRNAs identified as pro-survival miRNAs on a high-throughput screening assay specifically designed to identify miRNAs involved in survival of endothelial cells in ischemia;

2) Analyze the impact of pro-survival microRNAs on endothelial phenotype;

3) Identify the intracellular target genes of pro-survival microRNAs using transcriptomic and proteomic analysis;

4) Analyze the microRNA delivery potential of light-induced polymeric nanoparticles.

Materials and Methods

$CD34^+$ cells isolation from umbilical cord blood

All donors signed an informed consent form in compliance with Portuguese Legislation and the collection was approved by the ethical committee of Hospital Infante D. Pedro. Briefly, mononuclear cells were isolated from umbilical cord blood samples after density gradient separation using Ficoll (Histopaque-1077 Hybrid Max; Sigma Aldrich, St. Louis, USA). $CD34^+$ cells from mononuclear cells were positively selected (twice) using the mini-MACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer´s instructions.

Differentiation into $CD34^+$-derived ECs

Human $CD34^+$-derived endothelial cells (ECs) were derived as previously described (12 do paper). Following isolation, $CD34^+$ cells were seeded onto 1% (w/v) porcine skin type A gelatin-coated plates at $1x10^5$ cells/cm^2 in endothelial growth medium (EGM-2; *Lonza*, Gaithersburg, MD, USA) supplemented with 50 ng/mL vascular endothelial growth factor ($VEGF_{165}$; *PrepoTech Inc.*, Rocky Hill, USA) and 20% (v/v) foetal bovine serum (FBS) at 5% CO_2 and 37ºC. After 15-20 days of culture, the presence of endothelial markers was confirmed by immunocytochemistry and flow cytometry.

Endothelial cell culture

$CD34^+$-derived ECs were seeded onto 1% (w/v) porcine skin type A gelatin-coated plates in EGM-2, at passage four or five and at a final density of approximately 30.000 cells per cm^2. HUAECs and HUVECs were obtained from Lonza and

cultured in EGM-2 media according to the manufacturer´s instructions.

High-content screening (HCS) assay

$CD34^+$-derived ECs were seeded onto 1% (w/v) porcine skin type A gelatin coated 96-well plates at $1x10^4$ cells/well in EGM-2 medium without GA-1000 (Gentamicin, Amphotericin-B) and left to adhere overnight. The next day, cells were transfected with a library of miRNA mimics (Dharmacon miRIDIAN® microRNA Library – Human mimic miRBase version 19.0; 2080 miRNA mimics) according to the manufacturer´s instructions. Briefly, complexes miRNA:Lipofectamine RNAiMAX (50 nM miRNA: 0.3 μL lipofectamine final concentration per well) were prepared in EBM-2 medium, allowed to form during 30 minutes at room temperature and added to the cells. Transfection was allowed to proceed for 48 h upon which medium was replaced by EBM-2 medium containing 1% penicillin/streptomycin and plates were transferred to a hypoxia chamber (0.1% O_2 – from hereafter denominated ischemic conditions) and cultured for further 48 h. Per plate, untreated and lipofectamine-treated cells were used as a control.

The screening was repeated twice using two different pools of $CD34^+$-derived ECs (each pool consisting of a mixture of 5 different individuals). At the end of the experiment, medium was removed and the cell layer was washed twice with 200 μL of PBS and fixed with 4% paraformaldehyde. Next, cells were incubated with 1 μg/mL Hoechst 33342 (*Sigma Aldrich*) for 30 minutes at 37ºC and image acquisition was performed immediately.

Image acquisition and analysis

Image acquisition was performed using an automated high-content imager (InCell Analyzer 2200; *GE Healthcare*) at 20x magnification. A total of 8 randomly selected bright-field and fluorescent (nuclear staining) images were acquired per well and total nuclear count as well as nuclear morphometric quantification was performed using the InCell Investigator (*GE Healthcare*) and/or CellProfiler open-source software using a built-in nuclear segmentation algorithm. A miRNA was identified as a hit if both these conditions were fulfilled: (1) the total number of cells was higher than the mean plus two times the standard deviations of the lipofectamine-treated wells and (2) present on both replicas of the screening.

Confirmation of the miRNAs identified in High-content screening assay

$CD34^+$-derived ECs were seeded and transfected as described for HCS assay. Only the 25 miRNAs identified in HCS as capable of increasing $CD34^+$-derived ECs survival after exposure to ischemic conditions were used. Each miRNA was analyzed in triplicate and lipofectamine-treated cells were used as control. Image acquisition and analysis was performed as mentioned above. The 15 identified miRNAs are denominated as pro-survival miRNAs from hereafter.

Survival assay

$CD34^+$-derived ECs, HUVECs and HUAECs were plated as described above and three miRNA concentrations (10, 25 and 50 nM) were tested in triplicate for their capacity to

enhance cell survival in ischemic conditions. To account for differences in cell number before exposure to ischemic conditions, we performed a nuclear staining prior to transfer the plates to the hypoxia chamber and that value was used to calculate the cell survival percentage (**Figure 7**). Image acquisition and analysis was performed as described. Survival percentage is calculated, per well, according to:

$$\%\, survival = \frac{cell\ number\ after\ ischemic\ conditions}{average\ cell\ number\ in\ normoxia} \times 100$$

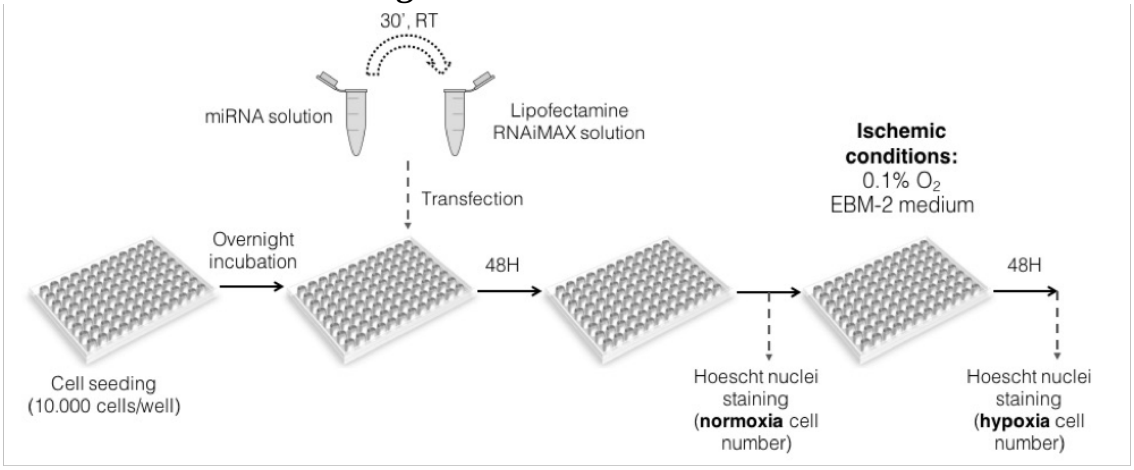


Figure 7. Experimental layout of survival assay using lipofectamine RNAiMAX to transfect miRNA into CD34⁺-derived ECs, HUVECs or HUAECs.

Immunocytochemistry

For immunocytochemistry (ICC), CD34$^+$-derived ECs were transfected with 25nM miRNA as described above for the survival assay. After 48 hours of transfection, cells were fixed with 4% paraformaldehyde and ICC staining was performed according to manufacturer's instructions. Briefly, VE-cadherin staining was performed after fixation, permeabilization with 0.1% TritonX-100 and blockage with 3% bovine serum albumin (BSA) for 1 hour. Next, cells were incubated for 2 h at room temperature with the primary antibody for VE-Cadherin (1:250; sc-9989, *Santa Cruz Biotechnology, Inc*), washed with PBS and incubated with

the secondary antibody (1:500; AlexaFluor 555 goat anti-mouse) for 1 hour at room temperature. Nuclei were stained with 1 *µ*g/mL Hoechst 33342 (*Invitrogen*) and images were acquired using the high-content imager as described above.

RNA isolation and quantitative real-time PCR for PTEN

Total RNA from $CD34^+$-derived ECs was extracted using the RNeasy kit (*Qiagen*) according to the manufacturer´s instructions. For the quantification of gene expression, total RNA was reverse transcribed. qRT-PCR for the putative miRNA target PTEN was performed using POWER SYBR Green PCR Master Mix (*Applied Biosystems*) and the detection was carried out in a 7500 Fast Real-time PCR System (*Applied Biosystems*). The housekeeping gene ß-actin was used for normalization and fold changes were determined using the $2^{\Delta\Delta Ct}$ method. Reverse primer sequence for PTEN used was GTTACTCCCTTTTTGTCTCTG and forward primer sequence was GGCTAAGTGAAGATGACAATC.

Angiogenesis assay

$CD34^+$-derived ECs were seeded onto 1% (w/v) porcine skin type A gelatin coated 6-well plates at $2x10^5$ cells/well in EMG-2 medium without GA-1000 and left to adhere overnight. Transfection was performed as described above using a ratio miRNA:Lipofectamine RNAiMAX of 25 nM miRNA: 6 *µ*L lipofectamine final concentration per well. Transfection was allowed to proceed for 48 h after which cells were harvested with tripLE express, counted and $1x10^4$ cells were seeded onto Ibidi *µ*-slides pre-coated with 10 *µ*L/well of Matrigel in 50 *µ*L EBM-2 medium containing 1%

(v/v) g according to the manufacturer´s instructions. For the positive control 50 ng/mL of $VEGF_{165}$ (PrepoTech Inc., Rocky Hill, USA) was added to the medium. The assay was performed in normoxia as well as in hypoxia (0.1% O_2) and after 4 h a bright-field image covering the area of the well was acquired as described above. Four replicas per experimental conditions were performed and the number of capillaries was determined by manually counting the number of completed formed capillary-like tubes.

Migration assay

$CD34^+$-derived ECs were transfected with 25 nM miRNA as described above for the HCS assay. After 48 h of transfection, cells were starved in EBM-2 medium. Next, wounds were created directly onto the 96-well plate by scratching the surface with a 200 μL pipette tip (Neubauer). Following, the cell layer was washed with EBM-2 to remove un-attached cells and EBM-2 medium containing 1% (v/v) GA-1000 was added to the wells. Medium containing 50 ng/mL of $VEGF_{165}$ (PrepoTech Inc., Rocky Hill, USA) was used as positive control. The assay was performed in normoxia as well as in hypoxia (0.1% O_2) and after 24 h one bright-field image was acquired as described above. Eight replicas per experimental conditions were performed and the percentage of wound area was determined according to:

$$\frac{wound\ size\ at\ t = 24\ h}{wound\ size\ at\ t = 0\ h} \times 100$$

Bioinformatics analysis

To identify the potential targets of each pro-survival miRNAs both experimentally validated and *in silico*-predicted gene-targets for each miRNA were retrieved from miRNA-gene target interaction databases and united in a single set.

Experimentally validated miRNA-target interactions were retrieved from *miRTarbase* (http://mirtarbase.mbc.nctu.edu.tw/) while computationally predicted targets were retrieved 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. In parallel, the same strategy was applied to the remaining 2065 miRNAs which did not increase $CD34^+$-ECs survival.

With both these data, we ranked each individual target according to a "pro-survival score". For a specific target, the pro-survival score is the ratio between the absolute frequency in the pro-survival target gene set and the absolute frequency in the non survival target gene set. This score was developed and applied assuming that a target involved in $CD34^+$-ECs survival will be enriched in the pro-survival miRNAs and not in the non-survival miRNAs. "Survival score" was then used to rank the target genes of miR-C and miR-G and the first 500 genes were selected for the subsequent analyses (**Figure 8**).

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 the 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 hypothesis testing using the Bonferroni correction. Pathways for which adjusted p-value is below 0.05 were identified for each miRNA.

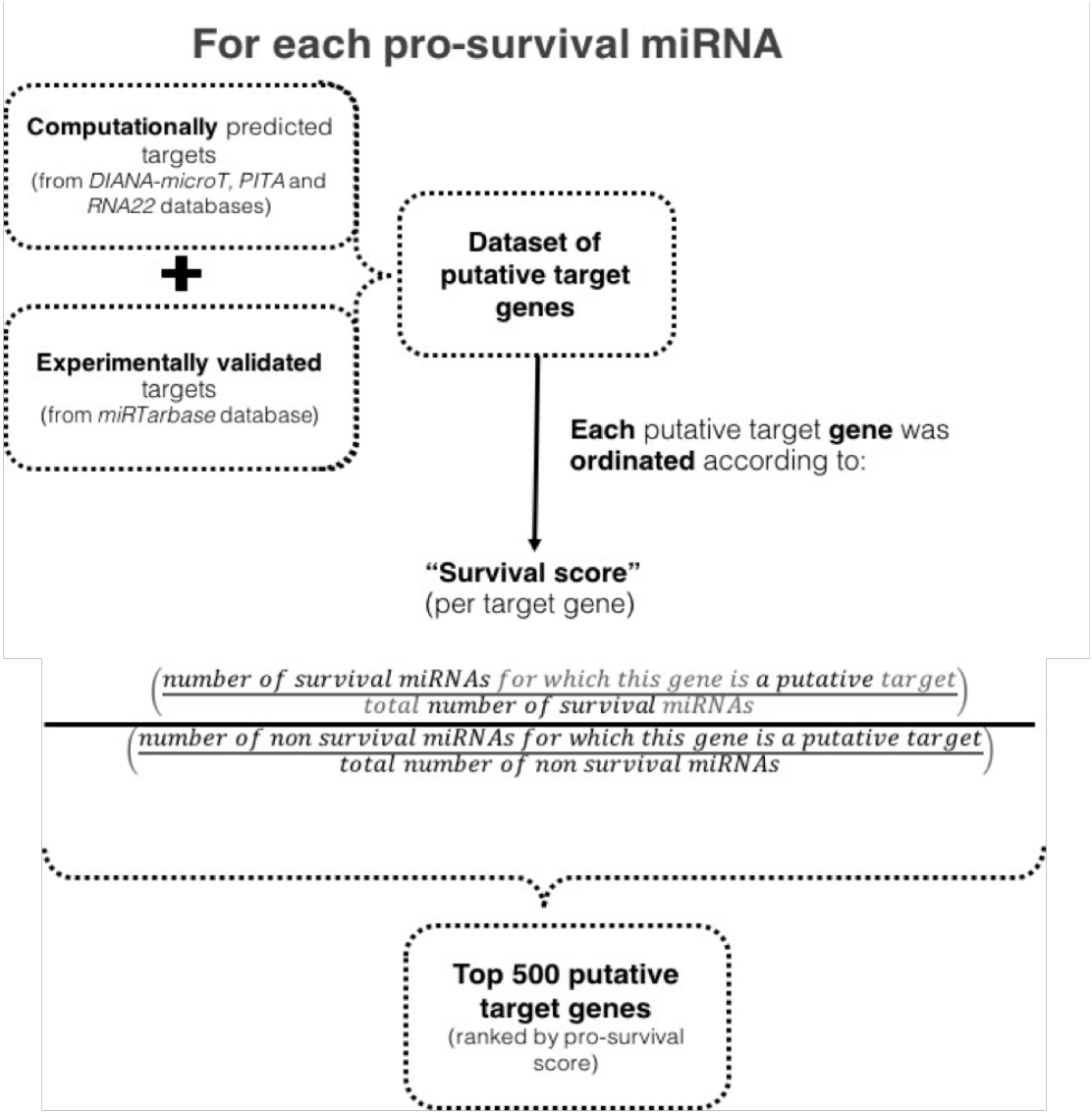


Figure 8. Schematic representation of the bioinformatic approach. For each of the 2080 miRNAs, a list of all putative gene targets was generated according to the referred databases. Then, each individual target gene of each list was assigned with a "survival score". This score consisted in the ratio between: the number of pro-survival miRNAs which had this gene as a predicted target dividing by the 15 survival miRNAs and the number of non-survival miRNAs which had this gene as a predicted target diving by the 2065 non-survival miRNAs. Higher survival scores are most likely attributed to genes involved in cell survival pathways and not involved in other non-survival pathways. The analysis was pursued with the top 500 target genes ranked according to their survival score for miR-C and for miR-G.

Luciferase reporter assay

Possible PTEN mRNA seed sequences for miR-C and miR-G were predicted by TargetScanHuman 7.0 software.

A plasmid containing the PTEN 3'UTR with the predicted seed sequences for both miRNAs and luciferase assay reagents were purchased from *Active Motif*. HeLa cells were seeded into 96-well plates at 7.500 cells/well in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS. The following day, cells were co-transfected with 12,5 ng 3'UTR PTEN plasmid and 25 nM miRNA using Lipofectamine 2000 (ratio of 1:1:2 per well, respectively). After 24 h, medium was exchanged for DMEM supplemented with 10% FBS and the luciferase assay was performed according to manufacturer's instructions. Luciferase signal was measured using a luminometer.

Western blotting

$CD34^+$-derived ECs were seeded onto 1% (w/v) porcine skin type A gelatin coated 6-well plates at $1.5x10^5$ cells/well in EMG-2 medium without GA-1000 and left to adhere overnight. Transfection was performed as described above and was allowed to proceed for 48 h after which medium was removed, cells were washed with room temperature PBS and lysed using 150 *µ*l RIPA buffer supplemented with Protease/Phosphatase Inhibitor Cocktail (#5872, *Cell Signaling*). Cells were lysed using a cell scraper and total lysates were centrifuged at 14 000 RCF for 15 minutes at 4ºC. Supernatant was transferred to pre-cooled tubes and immediately stored at -80ºC. Protein quantification was performed using Pierce™ BCA Protein Assay Kit (#23225, *ThermoFisher Scientific*) according to manufacturer´s instructions. Protein extracts (20 - 30 µg) were resolved in 8%-10% SDS-PAGE gels and transferred into PDVF membranes for 1 h30 min at 100 volts. Membranes were

blocked in 5% (w/v) BSA in TBST (0.2%) and probed against PTEN (PTEN, rabbit mAB; #9559 *Cell Signaling Technology*); Akt (Akt, rabbit mAB; #4691 *Cell Signaling Technology*); phospo-AKT (Phospho-Akt (Ser473) rabbit mAB, #4060 *Cell Signaling Technology*); Erk (p44/42 MAPK (Erk1/2) rabbit mAB; #4695 *Cell Signaling Technology*); phospho-Erk (Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), rabbit mAB, #4376 *Cell Signaling Technology*); FAK (Anti-FAK, #ab40794 *Abcam*); phospho-FAK (Phospho-FAK (Tyr397) rabbit antibody; #3283 *Cell Signaling Technology*) and VE-cadherin (VE-cadherin, mouse mAB, #sc-8999 *Santa Cruz Biotecchnology*) at a final dilution of 1:1000, overnight at 4°C. Membranes were then incubated with secondary peroxidase-conjugated antibodies (1:10 000) for 1 h at room temperature. The specific detection of probed proteins was achieved by a chemiluminescence reaction using WesternBright Quantum HRP substrate (*Advansta*).

RNA sequencing

$CD34^+$-derived ECs were transfected as described above. In brief, $CD34^+$-derived ECs were plated and transfected using a ratio miRNA:Lipofectamine RNAiMAX of 25 nM miRNA: 6 μL lipofectamine final concentration per well. Transfection was allowed to proceed for 48 h after which and total RNA was isolated using the RNeasy kit (*Qiagen*) according to the manufacturer´s instructions. RNA sequencing was performed by BGI, Hong Kong. After quality control check, BGI reported that oligo (dT) magnetic beads were used to select mRNA with polyA tail. The targetRNA was obtained after purification. The targetRNA frangment was reverse transcribed to double-strand cDNA (dscDNA). PCR was perfomed using two specific primers to amplify the ligation product. When finished, the PCR product was by

denatured by heat and the single strand DNA was cyclized by splintoligo and DNA ligase. At last, sequencing was performed on prepared library using the DNA probe.

Nanoparticles preparation and characterization

NPs were prepared as described by Boto et al. (2017) with small modifications. Briefly, PEI was derivatized with 4,5-dimethoxy-2-nitrobenzyl chloroformate (DMNC). PEI-DMNC was mixed with DMSO in aqueous solution and stirred for 15 minutes at room temperature. Dextran sulfate (DS) was added to this suspension to form NPs. To stabilize the NP suspension, zinc sulfate ($ZnSO_4$) was added and stirred for 45 minutes at room temperature. Then, NPs suspension was centrifuged at 400 RCF for 5 minutes to remove large aggregates. The supernatant was collected and centrifuged at 8 000 RCF for 10 minutes. Finally, NPs pellet was ressuspended in molecular grade H_2O and stored at 4 °C. Particle size and surface charge (zeta potential) were determined using dynamic light scattering via a Zeta PALS zeta potential analyzer and ZetaPlus Particle Sizing Software, v. 2.27 (*Brookhaven Instruments Corporation*), respectively.

Nanoparticles conjugation to miRNA

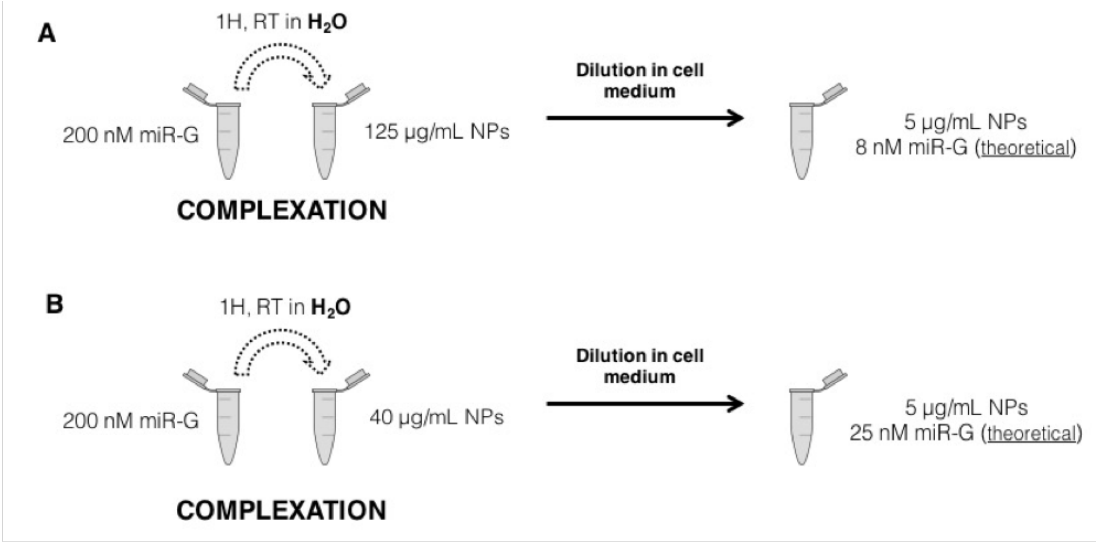
NPs were conjugated with 200 nM miRNA in molecular grade water for 1 h, at room temperature with mild agitation. NPs conjugated to miRNAs were centrifuged at 8 000 RCF for 10 min. Supernatant was collected and stained with SYBR Gold nucleic acid stain (*Invitrogen*) according to manufacturer's instructions. SYBR Gold is a very sensitive dye capable of binding to double- and single-stranded oligonucleotides. After 10 minutes of incubation, SYBR Gold was excited at 495 nm and emission measured at 537 nm.

miRNA complexation to NPs was measured by interpolation to a standard curve with defined concentrations of miRNA in NPs supernatant. Complexation rate was calculated according to:

$$\%\ \text{complexation} = 100 - \frac{\text{conjugated NPs supernatant} \times 100}{200}$$

Cell treatment with NPs conjugated to miRNA

CD34^{+}-derived ECs were seeded as described for the survival assay. In the following day, NPs were conjugated with miR-G in filtered molecular grade water for 1 h, at room temperature with mild agitation, just prior to transfection. Conjugation conditions were: 125 μg/mL NPs and 200 nM miR-G or 40 μg/mL NPs and 200 nM miR-G. NPs were diluted in EGM-2 medium without GA-1000 to a final concentration of 5 μg/mL NPs. Transfection was allowed to proceed for 4 h, after which the medium was removed and the cells were washed three times with EGM-2 medium. Cells were then irradiated by a blue laser (405 nm, 80 mW) for a total of 3 minutes. The following day (24 h after transfection) cells were placed under ischemic conditions for 48 h. Cell survival was measured as described for the survival assay: two wells were used to calculate the cell number in normoxia and three wells for calculate the cell number after ischemia (**Figure 9**). All plates tested included a control consisting of lipofectamine-treated wells transfected with 25 nM miR-G as mentioned for the survival assay.



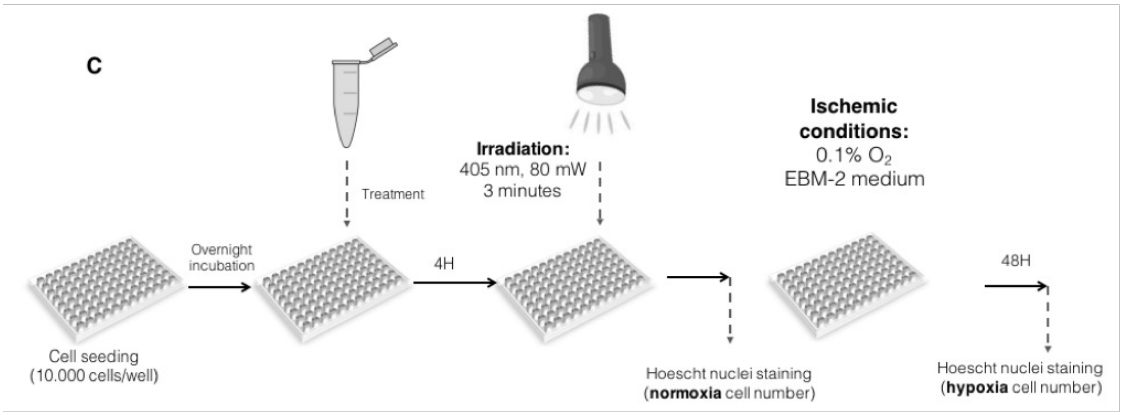


Figure 9. Experimental layout of: **A)** Complexation condition of 125 µg/mL NPs and 200 nM miRNA and final concentration in the well of 5 µg/mL NPs and 8 nM miRNA (theoretical). **B)** Complexation condition of 40 µg/mL NPs and 200 nM miRNA and final concentration in the well of 5 µg/mL NPs and 25 nM miRNA (theoretical). **C)** survival assay using NPs to deliver miRNA into $CD34^+$-derived ECs.

Statistical analysis

Statistical testing was performed using GraphPad® Prism 6.0 software. Results are presented as mean ± SD and were compared using ordinary one-way ANOVA followed by Tukey post-hoc test or unpaired t test. A probability value <0.05 was considered statistically significant.

Results

1. **Identification of "pro-survival" miRNAs**

A library of 2080 human miRNA mimics was screened for their capacity to enhance survival of $CD34^+$-derived ECs in ischemic conditions (EBM-2 and 0.1% O_2). Twenty-five miRNAs were identified as pro-survival miRNAs based on the fact that the total number of cells after 48 h exposure to ischemic conditions was higher than the mean plus two times the standard deviations of the lipofectamine-treated wells (**Supplementary Figure 1**). To validate the miRNAs identified in the primary screening, a counter screening was performed. This secondary screening was performed using the same experimental conditions as for the primary screening but now with three replicates for each miRNA. Fifteen miRNAs were confirmed as capable of significantly increase $CD34^+$-derived ECs survival under ischemic conditions and termed pro-survival miRNAs (**Figure 10**). From the validated pro-survival miRNAs, seven (blue bars) were already involved in cell survival related processes according to the literature. Pink bars correspond to miRNAs not described either in the cardiovascular context or in cell survival. "Pro-survival miR-C" and "pro-survival miR-G", from now referred to as miR-C and miR-G, were selected to proceed with the present work. Mir-C was selected due to its already described role in cell survival. As for miR-G, the selection criterion was based on novelty since there are no reports of miR-G in the human context.

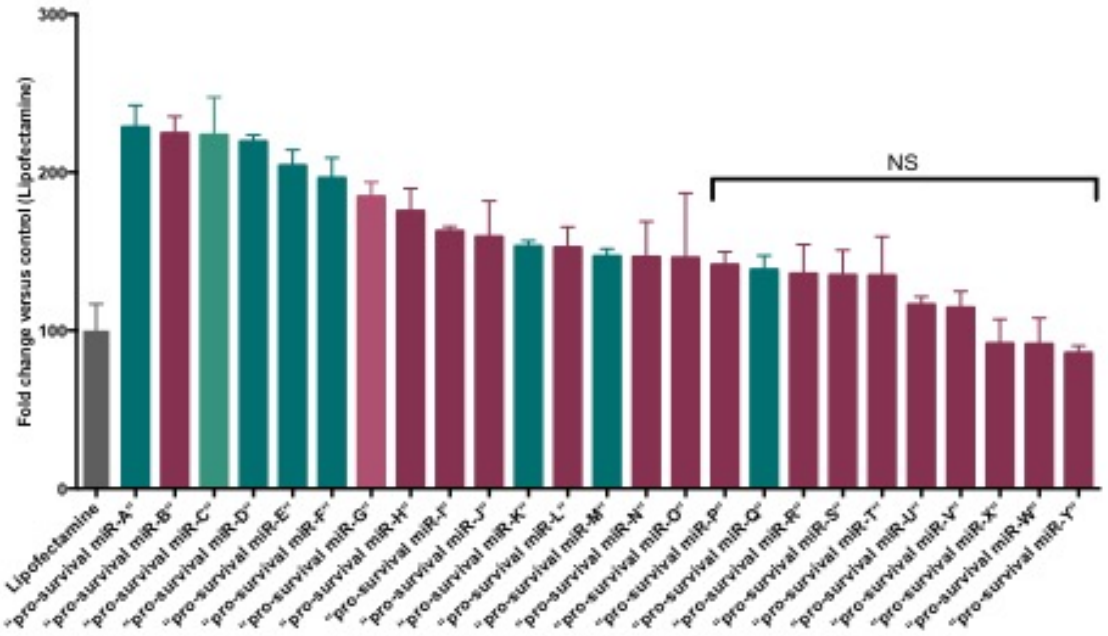


Figure 10. 15 miRNAs are confirmed as pro-survival from the 25 identified in the HCS. CD34⁺-derived ECs were transfected with 50 nM of all the represented miRNAs for 48 h. After ischemic treatment, the total number of cells was measured by Hoescht nuclei staining and compared to lipofectamine-treated wells. Ordinary one-way ANOVA followed by Tukey post-hoc test was used to determine statistical significance. Results are presented as mean ± SD. Blue bars correspond to miRNAs with an already known role either in the cardiovascular system and/or in cell survival. Pink bars correspond to novel microRNAs.

Next, the concentration of miRNA necessary to elicit a pro-survival effect was determined and subsequently the survival results obtained in $CD34^+$-derived ECs were validated in in two well accepted endothelial cell models – HUVECs and HUAECs. The results showed that for both miRNAs a concentration of 25 nM led to a statistically significant increase in the total number of cells after exposure to ischemic conditions regardless of the endothelial cell type used (**Figure 11**). For that, we used 25 nM from henceforward.

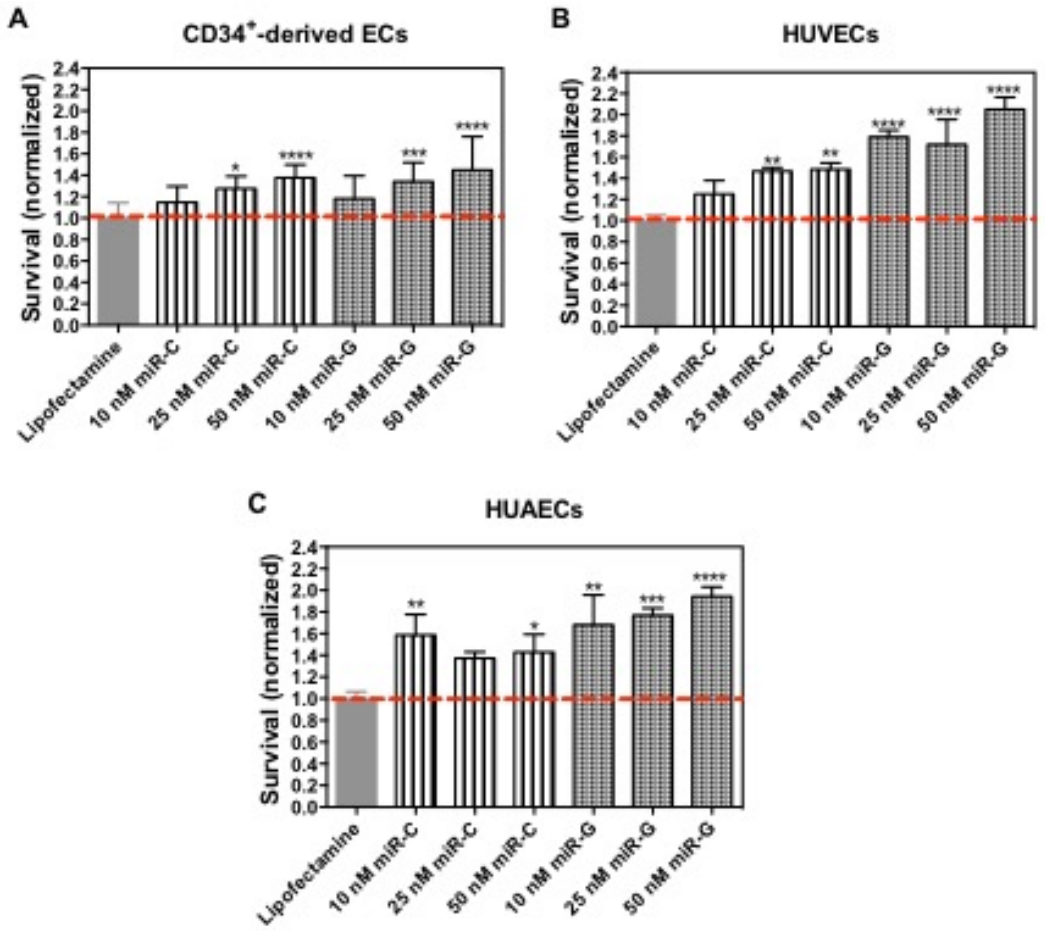


Figure 11. miR-C and miR-G enhance survival of endothelial cells. miR-C and miR-G identified in the primary screening were re-tested in triplicate using three different concentrations (10, 25 and 50 nM) and three different endothelial cell types (CD34+-derived ECs, HUAECs and HUVECs). CD34+-derived ECs were seeded onto 96 well plates in EGM-2 medium without GA-1000 and the next day transfected with the respective microRNAs for 48 h. Subsequently, cells were cultured for further 48 h in EBM-2 medium under 0.1 % O_2. Hoescht nuclear staining was performed before and after ischemia and images acquired using a high-content microscope (at least 8 fields per well were used for quantification) to calculate cell survival. Lipofectamine-treated cells were used as a control and survival was calculated relative to control. Results are presented as mean ± SD. Statistical significance was obtained using one-way ANOVA followed by Tukey´s multiple comparison test, *P<0.05, ** P<0.01, *** P<0.005, ****P<0.001.

2. **Pro-survival miRNAs and endothelial cell function**

To analyze if the miRNAs could alter endothelial phenotype, the expression of the endothelial marker VE-cadherin was evaluated upon transfection with the selected

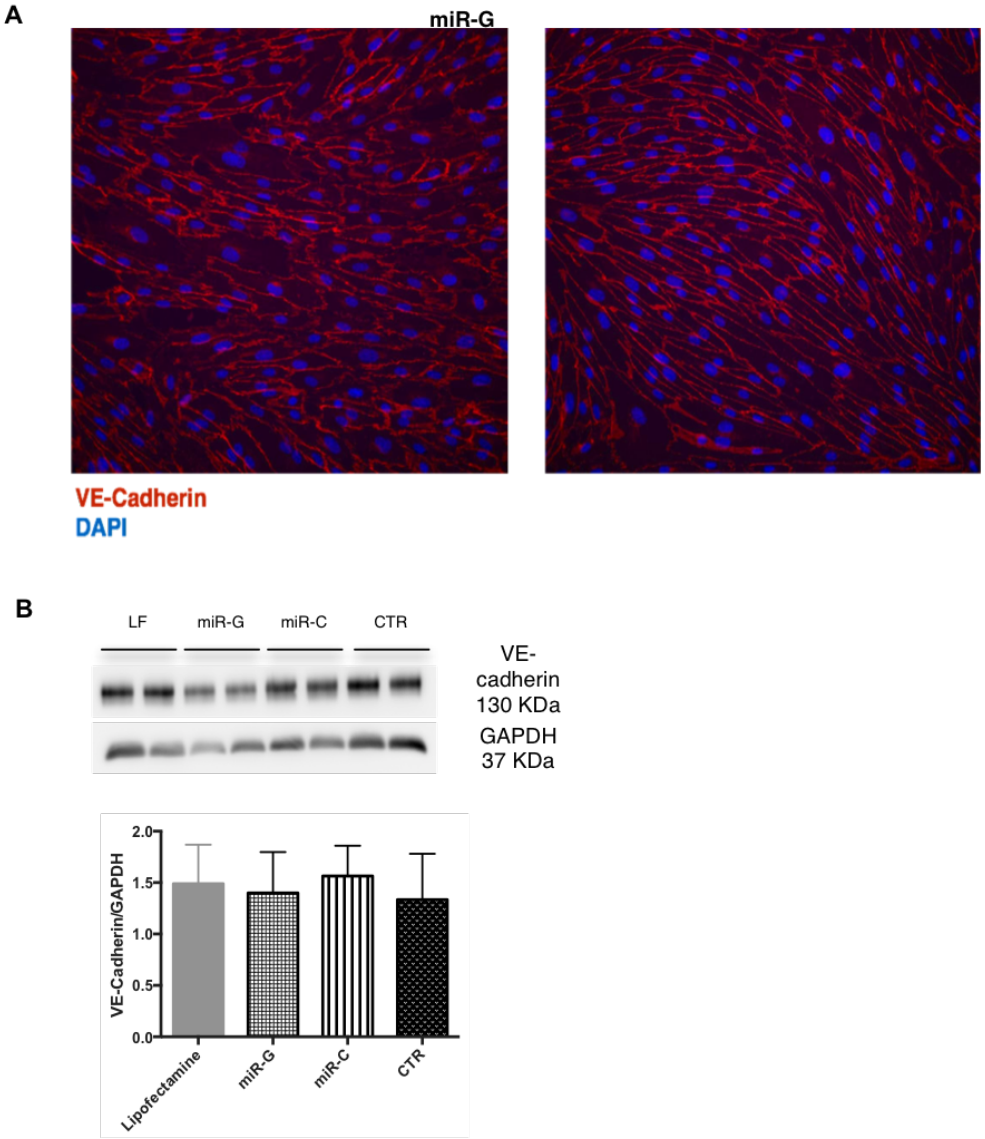


Figure 12. CD34⁺-ECs transfected with miR-C and miR-G maintain the location and expression of the classical EC marker VE-cadherin. A) CD34⁺-derived ECs were transfected as described above with both microRNAs and, upon fixation, immunocytochemistry was performed for VE-cadherin (red). Nuclei was stained with Hoescht (blue). **B)** CD34⁺-derived ECs were transfected with 25 nM of miR-C and miR-G. Total protein was extracted and blotted against VE-Cadherin. GAPDH was used as loading control. Bars represent mean±SD, n=2. Statistical significance was obtained using one-way ANOVA followed by Tukey´s multiple comparison test, *P<0.05, ** P<0.01, *** P<0.005, ****P<0.001.

miRNAs. VE-cadherin is a strictly endothelial specific adhesion molecule located at junctions between endothelial cells. Compared with the control, VE-cadherin expression levels were maintained and, as expected, localized at the cell membrane (**Figure 12**).

Additionally, the effect of both pro-survival miRNAs on EC cell function was analyzed. To that end, a migration and an angiogenesis assay were performed on ECs transfected with miR-C and miR-G and cell migration and tube formation were quantified after 24 h and 4 h, respectively.

Our results showed that both miRNAs were capable of accelerating $CD34^+$-derived ECs migration regardless of the culture conditions, hypoxia or normoxia (**Figure 13**). VEGF was used as a positive control in all the experiments and, as expected, given its pro-migratory effect on ECs, there was a statistically significant reduction in wound area upon exposure of ECs to 50 ng/mL VEGF. Our results also showed that for miR-C a statistically significant increase in cell migration was only observed for $CD34^+$-derived ECs (**Figure 13A**) but not for HUVECs (**Figure 13B**) and HUAECs (**Figure 13C**). The most pronounced effect was observed for the miR-G: regardless of the culture conditions and the ECs sub-phenotype, ECs transfected with miR-G displayed a statistically significant increase in cell migration compared with both controls (lipofectamine-treated and VEGF-treated cells) as well as miR-C treated cells.

Regarding angiogenesis, our results showed that neither lipofectamine-treated cells nor miR-C nor miR-G led to an increase in the number of tube-like structures compared with the positive control consisting of 50 ng/mL VEGF-treated ECs (**Figure 14**). In fact, regardless of the cell type or culture conditions tested (normoxia or hypoxia) there was a statistically significant reduction in the number of tube-like structures upon treatment of ECs with lipofectamine as well as miR-C and miR-G treated $CD34^+$-derived ECs (**Figure**

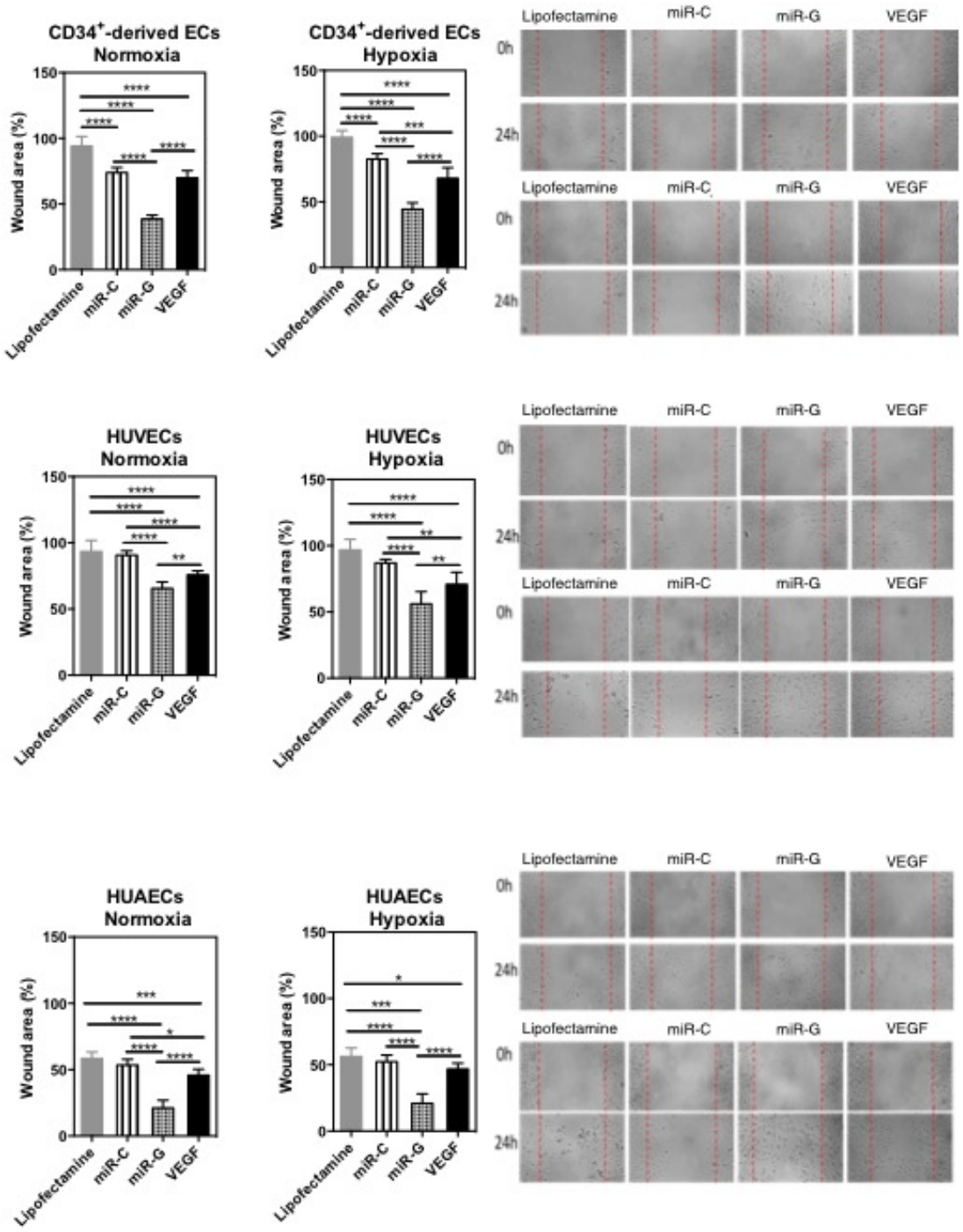


Figure 13. miR-C and miR-G enhance EC migration. CD34+-derived ECs (**A**), HUVECs (**B**) and HUAECs (**C**) were transfected with both microRNAs for 48h and subsequently starved in EBM-2 medium. A wound was created directly onto the 96-well plate, the cell layer was washed with EBM-2 to remove un-attached cells and EBM-2 medium containing 1% (v/v) GA-1000 was added to the wells. Medium containing 50 ng/mL of VEGF-165 was used as positive control. The assay was performed in normoxia as well as in hypoxia (0.1% O2) and after 24h one bright-field image was acquired. Eight replicas per experimental conditions were performed and the percentage of wound area was determined using the following formula: (wound size after 24h/wound size at 0h) x 100. Results are presented as mean±SD. Statistical significance was obtained using one-way ANOVA followed by Tukey´s multiple comparison test whereas * P<0.05, ** P<0.01, ** P<0.005, *** P<0.001 and **** P<0.0001.

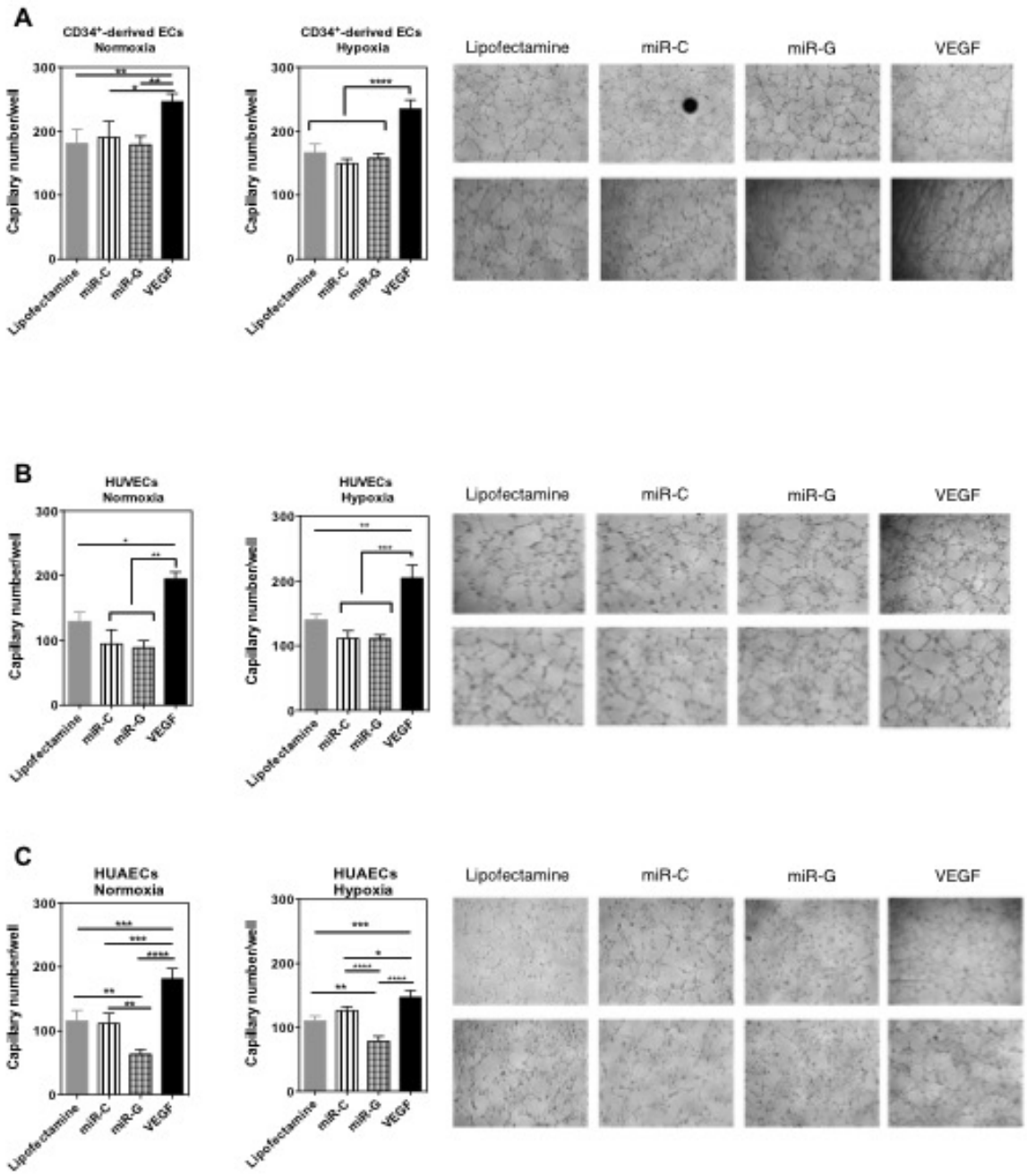


Figure 14. miR-C and miR-G do not enhance the formation of capillaries in ECs. CD34⁺-derived ECs (**A**), HUVECs (**B**) and HUAECs (**C**) were transfected with both microRNAs for 48h and subsequently harvested with tripLE express, counted and 1x10^4 cells seeded onto Ibidi μ-slides pre-coated with 10 μL/well of Matrigel in 50 μL EBM-2 medium containing 1% (v/v) gentamycin. Positive control consisted of cells treated with 50 ng/mL of VEGF-165. The assay was performed in normoxia and hypoxia (0.1% O2) and after 4h a bright-field image covering the area of the well was acquired. Four replicas per experimental conditions were performed and the number of capillaries was determined by manually counting the number of completed formed capillary-like tubes. Results are presented as mean±SD. Statistical significance was obtained using one-way ANOVA followed by Tukey´s multiple comparison test whereas * P<0.05, ** P<0.01, ** P<0.005, *** P<0.001 and **** P<0.0001.

14A), HUVECs (**Figure 14B**) and HUAECs (**Figure 14C**) when compared to VEGF treatment. Interestingly, miR-G behaved similarly to miR-C treated $CD34^+$-derived ECs **(Figure 14A**) and HUVECs (**Figure 14B**) but not HUAECs (**Figure 14C**). For HUAECs, miR-G led to a statistically significant reduction in the number of tube-like structures compared to lipofectamine-treated cells and miR-G treated cells. It is important to caveat that the main goal of these assays was to clarify if the migration and tube formation potential was not abolished.

3. **Pro-survival miRNAs target PTEN and modulate PI3K/Akt and MAPK signaling pathways**

The next step was to identify which are the intracellular targets of the miRNAs and how they can modulate cell survival signaling pathways. With this purpose, we used a computational approach to predict miRNA target genes. Databases such as TargetScan, miRWalk and DIANA-TarBase have their own algorithms to predict putative targets generally based in the energy association between miRNA and target or can collect information from experimentally validated interactions. We have developed a strategy to predict the miRNAs targets that could explain cell survival. For that, we implemented a pro-survival score that, for each putative target gene, reflects the probable interaction only with the miRNAs that increase cell survival.

According to the bioinformatics analysis, the only enriched pathway common to miR-C and miR-G is “Valine, leucine, isoleucine degradation” (**Figure 15**). However, while analyzing the individual list of top 500 predicted target genes for miR-C and miR-G, PTEN (phosphatase and tensin homolog) was a predicted target for both the miRNAs. Based on the survival score described above, PTEN had a pro-survival score of 457 for mir-G and 273 for miR-C. Due to its very well documented role in the literature as a master player

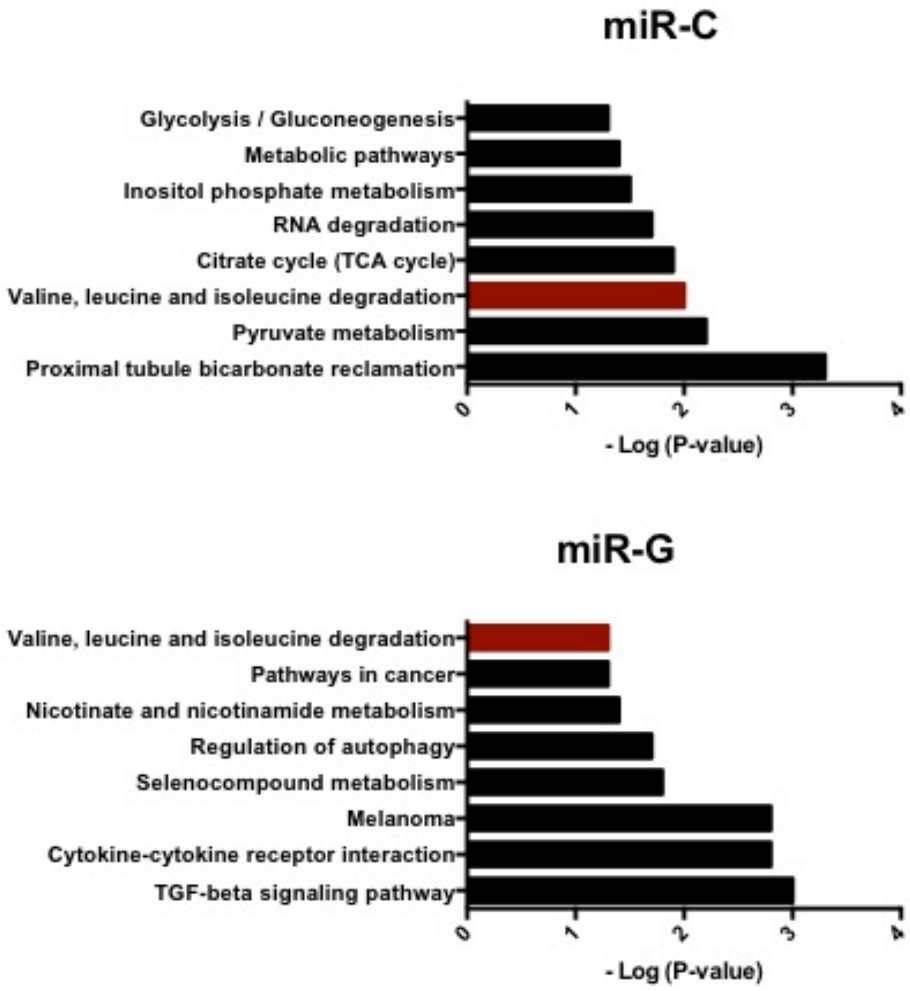


Figure 15. Bioinformatics analysis for miR-C and miR-G. Pathways enriched for miR-C and miR-G from which "Valine, leucine and isoleucine degradation" is the only one in common.

in cell survival we chose to confirm if PTEN was, in fact, a direct target for both miRNAs. TargetScan was used to evaluate the predicted pairing of target region with both miRNAs indicating a potential strong interaction between miR-C and miR-G and the 3´UTR from PTEN mRNA.

We used a luciferase reporter system assay to confirm if miR-C and miR-G directly interact with PTEN. In a luciferase reporter assay, a plasmid containing: the target mRNA sequence with the predicted nucleotide sequence of interaction; and the mRNA codifying luciferase is co-transfected with the miRNA and upon luciferase substrate addition, the resulting signal is detected in a luminometer.

When there is interaction between the miRNA and the plasmid mRNA, the luciferase intensity decreases as a reflection of the changes in the stability and/or translation efficiency of the luciferase transcript. We used a plasmid containing the 3'UTR of PTEN downstream to the mRNA that codifies the luciferase. Co-transfection of the plasmid with 25 nM of miR-C, miR-G and miR-26a-5p (positive control) led to a significant decrease in luciferase signal, confirming a direct interaction between PTEN and the miRNAs (**Figure 16A**).

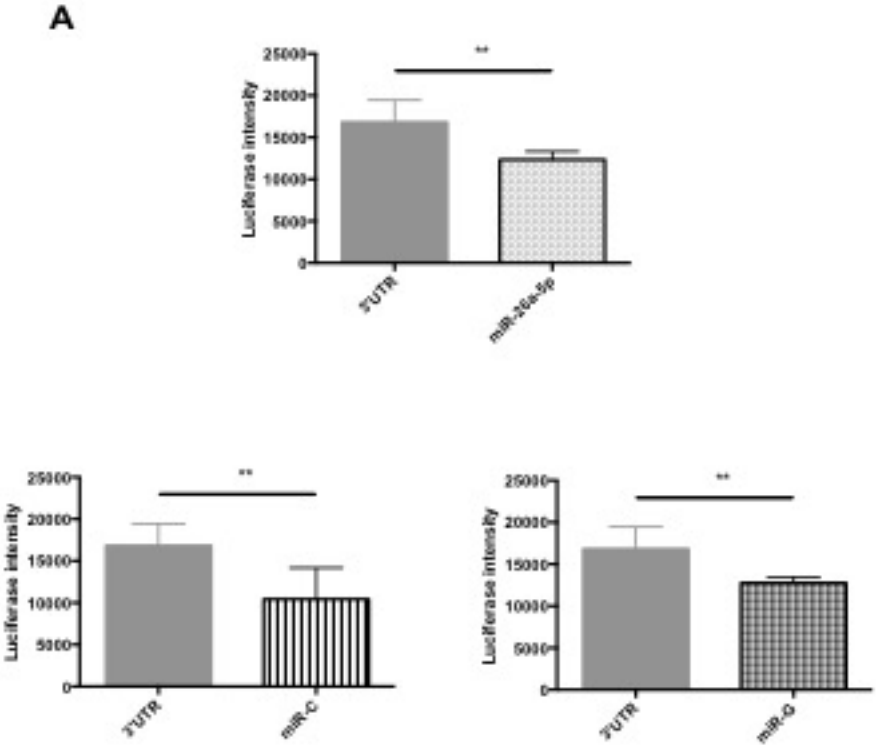


Figure 16. miR-C and miR-G target PTEN. A) Luciferase intensity in HeLa cells co-transfected with 12,5 ng 3'UTR PTEN plasmid and 25 nM miR-26a-5p, miR-C and miR-G compared to control, respectively. HeLa cells were seeded onto 96-well plates at 7500 cells/well in DMEM supplemented with 10% FBS. The following day, cells were co-transfected using Lipofectamine 2000 for 24 h. Six replicas per experimental condition were used and luciferase intensity was measured in a luminometer. **P<0.01 vs. control group using unpaired t test. Results are presented as mean±SD.

Furthermore, quantitative PCR and western blot showed a significant decrease of PTEN mRNA (**Figure 16B**) and total protein (**Figure 16B**), respectively, upon transfection of $CD34^+$-derived ECs with both miRNAs. Additionally, transfection of $CD34^+$-derived ECs with a siRNA against PTEN led to an increase in cell survival upon exposure to ischemic conditions suggesting the involvement of PTEN in the survival of $CD34^+$-derived ECs (**Figure 16D**).

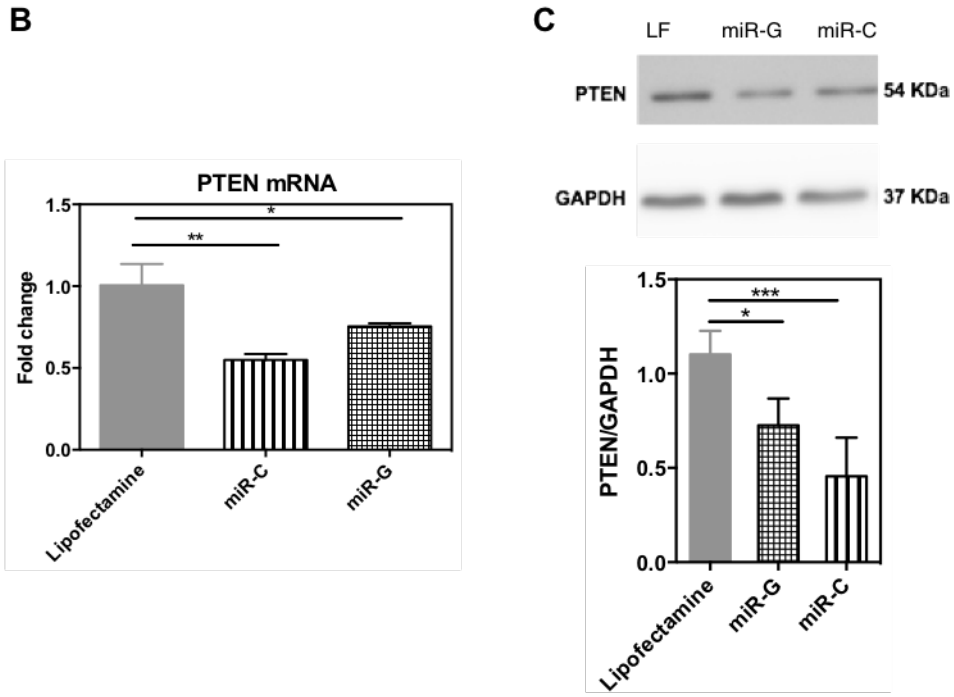


Figure 16 (continued). miR-C and miR-G target PTEN. $CD34^+$-derived ECs were transfected with 25 nM miR-C and miR-C during 48 h: **B)** total RNA was extracted and pRT-PCR was performed for PTEN. To determine fold change ΔΔCt method was used, PTEN mRNA is decreased in transfected cells when compared to lipofectamine (LF) treated cells. C) total protein was extracted and blotted against PTEN. GAPDH was used as loading control.

PTEN inhibits the conversion of PIP_2 (phosphatidylinositol 4,5-bisphosphate) to PIP_3 (phosphatidylinositol-3,4,5-trisphosphate) (Maehama *et al.* 2001). Intracellularly, PIP3 phosphorylates and activates effector proteins such as Akt

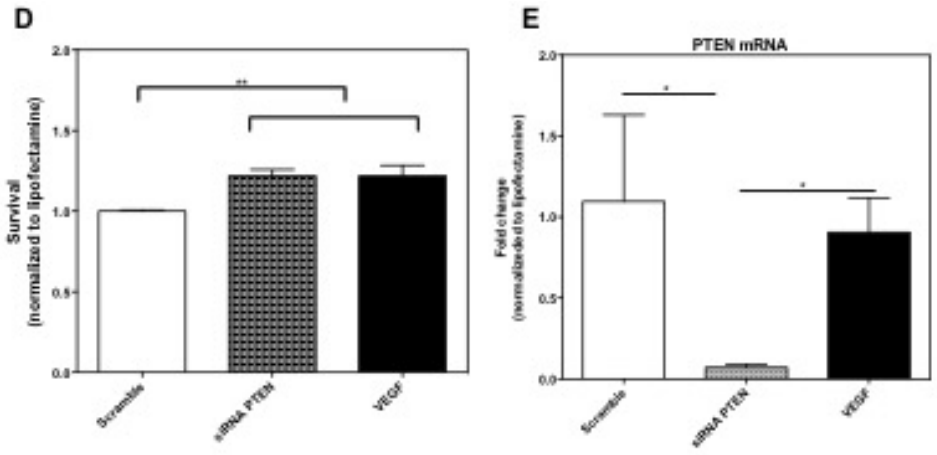


Figure 16 (continued). PTEN knockdown phenocopies miR-C and miR-G effects on survival. D) CD34+-derived ECs were transfected with a siRNA targeting human PTEN for 48 h upon which cells were placed in ischemic conditions (EBM-2 medium and 0.1% O_2) for 48 h. Hoechst nuclear staining was performed before and after ischemia to calculate cell survival. A scramble siRNA was used as a negative control and 50 ng/mL VEGF-165 as a positive control. **E)** CD34+-derived ECs were transfected with a siRNA targeting human PTEN for 48 h and PTEN expression was analyzed by qRT-PCR. PTEN expression was normalized to the reference gene B2M and fold change was calculated according to the ΔΔCt method. Results are presented as mean±SD. Statistical significance was obtained using one-way ANOVA followed by Tukey's multiple comparison test, *P<0.05, ** P<0.01, *** P<0.005, ****P<0.001.

(serine/threonine kinase 1) and promotes cell survival. To analyze how the inhibition of PTEN by miRNAs is modulating these intracellular pathways, the ratio of p-Akt to Akt protein levels was accessed by western blot. Transfection of CD34+-derived ECs with either miR-C or miR-G significantly increases Akt phosphorylation compared to lipofectamine-treated cells and untransfected cells. Interestingly, this increase is more pronounced for miR-G than miR-C (**Figure 17A**).

PTEN can also inhibit Shc protein (Src homology 2 domain containing protein) and therefore indirectly inhibits Erk1/2 (Mitogen-activated protein kinase 3/1) which is involved in the regulation of cell cycle progression (Yamada *et al.* 2001).

To analyse if our miRNAs modulate this pathway, the ratio of p-Erk1/2 to Erk1/2 protein levels was also analyzed by western blot. Both miRNAs significantly increase total levels of phosphorylated Erk1/2 (**Figure 17B**).

In parallel, we have verified that after $CD34^{+}$-derived ECs transfection with miR-C and miR-G the trypsinization time increased when compared to lipofectamine treated cells from 1 minute for control to 3 min or 2 minutes and 30 seconds for miR-C an miR-G, respectively. Interestingly, PTEN can also inhibit FAK (focal adhesion kinase) phosphorylation (Larsen *et al.* 2003). Upon phosphorylation, FAK is typically located at structures known as focal adhesions which are protein complexes connecting the cytoskeleton to the ECM. Given the observed effects on adhesion we decided to analyzed the ratio p-FAK to FAK by western blot and our results showed an increase in FAK phosphorylation only for miR-C (**Figure 17C**).

Besides PTEN, miR-C and miR-G are tought to regulate other molecular targets. To find those targets, RNA-sequencing (RNA-seq) was performed by BGI according to the experimental pipeline and subsequent bioinformatics analysis supplied in Supplementary Figure 2 and 3. From here after we will focus our analysis on miR-G since this miRNA has never been described in the literature. For miR-G treated cells, we have analyzed the top downregulated transcripts in relation to lipofectamine treated cells and used TargetScan to analyze if there was a predicted pairing between the mRNA and miR-G. From these genes, we have selected the ones involved in cell survival, angiogenesis or in the cardiac context. Selected mRNAs are represented in **Table 1** and include: RNF144A (ring finger protein 144A); PCDH10 (protocadherin-10); MYOZAP (myocardial zonula adherens protein); CXADR (Coxsackie Virus and Adenovirus receptor) and XB130 (actin filament associated protein 1 like 2).

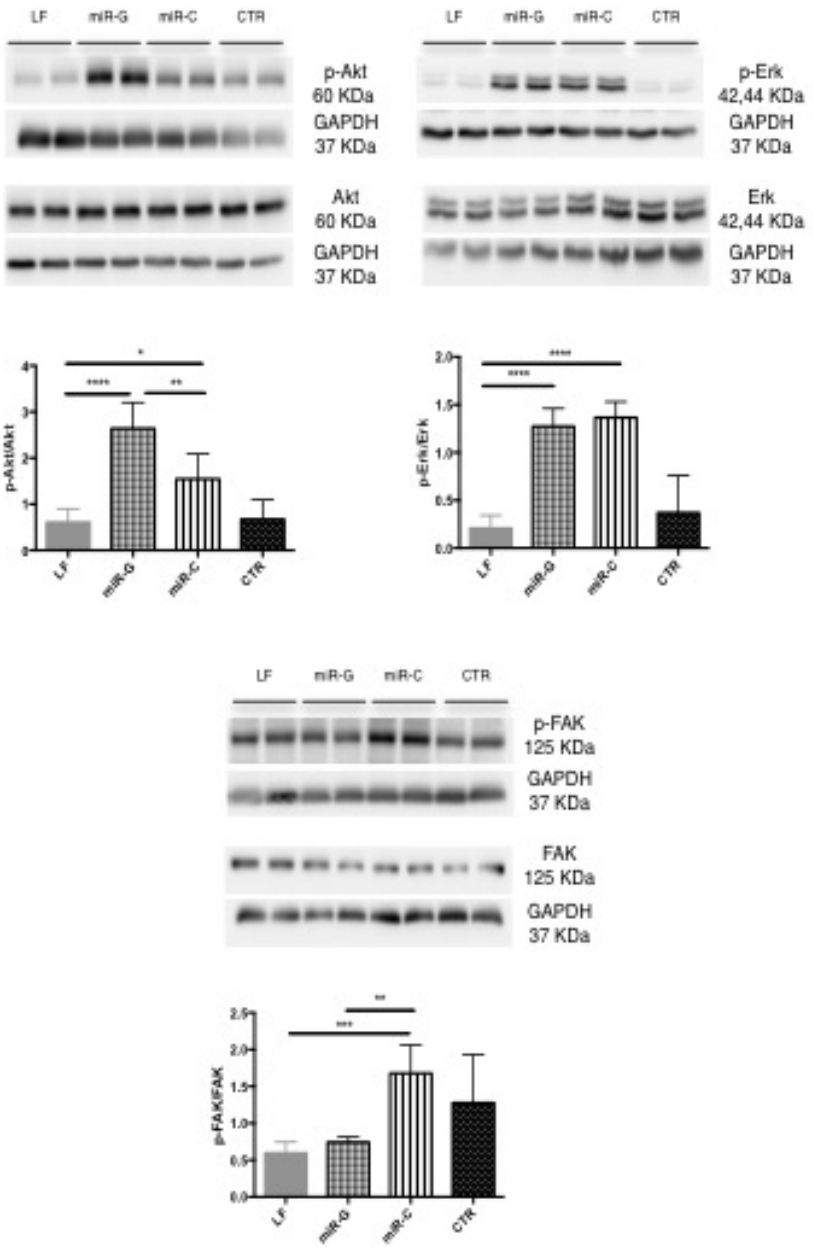


Figure 17. miR-C and miR-G modulate Akt, Erk and FAK phosphorylation in distinct magnitudes. CD34⁺-ECs were transfected with 25 nM miR-C and miR-G, respectively. Cells without treatment (CTR) and cells with transfection reagent (LF) were also used. Total cellular extracts were blotted against Akt, p-Akt, Erk, p-Erk, FAK, p-FAK. GAPDH was used as loading control.

Bars represent mean ± SD, n=3. Statistical significance was obtained using one-way ANOVA followed by Tukey´s multiple comparison test, *P<0.05, ** P<0.01, *** P<0.005, ****P<0.001. **A)** miR-C and miR-G increase ERK phosphorylation. **B)** miR-C and miR-G increase ERK phosphorylation. **C)** miR-C increases FAK phosphorylation.

4. **Delivery of miR-G using light-inducible polymeric nanoparticles (NPs):**

4.1 **Production and conjugation to miRNA**

NPs with an average size of 108.1 ± 9.9 nm (Boto *et al.* 2017) and an average zeta potential of 17.78 ± 1.46 mV were synthesized. Since these NPs were never described for miRNA delivery, we started by analyzing the complexation of miRNA to the NPs. Complexation rate was tested for a NP concentration of 125 μg/mL and a miR-G concentration of 200 nM and our results indicate a complexation rate of 86 ± 2.6%.

4.2. **Cell treatment**

Previously, Boto *et al.* (2017) have shown that NPs concentrations up to 10 *μ*g/mL were not toxic for HUVECs. Based on this, we started by using 1 *μ*g/mL and 5 *μ*g/mL NPs final concentration and we did not see any signs of cell death or apparent toxicity. To test the effect of the NPs conjugated with miRNA onto cell survival we conjugated our miR-G with the NPs and tested the effect of adding 1 μg/mL or 5 μg/mL of conjugated NPs to $CD34^+$-derived ECs during 4 h followed by irradiation of the cells with blue laser (405 nm, 80 mW) during 3 minutes. The following day (24 h after NPs administration), cells were placed under ischemic conditions and cell survival was assessed as previously described (**Figure 18A**). Our results showed a statistically significant increase in survival when $CD34^+$-derived ECs were exposed to 5 μg/mL NPs and therefore we decided to proceed our studies with this concentration of NPs. Interestingly, the final concentration of miR-G used in the transfection with NPs was significantly lower than the one previously tested with lipofectamine, from 8 nM to 25 nM, respectively. We decided

to repeat the experiment and increase the concentration of miR-G. To that end, we chose to repeat the complexation using 125 μg/mL with 200 nM miR-G and, in parallel, use 40 μg/mL NPs with 200 nM miR-G reaching a theoretical miR-G concentration of 8 nM and 25 nM, respectively. Furthermore, we also analyzed if irradiation upon addition of the NPs to the cells influences cell survival.

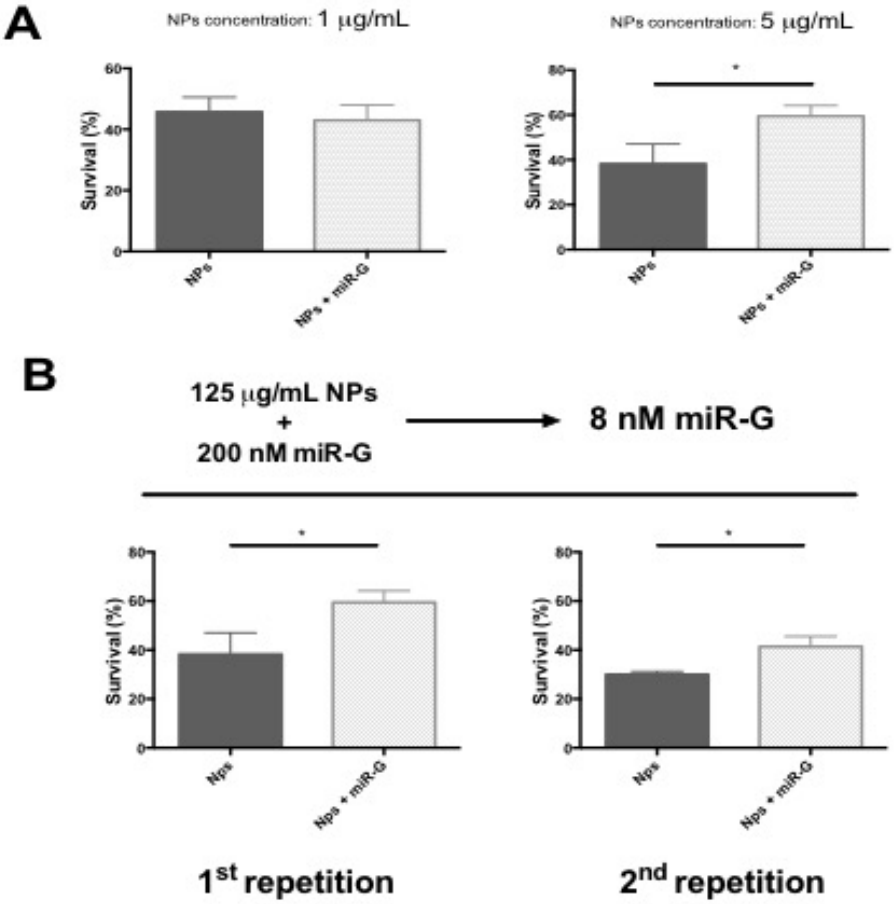


Figure 18. NPs modulate CD34+-derived ECs cell survival. A) CD34+-derived ECs were treated with 1 μg/mL NPs; 1 μg/mL NPs conjugated to miR-G or 5 μg/mL NPs; 5 μg/mL NPs conjugated to miR-G. After treatment (4 h), cells were irradiated with blue laser (405 nm, 80 mW) for 3 min. In the following day, CD34+-derived ECs were placed under ischemic conditions (EBM-2 medium and 0.1% O_2) for 48 h. Hoechst nuclear staining was performed before and after ischemia to calculate cell survival. Only 5 μg/mL NPs conjugated to miR-G increase cell survival when compared to cells treated with NPs alone. **B)** Two complexation conditions were used to calculate CD34+-derived ECs: 125 μg/mL and 200 nM of miR-G and 40 μg/mL and 200 nM of miR-G. Each condition was diluted in cell medium to a final concentration of 5 μg/mL NPs reaching a theoretical miR-G concentration of 8 nM and 25 nM, respectively. Cell treatment and survival assessment was performed as before. 125 μg/mL and 200 nM of miR-G complexation increased cell survival in two repetitions while 40 μg/mL and 200 nM of miR-G presented opposite results for the two repetitions.

Confirming our previous results, we observed that complexation of 200 nM of miR-G with 125 μg/mL NPs increased cell survival upon irradiation (**Figure 18B**). However, complexation of 200 nM of miR-G with 40 μg/mL NPs led to a significant increase in survival but, on a replicate experiment, failed to show the same effect (**Figure 18C**). Of

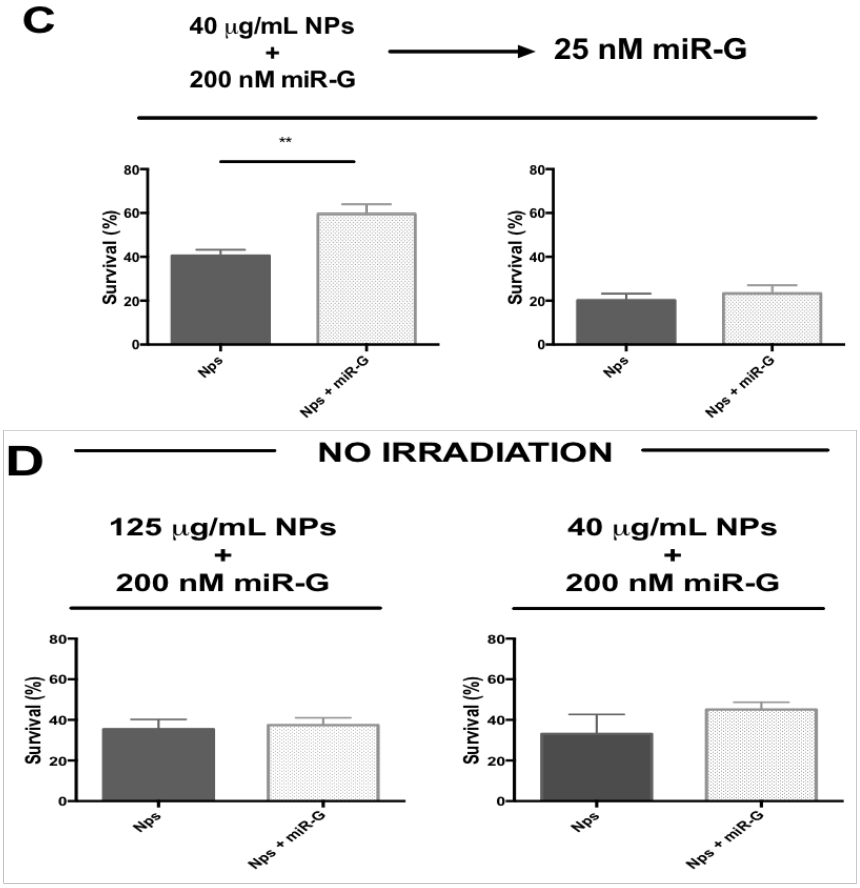


Figure 18 (continued). NPs modulate CD34⁺-derived ECs cell survival. C) Two complexation conditions were used to calculate CD34⁺-derived ECs: 125 μg/mL and 200 nM of miR-G and 40 μg/mL and 200 nM of miR-G. Each condition was diluted in cell medium to a final concentration of 5 μg/mL NPs reaching a theoretical miR-G concentration of 8 nM and 25 nM, respectively. After treatment (4h), cells were not irradiated. Survival assessment was performed as before. Without laser irradiation neither one of the complexation conditions increased cell survival. Bars represent mean ± SD (n=1). Statistical significance was obtained by unpaired t test. *P<0.05, **P<0.01 vs. NPs group.

note, in the second repetition, cell survival for NPs was around 20% indicating that, for an unknown reason, cells appear to have died a bit more under ischemic conditions than the usual.

Regarding laser irradiation, miR-G appeared to only induce a survival effect after light activation independently of the complexation condition (**Figure 18D**).

Discussion

Despite intense research on CVDs, cardiac regeneration remains poorly understood. Progresses have been made regarding the regeneration of the human heart, in particular with the discovery of cardiac stem cells and the proliferative potential of CMs (Xin *et al.* 2013). However, the potential of endogenous stem cells to differentiate into CMs is far from consensual and the proliferation of pre-existent CMs is insufficient to repopulate the lost tissue upon injury.

Cell-based therapies, despite deemed safe, have been inefficient in stimulating human heart regeneration and faced challenges of their own regarding survival and engraftment into the ischemic myocardium (Robey *at al.* 2008; Behfar *et al.* 2014). One approach to improve cell survival relies on preconditioning strategies such as heat shock, genetic modification or hypoxia (Wu *et al.* 2011). Interestingly, there is evidence that certain hypoxia regimens inhibit cell apoptosis and improved neoangiogenesis in the heart through upregulation of survival signaling pathways involving hypoxia-inducible factor (HIF)-1α and stromal cell-derived factor (SDF)-1, ultimately reducing the infarct size (Hu *et al.* 2008; Tang *et al.* 2009).

On the other hand, recent relevance has been given to the endothelium since ECs are the main cell type present in the heart and dynamically interact with CMs.

In the present work, we describe an approach to improve endothelial cell survival and hypothesize that targeting of ECs can be beneficial to the functional recovery of the ischemic myocardium.

1. **Identification of “pro-survival” miRNAs**

We have used endothelial cells derived from human donors, each composed of a pool of 5 different donors. $CD34^{+}$-derived ECs are a very well stablished model in our group with a well-defined phenotype and their functionality has been extensively studied (ref).

A miRNA library composed of 2080 human miRNAs mimics was used, over other strategies such as small molecules, due to the versatility of a single miRNA and its capacity to modulate simultaneously complementary pathways to promote cell survival. In fact, in our group this regimen of severe hypoxia and serum-deprivation has been already studied but it is the first time that miRNAs are studied in this context and in a high throughput manner.

All twenty-five miRNAs identified in the primary screening were validated and after statistical analysis we obtained fifteen pro-survival miRNAs. From them, seven were already reported in the literature as modulators of cell survival lending support to the results of the primary screening. We chose to pursue the work with two miRNAs, miR-C and miR-G ever since miR-C is a known regulator of cell survival while miR-G, in the opposite, has never been described in the literature.

2. **Pro-survival miRNAs and endothelial function**

In homeostasis, ECs are mostly quiescent but, upon injury, they are activated and, in a well-orchestrated process, start to migrate and originate new blood vessels. Our results point to an increase in migration and a decrease in capillaries number. The actual consequences of these are still unexplored but, regarding angiogenesis, it could be related with the adhesion to the substrate since this was the only

assay where a different substrate (Matrigel) has been used. Matrigel is composed of several ECM proteins and growth factors and is much more complex than gelatin. Angiogenesis will depend on the redistribution of cell-cell and cell-substrate adhesion molecules, cross talk between external ECM and internal cytoskeleton through focal adhesion molecules (Ghersi *et al.* 2008) and is highly dependent on substrate characteristics (Siavashi *et al.* 2016).

3. Pro-survival miRNAs target PTEN and modulate PI3K/Akt and MAPK signaling pathways

Using our bioinformatics approach, we obtained the biological pathways that are enriched for miR-C and miR-G, from which “Valine, leucine and isoleucine degradation” was the only one in common. Valine, leucine and isoleucine are branched-chain aminoacids (BCAAs) who have recently been implicated in HF. Sun *et al.* (2016) found that the most significantly downregulated genes in failing mouse hearts belonged to this pathway. In fact, one of the downregulated genes is a putative target gene for both miR-C and miR-G according to our bioinformatics analysis. The same authors also show that expression of BCAA mediators and measures of BCAA enzymatic activity are impaired in the myocardium of HF patients. Using a mouse model with genetic impairment of BCAA metabolism, Sun *et al.* (2016) showed that therapeutically boosting BCA flux prevented pathological remodeling and preserved ejection fraction. Furthermore, Tanada *et al.* (2015) showed that BCAA supplementation may ameliorate the progression of heart failure. Although beyond the scope of my thesis and of the research focus of our group, it would become interesting to understand how miR-C and miR-G can modulate BCAAs metabolism and its positive or negative impact in animal models of HF.

By analyzing individual putative target genes for miR-C and miR-G and by extensive literature mining we decided to confirm if PTEN is a direct target for both miRNAs. We validated miR-C and miR-G direct interaction with PTEN transcript resulting in its downregulation at mRNA and protein level. Additionally, when using a siRNA against PTEN we obtained a similar pro-survival effect confirming that PTEN inhibition is, at least in part, contributing to the enhanced survival. One must note that, the degree of inhibition of PTEN translation either by siRNA or miR-C and miR-G may not have the same magnitude. It would have been valuable to measure protein levels of PTEN after siRNA and compare them to miR-G and miR-C treatment.

PTEN is a key phosphatase involved in cell division, cell survival, apoptosis and migration (Hlobilková *et al.* 2003). In the plasma membrane, phosphatidylinositol 4,5-bisphosphate is phosphorylated by phosphoinositide 3-kinase (PI3K) into PIP_3 (Kim *et al.* 2016). PTEN is a negative regulator of PI3K, both in CMs and ECs (Oudit *et al.* 2004; Crackower *et al.* 2002; Hamada *et al.* 2005). By dephosphorylating the intracellular messenger PIP_3 PTEN can lead to Akt inactivation. Here we that our miRNAs, at least partially by downregulating PTEN, lead to Akt phosphorylation. Akt activation can phosphorylate pro-apoptotic protein Bad which becomes unable to block pro-apoptotic proteins such as Bcl-2 or Bcl-XL, leading to inhibition of apoptosis (Datta *et al*. 1997; Cardone *et al*. 1998). Furthermore, Akt activation can also inhibit GSK3β leading to cell cycle re-entry (Sherr *et al.* 1999).

In the cardiac context, PTEN is already well studied. As a matter of fact, loss of cardiac PTEN resulted in physiological-like hypertrophy (Crackower *et al.* 2002), was involved in ischemic preconditioning (Cai *et al.* 2005) and stimulated L-type Ca^{2+} channel current (Sun *et al.* 2006). In mechanical stress conditions as the aortic banding heart model, PTEN

deletion in muscle cells led to heart function improvement, diminished fibrosis, apoptosis and the hypertrophy caused by aortic banding (Oudit *et al.* 2004). Also, Siddall et al. (2008) have shown that genetic deletion of PTEN reduced the threshold of ischemic pre-conditioning and protected against I/R injury in isolated hearts. Pharmacological inhibition of PTEN *in vivo,* either before ischemia or reperfusion, led to a reduction in infarct size and an improvement in cardiac function with improved left ventricular function (Keyes *et al.* 2010).

Aside from dephosphorylating PIP3, PTEN can also inhibit Shc protein which will result in decreased Erk1/2 phosphorylation. Erk1/2 activation is directly associated with mitosis and post-mitosis functions which are closely related to cell migration, differentiation and survival (Kehat *et al.* 2010). Erk1/2 activates both numerous cytoplasmic proteins and nuclear transcription factors. We also show, by western blot that miR-C and miR-G increase Erk activation.

Interestingly, Erk1/2 has a dual effect on certain situations, for instance in the diabetic heart, Erk1/2 plays a detrimental role in oxidative stress, inflammation and apoptosis while its pro-survival effect has been protective against MI and I/R injury (Gross *et al.* 2007; Kim *et al.* 2010; Lambert *et al.* 2014). These opposite roles might be related to differences in the extent, subcellular compartmentalization and duration of Erk1/2 activation (Xu *et al.* 2016). In fact, a robust early phase of Erk1/2 signaling followed by a moderate sustained phase leads to G1 progression while a robust and prolonged activation of Erk1/2 causes G1 cycle arrest (Chambard *et al.* 2007). We analyzed the ratio of phosphorylated Erk1/2, 48 h after transfection with the miRNAs and we report a very strong signal by western blot. We may hypothesize that we are in the presence of a prolonged and robust activation of Erk1/2. Also, in the endothelial context, prolonged Erk1/2 activation can lead to a decrease in capillary bed sprouting

like we observe in our tube formation assays (Deng *et al.* 2013). It would be interesting to analyze the wave of Erk1/2 activation before 48 h and verify if our miRNAs can cause G1 cell cycle arrest, for example via flow cytometry. Furthermore, Chung *et al.* (2006) have showed in cancer cell lines that specifically nuclear PTEN downregulates cyclin D1 via down-regulation of Erk1/2 suppressing the cell cycle. We could also analyze by immunocytochemistry if our miRNAs lead to global PTEN downregulation or have a compartmentalized effect.

On the other hand, PTEN inhibition can also induce angiogenesis (Oudit *et al.* 2008) but our *in vitro* results only showed an acceleration of EC migration but not an improvement on tube formation. One must keep in mind, first the magnitude of PTEN inhibition and more importantly, and since we are talking about signaling pathways with several proteins involved and different cellular functions, that there are multiple regulation mechanisms which may be modulated by these miRNAs and are dependent on the cell type and context. Adding even more complexity, even though PTEN is a common target of both miRNAs, miR-C and miR-G must certainly differentially regulate distinct transcripts causing different signaling interactions. One classical example of a miRNA versatility is miR-21. MiR-21 targets PTEN therefore activating Akt and Erk1/2 signaling pathways. Besides it also targets programmed cell death 4 (PDCD4), sprouty1 (SPRY1) and sprouty2 (SPRY2) (Chen *et al.* 2010). Spry1 is not a miR-21 target gene in cardiomyocytes however, it is the target gene of miR-21 in cardiac fibroblasts (Buscaglia *et al.* 2011). Even more interestingly, miR-21 has been implied as a promoter of pathological hypertrophy and cardiac fibrosis while simultaneously, experimental overexpression of miR-21 can reduce the number of apoptotic cardiac myocytes and may also affect coupling of cardiac myocytes with other cardiac cells.

When working with miRNAs mimics we must be watchful to the different effects of the miRNA on other cell processes besides the ones being assessed and if they can be detrimental to other tissues. Furthermore, miRNA mimics can compete with endogenous miRNAs in binding to the RISC complex which could result in abnormal gene regulation (Vegter *et al.* 2016).

We used RNA-seq to try to find specific miR-G targets. The limitation of using RNA-seq to identify miRNA targets is that they are observed amongst a pool of indirect changes in transcript abundance (Thomson *et al.* 2011). To circumvent this, we have used TargetScan software to predict the interaction between the top downregulated mRNAs and miR-G and, after literature mining, the most potentially relevant mRNAs are represented in **Table 1**.

RNF144A is an E3 ubiquitin ligase which induced degradation of DNA-PKcs (DNA-dependent protein kinase, catalytics subunit) (Ho *et al.* 2014) via p53. DNA-PKcs is critical for DNA repair and can phosphorylate Akt (Feng *et al.* 2004). RNF144A depletion by miR-G could lead to increased levels of DNA-PKcs contributing to increased cell survival and phosphorylated Akt phenotype observed in our results.

PCDH10 functioned as a tumor suppressor gene inhibiting cell migration via p53 (Shi *et al.* 2015). Furthermore, PCDH10 inhibited the PI3K/Akt pathway and induced cell apoptosis in hepatocellular carcinoma cells (Ye *et al.* 2017). Thus, PCDH10 inhibition by miR-G could contribute to increase migration and activation of PI3K/Akt pathway.

Based on the above, RNF144A and PCDH10 are putative miR-G targets and worth pursuing and analyzing by western blot and luciferase assay if are indeed miR-G targets.

MYOZAP is widely expressed in cardiac tissue where it locates in the intercalated disks connecting CMs (Seeger *et*

al. 2010). Also, it co-localizes with adherent junctions connecting ECs from vascular and lymphatic systems (Pieperhoff *et al.* 2012). Mice with cardiac overexpression of myozap were more sensitive to pressure overload with severe loss of contractility and exaggerated cardiac hypertrophy (Frank *et al.* 2014). This model also led to cardiomyopathy. On the contrary, Rangrez *et al.* 2016 generated myozap-null mice that albeit not exhibiting a baseline phenotype, were also more sensitive to pressure overload with severe reduction in contractile function. Of note, in this MYOZAP null model, MAPK pathway was inhibited which is not in agreement with the increase in p-Erk/Erk ratio verified in our *in vitro* model.

Another putative target is CXADR. This membrane receptor has been studied in the heart. Human samples of patients who suffer from ischemia-induced ventricular fibrillation were associated with lower levels of cardiac mRNA for CXADR (Marsman *et al.* 2014). CXADR +/- mice hearts displayed ventricular conduction slowing during the early phase of acute myocardial ischemia following LAD ligation. Besides, hearts from these mice displayed increased arrhythmia susceptibility upon pharmacological electrical uncoupling.

From the future perspective of using miR-G in the MI context, it could be interesting to analyze if miR-G can target MYOZAP or CXADR and, if so, if miR-G administration would alter cardiac contractility or lead to arrhythmia in a mice model of pressure overload.

On the contrary, XB130 is a scaffold protein which regulates cell growth, survival and migration. Its downregulation causes more apoptosis, cell inhibition at G1 phase and less levels of p-Akt in cancer cells (Moodley *et al.* 2015; Shiozaki *et al.* 2012). This is not verified in our experimental model. Some explanations are possible: this phenotype is restricted to cancer cells and does not have the

same effect ECs; or despite XB130 being downregulated and causing less levels of p-Akt, other targets compensate and the global effect is the increase in p-Akt/Akt ratio.

Table 1. Putative miR-G targets according to RNA-sequencing analysis. RNA-sequencing data was analyzed and the top downregulated miR-G transcripts in relation to lipofectamine treated cells and used TargetScan to analyze if there was a predicted pairing between the mRNA and miR-G. From these genes, we have selected the ones involved in cell survival, angiogenesis or in the cardiac context.

Gene	Protein	Evidences	References
RNF144A	Ring Finger protein 144A	1) Degraded DNA damage kinase leading to cell apoptosis; 2) Phosphorylated Akt.	1) Ho et al. 2014 2) Feng et al. 2004
PCDH10	Protocadherin-10	1) Functions as tumor suppressor gene; 2) Inhibited cancer migration; 3) Inhibited the PI3K/Akt pathway and induced cell apoptosis in cancer cells.	1) Qiu et al. 2016 2) Shi et al. 2015 3) Ye et al. 2017
MYOZAP	Myocardial zonula adherens protein	1) Localized in the intercalated disks of CMs; 2) Localized in AJs connecting ECs of vascular systems; 3) Mice with cardiac overexpression of myozap developed cardiomyopathy and are more sensitive to pressure overload; 4) Myozap-null mice have accelerated cardiac hypertrophy and less contractile function.	1) Seeger et al. 2010 2) Pieperhoff et al. 2012 3) Frank et al. 2014 4) Rangrez et al. 2015
CXADR	Coxsackie Virus And Adenovirus Receptor	1) Less cardiac levels of CXADR mRNA were associated with human ischemia induced ventricular fibrillation; CXARD -/- mice hearts displayed ventricular ahrritmias shortly after MI.	1) Marsman et al. 2014
AFAP1L2/XB130	Actin filament associated protein 1 like 2	1) XB130 downregulation led to cell arrest at G1; increased apoptosis; decreased p-Akt; did not affect Erk1/2 in cancer line; 2) XB130 knockdown hindered transition of G1 to S phase in prostate cancer cell line.	1) Moodley et al. 2015 2) Chen et al. 2016

4. **Delivery of miR-G using light-inducible polymeric nanoparticles (NPs)**

Viral particles represent the majority of gene therapy trials, however significant effort has been made to develop non-viral strategies, in particular, in the nanomedicine field.

MiRNAs are susceptible to serum nucleases and since they are negatively charged, their cellular uptake is inefficient. Conjugation with nanocarriers gains relevance in this setting. For that, we tried to establish a suitable nanocarrier for miR-G.

In our lab, PEIDMNC:DS NPs have been successfully produced (Boto *et al.* 2017). As highlighted, PEI is a polymer that facilitates not only cellular internalization but also endosomal escape, while DMNC responds to light and its products show low cytotoxicity. Dextran sulfate allows NPs formation by electrostatic (PEI:DS) and hydrophobic interactions (DMNC:DMNC) forming the nanoparticle itself (Boto *et al.* 2017). Furthermore, PEI confers positive surface charge to the NPs allowing electrostatic interaction with miRNAs and is well documented in the literature as a suitable vehicle for nucleic acids, including siRNAs and miRNAs (Fernandez-Piñeiro *et al.* 2017). Accordingly, our NPs bind to miRNA showing high complexation rates.

The final miR-G concentration inside the cells will depend mainly on four factors:

1) miR-NPs complexation rate;

2) NPs internalization rate during transfection;

3) internalized NPs rate of endosomal degradation escape;

4) the rate of delivered miRNA that will be successfully processed by RISC machinery.

For each theoretical miRNA concentration (8 nM and 25 nM), cell survival assay was only repeated twice. For that reason, the following discussion will proceed with full awareness of the statistical limitations of the results. A theoretical miR-G concentration of 8 nM led to a mean increase in cell survival of 16%. For a theoretical miR-G concentration of 25 nM, we report in one of the assays a survival increase of 19% and no survival increase for the other one. In theory, we are increasing miR-G concentration 3-fold (from 8nM to 25 nM) but this does not translate in a 3-fold increase in cell survival, possibly because the complexation condition of 40 μg/mL NPs is reaching a saturation state and is not capable of complexing as much miR-G as 125 μg/mL NPs. It is necessary to repeat complexation measurement with SYBR Gold under 40 μg/mL NPs and 200 nM miRNA.

Our NPs were previously developed to deliver small molecules (Boto *et al.* 2017). In brief, following exposition to a blue laser, DMNC is photo-cleaved and the NPs disassembled and the small molecule is released. However, the small molecule is encapsulated inside the NPs while miR-G is complexed on the outer surface of the NP. Thus, we decided to analyze if irradiation would affect miR-G delivery by the NPs. The same NPs mixture was used either irradiated or not and even though we only tested irradiation condition once for each miR-G concentration, the survival effect was abolished when there was no irradiation, indicating that irradiation must be necessary for miRNA delivery. One may hypothesize that upon photo cleavage of DMNC, the miRNA can more easily escape endosomal degradation or be more efficiently released from PEI by breakage of PEI:miRNA interactions.

PEI-DMNC:DS NPs appear to be a strong potential candidate for miR-G delivery with the possibility of temporal delivery control using a non-toxic laser. Moreover, the

possibility of miR-G co-delivery with a small molecule is raised. Some reservations must be empathized. First, the experiments must be repeated. Of note, it would be relevant to analyze the alterations in miR-G levels upon NP transfection and compare them with the miR-G levels upon lipofectamine mediated transfection using qRT-PCR for miR-G. We could also use a fluorescent labelled miR-G and analyze its distribution along the transfection period and before exposure to ischemic conditions with or without irradiation.

Another approach could be to directly encapsulate miR-G upon NP assembly and analyze if this formulation could increase the amount of delivered miRNA to the cells, resulting in a more pronounced increase in cell survival.

Concluding remarks

Over the past 40 years, efforts to translate cardiac therapies from the bench to the bedside have yielded limited success (Xin *et al.* 2013). In parallel, miRNAs emerged as multifaceted modulators in physio- and pathological cardiac context.

Our strategy aimed at using miRNA mimics to enhance endothelial cell survival upon ischemic conditions.

We selected two pro-survival miRNAs according to a previous HTS and showed that both of them enhanced cell survival and migration.

Furthermore, we showed that PTEN is a common gene target of these miRNAs and that pro-survival PI3K/Akt and MAPK pathways are activated by our miRNAs. RNA-sequencing analysis opened the door for other targets, specifically for the novel previously reported miRNA, miR-G. The necessity of analyzing how miR-G would act upon cardiac delivery remains to be addressed. For instance, it would have been interesting to analyze miR-G effects on cardiomyocytes or other cell types of the heart as fibroblasts and smooth muscle cells.

Finally, we have used a previously developed in our group and well-studied light-induced nanocarrier and adapted it to miRNA deliver. Only preliminary studies have been made however these NPs are a promising suitable vehicle for miRNA controlled delivery. Strategies on how to maximize the amount of delivered miRNA were discussed and will be tested.

Overall our results, point for both miR-C and miR-G as relevant modulators of endothelial cell survival and worth pursuing its intracardiac delivery.

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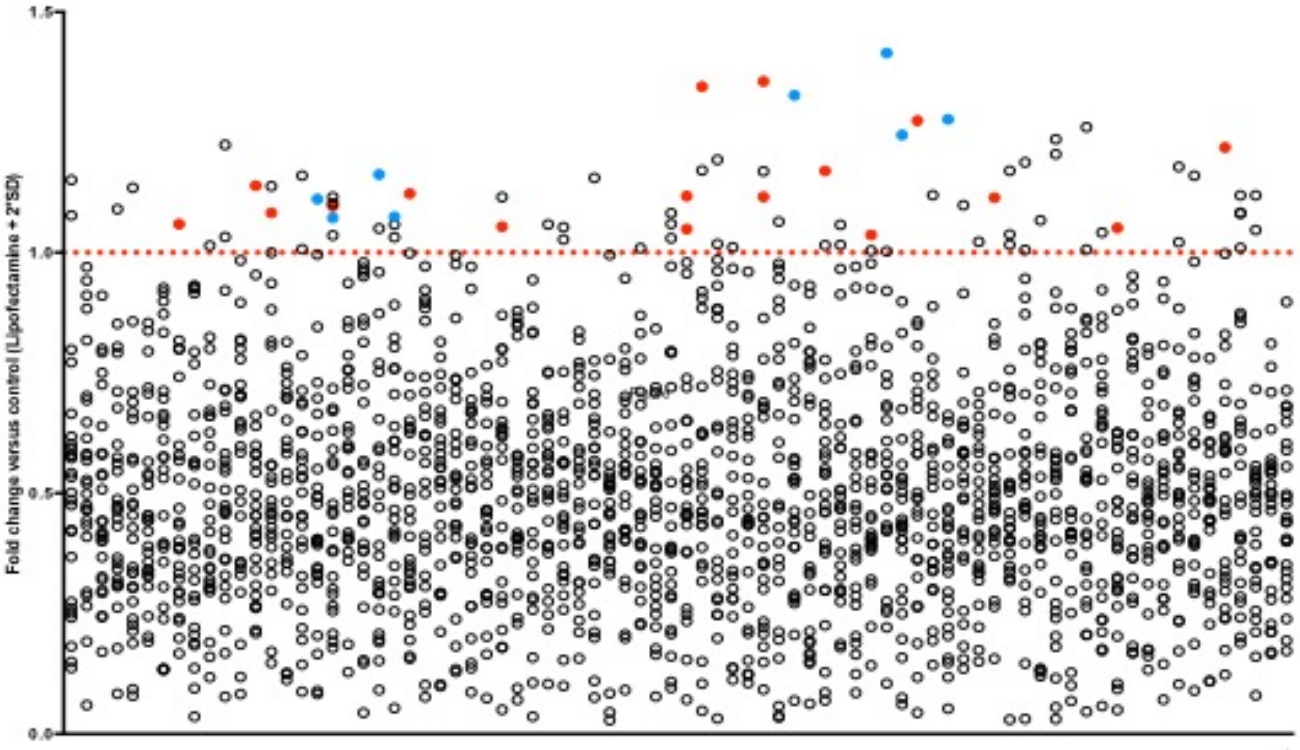
ATTACHMENTS



Supplementary Figure 1. High-content screening identifies microRNAs enhancing survival of CD34⁺-derived ECs. CD34⁺-derived ECs were seeded onto 96 well plates in EGM-2 medium without GA-1000 and the next day transfected with a library of 2080 human miRNA mimics (50 nM final concentration per well) for 48 h. Subsequently, the transfection medium was replaced by EBM-2 media and cells transferred to a hypoxia chamber (0.1% O_2) and cultured for further 48 h upon which nuclear staining was performed and images acquired using a high-content microscope (at least 8 fields per well were used for quantification). Per plate, untreated and lipofectamine-treated cells were used as a control. The screening was repeated twice using two different "donors" (each "donor" consisting of a pool of cells from 5 different donors) and a hit was identified if (1) the total number of cells after exposure to hypoxia was at least two times higher than the mean plus two times the standard deviation of lipofectamine-treated wells and (2) present on both donors. Data points in the graph represent the mean of both donors relative to lipofectamine. Blue dots represent microRNAs mimics previously associated with survival whereas red dots represent novel microRNAs.

Each step in experiment process (like sample test, library construction and sequecing) influences data quality and quantity, and then directly affect bioinformatics analysis results. To get high realiable sequencing data, we carry out strict quality control in each experiment step. The experiment pipeline is described as Figure1.

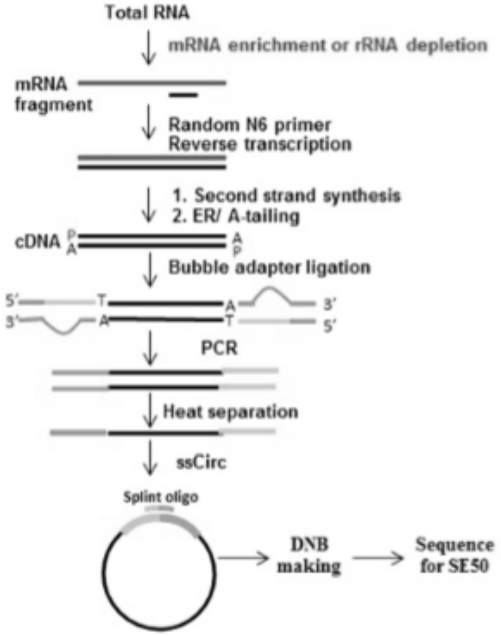


Figure 1 RNA-Seq experimental process.

1) There are two methods to treat total RNA. Oligo (dT) magnetic beads are used to select mRNA with polyA tail, or hybridize the rRNA with DNA probe and digest the DNA/RNA hybrid strand, followed by DNase I reaction to remove DNA probe. Then obtain the target RNA after purification. 2) Fragment the target RNA and reverse transcription to double-strand cDNA (dscDNA) by N6 random primer. 3) End repair the dscDNA with phosphate at 5' end and stickiness 'A' at 3' end, then ligate and adaptor with stickiness 'T' at 3' end to the dscDNA. 4) Two specific primers are used to amplify the ligation product. 5) Denature the PCR product by heat and the single strand DNA is cyclized by splint oligo and DNA ligase. 6) Perform sequencing on prepared library.

Supplementary Figure 2. Experimental pipeline used for RNA-sequencing. Briefly, RNA from lipofectamine treated $CD34^{+}$-derived ECs and transfected with 25 nM miR-C and miR-G for 48H was extracted using RNeasy kit (*Qiagen*) according to manufacturer´s instructions. BGI performed the RNA-sequencing as described in the figure.

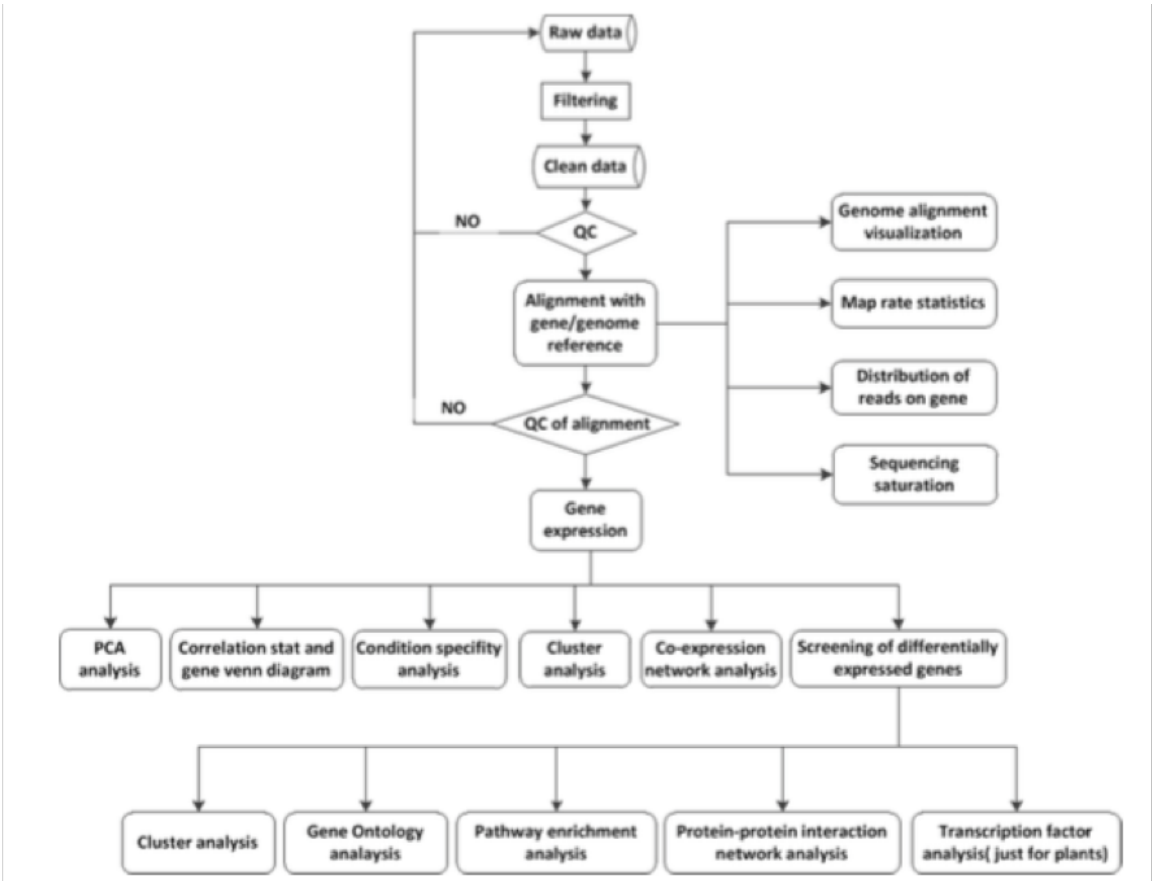


Figure 2 Bioinformatics analysis pipeline.

Primary sequencing data, called as raw reads, is subjected to quality control (QC) to determine if a resequencing step is needed. After QC, raw reads are filtered into clean reads which will be aligned to the reference sequences. QC of alignment is performed to determine if resequencing is needed. The alignment data is utilized to calculate distribution of reads on reference genes and mapping ratio. If alignment result passes QC, we will proceed with downstream analysis including gene expression and deep analysis based on gene expression (PCA/correlation/screening differentially expressed genes and so on). Further, we also can perform deep analysis based on DEGs, including Gene Ontology (GO) enrichment analysis, KEGG pathway enrichment analysis, cluster analysis, protein-protein interaction network analysis and finding transcription factor.

Supplementary Figure 3. Bioinformatics analysis pipeline. Data was analyzed by BGI and files containing Cluster; Gene Ontology; Pathway enrichment; protein-Protein interaction networks and Transcription factor analysis were produced.