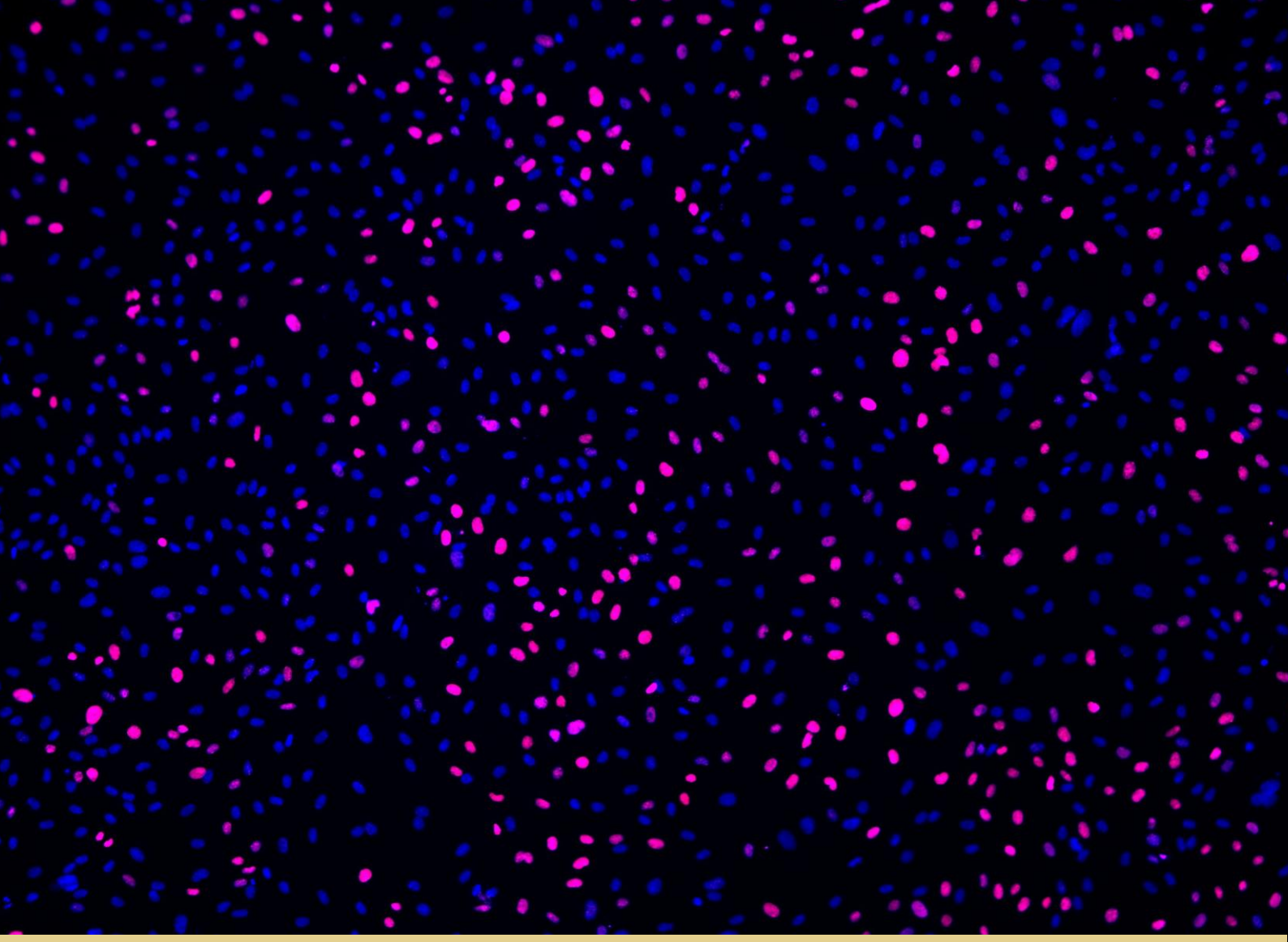

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MicroRNA inhibitor screening:
Searching for angiogenesis modulators in human endothelial cells

Dissertação de Mestrado em Biologia Celular e Molecular

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Cover figure legend: double staining for total nuclei (Hoechst 33342) in blue and for proliferative nuclei (EdU/Alexa 594) in red.

Acknowledgments

This year marked the end of a stage in my life and the beginning of another. This was the 5th year I was enrolled in university, the 2nd year of my Master's degree, and the first year that I spend almost exclusively working in the laboratory. It was also the first year I've lived abroad and far away from everyone and everything I had ever known. However, the people I met during my time in Maastricht have made this one of the most gratifying experiences I have lived and I am truly happy to have been part of such an environment. I could not let the chance of thanking everyone go by without a few words.

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Abbreviations

CM	Cardiomyocyte
CVD	Cardiovascular diseases
EC	Endothelial cell
eNOS	endothelial Nitric oxide synthase
FGF	Fibroblast growth factor
HCMVEC	Human cardiac microvascular endothelial cell
HF	Heart failure
hsa	Homo sapiens
HUVEC	Human umbilical vein endothelial cell
LNA	Locked nucleic acid
lncRNA	long non-coding RNA
miR	MicroRNA
mmu	Mus musculus
NO	Nitric oxide
pre-	Precursor
pri-	Primary
RISC	RNA induced silencing complex
TAC	Transverse aortic constriction
TC	Tip cell
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

Abstract

Cardiovascular diseases represent the highest mortality, morbidity and costs out of any medical condition in Europe and the US. The endpoint for most of these syndromes is heart failure, a stage of decompensated cardiac growth and dysfunction which results in lower cardiac performance. Despite the currently available treatments, the burden of the disease remains high. Therefore, novel strategies are required for its treatment, and while many studies have shown that the improvement of microvascular angiogenesis can have positive effects in the adaptive heart, no biomedical products have been produced yet. In this context, microRNAs appear as a novel, versatile and amenable platform for gene expression modulation, promising the ability of ameliorating heart failure or preventing it altogether.

Our group aimed to discover novel microRNAs involved in the regulation of the angiogenic potential of endothelial cells through a proliferation assay-based screening of a microRNA inhibitor library. We tested the effects of 753 microRNA inhibitors in human umbilical vein endothelial cells and evaluated their effects in terms of proliferation induction or repression through a dual staining for total nuclei (Hoechst 33342) and replicative nuclei/DNA (EdU).

Our results revealed a number of microRNAs, which are capable of drastically changing endothelial cell proliferation profiles. Among those, we chose to pursue the role of miR-219a-5p as our group had previously found this microRNA as having a role in the cardiac cells. We performed proliferation, scratch wound and tube formation assays in order to confirm the functional role of miR-219 in a human cell line. We observed that overexpression of this microRNA with a precursor resulted in slight proliferative increase compared to control, while inhibitor treatment decreased it, confirming the result from our screening.

We were then able to identify manic fringe as a potential target of miR-219, linking this microRNA to the Notch pathway, and thereby to endothelial cell proliferation and angiogenic capacity. Further studies will be necessary in order to validate this potential target as well as clarifying the mechanisms underlying the biological role and relevance of miR-219a-5p.

Resumo

As doenças cardiovasculares representam a condição médica com maior mortalidade, incidência e custos na Europa e nos EU. A maioria destas síndromes culmina em insuficiência cardíaca, um estado de crescimento cardíaco descompensado e disfuncional que resulta em performance reduzida. Apesar dos tratamentos actualmente disponíveis, o fardo da doença mantém-se elevado. Consequentemente, novas estratégias são requeridas e apesar de vários estudos terem demonstrado que o melhoramento da angiogénese microvascular pode ter efeitos benéficos no coração em adaptação, ainda não há produtos biomédicos disponíveis nesse sentido. Neste contexto, os microRNAs apresentam-se como uma plataforma versátil e modulável para a alteração de expressão génica, com a promessa de atenuar ou prevenir insuficiência cardíaca.

O nosso grupo pretendia descobrir microRNAs envolvidos na regulação do potencial angiogénico de células endoteliais através de uma selecção de uma "biblioteca" de inibidores de microRNAs baseada num teste de proliferação. Testámos o efeito de 753 inibidores de microRNAs em células endoteliais da veia umbilical humana e avaliámos os seus efeitos em termos de indução ou repressão de proliferação através de um protocolo de coloração dual para todos os núcleos (Hoechst 33342) e para núcleos/ADN replicativos (EdU).

Os nossos resultados revelaram vários microRNAs cuja inibição é capaz de alterar o perfil de proliferação endotelial. Entre estes, escolhemos continuar a estudar o papel do miR-219a-5p uma vez que este já tinha sido detectado previamente como relevante em células cardíacas. Efectuámos testes de proliferação, migração e tubulogénese de modo a confirmar a função do miR-219 em células humanas. Observámos que a sobreexpressão deste microRNAs com precursor resultou num aumento da proliferação de células endoteliais, relativamente ao controlo. Similarmente, a inibição deste microRNAs resultou na diminuição dos parâmetros angiogénicos testados, confirmando resultados prévios.

Conseguimos ainda identificar o gene "manic fringe" como um potencial alvo do miR-219, ligando este microRNAs à via de sinalização "Notch", e consequentemente à proliferação e angiogénese endoteliais. Estudos posteriores serão necessários de forma a validar este alvo bem como clarificar os mecanismos de acção inerentes à função biológica e relevância do miR-219a-5p.

1. Introduction

1.1. Heart Failure

1.1.1. Cardiovascular Pathologies – Epidemiological data

Cardiovascular diseases (CVD) are a group of pathologies pertaining to the malfunction of the cardiac and/or circulatory systems. Concerns over CVD prevalence and societal impact have been voiced for a long time, along with calls for research and prevention efforts to be increased. In 1969, the World Health Organization (WHO) dubbed the disease as "mankind's greatest epidemic"[1]. Forty years later, concerns were still being reiterated, with WHO reinforcing the idea that CVD are a "true pandemic"[2].

Nowadays, CVD are still the most predominant cause of death worldwide, in Europe[3] and the United States[4] CVD accounts for approximately 45% of all deaths. Additionally, CVD also make up for the most prevalent universal morbidity, with an average of over one in three people having cardiovascular complications[5]. Cardiovascular diseases are not only the leading cause of mortality globally, but they are also among the most costly, amounting to nearly four times the costs of cancer. Estimated annual expenditures for CVD in the US were over 300 billion USD in recent years, including direct costs associated with the treatment of the disease and indirect costs due to loss productivity[4,6]. In 2014, in Europe total direct costs for CVD averaged 13 billion euros per country, in six countries assessed. Additionally, it is estimated that there will be an increase of 20% over the next six years[7].

The term CVD comprises a wide range of pathologies, including coronary heart disease (CHD) and hypertension, which all may lead to heart failure (HF). This condition is characterized by an abnormal structural, functional, and biochemical remodeling of the heart leading to an inability to deliver enough blood to meet the metabolic needs of the body.

1.1.2. Cardiac remodelling

The human heart is comprised of different cell types, among which cardiomyocytes (CMs) account for the larger portion of its mass. Unlike other cardiac cells, CMs mostly lack the ability to proliferate in the adult organism. Thus, in response to a physiological or pathological stress, CMs usually undergo hypertrophy. This hypertrophic growth is not necessarily detrimental to the human heart, in fact, it is required for proper cardiac maturation from birth to early adulthood. Also in situations such as extreme exercise and pregnancy, a physiological increment in heart size is expected and reversible. Notably, both systolic and diastolic functions are generally preserved in these forms of cardiac hypertrophy[8]. As such, cardiomyocyte hypertrophy is regarded as a very common form of cardiac remodelling. Cardiac remodelling is broadly defined as the set of morphological and physiological changes that the heart and its vasculature can undergo, following a stimulus. This process entails a multi-cellular interplay, including CMs, cardiac fibroblasts, and cardiac endothelial cells (ECs) [9].

As CMs, and therefore the heart itself, get larger, their metabolism also changes. The healthy myocardium requires high levels of intracellular adenosine triphosphate (ATP) to function properly, but in a hypertrophic heart energy production is not able to meet with the increased need, rendering the heart energy-starved[10]. This energy imbalance leads to a shift from a fatty acid-driven metabolism to a glucose-reliant state, which is followed by a drastic change in the gene expression profiles of CMs, namely the upregulation of foetal genes. One of most notorious alterations is the conversion of adult myosin heavy chain (MHC) to a foetal-like phenotype. Myosin chains form the sarcomere, which are

the motors of contractile cells[11]. In turn, sarcomere properties determine the type of cardiomyocyte. Longer sarcomeres are usually a sign of dilated cells while thicker sarcomeres are more prevalent in classic hypertrophy. Both types of morphology imply impairment of heart function, either by a decrease in the volume of blood that the left ventricle pumps out in each cardiac cycle (ejection fraction) or by decrease in diastolic filling of the heart[12].

As previously mentioned, cardiac fibroblasts are also involved in the process of cardiac remodelling. In a maladaptive hypertrophic heart, fibroblasts receive profibrotic cues and start proliferating and secreting extracellular matrix more profusely, causing loss of contractibility and expansibility of the myocardium. Fibroblast-derived growth factors are also actively exported from fibrotic regions to CMs, potentiating their enlargement[9,13,14].

Endothelial cells are the primary constituents of the cardiac vasculature, forming an internal surface which is surrounded by either vascular smooth muscle cells (VSMCs) or pericytes, depending on the calibre of the vessel. In the post-natal life, the development of vasculature can occur through the branching of pre-existing vessels to form microvessels (angiogenesis), or, more rarely, through the *de novo* formation of vessels from stem cells (vasculogenesis)[15]. Angiogenesis is a complex process in which different signalling pathways converge in ECs. In stable adult vessels, ECs are in a quiescent state until they receive proangiogenic cues that change their behaviour. However, not all ECs are able respond to such stimuli, only a small subset of ECs is able to sprout in response to angiogenic cues, thus being known as "tip cells" (TCs)[16]. Once a proangiogenic signal is received, TCs loosen their intercellular junctions and profusely secrete proteases that degrade surrounding matrixes. These changes confer TCs an increased invasive and motile ability, required for the formation of new blood vessels. Then, the extension of filopodial protrusions from the TCs initiate a chemotaxic response that directs the newly formed vessel towards attractive signals in their microenvironment. This process ends once the TCs connect with a recipient vessel, as their phenotype reverts to that of a quiescent "stalk cell"[17].

The homeostatic endothelium is mostly composed of stalk cells, which make it anti-inflammatory, anti-thrombotic, anti-coagulant and anti-proliferative. These properties are achieved by intricate interplays of several pathways. Nevertheless, the activity of endothelial nitric oxide synthase (eNOS) is a major intervenient in blood vessel homeostasis. Accordingly, a major hallmark of healthy vasculature is the high bioavailability of nitric oxide (NO), the product of eNOS activity, in the endothelium. Normal NO levels are required for the pre-emptive protection against an atherosclerotic phenotype[18]. The transition to the TC phenotype is also regulated carefully, with mainly two other pathways at its centre. First, the vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis[19]. During angiogenesis, VEGF binds to its cognate receptor, activating multiple signalling pathways such as mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinases (PI3Ks), which in turn promote a proliferative, migratory and chemotaxic phenotype[20]. Secondly, the Notch signalling pathway is one of the main controllers of cell fate decisions and is therefore capable of directing ECs towards either a stalk or tip cell phenotype[21].

Cardiac insults can lead a decrease in cardiac function and thus signal for pathological cues that make the adult human heart compensate the lower blood output by undergoing a hypertrophic response[22]. Activated CMs are able to actively secrete VEGF, which augments cardiac microvascular vessel formation. It is thought that this interplay is meant to placate the increasing oxygen and nutritional demands of a hypertrophic heart by increasing cardiac irrigation. Nevertheless, the cardiac

microvasculature has limited angiogenic potential. Consequently, past the threshold when microvascular angiogenesis is able to support cardiac growth, an imbalance between metabolic demands and supply favours a pathological phenotype development[23]. Accordingly, it has been shown that in mice hearts, following transverse aortic constriction (TAC), there is an increase in blood vessel per cardiomyocyte ratio and also in VEGF levels. This adaptive change is then followed by a decrease in both indicators with time, at which point cardiac function is no longer preserved, and HF ensues[24]. Observations in the TAC mouse model also conclude that the crosstalk between cardiomyocytes and ECs during the early stages of HF accounts for the shift in microvessel phenotype. During the adaptive phase of cardiac growth, CMs are able to secrete pro-angiogenic cues, such as VEGF and hypoxia inducible factor (HIF-1α) which support microvascular development. However, repression of these factors leads to severe impairment of cardiac function and acceleration of HF progression[24]. Thus, it is theorized that abnormalities in myocardial perfusion owed to an angiogenic deficit may be at the very inception of HF.

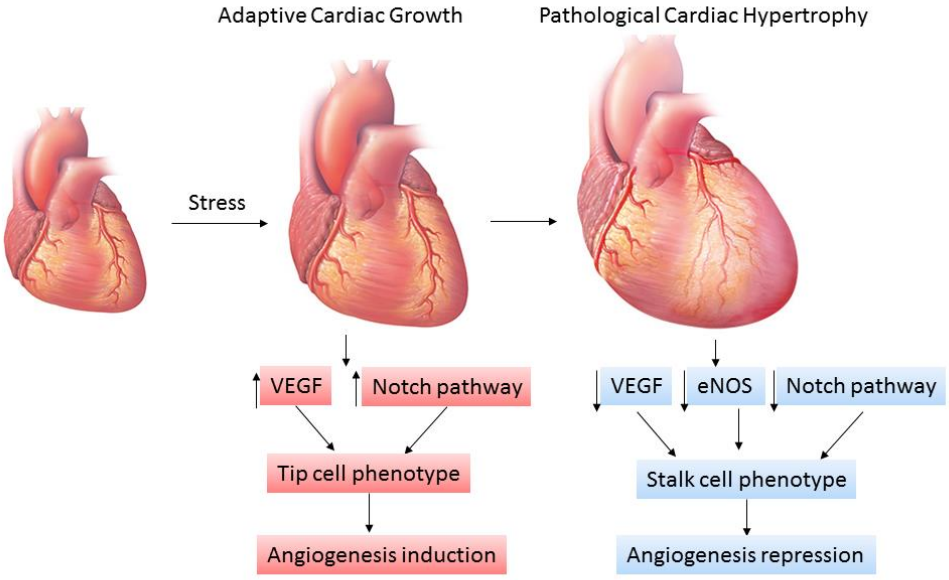


Figure 1. Microvascular phenotype switch between adaptive and pathological cardiac remodelling.

Despite the improvement of clinical practices and better outcomes for HF patients in the short term, around 12% of patients die within 6 months of a myocardial infarction, and long term chronic HF still affects a significant part of the population[4]. Given the need for more effective options, since the turn of the century, more than 150 clinical trials on HF have been completed, with over 5000 still ongoing[25,26]. Most trials conducted aim to test and approve compounds of varied chemistries to ameliorate HF symptoms, with the majority acting systemically and with significant side effects[27]. Other therapies include stem cell treatment, whereby the heart's regenerative capacity is increased through modulation of endogenous stem cell niches or through autologous graft of cardiomyocyte differentiated induced pluripotent stem cells (IPSCs). Albeit a promising technology, stem cell options have failed to deliver in terms of clinical results, so far[28].

In spite of recent findings pointing towards the importance of cardiac microvasculature in HF, there have not been successful therapies directed at this issue. A decrease in cardiac capillary density, known as capillary rarefaction, is a hallmark of HF. Therefore it stands to reason that in improving cardiac microvascular angiogenesis, it might be possible to ameliorate, slow or even prevent the progression of HF. A number of studies have been conducted in the past two decades highlighting the potential of angiogenic therapies for patients with vascular disorders. The majority of them have focused on specific gene therapy aimed at boosting endogenous angiogenic ability through overexpression of a potent proangiogenic signal such as VEGF or the fibroblast growth factor (FGF). Despite very optimistic pre-clinical trial data and robust results that prove the efficacy of such treatments, translational applications have yet to surpass randomized, large scale, double-blind, placebo-controlled phase II trials[29–32]. It is in this context that novel molecular therapies aiming to modulate gene expression in vivo are taking off. Among the most widespread research lines, the usage of non-coding RNAs is one of the most promising venues towards HF therapy, given their versatility and power to modulate gene expression endogenously.

1.2. MicroRNA basics

For decades it has been known that RNA is a highly flexible carrier of biological information, with messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) comprising the classical RNA species. A number of breakthroughs and technological advances have, however, improved our understanding of the subcellular workings of life, thereby shedding light on the enormous functional versatility of RNA. While non-coding RNAs are no novelty, since the discovery of rRNA and tRNA in the 1950s, it was not until the 1990s, when the existence of microRNAs was acknowledged, that the regulatory power of RNA was first hinted at[33,34].

MicroRNAs (also known as miRNAs or miRs) are small, single-stranded, non-coding ribonucleic acids approximately 22 nucleotides-long. By association with regulatory proteins and the formation of functional ribonucleic protein (RNP) complexes, microRNAs are able to modulate gene expression mostly by interacting with mRNA molecules. Even though the precise rules of microRNA-mRNA binding are not yet fully understood, it is consensual that the major determinant in mRNA targeting is a sequence of 6 to 8 nucleotides near the microRNA's 5' end, usually termed "seed" sequence, capable of base pairing with a cognate sequence in the 3' untranslated region (3' UTR) of the target transcript[35]. There are four types of conserved seeds, each with varying degrees of binding strength to their target. From higher to lower efficacy of binding, the seed can match the target binding region from nucleotides: 1 to 8 (8mer), 2 to 8 (7mer-m8), 2 to 7 followed by an A (7mer-A1) or 2 to 7 (6mer)[36]. Despite widespread consensus on the importance of the seed sequence, some authors also emphasize that other regions of the microRNA's sequence play a larger role in target binding than anticipated[37,38].

1.2.1. Nomenclature

At the time of the discovery of the first microRNA species, there was no set of rules specifying how to name them, thus they were named after their phenotype. For example, the first discovery microRNA, lin-4, was named for the fact that it directly targets LIN-14 in C. elegans[33]. More recent conventions have, however, established a nomenclature for microRNAs, mostly based on the sequential number

following their discovery. For instance, if the last published microRNA was miR-219, then the next one would be miR-220. Typically, this designation is preceded by the species abbreviation from where that microRNA was identified (i.e. hsa-miR-219 for human; mmu-miR-219 for mouse). Additionally, if multiple loci encode the same mature sequence of a microRNA, they are distinguished by a digit at the end (i.e. hsa-miR-219-1; hsa-miR-219-2). However, closely related mature sequences are distinguished by a letter (hsa-miR-219a; hsa-miR-219b). Moreover, microRNAs are derived from a hairpin-like precursor, which means the mature sequence could be encoded on the 3' or the 5' end of the hairpin. As such, mature microRNA forms can be named after the arm of the precursor from which they originate from (i.e. hsa-miR-219-3p; hsa-miR-219-5p). Finally, if one of those forms is known to be predominantly expressed over the other they can be distinguished through an asterisk instead (i.e. hsa-miR-219 for the predominant form; hsa-miR-219* for the opposite form)[39].

1.2.2. Categorization and Evolution

MicroRNAs can be broadly categorized according to their genomic location relative to introns and exons. The complexity of the eukaryote genomic landscape ensures that microRNA classification is not a trivial matter, as they can be present in either protein coding or non-coding transcripts, be intronic or exonic and be present in the sense or anti-sense strand in either case[40]. The expression of microRNAs present within the sense strand of protein coding genes is thought to be regulated by the expression profile of the host gene as well as splicing events[41,42]. It is noteworthy, however, that microRNA and host gene expression profiles can diverge. Recent studies have found that many intronic microRNAs are adjacent to transcription regulatory elements, which may account for independent transcription events[43–45].

The regulation of some microRNAs encoded within non-coding transcripts, also known as intergenic, is more debated as sometimes there are no obvious promoter regions adjacent to the transcriptional unit. Recently, some of these units have been categorized as long non-coding RNAs (lncRNAs). In light of their innate properties that distinguish them from canonical transcriptional units with highly conversed patterns, different standards for conservation of functional regions such as promoters have been called for. Thus, alternative RNA polymerase binding motifs may explain a fraction of lncRNA expression and thereby microRNAs encoded within. Moreover, RNA polymerase II is known to transcribe sequences adjacent to those intended through a "ripple-like" activity, which may account for the sporadic expression of some microRNAs. It is important to note that claims that the vast majority of the human genome is "putatively functional" due to the fact that transcriptomic studies have found that a vast portion is transcribed, are misleading[46]. Spurious, pervasive transcription of large portions of the genome may occur due to the low fitness impact it has to a cell versus the high cost of fine tuning the process. Furthermore, there is a wealth of accounts of poorly conserved microRNA species in human. However, conserved microRNAs are more highly expressed than non-conserved ones. The binding sites in mRNA molecules for microRNAs are also widely conserved[36]. It has been hypothesized that, through evolution, most poorly conserved microRNAs could be formed due to chance acquisition of features required for processing. Emerging microRNAs may have not yet attained sufficient expression levels that lead to a degree of biological relevance consistent with selective retention in the genome. As such, the vast majority of poorly conserved species should

disappear in time, as new ones keep emerging. Exceptions are also likely to occur, especially with reports of poorly conserved species being efficient in modulating target gene expression[47]. Thus, it is of paramount importance to ascertain not only the biological function of any microRNA species, but also its ultimate relevance to the affected pathways.

In addition to their genomic location, microRNAs can be classified into "families" according to their seed sequences. A family of microRNAs possesses the same binding region which should, theoretically, enable them to target the exact same transcripts. The expression profiles of different members of the same microRNA family can be widely divergent, as microRNAs can be spatially and temporally segregated, being expressed during different times in organism development or in different tissues. On the other hand, several microRNA species can be encoded in close proximity to one another and be regulated by the same promoter region. In this case, microRNAs are "clustered" and traditionally thought to be regulated as a whole. Interestingly, there seems to be a coordination between the microRNAs present in the same cluster, in terms of the function of their targets, which may suggest a common origin for different cluster members[48,49]. Moreover, through cluster and family coordination, microRNA function has been shown not only capable of fine tuning biological processes, but also to be fundamental for embryonic development and homeostasis maintenance. For example, several members of the clusters 17~92 and 106b~25 share the same highly conserved seeds. Their function has been shown to overlap, and their knockdown is highly deleterious for lung and heart development and function[47].

Regardless of the genomic complexity of microRNA circumstances, all of them are subject to standard transcriptional requirements. While there are several accounts of alternative transcription pathways being able to express microRNAs[50,51], the majority is transcribed by RNA polymerase II (RNA pol II). As products of RNA pol II activity, canonical microRNA primary transcripts (pri-microRNA) possess a 3' poly-adenine tail and a 5' methylguanosine cap. Structural chromosome conformations and epigenetic marks such as DNA methylation are also able to regulate the expression of a microRNA gene[52].

Following transcription, a pri-microRNA requires processing in order to become functional. Primary microRNAs are long, double stranded and contain a stem loop in which the mature microRNA sequences are embedded[53]. The main stem loop is flanked by single stranded extensions which possess conserved motifs required for proper pri-microRNA processing. The microprocessor, a protein complex composed of Drosha (a nuclear RNase III-type endonuclease) and its cofactor DiGeorge syndrome chromosome region 8 (DGCR8), is responsible for the cleavage of the pri-microRNA single stranded overhangs, generating the precursor (pre-microRNA) to the mature microRNA, with a 2 nucleotide extension at the 3' end[54]. As with most proteins, Drosha activity is regulated by a number of pre and post-translational cues and interactions[55–57]. Interestingly, Drosha and DGCR8 form a regulatory system with each other, as DGCR8 stabilizes Drosha while Drosha destabilizes the mRNA of DGCR8[58] (Figure 2). This negative feedback system ensures the maintenance of appropriate levels of the microprocessor. Moreover, Drosha requires additional factors in order to process certain pri-microRNAs[59,60] and recently it has been suggested that lncRNA are also able to interfere with Drosha function through binding complementarily to target pri-microRNAs[61]. Taken together, these mechanisms represent various layers of regulation over microRNA expression and their exact mechanisms and relevance must be ascertained.

The pre-microRNAs are exported, via exportin 5, to the cytoplasm where they will be further matured by Dicer, another RNase III-type endonuclease. Together with Dicer, Ago2 and TRBP form a ternary

complex called “RISC loading complex” (RLC), which is essential for pre-microRNA maturation.[62] Once the pre-microRNA is loaded into the RLC, Dicer usually binds to its 3’ end and is able to cleave the double stranded pre-microRNA at a fixed distance (around 22 nucleotides from the binding site), creating a short RNA duplex. If the precursor is too stable, Ago2 may cleave it at the 3’ arm in order to make it more amenable to Dicer processing[63]. Dicer is known to associate with several proteins containing double strand RNA binding domains (dsRBD) which are thought to facilitate the interaction with the pre-microRNAs[64,65]. The messenger RNA coding for Dicer is itself also a target of microRNA action, namely let-7. This microRNA is able to repress Dicer expression, again creating a homeostatic regulatory loop[66,67].

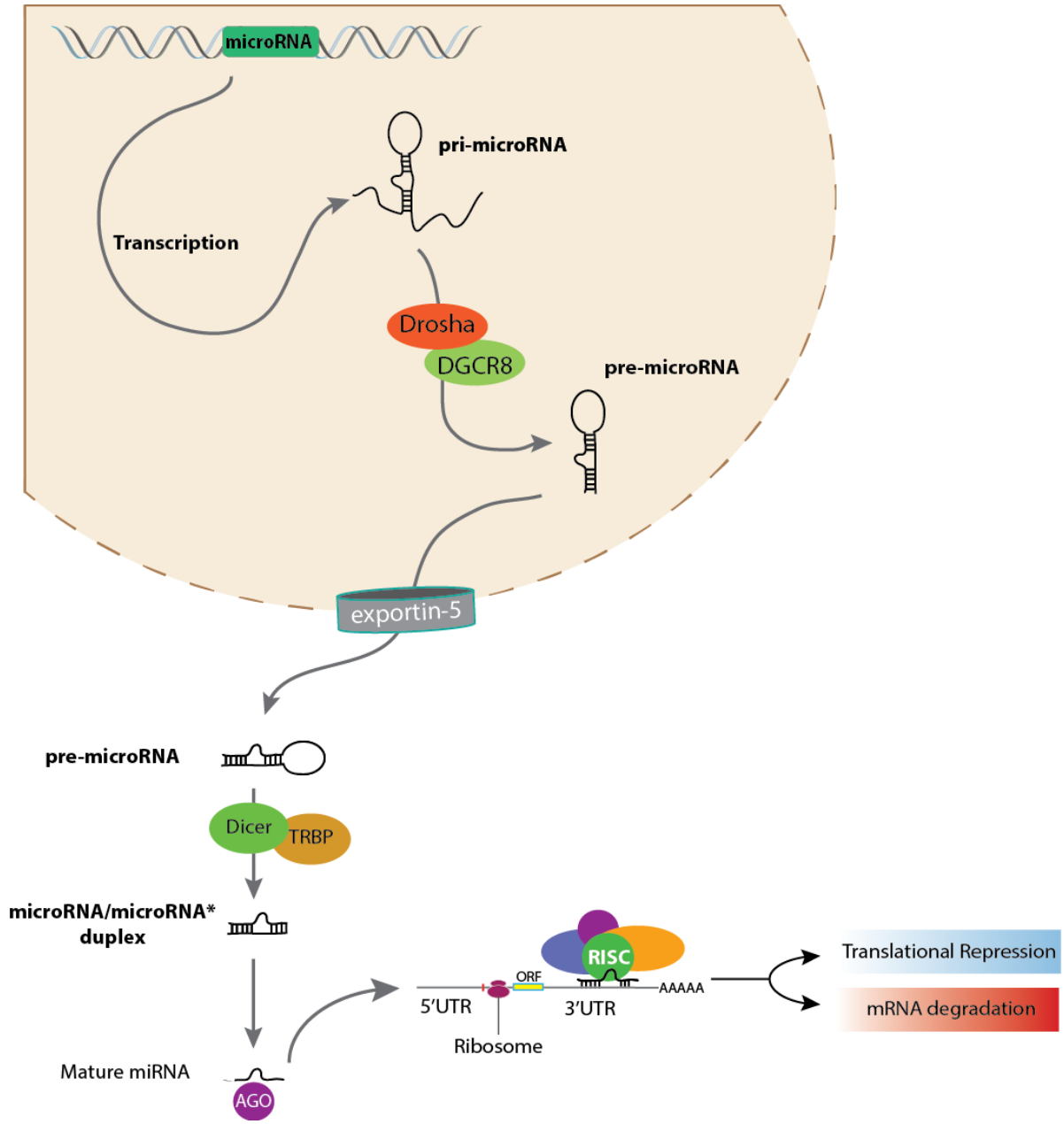


Figure 2. Canonical microRNA biogenesis and mode of action pathway.

Following Dicer action, the short dsRNA is loaded onto an Argonaut protein (AGO). Due to thermodynamic stability, the strand which is more unstable at its 5' end will typically become the guide, while the passenger strand (microRNA*) is quickly degraded. Guide strands starting with a uracil seem to be favoured by AGO[68,69] (Figure 2). The process of selection for the guide strand, which will constitute the mature microRNA, and exclusion of the passenger strand is not very strict. Furthermore, different AGO proteins have been shown to exhibit different selectivity of guide and passenger strands[70]. As such, it is possible that both strands are selected with similar frequencies and for both forms of the microRNA to coexist. Moreover, alternative Drosha cleavage can change the typical composition, and therefore stability, of the pre-miR, which may then lead to different selection of strands within the same organism, although the total contribution of these mechanisms in humans has yet to be clarified[68,69].

After selection of the guide strand by AGO and the detachment of the remaining RLC components, the RNA induced silencing complex (RISC), minimally composed of the mature form single stranded guide microRNA and Ago2, is formed. RISC is then able to target other transcripts based on the complementarity of the seed region of its microRNA (Figure 2). Mature microRNAs are generally stable molecules, with a half-life that may last several hours. Although no universal pathway for microRNA turnover has been discovered, there are several proteins that have been associated with microRNA degradation in human[71]. Still, the mechanisms underlying microRNase activity and microRNA turnover remain, in most cases, elusive. Other endogenous methods of microRNA regulation have been put forward, such as microRNA tailing, RNA editing and RNA methylation. However, it has been shown that microRNAs can be destabilized and degraded when in the presence of artificially synthesized highly complementary oligonucleotides. This technology has been used to exogenously manipulate microRNA levels in functional studies[72–74].

1.2.3. MicroRNA mechanism of action

While there are several hypothesized modes for microRNA action[75], the prevailing model emphasizes the complementarity of the seed with the binding site in a target transcript. In animals, when the microRNA sequence is completely complementary to its cognate in the target transcript, the transcript is cleaved by Ago. Most commonly though, if the sequences do not match perfectly, then there is an activation of the mRNA decay pathways and/or translational repression[76]. Translational repression of target mRNAs may occur through physical inhibition of several steps of the process, while mRNA decay often starts with decapping and deadenylation, which may occur concomitantly with P-body sequestration[76,77]. The contribution of each process to overall microRNA-induced gene silencing is not yet fully understood, although some studies seem to indicate that the properties of the 3' UTR of the target are what determine the path of microRNA action[78]. Interestingly, microRNA-induced gene silencing does not always decrease mRNA levels of the target, suggesting that translational repression acts independently from mRNA decay[79].

One of the major issues with the current knowledge on microRNA functional mechanics is the significant differences between *in vivo* systems and *in vitro* replicas often used for such studies. There are numerous RNA binding proteins that can act synergistically or antagonistically to microRNA in a complex biological environment[80–82]. Not only that, other non-coding transcripts, such as lncRNAs, can also serve as post-transcriptional regulators of both mRNA and microRNA molecules and vice-versa[83–

[86]. Thus, more effort needs to be put into properly engaging gene expression modulation networks as a whole, as opposed to studying individual, isolated molecules in artificial settings.

Given the versatility and widespread action of microRNAs, it is not surprising that there is an increasing body of literature describing them as key players in homeostatic and pathological development of numerous organs and tissues[87]. For example, in cancer, microRNAs that are particularly involved in tumour progression have been dubbed "oncomiRs" and they have gained such notoriety that large efforts have been undertaken in order to categorize cancer types to microRNA expression profiles[88,89]. In cardiovascular disease, microRNAs have also been gaining recognition as main instigators of cardiac dysfunction and heart failure progression[90–93]. However, the role of microRNAs in cardiac endothelial cell health and disease, and their significance for cardiac pathogenesis from an angiogenic perspective remains to be fully established.

1.3. MicroRNA in microvascular health and disease

Endothelial dysfunction has been associated with various cardiovascular risk factors such as hyperglycemia, insulin resistance, elevated free fatty acids, dyslipidemia, inflammation, and high blood pressure[94–97]. Thus, the notion that endothelial cell dysregulation is at the inception of heart failure is not a novel hypothesis[98–101]. However, the mechanisms that lead to a pathological endothelial phenotype, such as accumulation of oxidative damage following shear stress, are now known to also be modulated by microRNA action.

The mechanical forces that the blood exerts on vessels and the signaling events that ensue are collectively named "shear stress". Blood flow can be pulsatile or oscillatory depending on whether it is pumped rhythmically or irregularly, respectively. A laminar, pulsatile flow is responsible for enabling the endothelium to keep its anti-proliferative and anti-inflammatory features. Conversely, turbulent and/or oscillatory blood flow renders the endothelium easily permeable, prone to platelet aggregation, increases SMC growth and overall dysregulates vessel function and remodeling. Endothelial cells are sensitive to shear stress and thus are easily affected by blood flow dynamics[102,103]. One of the most important pathways contributing to shear stress-mediated endothelial cell behavior is the regulation of between nitric oxide availability by eNOS. In the heart, this interaction is key to controlling microvascular remodeling, which determines microcirculation and cardiac tissue perfusion. Interestingly, recent data shows that microRNAs are also involved in shear stress-mediated endothelial responses[104,105].

Transcription factor Krüppel-like factor 2 (KLF2) has been shown to be a master regulator mediating the response of microRNAs to shear stress. Pulsatile flow induces KLF2 expression, which is then able to downregulate miR-214. This microRNA is a negative modulator of eNOS, ergo, KLF2 is able to upregulate nitric oxide synthesis[106]. In addition, KLF2 regulates several other microRNAs, including miR-126, miR-30a, and miR-145[22,107]. Among them, miR-126 is endothelial cell specific and can enhance vascular endothelial growth factor signaling and promote angiogenesis[108].

On the other hand, some microRNAs are also able repress eNOS and are expected to be upregulated with oscillatory flow but downregulated with pulsatile flow[105] (Table 1), thereby promoting a pathological endothelial phenotype. In the microRNA cluster 17-92[109], miR-92a is especially interesting since it can directly target not only KLF2, but also cell cycle-related proteins such as MAP kinase kinase

4 (MKK4) and KLF4[110,111]. Similarly, miR-24 is able to also repress eNOS and p21-activated kinase PAK4 and the endothelial-enriched transcription factor GATA2. The two latter targets of miR-24 are at the center of multiple intersecting pathways regulating apoptosis, cell migration and cell proliferation. The in vivo suppression of either miR-92a or miR-24 has been shown to improve not only vascular function, but also, in the context of cardiac disease, amelioration of heart failure phenotypes following ischemic injury[111,112]. Thus microRNA in an endothelial context are immensely versatile, capable of inhibiting different pathways that ultimately lead to endothelial loss of fitness and dysregulation.

Other ischemia/reperfusion studies have shown that miR-210 is one of the most differentially expressed microRNAs in hypoxic conditions, and is also considered to be a valid biomarker for chronic heart failure[113,114]. The function of miR-210 is, however, debatable. Some groups advocate that this microRNA is pro-angiogenic and anti-apoptotic, through direct interaction with protein tyrosine phosphatase, non-receptor type 1 (Ptp1b) and ephrin A3 (Efna3) (Figure 3), thereby capable of improving cardiac function following artery ligation. In contrast, there is also evidence that this microRNA targets HIF-1α, inhibiting its action with a detrimental response in terms of angiogenesis under hypoxic conditions[115]. Interestingly, HIF-1α has also been proposed as a regulator of miR-210 expression[116]. These diverging results could be a product of different transfection methods and efficiencies and/or technical bias from the procedures[115,117].

Other microRNA species are also known to have profound impact on the cardiac system through modulation of endothelial cell angiogenic profiles. The inhibition of two recently described microRNAs, miR-26a and miR-377, has led to the recovery of left ventricular function after ischemic injury[118,119]. SMAD family member 1 (SMAD1) and serine/threonine kinase 35 (STK35), are respectively targeted by these microRNAs, and are both anti-angiogenic factors functioning through impairment of cell cycle progression (Figure 3)[118–120]. A similar role in the heart is also exerted by miR-34, where its inhibition increases capillary density post-myocardial infarction[121]. Interestingly, this microRNA is also a regulator of cardiomyocyte survival and function, suggesting that it bridges the interactions between microvascular endothelium and the cardiac muscle[122,123].

Another well-known endothelial-enriched pro-angiogenic microRNA, miR-126, is a fundamental player in the maintenance of vascular integrity and function[108]. This microRNA is encoded within the host gene epidermal growth factor like domain 7 (EGFL-7), which regulates tubulogenesis[124]. MiR-126 is able to potentiate endothelial pro-angiogenic pathways in multiple axes. This microRNA is able to directly inhibit sprout-related, EVH1 domain, containing 1 (SPRED1), which is an inhibitor of the extracellular regulated MAP kinase (ERK), and thus prevent its anti-angiogenic action. miR-126 is also a potent anti-apoptotic factor through regulation of the PI3K/Akt pathway[125–127]. Recent findings report a decrease in circulating levels of miR-126 after myocardial infarction, and correlate miR-126 expression profiles to cardiac function indexes, suggesting a vital role of miR-126 in the heart (Figure 3)[125,128].

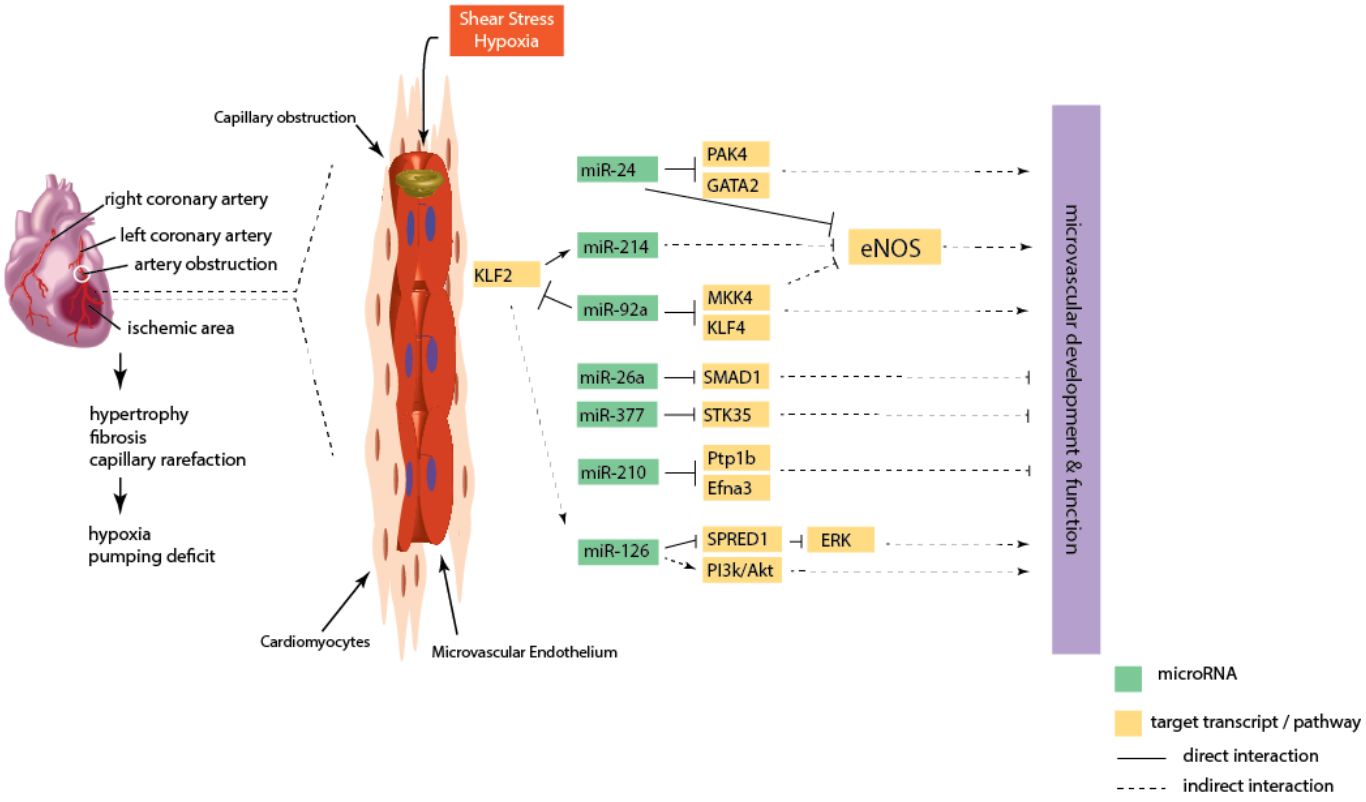


Figure 3. MicroRNA pathways affecting endothelial cell function and microvascular development. In the injured heart, endothelial cells are exposed to pathological stimuli such as hypoxia and abnormal shear stress. In response, endothelial cells change their gene expression profiles through microRNA action. The represented pathways are further discussed in the body of the text.

Several other microRNAs were reported to display differential expression and have biological relevance in ECs when exposed to different types of stress, such as alterations in the blood flow, inflammatory response, hyperglycaemia, and hypoxia (Table 1). While the significance of such findings to the regulation of cardiac microvasculature has not yet been established, one can speculate that many of these microRNAs may also play a role in the remodelling of the injured heart by impacting on EC function.

Table 1. Functional microRNAs in endothelial cells exposed to pathological stimuli prevalent in cardiovascular diseases.

MicroRNA	Targets	Pathological Stimuli	Model	Effect
miR-10a	MAP3K7 TRC	Dysregulated Flow Inflammation	HAEC	Anti-inflammation[129]
miR-15a	FGF2 VEGF	Ischemia	Murine Hindlimb	Anti-angiogenic[130]
miR-15b/16	TNFα/SOCS3*	Hyperglycemia	REC	Anti-apoptotic[131,132]
miR-17-3b	ICAM1	Inflammation	HUVEC	Anti-inflammation[133]
miR-19a	CCND1	Dysregulated Flow	HUVEC	Anti-proliferation[104]
miR-19/221/222	PGC-1α	Inflammation	HAEC	Pro-apoptotic[134]
miR-21	PTEN	Dysregulated Flow	HUVEC	Anti-inflammation[135] Anti-apoptotic
miR-21	PPARα	Dysregulated Flow	HUVEC	Pro-inflammation[136]
miR-23	E2F1*	Dysregulated Flow	HUVEC	Anti-proliferation[137]
miR-27b	Dll4/Notch* PPary*	Ischemia Inflammation	Murine MI Murine Hindlimb	Pro-angiogenic[138]
miR-31	E-SELE	Inflammation	HUVEC	Anti-inflammation[133]
miR-92	KLF2	Dysregulated Flow	HUVEC	Anti-angiogenic[110] Pro-inflammation
miR-100	mTOR	Ischemia	Murine Hindlimb	Anti-angiogenic[139]
miR-101	Cul3	Ischemia	HUVEC	Pro-angiogenic[140]
miR-101	mTOR	Dysregulated Flow	HUVEC	Anti-proliferation[141]
miR-106b-25	PTEN	Ischemia	Murine Hindlimb HUVEC	Pro-angiogenic[142]
miR-107	Dicer1	Ischemia	MCAO mice HUVEC	Pro-angiogenic[143]
miR-126	VCAM1*	Inflammation	HUVEC	Anti-inflammation[144]
miR-132/212	Rasa1 Spred1 Spry1	Ischemia	Murine Hindlimb	Pro-angiogenic[145,146]
miR-155	AT1R VEGFR2	Ischemia	MCAO mice	Anti-angiogenic[147,148]
miR-155	AT1R Ets1	Inflammation	HUVEC	Anti-inflammation[149] Anti-angiogenic
miR-200c	ZEB1	Ischemia	Murine Hindlimb	Anti-angiogenic[150]
miR-221/222	Ets1	Inflammation	HUVEC	Anti-inflammation[149]
miR-223	RPS6KB1	Ischemia	CMEC	Anti-angiogenic[151]
miR-365	Bcl2	Inflammation	HUVEC	Pro-angiogenic[152] Anti-inflammation
miR-424	Cul2	Ischemia	HUVEC	Pro-angiogenic[153]
miR-663	KLF4 CEBPB ATf3	Dysregulated Flow	HUVEC	Pro-inflammation[154]

MAP3K7= Mitogen-Activated Protein Kinase Kinase Kinase 7; TRC=Transfer RNA-Cys; FGF2=Fibroblast Growth Factor 2; VEGF=Vascular Endothelial Growth Factor; TNFα=Tumor Necrosis Factor alpha; SOCS3=Suppressor Of Cytokine Signalling 3; ICAM-1=Intercellular Adhesion Molecule 1; CCDN1=Cyclin D1; PGC-1α=Peroxisome proliferative activated receptor, Gamma, Coactivator 1 alpha; PTEN=Phosphatase and Tensin homolog; PPARα=Peroxisome Proliferative Activated Receptor alpha; E2F1=E2F transcription factor 1;Dll4=Delta-Like 4; PPARy=Peroxisome Proliferative Activated Receptor gamma; E-SELE=E-Selectin; KLF2=Krüppel-like factor 2; mTOR=mechanistic Target Of Rapamycin; Cul3=Cullin 3; Dicer1=Dicer ribonuclease 1; VCAM1=Vascular Cell Adhesion Molecule 1; Rasa1=RAS p21 protein activator 1; Spred1=Sprouty-related, EVH1 domain containing 1; Spry1=Sprouty RTK signalling antagonist 1; AT1R=Angiotensin Receptor 1; VEGFR2=Vascular Endothelial Growth Factor Receptor 2; Ets1=ETS proto-oncogene 1, transcription factor; ZEB1=Zinc finger E-box Binding homeobox 1; RPS6KB1=Ribosomal Protein S6 Kinase, Polypeptide 1; Bcl2=B-cell CLL/lymphoma 2; Cul2=Cullin 2; KLF4= Krüppel-Like Factor 4; CEBPB=CCAAT/enhancer binding protein beta; ATf3= activating transcription factor 3; HAEC=Human Aortic Endothelial Cells; REC=Retinal microvascular Endothelial Cells; HUVEC=Human Umbilical Vein Endothelial Cells; MCAO= Middle Cerebral Artery Occlusion.

2. Experimental Rationale

2.1. Project Objectives

The contribution of angiogenesis to the development of cardiac disease is often overlooked. With this project we meant to shed light on endothelial-specific molecular factors and pathways that may affect microvascular response to stress and ultimately play a role in modulating the angiogenic properties of cardiac ECs. MicroRNAs present themselves as potent and versatile regulators of cell fate and behaviour. Thus our research focused mostly on microRNA regulatory mechanisms of endothelial function.

Therefore we aimed to:

a) Develop and optimize a screening protocol to assess microRNA involvement in proliferation of ECs *in vitro*.
b) Discover novel microRNAs involved in the regulation of the angiogenic potential of endothelial cells.

2.2. Experimental Design

In vivo assessment of vessel properties and behaviour is a very costly and time-consuming ordeal. As such, we opted to perform an *in vitro* study to obtain preliminary data to be further tested in more complex models. However, even *in vitro*, angiogenic function is a complex process that requires multiple assays to evaluate. Having previously found that proliferation of ECs is often a good indicator of angiogenic potential, we used this parameter as a surrogate measure. In order to establish a universal protocol to test the effect of any given anti-microRNA/microRNA mimic in the proliferation of ECs we designed a system based on cell culture and DNA staining, in a 96-well plate format. Total DNA was stained with Hoechst 33342 while replicating DNA also incorporated 5-ethynyl-2-deoxyuridine (EdU), latter marked with a red fluorophore. Ultimately, our result readout relied on differential fluorophore colouring of proliferative nuclei (Figure 4). The cells were imaged with epifluorescence microscopy and the different parameters were analysed with the appropriate software.

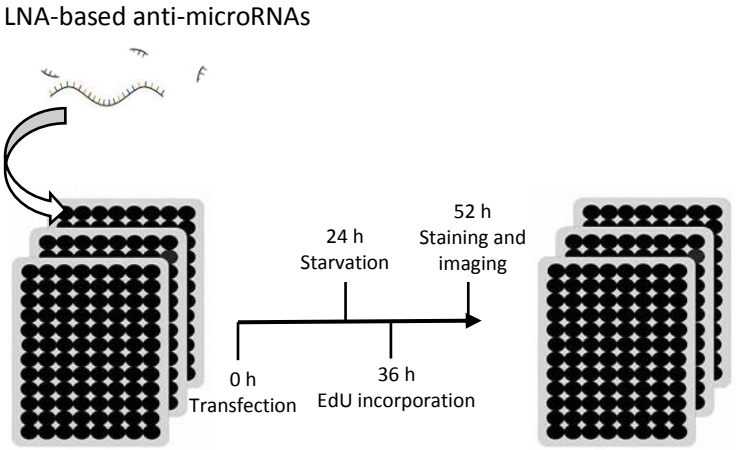


Figure 4. Experimental workflow for the anti-microRNA proliferation screening after seeding of ECs.

3. Materials and Methods

3.1. Optimization of functional screening protocol for microRNAs with proliferative effect on endothelial cells

3.1.1. Optimization of the cell growth medium

a) Cell culture, harvesting and seeding

Human umbilical vein endothelial cells (HUVEC – Lonza) were cultured from passage 2 as per the manufacturer's instructions. Briefly, cells were incubated at 37 ºC, 5% CO_2, in a 5 CellStar 75 cm^2 cell culture flask (Greiner Bio-one) in 10mL of Endothelial Growth Medium 2 (EGM2 – Lonza). Cell growth medium was refreshed 24h after seeding and every 48h thereafter.

Once cells reached approximately 90% confluency, they were harvested using a trypsin-based method. Briefly, medium was aspirated and each flask surface was washed with 5mL of Hank's Balanced Salt Solution (HBSS - ThermoFisher Scientific) and then exposed to 2.5mL of trypsin-ethylenediaminetetraacetic acid (T-EDTA - Lonza) for 3 minutes at 37ºC, 5% CO_2. Trypsin activity was stopped with 2.5mL of trypsin neutralizing solution (TNS – Lonza). The total volume was then taken into a sterile 15mL tube and the remaining contents of the flask were washed with 5mL of HBSS and added to the 15mL tube. The cells in suspension were pelleted through centrifugation at 500 RCF (relative centrifugal force) for 5 minutes. The supernatant was aspirated as before, cells were resuspended in 1mL of EGM2 and subsequently counted automatically.

Afterwards cells were seeded at a working cell density of 2000 cells per well in a 96-well black, clear, tissue culture treated plate (BD Falcon) in 100µL of EGM2.

b) Cell transfection with microRNA inhibitors

Cells were left to acclimatize overnight in the incubator, and transfected the following day as follows. Cell medium was replaced with 75µL of EGM2 per well and the transfection mix was prepared according to the formula:

A=12.4µL of Endothelial Basal Medium (EBM2 – Lonza) + 0.1µL of 50µM microRNA inhibitor (Exiqon)

B=12µL of EBM2 + 0.5µL of Oligofectamine (Invitrogen)

A+B=25µL

After preparing reagents A and B, they were homogenized and let repose for 5 minutes. The final transfection mix (A+B) was let repose for 25 minutes prior to being added to each well. The working concentration of microRNA inhibitor used was of 50nM per well. Each well was either transfected with anti-miR-17 or an inhibitor for a scrambled microRNA sequence as a negative control. Transfection occurred for 24h.

c) Working medium and EdU incorporation

Transfection was stopped by changing the transfection medium to a specific experimental condition medium composition (Table 2, Table 3), and incubating for 16h at 37°C, 5% CO_2.

Table 2. Different condition medium compositions used in the first optimization step of the anti-microRNA screening protocol.

Medium number	Condition Medium Composition	EdU
1	50µL EBM2	50µL EdU in EBM2 (1:1000)
2	49.5µL EBM2 + 0.5µL FBS	50µL EdU in EBM2 (1:1000)
3	49µL EBM2 + 1µL FBS	50µL EdU in EBM2 (1:1000)
4	45µL EBM2 + 5µL FBS	50µL EdU in EBM2 (1:1000)
5	49µL EBM2 + 1µL FGF	50µL EdU in EBM2 (1:1000)
6	48.5µL EBM2 + 0.5µL FBS + 1µL FGF	50µL EdU in EBM2 (1:1000)
7	48µL EBM2 + 1µL FBS + 1µL FGF	50µL EdU in EBM2 (1:1000)
8	44µL EBM2 + 5µL FBS + 1µL FGF	50µL EdU in EBM2 (1:1000)
9	49µL EBM2 + 1µL VEGF	50µL EdU in EBM2 (1:1000)
10	48.5µL EBM2 + 0.5µL FBS + 1µL VEGF	50µL EdU in EBM2 (1:1000)
11	48µL EBM2 + 1µL FBS + 1µL VEGF	50µL EdU in EBM2 (1:1000)
12	44µL EBM2 + 5µL FBS + 1µL VEGF	50µL EdU in EBM2 (1:1000)
13	48µL EBM2 + 1µL FGF + 1µL VEGF	50µL EdU in EBM2 (1:1000)
14	47.5µL EBM2 + 0.5µL FBS + 1µL FGF + 1µL VEGF	50µL EdU in EBM2 (1:1000)
15	47µL EBM2 + 1µL FBS + 1µL FGF + 1µL VEGF	50µL EdU in EBM2 (1:1000)
16	43µL EBM2 + 5µL FBS + 1µL FGF + 1µL VEGF	50µL EdU in EBM2 (1:1000)

Table 3. Stock and final concentrations of the components of the condition mediums tested.

Component	Stock concentration	Work concentration	Manufacturer
FBS	100%	0 to 5%	Gibco
FGF	10ng/µL	10ng/mL	Promega
VEGF	50ng/µL	50ng/mL	Peprotech
EdU	10mM	5µM	Life Technologies

The final setup of the 96-well plate is indicated in Figure 5. Each condition was performed in triplicate.

d) Staining protocol

After 16h in growth medium with EdU, cells were washed with 3% Bovine Serum Albumin (Sigma-Aldrich), fixated with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Igepal in PBS1X for 20 minutes. The labelling of EdU with the fluorophore Alexa 594 was done with the Click-IT kit (Life Technologies) as per the manufacturer's instructions.

Finally, cells were incubated for 10 minutes with 40µL of Hoechst 33342 (5µM, 1:1000 in PBS, Life Technologies C10339). Preservation

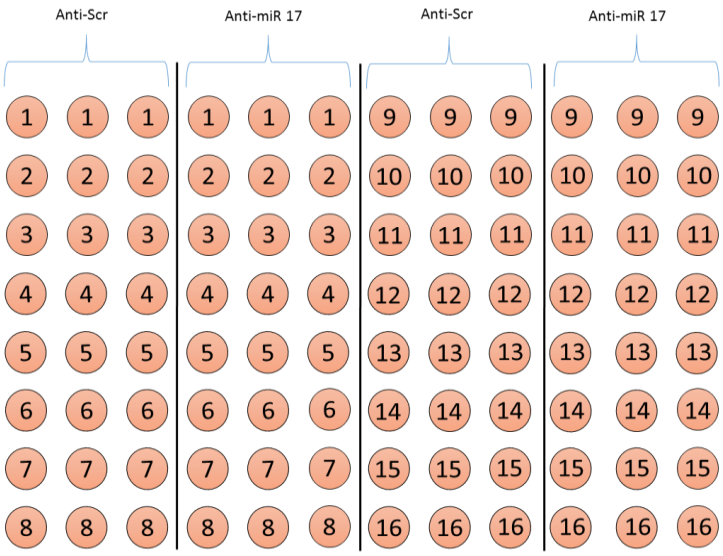


Figure 5. Experimental setup of the first optimization experiment, testing the effect of different condition medium compositions and the effect of anti-miR-17 in the proliferation of endothelial cells.

medium (10% Glycerol, 1% Gentamycin in PBS) was added to each well afterwards.

e) Image acquisition and analysis

Nikon ECLIPSE Ti epifluorescence microscope was used to acquire the images for the screening experiments. Excitation wavelengths were 549nm for the EdU staining and 390nm for Hoechst. Five pictures were acquired per well per wavelength. A 10x magnification was used at all times.

Images were analysed with FIJI software[155]. The number of nuclei was determined based on Hoechst staining, while the number of proliferative cells was determined based on EdU staining, through a custom-made macro for particle analysis:

```
dir = getDirectory("path");
list = getFileList(dir);

for (i=0; i<list.length; i++)
{
  if (endsWith(list[i], ".tif"))
  {
      open(dir + list[i]);
      run("Duplicate...", " ");
      run("8-bit");
      run("Gaussian Blur...", "sigma=2");
      setAutoThreshold("Default dark");
      getThreshold(lower,upper);
      setThreshold(41,255);
      run("Convert to Mask");
      run("Watershed");
      run("Analyze Particles...", "size=30-Infinity show=Nothing display clear
include");
      close();
      name=getTitle;
      IJ.renameResults(name);
      close();
      }
}
```

For all wells analysed, mean and standard deviation of total number of nuclei and total number of proliferative nuclei over 3 replicates was determined. A proliferation ratio was achieved for each condition after averaging the results of the replicates. Unpaired T-Test with a p value lower than 0.05 was deemed statistically significant.

3.1.2. Synchronization of the cell cycle

The protocol followed was identical to that described in 3.1.1. with the exception that an extra step was added after point b). After transfecting the cells for 24 hours, they were incubated at 37°C, 5%

CO_2, in starvation medium (EBM2 with 0.1% FBS) for 12 hours, in order to stop proliferation and synchronize cell cycles.

3.1.3. Optimization of the transfection time

The protocol followed was identical to that described in 3.1.2. with the exception that three 96-well plates were tested for different transfection times (24h, 48h and 72h). Additionally, the condition medium compositions tested comprised only the poorest medium (medium 1), an intermediate medium (medium 2) and the two most enriched mediums (medium 3 and 4) (Table 4). In addition to the transfection with anti-miR-17, cells were also transfected with anti-miR-19a, anti-miR-126a. An untransfected condition was also present (Figure 6).

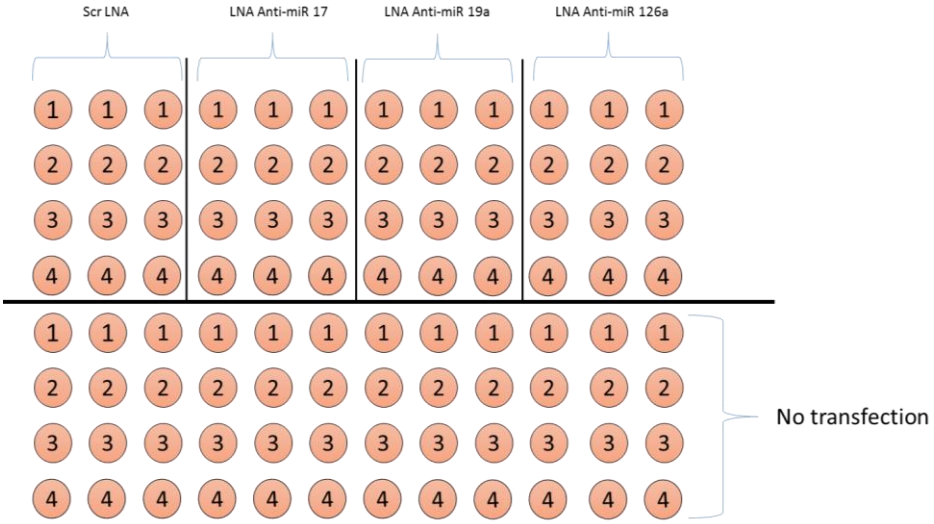


Figure 6. Experimental setup of the third optimization experiment, testing the effect of different medium compositions, different transfection times and the effect of anti-miR-17, anti-miR-19a and anti-miR-126a in the proliferation of endothelial cells.

Table 4. Different medium compositions used in the third optimization step of the anti-microRNA screening protocol.

Medium number	Working Growth Medium Composition	EdU staining
1	50µL EBM2	50µL EdU in EBM2 (1:1000)
2	49.5µL EBM2 + 0.5µL FBS	50µL EdU in EBM2 (1:1000)
3	43µL EBM2 + 5µL FBS + 1µL FGF + 1µL VEGF	50µL EdU in EBM2 (1:1000)
4	50µL EGM2	50µL EdU in EBM2 (1:1000)

3.1.4. Optimization of cell seeding density

The protocol followed was identical to that described in 3.1.3. for the conditions transfected for 24 hours, with the exception that only the two most enriched medium compositions were tested (mediums 3 and 4, Table 4). Additionally, four different cell seeding densities were tested (2000, 5000, 7500 and 10000 cells per well) (Figure 7).

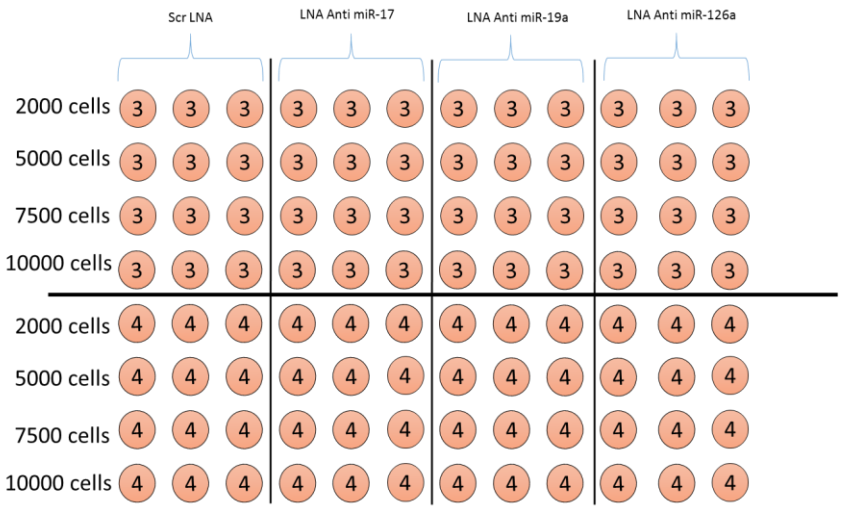


Figure 7.Experimental setup of the fourth optimization experiment, testing the effect of different medium compositions, different cell seeding densities and the effect of anti-miR-17, anti-miR-19a and anti-miR-126a in the proliferation of endothelial cells.

3.2. Functional screening

The final protocol used to screen through the miRCURY LNA™ microRNA Inhibitor Library – Mouse (Exiqon, #190202-2) was the sum of the previous optimization steps previously described. Briefly, HUVECs were grown until optimal confluency in EGM2 and were then seeded at a density of 5000 cells per well in all the wells of eleven 96-well plates corresponding to the eleven anti-microRNA plates in the inhibitor library. Cells were left to acclimatize overnight and were transfected for 24 hours with 5µL of 1µM stock concentration of microRNA inhibitors. After transfection, cells were starved for 12 hours and then incubated in condition medium (5% FBS, 50ng/mL VEGF and 10ng/mL FGF in EBM2) with EdU for 16 hours. The cells were then fixated, permeabilized and stained with Hoechst 33342 (all nuclei) and Alexa 594 (EdU). The imaging process was semi-automated, as the image acquisition was performed independently by the microscope while the changing of each plate was done manually. Exposure time for each picture was 100ms. Five pictures per channel, per well were acquired as previously described.

Image processing was done with ImageG, raw data was treated in Microsoft Office Excel and analysed with Prism (GraphPad). Total nuclei and proliferative nuclei were counted based on the respective staining and proliferation ratios were calculated for all conditions.

3.3. Screening validation and microRNA functional assessment

Based on the immediate availability of microRNA inhibitors/precursors and previous experimental data, miR-138-5p, miR-219a-5p, miR-335-5p and miR-1982-3p were selected for further study and validation of the functional screening. HUVECs were grown and transfected with the precursors to the listed microRNAs as described in 3.1.1. with the exception that cells were grown in 6-well plates and the quantities were scaled appropriately to maintain the same reagent working concentrations. The overview of the protocol used for all assays is displayed on Figure 8.

One extra plate per condition was prepared in the same way for RNA isolation for subsequent experiments.

3.3.1. Scratch Wound assay

In order to establish reference points for area measurements, the underpart of the wells in a 48-well plate were scratched with razor to draw two parallel stripes on, per row. Afterwards, 100000 transfected cells were seeded per well, in EGM2, and incubated in standard conditions overnight until confluent. Each condition was seeded in six replicate wells. The bottom of the confluent wells was scratched so that a rift was created between two hemispheres of cells. At the moment of the scratch, the medium on every three replicates was changed to either EBM2 with 5% FBS or EBM2 with 5% FBS, 50ng/mL VEGF and 10ng/mL FGF. For each well one picture was taken right after each scratch and 8 hours later. The distance closed by cell migration was measured as a percentage of the initial gap.

3.3.2. Proliferation assay

For the proliferation assay, 5000 transfected cells were seeded per well in EGM2 and left to acclimatize and adhere to the 96-well plate. After 5 hours, the medium on each three replicates was changed to either EBM2 with 5% FBS or EBM2 with 5% FBS, 50ng/mL VEGF and 10ng/mL FGF. EdU was added as previously described to each well. Cells were left to proliferate in standard conditions for 16 hours. The fixation and staining protocol was performed as described in 3.1.1. d) and image analysis was done as described in 3.1.1. e).

3.3.3. Tubulogenesis assay

An artificial extracellular medium (Matrigel – Cornings) was thawed overnight at 4°C and then 50uL was of the liquid matrix was plated on a 96-well plate prior to the start of the assay. After 1h of incubation at 37°C to solidify the matrix, cells were seeded in three replicates per condition, 20000 cells per well, in EGM2. After a 16h incubation in standard conditions, each well was analysed for the formation of vessel-like structures.

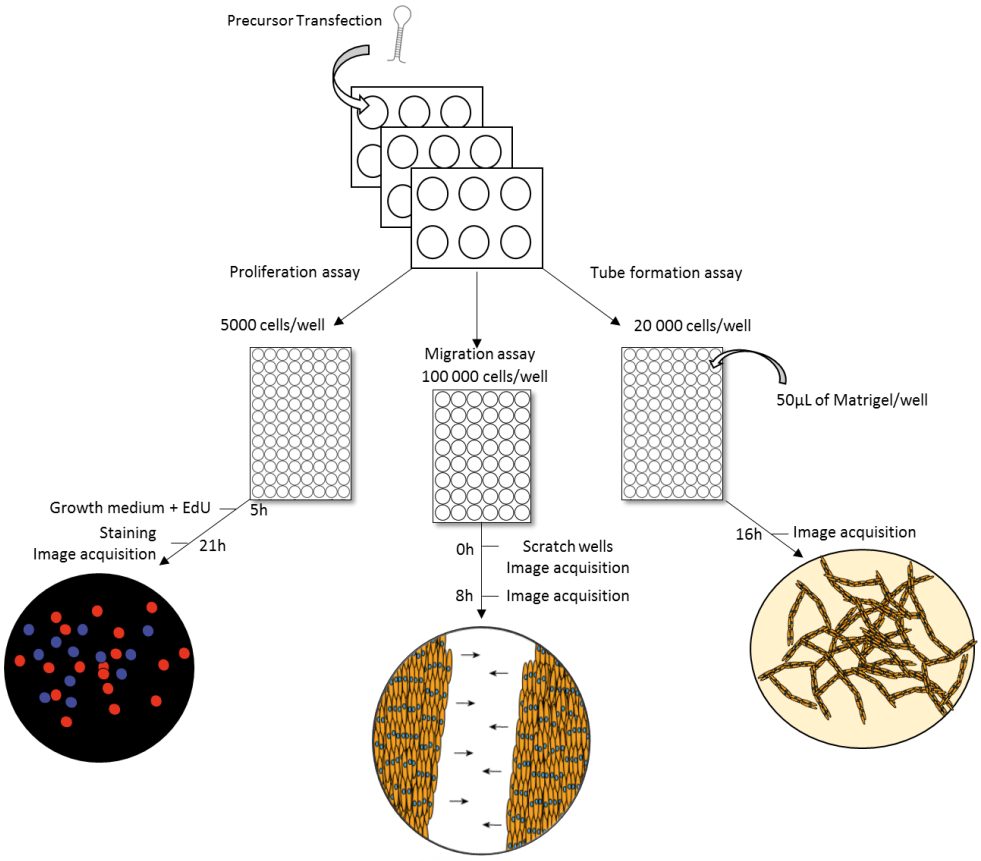


Figure 8. Overview of the protocols for the functional validation of the microRNA screening for angiogenic function.

3.3.4. microRNA target prediction and assessment

Functional targets for miR-219a-5p were predicted based on a bioinformatic analysis. First, miRwalk 2.0[156] was used to identify target genes predicted by a number of databases. Then, using Targetscan[157], we confirmed the number of conserved binding sites in human, mouse and rat for the predicted targets that were commonly identified by 6 or more databases. From those, we restricted the list by positively selecting genes that had been previously reported in the literature as having a role in autophagy and/or cell proliferation.

3.3.5. RNA isolation and gene expression assessment

For the analysis of gene expression, total RNA was isolated with the Direct-Zol RNA MiniPrep kit (Zymo Research, #R2050-2) through successive washing, purification and centrifugation of the total RNA in a column binding system. Approximately 20ng/reaction were used to generate cDNA with the with miRCURY LNA™ microRNA PCR, Polyadenylation and cDNA synthesis kit II (Exiqon, #203301) and 1ug/reaction was used for cDNA synthesis with the miScript II RT Kit (Qiagen, # 218160). qPCR was performed in duplicate by using both ExiLENT SYBR® Green master mix kit (Exiqon, #203421), for microRNA quantification, or the miScript II (Qiagen) kit, for mRNA amplification. Gene expression was normalized to either 5S for microRNA or L7 for mRNA.

4. Results and Discussion

4.1. Proliferation assay protocol optimization

Angiogenic assays are a powerful tool providing valuable information regarding the potential of any given compound towards vessel health and development. *In vitro* models are largely imperfect when compared to the complexity of *in vivo* assays, however they can provide useful clues as preliminary data that can be further pursued[158]. The three "classical" *in vitro* angiogenic assays are the migration, tube formation and proliferation assays. It would be unfeasible to assess the effect of all microRNA inhibitors in the library (Exiqon) with all assays, therefore we chose proliferation as a surrogate measure of overall angiogenic potential. Proliferation assay with EdU incorporation measures DNA replication, as EdU is an analogue of thymidine and is incorporated during active DNA synthesis. Other thymidine analogues such as bromodeoxyuridine (BrdU) require DNA to be denaturated in order to be tagged, making EdU the preferred reagent for this method. Moreover, while cell division itself is only one of the hallmarks of angiogenesis, the versatility and room for fine-tuning the protocol made it the most suitable for our purposes. Furthermore, the possibility of adjusting the assay to replicate it *in vivo* makes for a more consistent long-term approach[159,160].

Subsequently, our first objective was to develop a standard cell proliferation protocol and analysis so that the effect of microRNA inhibition would be emphasized. The starting point for this process was the identification of the optimal condition medium. We tested the effect of 16 different medium compositions (Table 2) on the proliferation of HUVECs (Figure 9a). Additionally, we set out to also evaluate the effect of microRNA inhibition in our experimental setup, through impairment of miR-17 and the appropriate scrambled control. MiR-17 is a particularly interesting microRNA in the context of endothelial proliferation due to a conundrum in the literature regarding its function. Some authors postulate that this microRNA, widely prevalent in cancer, is pro-angiogenic[161,162], while other studies refute this hypothesis[163,164]. We performed the staining of total nuclei with Hoechst 33342 and of proliferative nuclei with EdU, in order to calculate proliferation ratios for each condition (Figure 9a,b).

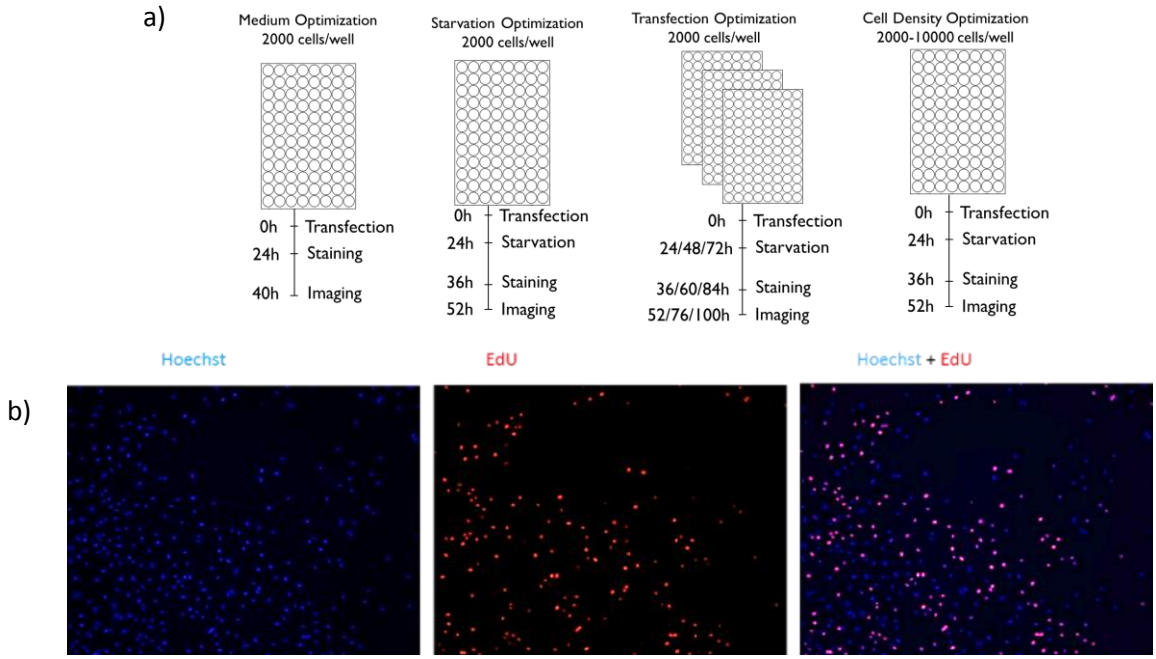
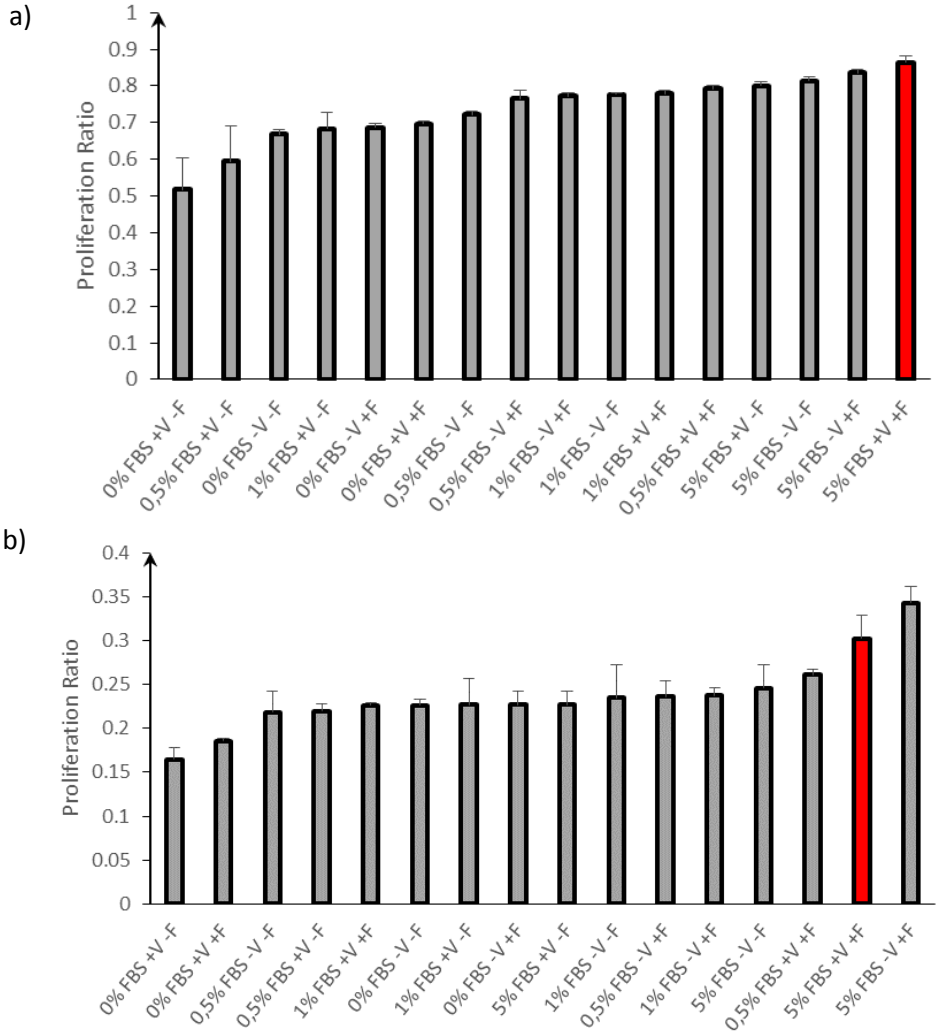


Figure 9. Optimization of the protocol for the proliferation assay-based anti-microRNA screening. (a) Workflow of the four different optimization steps. (b) Representative image of the double staining performed.

Our results showed that the proliferation ratios in all conditions were above 50% (Figure 10a). As all conditions exhibited high proliferation values, and the effect of anti-miR-17 could not be appreciated in any medium (Figure 10c), we suspected that the microRNA and/or medium effect could be masked. This led us to theorize that the cells were able to easily cope with the 16h incubation in different mediums due to the fact that they had been previously exposed to EGM2, a very rich medium, for long periods of time. The cell line in study is very sturdy, with high endogenous proliferative and survival rates. One way of increasing the time gap between medium changes and also synchronizing cell cycles is by starving the cells[165,166]. We hypothesised that by introducing a starvation period of 12h we would be able to decrease proliferation rates and thereby making the effects of medium compositions and microRNA more evident.

After starvation, proliferation values dropped dramatically (Figure 10b). Additionally, we observed that inhibition of miR-17 resulted in a decrease in proliferation dependent on medium richness (Figure 10d). The more complete the medium, the greater the effect of the microRNA inhibitor. Thus, our findings were in line with the hypothesis that miR-17 is indeed pro-angiogenic[167]. Moreover, the most enriched appeared to be the optimal for cell proliferation while potentiating microRNA effect analysis. Therefore, in subsequent experiments we focused on this medium composition (EBM2 with 5% FBS, 50ng/mL VEGF and 10ng/mL FGF), while comparing it with the commercially available EGM2 and also poorer compositions.



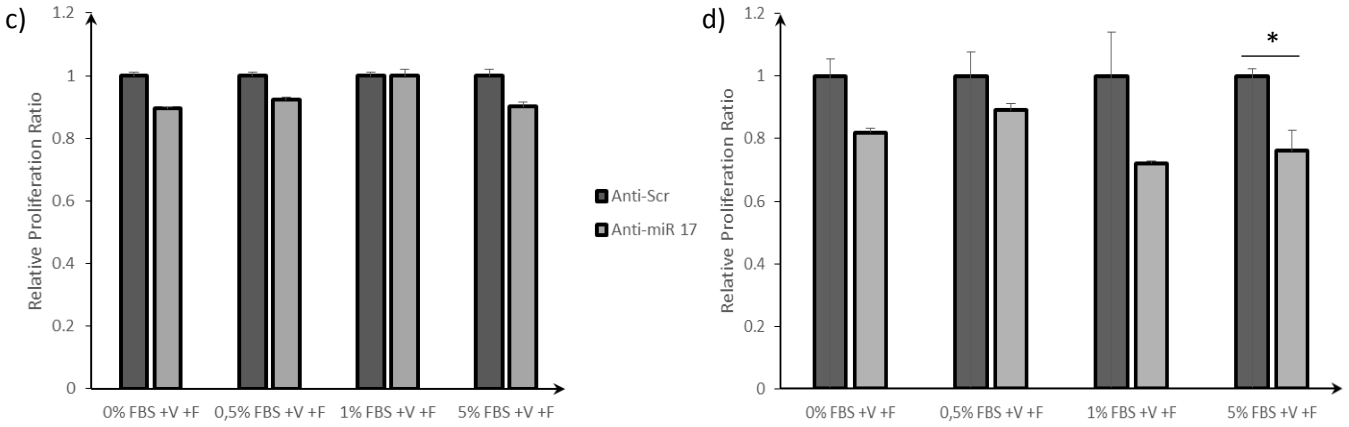


*Figure 10. Optimization of the protocol for the proliferation assay-based anti-microRNA screening. (a & b) Proliferation ratios in anti-scramble treated conditions for different medium compositions before and after starvation, respectively. Red highlights the most enriched mediums. (c & d) Proliferation ratios for anti-miR-17 versus anti-scramble treated conditions for enriched medium compositions before and after starvation, respectively. V=VEGF; F=FGF. *P<0.05, number of replicates = 3. Error bar represents S.E.M. Statistical significance assessed with unpaired Student's ttest.*

We also planned to find the optimal transfection time for microRNA inhibitor. Our protocol used a 24h incubation period with the anti-miR and the carrier molecule, but other studies also use longer transfection periods[168–170]. Thus we tested transfection times of 24h, 48h and 72h. Also, we wanted to test more microRNA inhibitors to confirm that our protocol is transversal. We decided to impair miR-19a, which shares the same cluster as miR-17, and miR-126a, an endothelial-enriched microRNA widely known to be a potent pro-angiogenic factor[171,172] (Figure 11a).

Overall proliferation values were in line with the previous optimization step, but only for the 24h transfection. Interestingly, proliferation rates decreased significantly from the shorter to the longer time points. Additionally, the proliferative phenotype of HUVECs where miR-17/19a were inhibited was the opposite of what was previously found, with an increase of proliferation above control levels. Nevertheless, impairment of miR-126a was sufficient to markedly repress proliferation, particularly with longer transfection times. Another notable consideration is that the levels of control proliferation in 48h and 72h transfection were very low (around or below 10%) (Figure 11a). It was plausible that a low endogenous control would skew our results too much, so while we strived to emphasize microRNA effect on proliferation, we chose to try developing a similar pattern, but with higher baseline proliferation rates. The fact that there were more cells but decreased proliferation rates led to us to believe that this was a side effect of higher confluency of the cells in longer transfections versus a lower cell density in shorter transfections. Thus, we hypothesized that higher cell densities were capable of reducing the intrinsic proliferative capacity of HUVECs and thereby emphasizing the microRNA effect. One possible mechanism that could account for this difference is the contact inhibition model, according to which if cells are already at a high confluency they will upregulate pathways repressing further growth[173]. Thus, it is also possible that the differences observed in the effect miR-17 inhibition were also due to a shift in overall endothelial phenotype, as miR-17 seems to be involved in proliferation and cell cycle progress, which are affected by high cell densities[174,175].

For our last optimization step, cells were seeded at different densities in order to check whether that would impact their proliferation as expected. Indeed, high seeding densities reduced proliferation significantly when compared to the condition where 2000 cells/well were previously used. However, it was unexpected that the baseline proliferation rates were considerably lower in this experiment compared to the previous assays. This result seems to indicate that there can be a large variability

between independent experiments, or possibly it hints at a technical error not accounted for. Very high seeding densities (7500 or 10000 cells per well) resulted in extremely low levels of control proliferation (less than 5%), greatly skewing microRNA inhibition results. Nevertheless, a seeding density of 5000 cells per well emphasized the largest differences between control and anti-microRNA action without fully compromising baseline proliferation (Figure 11b). Therefore, our final test condition consisted of a cell seeding density of 5000 cells per well followed by a 24h incubation with the transfection solution.

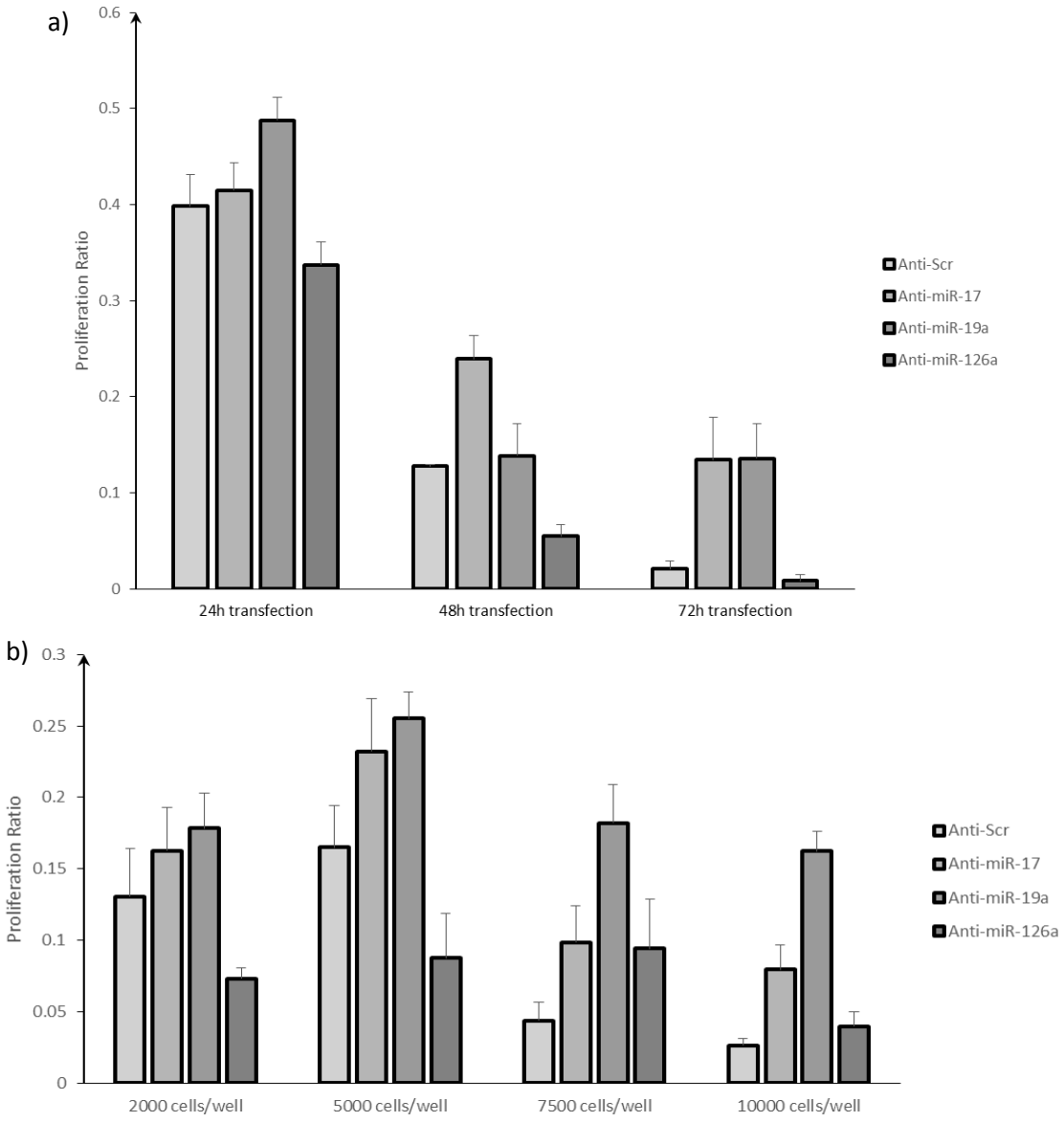


*Figure 11. (a) Proliferation ratios for anti-scramble, anti-miR-17, anti-miR-19a and anti-miR-126a for 5% FBS enriched with VEGF and FGF, after 24, 48 and 72h transfection. (b) Proliferation rates for anti-scramble, anti-miR-17, anti-miR-19a and anti-miR-126a for 5% FBS enriched with VEGF and FGF, with varying cell seeding densities. *P<0.05, number of replicates = 3. Error bar represents S.E.M. Statistical significance assessed with unpaired Student's t test.*

4.2. microRNA inhibitor screening

We screened through a microRNA inhibitor library (Exiqon) in order to discover novel microRNAs involved in the modulation of endothelial cell proliferation. The inhibitors in this library were synthesized to have a locked nucleic acid (LNA) structure. LNA-based oligonucleotides are modified RNA molecules which possess an extra bond between the 2' oxygen and the 4' carbon. This extra

bridge stops most duplex formation, making the ribose inaccessible, which increases the sensitivity and specificity of binding of an LNA molecule. As such, it is the preferred technology for microRNA-based assays, where the importance of the added specificity is essential when working with short sequences and low endogenous expression levels of many microRNAs[176,177].

The protocol followed was derived from the previous optimization experiments in order to magnify the effect of the inhibition of microRNA molecules (Figure 4, 12a). In each 96-well plate used for the screening, extra positive (anti-hsa-miR-126a-5p) and negative (anti-Scr) conditions were introduced. Additionally, an inhibitor against a scrambled sequence was tagged with a green fluorescent probe and tested in triplicate in a single plate, revealing a high transfection efficiency (Figure 12b). We obtained information on the effect of the 753 microRNA inhibitor compounds tested on the proliferation of HUVECs. The screening experiment met several technical issues that undermine the robustness of our analysis. Most worryingly, we suspect that our positive and negative controls did not work properly as the proliferation ratios found across these conditions was much higher than expected, even more so than baseline proliferation ratios of untransfected wells. However, as all 96-well plates had untransfected conditions, we used those as a pseudo-control in order to account for inter-plate variability. Moreover, it can be argued that the inhibition of a microRNA that boosts proliferation values above wild type cells or reduces it the most will be more suitable for further study. Thus, as the proliferation ratio for different conditions was obtained as an absolute number, that is, independent from the results of other conditions, only our comparative analysis became more stringent than previously anticipated when comparing each result to untransfected wells rather than anti-scramble.

Nevertheless, since the microRNA inhibitor library also included the previously tested inhibitors against microRNAs 126a, 19a and 17, we were able to find that our results were consistent with those observed in our optimization steps (Figure 12c,d). While the effect of the inhibition of miR-17 and miR-19a does not seem to be highly significant, it still remains above our control proliferation, supporting the notion that these microRNAs are by default anti-proliferative. On the other hand, despite our positive control, exogenous to the screening, not working, the inhibition of miR-126a found in the library resulted in almost 50% decrease of proliferation of wild type cells, confirming the pro-angiogenic function of this microRNA.

By plotting the proliferation ratios of each microRNA inhibitor tested as a fold variation of control proliferation one is able to draw several insights (Figure 12e). First and foremost, one can appreciate which microRNAs are more impactful to proliferation. For example, anti-miR-150-5p and anti-miR-191-5p are two of the microRNA inhibitors whose action results in a more pronounced induction of proliferation above control levels, with over twofold increase. Additionally, it is clear that a disproportionate majority of the anti-miRs tested reduces proliferation, as opposed to those that stimulate cell division. This pattern can be explained by the fact that many of the mature microRNA targeted by the inhibitors are not highly expressed in human ECs, rendering the inhibitor non-functional. However, transfection itself is known to decrease cell fitness, therefore, it is plausible that for all those microRNA inhibitors without a clear role in HUVECs, the detrimental impact of the transfection protocol is the cause of the reduced proliferative phenotype observed.

Moreover, raw proliferation ratios are not the only indicator of cell growth that one can derive from our screening experiment. By plotting the total number of cells (Hoechst positive) versus the total number of proliferative cells (EdU positive), one can better appreciate the impact that each condition

has, not only in proliferative ratios, but also in raw cell count (Figure 12f). It is interesting to observe that some microRNA inhibitors, despite inducing high proliferative ratios, decrease total cell count. Likewise, some other inhibitors are able to greatly increase the total number of cells but do yield higher proliferative ratios. This seemingly paradoxical effect can be explained either by technical reasons, biological phenomena or a combination of both. A different cell seeding density due to pipetting errors could induce this type of result, as previously mentioned. Similarly, the action of a microRNA inhibitor that allows for a cell to easily cope with the 12h starvation period could also result in a higher cell count prior to EdU incorporation, skewing our analysis. Therefore, it is essential that, in order to pick a good candidate microRNA for further studies, one takes into account the different cellular dynamics. The overall distribution of each microRNA inhibitor tested is represented and divided in four quadrants. These quadrants were drawn by extending the average of all control conditions across the experiment. Thus, microRNA inhibitors in quadrant I are those that are able to increase total cell count, albeit without significant increase in proliferation ratios; those in quadrant II are detrimental to both total cell count and proliferation; those in quadrant III are beneficial to both features and those in quadrant IV are beneficial to proliferation but some effect causes the total cell count to drop. An interesting example of this dichotomy is miR-150-5p which boasts the highest proliferation ratio in the experiment (2.34 fold increase over control) and one of the lowest total cell counts as well (1109 Hoechst positive cells). This microRNA has been reported to directly inhibit VEGF, which is an essential growth factor for endothelial cell proliferation and survival, and a major component of our medium compositions[178]. Additionally, it can also target other pro-angiogenic factors such as GRB2-associated-binding protein 1 (GAB1) and Forkhead box protein P1 (Foxp1), further attesting to its anti-angiogenic prowess[179]. Thus, by inhibiting this microRNA, it is expected that we have a high increase in proliferation, as observed, and therefore also a high total cell count. While it is possible that other targets of this microRNA are involved in cell death/survival and thus an abnormal combination of effects is reached (other reports relating this microRNA to cancer inhibition support this hypothesis[180–182]), it is more likely that the low cell count in this particular case is an experimental artefact and an aberration.

Other microRNAs, however, induce the expected phenotype, such as members of the let-7 family (Figure 12f). This large family of microRNAs is known to be pro-apoptotic and anti-proliferative, thus being very relevant in the context of cancer research[183–186]. Therefore, it is expected that upon its inhibition cell survival and mitosis are upregulated, as we can appreciate in our results where we find six members clustered together in quadrant III.

One of the more interesting results pertains to a microRNA that was also found in another screening previously performed by our group in order to find autophagy-related microRNAs. Inhibition of miR-219 was found to markedly increase autophagy in cardiomyocytes. There is a large body of evidence showing the inverse correlation between cell proliferation and autophagy regulation[187,188]. Since our results show that inhibition of miR-219 causes proliferation decrease (Figure 12e) they are, therefore, in line with the autophagy-related findings. Thus, we hypothesize that miR-219 might be involved in either or both of these pathways and decided to further assess its functional properties in endothelial cells.

Ultimately we decided to validate our results by repeating the proliferation assay for several microRNA and complementing them with other functional angiogenic assays.

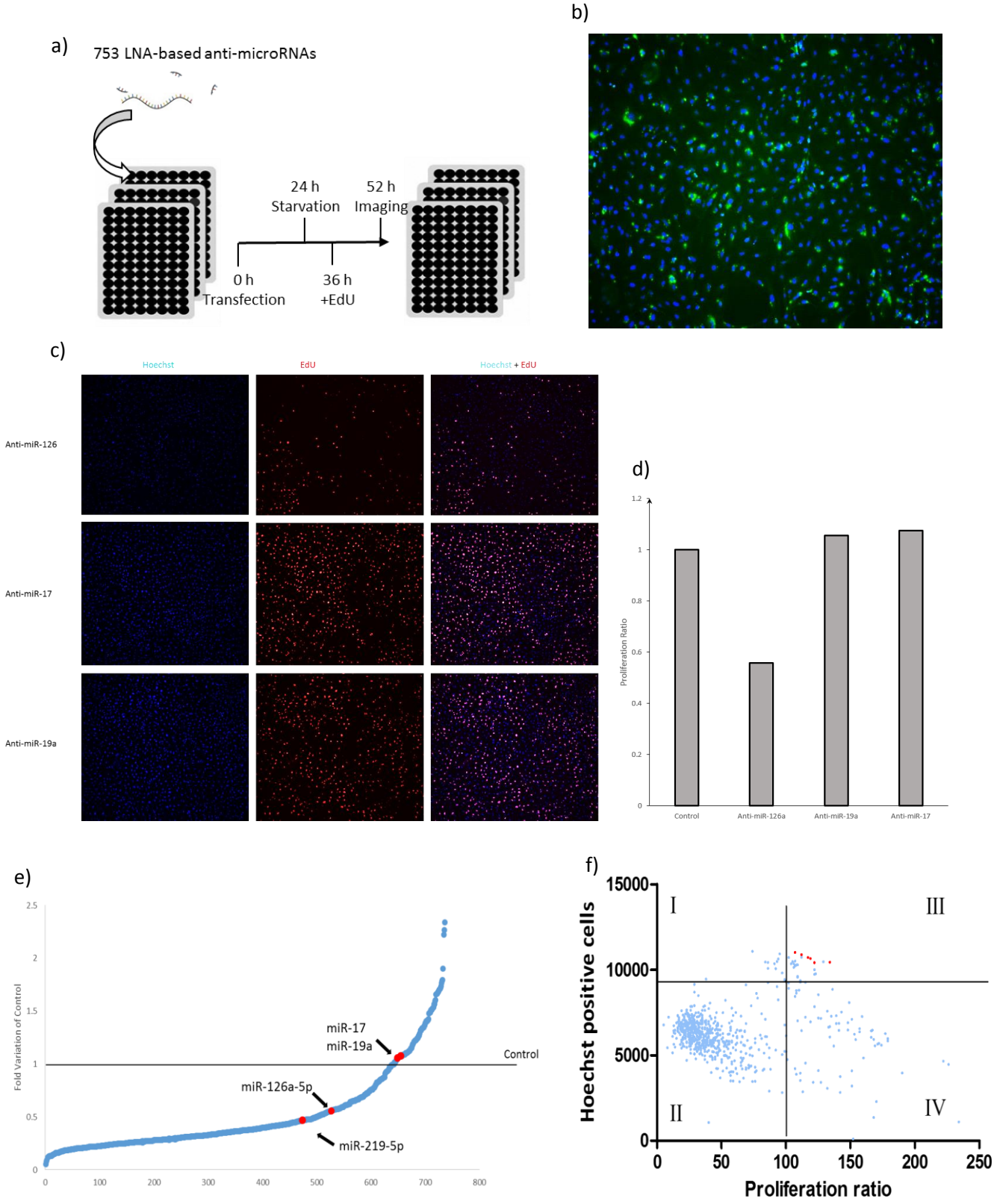
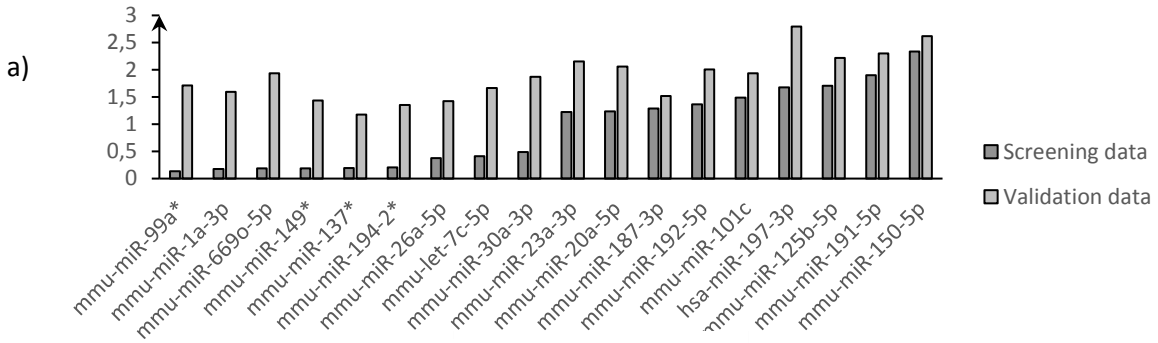


Figure 12. Functional proliferation screening of microRNA inhibitors on HUVECs. (a) Protocol overview. (b) [Transfection efficiency] (c&d) Representative image of the double staining performed for miR-126a-5p, miR-19a and miR-17-5p (c) and respective quantification of the proliferation ratios (d). (e) Fold variation of proliferation ratios for each microRNA inhibitor in the screening, relative to control. (f) Total cell count (Hoechst positive cells) versus proliferation ratio for each condition measured as fold variation of control. The dark lines represent an extension of the control results, in red let-7 family members.

4.3. Functional validation of the screening

We selected 18 microRNA inhibitors from our screening and repeated the proliferation assay as described, in order to confirm our results (Figure 13a). We calculated the proliferation value normalized to each plate's control for each condition in order to better compare the inter-experiment groups. The overall trend (pro or anti-angiogenic effect) was not very robust, as all of the microRNA inhibitors caused a pro-angiogenic phenotype in our validation assay. This is explained by a technical error since the final concentration of VEGF used was five times what had been used in our previous experiments, thus markedly increasing proliferation rates. Another reason why some of the results may be highly variable is the fact that for microRNAs which are endogenously expressed at very low levels (marked with *), the effect of their inhibition is by default extremely volatile. Interestingly, we were also able to determine that on this experiment, the inhibition of miR-150-5p did retain the expected high proliferation ratio and also a high total cell count, confirming our hypothesis that the low cell count previously observed was the product of a technical error and not the effect of the microRNA inhibitor itself.

The next logical step was to select a few microRNAs of interest based on the results of the screening and further validate them functionally through assessment of other angiogenic properties influenced by the modulation of microRNA levels. Due to logistic and resource limitations, we were only able to select 4 microRNAs, based on the list of those readily available in our laboratory. The microRNAs selected were: miR-138-5p, miR-219a-5p, miR-335-5p and miR-1982-3p. The cellular role of miR-219-5p was first hinted at in the autophagy screening performed by our group, as previously mentioned. We also chose miR-1982-3p because it is not expressed in human cells, therefore serving as a negative control. Finally, miR-138-5p and miR-335-5p were found to be moderately anti and pro-proliferative respectively, in our screening and could potentially serve to further confirm the quality of proliferation measurement as a surrogate for angiogenic potential. We transfected HUVECs with precursors of the aforementioned microRNAs and then performed proliferation, scratch wound and tube formation assays. The angiogenic assays were performed in the same conditions as the screening, with the clear difference that microRNA precursors, not inhibitors, were used this time. First we confirmed transfection through qPCR and observed high upregulation of all microRNA precursors tested (Figure 13b). In the proliferation assay (Figure 13c), as expected, overexpression of miR-219-5p resulted in significant increase in proliferation while the overexpression of miR-138-5p resulted in proliferation decrease. The effects of the pre-miR-335-5p treatment were not significant due to high inter-replicate variation, but there seems to be a trend towards proliferation increase. Surprisingly, overexpression of miR-1982-3p caused a significant decrease in cell proliferation. This result attests to the idea that overexpressing exogenous microRNAs in a cell is not always innocuous, with a risk for potential side effects. We expected comparable results in the remaining assays, so that overexpression of miR-219-5p and miR-335-5p would increase migratory and tubulogenic abilities of HUVECs, while miR-138-5p would antagonize them. However, the scratch wound assay (Figure 13d) and the tube formation assay (Figure 13e) results were largely inconclusive, with high intra-condition variances. We see, however, the same phenotypical trends in the scratch wound assay as in the proliferation assay. Further repetition of the experiment with a substantial increase in replicates could increase the coherence and robustness of the results, adding more statistical power to their computation. Out of all the microRNAs tested, miR-219 showed the most promising results, therefore we evaluated its function further through repetition of the angiogenic assays and also by determining its functional targets.


a)
3
2,5
2
1,5
1
0,5
0
mmu-miR-99a*
mmu-miR-1a-3p
mmu-miR-669o-5p
mmu-miR-149*
mmu-miR-137*
mmu-miR-194-2*
mmu-miR-26a-5p
mmu-let-7c-5p
mmu-miR-30a-3p
mmu-miR-23a-3p
mmu-miR-20a-5p
mmu-miR-187-3p
mmu-miR-192-5p
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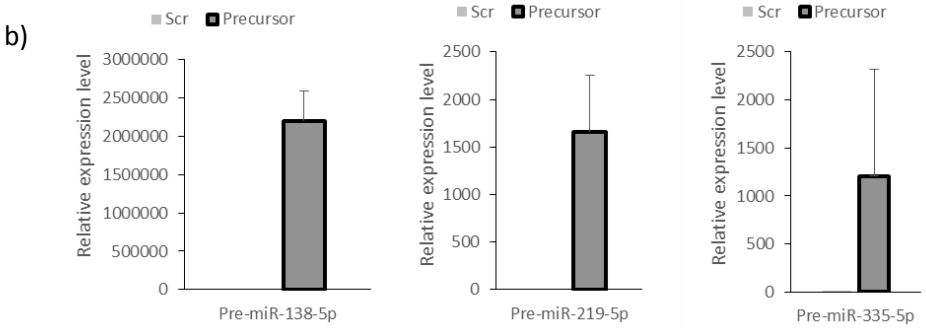
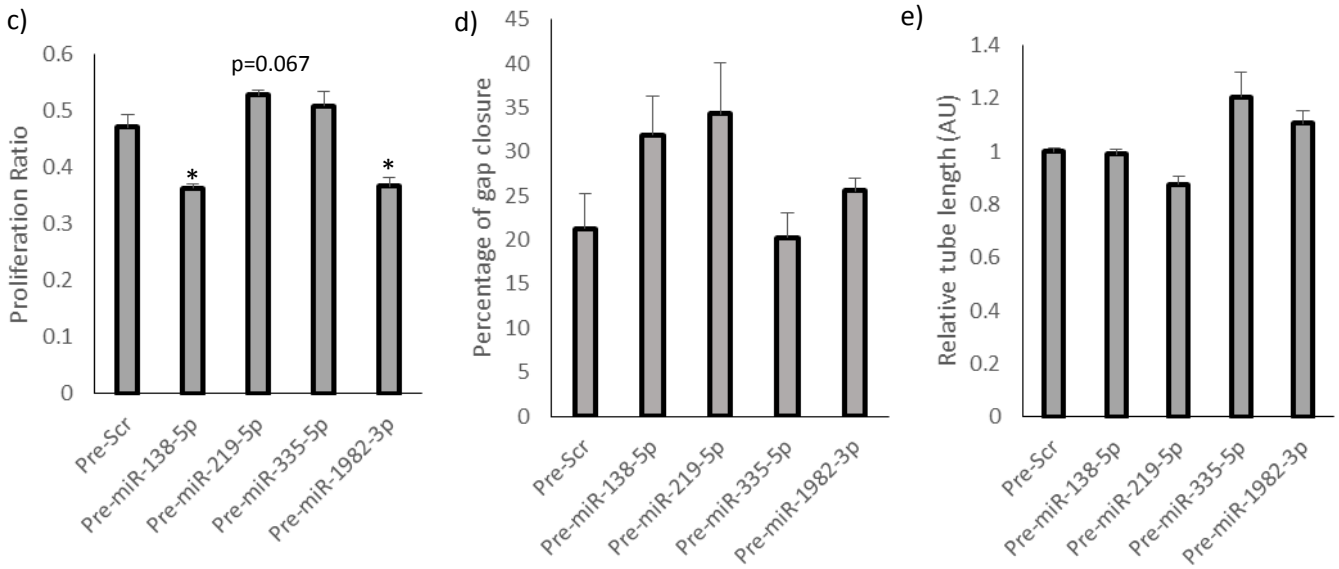
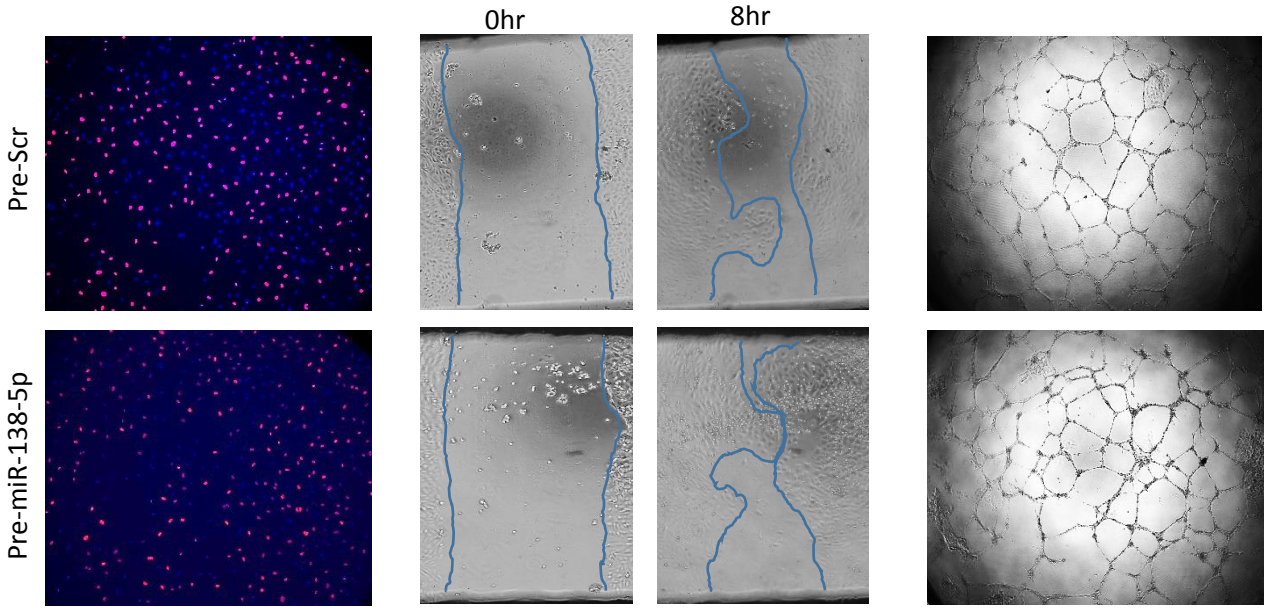
mmu-miR-150-5p
Screening data
Validation data


b)
Scr
Precursor
Relative expression level
3000000
2500000
2000000
1500000
1000000
500000
0
Pre-miR-138-5p
2500
2000
1500
1000
500
Pre-miR-219-5p
Pre-miR-335-5p


c)
Proliferation Ratio
p=0.067
*
*
0.6
0.5
0.4
0.3
0.2
0.1
0
Pre-Scr
Pre-miR-138-5p
Pre-miR-219-5p
Pre-miR-335-5p
Pre-miR-1982-3p
d)
Percentage of gap closure
45
40
35
30
25
20
15
10
5
0
e)
Relative tube length (AU)
1.4
1.2
1
0.8
0.6
0.4
0.2
0


0hr
8hr
Pre-Scr
Pre-miR-138-5p

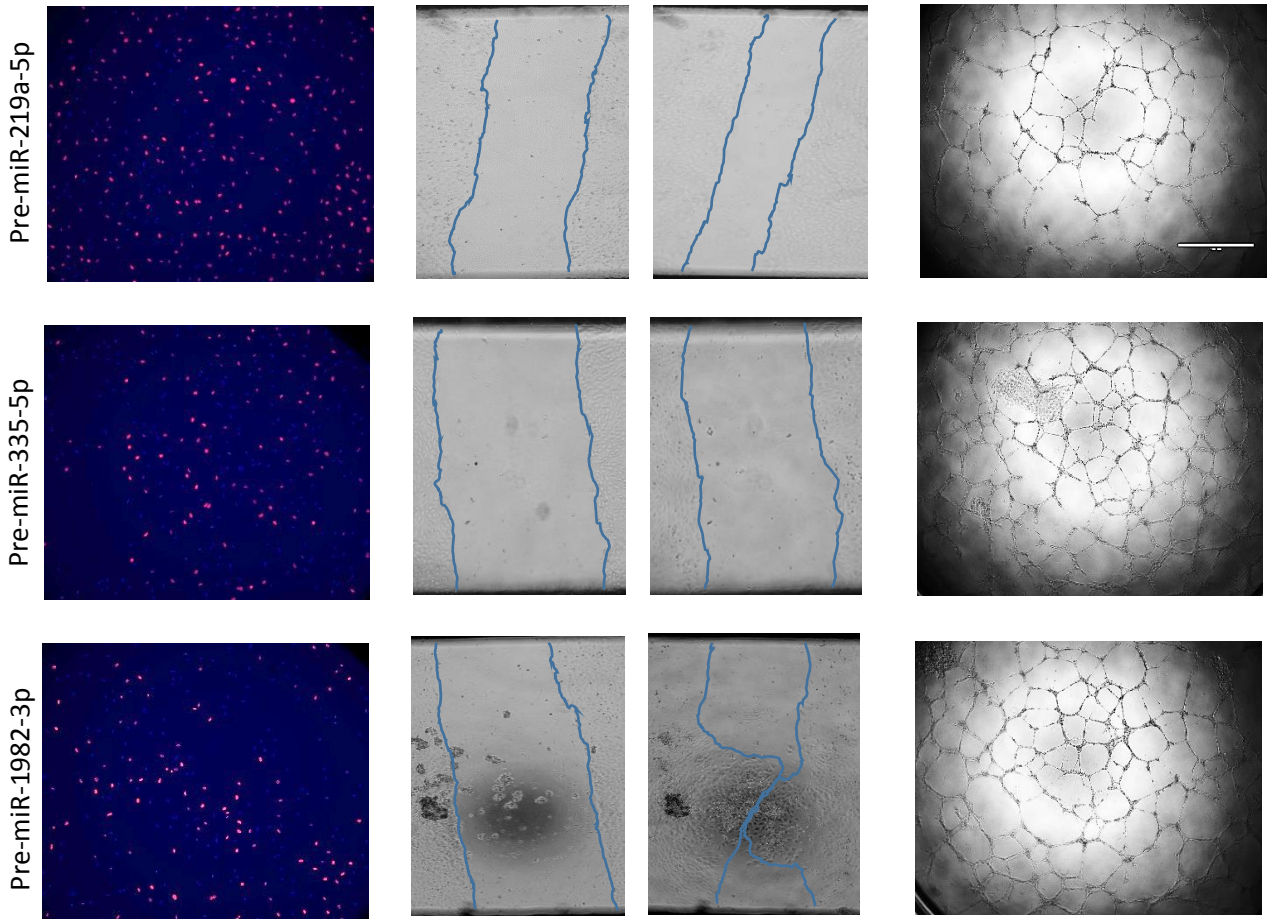


*Figure 13. Functional validation of the microRNA inhibitor screening. a) Proliferation rates of several microRNA in the screening experiment in a repetition assay. b) Quantification of qPCR for the microRNAs overexpressed through precursor transfection. c, d & e) Proliferation assay (c), migration assay (d) and tube formation assay (e) quantification (top) and representative pictures of each condition (bottom). All statistical comparisons are relative to the respective Pre-Scr conditions.*P<0.05, number of replicates = 3. Error bar represents S.E.M. Statistical significance assessed with unpaired Student's ttest.*

4.4. MicroRNA-219a-5p in angiogenesis

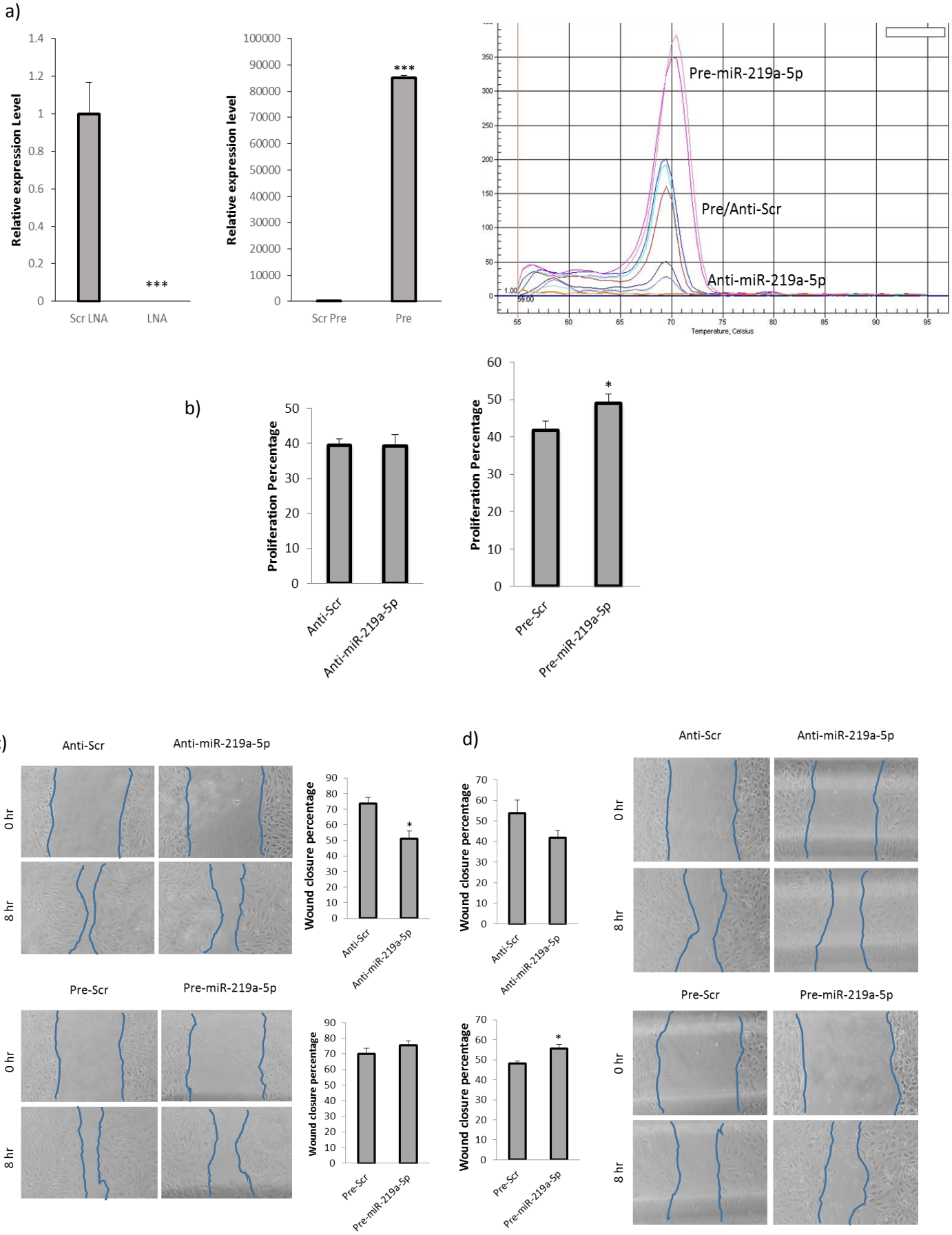
In order to clarify the functional role of miR-219a-5p in human ECs we repeated the angiogenic assays previously described in Figure 13. All assays were performed in the same manner, with the exception that for the scratch wound assay another, poorer, medium composition (5% FBS in EBM2 without growth factors) was also tested. The hypothesis was that, since the phenotype previously observed was not clear, by having a poorer medium then the potentially pro-angiogenic effect of the microRNA would be evidenced more easily. Not only that, but alongside overexpression of miR-219 through precursor, we also downregulated it through transfection with the appropriate LNA-based microRNA inhibitor, as described before.

We quantified the levels of miR-219a-5p in anti/pre-scramble conditions and anti/pre-miR-219 conditions through RT-qPCR. Our results show that we achieved very efficient downregulation and overexpression of miR-219, with the microRNA inhibitor and precursor, respectively (Figure 14a). Proliferation assay results were mostly in line with what we had seen in our screening and functional validation experiments. For the overexpression of miR-219, we observed a significant increase in cell proliferation, however, in inhibitor-treated cells we did not see a significant phenotypical change from

control (Figure 14b). Usually, precursor treatment tends to be much more efficient than inhibitor treatment with thousand-fold changes from endogenous conditions. However, from our qPCR data we see that inhibition has also been very efficient, therefore the only possible explanations for the discrepancy mentioned pertain to human error during the protocol or stochastic biological variance.

The data obtained from the scratch wound assay, however, has proven to be more robust than in our previous experiment. In rich medium conditions, the effect of the microRNA inhibitor is clear but the effect of the precursor is masked (Figure 14c). The opposite is true for the conditions in poor medium (Figure 14d). Our results indicate that inhibitor treatment is capable of decreasing wound closure percentage relative to control, eight hours after the scratch. Conversely, precursor treatment is capable of increasing relative wound closure. The complementary phenotype shown in poor and rich medium conditions can be a consequence of the cells own predisposition for proliferation induced by each medium. This is, in an enriched medium, HUVECs are easily able to proliferate greatly on their own, rendering mild pro-angiogenic stimuli undetectable, while evincing the role of anti-proliferative effects. The contrary would also hold true for poorer medium compositions, which may explain our results.

In the tube formation assay, our results show an interesting duality. In parameters related with the length of the vessel-like structures formed there is no apparent significance from either precursor or inhibitor treatment (Figure 14e). However, in parameters regarding to the nodes where those branches connect, the effects of both treatments are inducing the expected phenotype: anti-miR-219 decreases total node count while pre-miR-219 increases it. It is possible that physical constraints such as those imposed by the properties of the matrix where the cells have been embedded or the dimensions of the well itself are capable of influencing the length of the segments formed. Otherwise, it is also plausible that the segment length stably reaches a plateau that the range of effect of miR-219 is not capable of modulating.


a)
Relative expression Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
Scr LNA
LNA
Relative expression level
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
***
Scr Pre
Pre
Pre-miR-219a-5p
Pre/Anti-Scr
Anti-miR-219a-5p
Temperature, Celsius
b)
Proliferation Percentage
50
40
30
20
10
0
Anti-Scr
Anti-miR-219a-5p
Proliferation Percentage
60
50
40
30
20
10
0
*
Pre-Scr
Pre-miR-219a-5p
c)
Anti-Scr
Anti-miR-219a-5p
0 hr
8 hr
Wound closure percentage
90
80
70
60
50
40
30
20
10
0
*
Anti-Scr
Anti-miR-219a-5p
Pre-Scr
Pre-miR-219a-5p
0 hr
8 hr
Wound closure percentage
Pre-Scr
Pre-miR-219a-5p
d)
Wound closure percentage
70
60
50
40
30
20
10
0
Anti-Scr
Anti-miR-219a-5p
Anti-Scr
Anti-miR-219a-5p
0 hr
8 hr
Wound closure percentage
70
60
50
40
30
20
10
0
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Pre-Scr
Pre-miR-219a-5p
Pre-Scr
Pre-miR-219a-5p
0 hr
8 hr

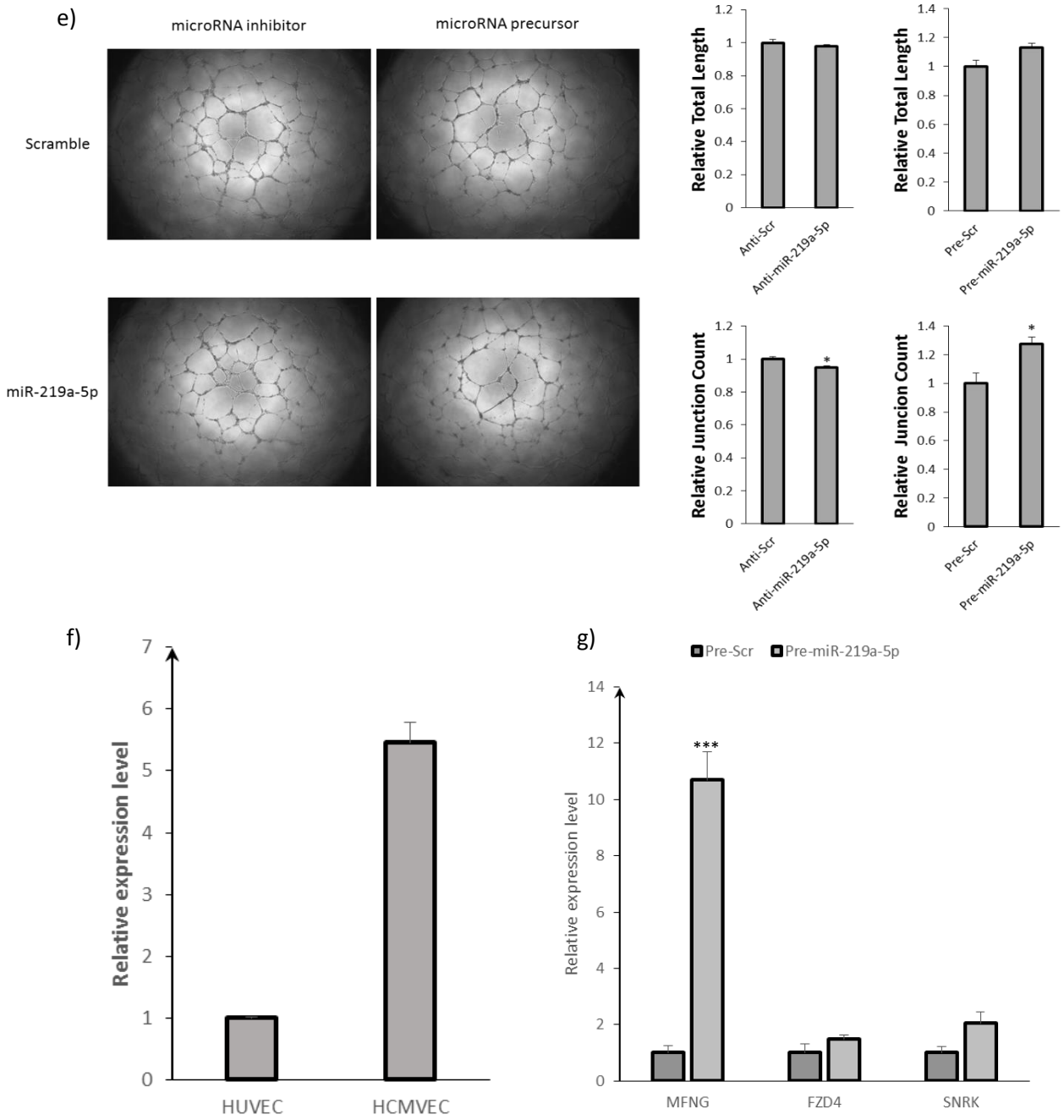

*Figure 14. Assessment of the role of miR-219 in angiogenesis. a) Quantification of qPCR data for the inhibitor (left) and precursor (middle) treatment for miR-219a-5p, with respective melting peaks for each condition (right). b) Proliferation assay quantification of the inhibitor treated (left) and precursor treated conditions (right). c &d) Migration assay representative figures and quantification of precursor and inhibitor treatment for miR-219a-5p in enriched medium (c) and poor medium (d). e) Tube formation assay representative figures and quantification of precursor and inhibitor treatment for miR-219a-5p. f) Quantification of qPCR data for the endogenous levels of miR-219a-5p in HUVEC and HCMVEC. g) Relative expression of the putative targets of miR-219a-5p after precursor treatment on HUVECs, normalized to L7. All statistical comparisons are relative to the respective Pre or Anti-Scr conditions.*P<0.05, ***P<0.001, number of replicates = 3. Statistical significance assessed with two-tailed, unpaired Student's ttest. Error bar represents S.E.M.*

Taken together, our experiments show that overexpression of miR-219 results in a mild pro-angiogenic effect overall, that is not just limited to proliferation. However, all our results have been drawn from HUVECs, which are, by nature, markedly different from adult ECs, especially microvascular cells. Therefore, we investigated the expression prolife of our microRNA of interest in human cardiac

microvascular endothelial cells (HCMVECs) through RT-qPCR (Figure 14f). Our results show that the expression of miR-219 in HCMVECs is over 5 times higher than in HUVECs. Therefore, we theorize that our results will not only be reproducible, but overall more pronounced in cardiac microvascular ECs.

In order to find the underlying mechanism of action of miR-219 we sought to find its direct target genes. Several bioinformatic tools and open-access databases (TargetScan, mirWalk) were used in order to narrow the list of potential targets. Ultimately, we arrived at a short list of 6 possible targets, all of them predicted by at least 6 independent databases and/or algorithms, with known expression in endothelial cells, at least one widely conserved binding site for miR-219a-5p and almost all of them with a previously reported role in either autophagy or cell proliferation (Table 5).

Table 5. List of predicted targets for miR-219a-5p. Conservation of binding sites was assessed only for human, rat and mouse.

Gene	Number of databases that predict it	Number of conserved binding sites	Proof of cellular function	Expressed in endothelial cells
MFNG	10	1	Angiogenesis[189]	Yes
CD164	9	1*	Angiogenesis[190]	Yes
PDGFRA	9	1	Angiogenesis[191] Autophagy[192]	Yes
FZD4	9	1	Angiogenesis[193]	Yes
SNRK	6	2	Angiogenesis[194] Autophagy[195]	Yes
RORB	7	3*	-	Yes
ATG14	6	1	Autophagy[196]	Yes

MFNG = MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase; CD164 = CD164 molecule; PDGFRA = Platelet derived growth factor receptor alfa; FZD4 = Frizzled class receptor 4; SNRK = SNF related kinase; RORB = RAR-related orphan receptor B; ATG14 = autophagy related 14

*This gene has more predicted binding sites in human, but not conserved in rat and mouse.

We tested the expression profile of these genes in HUVECs treated with either the precursor of miR-219a-5p or a scrambled negative control, with RT-qPCR. We were only able to amplify MFNG, FZD4 and SNRK. For these putative targets, the only significant result we observe pertained to MFNG.

Manic fringe (MFNG), along with lunatic fringe (LFNG) and radical fringe (RFNG) belong to a glycotransferase family capable of post-transcriptional regulation of Notch through glycosylation[197]. The Notch pathway is one of the most pervasive and widely conserved pathways in metazoans[198]. It has been reported to also be involved in angiogenesis and cardiac development[199]. Notch proteins are transmembrane receptors capable of binding to either of the five ligands of the Delta-Serrate-Lag (DSL) type (Jag1/2 and delta-like Dll1/3/4)[200]. However, the phenotype that binding to Jag or Dll ligands induces in ECs is quite opposite. When the Dll-Notch interaction is strengthened, the sprouting and overall angiogenic capabilities of ECs are reduced, whereas the opposite is true for Jag-Notch interactions[189]. Incidentally, fringe proteins control these interactions, thereby being capable of determining endothelial cell fate. Fringe family members are able to modify Notch in order to promote Dll binding and weaken Jag binding, thus potentiating an anti-angiogenic phenotype. Consequently, impairment of fringe proteins is expected to have the opposite effect and induce a pro-angiogenic phenotype. Thus, we hypothesize that it is through this mechanism that miR-219a-5p is capable of modulating proliferation and angiogenesis.

However, when we tested the levels of manic fringe after overexpression of miR-219a-5p we observed an upregulation, which is the opposite of what was expected (Figure 14g). This result was unexpected, especially since we see such a strong significance (p=0,00071) for over 10 times overexpression after precursor treatment. Thus, these results require further confirmation through repetition of the experiment to confirm their validity. Moreover, western blot should be used in order to determine the effect of miR-219 on the protein levels of MFNG and assess whether the same trend still holds true. However, there are a few plausible explanations that can justify the upregulation of this gene after overexpression of their inhibitor. As previously explained, microRNAs have multiple modes of action, with the two most common mechanisms being target mRNA cleavage and translational repression. It is possible that the prevalent mechanism in the case of miR-219a-5p/MFNG interactions is translational repression, which could mean that the mRNA itself is not signalled for degradation immediately, thereby accumulating within the cell, while protein levels decrease, however. A downstream signalling cascade perceiving the lack of MFNG at a protein level could ensue a simple negative feedback regulatory mechanism to increase manic fringe levels.

Another possible explanation would be the coincidental action of miR-219 over other proteins interacting with MFNG. In order to evaluate the validity of this possibility we investigated whether any of the genes whose protein interacts with manic fringe (Figure 15) has a binding site for miR-219a-5p. The result of the bioinformatic analysis was negative for all of them, and also for the other members of the fringe protein family.

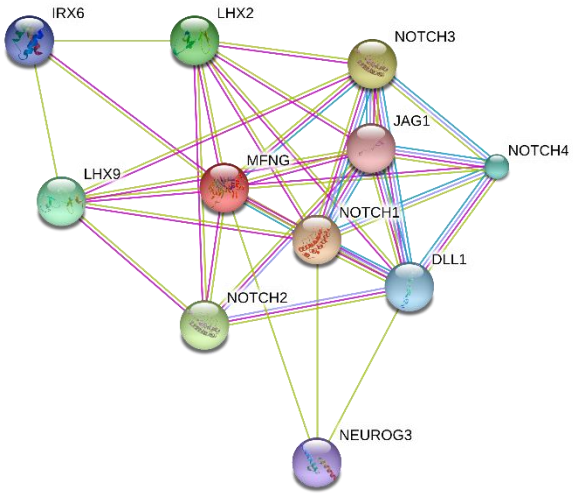

Figure 15. Predicted protein interaction network surrounding MFNG, from STRING database.

Interestingly, preliminary data from our group suggests that miR-219a-5p is downregulated in heart tissue after injury. This finding is very much in line with our hypothesis, so that in an injured heart, downregulation of miR-219 acts through the Notch pathway to downregulate angiogenic capacity in microvascular endothelial cells. Consequently, the ability of the cardiac microvasculature to cope with injury is reduced, furthering the heart failure phenotype. We speculate that overexpression of miR-219a-5p would be beneficial for heart remodelling in the context of cardiac failure after injury.

5. Conclusions

In the present work we sought to discover microRNAs involved in the regulation of an angiogenic phenotype in human umbilical vein endothelial cells. For this purpose we have developed a proliferation assay protocol in order to test the effect of microRNA-based treatments. Our protocol has shown to be capable of emphasizing the role of microRNAs in HUVECs with relative reproducibility. It is entirely possible to keep optimizing our assay through, for example, testing other microRNA inhibitor and precursor concentrations or different chemical composition. However, we believe we have achieved a satisfactory cost-efficacy ratio between the resources invested in the protocol optimization and how much more our readout can be perfected. Therefore, future efforts could be directed towards testing how transversal our assay can be to other small molecule treatments.

Previous studies have raised an interesting conflict in the literature regarding the function of the miR-17-92 cluster in angiogenesis. On the one hand it is a known oncogenic microRNA, with several groups advocating that its role is mostly pro-angiogenic[161,167]. On the other hand, several groups have claimed that this cluster may be anti-angiogenic, exhibiting different functions depending on cellular context[164,201,202]. According to the latter observation, we have described how miR-17 is capable of either decreasing HUVEC proliferation at low cell densities, or also increase it when cells are more confluent. This hypothesis is plausible given that microRNAs are able to modulate the expression of numerous target genes concomitantly. Thus, should the expression pattern of its targets shift as a consequence of exogenous stimuli then opposite effects can actually be appreciated. In accordance to this idea, a number of observations have noted that miR-17~92 is capable of inducing different cellular phenotypes regarding cell cycle progression and survival[201]. Further studies should be conducted in order to confirm that this microRNA cluster is able to cause different phenotypes and if so, then determine which are the mechanisms underlying the seemingly paradoxical function.

Our main objective, however, was the discovery of previously unreported microRNA in the context of endothelial cell proliferation. For this purpose we screened a library of microRNA inhibitors and quantified the effect of each on the total cell count and proliferation ratio of HUVECs. Our results revealed a number of microRNAs whose inhibition induced a drastic pro or anti-proliferative phenotype. We may pursue further hits in the future, in search of those that show the same function in vivo and in the cardiac system.

The finding of miR-219a-5p was one of the interesting results obtained from the screening that overlapped with the results from a previous autophagy related assay. Our results have shown that this microRNA is capable of inducing a mild pro-angiogenic phenotype in ECs. Interestingly, this microRNA is downregulated in the failing heart, but it remains to be determined whether there is any connection between these findings. If so, future studies would have to determine whether the differential expression of miR-219a-5p is a consequence or a cause of heart failure. Regardless, we expect that overexpression on this microRNA might be able to ameliorate a pathological heart phenotype through increase of microvascularization. Nevertheless, the mechanism through which miR-219 acts still remains elusive. We have found there to be an interesting correlation between the predicted target MFNG and overexpression of miR-219. However, we suspect that either technical complications or rare biological events might be behind the unexpected results. Still, the biological role of MFNG in the Notch pathway is in line with what we expected to observe from a target of a pro-angiogenic microRNA. Therefore, we speculate that miR-219 is able to act through the Notch pathway in order to

increase cell proliferation. Further validation of miR-219 function is required, at RNA level and especially at protein level. Future studies will aim to clarify the mechanism downstream of miR-219a-5p and investigate its role in other cell models, such as HCMVECs, where it is more highly expressed endogenously.

Our study is not, however, without limitations. The usage of HUVECs as a study platform for endothelial cell function and behaviour is far from being the perfect model. Despite being a stable, well defined and easy to work with cell type, its biological properties are very much different from other vascular endothelial cells[203]. Moreover, there are intrinsic limitations to *in vitro* models, such as the inability to accurately mimic physiological and pathological conditions in the living organism, such as the effect of shear stress. Therefore, all findings need to be confirmed in other endothelial cell models. For instance, our laboratory already has access to a Cre-recombinase-based endothelial specific reporter mouse model[204] to allow imaging of the vascular cardiac remodelling process, which will undoubtedly be key in pursuing our hypothesis *in vivo*.

Additionally, further repetitions of our proliferation screening are needed to have a higher statistical robustness for our results. As previously shown, proliferation-based assays are subject to high inherent variance, which may be reduced with a greater number of tests. Furthermore, using our microRNA inhibitor library to test for other angiogenic parameters, such as tube formation and migration capabilities, might also be desirable as a means to discover potential microRNAs involved in those processes but without significant function in proliferation itself. Finally, the usage of a mouse microRNA inhibitor library on a human cell line is also not ideal. Despite a high conservation among a majority of the microRNA between mouse and human, we have proved that exogenous microRNAs, such as miR-1982-3p can also induce phenotypical changes in human cells. Consequently, the acquisition of an up-to-date human microRNA inhibitor library and its assessment in the same fashion as we have shown here would be best.

Despite these shortcomings, we have been able to produce concrete evidence hinting at a biological role for miR-219a-5p and mostly completed our proliferation assay which may be further used for other comparable studies. Our research will thus continue pursuing the role and relevance of miR-219a-5p in the context of cardiac microvascular phenotype. It is also very likely that other microRNAs of interest will be subject of future efforts given their result in our screening experiments. Ultimately, albeit far from perfect, our research is sound and will hopefully contribute to the greater scheme of the development of biomedical technology to treat cardiovascular diseases.

6. References

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