The role of microRNA-126 in vascular homeostasis

Coen van Solingen

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CHAPTER 1

General introduction

Introduction

Cardiovascular diseases have emerged as the leading cause of death worldwide, particularly in developed countries. The World Health Organization has estimated that in 2030, almost 23.6 million people will die from cardiovascular diseases [1]. By the time that vascular problems are detected, the underlying cause is usually quite advanced, having progressed for decades. Therefore there is an increased emphasis on altering well-known risk factors by healthy diet, exercise, and avoidance of smoking. Moreover, to understand the exact influence of environmental risk factors on our vasculature it is of high relevance to fully understand the biological mechanisms that keep our vessels healthy and functional. This thesis covers several aspects on the fundamental gene regulation that is involved in maintaining vascular homeostasis. In particular, the role of endothelial enriched miRNA-126 in, so-called, angioadaptation has been described.

Part I: Vascular homeostasis

Vascular growth and regression plays a major role in both health and disease. In physiology, it contributes to embryogenesis, development and wound healing. Furthermore, it plays a role in the pathogenesis of many disorders, either by excessive vessel growth, for example in cancer, atherosclerosis, diabetic retinopathy, psoriasis and arthritis, or by insufficient vessel growth, for example in ischemic disease of heart, limb or brain, pre-eclampsia and osteoporosis [2]. However, development and maintenance of the vascular system requires not only the formation of new vessels, but also the continuous adjustment of vessels and network structures in response to functional needs, so called, angioadaptation [3]. Three types of neovascularizaton can be recognized in the formation of a vascular network; angiogenesis, vasculogenesis and arteriogenesis [4]. In each of these processes a complex interplay of growth factors, cytokines and the combined action of multiple cell types is required. The basic principles of these three vascular processes will be described in more detail in the first part of this general introduction.

Vasculogenesis

During embryogenesis the first endothelial and hematopoietic cells of the yolk sac arise from a common mesodermal progenitor, the hemangioblast, located in the blood islands [5, 6]. The formation of the capillary network that arises from the yolk sac is driven by embryonic endothelial progenitor cells which are located at the outer lining of these blood islands. The process, where blood vessels are formed when there is no pre-existing capillary network, is called vasculogenesis. Once the embryonic heart has developed, the first primitive blood cells start to circulate from the yolk sac into the embryo [5, 7].

Interestingly, adult blood circulation and tissues still contain small numbers of these endothelial progenitor cells (EPCs) [8-10] and the potential contribution of these circulating progenitor cells to the formation new blood vessels has gained much interest. Next to their presence in the circulation, it has also been demonstrated that EPCs could contribute to adult neo-vascularization (Figure 1) [11-15]. Using different quantification methods, many groups have shown that the number of EPCs (classified in human by expression of the markers CD34, CD133 and KDR/VEGFR-2) in the circulation reflects or correlates with disease state. As example, lower levels of EPCs are found in people at risk for cardio-vascular diseases [16-18]. On the contrary, an increase of these cells is found in patients with ischemic pathophysiologies [19, 20].

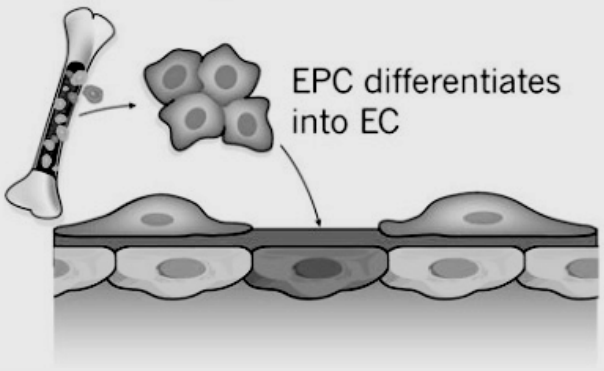

Figure 1. Vasculogenesis
Vessel formation can occur by the mobilization of bone marrow-derived endothelial progenitor cells that differentiate into endothelial cells [42]. Adapted from: Molecular mechanisms and clinical applications of angiogenesis, Carmeliet & Jain, Nature 2011.

Despite extensive research, the exact mechanisms how these bone marrow-derived cells exactly contribute to the formation of new blood vessels remain unclear. EPCs are mobilized by a variety of growth factors such as stromal cell-derived factor 1 (SDF-1) and VEGF21 and may by means of these mobilizers be attracted to sites of ischemia. However, incorporation of EPCs into the endothelial layer is rarely reported [15] leaving a paracrine function of cells with secretion of angiogenic factors or a supportive function as perivascular cells more probable mechanisms [22].

Regardless of the unknown, complex mechanisms how EPCs contribute to neovascularization, strategies for a stem cell-based therapeutical approach have been set out in humans and animals. The therapeutic effectiveness after injection of human CD34+ cells or cultured EPCs upon myocardial infarction has been demonstrated in mice [23], rats [24-28], swine [26, 29] and monkeys [30]. In humans several randomized, placebo-controlled studies showed effectiveness and safety of the infusion of bone marrow cells to treat (limb) ischemia [31-33].

Angiogenesis

Angiogenesis is the formation of new capillaries from endothelial cells that sprout from pre-existing vascular network and is regulated by a variety of pro- as well as anti-angiogenic factors [34]. A trigger for angiogenesis is most often the lack of oxygen (hypoxia) that induces the availability of hypoxia inducible factor 1 alpha (HIF-1α), a protein that is degraded when sufficient oxygen is available [35]. As a consequence HIF-1α is able to bind its counterpart HIF-1α. This homodimeric complex that adheres to hypoxia responsive elements in the promoter regions of target genes. As a result, elevated levels of this transcription factor complex lead to the increased production of pro-angiogenic cytokines and growth factors, such as vascular endothelial growth factor (VEGF) [36]. Subsequently, VEGF recruits myeloid cells that produce pro-angiogenic factors, like tumor necrosis factor alpha (TNF-α). Furthermore, VEGF activates endothelial cells to produce nitric oxide via endothelial nitric oxide synthase (eNOS), which induces vascular permeability [37]. Due to the increased levels of TNF-α and VEGF vascular junctions loosen and the endothelial cells detach from their neighboring cells, leading to the branching of tip cells towards the ischemic area [4, 37-39]. Endothelial cells, but also recruited inflammatory cells and fibroblasts produce proteases, such as matrix metalloproteinases (MMP) and urokinase-plasminogen activator that are essential in the degradation of the extracellular matrix and the liberation of growth factors. This degradation facilitates endothelial migration by tip and stalk cells that are essential by the formation of novel, primitive tube-like structures. Tip cells navigate in response to guiding VEGF-signals and adhere to the extracellular matrix to migrate. Stalk cells behind the tip cell proliferate, elongate and form a lumen, and sprouts fuse to establish a perfused neovessel. Proliferating stalk cells attract pericytes and deposit basement membranes to become stabilized. After fusion of neighboring branches by macrophages [40], lumen formation allows perfusion of the neovessel, re-establishment of junctions, deposition of basement membrane, maturation of pericytes and production of vascular maintenance signals (a schematic overview of angiogenesis by tip cells is shown in Figure 2). Consequently, blood will be able to flow augmenting perfusion and subsequent oxygen diffusion into the ischemic tissue [41, 42].

Therapeutically, the stimulation of angiogenesis by the administration of angiogenic growth factors into the circulation or the ischemic area is a promising approach and beneficial effects of methods using growth factors have been shown in a variety of ischemia models in animals. Early studies, where VEGF-A was used in small cohorts of patients suffering from advanced ischemic disease, were encouraging [43-46]. In contrast, larger randomized, placebo-controlled trials of therapeutic angiogenesis have been published more recently are inconsistent [47-50]. These results that have tempered the enthusiasm of developing new, single growth factor-based, angiogenic strategies. Therefore, to improve

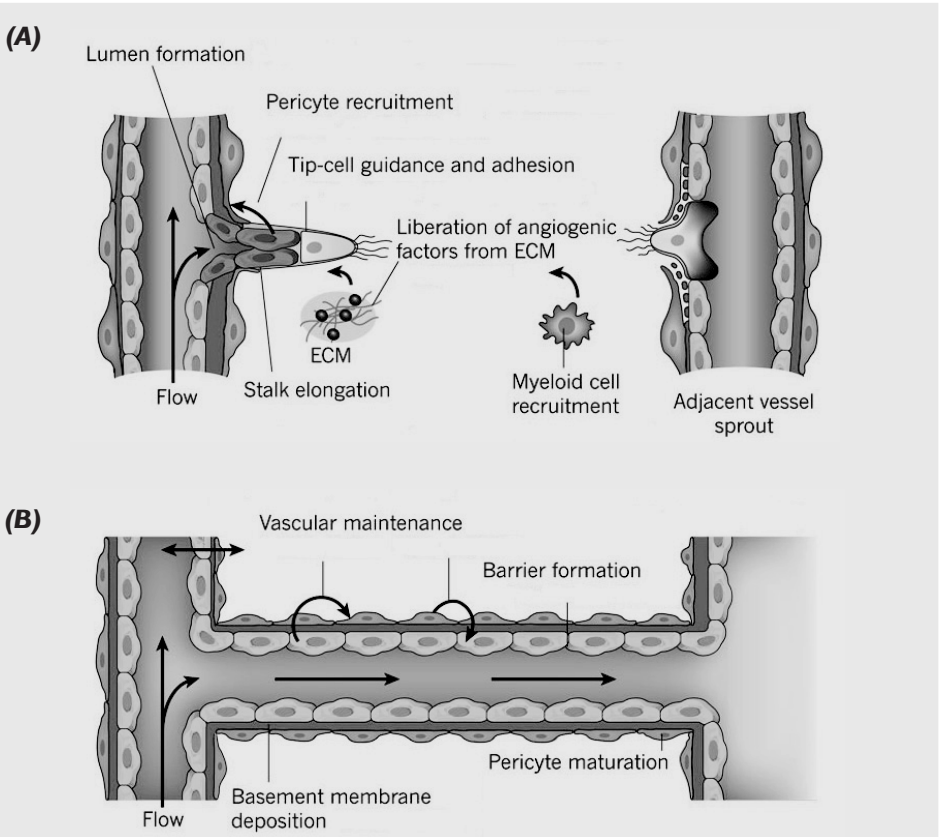

Figure 2. Angiogenesis - the consecutive steps of blood vessel branching ***(A)*** After stimulation with angiogenic or inflammatory growth factors, a vessel dilates and endothelial tip cells are formed that initiate the sprouting of new capillaries. Tip-cell formation requires degradation of the basement membrane, pericyte detachment and loosening of endothelial cell junctions. Increased permeability permits extravasation of plasma proteins) to deposit a provisional matrix-layer, and proteases remodel pre-existing interstitial matrix, all enabling cell migration. Tip cells navigate in response to guidance signals such as VEGF and adhere to the extracellular matrix to migrate. Stalk cells follow the tip cells, proliferate, elongate and form a lumen to support the growth of the new capillary. Finally, the novel sprouts fuse to establish a perfused neovessel. Proliferating stalk cells attract pericytes and deposit basement membranes to become stabilized. In the proces, recruited myeloid cells produce inflammatory and pro-angiogenic factors and guide the anastomosis of the capillaries. ***(B)*** Vessel maturation: After fusion of neighbouring branches, lumen formation allows perfusion of the neovessel, re-establishment of junctions, deposition of basement membrane, recruitment of pericytes and the production of vascular maintenance signals [42]. Adapted from: Molecular mechanisms and clinical applications of angiogenesis, Carmeliet & Jain, Nature 2011.

clinically interference with angiogenesis, more information is needed on the regulation of neovascularization in ischemic tissues.

Arteriogenesis

During arteriogenesis, unlike angiogenesis and vasculogenesis, no new blood vessels are formed, but the lumen of pre-existing capillaries is widened by remodeling and the vessels become mature collaterals. When a major artery is occluded, the formation of new capillaries is not enough to support sufficient oxygen levels in the risk region. This results in a redistribution of blood flow in the pre-existing, immature collateral arterioles. The trigger for arteriogenesis comes predominantly from the mechanic stimuli derived from the elevated flow and shear stress on the vascular endothelium following upstream occlusion. Endothelial cells become activated and up-regulate surface adhesion molecules. Furthermore endothelial cells start to produce growth factors like monocyte chemotractant protein-1, which attracts inflammatory cells [51, 52]. These inflammatory cells adhere to the endothelium via adhesion molecules like vascular adhesion molecule 1 (VCAM-1) and move through the endothelium to the collaterals and produce cytokines and growth factors that support collateral growth and remodelling [53]. Several inflammatory cell types have been identified

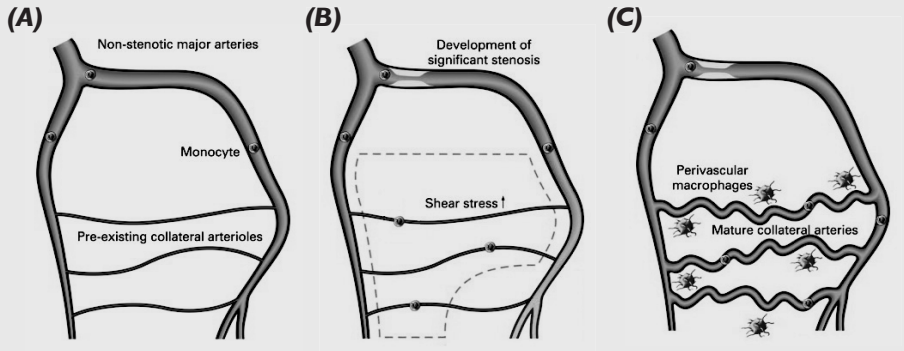

Figure 3. Mechanism of arteriogenesis

(A) Without stenosis, there is no pressure gradient over pre-existing collateral capillaries, which are small and barely carry blood. ***(B)*** Development of a significant arterial obstruction leads to a drop in pressure and oxygen saturation distal in the vascular bed, while proximal pressure and oxygen saturation proximal remain normal. The pressure gradient over the collateral circulation increases fluid shear stress in these arterioles. ***(C)*** Widened collateral arteries restore distal perfusion and provide sufficiently oxygenated blood to distal tissues [96]. Adapted from: Stimulation of collateral artery growth: travelling further down the road to clinical application, Schirmer et al, Heart 2009.

to contribute to arteriogenesis; monocytes [54] and natural killer cells play an early role, while a variety of T-cell subsets arrive in a later phase [55]. The

arteriogenic process is further finished by extra maturation of the vessels. Proteases, like MMPs, produced by the invading cells, participate by degrading extracellular matrix creating space for the growing collaterals. Finally smooth muscles are attracted to the vascular wall stabilizing the widened collaterals [56]. A schematic overview of this process is shown in Figure 3.

It has to be noted that the above-described processes all play an important role in vascular formation and usually occur at the same time. All three mechanisms share common mechanisms like the expression of growth factors and cytokines and the invasion of (inflammatory) cells. As described before excessive or insufficient neovascularization can lead to a wide variety of diseases. Therefore it is of critical importance that the expression of the genes that are involved in the processes of angioadaptation is tightly regulated. In the next part of this general introduction an overview will be given of the most important steps that are taken at the post-transcriptional level of gene regulation.

Part II: Gene regulation at the posttranscriptional level

Proteins, which are built out of amino acids, have an extraordinary versatility to catalyze chemical reactions and build cellular structures. Deoxyribonucleic acid (DNA) is the cellular library that holds the information that prescribes and links the right amino acid sequence for each individual protein. This information is arranged in genes, which are transcribed into so-called messenger ribonucleic acid (mRNA) out of which proteins are synthesized. Transcription of a gene by RNA polymerase consists of several steps. After binding of the RNA polymerase to a promoter region of the DNA, the DNA switches from a closed to an open configuration. Opening up of the DNA gives RNA polymerase the ability to move along to DNA further downstream and transcribe the full mRNA that codes for the gene-encoded protein. During this elongation process the guiding DNA stand is subjected to binding of a wide variety of factors, like specificity factors, repressors, activators and enhancers, that can either promote or repress the synthesis of mRNA [57]. In recent years a next, epigenetic level of gene regulation has been described. Epigenetics is the study of heritable changes in gene expression or cellular phenotype by modifications, such as DNA methylation and histone deacetylation, both of which serve to suppress gene expression without altering the sequence of the silenced genes. These modifications make the DNA more and less accessible for enzyme complexes that drive gene transcription [58]. Interestingly, diet and environmental exposures can alter the level and activation of methyltransferases and deacetylases. Variety in these levels may be an explanation why cells and organisms with identical genotypes can have dramatic phenotypical differences. In addition, epigenetic modulation of gene expression may provide a link between lifestyle, gene expression and risk

of disease [59, 60].

Surprisingly, genomes of 'higher eukaryotes', like that of man and mouse, harbor about the same number of protein coding genes (±15,000 and 20,000) as the genomes of the apparently less complex 'lower eukaryotes' such as the round worm *Caenorhabditis elegans*. A likely explanation for this discrepancy is that more complex species have evolved following the development of advanced systems to regulate and coordinate the expression of the gene pool at the posttranscriptional level. A major fraction of the mechanisms of post-transcriptional gene regulation involves non-coding RNA transcripts. As only 1-2% of the total genome is responsible for the coding of proteins there appears to be ample space for the coding of RNAs transcripts that support post-transcriptional regulation [61].

The four main mechanisms of posttranscriptional gene regulation involve: 5' capping, polyadenylation, splicing and the regulation by non-coding RNAs. The first three processes take place in the nucleus and are schematically shown in Figure 4 and shortly described below. The regulation by non-coding RNAs is described in more detail in the second part of this general introduction as this is related to the main topic of this thesis.

5' capping

To be protected against RNA-viruses, such as a retrovirus, the cell harbors a variety of exonucleases that can degrade primary unmodified RNA molecules. To protect endogenous mRNAs from these enzymes the 5' end of the mRNA

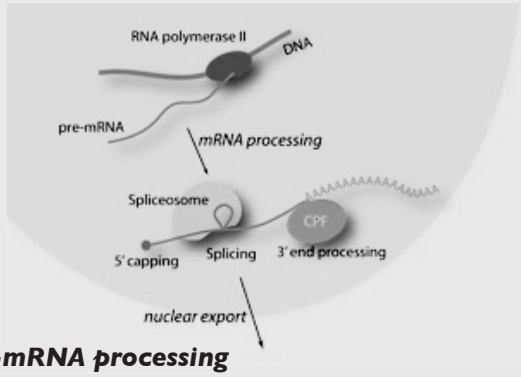

Figure 4. Pre-mRNA processing

Eukaryotic genes are normally transcribed by RNA polymerase II as pre-mRNAs that must be processed before they are exported from the nucleus and translated into proteins. To protect endogenous mRNAs from these enzymes the 5' end of the mRNA is capped. Next, the poly(A) tail is required for protection against as well as export of the mRNA into the cytoplasm, furthermore the poly(A) tail is linked to the spliceosome. Splicing is the final step in the formation of a mature, functional mRNA. During this process non-coding introns are removed and exons are spliced together. Adapted from: Passmore Lab, MRC Laboratory of Molecular Biology, Cambridge UK.

is capped. This cap mimics the 3' end of the mRNA and is therefore protected against exonucleases that target the 5' start of RNA. Next to the protection against these enzymes, 5' capping also supports the export of the capped mRNA out of the nucleus, where it can undergo translation in the endoplasmatic reticulum, where the 5' cap also functions as part of a docking station for the ribosome [57, 62, 63].

Polyadenylation

In animal cells, all mRNAs, have a 3' poly(A) tail, which consists of a homogenous long stretch of adenine bases. This poly(A) tail is important for the export of the mRNA out of the nucleus by poly(A) binding proteins and subsequent translation, furthermore the poly(A) tail protects the mRNA from enzymatic degradation [64]. Polyadenylation starts as soon as the transcription of the gene is completed. First, cleavage-and-polyadenylation specificity factor (CPF/CPSF) binds to a polyadenylation signal site (AAUAAA) and subsequently forms a loop with a GU rich sequence downstream of the polyadenylation signal [65]. Several cleavage factors then bind and stabilize this complex and then, with support of poly(A) polymerase, the RNA sequence is cleaved shortly downstream of the polyadenylation signal. Next, the cleavage factors and the downstream cleavage product are released and poly(A) polymerase adds residues to the 3' end of the RNA. This initial polyadenylation attracts the binding of poly(A)-binding protein II, which accelerates the rate of adenine addition, after around 250 adenine bases are added polymerization is stopped [66, 67].

Splicing

The polyadenylation complex is physically linked to the spliceosome, a complex that removes introns out of the RNA, in a process called splicing. Splicing is the final step in the formation of a mature, functional mRNA. During this process non-coding introns are removed and exons are spliced together. Splice sites are moderately conserved sequences at intron-exon boundaries, where the 5'-splice site consists mainly of GU bases and the 3'-splice site contains the complementary AG bases [68]. For splicing small nuclear ribonucleotide protein particles (snRNP) bind to the primary RNA transcript and to each other thereby catalyzing the transesterification of the 5'- and 3'-splice site. Upon this transesterification a subsequent reaction is catalyzed in which the intron in excised out of the primary RNA transcript and the two exons are fused. Upon fusion the snRNP-complex destabilizes and dissociates from the RNA [69].

Before splicing RNA transcripts can consist of multiple exons that may or may not all be needed for a functional protein. In this light, alternatively in- and exclusion of exons is very common in eukaryotic biology. Initially, the primary

transcripts of protein (isoforms) can be identical, while after complex splicing patterns several different mRNAs can be generated that lead to proteins with a different function, which also might be expressed at a totally different location [70, 71].

Non-coding RNAs

Until recently, 95% of our genome was considered to be 'junk-DNA', it was thought that this junk-DNA largely consisted of evolutionary artifacts and that untranscribable and untranslatable DNA was useful in a negative way (the importance of doing nothing) [72]. Large genomic and transcriptomic studies confirmed that only 1-2% of the mammalian genome corresponds to protein-coding genes and that the vast majority of the genome produces a wide variety of non-coding RNA transcripts. As such, RNA is not only a messenger operating between DNA and protein and might have more regulatory functions. Intriguingly, a linear relationship between the complexity of an organism and the number of produced non-coding RNAs has been indicated. This suggests that developmental complexity, which is not reflected in the number of protein coding genes, may be mediated by non-coding RNAs [73, 74].

A recently discovered group of large intergenic non-coding RNAs (lincRNAs) is receiving a growing recognition. At present only about a dozen lincRNAs are well characterized. These few lincRNAs show clear evolutionary conservation, confirming their potential important role in cell biology. Studies by Guttman et al. and Khalil et al. identified 3,289 lincRNAs and they speculate that their total number may well be in the range of 4,500. Although lincRNAs are among the least well understood of these transcript species, they cannot all be dismissed as merely transcriptional noise. Expression patterns suggest that lincRNAs are involved in diverse biological processes, including cell-cycle regulation, innate immunity and pluripotency. LincRNAs may act as molecular scaffolds that upon interaction with target proteins play regulatory roles in translation, splicing, nuclear organization and epigenetic gene expression. However, to date, clear mechanisms by which they have their influence are completely unknown [73, 75].

Short non-coding RNAs (microRNAs or miRNAs) have been described to play major roles in most, if not all, biological processes. MiRNAs are currently a well-established class of ~22 nucleotide endogenous, non-coding small RNAs that influence mRNA stability and translation [76]. MiRNA genes are transcribed by RNA polymerase II as capped and polyadenylated primary miRNA transcripts (pri-miRNA) [77, 78]. In the, so-called, canonical pathway, pri-miRNA processing occurs in two steps, catalyzed by two enzymes, Drosha and Dicer in cooperation with a dsRNA binding protein, DGCR8 [79]. In the first nuclear step, the Drosha–DGCR8 complex processes pri-miRNA into an ~70-nucleotide precursor hairpin (pre-miRNA), which is exported to the cytoplasm. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of

splicing and debranching, thereby bypassing the Drosha–DGCR8 step [80, 81]. The nuclear export of pre-miRNAs is mediated by the transport receptor exportin 5 [82]. In the cytoplasm the pre-miRNA is maturated by Dicer, assisted by a transactivation-responsive RNA-binding protein, into an imperfect RNA duplex [83]. The strand of the duplex with the weakest base paring at the 5' terminus is preferably loaded into the miRNA-induced silencing complex (miRISC) [84]. Subsequently, the miRNA guides the miRISC to bind to the 3'UTR of their target sequence, thereby is the seed sequence (first 2 to 8 nucleotides) the most important sequence for target recognition and the consequential silencing of the mRNA [85, 86]. Translation of the mRNA is inhibited after association of miRISC with its target sequence. Efficient mRNA targeting requires continuous base pairing of miRNA nucleotides 2 to 8 (the seed region). Ago-proteins and glycine-tryptophan protein of 182 kDa (GW182) proteins are directly associated with miRNAs, are core components of the miRISC and are needed for effective translational repression (a schematic overview of miRNA biogenesis is shown in Figure 5). The exact mechanisms of translational arrest by the miRNA:mRNA complex is still a matter of debate, however both initiation and elongation steps of translation are thought to be affected [87-89].

It has become clear that during development many miRNAs are expressed in a tissue specific manner indicating that miRNAs may be involved in specifying and maintaining tissue identity [90]. For instance, miRNA-1, miRNA-133 and miRNA-206 are enriched in muscle tissue [91], miRNA-122 is well conserved between species in the liver [90, 92, 93] and the expression of miRNA-126 is enriched in the endothelium of human, mice [94], zebrafish [90] and cows [95]. The conservation between species suggests that the biological pathways where miRNAs play a role also may have been conserved, making them research targets of high interest.

The enriched expression of miRNA-126 in endothelial cells combined with the fact that these cells are key players during the control of vascular integrity makes the detailed analysis of the biological mechanisms controlled by this miRNA a research topic of high interest and relevance. Understanding the role that miRNA-126 plays in vascular homeostasis may lead to new insights and possible new therapeutic developments.

Scope of thesis

This thesis details our studies assessing the role of the endothelial-enriched miRNA-126 in the regulation of vascular homeostasis. In **Chapter 2** the current insight in the role of miRNA-126 in vascular homeostasis is reviewed. **Chapter 3** focuses on the role of miRNA-126 in ischemia induced angiogenesis, followed by **Chapter 4** which describes the potential role of miRNA-126 the mobilization of vasculogenic progenitor cells upon ischemia.

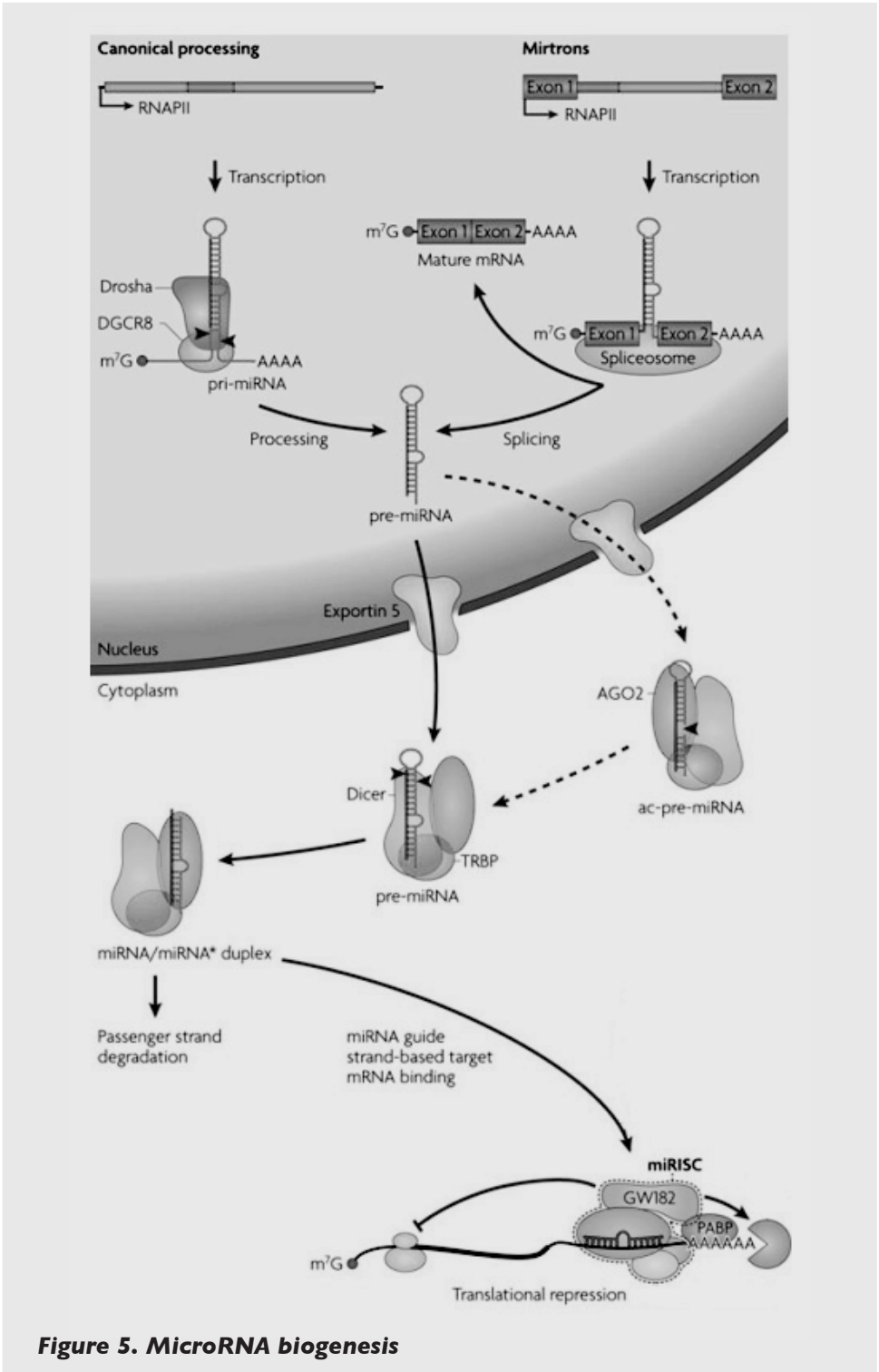

Figure 5. MicroRNA biogenesis

Figure 5. MicroRNA biogenesis
MicroRNAs (miRNAs) are processed from RNA polymerase II (RNAPII)-specific transcripts of independent genes or from introns of protein-coding genes. In the canonical pathway, primary precursor (pri-miRNA) processing occurs in two steps, catalyzed by two members of the RNase III family of enzymes, Drosha and Dicer in cooperation with dsRNA binding protein DGCR8. In the first nuclear step, the Drosha–DGCR8 complex processes pri-miRNA into an ~70-nucleotide precursor hairpin (pre-miRNA), which is exported to the cytoplasm. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of splicing and debranching, thereby bypassing the Drosha–DGCR8 step. In either case, cleavage by Dicer, assisted by transactivation-responsive RNA-binding protein (TRBP), in the cytoplasm yields an ~20 bp miRNA/miRNA* duplex. In mammals, Argonaute 2 (Ago2) support Dicer processing by cleaving the 3' arm of some pre-miRNAs, forming an additional processing intermediate called AGO2-cleaved precursor miRNA (ac-pre-miRNA). Following processing, one strand of the miRNA/miRNA* duplex (the guide strand) is preferentially incorporated into an miRNA-induced silencing complex (miRISC), whereas the other strand (passenger or miRNA*) is released and degraded, in some cases this passenger strand can also be loaded into miRISC to function as miRNAs. Most animal miRNAs imperfectly base-pair with sequences in the 3'-UTR of target mRNAs, and inhibit protein synthesis by either repressing translation or promoting mRNA deadenylation and decay. Efficient mRNA targeting requires continuous base-pairing of miRNA nucleotides 2 to 8 (the seed region). Ago-proteins and glycine-tryptophan protein of 182 kDa (GW182) proteins are directly associated with miRNAs, are core components of the miRNA-induced silencing complex and are needed for effective binding and subsequenial translational repression or a decay [80]. Adapted from: The widespread regulation of microRNA biogenesis, function and decay, Krol et al, Nature Reviews Genetics, 2010.

Both chapters utilize antagomir-technology to specifically silence miRNA-126 *in vivo*. This approach to silence miRNA-126 was also used in **Chapter 5** to elucidate the regulatory role of miRNA-126 in vascular cell adhesion molecule-1 expression in the kidney vasculature. **Chapter 6** details our findings that circulating miRNA-126 in the periphery is not exclusively derived from endothelial cells but can also originate from platelets. Consequently, the use of aspirin has to be taken into account when relating circulating miRNA-126 levels to the progression of cardiovascular disease. **Chapter 7** demonstrates that the angiogenic potential of miRNA-126 as described in Chapter 3 might reach beyond the presence of this pro-angiogenic miRNA in endothelium, but that neovascularization can also be supported by miRNA-126 expressed in circulating cells.

Finally, **Chapter 8** provides a summary of research presented in this thesis, presents the major conclusions that could be drawn and further discusses the role of miRNA-126 in vascular homeostasis.

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CHAPTER 2

The role of microRNA-126 in vascular homeostasis

Introduction

The vascular network comprises a large network of arteries, veins and capillaries that facilitates the circulation of blood to maintain homeostasis. The endothelium constitutes a thin layer of endothelial cells (ECs) that form a semi-permeable barrier between the circulating blood and the other structural compartments of the vascular wall. ECs are key mediators of vascular homeostasis and, therefore, the maintenance of a healthy endothelium is critical. Pathological conditions such as tissue ischemia, inflammation, hyperglycemia and hypercholesterolemia lead to EC activation, endothelial dysfunction and ultimately to EC apoptosis, which can accelerate the risk of premature atherosclerosis. Maintenance of endothelial integrity is of central importance for cardiovascular health and in large part determined by the balance between EC injury and repair [1]. One of the key repair mechanisms that aid in the replacement of damaged ECs is angiogenesis, the process by which new blood vessels bud from pre-existing capillary ECs. Angiogenesis is a tightly regulated process, that requires the coordination of numerous signaling pathways in which ECs act as both active participants and regulators [2]. The formation of novel capillaries may not be restricted to the sprouting capability of ECs as circulating, bone marrow-derived cells are also thought to be contributing to neovascularization and re-endothelialization [3]. This process that involves endothelial progenitor cells (EPCs) is called neo-vasculogenesis. The relative contribution and the exact phenotype of the cells involved in this process is a topic of active investigation. It has been demonstrated that EPCs can support the formation of independent functional vascular structures after migration towards a hypoxic region in the skin [4]. Others claim that the induced neovascularization mostly depends on stabilization of novel vascular structures by recruited supporting perivascular mural cells [5-6]. Although their role is elusive, it is clear that bone marrow-derived circulation cells contribute positively to maintain vascular integrity.

Insight into the cellular and molecular mechanisms that can control vascular homeostasis is of high relevance in pursuit of understanding and treatment of a broad range of diseases that involve deficient or aberrant neovascularization such as cardiovascular disease and cancer. It is becoming increasingly apparent that microRNAs (miRNAs) are key regulators of vascular homeostasis [19].

These short non-coding RNAs were initially discovered in 1993 [7]. However, the impact of miRNAs on cellular biology has only recently started to unfold. The genomic sequences encoding miRNAs are generally harboured within intronic regions of genes and have been found to be well conserved between species [8]. After synthesis and processing miRNAs are incorporated in the RNA-induced silencing complex (RISC), where the miRNA can guide the RISC to the 3'UTR of the designated target sequence. [9]. The seed sequence, defined by 2-8 nucleotides located at the 5' region of the miRNA, is critical for target recognition and

silencing of the mRNA [10-11]. Translation of the mRNA is repressed after association of a miRNA with its target sequence. The exact mechanism by which translational arrest is induced involves both degradation of the mRNA and the inhibition of the initiation and elongation steps of translation [12-14].

MiRNAs are expressed in a tissue- and cell-specific manner during development suggesting a role for miRNAs in specifying and maintaining tissue identity [15]. Also, there is growing recognition that one single miRNA can have multiple targets, and therefore impacts multiple pathways. These features also predict regulatory roles for miRNAs in the control of vascular homeostasis and recent studies identified a number of miRNAs with pro-angiogenic [16-19] as well as anti-angiogenic functions [20, 21].

Of particular interest with respect to a controlling role in neovascularisation is miRNA-126, a miRNA that was found to be highly enriched in the endothelium [22-23]. Initially it was thought that miRNA-126 was exclusively expressed in ECs, however miRNA-126 is also present in several cancer cell types [24-31], airway epithelium [32-34], circulating cells [35-39], and platelets [40-42]. Significant progress has been made in identifying its mRNA targets and function both in endothelial cells and other cell types that express this miRNA (listed in Table 1). Clearly, like many others, miRNA-126 appears to fulfil different functions in different stages of cell life and can work on several targets within the same cell.

Interestingly, miRNAs are also detected outside the cellular compartment as they can readily be detected in microvesicles in human plasma, such as apoptotic bodies [43] and exosomes[44] or 'free-floating' non-vesicle argonaute-2 (Ago2) complexes [45]. Unlike other miRNAs, miRNA-126 is not restricted to one of these groups and is present in each of these configurations [45].

More recently, miRNA levels have been quantitated in human plasma using miRNA-arrays and quantitative real-time PCR, and it is been reported that

Box 1.

Depending on the target prediction website and algorithm that is used to identify targets for (human) miRNA-126 the total of hits can vary between 17 in TargetScan (http://www.targetscan.org) and 937 in MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5). TargetScan was developed in 2001 to identify the targets of vertebrate miRNAs, the algorithm combines thermodynamics based modelling of RNA:RNA duplex with comparative sequence analysis to predict miRNA targets across multiple genomes [93]. MicroCosm Targets uses the Miranda algorithm, which follows about the same rules as the TargetScan algorithm. However, the genome of two other organisms, the zebrafish (***Danio rerio***) and the fugu (***Fugu rubripes***) was scanned for potential targets. In addition to the analysis of 3'UTRs, all protein coding regions for high scoring miRNA target sites are calculated, leading to far more possible target sites [94]. Despite numerous lists of potential targets per miRNA, only a small number of target sites on target genes have been experimentally verified.

lowered levels of miRNA-126 correlate with age, coronary artery disease (CAD) or subjects diagnosed with type II diabetes mellitus (DM2) [46-48]. These studies indicate a potential link between a reduction of this vascular miRNA and endothelial dysfunction. While the abundant expression of miRNA-126 by ECs suggests that these cells are the main source of circulating miRNA-126 in the circulation. However, other sources such as platelets [40-42] and bone marrow-derived circulating cells [38] express significant levels of miRNA-126 and can therefore also contribute to the circulating pool of miRNA-126.

MiRNA-126 in the endothelium

The locus encoding miRNA-126 resides within intron 7 of the EC-restricted epidermal growth factor like-domain 7 (EGFL7) [49]. EGFL7, that can be found on chromosome 9 or 2 in human and mice respectively [50], is a secreted protein of 41 kDa [51], that is up regulated after arterial injury *in vivo*. This augmentation of EGFL7 recruits ECs, angioblasts and supportive cells to sites of injury for vascular repair [52]. Upstream of the EGFL7/miRNA-126 locus are two E26 transformation-specific sequence (Ets) binding sites that are evolutionarily conserved. It has been established that the binding of Ets-1 or Ets-2 to an Ets binding site is required for the trans-activation of the EGFL7/miRNA-126 gene in ECs [53]. EGFL7 is restricted to the endothelium in adult mice and humans [50], and its expression can contribute to the presence of miRNA-126 in these cells, however, the presence of miRNA-126 in other cells, which do not express EGFL7 is counter-intuitive. A first explanation could be that the EGFL7 gene is also expressed shortly during early embryogenic development [51, 54], which might be the reason for the expression of miRNA-126 in other cells of the hematopoietic lineage. It is likely that the primary transcript of EGFL7 is post-transcriptionally silenced, independent of the nuclear and cytoplasmatic processing of miRNA-126. Furthermore, it has been described that the mRNA of EGFL7 harbours a binding site for miRNA-126 [55], indicating that miRNA-126 itself can block the translation of EGFL7. However, a positive or negative association for miRNA-126 with its host gene was absent in tumour samples taken from a cohort of 110 colon cancer patients [56]. These data indicate that miRNA-126 expression may react to different stimuli then those that lead to the expression of EGFL7.

Interestingly, the targeted deletion of miRNA-126, either via genetic deletion in animals [49, 57, 58] or following administration of antagomirs [59], perturbed vascular development [49, 57, 58], attenuated recovery after myocardial infarction [58], and impaired angiogenic capacity after ischemic hind limb injury [59]. Furthermore, mutant mice and morphant zebrafish demonstrated drastic vascular abnormalities, such as heart valve elongation defects [60], oedema, haemorrhaging and embryonic death [49, 57, 58]. As such, the diminished angiogenic capacity and vascular defects observed as a result of decreased

Table 1. Overview of validated targets and pathways of miRNA-126

Cell type / tissue	Target protein	Process	Ref.
Endothelium	Spred-1, PI3KR2, PAK1	vascular development angiogenesis	[57-59, 90]
Endothelium	VCAM-1	inflammation leukocyte adhesion EC heterogeneity	[23, 71]
Endothelium	RGS16	atherosclerosis Sca-1+ incorporation	[43]
Endothelium	CXCL12	ischemia mobilization of Sca-1+	[66]
CD4+ T cells	Dnmt1	DNA methylation	[39]
Hematopoietic stem cells	HOXA9, c-Myb, PTPN9	hematopoietic development erythropoiesis	[35, 36, 38]
Mast cells	Spred-1	mast cell differentiation cytokine production	[37]
Epithelium (lung)	?	allergen exposure Th2 response eosinophil recruitment	[32, 33]
Epithelium (lung)	TOM1	immune response modulation of TLRs	[34]
Epithelium (mammary)	PGR, β-casein	mammary gland development lactation	[91]
Breast cancer	IRS1, VEGF-A, PI3KR2	tumour development	[30, 31]
Colon cancer	p85β subunit	tumour development	[28]
Lung cancer	Crk, VEGF-A SLC7A5, EGFL7	tumour invasion tumour angiogenesis tumour cell proliferation	[24, 26, 27, 55]
Gastric cancer	SOX2, Crk	tumour cell proliferation tumour invasion	[25, 29]
Pancreatic cancer	Adam9	tumour invasion	[92]

miRNA-126 in ECs strongly suggests that miRNA-126 has an essential role in regulating EC responsiveness to angiogenic stimuli. Support for this notion can be derived from the fact that miRNA-126 regulates the angiogenic signalling pathways, downstream of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) by binding to the 3'UTRs of sprouty-related EVH1 domain containing 1 (SPRED-1) and phosphoinositide-3-kinase regulatory subunit 2 (PI3KR2) [57, 58]. By effectively blocking the expression of these aforementioned proteins by miRNA-126, the v-raf-1 murine leukaemia viral oncogene homolog 1 (RAF1) and phosphoinositide-3-kinase (PI3K) are able to trigger ECs to elicit a vascular response to injury [57, 58]. Therefore, it is likely that ECs require miRNA-126 to maintain the integrity of the vasculature during vascular development as well as in adult life. Recently, a role for miRNA-126 has been confirmed in zebrafish, where embryos were treated with the myosin ATPase inhibitor 2,3-butanedione 2-monoxime or the anaesthetic tricaine methanesulphonate to arrest the heart and block circulation [61], the expression of miRNA-126 and Kruppel-like factor 2a (KLF2a) were down regulated, leading to enhanced translation of SPRED-1. The resultant repression of VEGF-stimulated angiogenesis led to major (vascular) developmental defects [62].

Next to the role of miRNA-126 in regulating angiogenesis, a regulatory role has also been established for the development of atherosclerosis. In response to proapoptotic stimuli, ECs lining atherosclerotic plaques can generate apoptotic bodies [63-64]. Both the release and abundance of these apoptotic bodies have been found to be associated with endothelial dysfunction, suggesting that they may serve as a diagnostic marker of atherosclerotic vascular disease [63]. The incorporation of apoptotic bodies secreted by ECs by an acceptor cell can dramatically change its miRNA content, impacting cellular function. It has been established that miRNA-126 is the most abundant miRNA in these EC-derived apoptotic bodies [43]. The *in vitro* uptake of EC-derived apoptotic bodies by human umbilical vein ECs (HUVEC) resulted in a marked increase in intracellular expression and secretion of chemokine ligand 12 (CXCL12) [43]. However, the 3'UTR of CXCL12 is a direct target of miRNA-126, thus the increase in protein expression can not be the result of elevated miRNA-126 levels in the recipient cell. Regulator of G-protein signalling 16 (RGS16), a negative regulator of the CXCL12 receptor chemokine (C-X-C motif) receptor 4 (CXCR4), was identified to be a target of miRNA-126, and to be involved in the regulation of CXCL12 expression [65]. This was validated by intravenously injecting miRNA-126-containing EC-derived apoptotic bodies into ApoE-/- mice. After placing these mice on a high fat diet for a period of six weeks, mice that were administered miRNA-126-containing apoptotic bodies displayed a higher luminal incorporation of CXCR4-dependent Sca-1+ stem cells into the aortic root plaque than mice injected with non-EC derived apoptotic bodies. Prolonged treatment with EC-derived apoptotic bodies elevated CXCL12 levels

andreduced atherosclerotic plaque size in the aortic root [43]. These data implicate that the delivery of miRNA-126 by microparticles, such as apoptotic bodies, might play a key role in diet-induced atherosclerosis.

In addition, miRNA-126 can also directly influence CXCL12 expression by binding to 3'UTR of its mRNA. It has been established that attenuation of miRNA-126 with antagomirs increases the expression of CXCL12 in HUVEC. *In vivo* administration of antagomir-126 led to elevated protein levels in the circulation and ischemic tissue after inducing ischemic injury. The increase in CXCL12 expression triggered the mobilization of Sca-1+/Lin- stem cells into the circulation [66]. These findings suggest that miRNA-126 potentially plays an important role in regulating vasculogenesis after ischemic injury by targeting CXCL12.

It is well established that systemic inflammation leads to EC activation. Since miRNA-126 is a central regulator of EC function and homeostasis, it is likely that miRNA-126 might influence the EC response to inflammatory stimuli. A primary response to systemic inflammation is the augmentation of vascular cell adhesion molecule 1 (VCAM-1) expression, leading to the clustering of VCAM-1 on the endothelial surface. The formation of these clusters results in the transmission of numerous intracellular signals that facilitate adhesion, migration and diapedesis of leukocytes through the EC permeability barrier into the adjacent tissue [67-70]. A potential binding site for miRNA-126 was localized in the 3'UTR of the mRNA of VCAM-1, which suggests a role for miRNA-126 by controlling the expression of VCAM-1 upon inflammation. Indeed, over-expression of miRNA-126 in combination with the induction of an inflammatory response with tumour necrosis factor alpha (TNFα) in HUVEC suppressed the protein levels of VCAM-1 and the ability of these cells to adhere leukocytes [23]. Moreover, it has recently been shown that the expression of miRNA-126 in microvascular compartments is a governing factor in acute inflammation in the kidney. Upon induction of anti-glomerular basement membrane glomerulonephritis as well as TNFα, lipopolysaccharide or anti-myeloperoxidase-induced glomerulonephritis, VCAM-1 mRNA expression was highly increased in both arterioles and glomeruli, while the protein was only expressed to a limited extent in the glomerular compartment. Extensive RNA analysis in the glomerular and arteriolar vascular segments suggested that these two vascular compartments display different levels of miRNA-126. High miRNA-126 levels were found in the glomerular compartment and coincided with low VCAM-1 protein expression, while in the arterioles low miRNA-126 levels associated with increased VCAM-1 levels [71]. These elevated levels for miRNA-126 in glomerular ECs coincided with increased expression of Ets-1, an established transcriptional regulator of miRNA-126 [53]. The interaction between miRNA-126 and Ets-1 adds an extra level of complexity to the regulation of vascular inflammation. Vascular inflammation induces Ets-1

expression, thereby activating the transcription of pro-inflammatory proteins, including VCAM-1 [72]. In contrast, Ets-1 also induces miRNA-126, subsequently inhibiting the translation of VCAM-1 [23]. Through its influence on the expression and clustering of VCAM-1, miRNA-126 may contribute to the heterogenic response of ECs to inflammatory stimuli. The multi-regulating capacity of miRNA-126 in various stages of vascular development, neovascularisation and inflammation underlines the importance of miRNA-126 in ECs (summarized in Figure 1).

MiRNA-126 in progenitor cells

The function of miRNA-126 extends beyond its expression in endothelial cells. Several cancer types display elevated levels of miRNA-126 [24-31], and cells coming from the hematopoietic compartment also show expression of miRNA-126 [35-42]. For instance, elevated miRNA-126 expression has been detected in human CD34+ hematopoietic stem cells and progenitor cells following erythroid induction [36, 73], and mobilization with G-CSF [74]. Interestingly, miRNA-126 expression was found to be decreased during megakaryocytopoiesis [75]. Since differential expression of a miRNA highly impacts the expression of target genes, one can speculate that alterations in miRNA-126 expression in

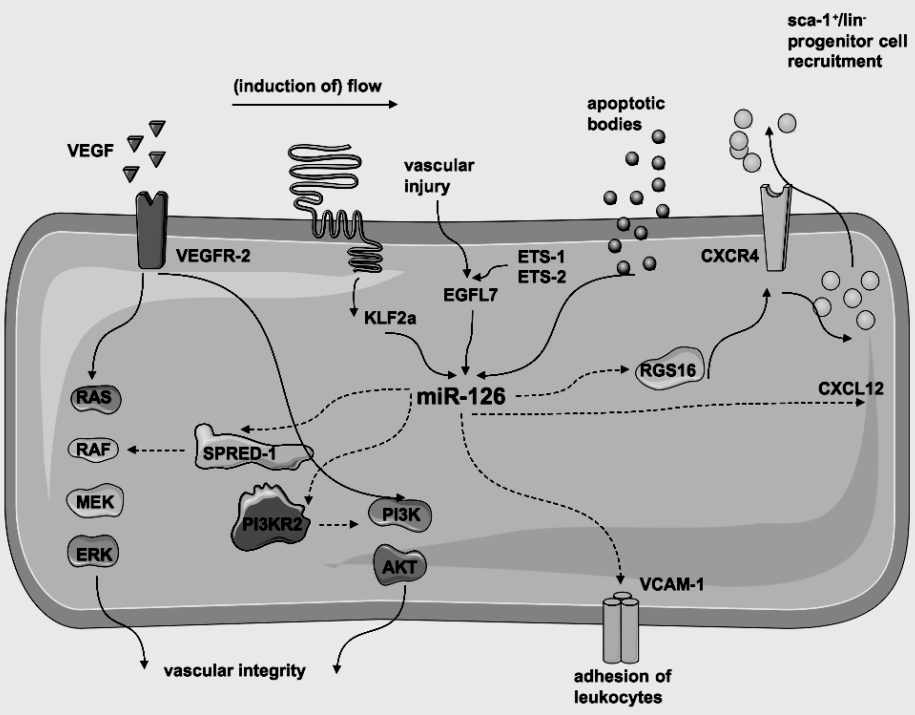

Figure 1. The multi-regulating capacity of miRNA-126 in endothelial cells

hematopoietic progenitor cells could profoundly impact cellular function.

Since the evolutionary conserved homeobox (HOX) genes play an important role during development and hematopoiesis[76-77], Shen and co-workers hypothesized that miRNA-126 could impact hematopoiesis by regulating HOX mRNA transcript stability [38]. However, only two HOX genes, namely HOXA3 (miRanda, http://www.microrna.org/) and HOXA9 (PicTar, http://pictar.mdc-berlin.de/), contain predicted binding sites for miRNA-126. *In vitro* experiments revealed that the abrogation of endogenous miRNA-126 in murine bone marrow cells increased the expression and activity of HOXA9 protein. Furthermore, it was demonstrated that the expression profile of miRNA-126 parallels HOXA9 mRNA expression in normal murine bone marrow. These findings suggest a potential role for miRNA-126 in controlling hematopoietic development by regulating the levels of HOXA9 protein [38].

In addition to murine bone marrow cells, human embryonic stem cells (hESCs) have been used to study the function of miRNA-126 in hematopoietic differentiation. For this, miRNA-126 was over-expressed in hESCs upon embryoid body formation, yielding a reduced number of erythroid colonies. Co-expression of tyrosine-protein phosphatase non-receptor type 9 (PTPN9), which contains a predicted binding site for miRNA-126, led to a partial recovery of erythropoiesis [36]. The inability to fully restore erythropoiesis suggests that another target of miRNA-126 might be found in the erythroid pathway. The role of miRNA-126 in erythropoiesis is also suggested by the notion that PTPN9 is hyper-activated in the erythroid progenitors in patients with polychytemia vera, a disease that results in erythrocyte overproduction. This hyper-activation is combined with the lack of miRNA-126 expression in the erythroid progenitors of these patients [78-79].

Circulating miRNA-126

Blood plasma samples harvested from subjects with cardiovascular disease (CVD) risk factors have extensively been studied for the presence of biomarkers. Interestingly, miRNAs could serve as novel biomarkers as they can be detected in the circulation, making it possible to readily assess the miRNA-profiles of healthy and diseased subjects. Importantly, miRNAs are surprisingly stable despite the high endogenous RNase activity in the circulation [80]. Circulating miRNAs are present in both serum as well as plasma and can be measured using quantitative real-time PCR [81]. To date, miRNAs have been detected in the circulation in two forms, notably as being carried by cell-released vesicles [82] or in association with Ago2 complex, the catalytic component of the RISC [45]. While most miRNAs exist in the circulation in only one of both forms, miRNA-126 has been found to be both vesicle-bound as well as in a complex with Ago2 [45].

In several different patient cohorts the circulating miRNA content was compared to healthy controls. In a limited study with 12 heart failure patients and

healthy controls, no differences were found in miRNA-126 levels [83]. In contrast, the presence of cardiovascular risk factors such as age, CAD and DM2 correlated with decreased expression of miRNA-126 as compared to healthy controls [46-48]. The loss of miRNA-126 could explain the observed impairment of angiogenic signalling in the periphery of patients diagnosed with CAD and DM2. It is likely that fine-tuning of miRNA-126 expression in CVD is essential to elicit the appropriate response in the case of acute endothelial activation. MiRNA-126 is abundantly expressed in ECs, and is required for the stimulation of neovascularization, while curtailing its expression in the face of chronic endothelial activation and injury, to avoid EC death.

This thesis sheds a light on cellular sources of miRNA-126 in the circulation, including ECs and circulating hematopoietic stem cells [35-39]. Recently, platelets have also been found to express miRNA-126 [41]. Although platelets have no nucleus and therefore do not possess the machinery to transcribe or generate mature miRNAs, miRNAs (including miRNA-126) are both abundant and functional in platelets [41]. The source of these mature miRNA-126 molecules is likely to be the megakaryocyte, since megakaryocytes have been found to express significant levels of miRNA-126 [75], which indicates that their miRNA content is transferred from the megakaryocyte to the budding platelets. Furthermore, it is possible that platelets actively endocytose vesicle-bound or Ago2-associated miRNAs from the periphery, thereby adding to their miRNA-126 content.

Upon endothelial injury platelets are exposed to collagens, von Willebrand factor and tissue factor derived from the subendothelium and get activated. This leads to platelet aggregation, a process that triggers the secretion of various cytokines and miRNA-containing microvesicles [82, 84, 85]. The notion that microvesicles and platelet-derived microvesicles can serve as a major source of circulating miRNAs for uptake by either ECs or other circulating cells [44, 86] further support the notion that miRNA-126 does not exclusively exert its effects in ECs, but could potentially function as a critical mediator of vascular homeostasis.

Concluding remarks

MiRNA-126 is abundantly expressed in ECs and plays an important role in neovascularisation by regulating the expression of various proteins driving both angiogenesis and vasculogenesis [43, 57-59, 66, 76]. Furthermore, a role for miRNA-126 in adjusting the expression and microvascular location of VCAM-1 in ECs upon inflammation has been demonstrated [23, 71]. In addition, miRNA-126 is expressed in bone marrow derived cells where it can determine the erythroid and hematopoietic fate of the cell [36-38].

The notion that on one side increased levels of miRNA-126 play a facilitating

role in angiogenesis and on the other side a lowered expression of miRNA-126 supports vasculogenesis may underline the importance of this vascular miRNA as a vasculogenic switch. This hypothesis is supported by the fact that lowered levels of miRNA-126 lead to the increased expression of VCAM-1 that may facilitate homing of leukocytes to the endothelium.

A major gap in current understanding of miRNA-126 biology is knowledge about the molecular mechanisms underlying the regulation of this miRNA in ECs. So far it has been demonstrated that binding of Ets-1 or Ets-2 to the EBS and induction of flow are needed to govern the expression of the EGFL7/miRNA-126 gene [53, 62]. Furthermore, it has been shown that EC-derived apoptotic bodies can increase the levels of miRNA-126 in ECs [43]. Despite these studies, a real understanding whether there are any extracellular factors that may contribute to an altered expression of miRNA-126 is unknown. For instance, cytokines like VEGF and TNFα that mediate endothelial activation, lead to an up regulation of a distinct subset of miRNAs, but not miRNA-126 [87]. It is therefore interesting to design studies to unravel the mechanisms that lead to an increase or abrogation of miRNA-126.

To date the source of miRNA-126 in the circulation is unknown. ECs, circulating cells and platelets can be considered as the major sources that can release miRNA-126 into the periphery. It is likely that these three cell types, and potentially other cell types contribute to the total miRNA-126 content observed in the circulation.

At present no molecular mechanisms has been linked to circulating miRNAs (including miRNA-126) and cardiovascular disease. Whether the source of circulating miRNA-126 is endothelium, circulating cells or platelets, the involvement of miRNA-126 in vascular biology will make it a key component to investigate in patients with cardiovascular risk factors. To date, the use of circulating miRNAs as predictive and/or monitory biomarkers is still in an early phase. However, in the future a spectrum of circulating miRNAs, miRNAs in urine samples [88] or other bodily fluids [89] will be highly informative about the disease status of a patient in the clinic.

Currently no clinical trials to enhance or antagonize miRNA-126 function are, to our knowledge, undertaken. Nevertheless, subjects with cardiovascular risk factors have decreased levels of miRNA-126 in their plasma [46-48], suggesting that mechanisms whereby miRNA-126 could be administered to these subjects could be an effective modality in the prevention of cardiovascular disease.

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CHAPTER 3

Antagomir-mediated silencing of endothelial cell specific microRNA-126 impairs ischemia-induced angiogenesis

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Abstract

MicroRNAs are negative regulators of gene expression that play a key role in cell-type specific differentiation and modulation of cell function and have been proposed to be involved in neovascularization. Previously, using an extensive cloning and sequencing approach, we identified miR-126 to be specifically and highly expressed in human endothelial cells. Here, we demonstrate EC-specific expression of miR-126 in capillaries and the larger vessels *in vivo*. We therefore explored the potential role of miR-126 in arteriogenesis and angiogenesis. Using miR-reporter constructs, we show that miR-126 is functionally active in EC *in vitro* and that it could be specifically repressed using antagomirs specifically targeting miR-126. To study the consequences of miR-126 silencing on vascular regeneration, mice were injected with a single dose of antagomir-126 or a control "scramblemir" and exposed to ischemia of the left hindlimb by ligation of the femoral artery. Although miR-126 was effectively silenced in mice treated with a single, high dose of antagomir-126, laser Doppler perfusion imaging did not show effects on blood flow recovery. In contrast, quantification of the capillary density in the gastrocnemius muscle revealed that mice treated with a high dose of antagomir-126 had a markedly reduced angiogenic response. Aortic explant cultures of the mice confirmed the role of miR-126 in angiogenesis. Our data demonstrate a facilitary function for miR-126 in ischemia-induced angiogenesis and show the efficacy and specificity of antagomir-induced silencing of EC-specific microRNAs *in vivo*.

Introduction

Endothelial cells (EC) play an essential regulatory role in the capacity of the vasculature to adequately respond to injury or hypoxia. In arteriogenesis, EC react to elevated shear stress by recruiting and activating leukocytes that mediate remodeling of small collateral arterioles. In tissue ischemia, novel capillaries are generated by proliferation and migration of EC that sprout from pre-existing capillaries. The molecular mechanisms underlying this directive role of EC in vascular plasticity have been extensively studied and involve numerous environmental cues that elicit complex, but tightly coordinated responses in the expression of genes controlling proliferation, migration and cell-differentiation [1]. MicroRNAs constitute a recently recognized class of short, non-coding RNA molecules (~ 21 nt) that could potentially regulate the activity of 30% of all genes at the post-transcriptional level [2]. The ability of microRNAs to regulate multiple targets provides a means for coordinated control of gene expression, while also making them especially attractive candidates for regulating both cell-type specific differentiation and modulation of cell function [3].

Recently, evidence supporting a role for endothelial microRNAs in the control

of neovascularization has been provided [4] with *in vitro* studies demonstrating both pro-angiogenic microRNAs (let 7b, miR-27b [5], miR-130a [6], miR-210 [7]) as well as microRNAs with anti-angiogenic actions (miR-221/222) [8, 9]. To study the role of endothelial microRNAs in neovascularization, we recently generated an inventory of known and novel microRNAs expressed by human microvascular EC and late outgrowth EC using extensive cloning and sequencing [10]. By comparing the microRNA expression profiles of various cultured EC with other tissues and cell types, we confirmed miR-126 to be highly abundant and specific for EC. MiR-126 is located in an intron of the epidermal growth factor-like-domain 7 gene (EGFL7) of human, mouse and zebrafish [11]. Due to the fact that EGFL7 expression is augmented in adult angiogenesis and vascular injury [12], we hypothesized that co-expression of miR-126 may also play a role in neovascularization. Recently, two papers have described a role for miR-126 in vascular development in mice [13], and zebrafish [14]. Targeted deletion of miR-126 resulted in vascular leakage, hemorrhaging and embryonic lethalility in a subset of the mutant mice and abnormal vessel morphology was observed in the zebrafish. In this study, we assessed the role of miR-126 in neovascularisation in the adult mouse and explored the effects of conditional silencing of this microRNA on arteriogenesis and angiogenesis *in vivo*. Previous research has demonstrated that intravenous injection of chemically modified and cholesterol-conjugated RNA analogs (antagomirs), specifically silences complementary microRNAs in mice for up to 23 days [15, 16]. Our studies using a mouse ischemic hind limb model demonstrate, for the first time, that antagomir-induced silencing of miR-126 impairs ischemia-induced angiogenesis *in vivo*.

Material and methods

Cells and cell culture

Human embryonic kidney cells (HEK-293T), HeLa cells and immortalized mouse endothelial cells (EC), originally derived from brain capillaries (bEnd3 cells) [28, 29] were cultured in DMEM (Gibco/Invitrogen, Breda, The Netherlands) supplemented with pen/strep and 10% fetal calf serum (FCS; Bio Whittaker/Cambrex, Verviers, Belgium). Immortalized human umbilical vein EC (EC-RF24) [30]. were cultured in M199 medium (Gibco/Invitrogen) supplemented with pen/strep, 20% FCS, 10 IU/mL heparin (Leo Pharma, Breda, The Netherlands), 2.5% HEPES buffer (Gibco/Invitrogen) and 12.5 µg/mL EC growth supplement (Sigma, St. Louis, MO). For the generation of monocyte-derived human dendritic cells (DC), human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy donors by Ficoll (Amersham, 's-Hertogenbosch, The Netherlands) density gradient centrifugation. Monocytes were positively selected by CD14-MACS microbeads (Miltenyi Biotech, Bergish Gladbach, Germany) and cultured for 7 days in RPMI medium (Gibco/Invitrogen)

containing 10% FCS, 2% pen/strep, human GM-CSF (5 ng/mL, Leucomax, Lelystad, The Netherlands) and human IL-4 (10 ng/mL, Peprotech, Rocky Hill, NJ) at a density of 1.5 x 106 cells/ well in a 6 wells plate (Costar, Cambridge, MA). For the generation of monocyte-derived human macrophages, PBMC were cultured with RPMI medium containing 10% FCS, 2% pen/strep and human GM-CSF (5 ng/mL). Human foreskin-derived microvascular endothelial cells (hMVEC) and human umbilical vein endothelial cells (HUVEC) were isolated, cultured and characterized as described previously [31-33].

Quantification of microRNA levels

Total RNA from different cell types and tissues was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands). Expression levels of selected microRNAs were validated in triplicate by quantitative RT-PCR (qRT-PCR). Reverse transcription was performed using a 5 minute 65°C incubation of 250 ng total RNA with dNTPs (Invitrogen) and oligo(dT) (U6, Invitrogen) or using specific Taqman® microRNA probes (miR-126 and miR-423, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). cDNA was synthesized using a M-MLV First-Strand Synthesis system (Invitrogen). Validation of miR-126 and miR-423 levels was performed using Taqman® miR assays and qRT-PCR. For normalization, a qRT-PCR on RNU6B was performed on cDNA obtained from the same RNA. The following primers were used for PCR: U6 (sense) CTCGCTTCGGCAGCACA and U6 (antisense) AACGCTTCACGAATTTGCGT. qRT-PCR conditions were the same as used for miR quantifications. Results were normalized using Gene Expression Analysis for iCycler IQ® RT-PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands).

In situ hybridizations

In situ hybridizations were performed as essentially described [18, 34, 35]. In brief, serial paraffin-embedded sections of the human kidney were used for *in situ* hybridization of miR-126. The sections were rehydrated, digested with proteinase K (5 µg/mL, 10 min, 37°C) and post-fixated with 4% formaldehyde. The sections were hybridized overnight in hybridization mix (50% formamide, 5 x SSC, 0.1% Tween-20, 500 µg/mL tRNA, 0.5% citric acid (92 mM) and 50 µg/mL heparin) at 53°C with a digoxigenin (DIG)-labeled locked nucleic acid (LNA) miR-126 probe (25 µM, Exiqon, Vedbaek, Denmark). The DIG-labels were visualized with sheep anti-DIG (Roche, Mannheim, Germany). MiR-126 stained blue after overnight incubation at 50°C in the dark with the substrate NCT/BCIP (Roche). After staining, the sections were dehydrated in a series of ethanol and xylene. Control sections were incubated with a LNA-probe that does not bind to any known miRNAs.

MiR-126 reporter assays

Cloning of one (pMIR-126M) or four (pMIR-126Q) target binding sites was performed into a pMIR-reportTM Expression Reporter Vector System (pMIR, Applied Biosystems), which contains an experimental firefly luciferase reporter gene. After cloning, the plasmids were sequenced to evaluate their fidelity. A renilla luciferase expressing plasmid (pRL-SV40, Promega, Leiden, The Netherlands) served as control for the efficiency of electroporation. Primer constructs used for correct cloning into pMIR-report vector were: pmiR-126M (sense) CTAGTCAGTGGCAGCAGCATTATTACTCACGGTACGATCAGTGGCAGCA; pmiR-126M (antisense) AGCTTGCTGCCACTGATCGTACCGTGAGTAATAATGCTGCTGCCACTGA; pmiR-126Q (sense) CTAGTAGGCGCGCCATATAGCATTATTACTCACGGTACGATATAGCATTATTACTCACGGTACGATATAGCATTATTACTCACGGTACGATATAGCATTATTACTCACGGTACGATATATAGCGCGCTACA; and pmiR-126Q (antisense) AGCTTGTAGCGCGCTATATATCGTACCGTGAGTAATAATGCTATATCGTACCGTGAGTAATAATGCTATATCGTACCGTGAGTAATAATGCTATATCGTACCGTGAGTAATAATGCTATATGGCGCGCCTA.

Design of antagomirs

Cholesterol-conjugated RNA analogs (antagomirs, Dharmacon RNA technologies, Lafayette, CO) were synthesized as previously described [15]. For antagomir-126 the following sequence was used: 5'-gscsauuauuacucacgguascsgsas-Chol-3'. As a control a 'scramblemir' was used, this RNA analog is constructed from a randomized nucleotide sequence which does not bind to any known microRNAs: 5'-asusgacuaucgcuauucgcsasusgs-Chol-3'. The lower case letters represent 2'-OMe-modified nucleotides; subscript 's' represents phosphorothiate linkage; 'Chol' represents a cholesterol-group linked through a hydroxyprolinol linkage (Manoharan, M., Kesavan, V., & Rajeev, K. G. SiRNA's containing ribose substitutes to which lipophilic moieties may be attached. U.S. Pat. Appl. Publ. (2005), US 2005107325).

Electroporation of EC-RF24cells

Fibronectin-adherent EC-RF24 cells (500.000) were detached by trypsin treatment and resuspended in 500 µL serum free medium (Optimem; Gibco/Invitrogen) and 1 µg specific pMIR-report and 100 ng pRL-SV40 was added. The cell suspension was chilled for 10 minutes at 4°C and electroporated in a Gene Pulser® cuvette (Bio-Rad Laboratories) using Gene Pulser II (Bio-Rad Laboratories). After 10 minutes recovery time at room temperature, a triplicate of 150.000 cells was plated in a 24-wells plate coated with fibronectin. After 24 hours the firefly-luciferase and renilla-luciferase signals were measured using a Dual-Luciferase® Assay Reporter System (Promega) in a Lumat LB9507 (EG&G Berthold, Bundoora, Australia) and the ratio of the firefly-luciferase expression

divided by renilla luciferase expression was calculated. Cells transfected with pMIR were taken as 100%. For inhibition studies, 5 µg/mL antagomir-126 or scramblemir was added on a near confluent cell layer, 16 hours prior to transfection.

Hind limb ischemia model

All animal experimental protocols were approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO). One day prior to surgery, C57BL/6 WT mice (n=6 per group, age=10 weeks, Charles River, Maastricht, The Netherlands) were injected intravenously (200 µL) with antagomir-126 at 1.0 mg/ animal (high dose, HD), 0.1 mg/ animal (low dose, LD), scramblemir at 1.0 mg/ animal (HD) or 0.1 mg/ animal (LD). Before surgery, mice were anesthetized intraperitoneally with a combination of Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion Corporation, Turku, Finland) and Fentanyl (0.05 mg/kg, Janssen Pharmaceutica, Tilburg, The Netherlands). Ischemia of the left hind limb was induced by electrocoagulation of the left common femoral artery, proximal to the bifurcation of superficial and deep femoral artery, as described [36]. The blood flow in the ligated and non-ligated hind limb was measured using laser Doppler perfusion imaging (Moor Instruments, Milwey, UK), as previously reported [37]. Measurements were performed immediately after surgery and serially up to 10 days. To control for temperature variability, animals were kept in a double-glassed vessel filled with water at constant temperature of 37°C for 5 minutes and during subsequent measurements. Perfusion was expressed as the ratio of left (ischemic) to right (non-ischemic) limb.

Upon sacrifice of the mice, the gastrocnemius muscle of both hind limbs was dissected and fixated overnight in 4% formaldehyde. After paraffin embedding, 4 µm thick serial cross sections were made for immunohistochemical analysis. Lungs were harvested and stored at -80°C for RNA analysis.

Immunohistochemistry (IHC)

Four µm-thick sections of human kidney or murine gastrocnemius muscle were re-hydrated and endogenous peroxidase activity was blocked. Antigen-retrieval in the human kidney was performed by heat-treatment, the mouse gastrocnemius muscle was incubated with 10 mM TRIS/1 mM EDTA. EC were visualized with diaminobenzidine (DAB substrate KIT, Pierce, Rockford, IL) after incubation with monoclonal antibodies (moab) directed against CD31 (clone M0823 (Dako Cytomation, Eindhoven, The Netherlands) for human kidney and moab (Santa Cruz Biotechnology, Santa Cruz, CA) for the murine gastrocnemius muscle), followed by peroxidase-labeled rabbit anti-mouse-IgG (Mouse Envision Labeled Polymer horseradish peroxidase system, DakoCytomation). As negative control, primary antibodies were omitted. Sections of the human kidney were counter-

stained with hematoxylin.

Quantification of angiogenic response

Two slides (200 µm apart) were taken of each murine gastrocnemius muscle. Several pictures were taken randomly and the percentage coverage was measured and expressed as the total area of CD31-positive cells per image using the Scion Imaging software (Scion Corporation, Fredrick, MD). Scramblemir (LD) was taken as 100%.

Aortic explants cultures

Eleven days after antagomir-treatment, the thoracic to abdominal aorta was removed [38] and transferred to a petri dish containing M199-medium (Gibco) supplemented with pen/strep, 20%FSC, 10 IU/mL heparin (Leo Pharma) and 25 mg bovine pituitary extract (Gibco). The surrounding tissue was carefully removed and the aorta was flushed several times with the same medium. The aorta was cut into small pieces and transferred to a fibronectin-coated 24 wells plate (Costar). After firm attachment of the explants (after 24 hours) extra medium was added. After 5 and 10 days in culture, pictures were taken from the explants (Leica DMI6000, Nieuw Vennep, The Netherlands) and the total surface area covered with outgrowing cells was measured in a representative area (1.5×10^5 µm^2).

In vitro capillary formation

The role of miR-126 in the capacity of EC to form capillary-like structures was assessed using an *in vitro* angiogenesis assay kit (Chemicon, Temecula, CA) and HUVEC. HUVEC were incubated *in vitro* overnight with 5 µg/mL antagomir-126 or scramblemir and added to the matrigel. After 8h, tube formation was measured by staining the viable cells with calcein-AM (5 µg/mL, Molecular Probes, Leiden, The Netherlands). Total tube area and length were determined using images obtained with an inverted fluorescence microscope and the Scion Imaging software.

Scratch-wound assay

HUVEC were cultured in a 12-wells plate (Costar) and incubated with antagomir-126 or scramblemir (5 µg/mL, 4 wells per condition, 16 hours). Next, the medium was removed and stored on ice and a scratch was made of approximately 800 µm in the EC-monolayer using a pipette-tip. Cells were washed to remove cell-debris and the medium containing antagomir-126 or scramblemir was transferred back to the appropriate wells. Several images (LeicaDMI6000) were taken within 24 hours and the distance of the scratch was measured (expressed in µm).

Statistical analysis

Results are expressed as standard error of the mean (SEM). Statistical analysis was performed using the Mann-Whitney T-test. $P<0.05$ were considered statistically significant.

Results

miR-126 is specifically expressed in endothelial cells in vitro and in vivo

To validate the expression of miR-126 in EC, we first quantified the presence of the mature form of miR-126 in endothelial and non-endothelial cell types using a TaqMan real-time PCR assay (Figure 1A). After normalization of the signals to U6 small nuclear RNA, we observed that human CD14-derived dendritic cells (DC), human macrophages (MΦ), HeLa cells and embryonic kidney cells (HEK-293T) expressed only background levels of miR-126. As expected, both human (EC-RF24, MVEC, HUVEC) and murine (bEnd3) EC displayed over two orders of magnitude more miR-126, confirming that miR-126 is highly expressed in microvascular and macrovascular EC *in vitro*.

In contrast to the expression of miR-126 in cultured EC [5, 10. 17], little is known about the tissue specific expression of miR-126 *in vivo*. Therefore, we performed *in situ* hybridization analysis on human renal sections using digoxigenin-labeled locked nucleic acid (LNA) probes [18]. To localize the endothelium in these sections, we co-stained for the endothelial marker CD31

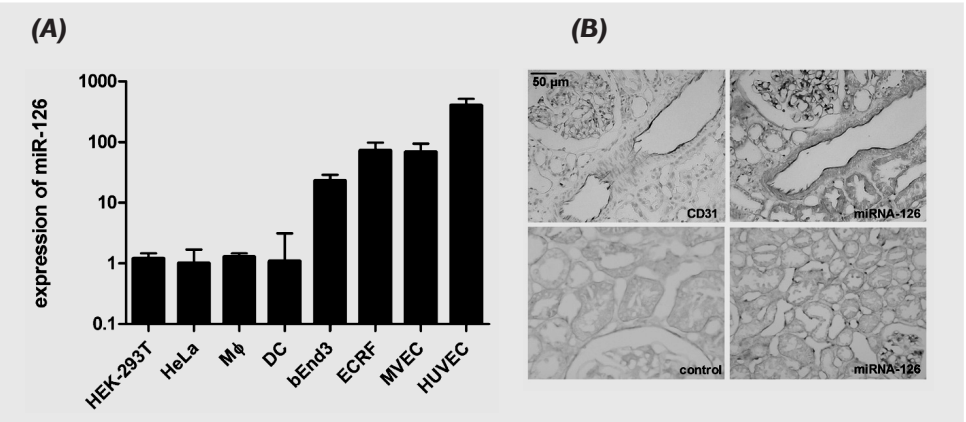

Figure 1. miR-126 is specifically expressed in EC

(A) Total RNA was harvested from different cell types and analyzed by qRT-PCR on miR-126. Obtained values were normalized by qRT-PCR on U6 snRNA. ***(B)*** Representative microscopic images of immunohistochemistry of CD31 on human renal sections and ***in situ*** hybridization of miR-126 with LNA-probe or a control LNA-probe. Upper two pictures represent consecutive sections.

(Figure 1B). *In situ* hybridization of consecutive human renal sections with the LNA probe for miR-126 demonstrated expression of miR-126 in the CD31-positive endothelium of the glomerular and peritubular capillaries as well as the endothelium of the larger vessels. These findings confirm the EC-specificity of miR-126 expression *in vivo*.

miR-126 is functionally active in EC

Next, we sought to investigate whether miR-126 is functionally active in EC. For this, we constructed reporter plasmids containing the firefly luciferase gene under the control of the constitutive CMV promoter and no (pMIR), one (pMIR-126M) or four (pMIR-126Q) perfect miR-126 target sites in the 3' untranslated region of the luciferase reporter gene. Twenty four hours after electroporation of the reporter plasmids into the target cells, firefly luciferase activity was measured and normalized for electroporation efficiency using renilla luciferase activity derived from a co-electroporated expression plasmid. In HEK-293T cells, which lack miR-126 expression, luciferase expression was identical for all three reporters, indicating that the incorporation of the miR-126 target sites did not impact the luciferase transcript translation efficiency (Figure 2A).

In contrast, EC-RF24 cells displayed markedly reduced luciferase activity, with the presence of one or four miR-126 target sites reducing luciferase expression to 13% ($p<0.01$) and less then 1% ($p<0.01$), respectively. These data clearly demonstrate a potent negative regulatory role of miR-126 in cultured EC.

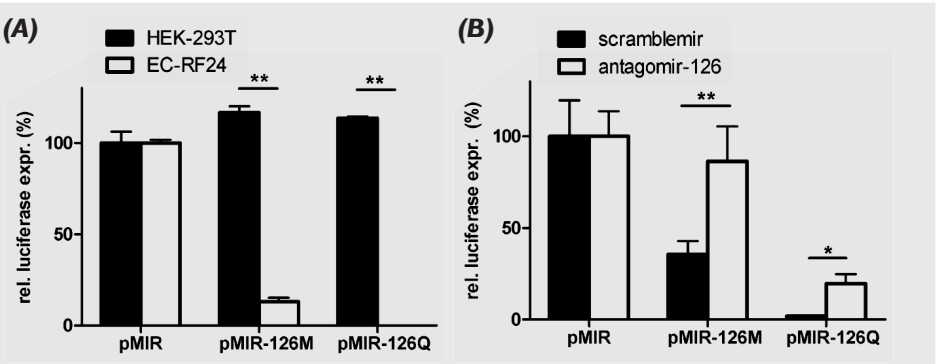

Figure 2. miR-126 is functionally active in vitro and can be specifically repressed by antagomir-126 in vitro and in vivo

(A) Relative firefly luciferase expression of HEK-293T and EC-RF24 transfected with a luciferase reporter plasmid harbouring 0 (pMIR), 1 (pMIR-126M) or 4 (pMIR-126Q) perfect match target sites for miR-126 in the 3'UTR of the luciferase transcript (** = $p<0.01$). ***(B)*** Relative firefly luciferase expression of transfected EC-RF24 incubated for 16 hours with 5 µg/mL scramblemir or antagomir-126 (*=$p<0.05$ and **=$p<0.01$).

Antagomir silencing of miR-126 in EC

Antagomirs have been used to specifically silence microRNAs [15], which prompted us to determine whether antagomirs can also be used to silence miR-126 in EC. Therefore, we designed both a cholesterol-conjugated modified 21 nucleotide RNA, complementary to mature miR-126 (antagomir-126) as well as a control RNA analog of identical composition and length, but with a random sequence (scramblemir). Using a bioinformatic approach, this sequence was chosen due to the fact that it did not match any known miR or mRNA. Addition of 5 µg/mL scramblemir to the culture medium of EC-RF24 cells, 16 hours before electroporation with the miR-126 reporter plasmids (Figure 2B), had little effect on the miR-126-repressed luciferase levels of pmiR-126M (35%) and pmiR-126Q (2%). In contrast, pre-culturing the cells with 5 µg/mL antagomir-126 restored luciferase levels to 86% ($p<0.01$) and 20% ($p<0.05$) for pmiR-126M and pmiR-126Q, respectively. This supports the notion that antagomirs can be efficiently taken up by EC and that antagomir-126 can be used to specifically counteract the negative regulation of target gene expression by miR-126.

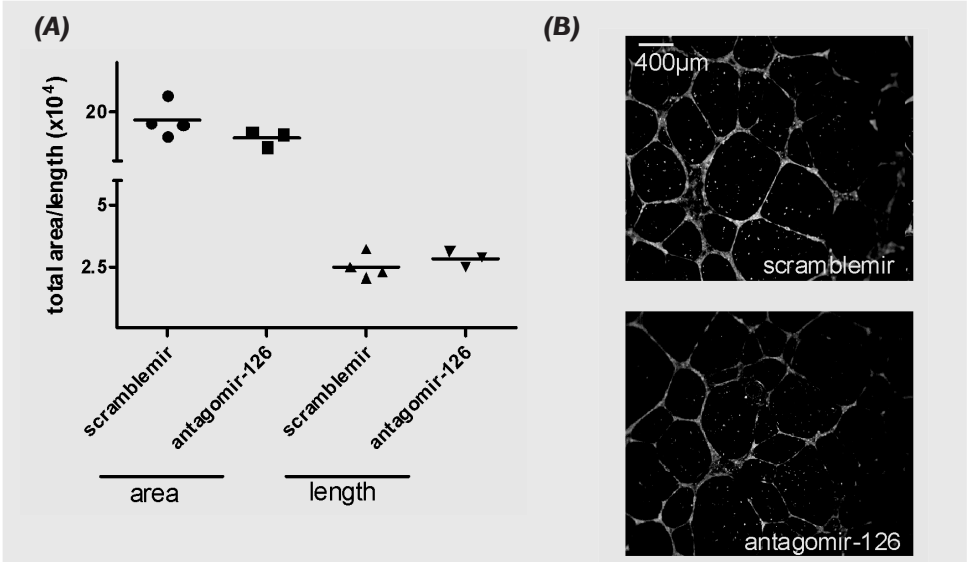

Figure 3A-B. Repression of miR-126 in vitro does not influence the formation of capillary structures or EC proliferation and migration
(A) Quantification of area and length of capillary structures after 8h of culture on matrigel (data expressed in arbitrary units). ***(B)*** Representative microscopic images of the formation of capillary-like structures by HUVEC.

Antagomir silencing of miR-126 has no effect on in vitro angiogenesis and migration and proliferation

To assess whether miR-126 plays a role in the capacity of EC to form capillary-like stuctures on matrigel, HUVEC were incubated overnight with 5 µg/mL

antagomir-126 or scramblemir. Eight hours after cell seeding, tube formation was not affected by silencing of miR-126 (Figure 3A-B). Next, similarly-treated HUVEC were cultured to confluence and their capacity to migrate and proliferate was assessed by measuring the degree to which a 800 µm scratch was re-populated by the cells in 24h. Again, we observed no effects of miR-126 silencing on proliferation and migration of the cells (Figure 3C-D).

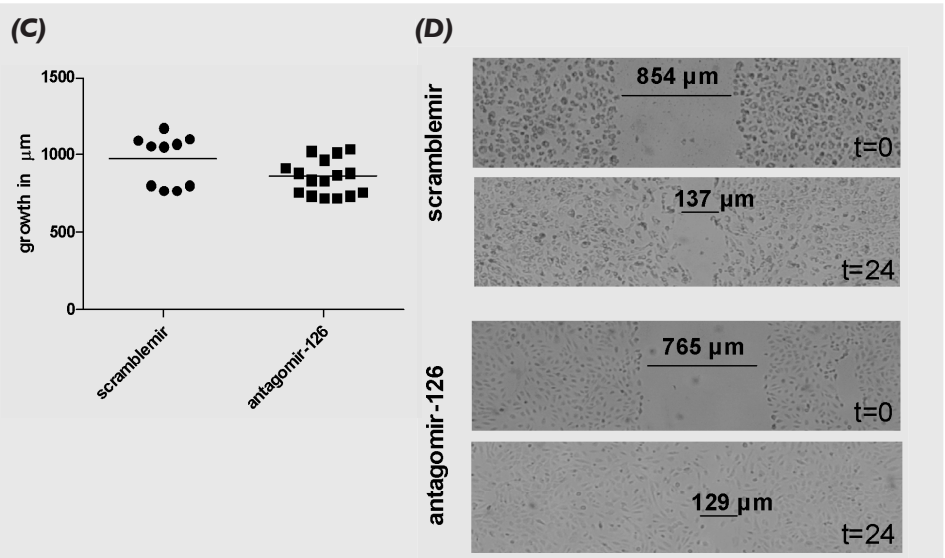

Figure 3C-D. Repression of miR-126 in vitro does not influence the formation of capillary structures or EC proliferation and migration
(C) Quantification of the degree of EC regrowth in a scratch-wound assay (expressed in µm from scratch border). ***(D)*** Representative microscopic images of HUVEC incubated with antagomir-126 or scramblemir before and 24h after the scratch-wound assay.

Antagomir silencing of miR-126 does not affect arteriogenesis

The murine ischemic hindlimb model makes it possible to assess the consequences of interventions on collateral formation around the ligated femoral artery as well as on the hypoxia induced angiogenic response in the distal calf muscle [19]. To investigate the role of miR-126 in neovascularization and the use of antagomirs to silence endothelial microRNAs *in vivo*, we injected four groups (n=6) of male C57Bl/6 WT mice in the tail vein with either a low dose (0.1 mg, LD) or a high dose (1.0 mg, HD) of antagomir-126 or scramblemir. After 24 hours, unilateral hindlimb ischemia was induced by electrocoagulation of the left common femoral artery and blood flow recovery was measured over 10 days using laser Doppler perfusion imaging. As shown in Figure 4C, the progression of blood flow recovery was similar for all treated groups, reaching normal levels between day 7-10.

To verify that the injected antagomir-126 had indeed silenced miR-126 in the endothelium of the mice at the end of the experiment, the lungs of the mice were harvested and the expression levels of miR-126 and a control EC-enriched microRNA miR-423, were quantified by real-time PCR (Figure 4A-B). Whereas the mice treated with antagomir-126 (LD) show only a marginal reduction in miR-126 expression (1.9 fold, $p<0.05$ versus scramblemir (LD)), mice treated

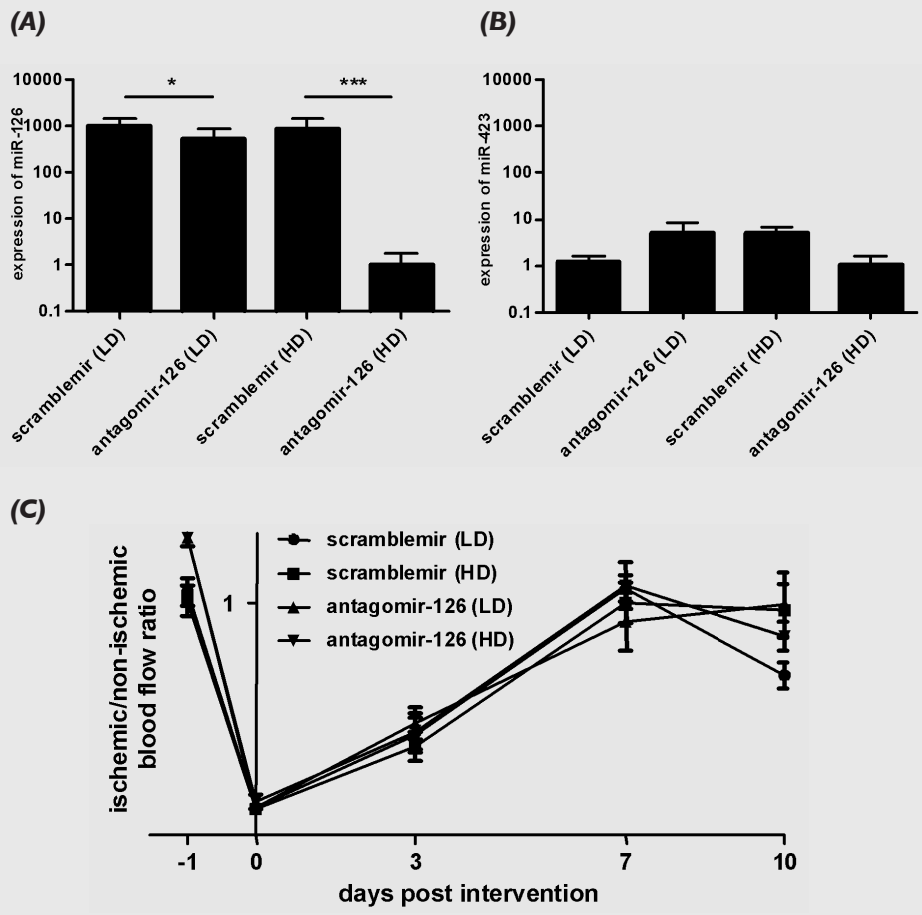

Figure 4A-C. miR-126 silencing has no effect on collateral formation, but miR-126 facilitates ischemia-induced angiogenesis
(A-B) Total RNA was harvested from lungs of mice after 11 day treatment with high and low doses of antagomir-126 or scramblemir (LD or HD). Samples were analyzed by qRT-PCR for A, miR-126 and B, miR-423 expression. Obtained values were normalized by qRT-PCR on U6 snRNA (* = $p<0.05$ and *** = $p<0.001$). ***(C)*** Quantitative evaluation of blood flow recovery measured before and after induction of ischemia by laser Doppler perfusion imaging and expressed as a ratio of the left (ischemic) and right (non-ischemic) limb.

with antagomir-126 (HD) displayed an over 1000-fold reduction in miR-126 expression compared to the scramblemir-treated mice ($p<0.001$). In contrast, no significant differences were observed for the miR-423 levels in all groups. Our data demonstrate that *in vivo* silencing of the endothelial miR-126 remains readily detectable ten days after administration of a single dose of 1.0 mg of antagomir-126. Moreover, we conclude that miR-126 is not directly involved in arteriogenesis.

Antagomir silencing of miR-126 impairs ischemia-induced angiogenesis

To assess the effect of miR-126 silencing on the ischemia-induced angiogenic response, we performed a detailed quantitative analysis of CD31 stained capillaries in sections of gastrocnemius muscle of all treated mice (Figure 4D-E). Mice treated with a single dose antagomir-126 (HD) showed a markedly lower density of capillary vessels compared to antagomir-126 (LD) or both control groups (35% versus 118% ($n=6$, $p<0.05$), 109% ($n=6$, $p<0.001$) and 100% ($n=6$, $p<0.01$)). These studies demonstrate that silencing of miR-126 impairs the angiogenic response to ischemia.

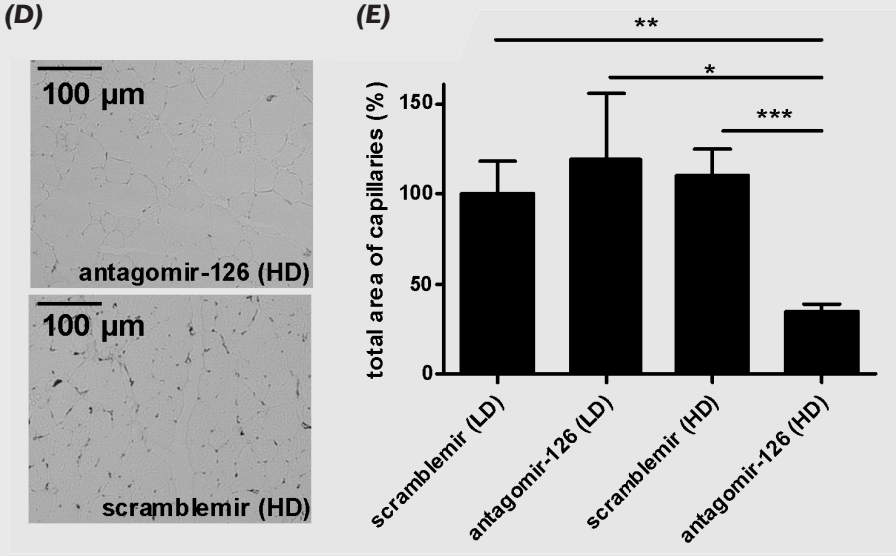

Figure 4D-E. MiR-126 silencing has no effect on collateral formation, but miR-126 facilitates ischemia-induced angiogenesis

(D) Representative microscopic images of CD31 staining in ischemic murine gastrocnemius muscles. ***(E)*** Quantification of the total area of capillaries in sections of the gastrocnemius muscle 10 days after induction of ischemia (* = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$).

Antagomir silencing of miR-126 impairs EC outgrowth in aortic explant cultures

The degree of EC outgrowth from freshly dissected pieces of abdominal aorta has been used as an assay for the potency of compounds to induce angiogenic sprouting of the aortic EC. Therefore, we cultured aortic explants on fibronectin-coated plates in angiogenic medium and assessed the endothelial outgrowth after 11 days from mice of all four treatment groups. As shown in Figure 5, endothelial outgrowth was strongly impaired only in aortic explant cultures derived from mice treated with antagomir-126 (HD). These data support our finding of the endothelial miR-126 is required for an appropriate angiogenic response.

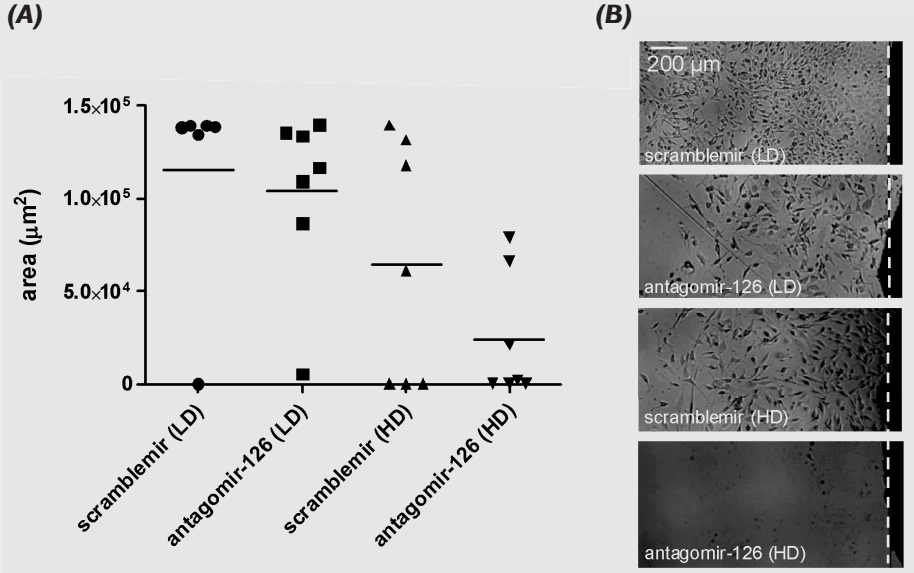

Figure 5. Repression of miR-126 impairs endothelial outgrowth or aortic explants

(A) Quantification of total surface (in μm^2) covered by endothelial outgrowth of aortic explants. ***(B)*** Representative microscopic images of endothelial outgrowth from aortic explants.

Discussion

Previously, miR-126 was found to be expressed in the heart and blood vessels of zebrafish embryos [18]. We demonstrate here that miR-126 is specifically expressed in EC of capillaries and arterioles *in vivo*. To gain insight into a possible regulatory role for this microRNA in neovascularization, we aimed to obtain a specific miR-126 inhibitor for conditional silencing of miR-126 in the vascular endothelium. Recent work has resulted in the development of two potent

approaches for the *in vivo* silencing of microRNAs, notably: 1) Locked nucleic acid (LNA)-modified oligonucleotides for the efficient and long lasting silencing of miR-122 function in the liver of mice and non human primates [20, 21]. 2) Chemically modified and cholesterol-conjugated RNAs termed antagomirs for the rapid and specific degradation of microRNAs in multiple tissues after tail vein injection [15]. As cholesterol uptake is a salient feature shared by virtually all cells, including EC, we designed an antagomir directed to miR-126. We provide evidence that a dose of 5 μg/mL of antagomir-126 specifically silenced miR-126 function in the reporter assay in cultured EC whereas the scramblemir had little effect. Higher doses of scramblemir (>50 μg/mL) resulted in non-specific silencing of miR-126 (data not shown). This effect is likely the result of excessive cellular uptake of small single-stranded RNA analogs leading to non-specific interference with microRNA repression. These results emphasize the need for equally dosed scramblemir controls, for studies assessing microRNA silencing. This may be particularly relevant for *in vivo* studies where there is less control over the distribution of antagomirs over the different tissues.

In mice treated with antagomir-126, we validated the specificity of miR-126 silencing by quantifying the level of mature miR-126 in total lung tissue. This was based on previous observations that, of all organs profiled for microRNA-expression by extensive cloning and sequencing, the lung displays the highest levels of miR-126 expression [13, 22] (e.g. 3 fold higher than skin, 8 fold higher than in heart and 130 fold higher than in total brain tissue [10]). We observed that 10 days after administration of a single, 1.0 mg injection of antagomir-126 per mouse, was sufficient to almost completely abrogate miR-126 expression in lung tissue, whereas miR-126 remained readily detectable in the low dose group as well as the control scramblemir groups. As a single injection of 1.0 mg is low compared to the reported dose needed for silencing of the liver specific miR-122 (3 consecutive injections of 2 mg per mouse), we conclude that EC readily take up antagomirs from the circulation and may therefore be highly useful for studying endothelial microRNA function *in vivo*.

Leukocyte recruitment by the endothelium also plays a critical role in arteriogenesis [1]. Recently, it has been reported that vascular cell adhesion molecule 1 (VCAM-1) is a target for miR-126 in HUVEC and that decreasing miR-126 levels increased the adherence of leukocytes *in vitro* [23]. However, we did not observe any differences in blood flow recovery after femoral artery ligation in either of the treated groups. Therefore, we conclude that miR-126 regulation of VCAM-1 expression probably is likely not a rate-limiting factor for *in vivo* arteriogenesis.

Our data do support, however, a role for miR-126 in the angiogenic response. The reduction of tissue miR-126 expression in the high dose treated mice is associated with a reduction in capillary density in gastrocnemius muscle compared to the scramblemir-treated mice. Likewise, we observed impaired out-

growth of EC from aortic sections of miR-126-silenced mice.

Surprisingly, *in vitro* experiments designed to assess the relatively short term effects of antagomir-126 silencing in HUVEC revealed no differences in the formation of capillary-like structures, or cellular migration and proliferation (scratch/wound assay). This observation is compatible with the notion that different mechanisms are required for 3-dimensional sprouting of EC into a matrix compared to 2-dimensional cell movement in a culture dish [24]. The effects of miR-126 on angiogenesis most likely involve mechanisms operational in EC in the *in vivo* context that involves the interaction with pericytes, EC matrix and the basement membrane. For instance, physical contact between EC and pericytes is thought to induce a quiescent, non-sprouting phenotype [25]. Initiation of angiogenic sprouting is preceded by the formation of so-called tip-cells that lead the sprouting, while the trailing EC must maintain their connection to the patent vasculature [26]. Tip-cell formation in both mice and zebrafish is regulated by Notch signaling pathways and vascular endothelial cell growth factor receptor-3 (VEGFR-3) is involved in the generation of tip-cells. In mice, it has been established that the Sprouty-related Ena/VASP homology 1 domain containing protein (Spred-1) can function as a potent repressor of VEGFR-3 [26]. Spred-1 and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2, p85-β), another protein actively involved in the negative regulation of VEGF signaling [27], are both predicted targets of miR-126 (http://www.targetscan.org). Consequently, upregulation of miR-126 would thus facilitate angiogenesis by reducing the expression of both repressors of VEGF signaling whereas low levels of miR-126 would be associated with elevation of Spred-1 or PIK3R2and repress angiogenic signaling. Indeed, two recent studies reported that targeted deletion of miR-126 in mice and zebrafish impairs angiogenesis likely through dysregulation of Spred-1 and PIK3R2 expression suggesting a critical role for this microRNA in angiogenic signaling events during embryogenesis [13,14]. Here we demonstrate that miR-126 also plays a key role in the regulation of ischemia-induced angiogenesis in adult mice. Using qRT-PCR we observed that antagomir-mediated silencing of miR-126 in murine endothelial cells (bEnd3) led to a concomitant four fold upregulation of Spred-1 mRNA (data not shown). Therefore, it is tempting to speculate that upregulation of Spred-1 and PIK3R2 levels also underlies the anti-angiogenic effects observed in our study.

Taken together, we have demonstrated that functional activity of miR-126 is required for the ischemia-induced angiogenic response *in vivo*. In addition, our study supports the potential therapeutic use of antagomir-based approaches for conditional silencing of microRNAs in the endothelium *in vivo*.

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CHAPTER 4

MicroRNA-126 modulates endothelial SDF-1 expression and mobilization of Sca-1^{+}/Lin^{-} progenitor cells in ischemia

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Abstract

MicroRNA-126 (miR-126), which is enriched in endothelial cells, plays a role in angiogenesis. Based on the seed sequence, miR-126 can also be predicted to regulate vasculogenesis by modulating the endothelial expression of stromal cell-derived factor-1 (SDF-1).

Using miR-reporter constructs, we first validated that miR-126 inhibits SDF-1 expression in endothelial cells *in vitro*. Next, we investigated the potential relevance of this observation with respect to the mobilization of progenitor cells. For this, we studied the migration of human $CD34^+$ progenitor cells towards chemotactic factors present in endothelial cell-conditioned medium. Antagomir-induced silencing of miR-126 elevated SDF-1 expression by human umbilical vein endothelial cells and enhanced migration of the $CD34^+$ cells. In a murine model of hind limb ischemia, a striking increase in the number of circulating Sca-1+/Lin- progenitor cells in antagomir-126-treated mice was observed as compared to scramblemir-treated controls. Immuno-histochemical staining of capillaries in the post-ischemic gastrocnemius muscle of miR-126-silenced mice revealed elevated SDF-1 expressing CD31-positive capillaries, whereas a mobilizing effect of miR-126 inhibition was not detected in healthy control animals.

miR-126 can regulate the expression of SDF-1 in endothelial cells. In the context of an ischemic event, systemic silencing of miR-126 leads to the mobilization of Sca-1^+/Lin^- progenitor cells into the peripheral circulation, potentially in response to elevated SDF-1 expression by endothelial cells present in the ischemic tissue.

Introduction

Integrity of the vascular endothelium is central to vascular homeostasis and is determined by the balance between endothelial injury and repair [1]. In the context of adverse hemodynamic and metabolic risk factors, endothelial cells (ECs) are damaged and constantly replaced by the proliferation of adjacent mature ECs. In the rat aorta, ECs in areas resistant to atherosclerosis have a low rate of cellular replication and have been estimated to have a lifespan of approximately 12 months. In contrast, EC turnover in lesion-prone sites is accelerated to weeks or less as animals age [2]. To maintain the integrity of the endothelium throughout life, endothelial cells in the arterial wall would therefore have to replicate well over a thousand times. However, primary endothelial cell cultures already become senescent after a limited number of passages due to a progressive shortening of telomeres that takes place upon each cell division. Moreover, telomere erosion is accelerated by chronic exposure of endothelial cells to oxidative stress [3]. Consequently, it has been suggested that mature ECs

are insufficiently capable of repairing the chronically damaged endothelium throughout life. Support for this notion has been provided by several studies identifying a significant contribution by endothelial progenitor cells as an alternative cellular source for re-endothelialization of the damaged artery wall [4-6].

Stromal cell derived factor-1 (SDF-1 or CXCL12) has been demonstrated to facilitate the homing of progenitor cells from the peripheral circulation to sites of vascular injury or tissue ischemia. SDF-1 can be actively secreted by injured endothelial cells[7] or activated platelets[8], leading to the homing of bone marrow-mobilized progenitor cells to the site of injury [9, 10]. The beneficial role of SDF-1 in recruiting bone marrow cells to ischemic tissue has been established in several animal models to improve recovery after an ischemic event [7, 11-13]. Therefore, SDF-1 is believed to play a central role in regulating both vascular integrity and homeostasis, implicating that its expression should be tightly controlled. Indeed SDF-1 expression is reported to be regulated at multiple levels including transcription and post-translation [7].

At the post-transcriptional level, SDF-1 expression could be regulated by microRNAs (miRNAs). MiRNAs constitute a class of highly conserved non-coding RNAs that control gene expression by inhibiting the translation of mRNA [14]. The ability of miRNAs to regulate multiple targets provides a means for the coordinated control of gene expression and make these molecules especially attractive candidates for regulating cell type-specific differentiation and modulating cell function [15]. In recent years, miRNA expression-profiles of ECs have been analyzed in detail and recent studies demonstrated both pro-angiogenic [16-19] as well as anti-angiogenic functions for endothelial miRNAs [20-21].

In silico analyses (http://www.microrna.org) for miRNAs targeting the 3' untranslated region (3' UTR) of the SDF-1 mRNA identified miR-126 as a potential post transcriptional regulator of SDF-1 expression. MiR-126 is highly enriched in ECs and has been demonstrated to regulate (ischemia-induced) angiogenesis by blocking the expression of SPRED-1 and PI3KR2 [22-24]. Here, we validated the inhibitory actions of miR-126 on expression and function of SDF-1 in ECs *in vitro*. Furthermore, we demonstrate that antagomir-induced silencing of miR-126 prior to acute ischemia leads to the augmentation of SDF-1 levels in both the circulation and ischemic tissue. This is associated with increased mobilization of bone marrow-derived progenitor cells *in vivo*. Our data demonstrate that endothelial miR-126 can regulate both features of angiogenesis as well as vasculogenesis and suggest a role for miR-126 in the maintenance of endothelial homeostasis.

Material and methods

Cells and cell culture

Immortalized human umbilical vein endothelial cells (EC-RF24) [25] were cultured in M199 medium (Gibco, Breda, the Netherlands) supplemented with penicillin/streptomycin (Gibco/Invitrogen), 20% fetal calf serum (FCS, Bio Whittaker/Cambrex, Verviers, Belgium), 10 IU/mL heparin (Leo Pharma, Breda, the Netherlands), 2.5% HEPES buffer (Gibco) and 12.5 µg/mL endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO). Human umbilical vein endothelial cells (HUVEC) were isolated, cultured and characterized as described previously [26]. In short, ECs were isolated from freshly obtained human umbilical cords by trypsin/EDTA digestion of the interior of the umbilical vein. The cells were cultured in M199 medium supplemented with penicillin/streptomycin, 20% FCS, 10 IU/mL heparin, and 5% bovine pituitary extract (Gibco). $CD34^+$ cells were isolated from umbilical cord blood using Ficoll (Amersham, 's-Hertogenbosch, the Netherlands) density gradient centrifugation and positive selection using CD34-MACS microbeads (Miltenyi Biotech, Bergish Gladbach, Germany). Umbilical cord blood and umbilical cords were collected non-identifiable and with informed consent. Since the human tissues were unidentifiable, approval from the university ethics review board was not necessary

SDF-1 3'UTR reporter assays

Synthetic, double-stranded oligonucleotides spanning a 60 base-pair region of the murine 3'UTR of SDF-1 mRNA containing the putative miR-126 binding site with (pSDFmm) or without (pSDF) a C to G mismatch at position 3 of the seed-sequence were cloned into the pMIR-reportTM Expression Reporter Vector System (Applied Biosystems, Amsterdam, the Netherlands; see supplementary file for oligonucleotide sequences). All plasmids were sequenced to confirm their structure and exclude cloning artefacts. A renilla luciferase expression plasmid (pRL-SV40, Promega, Leiden, the Netherlands) and a plasmid containing a single, perfect match miR-126 binding site served as controls [23].

Design of antagomirs

Antagomir-126 and a control scramblemir (Dharmacon RNA technologies, Lafayette, CO) were synthesized as previously described [23].

Luciferase assay

Antagomir-126 or control scramblemir (5 µg/mL) was added to a near confluent layer of fibronectin-adherent EC-RF24 cells. Twenty four hours after antagomir treatment, the EC-RF24 cells were detached by trypsinization and resuspended in 500 µL serum-free Optimem culture medium (Gibco) and 1.5 µg specific pMIR-report and 150 ng pRL-SV40 were added. The cell suspension

was chilled for 10 minutes at 4°C and electroporated using the Gene Pulser II (Bio-Rad Laboratories, Veenendaal, the Netherlands). After 10 minutes recovery time at room temperature, 1.5 x 10^5 cells were plated in a 24-wells plate coated with fibronectin in triplicate. After 24 hours, the firefly-luciferase and renilla-luciferase signals were measured using a Dual-Luciferase® Assay Reporter System (Promega) in a Lumat LB9507 luminometer (EG&G Berthold, Bundoora, Australia).

Western blot

HUVEC were incubated for 48 hours with 5 µg/mL antagomir-126 or scramblemir. Culture medium was aspirated and cells were washed two times with PBS, cellular lysates were harvested using lysis buffer (50 mM Tris-HCl (pH=7.5), 150 mM NaCl, 1% SDS, 0.5% sodiumdeoxycholate and 0.5% Triton X-100 (Sigma-Aldrich). Protein levels for SDF-1 and β-actin were assessed by Western blot analysis with chemiluminescence detection. Equal amounts of protein were resolved on 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA). SDF-1 was detected using a polyclonal rabbit antibody against human SDF-1 (0.5 ug/mL, Abcam, Cambridge, UK) and for β-actin a polyclonal rabbit antibody against human β-actin (0.1 ug/mL, Abcam) was used. Horseradish peroxidase conjugated goat-anti-rabbit IgG (0.05 ug/mL, DakoCytomation, Enschede, the Netherlands) was used as secondary antibody. Bound fragments were detected with chemiluminescent reagents (Supersignal West Dura Extended Duration Substrate, Thermoscientific) and exposed on Hyperfilm ECL (Amersham). Quantitative analysis of the SDF-1 band intensity on Western blot was performed using imageJ software and normalized for β-actin. The ratio of the SDF-1 band intensity over the β-actin band intensity of the antagomir-126 treated HUVEC was arbitrarily set at 100%.

Migration assay

HUVEC were incubated in serum-free, cell-specific medium with 0.1% insulin-transferrin-sodium selenite media supplement (ITS, Sigma-Aldrich) containing antagomir-126 or scramblemir (5 µg/mL) at 37°C. After 48 hours, cell supernatants were harvested and placed into the lower compartment of a transwell system with a pore size of 5 µm (Corning B.V. Life Sciences, Amsterdam, the Netherlands). Human $CD34^+$ cells were added to the upper chamber and migration was followed over a period of 4 hours at 37°C. As a positive control 50 ng/mL recombinant human SDF-1(R&D Systems) was added in the lower compartment. The SDF-1-receptor, CXCR4, was neutralized by incubation of $CD34^+$ cells with a blocking antibody against CXCR4 (8 µg/mL, R&D Systems) for 30 minutes at 4°C. After 4h, the cell-suspensions in the lower compartment were harvested, spun down and incubated for 30 minutes at 4°C in FACS buff-

er (PBS + 1% bovine serum albumin (BSA, Sigma-Aldrich)) with directly conjugated antibodies against human CD34 (PerCP-Cy5, BD Biosciences, Breda, the Netherlands) and human CD133 (APC, Miltenyi Biotec). A fixed number of $CD34^+$ cells was fluorescently labeled by incubation with calcein-AM (R&D Systems, Minneapolis, MD) and added prior to FACS analysis.

Hind limb ischemia model

All animal experimental protocols were approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO, Leiden, the Netherlands) and conform the Directive 2010/63/EU of the European Parliament.One day prior to surgery, C57BL/6 WT mice (n=6 per group, age=10 weeks, Charles River, Maastricht, the Netherlands) were injected intravenously (200 µL) with antagomir-126 (1.0 mg/animal) or scramblemir (1.0 mg/animal). Before surgery, mice were anesthetized intraperitoneally with a combination of Midazolam (5 mg/kg, Roche Diagnostics, Almere, the Netherlands), Medetomidine (0.5 mg/kg, Orion Corporation, Turku, Finland) and Fentanyl (0.05 mg/kg, Janssen Pharmaceutica, Tilburg, the Netherlands). Ischemia of the left hind limb was induced by electrocoagulation of the left common femoral artery, proximal to the bifurcation of superficial and deep femoral artery, as described [27]. To sacrifice the mice, blood was withdrawn for FACS-analysis (EDTA-anti-coagulated) by heart puncture and the tibia and femur of both legs were kept for isolation of bone marrow cells. Additionally, the gastrocnemius muscle of both hind limbs was dissected and placed in 4% formaldehyde overnight. After paraffin embedding, 4 µm thick serial cross-sections were made for immunohistochemical analysis.

Whole blood analysis

Whole blood was collected by incision of the tail vein or heart puncture and analyzed by semi-automatic hematology analyzer F-820 (Sysmex; Sysmex Corporation, Etten-Leur, the Netherlands), FACS-analysis or ELISA. Hematological values obtained were white blood cell counts (WBC, $n*10^6$/mL), red blood cell counts (RBC, $n*10^9$/mL), platelets (PLT, $n*10^6$/mL), hematocrit (HCT, %/%) and haemoglobin (HGB, mmol/L). For FACS analysis, we incubated 50 µL of whole blood for 60 minutes at 4°C with directly conjugated antibodies directed against Sca-1 (FITC, BD-Biosciences), Flk-1 (PE, BD-Pharmingen, San Diego, CA) and a cocktail against lineage-positive cells (APC, BD-Pharmingen). In a separate tube, 50 µL of whole blood was incubated with an appropriate cocktail of isotype controls, to identify the threshold for lineage-positivity. SDF-1 levels in serum were assessed with ELISA (R&D Systems).

Immunohistochemistry (IHC)

Four µm-thick sections of murine gastrocnemius muscle were re-hydrated

and incubated with antigen-retrieval buffer (0.1% trypsin/EDTA in PBS). Next, sections were incubated with specific antibodies against murine SDF-1 (rabbit polyclonal IgG, Thermoscientific, Rockford, IL) and murine CD31 (mouse monoclonal IgG2b, Santa Cruz, Heidelberg, Germany) followed by secondary antibodies against goat-anti-rabbit-IgG labeled with Alexa-488 or goat-anti-mouse IgG labeled with Alexa-568 (Molecular Probes). As a negative control, isotype-matched IgGs were used. Images were made using a confocal microscope (Carl Zeiss, Sliedrecht, the Netherlands).

FACS analysis

All samples obtained for FACS analysis were either immediately analyzed by flow cytometry analysis (FACS LSRII, BD Biosciences) or were fixed in 1% paraformaldehyde and analyzed within 24 hours after preparation. Data were analyzed using FACSDiVa software (BD Biosciences).

Statistical analysis

Results are expressed as standard error of the mean (SEM). Statistical analysis was performed using the Mann-Whitney T-test. $P<0.05$ was considered statistically significant.

Results

MiR-126 affects the expression and function of SDF-1 in vitro

Using an online miRNA target search tool (http:// www.microrna.org) we identified a putative miR-126 binding site in the 3'UTR of the SDF-1 mRNA. To assess its functionality, we cloned this binding site into the 3'UTR of a luciferase reporter gene driven by a constitutive promoter (pSDF) and analyzed luciferase expression and of a control plasmid in the human endothelial cell line EC-RF24. In these miR-126 expressing cells, luciferase expression driven from a reporter construct lacking a miRNA seed sequence (pMIR) was not affected when miR-126 was silenced using an antagomir approach (anta-126). In contrast, a reporter gene carrying a single perfect miR-126 binding site (pMONO) was fully active when miR-126 was silenced but its expression was strongly reduced in the presence of a control scramblemir (scr). These studies confirm the functionality of miR-126 in EC-RF24 cells. Likewise, the presence of the putative SDF-1 miR-126 binding site in the reporter construct led to a 40% reduction of luciferase-expression in miR-126 expressing cells (Figure 1A, scramble versus antagomir-126, $P<0.01$). A single mismatch in the seed sequence of the putative SDF-1 miR-126 binding site (C to G at position 3 of the seed sequence, pSDF-mm) alleviated miR-126-dependent repression of reporter gene expression, confirming the presence and specificity of the miR-126 binding site in the 3'UTR of the SDF-1 mRNA. To validate the regulatory effect of miR-126 on endogenous

SDF-1 protein expression in endothelial cells, human umbilical vein endothelial cells (HUVEC) were treated with antagomir-126 or scramblemir, after which SDF-1-protein expression was determined by Western blot analysis. A marked increase of SDF-1 protein expression was observed in HUVEC treated with antagomir-126 as compared to scramblemir (Figure 1B).

Next, we assessed the potential of miR-126 to regulate the extent by which endothelial cell-derived SDF-1 attracts progenitor cells. To this end, conditioned medium derived from HUVEC, incubated with antagomir-126 or scramblemir, was applied to the lower compartment of a transwell-system and umbilical cord blood-derived $CD34^+$ cells were placed in the upper compartment. As shown in Figure 1C, silencing of miR-126 in HUVEC-enhanced migration of $CD34^+$ cells compared to the scramblemir-control ($P<0.001$). Moreover, neutralization of CXCR4 expressed by the $CD34^+$ cells with a function-blocking antibody (αCXCR4, 8 μg/mL) decreased cell migration towards the conditioned supernatant derived from antagomir-126 treated HUVEC back to the scramble control levels ($P<0.05$). As a control, maximal cellular migration was observed using medium containing recombinant human SDF-1 (50 ng/mL), which was significantly reduced by αCXCR4 (Figure 1C, $P<0.01$). These data suggest that suppression of miR-126 in endothelial cells *in vitro* can augment expression of functional SDF-1, leading to elevated progenitor cell migration.

MiR-126 affects progenitor cell mobilization via SDF-1 in vivo

Endothelial miR-126 expression and functionality is conserved in murine EC, both *in vivo* [23] and *in vitro* (supplementary Figures S3-1 and S3-2). To investigate the regulatory role of miR-126 on SDF-1-dependent progenitor cell mobilization *in vivo*, we applied single tail vein injections of antagomir-126 or the control scramblemir into wild type (WT) male C57Bl/6 mice (n=6) Ten days after injection, we enumerated the number of circulating Sca-1-positive and lineage-negative ($Sca\text{-}1^+/Lin^-$) cells by FACS analysis. Surprisingly, when we expressed the circulating $Sca\text{-}1^+/Lin^-$ cell numbers as a percentage of Lin^+ leukocytes, we observed a significant decrease in circulating $Sca\text{-}1^+/Lin^-$ cells in the mice in which miR-126 had been silenced by antagomir-126 administration as compared to the scramblemir-treated controls (Figure 2A, $P<0.05$). This observation could be explained by the fact that antagomir-126 treated mice also displayed a significant increase in total white blood cell (WBC) count (Figure 2B, $P<0.01$). Such an increase is consistent with reports showing that increased SDF-1 levels in the circulation result in an elevation of WBCs [9]. As such, when we expressed the number of circulating $Sca\text{-}1^+/Lin^-$ cells per mL blood, the differences between antagomir-126 and scramblemir-treated animals was lost, indicating that there is no systemic role for miR-126 in $Sca\text{-}1^+/Lin^-$ progenitor cell mobilization *in vivo* (Figure 2C).

Since ischemic endothelial cells have been established to up regulate SDF-1[7].

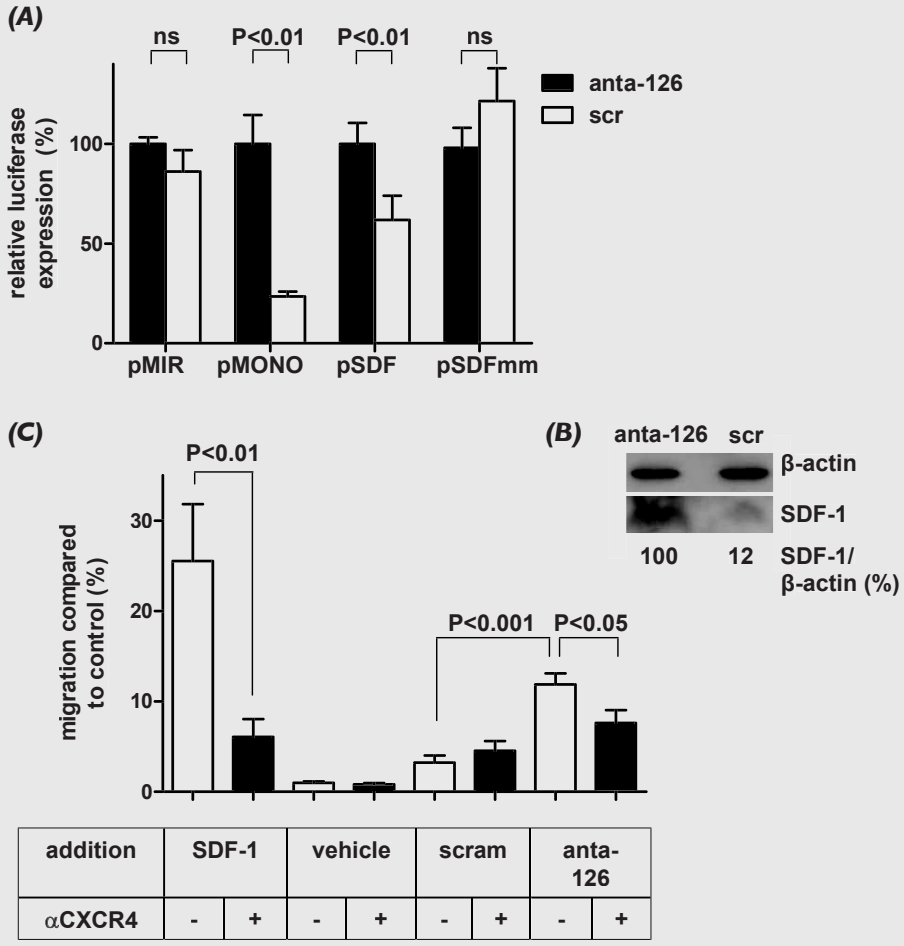


Figure 1. miR-126 affects the expression and function of SDF-1 in vitro

(A) EC-RF24 were pre-incubated with scramblemir or antagomir-126 and transfected with pMIR as negative control, pMONO as positive control, pSDF with the sequence of the SDF-1 miR-126 binding site or a variant with a single mismatch in the seed sequence (pSDFmm). Experiment were performed in triplicate and data are expressed as mean values ± standard error of the mean. ***(B)*** HUVEC were incubated for 48 hours with antagomir-126 or scramblemir and conditioned cell supernatants subjected to Western blot analysis. Quantitative analysis of SDF-1 band intensity on Western blot were performed using Image-J and normalized for β-actin with the antagomir-126 band set at 100%. ***(C)*** HUVEC (n=4) were treated with antagomir-126, scramblemir or vehicle and the conditioned media were used to study the chemotactic activity towards human hematopoietic $CD34^+$ cells in vitro. Recombinant SDF-1 was used as positive control and the contribution of the SDF-1 receptor (CXCR4) was studied with the use of a function-blocking antibody (αCXCR4) (n=2).

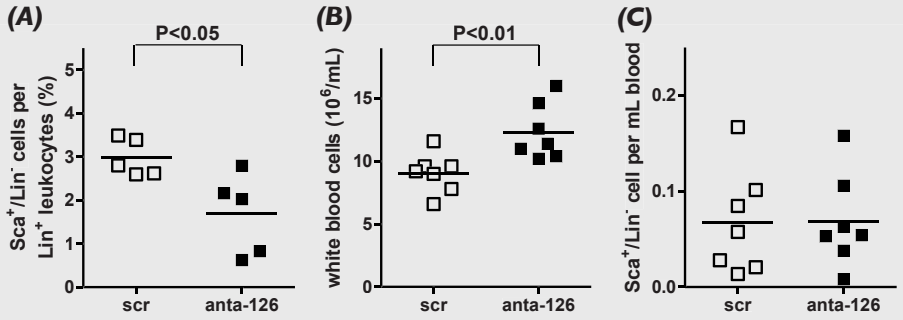


Figure 2. miR-126 does not solely affect mobilization of Sca-1$^+$/Lin$^-$ cells in vivo

(A) Ten days after injection of scramblemir or antagomir-126, Sca-1$^+$/Lin$^-$ cells in whole blood were analyzed by FACS, expressed as percentage of total Lin$^+$ leukocytes. ***(B)*** Ten days after injection of scramblemir or antagomir-126, total white blood cell counts per mL was determined. ***(C)*** Ten days after scramblemir- or antagomir-126-injection Sca-1$^+$/Lin$^-$ cells were analyzed by FACS in whole blood expressed as total number in mL blood.

(and supplementary Figure S2-1). We investigated whether an ischemic stimulus in combination with attenuation of miR-126 levels would modulate progenitor cell levels in mice. Therefore, we injected male WT mice (n=6) with either antagomir-126 or scramblemir and, the next day, this procedure was followed by the induction of unilateral hind limb ischemia (HLI). Ten days after induction of HLI, the animals were sacrificed and serum levels of SDF-1 were assessed by ELISA. Although systemic SDF-1 levels were elevated in antagomir-126-treated mice as compared to controls, no significance was reached (Figure 3A, P=0.19). Nevertheless, when we examined the *in vivo* mobilization of Sca-1$^+$/Lin$^-$ cells to the circulation after ischemia, we observed an 8-fold increase of Sca-1$^+$/Lin$^-$ cells expressed as percentage of total Lin$^+$ leukocytes in the blood of antagomir-126-treated animals as compared to scramblemir-treated control animals (Figure 3B, P<0.005). The absolute number of Sca-1$^+$/Lin$^-$ cells per mL blood was raised from 0.24 ± 1.4 in the control group to 2.1 ± 1.4 in the antagomir-126 treated animals. As the observed effects suggested an ischemia-dependent elevation of SDF-1, we next investigated whether the ischemic gastrocnemius muscle could be a potential contributor to the elevated levels of circulating SDF-1. Therefore, we performed both detailed qualitative and quantitative immunohistochemical analysis of SDF-1 expression in relation to CD31-positive capillaries. Indeed, we observed a clear co-localization of SDF-1 and CD31 expression in sections of the ischemic muscle (Figure 3C). Furthermore, the ischemic muscle of antagomir-126-treated animals showed a higher percentage of double-positive capillaries (Figure 3D, P<0.01) as opposed to scramblemir-treated controls. These data imply that the mildly elevated levels of SDF-1 protein in the circulation

upon antagomir-126-treatment may be a reflection of the locally enhanced expression of SDF-1 protein in the vessels of ischemic tissue.

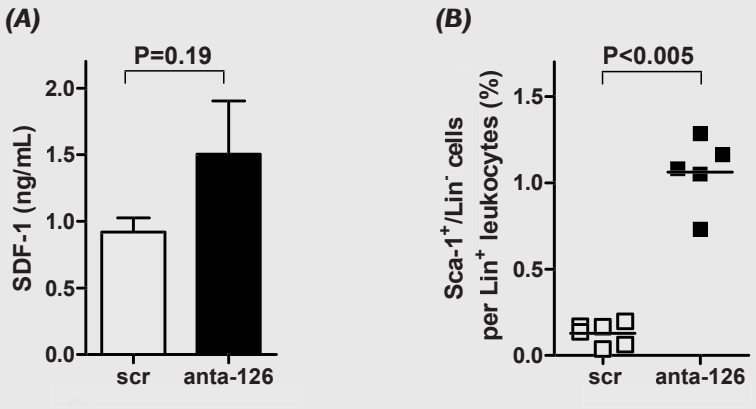


Figure 3A-B. miR-126 can alter SDF-1-expression and induce progenitor mobilization after ischemic injury in vivo

(A) Ten days after HLI antagomir-126-treated mice showed non-significantly elevated levels of SDF-1 in the serum as compared to scramblemir-treated controls (n=6). ***(B)*** Ten days after HLI and injection of scramblemir or antagomir-126, Sca-1$^+$/Lin$^-$ cells were measured in whole blood, expressed as percentage of total Lin$^+$ leukocytes.

Discussion

A function for miR-126 in angiogenic processes in vascular maintenance and during development has been shown in a number of publications demonstrating that loss of miR-126, either in knockout models or mediated by treatment with antagomirs, leads to structural impairment of the vascular bed [22-24]. In the current study, we provide evidence for a vasculogenic role for miR-126 in regulating the mobilization of endothelial progenitor cells via the release of chemokine SDF-1 from ischemic endothelial cells. *In vitro*, the increased secretion of SDF-1 upon silencing of miR-126 was sufficient to stimulate the migration of human CD34$^+$ stem cells. In mice however, systemic silencing with a single tail vein injection of antagomir-126 was not sufficient to raise the levels of circulating murine Sca-1$^+$/Lin$^-$ progenitor cells. However, in combination with the ligation of the femoral artery, we demonstrated an increase in circulating Sca-1$^+$/Lin$^-$ cells following miR-126 silencing, strongly suggesting that tissue ischemia is needed to reveal the regulatory role of miR-126 *in vivo*.

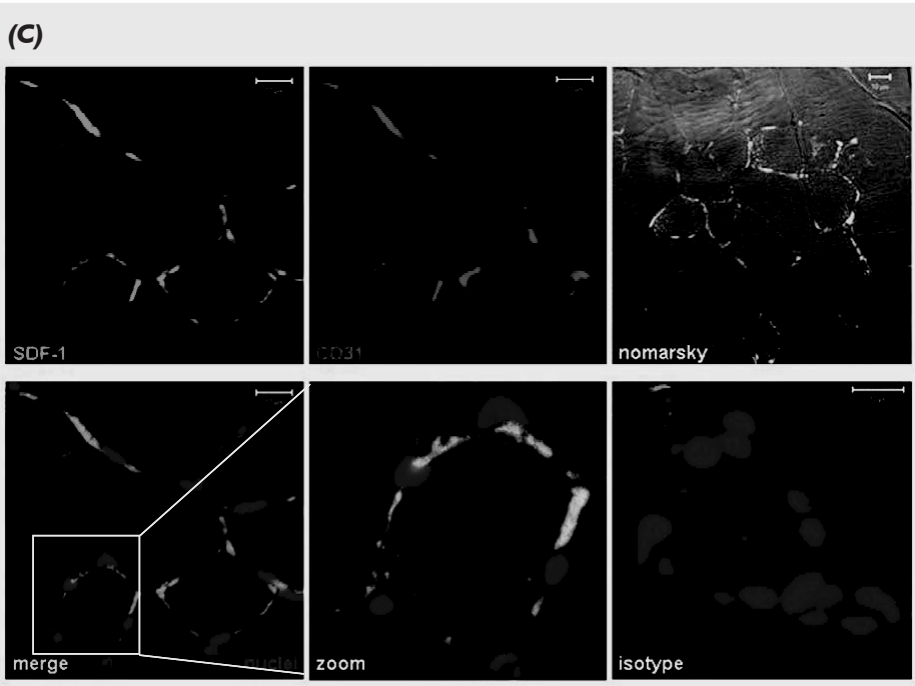
Our data suggest that elevated numbers of circulating Sca-1$^+$/Lin$^-$ cells in the antagomir-treated animals are the result of SDF-1 mediated mobilization of these cells following ischemia. This is supported by the fact that SDF-1 protein expression is also up regulated in the endothelial cells of the ischemic tissue as

well as in the peripheral circulation. Nevertheless, definitive proof of a causal role of miR-126 in SDF-1 dependent mobilization of progenitor cells would require an *in vivo* blockade in the SDF-1/CXCR4 axis. For this, inhibitors of the interaction of SDF-1 with CXCR4, such as AMD3100, could be administrated to antagomir-126 treated mice and used to alleviate the elevated progenitor cell mobilization following ischemia. This approach is however complicated by the direct effects of these inhibitors on the egress of progenitor cells from the bone marrow which could potentially override the SDF-1 effects elicited by the endothelium in the ischemic tissues in the periphery [28]. Also, impaired progenitor homing to the ischemic tissue or the spleen cannot be fully excluded [29].

An interaction between miR-126 and SDF-1 has previously been shown to increase miR-126 uptake of endothelial cell-derived apoptotic bodies by endothelial cells. This resulted in increased SDF-1 expression through inhibition of Regulator of G-protein signaling 16 (RGS16) [30]. In contrast, our studies implicate that the abrogation of miR-126 is associated with increased expression of SDF-1, suggesting that miRNAs could serve as a biological rheostat, with the response magnitude of biological pathways being dependent on the context and source of the external stimulus.

Recently, it has been demonstrated that miRNAs are present in the circulation and that alterations in the profile of plasma or serum miRNAs can be associated with disease states [31, 32]. These early reports mainly displayed a link between circulating miRNAs and cancer, while subsequent studies have also revealed a clear association of circulating miRNAs with cardiovascular disease [33-35]. Since endothelial injury is considered one of the hallmarks of patients at risk for cardiovascular disease and, upon injury, endothelial cells can secrete miRNA-containing microvesicles [36-38], it is of interest to address the value of endothelial cell-derived circulating miRNAs as biomarkers for cardiovascular disease. Indeed, two recent clinical studies revealed a decrease in circulating miR-126 levels in patients with coronary artery disease (CAD) [39] and diabetes mellitus 2 (DM2) [40]. It was suggested that lowered levels of miR-126 in DM2 patients might be explained by the fact that high glucose levels can lead to a decrease of the miR-126 content in the endothelial particles, while cellular miRNA levels remained unaltered [40]. One may speculate that lowering of circulating miR-126 levels might be a specific signal of the injured endothelium to increase the expression levels of distinct miR-126 targets that are critical for the integrity of the endothelium.

We suggest here that miR-126 functions as a regulator of endothelial homeostasis (Figure 4). In the healthy, viable endothelium, miR-126 is readily expressed and serves to down regulate SPRED-1 and PI3KR2, both of which are inhibitors of angiogenic and cell survival signals in response to vascular endothelial cell growth factor (VEGF) [22, 24]. This condition favors the



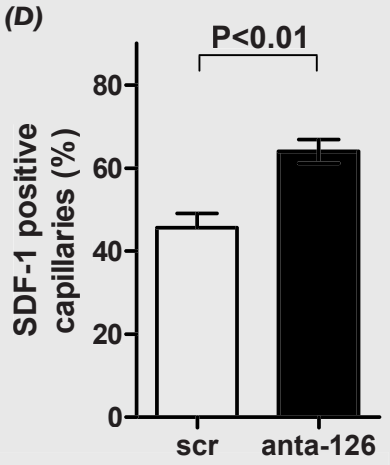


Figure 3C-D. miR-126 can alter SDF-1-expression and induce progenitor mobilization after ischemic injury in vivo

(C) Immunohistochemical micrographs of immunohistochemistry of the gastrocnemius muscle after HLI showed colocalization of SDF-1 and CD31. SDF-1 was visualized with alexa-488 (green) and CD31 with alexa-568 (red). Nuclei were stained with DAPI and shown in blue, Nomarsky contrast images show muscle tissue. Scale bars represent 10 μm. D, Quantification of microscopic images displayed that antagomir-126-treated mice have increased levels of SDF-1 positive capillaries as percentage of total number of capillaries (n=6).

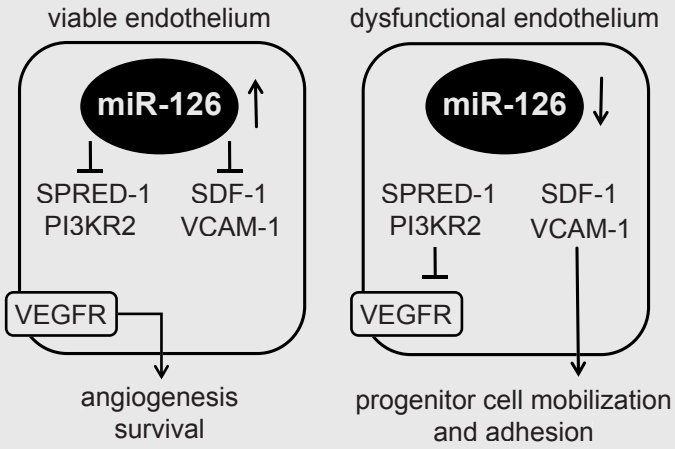


Figure 4. miR-126 acts as a vasculogenic switch

In viable endothelium miR-126 inhibits the expression of SPRED-1 and PI3KR2 thereby facilitating VEGF-dependent angiogenesis. Furthermore, the expression of SDF-1 and VCAM-1 are inhibited. When miR-126 is lost, SPRED-1 and PI3KR2 are up regulated thereby blocking angiogenesis, at the same time SDF-1 and VCAM-1 levels are up regulated and this elevation subsequently leads to an increased mobilization and adhesion of bone marrow derived progenitors.

angiogenic response to injury, as SDF-1 expression is concomitantly repressed by miR-126. On the other hand, under conditions associated with endothelial cell dysfunction or senescence, a decrease in miR-126 levels would inhibit angiogenic and cell survival signals. Furthermore, this condition favors the expression of SDF-1 and VCAM-1 [41]. of the affected endothelial cells and support re-endothelialization by a vasculogenic response through the recruitment and subsequent adhesion of vascular progenitor cells. As bone marrow-derived $CD34^+$ cells were recently demonstrated to represent a more functional EPC population than $Sca\text{-}1^+/Lin^-$ progenitor cells, future studies may include these progenitor cell population as well [42].

Although speculative, our model is supported by the observed decreased levels of circulating miR-126 in DM2 and CAD patients [39, 40] and elevated levels of SDF-1 in patients with acute coronary syndrome (ACS) [43]. Future studies in patient cohorts will provide insight as to whether the down regulation of circulating miR-126 can be correlated with increased levels of SDF-1.

In conclusion, both *in vitro* and *in vivo*, miR-126 can regulate the expression of SDF-1 in endothelial cells, which may lead to the mobilization of $Sca\text{-}1^+/Lin^-$ progenitor cells into the peripheral circulation following conditions of acute ischemia. As the endothelial miR-126 regulates both features of angiogenesis as well as vasculogenesis, a regulatory role for this miRNA in endothelial homeostasis is proposed.

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CHAPTER 5

MicroRNA-126 contributes to renal microvascular heterogeneity of VCAM-1 protein expression in acute inflammation

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Abstract

Endothelial cells in different microvascular segments of the kidney have diverse functions and exhibit differential responsiveness to disease stimuli. The responsible molecular mechanisms are largely unknown. We previously showed that during hemorrhagic shock VCAM-1 protein was expressed primarily in extra-glomerular compartments of the kidney, while E-selectin protein was highly induced in glomeruli only. Here, we investigated the molecular control of expression of these endothelial cell adhesion molecules in mouse models of renal inflammation. Microvascular-segment specific responses to the induction of anti-glomerular basement membrane (anti-GBM) glomerulonephritis and systemic TNFα treatment showed that E-selectin expression was transcriptionally regulated, with high E-selectin mRNA and protein levels preferentially expressed in the glomerular compartment. In contrast, VCAM-1 mRNA expression was increased in both arterioles and glomeruli, while VCAM-1 protein expression was limited in the glomeruli. These high VCAM-1 mRNA/low VCAM-1 protein levels were accompanied by high local microRNA (miR)-126 and Egfl7 levels, as well as higher Ets1 levels compared to arteriolar expression levels. Using miR-reporter constructs functional activity of miR-126 in glomerular endothelial cells could be demonstrated. Moreover, *in vivo* knock-down of miR-126 function unleashed VCAM-1 protein expression in the glomeruli upon inflammatory challenge. These data imply that miR-126 has a major role in the segmental, heterogenic response of renal microvascular endothelial cells to systemic inflammatory stimuli.

Introduction

It is well recognized that endothelial cells in different vascular segments of the kidney differ in function [21]. Renal arteries branch repeatedly into small-diameter afferent arterioles in which the endothelial cells are covered by smooth muscle cells, forming the primary site of vascular resistance in the kidney. Endothelial cells lining the glomerular capillaries are fenestrated and together with podocytes, mesangial cells, and the glomerular basal membrane, regulate the glomerular filtration of water and small molecules into the urine, while preventing loss of large serum proteins. After glomerular filtration, blood leaves the glomerular capillary networks by efferent arterioles to peritubular capillaries and postcapillary venules [20].

In spite of the broad knowledge of structural and functional heterogeneity of endothelial cells in the kidney, the underlying molecular basis for microvascular endothelial heterogeneity is largely unknown. Also in acute and chronic renal diseases such as glomerulonephritis, vasculitis, and ischemia related acute renal failure, knowledge on how distinct microvascular endothelial cells subsets

respond at the molecular level to disease stimuli is almost non-existent. Molecular heterogeneity of endothelial cells can be controlled at multiple levels, ranging from the heterogenic expression of transmembrane signaling receptors and local concentrations of their ligands, to differentially activated signal transduction cascades and differentially controlled ubiquitinylation-dependent protein degradation pathways [18].

In recent years, the importance of post-transcriptional regulation of inflammation by microRNAs (miRs) has become increasingly apparent [28]. Mature miRs are short non-coding RNAs that bind to (partially) complementary sequences, most commonly found in the 3'UTR (untranslated region) of target mRNAs, which results in inhibition of protein synthesis by degradation or translational repression of the target mRNA. MicroRNAs play a role in endothelial biology [29, 38], and also have been associated with pathogenesis and progression of various kidney diseases [16, 31, 37, 39]. Harris et al, and more recently Salvucci et al. demonstrated a link between miR expression in endothelial cells and inflammation, by showing that high miR-126 levels related to low VCAM-1 protein expression in human umbilical vein endothelial cells (HUVEC) *in vitro* [10, 26]. MiR-126 is highly enriched in the endothelium [5], exerts a regulatory function in vascular integrity and vascular pathology [9, 30, 34, 35, 40], and its expression was shown to be partly driven by vascular associated Ets transcription factors [11, 23].

The current study describes two distinct molecular mechanisms for two eminent pro-inflammatory endothelial adhesion molecules, namely post-transcriptional, miR-126 controlled expression of VCAM-1, and transcriptional regulation of E-selectin, that contribute to heterogeneity in renal microvascular endothelial engagement in response to an inflammatory challenge. Previously, we reported that exposure of mice to hemorrhagic shock resulted in a highly compartmentalized microvascular segment restricted expression of adhesion molecules in the kidney. While E-selectin protein was primarily expressed in the glomerular compartment, VCAM-1 protein was predominantly expressed in the arteriolar and peritubular endothelial cells [33]. A similar microvascular segment restricted VCAM-1 protein expression pattern was previously reported in a rat renal allograft model [36]. Based on the *in vitro* results reported by Harris et al [10]. we hypothesized that differential expression of miR-126 in microvascular segments of the kidney might be involved in the heterogenic expression of VCAM-1 protein in the kidney. As different microvascular segments engage in different (renal) diseases [19], understanding the contribution of transcriptional respectively posttranscriptional miR control will provide a basis for further studies into their role in heterogenic microvascular responsiveness in disease development.

Materials and methods

Cell cultures

The conditionally immortalized human glomerular endothelial cell line ciGEnC and the glomerular podocyte cell line AB8/13 were cultured at 33°C for propagation of cells. Unless otherwise stated, 5 and 14 days prior to experiments, respectively, ciGEnC and AB8/13 cells were transferred to 37°C to inactivate the SV40 T antigen and allow the cells to differentiate [24, 27]. Human glomerular mesangial cells were cultured at 37°C as described previously [4].

Animal models of acute inflammation

All animal experiments were performed according to national guidelines and upon approval of the local Animal Care and Use Committees. C57bl/6 mice (8–10 weeks) were purchased from Harlan (Zeist, The Netherlands). Animals were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-hr light/dark cycle. Induction of anti-GBM glomerulonephritis was performed as described previously [2]. Mice were sacrificed at 2 h. Alternatively, inflammation was systemically induced by i.v. injection of recombinant mouse TNFα (BioSource Europe, Nivelles, Belgium) at 200 ng dose. Mice were sacrificed 2h after TNFα administration. To induce hemorrhagic shock, mice under anesthesia, breathing spontaneously and on a temperature controlled surgical pad (37-38°C), were subjected to blood withdrawal until a reduction of the MAP to 30 mmHg within 15-30 minutes as described previously [33]. Mice were sacrificed at 90 minutes after MAP of 30 mmHg was achieved. Organs from all treatment groups were harvested, snap-frozen in liquid nitrogen and stored at -80°C prior to RNA isolation, laser microdissection, and/or immunohistochemical staining.

Antagomir-126 effects on protein expression in vivo

Five days prior to TNFα challenge, C57bl/6 mice were injected in the orbital plexus with 200 µL antagomir-126 or scramblemir in saline at 1.0 mg/animal, or saline, 2 mice per group [34]. At the day of sacrifice, mice were injected in the orbital plexus with 70 µL TNFα at 200 ng per animal in saline/BSA, or with vehicle only. After 2 hours, mice were sacrificed and relevant organs were taken out, snap frozen on liquid nitrogen, and stored at -80°C for further analysis.

Immunohistochemical staining

Five-µm cryosections were fixed in acetone for 10 min and incubated for 45 min with monoclonal antibodies: rat anti-mouse CD31 (PECAM-1; BD Pharmingen, Alphen a/d Rijn, the Netherlands), rat anti-mouse E-selectin (MES-1; kindly provided by Dr. D. Brown, UCB Celltech, Slough, UK) and rat anti-mouse VCAM-1 (CD106, BD Biosciences, Breda, The Netherlands). Antibody dilution and

washing steps were performed with PBS/5% fetal calf serum (FCS). Staining was carried out with Envision+ system-HRP kit (DakoCytomation, Denmark), according to manufacturer's protocol, including a blocking step for endogenous peroxidase activity. Sections were incubated for 45 min with rabbit anti-rat antibodies (AI-4001, Vector Laboratories Inc., Burlingame, CA, USA; diluted in PBS/5% FCS/5% normal mouse serum/5% normal sheep serum) followed by 30 min incubation with Envision+ system-HRP anti-rabbit polymer. Detection was performed with 3-amino-9-ethylcarbazole (AEC) and sections were counter-stained with Mayer's hematoxylin. Between all incubation steps sections were washed extensively with PBS. Isotype matched controls were consistently found to be devoid of staining.

Quantification of VCAM-1 protein levels by ELISA

To quantify VCAM-1 protein, snap frozen 10 µm cryostat cut kidney and liver sections were homogenized in ice cold RIPA-buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5% sodium deoxycholate and 0.1% SDS) containing protease inhibitors (Complete Mini, Roche Applied Science, Mannheim, Germany), phosphatase inhibitors (PhosSTOP, Roche Applied Science) and 1 mM sodium orthovanadate. The homogenates were sonicated and centrifuged at 13,000g for 10 minutes at 4°C. Total protein was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, USA), before quantification of VCAM-1 by ELISA (mouse sVCAM-1/CD106 (MVC00), R&D Systems Inc. Minneapolis, USA) according to the manufacturer's instructions. VCAM-1 levels were normalised to total protein concentrations in the tissue homogenate and expressed as pg VCAM-1 per µg total protein.

Laser microdissection of renal microvascular segments

Seven hundred glomeruli (area ~3*106 µm2) and arteriolar vascular segments (area ~6*105 µm2) were laser microdissected from 9-µm hematoxylin stained cryosections using the Leica Microbeam System. Glomeruli were dissected through the Bowmans capsule in order to only obtain cells within the glomeruli. Arterioles were identified based on their morphology, the inner layer of endothelial cells was laser microdissected from the tissue [3].

RNA isolation and quantitative RT-PCR

Total RNA (18 nt and larger) was isolated by miRNeasy Mini kit RNA and included a DNase treatment on the column (Qiagen Benelux B.V., Venlo, The Netherlands). RNA integrity for high RNA yield samples was determined by 28S/18S ratio detection on an agarose gel. For laser microdissected samples, RNA integrity was determined on an Experion Automated Electroforeses System using RNA HighSens Chips (Bio-Rad Laboratories, Hercules CA, USA). For gene expression analysis RNA was reverse transcribed using Superscript III

reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). For expression analysis of miRs, RNA was reverse transcribed using Taqman miRNA reverse transcription kit (Applied Biosystems, Applera Nederland, Nieuwerkerk a/d IJssel, The Netherlands) in the presence of specific miR reverse transcription primers.

Quantitative PCR (qPCR) amplifications of each sample were performed in duplicate or triplicate according to manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System with gene- and miR-specific Taqman primers/probe from Applied Biosystems. Gene expression of E-selectin (Mm00441278_m1 and Hs00174057_m1), VCAM-1 (Mm00449197_m1 and Hs00365486_m1), CD31 (Mm00476702_m1 and Hs00169777_m1), Egfl7 (Mm00618004_m1 and Hs00211952_m1), and Ets-1 (Mm00468970_m1 and Hs00428293_m1) was determined as relative to the house keeping gene GAPDH (Mm99999915_g1 and Hs99999905_m1) based on the comparative Ct method. Similarly, the expression of miR-126 (assay 002228) and miR-31 (assays 000185 and 002279) was related to the expression of sno202 (assay 001232) for mouse samples, and of RNU48 (assay 001006) for human samples. The expression of the pan-endothelial marker gene CD31 was used to correct for the relative content of endothelial cells in laser microdissected arterioles and glomeruli in the case of analysis of endothelial restricted miR and genes.

miR-126 reporter assay

The human VCAM-1 3'UTR was amplified by PCR using the following primers: VCAM-1 (sense): TATAAAACTAGTCAAGCCATGCATTCAGACTTC and VCAM-1 (antisense): TATAAAAAGCTTGATGACTATGCTAGGCTCCTG.

These primers were designed to generate two flanking enzyme restriction sites (SpeI and HindIII) that enabled direct cloning into the pMIR-reportTM Expression Reporter Vector System (Applied Biosystems), which contains an experimental firefly luciferase reporter gene. After cloning, the plasmids were sequenced to evaluate their fidelity. To determine the regulatory capacity of miR-126 in ciGEnC on the 3'UTR of VCAM-1, ciGEnC were seeded at 5×10^5 cells per well in 6-wells plates at 33 °C. Five µg/mL cholesterol-conjugated antagomir-126 (5'-gscsauuauuacucacgguascsgsas-Chol-3') or control (scramblemir), that was selected to lack complementarily to the human and mouse transcriptome (5-asusgacuaucgcuauucgcsasgs-Chol-3') were added to the cells 16 hours prior to transfection. Transfection was performed by electroporation in serum free medium (Optimem; Gibco/Invitrogen) as previously described [34]. A Renilla luciferase expressing plasmid (pRL-SV40, Promega, Leiden, The Netherlands) served as control for the efficiency of electroporation. In short, 1.5 µg pMIR- VCAM-1 3'UTR or pMIR-Report control plasmid was mixed with 150 ng pRL-SV40 and added to the cells. The cell suspension was incubated for 10 min at 4°C and electroporated with Gene Pulser II (Bio-Rad Laboratories, Veenendaal, The

Netherlands). Cells were recovered for 10 min at room temperature, plated in triplicate at 1.5 x 10^5 cells per well in 24-wells plate and incubated for 24 h at 33°C. Firefly luciferase and Renilla luciferase signals were measured using Dual-Luciferase Assay Reporter System (Promega) and Lumat LB9507 (EG&G Berthold, Bundoora, Australia). The ratio of firefly/Renilla luciferase signals was calculated. To evaluate the relative luciferase signals, ciGEnC that were incubated with antagomir-126 prior to transfection were arbitrarily set at 100%.

Statistical analysis

Statistical significance of differences in gene and miR expression was performed by means of two-sided Student's t-test, assuming equal variances. Differences were considered to be significant when $p<0.05$.

Results

Expression of cell adhesion molecules in response to disease induction

Induction of anti-GBM glomerulonephritis in mice as well as *in vivo* exposure to i.v. TNFα resulted in a rapid activation of endothelial cells, as represented by a strong upregulation of E-selectin and VCAM-1 mRNA expression in the kidney (Fig. 1A – anti-GBM, Fig. 1B – TNFα). In both models, E-selectin protein was predominantly expressed in glomerular endothelial cells, while expression of VCAM-1 protein was highest in arterioles, and expressed to a limited extent in the glomeruli (Fig. 1C). Also in a third inflammation model, i.c., induced by hemorrhagic shock, a similar pattern of restricted expression of E-selectin in glomerular capillaries and high VCAM-1 protein expression in arterioles with concomitant low expression in glomeruli was observed (Fig. 1C). This implies that heterogenic endothelial responsiveness to acute inflammatory stimuli in different renal microvascular segments is a general response irrespective the nature of the stimulus.

E-selectin and VCAM-1 gene expression in renal microvascular segments

To analyze the vascular segment restricted effects of inflammatory stimuli on gene expression, we isolated glomeruli and arterioles from the kidney by laser microdissection prior to qRT-PCR analyses. This protocol combines preservation of RNA levels with providing information on the original location of the vascular segments in the kidney. Using this, we could show high and preferential up regulation of E-selectin mRNA in glomerular capillaries, both in the anti-GBM model (Fig. 2A) and in the i.v. TNFα challenge model (Fig. 2B). Together with the protein data from Figure 1, this indicates regulation of E-selectin expression in the glomerular compartment at the transcriptional level. Surprisingly, VCAM-1

mRNA levels were upregulated to a similar extent in both glomeruli and arterioles in both models (Fig. 2A and 2B) revealing a marked discordance in VCAM-1 gene versus protein expression in the glomeruli that suggested a marked regulation of VCAM-1 expression at the post-transcriptional level.

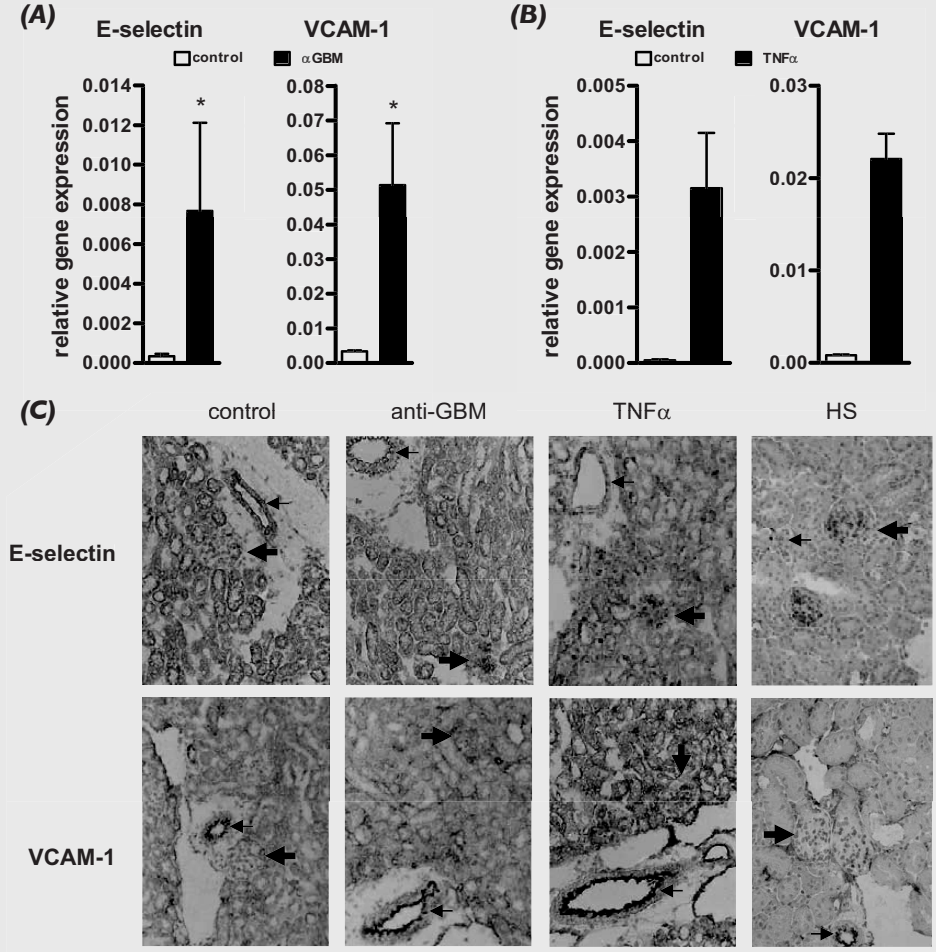

Figure 1. Expression of E-selectin and VCAM-1 in the kidney

(A) Gene expression of E-selectin and VCAM-1 is upregulated in the kidney at the onset of anti-GBM glomerulonephritis, and ***(B)*** in response to systemic TNFα administration. Quantitative gene expression in kidney samples of untreated mice (control), and at 2 h after induction of anti-GBM-glomerulonephritis (αGBM) respectively TNFα administration relative to GAPDH house keeping gene. Data are presented as mean values ± SD of 3-6 animals per treatment; *, $p<0.05$. ***(C)*** Immunohistochemical staining of E-selectin, VCAM-1 in kidney-cryosection of untreated mice (control), of anti-GBM glomerulonephritis mice (anti-GBM), after i.v. administation of TNFα, and after induction of hemorrhagic shock (HS). Small arrow heads indicate the location of arterioles, big arrows the location of glomeruli.

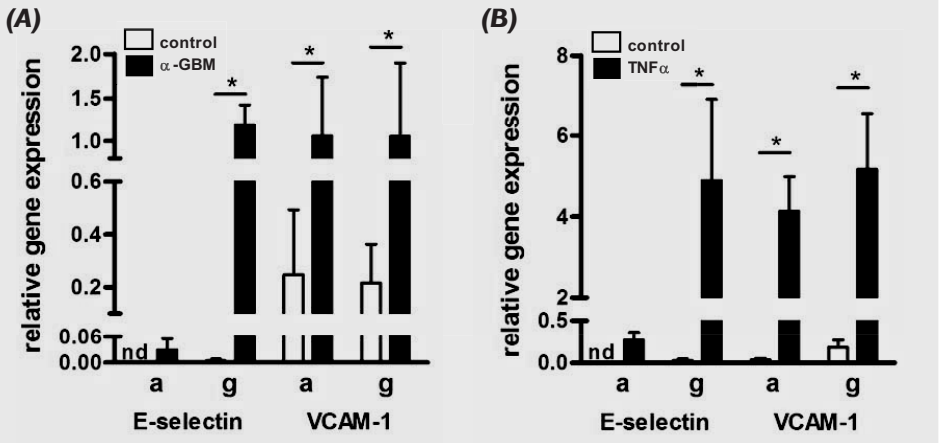

Figure 2. E-selectin and VCAM-1 gene expression in vivo are compartmentalized in different microvascular segments in the kidney

Both in anti-GBM glomerulonephritis ***(A)*** and in response to systemic TNFα treatment ***(B)***, gene expression of E-selectin was highly and specifically upregulated in glomerular endothelial cells (g) while hardly affected in the arterioles (a), while VCAM-1 gene expression was induced to a similar extent in arterioles (a) and in glomeruli (g). Mice were sacrificed at 2h after disease induction and vascular segments were laser microdissected prior to qRT-PCR analysis as described in M&M. Data are presented as mean values ± SD of 3-6 animals per treatment; *, gene expression in mice subjected to disease stimulus was significantly different compared to untreated control mice, $p<0.05$; nd - not detected.

miR-126 levels of expression follow the expression patterns of Egfl7 and Ets1

Based on the recent data published by Harris et al. [10] regarding the role of miR-126 in posttranslational regulation of VCAM-1 expression, we hypothesized that in acute inflammation, the observed limited glomerular VCAM-1 protein was related to a high expression of miR-126. To test this hypothesis, we analyzed miR-126 levels *in vivo* in arterioles and glomeruli obtained by laser microdissection, in which the endothelial cell input in each sample was normalized using the pan-endothelial marker CD31. miR-126 expression was significantly higher in the glomerular segments than in the arteriolar segments (Fig. 3A, white bars). These levels did not change in response to the induction of acute inflammation (Fig. 3A, black bars). In contrast, the non-endothelial restricted microRNA miR-31 was expressed at similar levels in the two renal microvascular segments both in quiescent and in inflammatory conditions (Fig. 3B).

Because miR-126 is an intronic product of the gene Egfl7 (epidermal growth factor-like domain 7, also known as vascular endothelial statin [22]), and miR-126 was previously reported to follow endothelial restricted expression patterns of

Egfl7[22, 38] we also analyzed Egfl7 expression in the two microvascular beds. This analysis revealed a similarly higher expression of Egfl7 in the glomerular compartment compared to its expression in the arteriolar compartment, both in control conditions and in response to the inflammatory challenge (Fig. 3C). In contrast to the others, Egfl7 expression was modestly downregulated in both compartments under acute inflammatory conditions.

While performing our study, Harris et al. reported that Ets1 is one of the main transcription factors in control of miR-126 expression in endothelial cells *in vitro* [11]. Analyzing Ets1 expression levels in the two vascular compartments under study revealed a significant, 6-fold higher expression level of this transcription factor in the glomeruli as compared to the arterioles, both of which were not affected by the inflammatory process (Fig. 3D).

miR-126 expression is only expressed in the endothelial cells of glomeruli

Since laser microdissected glomeruli consist of more cell types besides

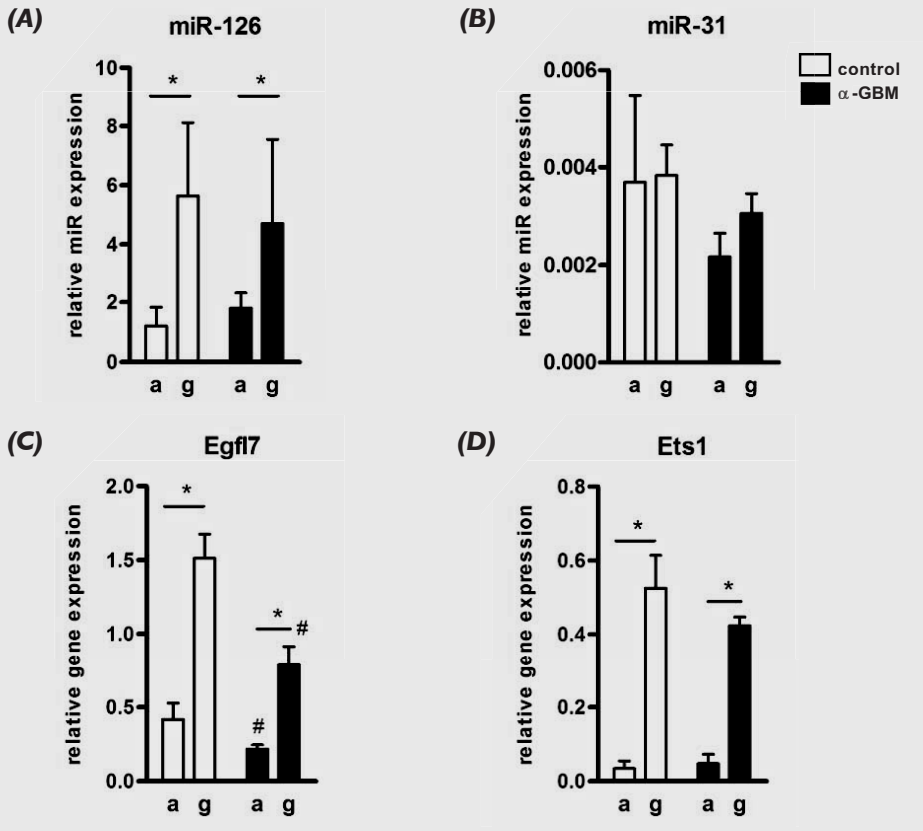

Figure 3. Differential expression of miR-126 in renal microvasculature

Figure 3. Differential expression of miR-126 in renal microvasculature

MicroRNA respectively mRNA expression levels in arterioles (a) and glomerular (g) compartments, in control mice (white bars) and mice subjected to the anti-GBM glomerulonephritis model (black bars). Levels were quantified after laser microdissection of the respective microvascular beds from frozen mouse kidney biopsies as described in *Materials and methods*. The level of expression of miR-126 ***(A)*** was significantly higher in the glomerular compartment than in the arteriolar compartment, and not affected by the inflammatory process, while the non-endothelial restricted miR-31 was expressed in both compartments to a similar extent and also not affected by the inflammation ***(B)***. The expression patterns of Egfl7 ***(C)***, the host gene of miR-126, as well as of Ets1 ***(D)***, a transcription factor shown to control miR-126 expression, followed the same spatial expression pattern as observed for miR-126. While Egfl7 was modestly decreased by the inflammation induced, Ets1 was not affected. Data are presented as mean values ± SD of 3-6 animals per group; *, $p<0.05$ arterioles vs. glomeruli, #, $p<0.05$ control vs. anti-GBM.

endothelial cells, we analyzed the expression of miR-126, miR-31, Egfl7, and Ets1 in cultured glomerular endothelial cells, mesangial cells and podocytes. The data presented in Figure 4A support the notion that miR-126 expression is endothelial cell restricted, with detectable but very low levels in mesangial cells and podocytes, while the non-endothelial restricted miR-31 was found to be expressed at similar levels in all three glomerular cell types. Also Egfl7 exhibited an endothelial restricted expression profile, while Ets1 was expressed to a similar extent in all three cell types (Fig. 4A). TNFα mediated regulation of the miRs and genes in glomerular endothelial cells was only observed for Egfl7 and Ets1. Egfl7 was downregulated to 50% of control levels at a later stage of TNFα mediated activation (24h), while Ets1 expression increased approx. 4.5 fold versus control within 4h after start of TNFα exposure (Fig. 4B).

miR-126 is functionally active in glomerular endothelial cells

To investigate whether miR-126 can be functionally active in the glomerular endothelial cells, we used reporter analysis to validate binding to the 3'UTR region of VCAM-1 (Fig. 5A). Prior to the transfection experiments, glomerular endothelial cells were pre-cultured overnight with a 21 nucleotide cholesterol-conjugated random RNA sequence (scramblemir) or with identically sized and modified RNA complementary to mature miR-126 (antagomir-126). While in scramblemir treated cells the relative firefly luciferase expression of the reporter plasmid harboring the 3'UTR region of VCAM-1 (pMIR-VCAM-1 3'UTR) was low compared to a control reporter plasmid lacking these sequences (pMIR-report; Fig. 5B), incubation with antagomir-126 restored luciferase levels of pMIR-VCAM-1 3'UTR without affecting luciferase levels of the pMIR-reporter. These results imply that miR-126 is functional in glomerular endothelial cells as

a negative regulator of VCAM-1 expression.

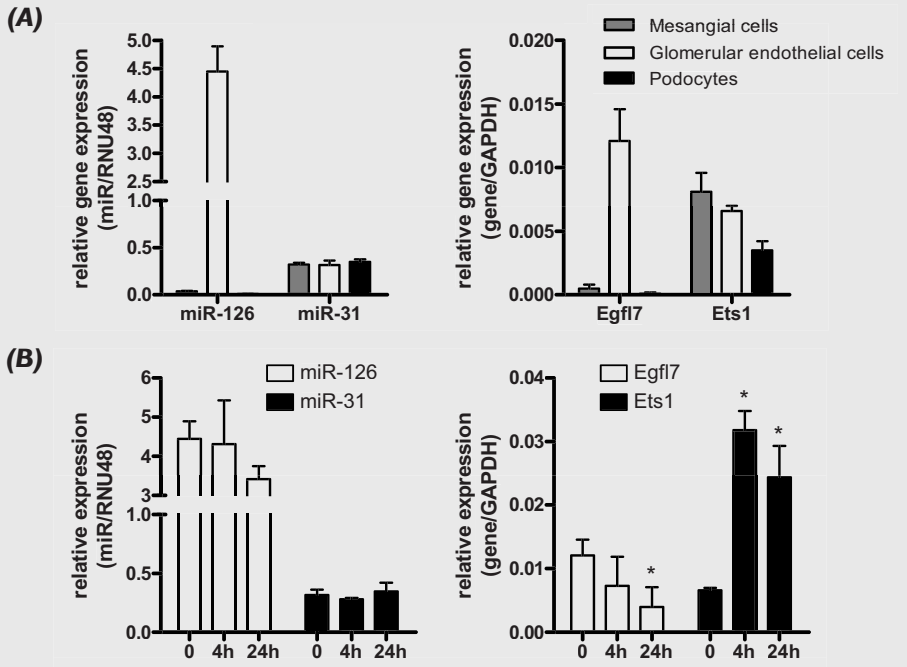

Figure 4. Expression of miR-126, miR-31, Egfl7 and Ets1 in glomerular cells in vitro, and their regulation in glomerular endothelial cells by TNFα exposure

(A) Basal expression levels of the microRNAs and mRNAs were determined as described in *Materials and methods* by qRT-PCR, and normalized to RNU48 respectively GAPDH. ***(B)*** Regulation of the expression of the microRNAs and mRNAs in glomerular endothelial cells exposed to 10 ng/mL TNFα for 4h and 24h. Data are presented as mean values ± SD; n=3–6, *, $p<0.05$.

In vivo silencing of miR-126 by antagomir-126 resulted in increased VCAM-1 protein expression upon TNFα challenge

The above data suggested that miR-126 can play a role in repressing glomerular VCAM-1 protein expression upon an inflammatory challenge and that this post-transcriptional role by miR-126 could explain the observed heterogenic VCAM-1 protein expression patterns in the microvasculature of the kidney. To study whether such a relation exists, we treated mice with antagomir-126 and scramblemir control oligonucleotides prior to an acute challenge by i.v. administration of TNFα. As a control for proper induction of inflammation, we followed the expression of E-selectin protein by immunohistochemistry (Fig. 6A) and observed that, as expected, the expression of this protein was not affected by antagomir-126 or scramblemir treatment. In contrast, we observed a marked increased expression of VCAM-1 protein in the glomerular compartment of

the mice treated with the antagomir-126 (Fig. 6). Moreover, non-glomerular segments also displayed an antagomir-126 treatment related increased expression of VCAM-1 protein, which is most likely due to the presence of functional, albeit lower levels, of mir-126 in the other microvascular segments, as we previously showed qualitatively by *in situ* hybridization [34]. Scramblemir treatment did not affect VCAM-1 protein expression levels in any of the vascular segments. Quantification of VCAM-1 protein levels in whole kidney protein isolates by ELISA also showed that VCAM-1 protein levels were increased by the antagomir-126 treatment, while scramblemir treatment did not affect the levels (Fig. 6B).

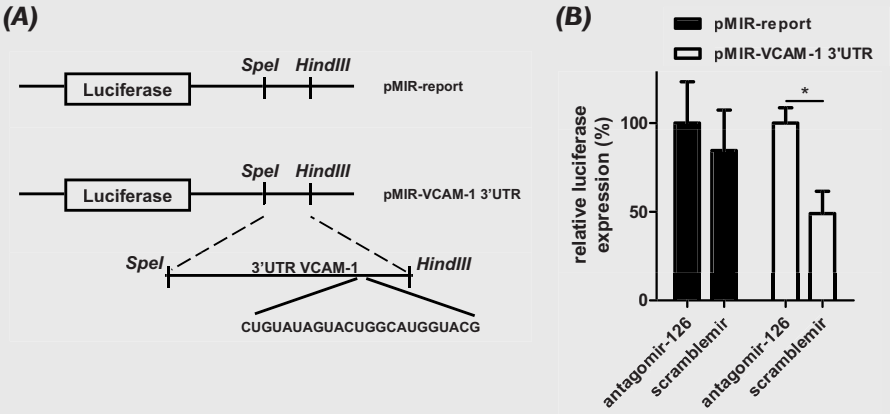

Figure 5. MiR-126 is functionally active in glomerular endothelial cells

(A) Schematic representation of pMIR-report and pMIR-VCAM-1 3'UTR plasmids. Both plasmids contain the firefly luciferase gene. In the latter, the 762 nt of VCAM-1 3'UTR were cloned into the multiple cloning site downstream of the luciferase gene. The sequence given below the pMIR-VCAM-1 3'UTR is the binding site for miR-126. ***(B)*** Relative firefly luciferase expression in glomerular endothelial cells pre-incubated with antagomir-126 and control scramblemir, respectively. Cells were transfected with either the pMIR-report which lacks the miR-126 consensus or the pMIR-VCAM-1 3'UTR, both schematically shown in panel A. Data are presented as mean values ± SD; n=5-6; *, $p<0.05$.

In parallel, we analyzed the microvascular responsiveness to antagomir-126 administration in the liver, where we also observed prominently enhanced VCAM-1 protein expression in the microvascular sinusoidal endothelial cells, which was further substantiated by ELISA quantification of VCAM-1 protein content of the liver (Fig. 7). In contrast, the capillaries of the lungs and heart were devoid of such a response (data not shown). This implies that not only in the kidney, but also in other organs, yet not in all, miR-126 exerts a role in VCAM-1 protein expression control in response to an acute inflammatory stimulus.

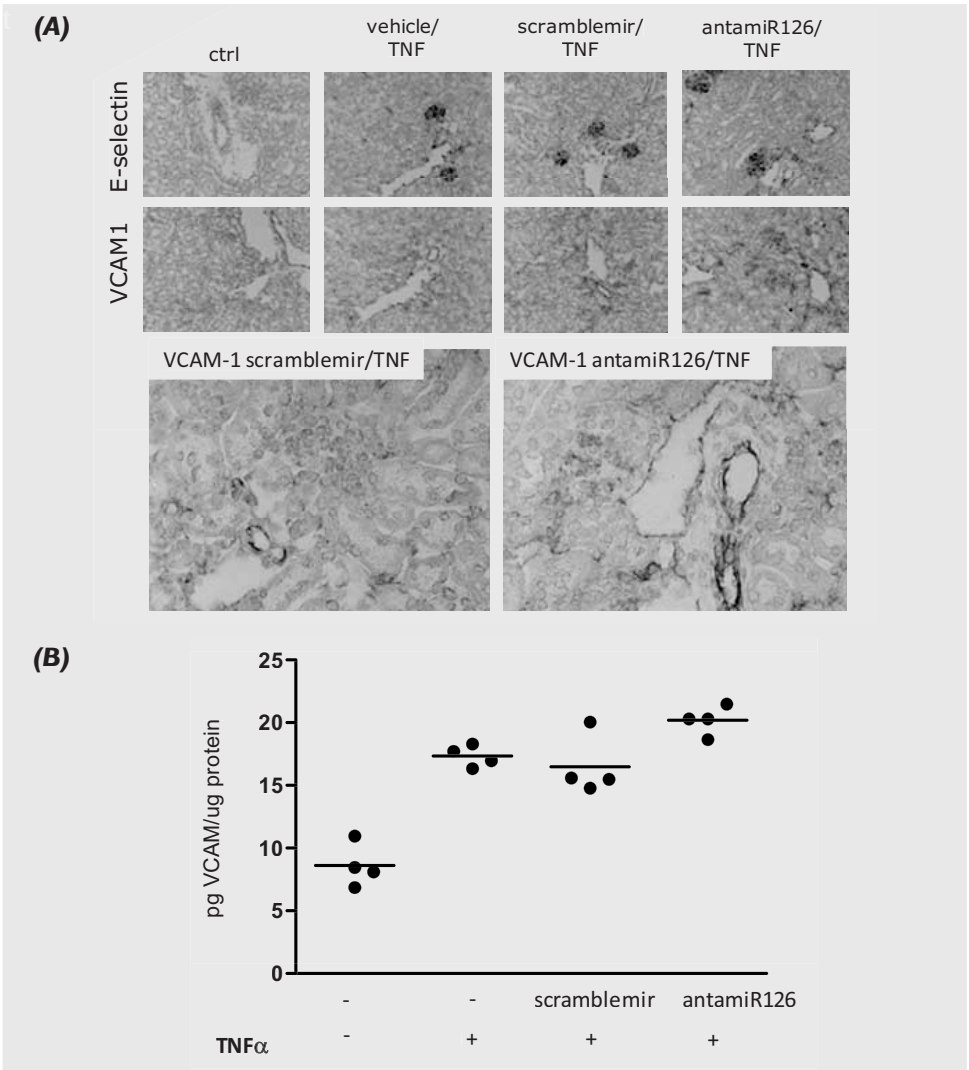

Figure 6. In vivo treatment with antagomir-126 prior to TNFα challenge leads to exaggerated VCAM-1 protein expression

Five days after i.v. injection of, respectively, antagomir-126 (antamiR126), scramblemir, and vehicle, mice were challenged by i.v. TNFα administration and pro-inflammatory protein expression was compared to untreated, unchallenged controls. ***(A)*** Immunohistochemical analysis of E-selectin shows proper microvascular endothelial cell activation upon TNFα exposure (upper row). VCAM-1 staining in the scramblemir treated mice was comparable to that in vehicle pretreated mice, while antagomir-126 treated mice demonstrated induced VCAM-1 protein expression in glomeruli as well as in the other microvascular compartments (middle and lower row). ***(B)*** Quantitative analysis of VCAM-1 protein in whole kidney protein extracts (right) confirmed this increase in VCAM-1 protein expression in antagomir-126 treated animals. Two mice per group, each point represents one kidney.vv

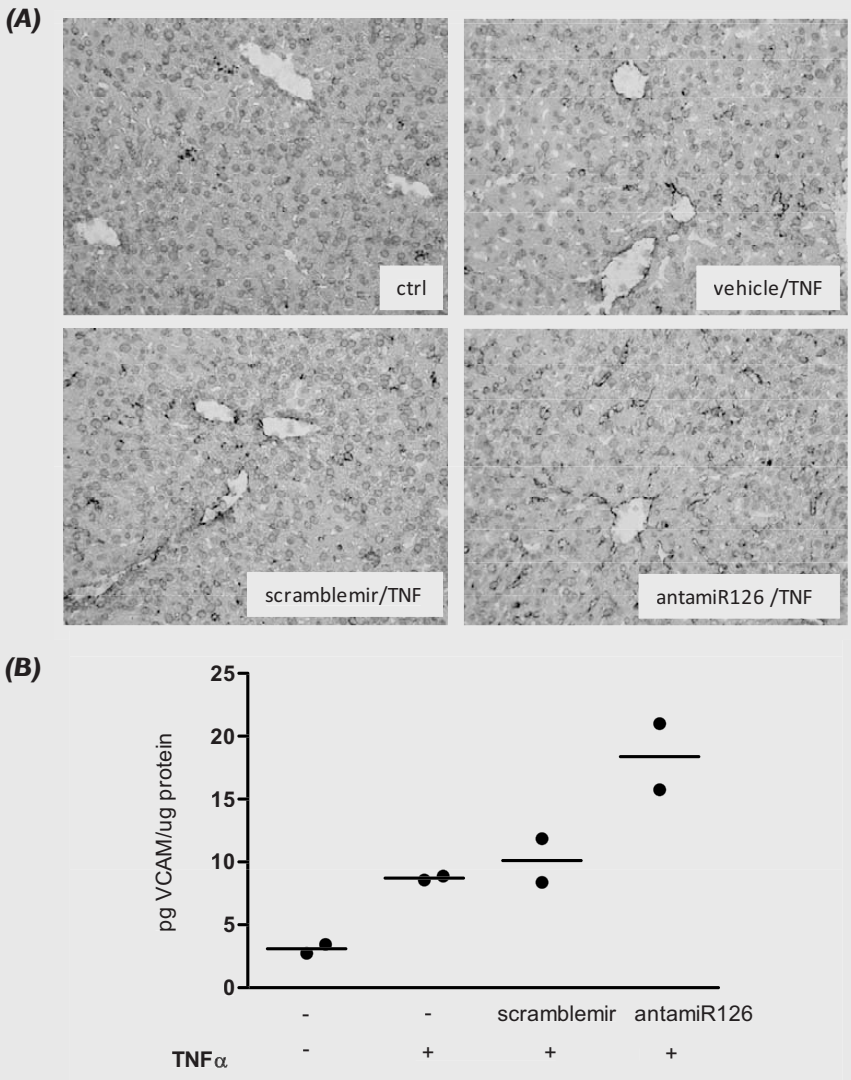

Figure 7. In vivo treatment with antagomir-126 prior to TNFα challenge leads to exaggerated VCAM-1 protein expression in the liver microvasculature

Five days after i.v. injection of, respectively, antagomir-126 (antamiR126), scramblemir, and vehicle, mice were challenged by i.v. TNFα administration and pro-inflammatory protein expression was compared to untreated, unchallenged controls. ***(A)*** Immunohistochemical analysis of VCAM-1 expression in the scramblemir treated mice was comparable to that in vehicle pretreated mice, while antagomir-126 treated mice demonstrated induced VCAM-1 protein expression upon TNFα challenge. ***(B)*** Quantitative analysis of VCAM-1 protein in whole liver protein extracts confirmed this increase in VCAM-1 protein expression in antagomir-126 treated animals. Two mice per group, each point represents one liver.

Discussion

The molecular basis for endothelial heterogeneity in renal microvascular segments is rather elusive, though knowledge thereof forms an essential fundament for understanding segmental involvement in disease and (lack of) responsiveness to drug intervention strategies [14, 18]. In the current study we show that expression of miR-126 in the glomerular microvascular compartment is a governing factor in the control of VCAM-1 protein expression in response to acute inflammation. High miR-126 levels in the glomerular compartment coincided with low VCAM-1 protein expression, while mRNA levels were highly induced. In contrast, in arterioles low miR-126 levels were associated with high VCAM-1 protein levels (see Figure 8 for a schematic presentation of this molecular concept). This posttranscriptional control mechanism is clearly distinct from the transcriptional control of E-selectin.

This is one of the first studies that show the validity of the concept of an inverse, causal relation between miR-126 expression in endothelial cells and VCAM-1 protein expression in an *in vivo* setting. Upon inhibiting miR-126 function using antagomir-126, we observed increased VCAM-1 protein expression in response to TNFα challenge in glomeruli and other renal microvascular segments as well as in the liver microvasculature (Fig. 6 and Fig. 7, respectively). This is in line with studies by Krutzfeldt and colleagues who

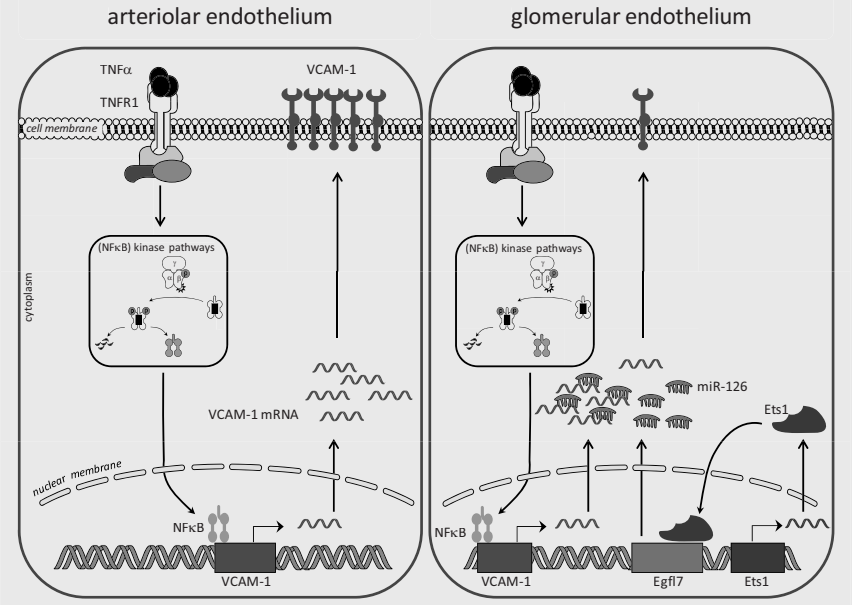

Figure 8. Schematic presentation on how microvascular endothelial subset specific microRNA-126 expression may contribute to heterogenic VCAM-1 protein expression

Figure 8. Schematic presentation on how microvascular endothelial subset specific microRNA-126 expression may contribute to heterogenic VCAM-1 protein expression

The expression of microRNA-126 (miR-126), an intronic product of the endothelial restricted Egfl7 gene, is substantially higher in glomerular endothelium than in arteriolar endothelium in the kidneys of C57bl/6 mice, as shown in this study. One consequence of this differential expression is that one of its target genes, the pro-inflammatory adhesion molecule VCAM-1, is translationally repressed primarily in the glomerular compartment. In both microvascular compartments, inflammatory activation, e.g., via TNFα binding to its receptor TNFR1, leads to activation of kinase signaling pathways which includes Nuclear Factor κ-B (NFκB) activation. NFκB mediated VCAM-1 transcription is initiated in both endothelial subsets upon glomerulonephritis induction or TNFα administration, but in the glomerular endothelial cells the higher miR-126 levels represses VCAM-1 protein transcription. As a consequence, in reaction to an inflammatory stimulus, less VCAM-1 protein is being produced in the glomerular compartment.

reported access of the inhibitory nucleotides to all tissues due to the cholesterol derivatization [13], although proper *in vivo* biodistribution studies to substantiate their accumulation in all microvascular beds are at present lacking. Ideally, endothelial specific miR-126 inhibitory nucleotides or transgenic mice should be used to prove this causal relation, to exclude indirect extra-endothelial effects being the cause of the observations reported here. Knowledge regarding genes that are restrictedly expressed in the endothelial subsets under study to identify useful promoters to make these transgenic mice is, however, missing. Although we previously showed that endothelial subset specific drug delivery systems can be designed and applied successfully to interfere with endothelial cells in glomerulonephritis [2], systems specific for quiescent endothelial subsets have not been reported yet [12]. Still, in our study set-up, the molecular target of the inhibitory nucleotides, miR-126, creates a certain level of specificity as miR-126 is restrictedly expressed in the endothelium [5, 9, 34].

Intuitively, one would expect miR-126 levels to drop after antagomir treatment. We, however, did not observe this, either in arteriolar or in glomerular segments of antagomir-126 treated mice (data not shown). Quantifying miRs after antagomir approaches by PCR-based methods is dependent on the timing of analysis after administration and the nature of the tissue analyzed. Moreover, after the antagomir inhibits its miR target by forming a duplex structure, the exact fate of miR is unknown. Torres et al. [32] recently suggested that upon binding the miR:antagomir-duplex is secluded, but not degraded. Furthermore, the fate of the duplex may depend on its binding chemistry, and it is not unlikely that different pathways prevail in different cell types. Technical issues may complicate this matter when using quantitative RT-PCR for miR detection, which includes several heating steps that can melt the duplex. By this means, the silenced miR may be

unleashed and can bind to complementary primers during the PCR reaction. In addition, Davis et al. [7] reported that currently available detection techniques of inhibited miR can be non-informative, since non-complexed antagomirs can interfere with miR levels. Therefore, the identification of a secondary end-point, the miR target gene, in our case VCAM-1, is absolutely critical for the interpretation of a miR-inhibition study.

Within the broader concept of endothelial heterogeneity [1], it is of interest to note that in the other three organs examined – liver, lungs and heart – only in the liver induced expression of VCAM-1 protein was observed after antagomir-126/TNFα treatment. This implies that not in every organ in the microvasculature miR-126 is in control of VCAM-1 protein expression and that expression control at other molecular levels is plausible. Competing endogenous RNAs, including other microRNAs and long non-coding RNAs, may contribute to a more complex control of VCAM-1 protein expression [25]. The observed divergence between Ets1 and Egfl7/miR-126 expression in the glomerular cells, in addition, points to another level of complexity of (endothelial subset restricted) gene expression control, that in the case of Efgl7 may involve transcription factors other than Ets1, such as Erg, GATA-2 [15] and Ets2 [11].

The observed glomerular specific induction of E-selectin, both at the mRNA and protein level, also instigates the question of how this highly compartmentalized expression is controlled, as all models applied have a systemic inflammation component. Negative regulation of E-selectin expression by miR-31 was recently described by Suarez and colleagues [30]. As similar levels of miR-31 were detected in glomeruli and arterioles (Fig. 3), we could not directly associate differential expression levels of miR-31 with preferential E-selectin expression in the glomeruli. Alternatively, restricted glomerular expression of E-selectin is associated with specific modifications of the chromatin architecture of the E-selectin promoter [8]. Which mechanisms are exactly involved in the distinct microvascular segment-specific, inflammation induced E-selectin expression *in vivo* remains, for now, unknown.

It needs to be established which other targets are directly or indirectly affected in time by antagomir treatment, to better understand the role of miR-126 in the molecular complexity of the changes that occur and the functional consequences thereof. To address this, less complex models such as cell culture systems are first choice, for both analytical and experimental reasons. We were, however, not able in endothelial cell cultures to show a miR-126 / VCAM-1 relation in a direct fashion: pre-incubation of glomerular endothelial cells and HUVEC with antagomir-126 followed by TNFα activation did not consistently result in induced VCAM-1 protein expression (data not shown). Both in our studies as well as in that of Harris and others [9, 10] molecular tools including pre-miR-126 expression plasmids and VCAM-1 reporter plasmids were employed to demonstrate a relation. Taking endothelial cells from an organ into a culture

system leads to a major drift of genes [6], and major changes in responsiveness to e.g., proinflammatory stimuli [17], which may underlie the fact that we cannot directly recapitulate the *in vivo* observations in an *in vitro* context. This may also play a role in the observed differences between Egfl7 expression control under inflammatory conditions in the cells in culture and *in vivo*, and emphasizes the importance of studying both molecular processes and the functional consequences in an *in vivo* context.

In summary, we here showed that glomerular expression of E-selectin in response to an acute inflammatory stimulus is transcriptionally controlled, while the contained, limited expression ofVCAM-1 in this microvascular compartment is posttranscriptionally controlled. Understanding the more detailed microvascular segment specific mechanisms of control and functional consequences for endothelial engagement in disease is a prerequisite to identify whether these processes will herald new venues for renal microvascular segment targeted therapeutic intervention strategies.

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CHAPTER 6

Aspirin treatment hampers the use of plasma microRNA-126 as biomarker for the progression of vascular disease

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Abstract

MiR-126 facilitates angiogenesis and regulates endothelial cell function. Recent data suggest that miR-126 can serve as a biomarker for vascular disease. Although endothelial cells are enriched for miR-126, platelets also contain miR-126. In this paper, we investigated the contribution of platelets to the pool of miR-126 in plasma from patients with type 2 diabetes and how this is affected by aspirin.

In vitro platelet activation resulted in the transfer of miR-126 from the platelet- to the plasma-compartment, which was prevented by aspirin. *In vivo* platelet activation, monitored in patients with type 2 diabetes by measuring soluble P-selectin, correlated directly with circulating levels of miR-126. Administration of aspirin resulted both in platelet inhibition and concomitantly reduced circulating levels of platelet derived microRNAs including miR-126.

Platelets are a major source of circulating miR-126. Consequently, in patho-physiological conditions associated with platelet activation, such as diabetes type 2, administration of aspirin may lead to reduced levels of circulating miR-126. Thus, the use of platelet inhibitors should be taken into account when using plasma-levels of miR-126 as a biomarker for the progression of vascular disease.

Introduction

MicroRNAs (miRNAs) are short non-coding RNAs that function as post-transcriptional, negative regulators of gene expression. While native RNA molecules are rapidly degraded in plasma, miRNAs display exceptional stability in the circulation due to their association with argonaute protein [1], high density lipoprotein [2] or their inclusion into exosomes or microparticles [3]. Since many miRNAs are tissue-specific and differentially expressed in patho-physiology, miRNA-profiles in the circulation may serve as biomarkers for disease progression. Indeed, altered levels of selected miRNAs have been reported in various cardiovascular diseases, such as acute myocardial infarction [4], myocarditis [5], acute and chronic heart failure [6]. MiR-126, which has been shown to be enriched in endothelial cells [7], has received particular interest. Alterations in circulating miR-126 have been proposed as a marker for endothelial dysfunction in type 2 diabetes (DM2) [8] and coronary artery disease (CAD) [9]. However, next to its endothelial origin, miR-126 also constitutes one of the most abundantly expressed miRNAs in platelets [10, 11]. Here, we investigated whether platelets are a possible source of circulating miR-126 and how aspirin treatment affects its plasma level in patients with DM2.

Material and methods

In vitro platelet activation

To establish a relation between the activation of platelets and miR-126, sodium citrate-anticoagulated (3.8% Na-citrate containing vacuum tubes, Becton Dickinson) whole blood samples were obtained from four healthy, male volunteers. From these samples, platelet-rich-plasma (PRP) was obtained by centrifugation (15 min at 150 g at room temperature, no brake) and divided into three samples (60 µL per sample). One sample served as non-stimulated control. The other two samples were incubated with arachidonic acid (AA, 1.5 mmol/L, Hart Biologicals, UK) in the absence or presence of aspirin (asp, 330 µmol/L, Sigma). This concentration of aspirin is equivalent to the amount that patients receive when they are treated with 300 g/day of aspirin. We chose for AA, since this platelet activator is inhibited optimally by aspirin whereas two other routinely used platelet-activators collagen or adenosine diphosphate are only inhibited by aspirin for approximately 50% [12]. After AA-incubation (10 min at 37°C), 50 µL of the PRP was transferred to 450 µL of paraformaldehyde (1% w/v, PFA) to fixate the platelets. From the remaining sample, platelet-free plasma (PFP) and a platelet-pellet was obtained by centrifugation (5 min at 5000 rpm). To check whether the PFP preparations were cell-free, all samples were analyzed with an automated cell-counter (Sysmex®) and by FACS. To illustrate that the PRP did not contain any leukocytes, the forward (FSC)/ side scatter (SSC) FACS-plots (logarithmic scales) are shown from one donor (Supplemental Figure S1): non-incubated (tube A), incubated with arachidonic acid and aspirin (+AA/+asp; tube B) or AA alone (+AA, tube C), as indicated. Each tube contained a small portion of small particles (debris in grey), while the platelets (anthracite) are clearly distinguishable from the debris, not only on the FSC/SSC-plot, but especially when the expression of P-selectin is measured (second row of dot plots) after activation with AA in the absence of aspirin (tube C). The number of events in the debris- and platelet-gate accounted for 99.4 % (tube A), 99.3 % (tube B) and 99.4% (tube C) of the total number of events, of which 99% could be identified as P-selectin positive when incubated with arachidonic acid (histogram, tube C). This indicates that leukocytes were not detectable in these PRP samples, not by cell-counter (not shown), nor by FACS, and also not in the PFP samples obtained from these PRP-samples at a later stage. Samples were stored at -80°C until further analysis. All *in vitro* experiments were performed in triplicate. Representative results are shown.

Surface-expression of P-selectin by platelets

To assess the expression of P-selectin by platelets as a measure of AA-activation and to assess the inhibitory effect of aspirin treatment, PFA-fixed platelets were washed with FACS-buffer (PBS supplemented with 1% bovine serum albumin and 0.05% Na-azide), incubated with mouse IgG directed against human P-selec-

tin or an isotype-matched control IgG (both 5 µg/mL, BD Biosciences), washed with FACS buffer and incubated with goat-anti-mouse IgG conjugated with Alexa-488 (Molecular Probes). P-selectin expression was measured using the LSRII (Becton Dickinson). Platelets incubated with isotype-matched control IgG, were gated (gate P2), which represents background staining. Gate P3 was set to detect fluorescent signals above background and represents P-selectin-positive platelets. Mean fluorescent intensity (MFI) was measured from the platelets in P3.

Measurement of soluble P-selectin and von Willebrand factor

Soluble P-selectin (sP-sel)was measured in PFP using a commercially available ELISA kit, according to the instructions of the manufacturers (R&D Systems). Measurements were performed in duplicate and average values were used. Von Willebrand factor (vWF) was measured routinely at the Clinical Chemistry Laboratory of the Leiden University Medical Center according to standard procedures.

MiRNA-expression levels

Total RNA from EDTA-samples was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands). To serve as a technical control 5 fmol synthetic, exogenous C.Elegans miR-238 (Biolegio, Nijmegen, The Netherlands) was spiked into 200 µL plasma or platelet isolates. Subsequently, RNA was isolated and expression levels of miR-126, miR-16, miR-223, miR-423 and miR-238 were validated in triplicate by quantitative RT-PCR (qPCR). Reverse transcription was performed using a 5 minute incubation at 65°C of 2.0 uL (plasma samples) or 250 ng total RNA (platelet isolates) with specific Taqman® microRNA probes (miR-126, Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). cDNA was synthesized using a M-MLV First-Strand Synthesis system (Invitrogen). Results were normalized using Gene Expression Analysis for iCycler IQ® RT-PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands). To compare miRNA-levels, the concentration (fmol/mL) of miRNAs was calculated by correlating the mean miR-238 CT-values with the spiked concentration of miR-238 added to the samples or miRNA-expression was shown as "fold change", calculated with standard $\Delta\Delta$CT method.

Plasma samples of type 2 diabetes mellitus patients

Platelet-free plasma samples were acquired from patients diagnosed with DM type 2 (DM2), who had entered a prospective, randomized study with a placebo-controlled, double-blind, crossover design. A detailed description of the inclusion criteria of these patients and the study has been published previously [12, 13]. All subjects gave written informed consent and the study was approved by the institutional review committee and performed in accordance with the Declaration of Helsinki [12, 14]. In short, all subjects (n=40) received one period placebo

and the other period aspirin (100 or 300 mg/day). The first treatment period with aspirin or placebo for 6 weeks was followed by a washout period of 4 weeks. Thereafter, those assigned to placebo in the first period received aspirin for 6 weeks and those assigned to aspirin received placebo for additional 6 weeks. At each visit, EDTA-anticoagulated peripheral blood samples were drawn from antecubital veins. All plasma-samples were stored at -80°C until further analysis. Based on our definition of aspirin-responders and non-responders (see main text), 19 patients were considered responders, 19 patients were non-responders and for 2 patients the aspirin-responsiveness could not be determined, since a sP-selectin value was missing. These patients were excluded. For 2 patients insufficient amounts of plasma were present to determine levels of miR-126, yielding 18 miR-126 values for both the "responder" and the "non-responder" group.

Statistical analysis

All calculations were performed with Graphpad Prism software. For linear regression analysis P-, β- and r^2-values are reported. To calculate significant differences ($P<0.05$), paired T-tests were used.

Results

In vitro platelet activation

To investigate the correlation between platelet activation and miR-126 in plasma, peripheral blood was withdrawn from healthy volunteers. After obtaining PRP, platelet activation was induced with AA in the absence or presence of aspirin. Of note, PRP samples did not contain contaminating leukocytes as was determined with an automated cell-counter (data not shown) and by FACS (Supplemental Figure S1).

Platelet activation was monitored by surface-expression of P-selectin or by shedding of the soluble form (sP-sel) into the plasma. Non-activated platelets did not express P-selectin (Figure 1B). AA induced P-selectin expression on all platelets (99%, Figure 1D) and in all donors (Figure 1A, left axis). Aspirin (+asp) inhibited the AA-induced expression of P-selectin significantly ($P=0.001$), although this expression was not inhibited on all platelets (Figure 1C) and was not inhibited to non-stimulated levels (Figure 1A, $P<0.001$). AA-stimulation resulted in a significant increase of sP-sel in the plasma-compartment (PFP) obtained from PRP (Figure 1A, right axes, $P=0.025$). Addition of aspirin alone did not alter expression of P-selectin on the platelets or sP-sel levels in the PFP (data not shown). When aspirin was added during AA-stimulation, shedding of sP-sel was completely inhibited to basal levels in each donor. Apparently, aspirin is able to prevent the shedding of sP-sel from the platelets, while membrane expression of P-selectin is not inhibited completely.

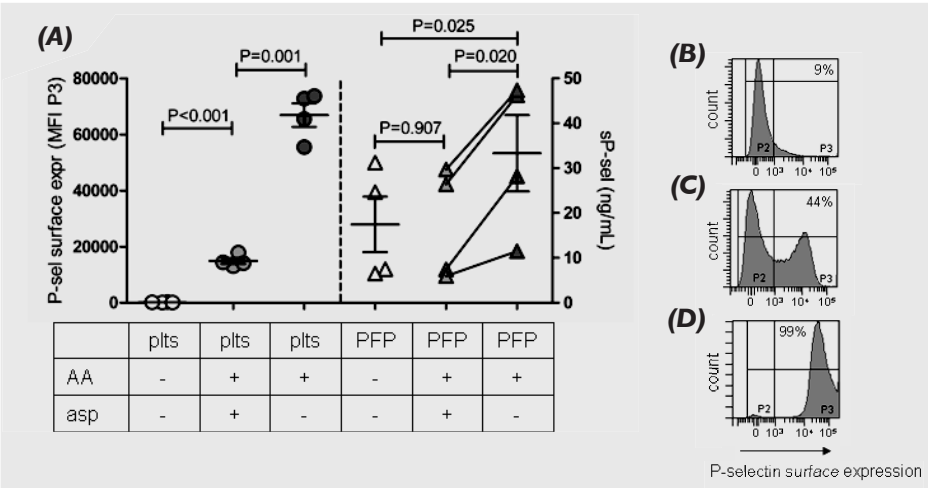


Figure 1. In vitro platelet activation leads to transfer of miR-126 from the platelet- to the plasma-compartment

(A) PRP obtained from healthy volunteers was incubated with arachidonic acid (+AA) in the absence (-asp) or presence of aspirin (+asp). Surface expression of P-selectin, expressed in mean fluorescent intensity (MFI of P3-gated platelets) was measured (left axis), on plts/-asp/-AA ***(B)***, plts/+asp/+AA ***(C)*** or plts/-asp/+AA ***(D)***. Soluble P-selectin (sP-sel; right axis) was measured in platelet free plasma (PFP) after AA-induced platelet activation plus or minus aspirin.

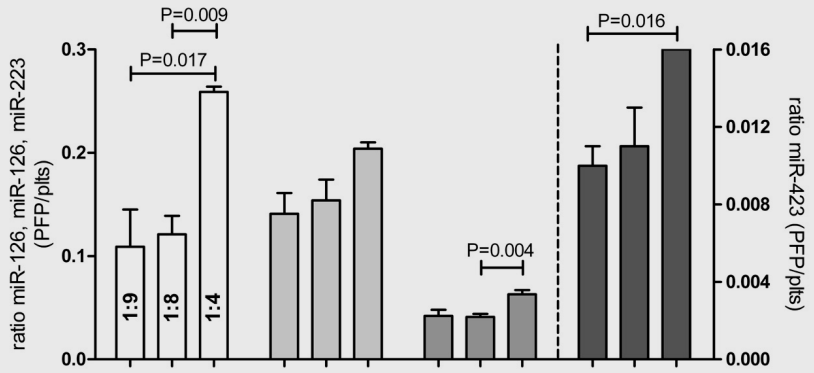
miR-126 levels were measured in PFP or the platelet-pellet, both isolated from the same PRP-sample and the relative distribution ratio (PFP/plts) was calculated. Non-activated PRP showed a distribution ratio of approximately 1:9 (PFP/plts, 0.109±0.036, Figure 2) and AA-activation resulted in a significant transfer of miR-126 (P=0.017) from the platelet- to the plasma-compartment, yielding a relative ratio of 1:4 (+AA, 0.259±0.05). AA-activation in the presence of aspirin prevented the transfer of miR-126 towards the plasma-compartment (P=0.009), which showed a similar ratio of 1:8 (0.121±0.018) as non-activated PRP. As three other platelet-enriched miRNAs (miR-16, miR-223 and miR-423) were released from activated platelets in a similar fashion (Figure 2) our data imply that platelet activation leads to shedding of P-selectin from the platelet-membrane and release of miRNAs from intracellular platelet-stores.

In vivo platelet activation

To investigate the relation between plasma levels of miR-126 and *in vivo* platelet activation, we studied patients with DM2, who exhibit a disease-mediated platelet activation [15]. These patients had participated in a placebo-controlled cross-over study, in which 40 patients were randomly assigned to a period of aspirin-treatment (100 or 300 mg/day) or placebo [12]. This resulted in a wide range of

DM2-induced *in vivo* platelet activation, monitored by sP-sel [16], which is consistent with increased platelet aggregability in chronic vascular diseases, such as DM2 [15].

Figure 3A shows that the absolute level of sP-sel yielded a positive linear regression with circulating levels of miR-126 (expressed as fold change) (P=0.006, β=1.123, r^2=0.197), irrespective of treatment and aspirin-dose. When treatment was taken into account, linear regression analysis showed that the change of sP-sel (delta sP-sel; asp-plac) positively correlated with the change in miR-126 levels (ratio asp/plac, Figure 3B, P=0.010, β=1.992, r^2=0.180).



miR	miR-126			miR-16			miR-223			miR-423		
AA	-	+	+	-	+	+	-	+	+	-	+	+
asp	-	+	-	-	+	-	-	+	-	-	+	-

Figure 2. The relative distribution ratio of miRs
miR-126, miR-16 and miR-223 (left axis) and miR-423 (right axis) in PFP versus platelets (PFP/plts) is shown in treated versus non-treated PRP obtained from healthy volunteers. As negative control miR-263, not present in platelets, was measured.

As shown previously [14], patients were considered aspirin-responders, when their sP-sel level decreased upon aspirin treatment, while patients were defined as non-responders when their sP-sel was either not affected or was even increased. When discriminating for responders (resp) and non-responders (non-resp), the expected maximal difference for delta sP-sel (Figure 4A) coincided with a highly significant difference in the ratio of miR-126 (Figure 4B; P<0.001). Since *in vivo*, endothelial cell activation may contribute to the plasma pool of sP-sel, we also measured the established endothelial cell marker von Willebrand factor (vWF) in the plasma [17]. When calculating delta vWF-values (asp-plac), no difference was observed between the responder and non-responder group (Figure 4C, P=0.350) and linear regression analysis for delta-vWF values and the ratio of miR-126 showed no significance (Figure 3C, P=0.450, β=-0.008, r^2=0.017), indicating that sP-sel levels, and thus miR-126 levels, were not changed due to activation/damage of the endothelium.

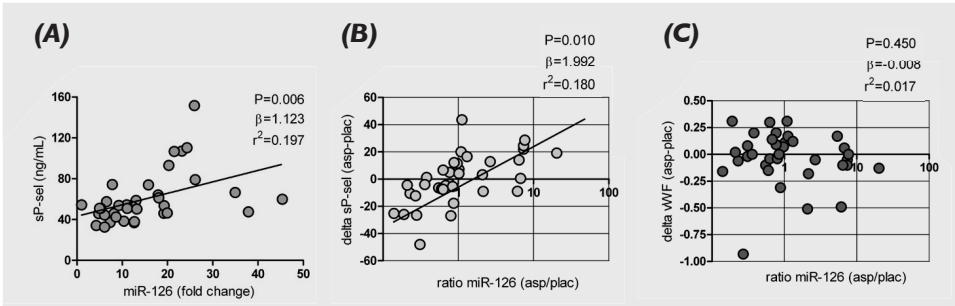


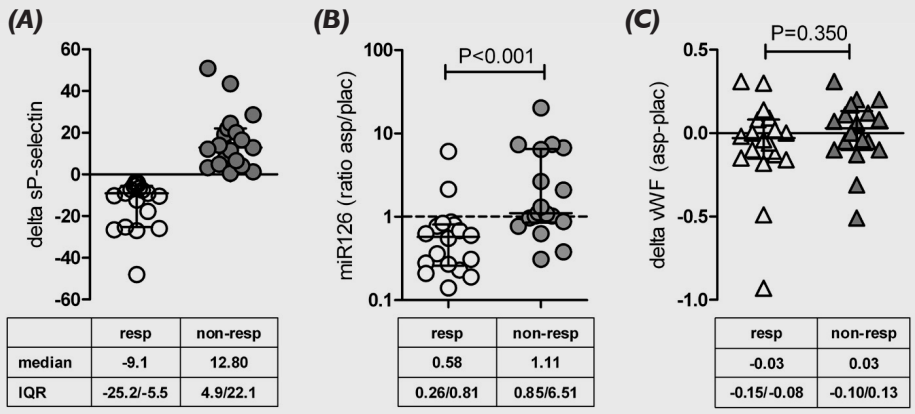
Figure 3. In vivo platelet activation in DM2-patients shows a correlation with miR-126 levels

Linear regression analysis was performed on ***(A)*** miR-126 (fold change) versus sP-sel (ng/mL) in plasma of DM2 patients (n=36) and ***(B)*** the change in miR-126 (ratio asp/plac) versus delta sPsel values (asp-plac) or ***(C)*** delta vWF values (asp-plac) in response to aspirin treatment. Note the logarithmic scale for miR-126 in panels (B) and (C).

Discussion

Previous reports show that miRNAs in plasma display exceptional stability in the circulation due to their association with argonaute protein [1], high density lipoprotein [2] or their inclusion into exosomes or microparticles [3]. In fact, it has recently been shown that the main fraction of miRNAs in human plasma is localized in microparticles of which 41-45% originates from platelets [18]. These miRNA-containing microparticles may actually play a role in cardiovascular diseases by transferring their miRNA-content to target cells. For example, Zernecke et al. have shown that miR-126-containing apoptotic bodies were able to mediate athero-protective effects in mouse models of atherosclerosis [19] and miR-126-containing microvesicles protected the kidney against ischemia/reperfusion injury in mice [20]. These functional properties and the notion that circulating miRNAs may be derived from cells in the vascular system has stirred current interest in the use of circulating miRNA profiles as diagnostic and maybe even prognostic biomarkers for the progression of cardiovascular disease [4-6].

The pathophysiological mechanisms involved in vascular diseases, such as DM2 and CAD, include EC-dysfunction [21] and ongoing vascular injury [22]. Since miR-126 is enriched in endothelial cells [7], these vascular diseases would predict a release of endothelial-derived miR-126 into the circulation. However, in a recent study, a counter-intuitive decrease of miR-126 was observed in CAD patients in comparison to normal age- and gender-matched controls [9]. Interestingly we noticed that all CAD-patients were treated with aspirin, while none of the healthy controls used aspirin. According to our observations, this



	resp	non-resp
median	-9.1	12.80
IQR	-25.2/-5.5	4.9/22.1

resp	non-resp
0.58	1.11
0.26/0.81	0.85/6.51

resp	non-resp
-0.03	0.03
-0.15/-0.08	-0.10/0.13

Figure 4. Classification in subgroups of DM2 patients in aspirin-responders and non-responders

(A) Based on their delta sP-sel levels. Patients subgroups show a significant difference in ***(B)*** the change in miR-126 (ratio asp/plac), but not in ***(C)*** delta vWF levels (asp-plac). Note the logarithmic scale for miR-126 in panel (B). Absolute values represent median and interquartile range (IQR).

could explain the decreased miR-126 levels. Furthermore, an overall systemic inhibition of platelet activation would predict a decrease of other platelet-derived miRNAs as well. Indeed, our *in vitro* platelet activation assay showed that the transfer from the platelet- to the plasma-compartment of 3 other miRNAs that are abundantly present in platelets [11], miR-16, miR-223 and miR-423, were likewise inhibited in the presence of aspirin.

Consistent with our current findings [22], of the 25 miRNAs that were reported to be downregulated to the highest extent (0.50-0.76 fold) in the aspirin-treated CAD patients [9] were located in the top highest-expressed platelet-miRs [10, 11] of which miR-126 was ranked number 5. This is further illustrated in Figure 5, in which the platelet-miRNA-profiles from 2 independent papers are combined [10, 11] displaying a very similar rank-order with a highly significant regression for quantified signals of the miRNA-arrays ($P<0.0001$, $\beta=0.607$, $r^2=0.337$). In contrast, of the 20 miRNAs reported to be upregulated to the highest extent in the aspirin-treated CAD patients [9], only 4 miRNAs are present in the list of platelet-associated miRNAs and these 4 miRNAs are even located in the lowest region of rank-order [11].

These data suggest that when *in vivo* platelet activation is inhibited, as was the case in our DM2 study [14] and the CAD study [9], the release of platelet-derived miRNAs in general and miR-126 in particular, is inhibited accordingly. In fact, the CAD-study showed an actual negative influence of aspirin on circulating miR-126 ($P<0.001$, $R=-0.469$) [9].

Interestingly, in patients with troponin-positive acute coronary syndrome

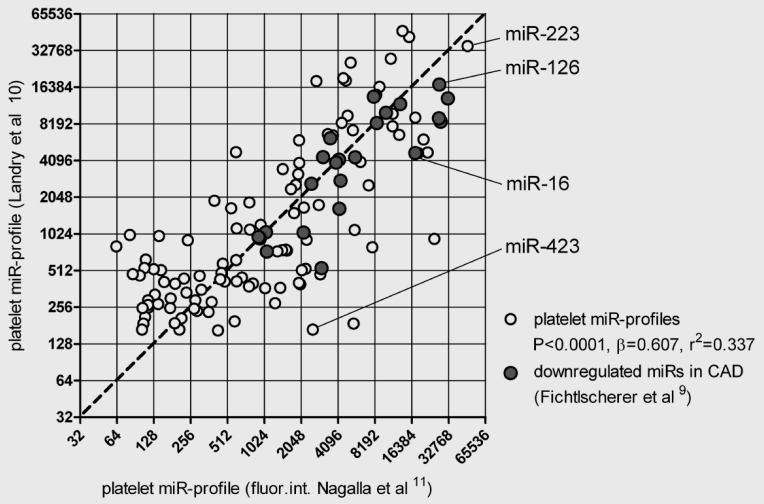


Figure 5. Rank correlation analysis of platelet-derived miRs
Expressed in fluorescence intensities (fluor. int; log 2 scales) as reported by Nagalla et al [11] (X-axis) and by Landry et al [10] (Y-axis), show a significant linear regression ($P<0.0001$). The top 23 miRs that were shown to be significantly down-regulated in the plasma of aspirin-treated CAD patients [9] are shown in grey circles. The location of miR-126, miR-223, miR-16 and miR-423 are shown separately. The optimal regression curve ($\beta=1.0$) is depicted as dotted line.

(ACS), which implies extensive myocardial injury, increased concentrations of systemic miR-126 were documented as compared to patients with CAD, displaying non-elevated troponin levels23. In addition, a significant increase was reported for sP-sel in troponin-positive ACS patients as compared to troponin-negative patients and a positive correlation was found between troponin levels and sP-sel [24].

Of note, in both studies, all patients (CAD and ACS) were treated with aspirin. Apparently, in the acute phase of the coronary syndrome platelet activation exceeds the inhibitory effect of aspirin, leading to increased levels of both sP-sel and miR-126, while in the chronic phase of the disease, as in CAD, effective platelet inhibition by aspirin may lead to corresponding reduced levels of both miR-126 and sP-sel.

In conclusion, aspirin-use should be taken into account when using circulating miR-126 and probably other platelet-associated miRNAs, as diagnostic biomarker for cardiovascular diseases or when studying a possible role of these miRNAs as mediators of cardiovascular disease and/or in athero-protective effects.

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CHAPTER 7

MicroRNA-126 overexpression in lineage depleted bone marrow cells leads to increased neovascularization

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Abstract

It has been demonstrated that bone marrow (BM) cells play an important role in the formation of new capillaries. Whether there is a direct differentiation of these cells in to endothelial cells or whether there is only have a supporting role is still unclear. Therefore, the molecular mechanisms that underlie the contribution of BM cells are of high interest. It is well established that microRNAs (miRs) play a key role in cell differentiation as well as cell fate. The endothelial cell enriched miR-126 has been described to be a key factor for angiogenesis, furthermore it has been demonstrated that bone marrow cells also express relevant levels of miR-126. Here, we investigate whether over-expression of the miR-126 in bone marrow cells can have a potential positive effect on neo-vascularization.

Using a lentiviral construct we successfully over-expressed miR-126 in lineage depleted bone marrow cells. Subsequently these cells were intravenously injected into lethally irradiated mice. Seven weeks after reconstitution of the bone marrow an angiogenic matrigel plug containing VEGF and SDF-1 was inserted into the flanks of the animal. After seven days vascularization of these plugs was visualized and a significant increased vessel length was observed in the plugs of the animals that were transplanted with bone marrow cells that over-expressed miR-126 Furthermore, FACS analysis revealed an up-regulation of total white blood cells in the periphery of the mice transplanted with the BM cells that over-expressed miR-126. This enhanced migratory capacity of white blood cells was observed for almost all subpopulation of leukocytes that were tested. Both enhanced neovascularization as well as the increased levels of white blood cells where highly correlated to the expression levels of miR-126 in the bone marrow.

Our data demonstrates that miR-126 has an angiogenic potential that reaches further then its presence in EC. Although the exact mechanisms leading to increased neovascularization and elevated levels of leukocytes is unknown, it is likely that these two effects are linked.

Introduction

It has been demonstrated that the formation of novel capillaries may not only be restricted to the sprouting capability of endothelial cells (ECs) but that bone marrow (BM)-derived progenitor cells also play a facilitary role in neovascularization [1]. Whether these precursor cells can differentiate into mature ECs is extensively studied, but remains unclear [2]. Nevertheless it has been reported that BM-derived endothelial progenitor cells (EPC) can form independent functional vascular structures after migration towards a hypoxic region in the skin [3]. Furthermore, others claim a capability of BM-derived cells to promote vascular growth in (tumor)-ischemia, however this support is not generated by

differentiation of these EPC in to mature endothelium but involves perivascular stabilization of the neovasculature and paracrine effects of these cells [4, 5]. Based on these observations, the contribution of BM-derived progenitor cells might depend on depth of ischemia, release of cytokines and sort of injury.

The molecular mechanisms underlying these processes of vascular homeostasis involve the tight regulation of genes that control of cell proliferation, migration and differentiation [6]. MicroRNAs (miRs) are small regulatory RNAs that function as negative regulators of gene expression [7]. The discovery that miRs are expressed in a tissue- and cell-specific manner during development suggest that miRs could play a role in specifying and maintaining tissue identity [8]. Furthermore, miRs possess the capacity to regulate multiple targets, and can thereby influence the activity of diverse signalling pathways. To this end, ECs, that play a key role in the initiation of neovascularization, have been thoroughly analyzed for the presence of miR. Indeed, several miRs were found to have both pro-angiogenic [9-12]. as well as anti-angiogenic functions in ECs [13, 14].

Of particular interest, with respect to a controlling role in neovascularization, is the endothelial-enriched miR-126. It has been demonstrated that miR-126 targets proteins that play key roles in angiogenesis, vasculogenesis and inflammation [5, 15-19]. MiR-126 is highly expressed in EC and targets two potent repressors of pro-angiogenic signaling sprouty-related EVH1 domain containing 1 (SPRED-1) and phosphoinositide-3-kinase regulatory subunit 2 (PI3KR2) [19, 20]. Therefore, it is likely that ECs require constitutive levels of miRNA-126 to maintain the integrity of the vasculature both during vascular development as well as in adult life. In addition, in experimental ischemia, miRNA-126 can also directly modulate CXCL12/SDF-1 expression and drive the mobilization of vasculogenic Sca-1+/Lin- stem cells into the circulation [18].

Next to its abundant presence in EC, miRNA-126 also circulates in plasma in a complex with Ago2, but also in vesicles or exosomes [21]. Exosomes are vesicles that are secreted as a consequence of the fusion of multivesicular bodies with the plasma membrane and are loaded with distinct sets of miRNAs. Following their release, exosomes can fuse with target cells thereby facilitate functional repression of target cell mRNAs by the exosomal miRNAs [22]. Recently, it was shown that circulating levels of miR-126 decreased during transcoronary passage in patients with evidence of myocardial injury suggesting a role for circulating miR-126 in vascular homeostasis [23].

Although the EC is a likely source for circulating vesicles containing miR-126 [24, 25], significant levels of miR-126 are also expressed in circulating cells hematopoietic cells and platelets [26-29]. Moreover, these cells have been demonstrated to be highly capable of exosome/vesicle secretion allowing them to exert transcellular regulation of gene expression [30]. In addition, hematopoietic cells, in particular myeloid cells have been demonstrated to play a rate limiting role on neovascularization facilitating angiogenesis [31] as well as arteriogenesis

[32]. To investigate the role of hematopoietic cell expressed miR-126 in vascular homeostasis we over expressed miR-126 in the hematopoietic compartment of mice. Seven weeks after transplantation, a VEGF- and FGF-rich matrigel plug was inserted to assess the effect of hematopoietic overexpression of miRNA-126 on leukocyte mobilization and neovascularization. Our findings underline a potential role for miR-126 in neovascularization an indicates that this regulatory capacity is not limited to the EC, but may also involve a role for hematopoietic cells via transcellular or direct effects.

Material and methods

Mice

C57BL/6J wild type (WT) and B6.SJL-Ptprca Pepcb/BoyCrl (Ly5.1) were purchased from Charles River Nederland (Maastricht, the Netherlands). Mice were housed under a 12-h light/dark cycle, standard chow diet and drinking water were provided *ad libitum*. All animal experimental protocols were approved by the animal welfare committee of the veterinary authorities of the Leiden University Medical Center.

Lentiviral constructs

The vesicular stomatitis virus G protein-pseudotyped self-inactivating (SIN) HIV type 1 (HIV-1)-based vectors LV-miR126 and LV-control were generated in 293T cells with the aid of the packaging plasmids psPAX2 (Addgene, Cambridge, MA) and pLP/VSVG (Invitrogen, Breda, the Netherlands) as specified before [33]. To concentrate and purify lentivirus vector particles, producer cell supernatants were layered onto 5 mL cushions of 20% (wt/vol) sucrose (Merck, Whitehouse Station, NJ) in phosphate-buffered saline and centrifuged at 15,000 rotations per minute for 2 hours at 10°C in an SW28 rotor (Beckman Coulter, Woerden, the Netherlands). Prior to ultracentifugation, producer cell supernatants were subjected to filtration through 0.45-mm pore-sized cellulose acetate filters (Pall, Port Washington, NY).

Cells and cell culture

Bone marrow (BM) cells were isolated from the femora and tibia of euthanized WT mice. Following isolation, cells were cultured in StemSpan-SFEM (Stemcell Technologies Inc, Vancouver, BC, Canada) supplemented with 50 ng/mL recombinant mouse stem cell factor (rmSCF), 10 ng/mL recombinant mouse thrombopoietin (rmTPO) and 50 ng/mL recombinant mouse fms-related tyrosine kinase 3 ligand (rmFLT3-L) (all R&D Systems, Minneapolis, MI) at 37°C and 5% CO2. After 24 hours the BM cells were transduced by spin occulation in the presence of 4 µg/mL proteamine sulphate (Sigma Aldirch, St Louis, MO) at 800g and at 32°C for 1 hour. Cells were transduced at a multiplicity of infection

(MOI) of 5 with either LV-control or LV-miR126 and kept in culture. Microscopic images were made daily and after five days cells were harvested for FACS analysis and RNA analysis.

Transduction and transplantation of bone marrow cells

BM cells were isolated from the femora and tibia of euthanized CD45.1+ mice (Charles River, aged 8–10 weeks). Upon isolation, lineage negative cells were negatively selected (Lineage Cell Depletion Kit (mouse), Miltenyi Biotec, Bergish Gladbach, Germany) and cultured for 1 day as described before and subsequently transduced at a MOI of 5 with either LV-control or LV-miR126. After transduction, cells were maintained for another 24 hours in the presence of cytokines. Transduced cells (300 000/mouse) were mixed with supportive spleen cells (500 000/mouse) and injected into the tail vein of lethally irradiated (8 Gy) male C57BL/6J recipient mice (n=12 per group, Charles River, aged 8–10 weeks).

Matrigel plug assay

Seven weeks after irradiation, mice (n=10 per group) were anesthetized with isoflurane and injected subcutaneous into the flank with 0.5 mL ice-cold matrigel (BD Biosciences, Breda, the Netherlands). Matrigel was supplemented with 100 ng/mL recombinant mouse SDF-1 (Invitrogen) and 50 ng/mL recombinant mouse VEGF (Invitrogen). After 7 days, mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg), and atropine (125 µg/kg) and the skin was opened to visualize the vasculature of the implant with a Sidestream Dark Field (SDF)-camera. Thereafter, implants were extracted, imaged with a Leica microscope (DMI6000, Nieuw Vennep, the Netherlands) and subsequently fixed in 4% paraformaldehyde and snap frozen at -80°C. From all microscopic images of the matrigel implants the number of visual vessels was established on both sides of the implants. To obtain total length of the vessels pictures were digitalized and the total pixel area of the vessels was calculated using ImageJ software.

Immunohistochemistry on matrigel plugs

Twenty µm-thick sections of matrigel plugs were fixed with methanol on a glass side and subsequently blocked with 2% fetal calf serum (FCS, Bio Whittaker/Cambrex, Verviers, Belgium) 3% bovine serum albumin (BSA, Sigma Aldirch) in PBS. Next, sections were incubated with specific antibodies against murine vWF (Dako Netherlands, Heverlee, Belgium) or F4/80 (Abcam, Cambridge, UK) followed by secondary antibodies against goat-anti-rabbit-IgG labeled with Alexa-488 (Molecular Probes). Ly6C (ABD Serotec, Düsseldorf, Germany) was directly labeled with Alexa-488. As a negative control, isotype-matched IgG were used.

Whole blood and bone marrow analysis

Whole blood was collected by incision of the tail vein or heart puncture and analyzed by semi-automatic hematology analyzer F-820 (Sysmex; Sysmex Corporation, Etten-Leur, the Netherlands) microscope (Leica) and flow cytometry (FACS, LSR II, BD Biosciences). Hematological values obtained were white blood cell counts (WBC, n*10^6/mL), red blood cell counts (RBC, n*10^9/mL), platelets (PLT, n*10^6/mL), hematocrit (HCT, %/%) and hemoglobin (HGB, mmol/L). For microscopic images a blood smear was made on a glass cover and images were made using a fluorescence microscope. For BM cells the same procedure was followed For FACS analysis, we incubated 35 µL of whole blood or 106 bone marrow cells for 30 minutes at 4°C with directly conjugated antibodies directed against CD45.1 (PE-Cy7, eBioscience, Vienna, Austria), CD45.2 (FITC, BD Biosciences) to analyze the percentage of chimerism of the mice after BM transplantation, CD11b (PercP, BD Biosciences), CD3e (APC, BD Biosciences) and B220 (APC-eFluor780, eBioscience). A different sample was prepared with the same amount of cells and antibodies directed against Ly6G (PE, BD Biosciences), CD115 (biotin, eBioscience), CD11b (APC, BD Biosciences) and MP20 (FITC, kindled provided by Erasmus University Rotterdam). These cells were subsequently incubated with secondary antibody with streptavidin (PercP-Cy5.5, BD Biosciences) to visualize the biotin labeled antibody. Furthermore, a third mix was prepared with 50 µL of whole blood or 106 bone marrow cells with antibodies directed against Sca-1 (FITC, BD Biosciences), CD117 (PE-Cy7, BD Biosciences) and a cocktail against lineage-positive cells (APC, BD-Biosciences). In a separate tube, 50 µL of whole blood was incubated with an appropriate cocktail of isotype controls.

Quantification of miR levels

Total RNA from BM cells was isolated using Trizol reagent (Invitrogen). Expression levels of miR-126 were validated by quantitative RT-PCR (qRT-PCR). Reverse transcription was performed using a 5 minute 65°C incubation of 250 ng total RNA with specific Taqman® miR probes for miR-126 (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). cDNA was synthesized using a M-MLV First-Strand Synthesis system (Invitrogen). Validation of miR-126 levels was performed using Taqman® miR assays and qRT-PCR. For normalization, a qRT-PCR on RNU6B was performed on cDNA obtained from the same RNA. The following primers were used for PCR: U6 (sense) CTCGCTTCGGCAGCACA and U6 (antisense) AACGCTTCACGAATTTGCGT. Results were normalized using Gene Expression Analysis for iCycler IQ® RT-PCR Detection System (Bio-Rad Laboratories, Veenendaal, the Netherlands).

FACS analysis of blood and bone marrow

All samples obtained for FACS analysis were either immediately analyzed by

flow cytometry analysis (FACS, LSRII) or were fixed in 1% paraformaldehyde and analyzed within 24 hours after preparation. Data were analyzed using FACSDiVa software (BD Biosciences).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Mann-Whitney or a standard T-test. $P<0.05$ was considered statistically significant.

Results

Transduction of BM cells with LV-miR126 leads to overexpression of miR-126 in vitro

The hairpin for miR-126 was cloned into a pLV-U6 construct that also contained the sequence for dsRED behind a PGK promoter. A control was used that lacked the hairpin structure (Figure 1A). Lineage-depleted BM cells were transduced with LV-miR126 and LV-control at a MOI of 5. As compared to non-transduced cells a high number of fluorescent dsRED-positive BM cells were observed 5 days after transduction with either viral construct. Quantification by flow cytometry indicated a transduction efficiency of around 30% for both constructs (Figure 1B). Quantitative real-time PCR analysis (qPCR) revealed a 30 fold increase in miR-126 expression levels in the BM cells transduced with the lentiviral construct harboring the gene for miR-126 (LV-126, n=3, Figure 1C) as compared to non-transduced BM cells (mock) and BM cells transduced with control virus (LV-C).

Transduction of bone marrow cells with LV-miR126 leads to over-expression of miR-126 in vivo after eight weeks

To generate mice overexpression miR-126 in the hematopoietic compartment, ex vivo transduced BM cells were intravenously injected into lethally irradiated mice. Eight weeks after transplantation mice were sacrificed and blood and bone marrow samples were obtained. Smears of blood and BM suspensions isolated from the animals transplanted with transduced BM showed dsRED positivity, validating successful transplantation. Blood and BM smears derived from control donor mice showed no dsRED positivity (Figure 2A and 2B). To determine the degree of chimerism, the ratio between congenic markers CD45.1 (donor) and CD45.2 (acceptor) in blood and BM cells was assessed by FACS analysis. As shown in Figure 2C and D, the percentage of $CD45.1^+$ cells in total $CD11b^+$ leukocytes (top left quadrant of FACS plot) of the animals was between 90% and 100%. The overall percentage of CD45 cells in either blood (~90% $CD45^+$ cells) as well as in BM (~75% $CD45^+$ cells) was comparable in transduced with LV-miR-126 as well as LV-control, indicating that the BM of the trans-

planted animals was successfully reconstituted after whole body irradiation and subsequent bone marrow transplantation. A small trace of CD45.2$^+$ cells was still found in blood as well as bone marrow of the transplanted mice, however the presence of a high percentage of CD45.1$^+$ cells in blood as well as BM indicates that these cells have been responsible for the repopulation of the bone marrow compartment.

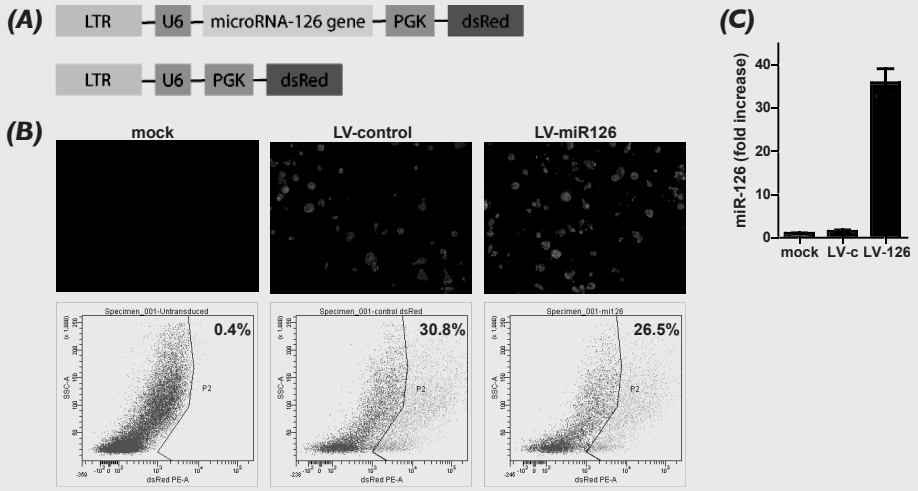

Figure 1. Overexpression of miR-126 in bone marrow in vitro

(A) Schematic representation of either the miR-126 over expression virus (LV-126, upper scheme) or the control virus (LV-C, lower scheme). The DNA encoding the precursor RNA hairpin of miR-126 was cloned behind the constitutive U6 promoter. Both viruses contain the dsRED reporter gene behind a PGK promoter to be able to detect transduced cells with a fluorescence microscope. ***(B)*** Fluorescence microscopic images show dsRED positivity in bone marrow cells indicating successful transduction by LC-V and LV-126. Non-transduced (mock) cells are shown in left panel as a negative control. FACS plots depict the percentage of dsRED positive cells of total number of cells. ***(C)*** qRT-PCR on RNA isolated from total BM cells (n=3) demonstrates up regulation of mir-126 expression in LV-126 transduced cells when compared to cells transduced with LV-C or non-transduced cells (mock).

Quantitative PCR on total RNA isolated from BM suspension cells revealed a 8.2-fold increase in miR-126 expression levels in the BM cells from the animals transplanted with miR-126 over expressing BM (LV-126) as compared to animals transplanted with BM cells transduced cells with control virus (LV-C) and BM from CD45.1$^+$ donor animals $P<0.0001$, n=11, Figure 2E).

Sidestream dark field imaging can be used to show functional vessels in an angiogenic matrigel plug in vivo

Seven weeks after BM transplantation, matrigel plugs containing recombinant

SDF and VEGF were inserted in the flank of mice. After 7 days, mice were anesthetized and the skin was opened to visualize the vasculature in the implant by sidestream dark field camera. Figure 3B shows a still of one of the movies that showed active flow of red blood cells through the vessels that had grown into the angiogenic plugs. The movies confirmed neovascularization of the matrigel SDF-1/VEGF supplemented plugs.

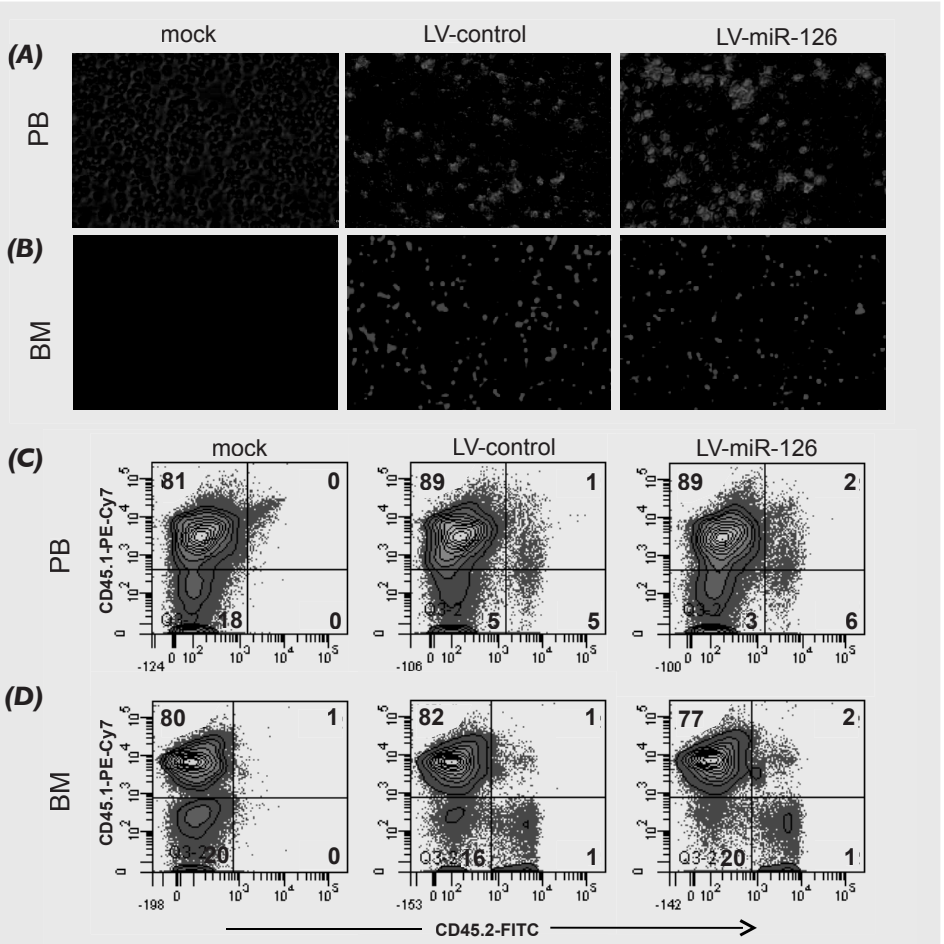

Figure 2A-D. Overexpression of miR-126 in blood and bone marrow cells in vitro

(A) Microscopic images of blood smears show clear dsRED positive blood cells in animals transduced with either LV-C or LC-126 when compared to cells of non-transduced animals. ***(B)*** Microscopic images of bone marrow cells show clear dsRED positive blood cells in animals transduced with either LV-C or LC-126 when compared to cells of non-transduced animals. ***(C,D)*** Representative picture for FACS plots that demonstrate successful chimerism on CD45.1 (donor) and CD45.2 (acceptor) levels in blood and bone marrow cells. All animals were over 95% chimeric.

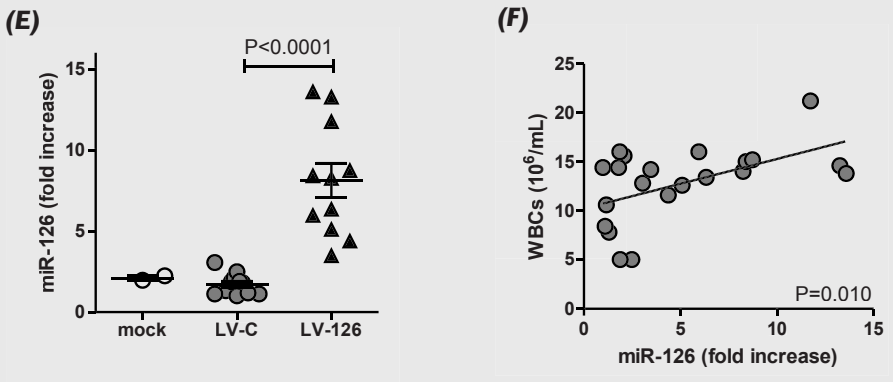

Figure 2E,F. Overexpression of miR-126 in blood and bone marrow cells in vitro

(E) qRT-PCR on bone marrow cells (n=10-12) demonstrates up regulation of mir-126 in LV-126 transduced when compared with cells transduced with LV-C or non-transduced cells (mock). ***(F)*** Overexpression of miR-126 leads to increased levels of white blood cells in the periphery. A correlation was found between the levels of white blood cells in the periphery and the expression levels of miR-126 in the bone marrow (n=10-12).

Overexpression of miR-126 in the hematopoietic compartment leads to increased neovascularization in angiogenic matrigel plugs

To quantify the impact of hematopoietic overexpression of miR-126 on neovascularization microscopic images were taken from both sides of the angiogenic plugs that had been implanted for 7 days. Figure 3A shows a panel of representative microscopic images of the plugs and the arrowheads indicate the blood filled microvascular structures that had grown into the matrigel plugs following implantation. The number of ingrowth vessels per matrigel implant was determined by counting the vessels present on the photographs taken from both sides of the matrigel plug. A nearly significant up regulation was found in the number of vessels of the animals transplanted with miR-126 as compared to the control-transplanted animals (Figure 3C, n=10-12, P=0.053). However, when the images were digitalized with imageJ, quantification of the total microvascular surface revealed a significant 1.4-fold increase in vascularization confirming augmented neovascularisation in the mice overexpressing mir-126 in the hematopoietic compartment (Figure 3D, n=10-12, P<0.02). Finally, we observed a near significant direct correlation between the total length of the vessels per mice and the levels of BM miR-126 expression (Figure 3E, n=22, P=0.054). Our data indicate that over expression of miR-126 in the hematopoietic compartment augments neovascularization of angiogenic plugs that were subcutaneously implanted in mice for 7 days.

Immunohistochemical analyses of the vascularized matrigel plugs demonstrated

a profound infiltration of dsRED positive cells directly adjacent to the infiltrating von Willebrand factor positive microvessels (Figure 4A and 4B), indicating that the BM-derived cells merely act as pericytes to support the ingrowth of endothelial cells into the matrigel plug. Further immunohistochemical analyses shows that there was co-expression of the dsRED positive with macrophage and monocyte markers such as F4/80 and Ly6c (Figure 4C-D, indicated with asterisks in Figure 4E-F). These results may suggest that leukocytes that are derived from the bone marrow may create an angiogenic environment in the plug into which endothelial cells can sprout and be subsequently supported by pericytic function

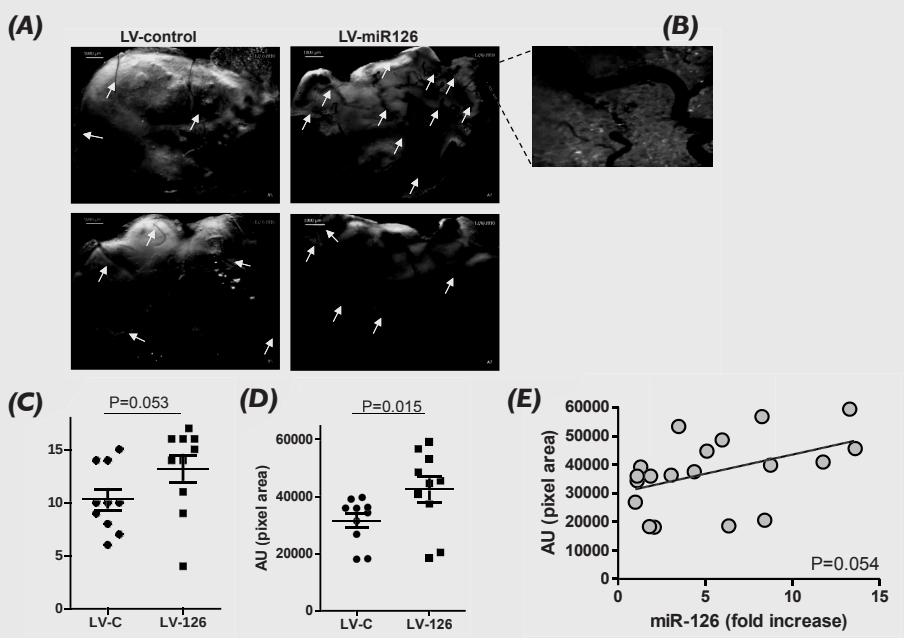

Figure 3. Overexpression of miR-126 in BM cells leads to increased neovascularization

(A) Representative microscopic images of both sides of matrigel plugs seven days after implantation in the flank of mice. Right panels show matrigel plugs obtained from animals transplanted with bone marrow cells transduced with LV-126, left panels demonstrate control animals. Arrowheads show penetrated vessels. ***(B)*** Movies were shot with SDF-camera 7 days after insertion of the matrigel plug in to the flank of the mice. A still of one of the movies is shown that displayed the active flow of red blood cells through the vessels that had grown into the angiogenic plugs. ***(C)*** Number of vessels was counted per plug and plotted. Animals transplanted with bone marrow cells that were transduced with LV-126 show higher number of vessels (n=10-12). ***(D)*** Vessels were 'digitalized' and the total length (in pixel area) was calculated in imageJ. LV-126-animals demonstrated a significant higher total pixel area as compared to LV-C-animals (n=10-12). ***(E)*** A correlation was found between the total length of vessels in the plug and the expression levels of miR-126 in the bone marrow (n=10-12).

of the bone marrow cells.

Overexpression of miR-126 in bone marrow leads to increased levels of white blood cells in the circulation

To begin to determine whether over expression of miR-126 in the bone marrow augmented matrigel vascularization due to elevated leukocyte infiltration we assessed the distribution of circulating cells in the periphery by sysmex and FACS analyse. Indeed we observed that the total number of white blood cells per mL blood was significantly correlated with the miR-126 expression levels found in the bone marrow (WBC, Figure 2F, n=22, $p<0.01$). FACS analysis of the various circulating hematopoietic subsets was performed to establish whether elevated numbers of circulating WBC was caused by a general mobilization effect of all WBCs or whether overexpression of miR-126 affected the differentiation of specific populations of the white blood cells, such as granulocytes, eosinophils and monocytes. When we plotted the number of cells per mL we observed that mice that overexpressed miR-126 displayed a higher number of circulating WBC and that the mobilizing effect of placing a VEGF/SDF-1 rich plug was also only observed in the mice overexpressing miR-126 in the hematopoietic compartment. In particular, in monocytes this mobilizing effect reached statistical significance (Table 1, n=22). When the number of the circulating subset cells are as percentage of total number of white blood cells we did not observe selective enrichments for any of the subpopulations indicating that, when assessed in the periphery, overexpression of miR-126 did not have a major impact on hematopoietic lineage specification (Table 1, n=22).

Discussion

Various studies have demonstrated a role for endothelial miR-126 in angiogenesis and the mobilization of vasculogenic cells [18-20]. However, significant expression of this miR has been demonstrated in circulating hematopoietic cells and platelets [26-29]. In this study we have set out to determine whether miR-126 expression in the hematopoietic compartment can also affect neovascularisation. As previously used methods to silence miR-126 with, for example, antagomirs [17, 18] act systemically and do not allow to assess the function of hematopoietic miR-126 independently from that of endothelial miR-126 we have chosen to overexpress miR-126 in the hematopoietic compartment specifically. To that end, we transduced lineage-depleted BM from donor mice with a lentiviral vector that drives the expression of miR-126 by a constitutive U6 promoter. Subsequently these BM cells were used to reconstitute the BM of lethally irradiated mice. After eight weeks chimeric mice displayed an over 8-fold elevation of miR-126 expression in the BM and consequently in all hematopoietic cells while we observed no major changes in weight, behavior

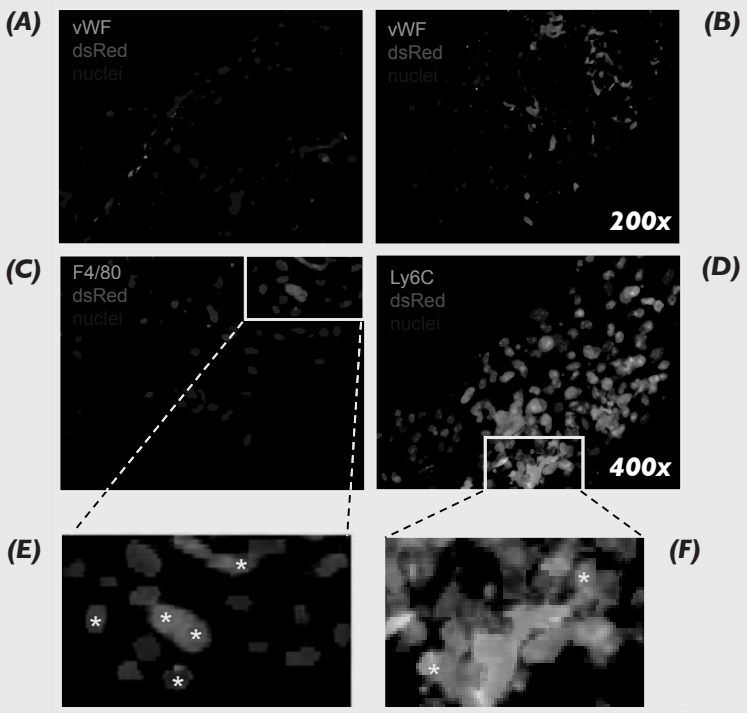

Figure 4. Matrigel plug slides were stained with endothelial marker von Willebrand Factor, macrophage marker F4/80 or monocytic marker Ly6C

(A,B) Microscopic images shows a positive staining for von Willebrand Factor adjacent to dsRED positive cells indicating a percytic role for infiltrated bone marrow. Macroscopic images show double positive staining for dsRED as well as ***(C)*** Ly6C and ***(D)*** F4/80 suggesting that monocytes and macrophages in the plug are derived from the bone marrow. Magnification of images show distinct double positive cells for dsRED and for ***(E)*** Ly6C and ***(F)*** F4/80 indicated with asterisks.

other health indicators. To begin to elucidate the relevance of hematopoietically expressed miR-126 to neovascularization we implanted VEGF and SDF-1 rich matrigel plugs subcutaneously in these mice. Surprisingly, after seven days, both image analysis as well as visual inspection of the matrigel plugs with a sidestream dark field camera revealed an almost two fold increase in vascularization of the plugs in mice overexpressing miR-126 in the hematopoietic cells.

Over expression of miR-126 in the transplanted bone marrow also led to an up regulation of total white blood cells in the circulation as compared to animals that were transplanted with control BM cells. As we showed this was a general effect on mobilization and this effect was directly related to the BM miR-126 levels in the tested mice, we concluded that miR-126 in the hematopoietic compartment serves to modulate VEFG and/or SDF-1 driven mobilization of BM cells to the

Table 1. Overview of FACS results and the correlation per population of leukocytes in the periphery

Population	% of cells (miR-126 versus control)	# of cells/mL (miR-126 versus control)	Correlation with miR-126 in BM
Lymphoblasts	Not upregulated	Upregulated (ns)	P<0.10
Lymphocytes	Not upregulated	Upregulated (ns)	P<0.15
Activated T-cells	Not upregulated	Not upregulated	-
Eosinophils	Not upregulated	Upregulated (P<0.05)	P<0.05
Granulocytes	Not upregulated	Upregulated (P<0.05)	P<0.10
Monocytes	Not upregulated	Upregulated (P<0.05)	P<0.02
NK-cells	Not upregulated	Upregulated (P<0.001)	P<0.17

periphery. Interestingly, a recent paper demonstrated that the general mobilizer GCSF promotes the accumulation of miR-126 loaded microvesicles in the BM. Subsequently, these vesicles are proposed to fuse to BM progenitor, stromal and endothelial cells and reduce the expression of vascular cell adhesion molecule-1 (VCAM-1) by these cells in a miR-126 dependent fashion. As VCAM-1 is critical to the retention of hematopoietic stem and progenitor cells in the BM, loss of VCAM-1 expression would drive the mobilization of these cells to the periphery [34]. As granulocytes are the main target of GCSF and express relatively high levels of miR-126 (van Solingen et al, unpublished data) and granulocytes might also be responsive to both VEGF and SDF-1 increasing miR-126 levels in these mobilizing vesicles could provide an explanation for the general mobilizing effect we observed when implanting VEGF/SDF-1 rich matrigel plugs into the miR-126 overexpressing mice. Alternatively, overexpression of miR-126 in BM cells could inhibit the expression of miR-126 target genes PI3KR2 and SPRED-1[19, 20] two major negative mediators of the VEGF receptor and CXCR4 signaling. Therefore, overexpression of miR-126 may render the hematopoietic cells more responsive to VEGF and SDF-1 gradients and lead to an increased migratory capacity.

Increased recruitment of leukocyte subsets to the matrigel plug could also contribute to the observed increase in vascularization. For instance, granulocytic neutrophils are able to infiltrate a foreign body [35] and are major sources of matrix metalloprotease type 9 (MMP-9) a protein that, by breaking down the extracellular matrix, functionally contributes to angiogenesis [36]. Furthermore, the secondary granules of eosinophils contain large amounts of preformed VEGF [37] and when these cells are in direct contact with ECs they enhance EC

proliferation and as such, angiogenesis [38] also, high levels of SDF-1 and VEGF have been demonstrated to be chemotactic for monocytes [39, 40] and upon extravasation into a foreign body these cells can differentiate into macrophages that then can support angiogenesis. Subsequently, macrophage can contribute to angiogenesis by producing angiogenic factors like VEGF and TGFβ [41, 42] or facilitating anastomosis of the forming vessels [43]. Taken together, the elevated levels of any of these cellular populations may affect the number and length of the vessels in the angiogenic plugs.

We also observed that the vascularization of the matrigel implants is closely associated with a profound infiltration of BM derived cells that organize alongside the von Willebrand factor-positive microvasculature suggesting that these cells fulfill a perivascular role supporting the ingrowth of the vessels. A mere higher number of possible pericytes that can invade the plug may also augment the vascular in growth either via stabilizing the growing vessels or in a paracrine way that may involve the secretion of miR-126 containing exosomes that can be taken up by ECs [30]. Whether such pro-angiogenic exosomes are also elevated in the circulation in the miR-126 overexpressing mice is the topic of current investigations.

In conclusion, overexpression of miR-126 in the hematopoietic compartment leads to increased neovascularization and has a general augmenting effect on the mobilization of of BM-derived to the peripheral circulation. While the exact mechanism by which hematopoietic miR-126 increases neovascularization is yet unclear we demonstrates a novel role for hematopoietic miR-126 in vascular homeostasis.

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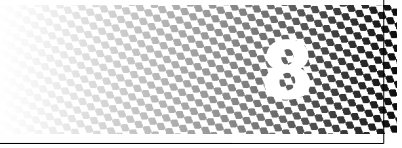
CHAPTER 8

General discussion and summary



In this thesis, studies are described in which the role of miRNA-126 in vascular biology is investigated. While initially considered to be exclusively restricted to endothelial cells, it is now appreciated that significant levels of miRNA-126 can be found in platelets, epithelial cells and circulating hematopoietic cells [1-3]. Moreover, 'free-floating' miRNA-126 molecules have now been detected in the periphery [4]. The presence of miRNAs in the circulation may underline the importance of these molecules as potential biomarkers. While native RNA molecules are rapidly degraded in plasma, miRNAs display exceptional stability in the circulation due to their association with argonaute protein [4], high density lipoprotein (HDL) [5] or their inclusion into exosomes or microparticles [6]. Considering the tissue-specific nature of miRNAs and their stable presence in the periphery, circulating miRNAs may give a reflection of the health status of tissue connected to the vascular bed. So far, only circumstantial evidence has been reported, the levels of miRNA-126 might be an indicator of ongoing endothelial injury in the body during cardiovascular disease [7-9]. To date, an exact role for these free-floating molecules has not been established, however, they might become increasingly important to serve as new biomarkers.

A functional role of cellular miRNA-126 was not established until the binding of miRNA-126 to the 3' untranslated-region (UTR) of vascular cell adhesion molecule-1 (VCAM-1) was revealed. The binding of miRNA-126 subsequently led to the interference with the function of VCAM-1 [10]. Soon after, targeted deletion of miRNA-126 in endothelial cells, by either genetic deletion or use of cholesterol-conjugated antisense oligonucleotides (antagomir) showed the facilitating role of miRNA-126 in vascular development and ischemia-induced angiogenesis [11-13].

To date, a limited number of pathways has been associated with the functional targeting of mRNAs by endothelial miRNA-126. It has been determined that miRNA-126 is an important mediator in vascular homeostasis by targeting key proteins involved in angiogenesis, vasculogenesis and inflammation [10-15]. This chapter summarizes and discusses the contribution of the research described in this thesis to the understanding of the role of miRNA-126 in vascular homeostasis.

Silencing of miRNAs in vitro and in vivo

Previously, miRNA-126 was found to be expressed in the heart and blood vessels of zebrafish embryos [16]. We demonstrate in Chapter 3 that miRNA-126 is specifically expressed in endothelial cells of capillaries and arterioles *in vivo*. To gain insight into a possible regulatory role for this miRNA in neovascularization, we aimed to obtain a specific miRNA-126 inhibitor for conditional silencing of miRNA-126 in the vascular endothelium. Several different methods to

silence miRNAs *in vitro* and *in vivo* have been established. (1) Locked nucleic acid (LNA)-modified oligonucleotides for the efficient and long lasting silencing of miRNA-122 function in the liver of mice and non-human primates [17, 18]. This LNA-based method is momentarily being evaluated in the first human clinical trials of miRNA inhibition (Santaris Pharma, ClinicalTrials.gov). (2) Chemically modified and cholesterol-conjugated antisense oligonucleotides (antagomirs) bind to miRNAs and block their function in multiple tissues after tail vein injection [19]. The use of antagomirs has been extensively studied and has been widely used in a variety of *in vitro* and *in vivo* studies. Although both the antagomir and LNA-modified oligonucleotides can effectively target a miRNA, the LNA-modified chemistries require lower doses based on their higher binding affinity. (3) Recently, the use of 8-mer LNA-anti-miR has been described [20]. The 8-mer fully modified LNA-oligomer is directed against the seed region of a miRNA and can additionally be functional for targeting multiple miRNA family members at once. Gene expression analysis indicates that the shorter LNA-containing chemistries do not induce off-target gene expression changes as opposed to the longer LNA-anti-miRs and antagomirs [18]. This off-target targeting is probably due to the fact that when too many RNA-oligonucleotides are incorporated into a cell, all cytoplasmatic RNA binding proteins, including the RNA Induced Silencing Complex, present might be saturated with RNA molecules. This oversaturation may then lead to an overexpression of all miRNA-regulated proteins. Therefore, to be able to analyze the effects of antagomirs properly, we have used a control RNA analog of identical composition and length, but with a random sequence (scramblemir) in all *in vitro* and *in vivo* experiments.

So, although the possibility of off-target targeting exists while using antagomirs, we chose to use this method due to its high potential to inhibit miRNA expression and function in almost all organs [19]. Furthermore, as cholesterol uptake is a salient feature shared by virtually all cells, including endothelial cells, we designed an antagomir directed to miRNA-126. In mice treated with antagomir-126, we validated the specificity of miRNA-126 silencing by quantifying the level of mature miRNA-126 in total lung tissue. This was based on previous observations that, of all organs profiled for miRNA-expression by extensive cloning and sequencing, the lung displays the highest levels of miRNA-126 expression [13, 21, 22]. We observed that 10 days after administration of a single, 1.0 mg injection of antagomir-126 per mouse, was sufficient to almost completely abrogate miRNA-126 expression in lung tissue, whereas miRNA-126 remained readily detectable in the control scramblemir groups. As a single injection of 1.0 mg is low compared to the reported dose needed for silencing of the liver specific miRNA-122 (3 consecutive injections of 2 mg per mouse), we conclude that endothelial cells readily take up antagomirs from the circulation and may therefore be highly useful for studying endothelial miRNA-function *in vivo*. In addition, the data described in **Chapters 3, 4 and 5** supports the poten-

tial therapeutic use of antagomir-based approaches for conditional silencing of miRNAs in the endothelium *in vivo*.

MiRNA-126 and angiogenesis

Endothelial cells are key mediators in vascular integrity and, as such, the maintenance of the endothelial cell layer in the periphery is of high relevance. Pathological conditions such as tissue ischemia and inflammation lead to the activation of endothelial cells and ultimately to endothelial cell apoptosis [23]. The loss of endothelial function is a hall mark of vascular disease and is an early event in development of atherosclerosis and, furthermore, shown to be predictive of future adverse cardiovascular events. To keep the endothelium healthy is, therefore, a crucial aspect for vascular integrity and mechanisms, like angiogenesis and vasculogenesis, that help to overcome endothelial cell dysfunction have been intensively studied. The execution of these tightly regulated programs depends on a vast array of factors whose identification has been a prime focus of cardiovascular research in the last two decades [24]. Since the role for miRNAs in gene regulation has been widely acknowledged and evidence supporting a role for endothelial miRNAs in the control of neovascularization has been provided for a high number of miRNAs [25-31], we have studied the role for endothelial miRNA-126 in vascular integrity.

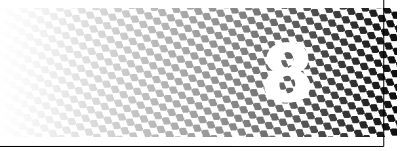
In **Chapter 2** we demonstrate data that supports a role for miRNA-126 in an angiogenic response induced by ischemia. To investigate the role of miRNA-126 in neovascularization, we injected C57Bl/6 WT mice in the tail vein with either antagomir-126 or scramblemir. Subsequently, we subjected these animals to unilateral hind limb ischemia by electrocoagulation of the left common femoral artery. Using this model we were able to assess the hypoxia induced angiogenic response in the distal calf muscle [32]. The mice treated with antagomir-126 showed a strongly reduced capillary density in gastrocnemius muscle as compared to the scramblemir-treated mice. Likewise, an impaired *ex vivo* outgrowth of endothelial cells from aortic sections of miRNA-126-silenced mice was observed. Surprisingly, *in vitro* experiments designed to assess the relatively short term effects of antagomir-126 silencing in human umbilical vein endothelial cells (HUVEC) revealed no differences. The effects of miRNA-126 on angiogenesis likely involve mechanisms operational in endothelial cells in the *in vivo* context.

Two other studies reported that targeted deletion of miRNA-126 in mice and zebrafish impairs angiogenesis, likely through dysregulation of Sprouty-related Ena/VASP homology 1 domain containing protein (Spred-1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2, p85-β) expression [11, 13]. Spred-1 and PIK3R2 are actively involved in the negative regulation of vascular endothelial growth factor (VEGF) signaling [33] and are both predicted targets of miRNA-126 (http://www.targetscan.org). Consequently, upregulation of miRNA-126 would thus facilitate angiogenesis by reducing the expression of

both repressors of VEGF signaling, whereas low levels of miRNA-126 would be associated with elevation of Spred-1 or PIK3R2 and repress angiogenic signaling. This makes them likely targets to be associated with diminished capacity of endothelial cells to overcome ischemia-induced angiogenesis as demonstrated in our studies.

The angiogenic potential of miRNA-126 was underlined in a different study, which is described in **Chapter 7**. Here, we demonstrate that the angiogenic potential of miRNA-126 may reach beyond its presence in endothelial cells, supporting the potential therapeutic use of this miRNA. We over-expressed the endothelial, pro-angiogenic miRNA-126 in bone marrow cells, which subsequently were used to successfully reconstitute the bone marrow of lethally irradiated mice. Over-expression of miRNA-126 in the transplanted bone marrow lead to an upregulation of white blood cells in the circulation when compared to animals that were transplanted with bone marrow cells transduced with control lentiviral particles. This upregulation of white blood cells coincided with expression levels of miRNA-126 in the bone marrow. Furthermore, the animals that were transplanted with miRNA-126 over-expressing bone marrow cells showed increased capillary infiltration of an angiogenic matrigel plug, which was inserted in the flank of mice. The injected matrigel plug contained high levels of recombinant VEGF and stromal cell-derived factor-1 (SDF-1) that is released slowly into the blood stream. Over-expression of miRNA-126 in bone marrow cells will inhibit the translation of PI3KR2 and SPRED-1 [11, 13] that are two major negative mediators of the VEGF-receptor and SDF-1/CXCR4 signaling pathways in endothelial cells. Therefore, these miRNA-126-transduced cells may have become more responsive to a gradient of VEGF and SDF-1 in the periphery, leading to an increased migratory capacity as compared to cells transduced with a control virus.

It has been described that leukocytes can contribute to angiogenesis in a variety of molecular mechanisms [19, 34-41]. Therefore, the variable representations of leukocytes that are upregulated in the circulation might explain the increased number and length of the vessels in the angiogenic plug. Moreover, endothelial cells that are proximal to the matrigel plug are exposed to high local concentrations of VEGF and SDF-1, which could accelerate their invasion of the plug after insertion. Alongside this process, bone marrow cells will enter the angiogenic plug and support the endothelial cells to vascularize the plug, by perivascular stabilization of the newly formed capillaries. Also, invading, (perivascular) bone marrow cells have the possibility to release microvesicles that contain (elevated) levels of miRNA-126 that can be taken up by endothelial cells [42]. After uptake, miRNA-126 levels will increase in the angiogenic endothelial cells and this leads to an increased potential of capillary formation [12]. Indeed, we found a number of ds-Red-positive cells in the matrigel plug that align vessel-like structures (shown by a positive staining for endothelial cell-marker



von Willebrandfactor), indicating that these act as perivascular cells that support capillary ingrowth. Since we also saw an increase of total white blood cells in the circulation, one can speculate on the miRNA-126 based mechanisms that underlie these findings. The study described in **Chapter 7** underlines the angiogenic capacity of miRNA-126 and that this miRNA might be used in pro-angiogenic therapies.

MiRNA-126 and vasculogenesis

The loss of miRNA-126, either in knockout models or mediated by treatment with antagomirs, leads to structural impairment of the vascular bed [11-13]. In **Chapter 4**, we provide evidence for a vasculogenic role for miRNA-126 by regulating the mobilization of endothelial progenitor cells via the release of chemokine SDF-1 from ischemic endothelial cells. *In vitro*, the increased secretion of SDF-1 upon silencing of miRNA-126 was sufficient to stimulate the migration of human CD34+ stem cells. In mice, however, systemic silencing with a single tail vein injection of antagomir-126 was not sufficient to raise the levels of circulating murine Sca-1+/Lin- progenitor cells. However, in combination with the ligation of the femoral artery, we demonstrated an increase in circulating Sca-1+/Lin- cells following miRNA-126 silencing, strongly suggesting that tissue ischemia is needed to reveal the regulatory role of miRNA-126 *in vivo*. The elevated numbers of circulating Sca-1+/Lin- cells in the antagomir-treated animals are the result of SDF-1 mediated mobilization of these cells following ischemia. This is supported by the fact that SDF-1-protein expression is also up regulated in the endothelial cells in the ischemic tissue as well as in the peripheral circulation. Interestingly, interaction between miRNA-126 and SDF-1 has previously been shown to increase miRNA-126 uptake of endothelial cell-derived apoptotic bodies by endothelial cells. This resulted in increased SDF-1 expression through inhibition of Regulator of G-protein signaling 16 (RGS16) [43]. In contrast, our studies implicate that the abrogation of miRNA-126 is associated with increased expression of SDF-1, suggesting that miRNAs could serve as a biological switch, with the response magnitude of biological pathways being dependent on the context and source of the external stimulus.

Since the functional repression of miRNA-126 leads to impaired angiogenesis in one hand, and to increased vasculogenesis in the other, it has been proposed that this elevation of stem cells, upon ischemia, can be seen as a vascular rescue mechanism to overcome impaired angiogenesis after losing expression of endothelial miRNA-126. This suggestion underlines the importance of miRNA-126 in vascular homeostasis.

MiRNA-126 and vascular inflammation

It is well established that systemic inflammation leads to endothelial cell activation and subsequent leukocyte recruitment [44]. In contrast, knowledge

on how distinct microvascular endothelial cells subsets respond molecularly to inflammatory stimuli is almost non-existent in acute and chronic renal diseases, like glomerulonephritis, vasculitis, and ischemia related acute renal failure. Since miRNA-126 is a central regulator of endothelial cell function and homeostasis, it is likely that miRNA-126 might influence the microvascular endothelial cell response to inflammatory stimuli in the kidney. In **Chapter 5** we describe that the expression of miRNA-126 in the glomerular microvascular compartment is a governing factor in the control of VCAM-1 protein expression in response to acute inflammation. High miRNA-126 levels in the glomerular compartment coincided with low VCAM-1 protein expression. Furthermore, in arterioles low miRNA-126 levels were associated with high VCAM-1 protein levels. Previously, the relation between miRNA-126 and VCAM-1 has been investigated in HUVEC, predominantly in conditions in which miRNA-126 was exogenously over-expressed [10]. We showed that *in vivo* target deletion of miRNA-126 by antagomir-126 injection resulted in increased VCAM-1 protein production in all renal microvascular segments in response to a challenge with tumor necrosis factor-alpha (TNFα).

The role of miRNA-126 in response to inflammation in the kidney and hypoxia is of high interest in the search for therapeutic targets. Our discovery that miRNA-126 is a governing factor of VCAM-1 expression in the heterogenic response of the renal vascular bed to an inflammatory stimulus provides an interesting link between miRNA-126 and the inflammatory response, via its target VCAM-1. However, our finding that antagomir-126 administration did not result in increased blood flow recovery after femoral artery ligation when compared to scramblemir-treated animals shows that regulation of VCAM-1 expression by miRNA-126 is unlikely to be a rate-limiting factor for *in vivo* arteriogenesis.

MiRNA-126 in the circulation

Endothelial cells, circulating cells and platelets can be considered as sources that can release miRNA-126 into the periphery. To date, the exact quantitative contribution of each cell type has not been elucidated. Nonetheless, it is likely that all three cell types might release miRNAs into the circulation during their life time. In **Chapter 6** we demonstrate that platelets store significant amounts of miRNA-126 and that upon platelet-activation miRNA-126 is secreted into the surrounding plasma *in vitro*. In parallel with those findings, we show that when the activation of platelets is blocked by aspirin, the release of miRNA-126 by platelets is hampered. These data were underlined by a patient study where we investigated the relation between plasma levels of miR-126 and *in vivo* platelet activation. We studied miRNA-126 levels in plasma of patients with type 2 diabetes mellitus, who had participated in a prospective, randomized, placebo-controlled, double-blind, crossover study, in which patients were assigned to a period of aspirin-treatment or placebo [45, 46]. As shown previously [47], only

50% of the aspirin-treated patients were considered 'aspirin-responders', defined by a decrease in the platelet-activation marker, soluble P-selectin (the other 50% were so called 'non-responders'). When discriminating for responders and non-responders a significant difference between the miRNA-126 plasma levels was measured between both groups.

At present, no molecular mechanisms are linked to circulating miRNAs (including miRNA-126) and cardiovascular disease. Whether the source of circulating miRNA-126 is endothelium, circulating cells or platelets, the established involvement of miRNA-126 in vascular biology will make it a key component to investigate in patients with cardiovascular risk factors. To date, the use of circulating miRNAs as predictive and/or monitory biomarkers is still in an early phase. However, in the future a spectrum of circulating miRNAs, miRNAs in urine samples [48] or other bodily fluids [49] will be highly informative about the disease status of a patient in the clinic.

Currently no clinical trials to enhance or antagonize miRNA-126 function are, to our knowledge, undertaken. Nevertheless, subjects with cardiovascular risk factors have decreased levels of miRNA-126 in their plasma [7, 8, 50], suggesting that mechanisms whereby miRNA-126 could be administered to these subjects could be an effective modality in the prevention of cardiovascular disease.

Future perspectives

As has been shown in this thesis, miRNA-126 is abundantly expressed in endothelial cells, circulating cells and platelets, and plays an important role in neovascularisation by regulating the expression of various proteins involved in driving both angiogenesis and vasculogenesis [11-13, 15, 43]. Although the role of angiogenic miRNAs such as miRNA-126 in vascular maintenance and repair is now well-established, surprisingly little is known about the molecular mechanisms underlying the regulation of these regulators. So far it has been demonstrated that binding of Ets-1 or Ets-2 to the EBS and induction of flow are needed to govern the expression of the EGFL7/miRNA-126 gene [51, 52]. Furthermore, miRNA-126 levels can be increased in endothelial cells as a result of microvesicle endocytosis [43]. In contrast, extracellular factors that lead to endothelial cell activation and also potentially modulate miRNA-126 levels are currently unknown. For instance, cytokines like vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNFα) that mediate the activation of endothelial cells, lead to an upregulation of a distinct subset of miRNAs, but appear to have no impact on the expression of miRNA-126 [29, 53]. In order to fully understand the role of miRNAs in neovascularisation and inflammation it is of particular interest to unravel the molecular mechanisms that modulate the expression of these miRNAs.

To study the impact of extracellular factors on endothelial miRNA-expression and explore the intracellular mechanisms that control the distribution of

miRNAs after endothelial cell activation, future studies could seek to:

1) Identify extracellular factors that modulate the expression of miRNA-126 in endothelial cells

To gain insight into the regulation of miRNA-126 expression in endothelial cells, the endothelial response program can be triggered by mechanic (shear) stress, oxidative stress or a variety of soluble growth factors. Recent work has established that the absence of pulsatile flow leads to severely diminished levels of miRNA-126 in zebrafish [52]. Furthermore, preliminary studies have identified that continuous flow (15 dyne/cm^2 for 4 days) and hypoxia (2.5% O_2 for 6-48 hours) on HUVEC leads to increased miRNA-126 expression (Van Solingen et al, unpublished data). Varying parameters such as the time the cells are exposed to flow, flow velocity, and the degree of hypoxia will likely provide key insight into conditions that attenuate miRNA-126 levels.

Since stimulation of endothethelial cells with VEGF or TNFα has a minimal impact on miRNA-126 expression [29, 53], HUVEC can be exposed to additional pro-angiogenic cytokines such as TGFβ, FGF, IGF-1, angiopoietin-1 or -2 to determine whether these stimuli modulate miRNA-126 levels.

2) Unravel signal transduction pathways associated with differential expression of angiogenic miRNAs in endothelial cells

It has been demonstrated that miRNA biogenesis can be regulated a) at the level of transcription; b) during miRNA-processing; c) by altering stability; and d) through activation of secondary signalling elements and downstream transcription factors [54, 55]. Preliminary studies reveal that the stimulation of endothelial cells with VEGF leads to a striking up regulation of several miRNAs (miRNA-16, -155, the miRNA-cluster -17~92, Suarez et al, unpublished data) that are implicated in the control of angiogenesis and/or endothelial cell proliferation [53, 56, 57]. It would be, therefore, of high interest to determine on what level pro-angiogenic miRNAs, including miRNAs, are regulated.

To determine if the observed increase of VEGF-induced miRNAs is the result of transcriptional regulation, expression levels of primary miRNA transcripts can be examined.

To assess to which degree miRNA expression is regulated at the level of pre-existing primary transcript processing, proteins involved in this processing (including Dicer, Ago2 and exportin5 [55]) can be silenced using a siRNA approach. Upon this knock down endothelial cells can be stimulated and the expression of the mature miRNA forms.

Treatment of endothelial cells with VEGF may influence the miRNA stability. The generation of new miRNA transcripts can be arrested using actinomycin D. Subsequently, endothelial cells can be treated with VEGF and the miRNA primary transcript expression profile can be assessed.

The contribution of the VEGF-regulated signalling pathway, as well as the participation of downstream transcription factors to the regulation of miRNAs can be tested. A siRNA approach for the identification of proteins that regulate the expression of a miRNA of interest can be used to study the importance of this aspect of miRNA-biogenesis.

The previous two objectives will discern the transcriptional and post-transcriptional regulation of miRNAs in endothelial cells after stimulation with VEGF. Finally it will be highly interesting to study the possible therapeutic potential of angiogenic miRNAs. Therefore the determination of a functional role of attenuated miRNAs in angiogenesis can be tested by modulating their expression levels *in vitro* as well as *in vivo* by means of treatment with either miRNA-mimics or antisense oligonucleotides

Conclusively, identifying the mechanisms that regulate the expression of, among others, the pro-angiogenic miRNA-126 could provide critical insight into the role of this miRNA in regulating endothelial cell homeostasis and in particular, the response to injury to the endothelium. Furthermore, the identification of factors that trigger angiogenic miRNA expression could potentially lead to the generation of novel therapeutic approaches to maintain a healthy endothelium.

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Nederlandse samenvatting

Achtergrond

In dit proefschrift zijn studies beschreven waarin de rol van microRNA-126 in de biologie van de vaatwand is onderzocht. Toen dit project startte in november 2006 was het enige dat was gerapporteerd over deze microRNA dat het in grote hoeveelheden voorkwam in de cellen die de vaatwand bedekken (het endotheel). Verder bleek microRNA-126 erg goed geconserveerd te zijn tussen verschillende diersoorten, wat een indicator kan zijn van een relevante rol in de biologie.

Aanvankelijk werd gedacht dat microRNA-126 alleen maar aanwezig was in het endotheel, maar inmiddels is duidelijk geworden dat deze microRNA ook voorkomt in het epitheel van de longen, circulerende hematopoietische (stam)cellen en bloedplaatjes. Heel recentelijk zijn zelfs 'losse' microRNA-126 moleculen gedetecteerd in het bloed. Een exacte rol voor deze vrij circulerende microRNAs is onduidelijk, maar aangezien hun kwantitatieve aanwezigheid is gerelateerd aan verscheidene risicofactoren van hart- en vaatziekten kunnen ze wellicht dienen als nieuwe biomarkers.

Een functionele rol voor microRNA-126 was onbekend totdat duidelijk werd dat microRNA-126 kan binden aan het messenger RNA (mRNA) van vascular cell adhesion molecule-1 (VCAM-1) en dat deze binding leidt tot een verandering van de functie van dit eiwit *in vitro*. Kort daarop werd aangetoond dat, wanneer de expressie van microRNA-126 werd voorkomen, zowel in muizen als zebravissen de vasculaire ontwikkeling sterk was verstoord. Mede uit onderzoek beschreven in dit proefschrift bleek dat de functionele remming van microRNA-126 in volwassen muizen ervoor zorgt, dat in deze dieren de angiogene respons op hypoxie volledig is gestoord.

Inmiddels is er een aantal vasculaire pathways bekend waarin microRNA-126 een belangrijke rol speelt. MicroRNA-126 kan, onder andere, binden aan mRNAs die een sleutelrol spelen in angiogenese, vasculogenese en een ontstekingsreactie. In **Hoofdstuk 2** wordt de huidige kennis over de biologie van microRNA-126 in detail besproken.

Het remmen van microRNAs in vitro en in vivo

In **Hoofdstuk 3** is beschreven dat microRNA-126 voornamelijk voorkomt in de endotheelcellen van de capillairen en arteriolen in de nier. Om de functionele rol van microRNA-126 te bestuderen hebben we gezocht naar een manier om specifiek deze microRNA te remmen. In de literatuur zijn verscheidene manieren beschreven om de functie van microRNAs te remmen. In de experimenten beschreven in dit proefschrift hebben wij voornamelijk gebruik gemaakt van zogenaamde antagomirs. Deze antagomirs hebben een nucleotidevolgorde complementair aan een target-microRNA. Tevens is de affiniteit met de complementaire RNA-structuren en de stabiliteit van de antagomirs *in vivo* verbeterd door toepassing van gemodificeerde nucleotiden. Van groot belang voor de werkzaamheid van antagomirs is de conjugatie van het oligonucleotide met een

cholesterolmolecuul. Dit molecuul zorgt ervoor dat vrijwel alle lichaamscellen de antagomirs gemakkelijk opnemen. Eenmaal in de cel inactiveert de antagomir het complementaire target-microRNA door het vormen van een stabiele hybride.

In **Hoofdstuk 3, 4 en 5** beschrijven we dat antagomir-126 een effectief hulpmiddel is om de functie van microRNA-126 in de endotheelcellen van muizen te achterhalen. De resultaten beschreven in deze hoofdstukken suggereren dat antagomirs wellicht ook voor therapeutische doeleinden zouden kunnen worden gebruikt.

MiRNA-126 en angiogenese

In **Hoofdstuk 3** beschrijven we dat microRNA-126 een rol speelt in de angiogene respons bij hypoxie. Muizen die behandeld werden met antagomir-126 en waarvan vervolgens de dijbeenslagader operatief was verwijderd, reageerden met een verminderde ingroei van capillairen naar het hypoxische gebied in de kuitspier. Verder was er minder endotheliale uitgroei te zien bij aorta's van de dieren, die *ex vivo* waren gegroeid in een petrischaaltje. Rond hetzelfde moment dat wij dit onderzoek ter publicatie aanboden, kwamen er twee publicaties uit die de rol van microRNA-126 in angiogenese beschreven en onze hypothesen aangaande de betrokken mechanismen bevestigden.

Spred-1 en PI3KR2 spelen een negatieve sleutelrol in groeifactorgeïnduceerde angiogenese. De synthese van deze eiwitten blijkt door microRNA-126 te worden geremd. Bij remming van microRNA-126, door het gebruik van antagomir-126, worden deze twee eiwitten in hoge mate tot expressie gebracht. Hierdoor is de vascular endothelial growth factor (VEGF) gemediëerde signaaltransductie geremd en blijft de angiogene respons van het endotheel achterwege. Uit deze studies blijkt dat microRNA-126 een facilitaire rol speelt bij angiogenese, mede door het in bedwang houden van de expressie van deze twee remmende eiwitten.

De angiogene rol van microRNA-126 wordt verder uit de doeken gedaan in **Hoofdstuk 7**, hier beschrijven we dat deze functie verder reikt dan alleen in endotheelcellen. In deze studie hebben we microRNA-126 tot overexpressie gebracht in beenmergcellen en vervolgens gebruikt voor een beenmergtransplantatie in subletaal bestraalde muizen. Het was mogelijk deze getransduceerde cellen te gebruiken om het beenmerg van deze bestraalde muizen te reconstitueren. Om vervolgens de bijdrage te bestuderen van het hematopoietische systeem aan neovascularisatie, hebben we bij deze dieren een angiogene matrigelplug onder de huid ingebracht. In de pluggen van muizen die beenmergcellen hadden ontvangen die microRNA-126 tot overexpressie brachten zagen we meer capillaire ingroei dan in de pluggen van muizen die beenmergcellen zonder extra microRNA-126 ingespoten hadden gekregen. Op het moment van schrijven hebben we nog geen exact mechanisme vastgesteld dat zorgt voor deze bijdrage, wel ontdekten we dat in de circulatie van de 'microRNA-126

overexpressie-dieren' veel meer witte bloedcellen waren terug te vinden. Aangezien microRNA-126 werkt op de pathways die te maken hebben met de mobilisatie van cellen vanuit het beenmerg, is het mogelijk dat de overexpressie van microRNA-126 ten grondslag ligt aan de vermeerdering van deze cellen in het bloed. Gezien de vele publicaties over de rol van witte bloedcellen bij neovascularisatie, is het tevens aannemelijk dat de verbeterde neovascularisatie van de plug is toe te schrijven aan de kwantitatieve bijdrage van de witte bloedcellen.

In de herfst van 2011 is er nog een tweede beenmergtransplantatieonderzoek in combinatie met een ischemie/reperfusie schademodel in de nieren gestart. Hopelijk kan deze studie nieuw inzicht verschaffen in de rol van microRNA-126 bij stamcelmobilisatie, neovascularisatie en schadeherstel.

MicroRNA-126 en vasculogenese

Zoals beschreven in **Hoofdstuk 3 en 7** is er een duidelijke rol voor microRNA-126 in angiogenese. Zowel in het endotheel als in beenmergcellen draagt de expressie van microRNA-126 bij aan de integriteit van het vasculaire systeem. In **Hoofdstuk 4** benadrukken we een andere rol van microRNA-126 in de vasculaire biologie. Hier laten we zien dat microRNA-126 ook een rol speelt bij de ischemie/reperfusie geassocieerde mobilisatie van stam- en progenitorcellen. Deze verhoging van stamcellen in het bloed ging gepaard met een verhoging van stromal cell-derived factor-1 (SDF-1) in het bloed, alsmede met een verhoging van SDF-1-expressie in de ischemische kuitspier. De rol van microRNA-126 bij de mobilisatie van stamcellen is ook onderzocht in een humane *in vitro* setting. Ook hier konden we aantonen dat er een link bestaat tussen het uitschakelen van microRNA-126 en de verhoogde expressie van SDF-1 in het endotheel.

De *in vivo* remming van microRNA-126 leidt tot een verminderde angiogene capaciteit aan de ene kant, maar tot een verhoogde stamcelmobilisatie aan de andere kant. Deze conclusie ondersteunt het belang van microRNA-126 in de vasculaire homeostase. Onze huidige werkhypothese stelt dat, in de aanwezigheid van microRNA-126, re-endothelialisatie plaats kan vinden middels de proliferatie van lokale mature endotheelcellen in een proces dat lijkt op angiogenese. Echter, bij endotheelcel-dysfunctie of verlaging van microRNA-126 zal vaatwandherstel van een VEGF-afhankelijk angiogeen proces overgaan naar een SDF-1-afhankelijk vasculogeen proces; de mobilisatie van circulerende myeloide of vasculaire progenitorcellen.

MicroRNA-126 en ontsteking

Een lokale ontstekingsreactie leidt tot activatie van het endotheel en vervolgens tot de expressie van adhesieve eiwitten die het mogelijk maakt dat ontstekingscellen kunnen hechten. Deze cellen kunnen dan via het endotheel migreren in de richting van de bron van de ontsteking. Een van de belangrijke

endotheliale adhesiereceptoren is vascular endothelial adhesion molecule-1 (VCAM-1), een gevalideerde target van microRNA-126. In **Hoofdstuk 5** beschrijven we dat, in de nier, VCAM-1 niet in alle microvasculaire vaatbedden in dezelfde mate tot expressie komt. Na de experimentele inductie van een inflammatoire respons met behulp van tumor necrosis factor-α (TNFα) zagen we dat VCAM-1 wel tot expressie kwam in het arteriolair endotheel van de nier, maar niet in de glomerulaire capillairen. Door met behulp van laser-capture-microdissectie endotheelcellen te isoleren van zowel de glomeruli als de arteriolen, bleken beide celsoorten, na TNFα-behandeling, vergelijkbare hoeveelheden VCAM-1-mRNA tot expressie te brengen. Posttranscriptionele regulatie van VCAM-1 door microRNA-126 was een reële verklaring voor de heterogene inflammatoire respons van het renale vaatbed. In **Hoofdstuk 5** hebben we deze hypothese bevestigd door wederom met antagomir-126 de functie van microRNA-126 te remmen en vervolgens bij deze muizen een ontstekingsreactie te starten met TNFα. Het bleek dat in dit geval VCAM-1 wel tot expressie kwam in de endotheelcellen van zowel de glomeruli als de arteriolen.

Uit deze studie blijkt dat microRNAs, zoals microRNA-126, een belangrijke rol kunnen spelen bij de vaatbedspecificatie van het nier-endotheel.

Toekomstperspectief

In dit proefschrift zijn enkele 'vasculaire' functies van microRNA-126 beschreven. MicroRNA-126 speelt een belangrijke rol in endotheelcellen waar het de expressie reguleert van eiwitten die van belang zijn in angiogenese, vasculogenese en ontsteking. Verder is beschreven dat microRNA-126 tot expressie komt in beenmergcellen en bloedplaatjes. Een overexpressie van deze microRNA leidt in beenmergcellen tot een verbeterde angiogenese en de aanwezigheid van microRNA-126 in plaatjes en in de circulatie kan wellicht als biomarker dienen voor de diagnose van de impact van cardiovasculaire risicofactoren op de vaatwand.

Tot op heden is het echter nog onduidelijk welke processen vooraf gaan aan de expressie van microRNA-126. Een aantal studies heeft aangetoond dat de binding van de transcriptiefactoren Ets-1 en Ets-2 en de stroming van het bloed kunnen leiden tot een verhoogde expressie van microRNA-126 in het endotheel. Het is echter onbekend of extra- en intracellulaire factoren, die leiden tot de activatie van het endotheel, vervolgens ook kunnen leiden tot een verhoogde (of verlaagde) expressie van microRNA-126. Om de rol van microRNA-126 in de vasculaire biologie in zijn totaliteit te kunnen begrijpen is het van belang dat er onderzoek wordt gedaan naar de moleculaire mechanismen die de expressie van microRNA-126 moduleren. Wanneer dit bekend is, kan het onderzoek zich verbreden door deze moleculaire mechanismen (proberen) te koppelen aan risicofactoren. In de toekomst is het dan wellicht mogelijk om therapeutische mogelijkheden te ontwikkelen die via microRNA-mechanismen kunnen bijdra-

gen aan het gezond houden van het endotheel.

Curriculum vitae

The author of this thesis was born on June 4, 1983, in Naaldwijk, the Netherlands. After completing secondary education with VWO in 2001, the author continued his education in the same year with the study Life Science and Technology at Leiden University and the Technological University of Delft. During this study two research projects were completed; at the Department of Rheumatology, Leiden University Medical Center, under the supervision of dr. F.A.S. Kurreeman and at the Department of Virology, Erasmus University Rotterdam, under the supervision of dr. B.G. van den Hoogen. He received his *M.Sc.* in Life Science and Technology in September 2006. In November of the same year he started as a *Ph.D.* student in the Department of Nephrology, Leiden University Medical Center, under supervision of prof. dr. A.J. van Zonneveld. During this *Ph.D.* project he mainly focused on the role of microRNA-126 in vascular homeostasis. Starting in February 2012, he will work as a post-doctoral fellow at the Leon Charney Division of Cardiology at New York University School of Medicine, under the supervision of dr. Y. Suárez. Here, he will continue his work on microRNAs in endothelial cell biology by unraveling the molecular mechanisms that control the expression microRNAs in endothelial cells.

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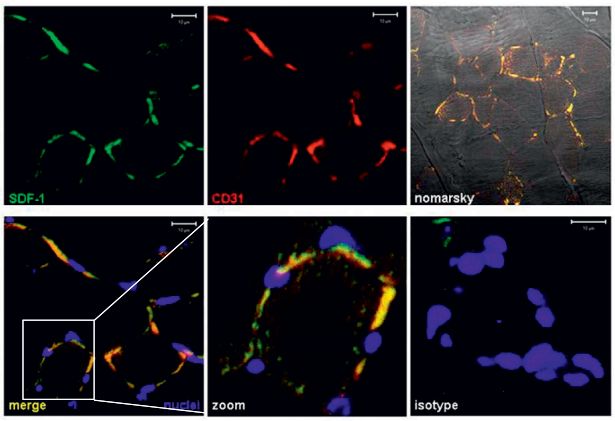
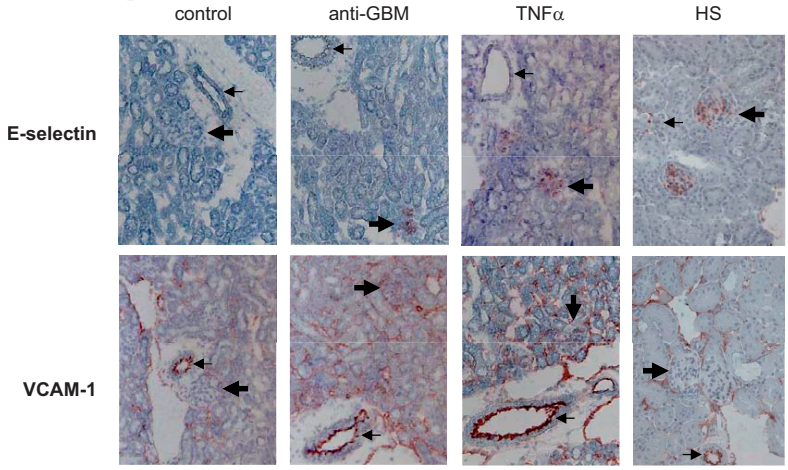
Color figures

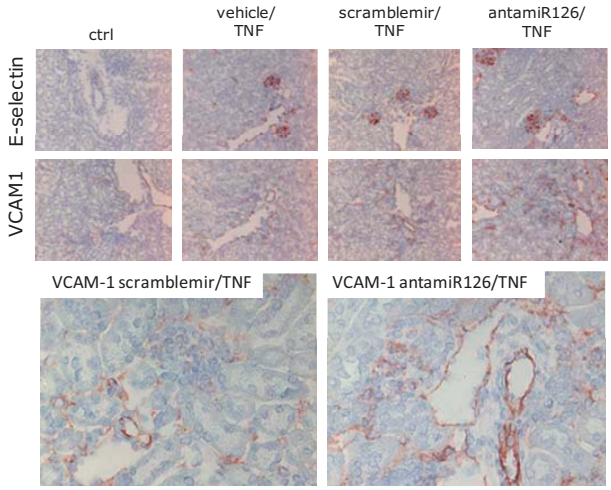
Chapter 4: Figure 3C



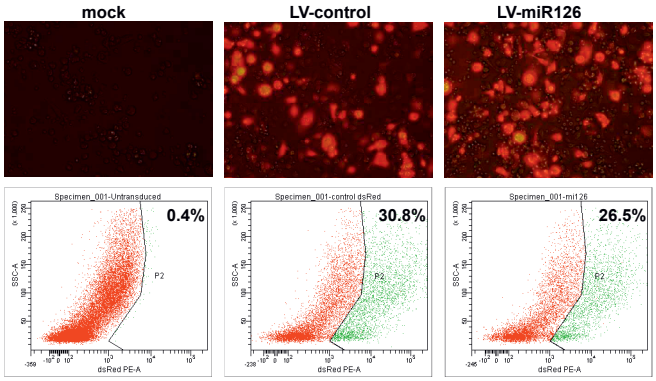
Chapter 5: Figure 1C



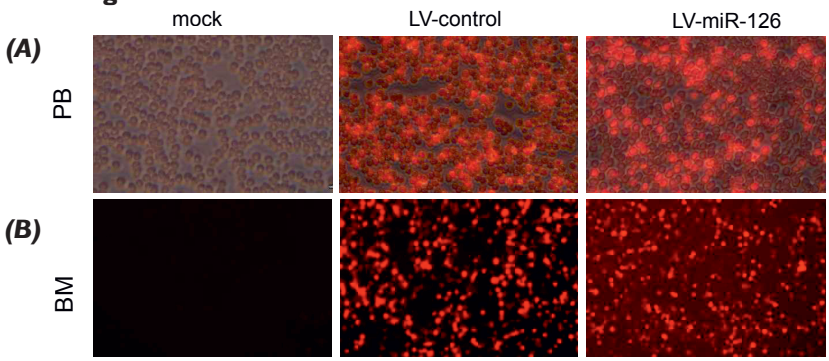
Chapter 5: Figure 6A



Chapter 7: Figure 1B



Chapter 7: Figure 2A-B



Chapter 7: Figure 4A-F