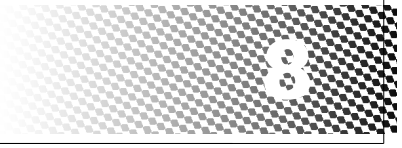
CHAPTER 8

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.

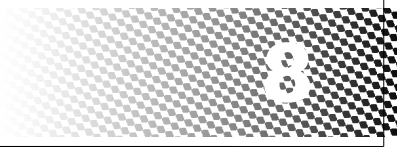
In **Chapter 2** we demonstrate data that supports a role for miRNA-126 in an angiogenic response induced by ischemia. To investigate the role of miRNA-126 in neovascularization, we injected C57Bl/6 WT mice in the tail vein with either antagomir-126 or scramblemir. Subsequently, we subjected these animals 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.

References

1. Edelstein LC, Bray PF. MicroRNAs in platelet production and activation. Blood 2011.
2. Mattes J, Collison A, Plank M, Phipps S, Foster PS. Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. Proc Natl Acad Sci U S A 2009;106:18704-9.
3. Shen WF, Hu YL, Uttarwar L, Passegue E, Largman C. MicroRNA-126 regulates HOXA9 by binding to the homeobox. Mol Cell Biol 2008;28:4609-19.
4. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 2011;108:5003-8.
5. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 2011;13:423-33.
6. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 2008;3:e3694.
7. Fichtlscherer S, De RS, Fox H, Schwietz T, Fischer A, Liebetrau C, et al. Circulating MicroRNAs in Patients With Coronary Artery Disease. Circ Res 2010.
8. Fukushima Y, Nakanishi M, Nonogi H, Goto Y, Iwai N. Assessment of plasma miRNAs in congestive heart failure. Circ J 2011;75:336-40.
9. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res 2010;107:810-7.
10. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci U S A 2008;105:1516-21.
11. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell 2008;15:272-84.
12. van Solingen C, Seghers L, Bijkerk R, Duijs JM, Roeten MK, van Oeveren-Rietdijk AM, et al.

Antagomir-mediated silencing of endothelial cell specific microRNA-126 impairs ischemia-induced angiogenesis. J Cell Mol Med 2009;13:1577-85.
13. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell 2008;15:261-71.
14. Ásgeirsdóttir SA, van Solingen C, Neng Fisheri K, Zwiers PJ, Heeringa P, van Meurs M, et al. MicroRNA-126 contributes to renal microvascular heterogeneity in VCAM-1 protein expression in acute inflammation. Am J Physiol Renal Physiol (Submitted) 2011.
15. van Solingen C, de Boer HC, Bijkerk R, Monge M, van Oeveren-Rietdijk AM, Seghers L, et al. MicroRNA-126 modulates endothelial SDF-1 expression and mobilization of Sca-1+/Lin- progenitor cells in ischemia. Cardiovasc Res 2011.
16. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, et al. MicroRNA expression in zebrafish embryonic development. Science 2005;309:310-1.
17. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, et al. LNA-mediated microRNA silencing in non-human primates. Nature 2008;452:896-9.
18. Elmen J, Lindow M, Silahtaroglu A, Bak M, Christensen M, Lind-Thomsen A, et al. Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. Nucleic Acids Res 2008;36:1153-62.
19. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature 2005;438:685-9.
20. van Rooij E., Marshall WS, Olson EN. Toward microRNA-based therapeutics for heart disease: the sense in antisense. Circ Res 2008;103:919-28.
21. Berezikov E, van Tetering G., Verheul M, van de Belt J., van Laake L., Vos J, et al. Many novel mammalian microRNA candidates identified by extensive cloning and RAKE analysis. Genome Res 2006;16:1289-98.
22. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002;12:735-9.
23. Rabelink TJ, de Boer HC, van Zonneveld AJ. Endothelial activation and circulating markers of endothelial activation in kidney disease. Nat Rev Nephrol 2010;6:404-14.
24. Nikolic I, Plate KH, Schmidt MH. EGFL7 meets miRNA-126: an angiogenesis alliance. J Angiogenes Res 2010;2:9.
25. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. Blood 2008;111:1217-26.
26. Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem 2008;283:15878-83.
27. Kuehbacher A, Urbich C, Zeiher AM, Dimmeler S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. Circ Res 2007;101:59-68.
28. Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, et al. MicroRNAs modulate the angiogenic properties of HUVECs. Blood 2006;108:3068-71.
29. Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. Circ Res 2007;100:1164-73.
30. Suarez Y, Fernandez-Hernando C, Yu J, Gerber SA, Harrison KD, Pober JS, et al. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Proc Natl Acad Sci U S A 2008;105:14082-7.
31. Urbich C, Kuehbacher A, Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. Cardiovasc Res 2008;79:581-8.
32. Madeddu P, Emanueli C, Spillmann F, Meloni M, Bouby N, Richer C, et al. Murine models of myocardial and limb ischemia: diagnostic end-points and relevance to clinical problems. Vascul Pharmacol 2006;45:281-301.
33. Jiang BH, Zheng JZ, Aoki M, Vogt PK. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proc Natl Acad

Sci U S A 2000;97:1749-53.
34. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood 1996;87:3336-43.
35. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737-44.
36. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 2006;124:175-89.
37. Horiuchi T, Weller PF. Expression of vascular endothelial growth factor by human eosinophils: upregulation by granulocyte macrophage colony-stimulating factor and interleukin-5. Am J Respir Cell Mol Biol 1997;17:70-7.
38. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood 2004;104:2224-34.
39. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci U S A 2006;103:12493-8.
40. Plaks V, Birnberg T, Berkutzki T, Sela S, BenYashar A, Kalchenko V, et al. Uterine DCs are crucial for decidua formation during embryo implantation in mice. J Clin Invest 2008;118:3954-65.
41. Puxeddu I, Alian A, Piliponsky AM, Ribatti D, Panet A, Levi-Schaffer F. Human peripheral blood eosinophils induce angiogenesis. Int J Biochem Cell Biol 2005;37:628-36.
42. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654-9.
43. Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal 2009;2:ra81.
44. Luscinskas FW, Gimbrone MA, Jr. Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment. Annu Rev Med 1996;47:413-21.
45. Hovens MM, Snoep JD, Groeneveld Y, Tamsma JT, Eikenboom JC, Huisman MV. High levels of low-density lipoprotein cholesterol and triglycerides and suboptimal glycemic control predict diminished ex vivo aspirin responsiveness in patients with Type 2 diabetes. J Thromb Haemost 2007;5:1562-4.
46. Hovens MM, Snoep JD, Groeneveld Y, Frolich M, Tamsma JT, Huisman MV. Effects of aspirin on serum C-reactive protein and interleukin-6 levels in patients with type 2 diabetes without cardiovascular disease: a randomized placebo-controlled crossover trial. Diabetes Obes Metab 2008;10:668-74.
47. de Boer HC, Verseyden C, Ulfman LH, Zwaginga JJ, Bot I, Biessen EA, et al. Fibrin and activated platelets cooperatively guide stem cells to a vascular injury and promote differentiation towards an endothelial cell phenotype. Arterioscler Thromb Vasc Biol 2006;26:1653-9.
48. Hanke M, Hoefig K, Merz H, Feller AC, Kausch I, Jocham D, et al. A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. Urol Oncol 2009.
49. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The MicroRNA Spectrum in 12 Body Fluids. Clin Chem 2010.
50. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, et al. Plasma MicroRNA Profiling Reveals Loss of Endothelial MiR-126 and Other MicroRNAs in Type 2 Diabetes. Circ Res 2010.
51. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci U S A 2008;105:1516-21.
52. Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE, Lawson ND. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. Nature 2010.

53. Suarez Y, Wang C, Manes TD, Pober JS. Cutting edge: TNF-induced microRNAs regulate TNF-induced expression of E-selectin and intercellular adhesion molecule-1 on human endothelial cells: feedback control of inflammation. J Immunol 2010;184:21-5.
54. Davis BN, Hata A. Regulation of MicroRNA Biogenesis: A miRiad of mechanisms. Cell Commun Signal 2009;7:18.
55. Siomi H, Siomi MC. Posttranscriptional regulation of microRNA biogenesis in animals. Mol Cell 2010;38:323-32.
56. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science 2009;324:1710-3.
57. Doebele C, Bonauer A, Fischer A, Scholz A, Reiss Y, Urbich C, et al. Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. Blood 2010;115:4944-50.