The role of microRNA-126 in vascular homeostasis

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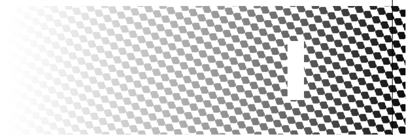
CHAPTER 1
General introduction



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].

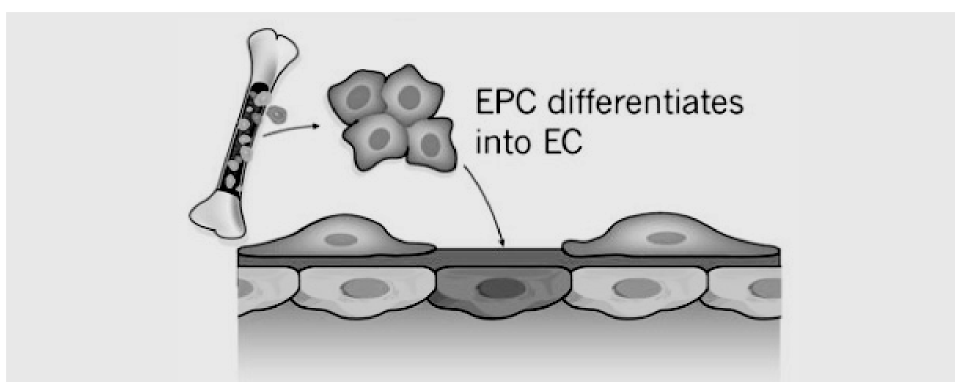
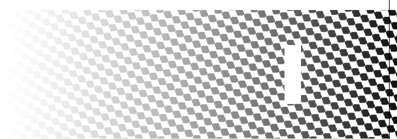


Figure 1. Vasculogenesis
Vessel formation can occur by the mobilization of bone marrow-derived endothelial progenitor cells that differentiate into endothelial cells [42]. Adapted from: Molecular mechanisms and clinical applications of angiogenesis, Carmeliet & Jain, Nature 2011.

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].

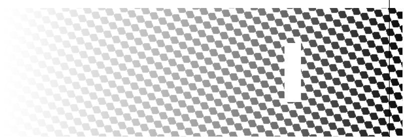


Angiogenesis

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 neo-vessel. 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

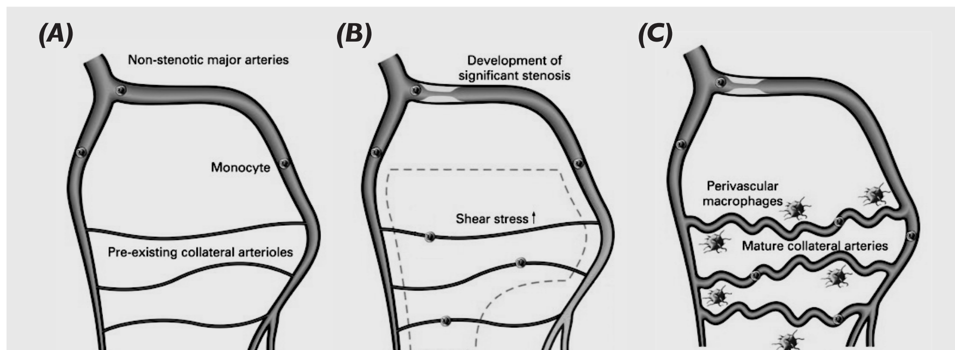


Figure 3. Mechanism of arteriogenesis

(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.

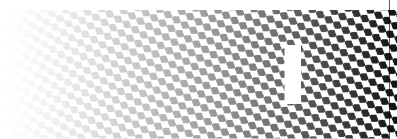
to contribute to arteriogenesis; monocytes [54] and natural killer cells play an early role, while a variety of T-cell subsets arrive in a later phase [55]. The

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 deacetlylases. 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

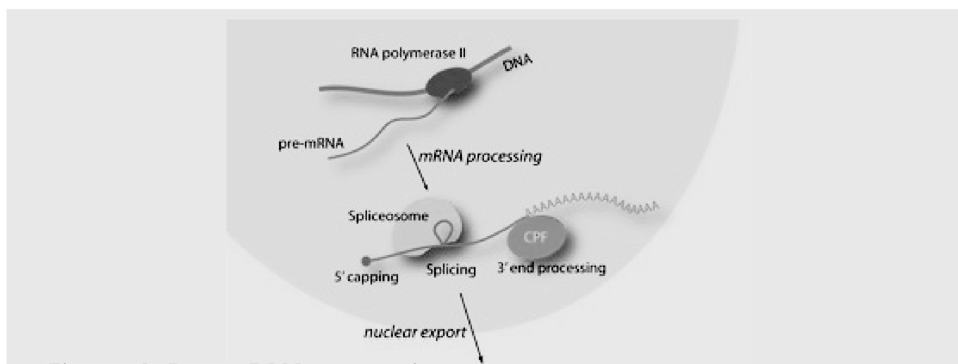


Figure 4. Pre-mRNA processing

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].

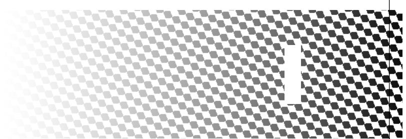
Polyadenylation

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

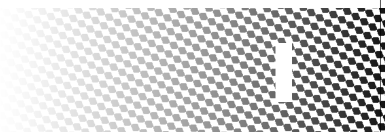
splicing and debranching, thereby bypassing the Drosha–DGCR8 step [80, 81]. The nuclear export of pre-miRNAs is mediated by the transport receptor exportin 5 [82]. In the cytoplasm the pre-miRNA is maturated by Dicer, assisted by a transactivation-responsive RNA-binding protein, into an imperfect RNA 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.



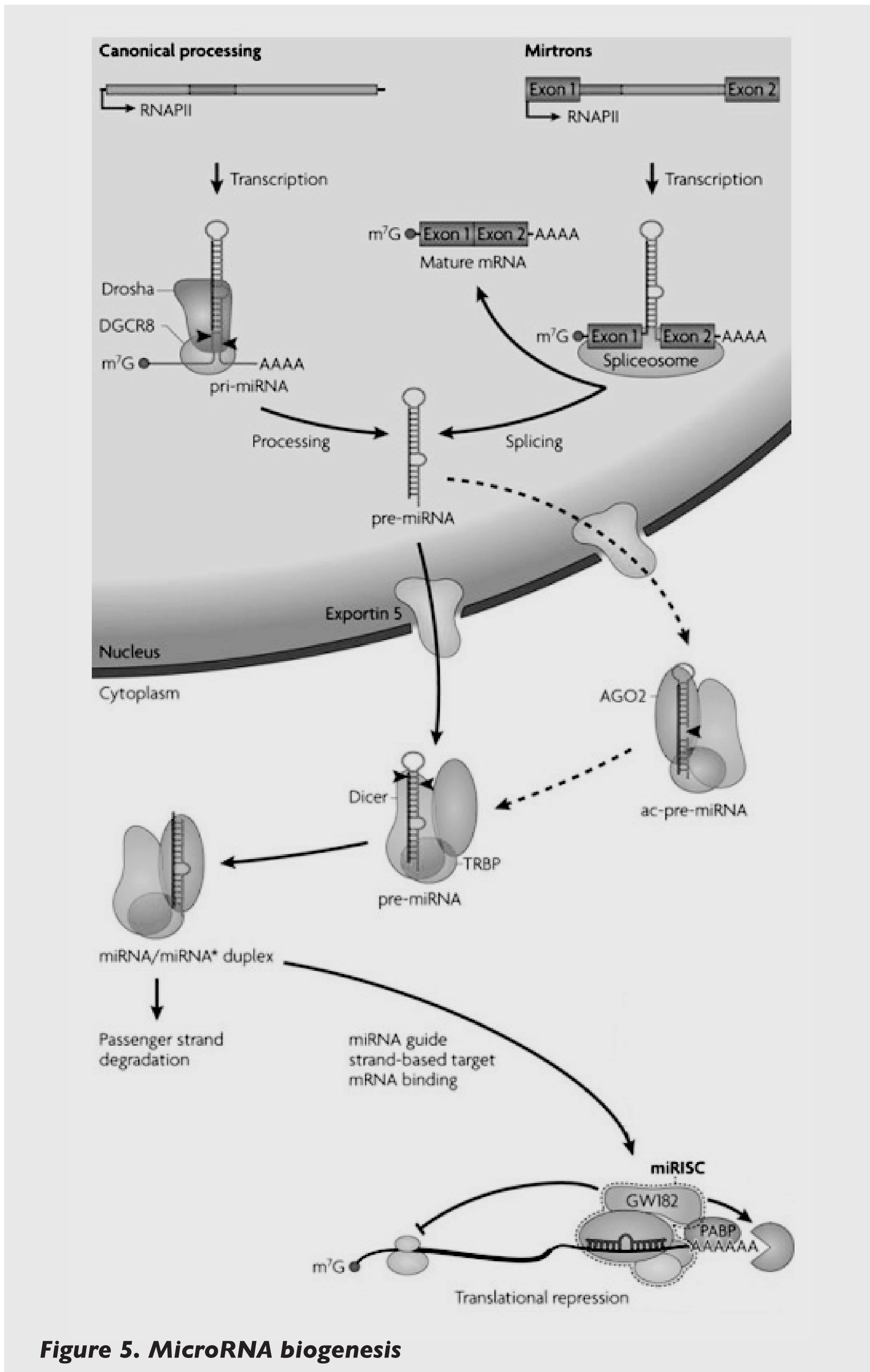
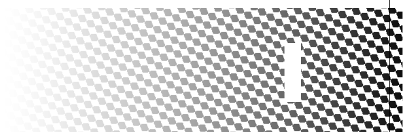


Figure 5. MicroRNA biogenesis

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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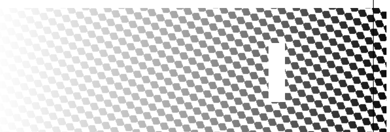

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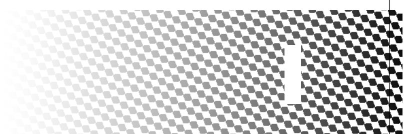

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