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microRNAs as novel regulators of angiogenesis

Yajaira Suárez^{1,3} and William C. Sessa^{2,3}

¹Department of Immunobiology, Yale University School of Medicine, New Haven, Connecticut, USA

²Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut, USA

³Vascular Biology and Therapeutics Program, Yale University School of Medicine, New Haven, Connecticut, USA

Abstract

MicroRNAs are short non-coding RNAs that function as negative regulators of gene expression. Posttranscriptional regulation by miRNAs is important for many aspects of development, homeostasis and disease. Endothelial cells are key regulators of different aspects of vascular biology including the formation of new blood vessels (angiogenesis). Here we review the approaches and current experimental evidence for the involvement of miRNAs in the regulation of the angiogenic process and their potential therapeutic applications for vascular diseases associated with abnormal angiogenesis.

Keywords

Angiogenesis; miRNA; endothelial cells; Dicer; gene expression; VEGF; cancer

Introduction

All blood vessels are lined by the vascular endothelium, a critical barrier between the circulating blood and tissue. The non-thrombogenic surface of the endothelium permits the flow of blood to meet the metabolic demands of tissues and alterations in flow patterns are determined by the changes in pressure and vascular resistance in a given vascular segment¹. The *de novo* generation and remodeling of blood vessels is essential to embryonic growth and throughout postnatal life. With regard to the latter, dynamic regulation of vascular density is critical for physiological organ repair during wound healing, post-ischemic tissue restoration and the menstrual cycle. During adulthood, the endothelium remains essentially quiescent, to fulfill their main function to conduct nutritive blood flow to organs, with turnover rates on the orders of months to years and rapid changes in their proliferation rates occur following activation of endothelium by angiogenic cytokines². The loss of typical endothelial quiescence and barrier function is a common feature of conditions such as inflammation, tumor progression, atherosclerosis, restenosis, and various vasculopathies³.

The formation of the vascular system starts with the assembly of embryonic progenitors cells to form the vascular plexus of small capillaries in a process known as vasculogenesis. This phase is followed by angiogenesis resulting in the expansion of the nascent vascular plexus by sprouting and remodeling into a highly organized and stereotypic network of larger arterial

Address correspondence: William C. Sessa, Yale University School of Medicine, Amistad Research Building, 10 Amistad Street, New Haven, CT06519, USA. Fax: 203-737-2290. william.sessa@yale.edu.

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and venous vessels ramifying into smaller ones^{3,4}. Therefore, vasculogenesis and angiogenesis are physiological processes during development that are down-regulated in the healthy adult – except for the organs of the female reproductive system – and are almost exclusively associated with pathology when angiogenesis is induced by microenvironmental factors like hypoxia or inflammation^{2,5,6}. The pathological processes associated with or induced by angiogenesis include diseases as diverse as cancer, macular degeneration, psoriasis, diabetic retinopathy, thrombosis, and inflammatory disorders including arthritis and atherosclerosis. Moreover, insufficient angiogenesis is characteristic of ischemic heart disease, peripheral vascular disease and pre-clampsia³. The above examples represent the broad array of diseases that are associated with the activated endothelial cell (EC) phenotype.

Upon angiogenic activation, EC proliferate, degrade extracellular matrix, change their adhesive properties, migrate, avoid apoptosis, form tube like-structures and eventually mature into new blood vessels. Therefore, the growth of vessels is a complex process involving a number of molecular and cellular events that require to be temporal and spatial orchestrated by a finely tuned balance between stimulatory and inhibitory signals⁷. Finally, all these processes are controlled by signals received by EC from their microenvironment, signals whose transduction pathways lead specific programs of gene expression to assure an adequate angiogenic response^{8,9}.

MicroRNAs (miRNAs) have emerged as crucial players regulating the magnitude of gene expression in a variety of organisms¹⁰. This class of short (≈ 22 nucleotides) non-coding RNA molecules have been shown to participate in almost every cellular process investigated so far¹¹, and their dysregulation is observed in -and might underlie- different human pathologies including cancer, heart disease and neurodegeneration¹²⁻¹⁵. These new molecular regulators have been identified in ECs and their role in the regulation of different aspects of the angiogenic process has been recently investigated in a variety of laboratories¹⁶⁻²². The present review focuses on the approaches and current experimental evidence for the involvement of miRNAs in the angiogenic process and their potential therapeutic applications for vascular diseases associated with abnormal angiogenesis.

miRNAs: biogenesis and modus operandi

Since the discovery of the first two miRNAs *-lin-4* and *let-7*²³⁻²⁵ hundreds of miRNAs have been identified in plants, animals and viruses by molecular cloning and bioinformatics approaches²⁶. miRNAs constitute a family of short non-coding RNA molecules of 20-25 nucleotides in length that regulate gene expression at the post-transcriptional level^{27,28}. They generally repress target mRNAs through an antisense mechanism. In animals, miRNAs typically target sequences in the transcript 3' untranslated regions (3'UTR) that are only partially complementary to the miRNA, causing a repression of the protein synthesis²⁹. They are involved in the control of a wide range of biological functions and processes such as development, differentiation, metabolism, growth, proliferation and apoptosis^{11,12,14,27,28,30} and are the center of attention in molecular and cell biology research. More than 700 human miRNAs have been cloned and bioinformatic predictions indicate that mammalian miRNAs can regulate approximately 30% of all protein-coding genes^{29,31}.

Most miRNAs are transcribed by RNA polymerase II from individual miRNAs genes, from introns of protein coding genes, or from polycistronic transcripts that often encode multiple related miRNAs³². These long –thousands of nucleotides- primary transcripts generate a stem-loop containing primary miRNA (pri-miRNA). The pri-miRNA is processed within the nucleus by a ribonuclease III (RNase III), called Drosha³³, along with an RNA-binding protein DGCR8/Pasha³⁴ (Figure 1). Most mammalian miRNAs that are encoded in introns are processed before splicing, however there is a subset of intronic miRNAs called “miRtrons” that circumvent the

Drosha pathway³⁵. The product of the Drosha cleavage event is a 70-100 nucleotides hairpin-shaped precursor (pre-miRNA) that is transported to the cytoplasm via an Exportin-5 and Ran-GTP dependent mechanism³⁶. Then the pre-miRNA is further cleaved to produce the mature \approx 22-nt miRNA:miRNA* duplex by another RNaseIII enzyme, Dicer³⁷ (Figure 1). The miRNA duplex is incorporated into the effector ribonucleoprotein complex RISC (RNA induced silencing complex)^{38,39} whose key components are proteins of the Argonaute (AGO) family⁴⁰ (Figure 1). The miRNA duplex is unwound into the mature single-stranded form (guide strand) and its complementary strand (passenger strand or miRNA*) that is typically degraded. The stem loop in pre-miRNAs contributes to the strand selection, however the miRNA* also has chance to be selected and used in gene regulation⁴¹. As part of the RISC, the miRNA guides the complex to its RNA targets by Watson-Crick base-pairing interactions. In cases of perfect or near-perfect complementary to the miRNA, target mRNA 3'UTR can be cleaved and degraded. In most cases, animal miRNAs pair imperfectly with their targets and their translation is repressed⁴². The mechanism(s) of translational repression by miRNAs remains unclear and can also affect mRNA stability. These include sequestration from ribosomes (by relocation into P bodies), blockage of translational initiation, translational repression after initiation and target deadenylation coupled to transcript degradation^{42,43} (Figure 1). Recent reviews covering these topics and more general information about biogenesis and mechanisms of action can be found^{11,12,29,44}.

Elucidation of the function of a miRNA requires identification of putative mRNA targets that it regulates and this is very challenging since miRNA usually are imperfectly complementary to their targets. In mammals, the most consistent requirement, although not always essential, of miRNA:target interaction is a contiguous and perfect base pairing of the miRNA nucleotides 2-8, representing the “seed” region. In many cases, the seed seems to determine this recognition; in other cases, additional determinants are required, such as reasonable complementarity to the miRNA 3' half to stabilize the interaction, mismatches must to be present in the central region of the miRNA-mRNA, among others^{12,29,45,46}. It is important to note that identifying functionally important miRNAs targets is crucial for understanding miRNA functions. However, the possibility that a single miRNA may target multiple transcripts within a cell type and that individual transcripts may be subject to regulation by multiple miRNAs amplifies the scope of putative miRNA regulation of gene expression, and indicates that the particular cellular context of a given miRNA will determine its function in a specific cell type.

Regulation of Angiogenesis by miRNAs: global approaches

An approach to examine the spectrum of the biological significance of miRNAs is to remove all miRNAs by mutation or disruption of *Dicer*, the rate limiting enzyme involved in the maturation of miRNAs. *Dicer* loss of function results in profound developmental defects in both zebrafish and mice^{47,48}. Zebrafish lacking *Dicer* undergo to a relative normal morphogenesis and organ development but die two weeks after fertilization due to a general growth arrest⁴⁸. The survival to this stage likely reflects the presence of maternal *Dicer*. When an offspring of fish that lack both maternal and zygotic *Dicer* was created⁴⁹, these *Dicer*-null embryos exhibited severe defects most prominently in gastrulation, brain morphogenesis, cardiac development associated with a disrupted blood circulation. Reminiscent of the zebrafish *dicer*-null phenotype, loss of *Dicer* in mice by replacement of exon 21 with a neomycin-resistance cassette leads to lethality early in embryogenesis at day 7.5 and the embryos were depleted of pluripotent stem cells⁴⁷. Another group generated *Dicer*^{ex1/2} mice have a deletion of the amino acid sequences from the first two exons of the *Dicer* gene⁵⁰. *Dicer*^{ex1/2} homozygous embryos die between days 12.5 and 14.5 of gestation, again demonstrating that *Dicer* is necessary for normal mouse development. To further explore the consequences of *Dicer* deletion, several laboratories have generated mice harboring

conditional *Dicer* alleles. Tissue-specific inactivation of *Dicer* has led to the conclusion that *Dicer* is essential for proper limb formation, lung and skin morphogenesis, the maintenance of hair follicles, T cell development, differentiation and function, neuronal survival, skeletal muscle development, chondrocyte proliferation and differentiation, germ cell development and spermatogenesis, autoimmunity and antibody diversity and B lymphocytic lineage survival⁵¹⁻⁶⁴. At the level of the cardiovascular system, cardiac specific deletion of *Dicer* produces dilated cardiomyopathy associated with heart failure in neonates⁶⁵ and spontaneous cardiac remodeling when *Dicer* deletion was induced postnatally in the myocardium⁶⁶.

Recent reports, both *in vitro* and *in vivo*, also indicate a role for Dicer-dependent miRNAs in vascular signaling and functions related to angiogenesis^{16,19,20,50,67}. In fact, the early embryonic lethality observed in *Dicer*^{ex1/2} mice has been suggested to be a consequence of defective blood vessel formation and maintenance⁵⁰, data that were in accordance with the disrupted blood circulation observed in zebrafish *Dicer*-null embryos⁴⁹. The defects observed in *Dicer*^{ex1/2} embryos and yolk sacs were associated with altered expression of vascular endothelial growth factor (VEGF), its receptors KDR (VEGFR2) and FLT-1 (VEGFR1) as well as the putative angiopoietin-2 receptor, Tie-1. This study suggests that *Dicer* has a role in embryonic angiogenesis, probably through processing of miRNAs that regulate expression levels of key angiogenic regulators⁵⁰. These observations give rise for a series of studies relating to miRNA and endothelial cells functions relevant to angiogenesis.

The functional role of miRNAs in endothelial cells was assessed by specifically silencing Dicer using short interfering RNA (siRNA) in human umbilical endothelial cells (HUVECs) and EA.hy.926 cells. Depletion of Dicer impairs the development of capillary-like structures and exerts an antiproliferative effect^{16,19,20}. The knockdown of Dicer in human microvascular endothelial cell (HMECs) shows diminished tube formation and cell migration⁶⁷. Accordingly, migration was also impaired in Dicer knockdown HUVECs when fibronectin was used as matrix¹⁶. As expected, the knockdown of Dicer in EC alters constitutive protein expression patterns, largely affecting proteins that play a role in endothelial cell biology and angiogenic responses, such as Tie-2/TEK, VEGFR2, endothelial nitric oxide synthase (eNOS), IL-8 and angiopoietin like 4 (ANGPTL4)¹⁹. Some of the upregulated transcripts/protein were consistent with the reported *Dicer*^{ex1/2} embryos, such as of Tie-2/TEK and VEGFR2^{19,50}. The decrease in growth and morphogenesis observed in EC after Dicer silencing^{16,19,20} was consistent impaired vascular development in *Dicer*^{ex1/2} embryos and regardless of the paradoxical upregulation of VEGFR1 and VEGFR2 observed in *Dicer*^{ex1/2} embryos⁵⁰ and VEGFR1, VEGFR2, Tie-2 and eNOS observed in Dicer knockdown ECs¹⁹. Interestingly, the Dicer silencing in EC increased the expression of thrombospondin-1 (Tsp1)^{16,20}, a multi-domain matrix glycoprotein that has been shown to be a natural endogenous inhibitor of angiogenesis, which may explain, in part, the antiangiogenic phenotypes observed *in vitro*. Furthermore, the knockdown of Dicer in HMECs also reduces the expression of miRNAs that control the expression of the HMG-box protein 1 (HBP1) transcriptional suppressor, which negatively regulates p47phox of the NADPH oxidase complex, decreases the basal production of reactive oxygen species (ROS) impairing aspects of redox regulation during an angiogenic response⁶⁷.

The early embryonic lethality of *Dicer* null alleles in mice^{47, 50} has limited the ability to address the role of *Dicer* in normal mouse growth and development. The global effect of *Dicer* deficiency in adult mice was investigated by using a *Dicer* hypomorphic mouse (*Dicer*^{d/d}), obtained by a gene-trap method⁶⁸. *Dicer*^{d/d} female mice are infertile due to corpus luteum (CL) insufficiency and defective ovarian angiogenesis. CL is formed from the ovulated follicle and plays a critical role in the secretion of progesterone, a hormone needed for the maintenance of early pregnancy and requires intense angiogenesis. Impaired CL angiogenesis was partly explained by the lack of miR-17-5p and let7b, miRNAs that participate in angiogenesis via

targeting the anti-angiogenic factor tissue inhibitor of metalloproteinase 1 (TIMP1)⁶⁹. Although CL angiogenesis was reduced, embryonic vasculogenesis and angiogenesis were not affected in *Dicer^{del/d}* mice, indicating that angiogenesis in different tissues has different sensitivities to the levels of Dicer protein^{68,69}.

The first attempt to show the role of endothelial miRNAs in angiogenesis *in vivo* was performed by subcutaneous injection of Dicer knockdown HUVEC (suspended in a matrigel plug) into nude mice, demonstrating reduced sprout formation¹⁶. Finally, the requirement of endothelial miRNAs for post-natal angiogenesis was recently tested by the generation of two EC-specific *Dicer* knockout mouse lines, conditional Tie2-Cre;*Dicer^{lox/lox}* mice and the tamoxifen (TMX) inducible VECad-Cre-ER^{T2};*Dicer^{lox/lox}* mice²⁰. Despite the fact that Dicer protein was reduced and miRNA production diminished (e.g. miR-126 and miR-31) in EC isolated from Tie2-Cre;*Dicer^{lox/lox}* mice, they were viable and overtly normal, suggesting the mice were hypomorphic for Dicer expression in the endothelium. However, the lack of lethality allowed investigation of the relevance of endothelial miRNAs in post-natal angiogenic responses using several models of angiogenesis. VEGF (VEGF-A) is a major pro-angiogenic factor whose main functions are to promote EC survival, induce EC proliferation, enhance cell migration and invasion of EC, all phenotypes that promote angiogenesis. As shown previously in human EC transfected with Dicer siRNA^{16,19} VEGF driven angiogenesis is reduced in mice that are conditional EC-specific Dicer hypomorphs²⁰. Altered miRNA expression has been implicated in tumor formation via miRNA modulation of critical genes involved in tumor cells proliferation or survival^{14,70}. Importantly, angiogenesis is necessary for adequate delivery of nutrients and oxygen to growing tumors⁷¹. Interestingly, the participation of endothelial miRNAs in the tumor-induced neovascularization, was examined by post-natal inactivation of *Dicer* in the endothelium prior to tumor implantation. Tumor growth as well as the tumor-induced microvessel formation was reduced in VECad-Cre-ERT2;*Dicer^{lox/lox}*²⁰. Taken together, miRNAs participate and are required for tumor cell proliferation plus angiogenesis. The pathophysiological relevance of endothelial miRNAs was also further investigated in response to limb ischemia and wound healing²⁰. The vascular supply to limbs and peripheral tissues is essential for normal physiological functions. Under certain pathologic conditions, however, vascular supply may be reduced to such an extent that it leads to necrosis of the tissue. After ischemia, inactivation of *Dicer* in EC reduced the angiogenic response to limb ischemia indicated by a reduction in capillary densities and blood flow recovery. The reduced flow impaired lower limb function and resulted in higher ischemic damage scores²⁰. Recent works on the significance of miRNA in skin morphogenesis and development provide important insight that lays the foundation for wound healing process^{53,54}, the potential significance of miRNAs in cutaneous wound angiogenesis has also been discussed⁷². As mentioned before, angiogenesis is necessary for wound repair since the new vessels provide nutrients to support the active cells, promote granulation tissue formation and facilitate the clearance of debris. Cutaneous wound-healing was delayed when *Dicer* was inactivated in EC. Tie2-Cre;*Dicer^{lox/lox}* and post-natally in VECad-Cre-ERT2;*Dicer^{lox/lox}* showing larger areas of granulation tissue devoid of hair follicles with less granulation tissue deposition and collagen accumulation²⁰, hallmarks of an angiogenic response.

The knockdown of Drosha –involved in the processing of pri-miRNAs- was also undertaken to globally reduce miRNAs. The silencing of Drosha in EC produces less pronounced effects on angiogenesis than Dicer silencing^{16,19}, although capillary sprouting and tube forming activity were blunted; the knockdown of Drosha does not exert significant effects on the *in vivo* matrigel plug model¹⁶. This may be explained by the processing of pri-miRNA independent of Drosha³⁵. On the other hand, the angiogenic potential of EC disappeared when Ago2 –component of the RISC- was knockdown⁷³. Of the four mammalian Argonautes (Ago1-4), only Ago2 functions in the RNA interference pathway^{74,75}, whereas all four seem

to participate in the miRNA-mediated repression, indicating that the global impairment of repression by impeding miRNA-mRNA interaction also affect EC angiogenic responses.

These experimental approaches likely reveal the consequences of a block in miRNA biogenesis, however it is important to consider when interpreting the data, the fact that Dicer may participate in other processes unrelated to miRNA biology, such as formation of the heterochromatin⁷⁶ and that there are alternative Drosha-independent pri-miRNA processing pathways³⁵. Deciphering the miRNA network responsible for the modulation of the angiogenesis might lead to novel therapeutic approaches for cancer, wound healing and ischemic conditions such myocardial ischemia, peripheral vascular disease and vascular diabetic complications.

Role of individual miRNA in angiogenesis

Although the previous studies emphasize the importance of the miRNA pathway in several aspects of the angiogenic process, the majority does not provide information regarding the functions of specific miRNAs. Studies aimed at elucidating the role of individual miRNAs in the regulation of angiogenesis are increasingly being performed and most of the examples that illustrate principles of miRNA function in angiogenesis are presented here.

Many miRNAs exhibit striking organ specific expression patterns suggesting cell type-specific functions⁷⁷⁻⁷⁹. Consequently, dysregulation of miRNA expression and function may lead to human diseases¹². The first large-scale analysis of miRNA expression in EC was carried out in HUVECs and identified 15 highly expressed miRNAs with receptors of angiogenic factors (Flt-1, Nrp-2, Fgf-R, c-Met as c-kit) as putative mRNA targets, according to prediction algorithms¹⁸. Additional studies also profiled the expression of miRNAs in ECs^{16,19}. The highly expressed miRNAs that were common in at least two out of the three studies, included miR -15b, -16, -20, -21, -23a and b, -24, -29a and b, -31, -99a, -100, -103, -106, 125a and b, -126, -130a, -181a, -191, -221, -222, -320, -let-7, let-7b, let-7c and let-7d^{16,18,19}. However, their specific targets and functions in EC related to angiogenesis has only been characterized for a few of them (Table 1).

The prediction algorithms utilized to find receptors for angiogenic factors that may potentially be targeted by some of the miRNAs identified miR-221 and miR-222 to target c-kit¹⁸. c-kit is a tyrosine kinase receptor for stem-cell factor (SCF), and has been shown to promote survival, migration and capillary tube formation in HUVECs⁸⁰. Interestingly, transfection of HUVECs with miR-221/222 inhibits tube formation, migration and wound healing in response to SCF¹⁸. miR221/222 were shown to control the growth of erythropoietic and erythroleukemic cells, through the regulation of c-kit expression at translational level⁸¹. Accumulating evidence suggests that bone marrow-derived circulating precursors contribute to vascular repair, remodeling, and lesion formation under physiological and pathological conditions⁸². Interestingly, the interaction between miR-221/222 and the c-kit 3'UTR was also demonstrated in ECs and thus the antiangiogenic activity of these miRNAs¹⁸. miR221/222 overexpression in Dicer knockdown ECs restored the elevated eNOS protein levels eNOS induced by after Dicer silencing¹⁹. NO synthesized by eNOS is necessary for EC survival migration and angiogenesis⁸³. However, prediction sites for these miRNA were not found in eNOS 3' UTR, suggesting that the regulation of eNOS protein levels by miR-221/222 is likely to be indirect. Collectively, these reports suggest an antiangiogenic action for these miRNAs and then might be a potential tool to block angiogenesis. However, it is important to note that miR221/222 can also promote cancer cell proliferation through the regulation of p27(Kip1) tumor suppressor⁸⁴, indicating that the regulation of proliferation by these miRNAs appears cell type specific. Therefore, cell specific targeting with miRNAs is an important area of investigation to be developed.

Other miRNAs expressed in EC, let-7f and miR-27b, have been shown to exert pro-angiogenic effects, as revealed by the blockade of *in vitro* angiogenesis with 2'-O-methyl oligonucleotides inhibitors¹⁶, although their targets in ECs have not been already characterized.

The best characterized EC-specific miRNA is miR-126^{17,22,85}. miR-126 is a highly conserved miRNA (<http://microrna.sanger.ac.uk/sequences/index.shtml>). In both mouse and zebrafish miR-126 is enriched in tissues with a high vascular component such as the lung and the heart^{86,87}. In mammals, it is encoded by intron 7 of the EGF-like domain 7 (*Egfl7*) gene also known as VE-statin, which encodes an EC-specific secreted peptide that acts as a chemoattractant and inhibitor of smooth muscle cell migration^{88,89}. Location of miRNAs within non-coding regions of specific genes represents a common mechanism of co-regulation. Although the intronic miRNAs and their host genes could be regulated independently, it is possible that the signals that activate the transcription of the host gene lead to the transcription of the intronic miRNA. These miRNAs can in turn mediate the regulation of its host protein-coding gene or the regulation of other proteins whose expression is inappropriate for the stimulated process. In this regard, miR-208, is a cardiac specific miRNA encoded by an intron in the gene that encodes α -myosin heavy chain and function within a regulatory network to control cardiac stress response⁷⁹. Thus, the expression pattern of miR-126 in tissues and cells lines²² parallels to of *Egfl7*^{90,91}. Additionally, miR-126 has been shown to be enriched in embryonic bodies-derived Flk1 positive cells. Indeed, the expression of *Egfl7* and miR-126 largely matched that of EC markers during embryoid body formation, being highly enriched in Flk1-positive vascular progenitors at d4 as well as in mature CD31- expressing ECs at d7¹⁷. Although enriched in vascular progenitors, it is not sufficient to promote the differentiation of pluripotent cells towards an EC lineage¹⁷. *In vitro*, miR-126 regulates many aspects of EC biology, including cell migration, organization of the cytoskeleton, capillary network stability and cell survival¹⁷. *In vivo*, the knockdown of miR-126 in zebrafish resulted in the loss of vascular integrity and hemorrhage during embryonic development¹⁷. Furthermore, targeted deletion of miR-126 in mice causes partial embryonic or perinatal lethality (40% of *miR-126*^{-/-} mice). The embryonic lethality was due to a severe systemic edema, multifocal hemorrhages and rupture of blood vessels. Of the *miR-126*^{-/-} mice that survived to birth, 12% died by P1 and contained excessive protein rich fluid in the pleural space of the thoracic cavity, indicating a severe edema. The surviving *miR-126*^{-/-} mice appeared normal to adulthood and displayed no obvious abnormalities²², indicating that miR-126 plays an important role in the maintenance of vascular integrity during embryogenesis but not for vascular homeostasis after birth. Interestingly, ECs from adult *miR-126*^{-/-} mice showed diminished angiogenic responses, suggesting a role for miR-126 in neoangiogenesis of adult tissues in response to injury. Indeed, when *miR-126*^{-/-} were subjected to myocardial infarction, 50% died after 1 week and nearly all of them die by 3 weeks, in contrast, 70% of wild type (wt) mice survive at least for 3 weeks²². In fact, PECAM staining revealed extensive vascularization in the injured myocardium of wt mice, whereas there it was reduced in *miR-126*^{-/-} mice²². Vascular cell adhesion molecule-1 (VCAM-1) was the first target identified for repression by miR-126 *in vitro*⁸⁵, however additional targets have been identified^{17,22}. Gene expression profiles by microarray analysis of EC isolated from adult kidneys of wt and *miR-126*^{-/-} mice²² or from miR-126 zebrafish morphants or from HUVECs in which miR-126 was knockdown¹⁷ were performed to identify genes regulated by miR-126. Genes implicated in endothelial cell biology, angiogenesis, cell cycle, inflammation, cytoskeleton and growth factors were dysregulated in the absence of miR-126^{17,22}. Bioinformatic analysis, predicted integrin α -6, VCAM-1 and Sprouty-related protein-1 (*Spred-1*)^{17,22} as well as phosphoinositide-3-kinase, regulatory subunit 2 (PIK3R2) also known as p85- β , regulator of G protein signaling 3 (RGS3) and CRK¹⁷. miR-126 directly targets the 3' UTR of *Spred-1*^{17,22}, VCAM-1^{17,85} and PIK3R2¹⁷ for repression. *Spred-1* and PIK3R2 have been shown to function as negative regulators of VEGF / FGF signaling via MAP-kinase and PI3 kinase pathways, respectively. Thus, miR-126 promotes growth factor (VEGF/FGF) signaling, angiogenesis and vascular

integrity by inhibiting endogenous repressors of growth factors within ECs^{17,22}. This findings illustrate that a single miRNA can regulate vascular integrity and angiogenesis, providing a new target for either pro- or antiangiogenic therapies.

As mentioned before, numerous factors are implicated in vessel growth. Among these factors, angiotensin II (Ang II), the main effector peptide of the renin-angiotensin system, appears to be implicated in the regulation of the angiogenic process⁹². ANG II has been shown to work through both type 1 (AT1R) and type 2 (AT2R) receptors, which display opposing vasomotor and angiogenic actions⁹³. AT1R receptor activation is known to stimulate vascular growth and microvascular angiogenesis in nonneural tissues such as skeletal and cardiac muscle, whereas AT2R activation was recently shown to antagonize these actions⁹⁴. miR-155 is expressed in ECs and VSMC^{20,95} and has been shown to specifically interacts with the 3'UTR of the human AT1R mRNA, thereby reducing the endogenous expression of the hAT1R and consequently Ang II signaling⁹⁵. Translational repression by miR-155 provides yet another mechanism by which AT1R expression can be modulated. In this regard, it has been reported that Ang II induces in a dose dependent manner the expression VEGFR2 and significantly enhances VEGF-induced cell proliferation and tube formation, mediated by AT1 receptor⁹⁶ and suggesting that AT1 receptor may contribute to the development of diabetic retinopathy by enhancing VEGF-induced angiogenic activity. Then, the downregulation of AT1R by miR-155 suggest an antiangiogenic function for this miRNA in ECs. However, its role in EC angiogenesis has not been specifically addressed. Stimulation of human fibroblast with transforming growth factor β -1 (TGF- β 1) decreased the expression of miR-155 and increased the expression of hAT1R. Furthermore, miR-155 is induced in macrophages by cytokines such as tumor necrosis factor α (TNF α) and interferon β (IFN β)⁹⁷. Interestingly, angiogenic stimulation of EC with VEGF increases the expression of miR-155²⁰ suggesting VEGF may control the levels of ATR1 via miR-155. Nevertheless, the oncogenic potential of miR-155 has been confirmed in mice, where its overproduction leads to spontaneous B-cell malignancy, showing the complexity of miRNA-mediated regulation, given that the same miRNA may have opposite effects in different biological contexts.

Regulation of miRNA expression in angiogenesis

It is well accepted that miRNAs post-transcriptionally govern the levels of gene expression. However an important burgeoning area of investigation is to elucidate how the levels of miRNAs, per se, are regulated. The information about specific regulation of miRNAs has comparatively lagged behind, in contrast to the wealth of publications about their biological effects. Indeed, extracellular factors can modify the activity of a miRNA by affecting its expression, stability (by controlling synthesis or degradation or cellular localization⁹⁸⁻¹⁰²). In this regard, promoter elements that could contribute to the expression of the muscle-specific miR-1/miR-133 cluster have been identified¹⁰³ as well as the implicated in miR-223 during granulopoiesis⁹⁸. The oncogenic transcription factor c-Myc activates the miR-17-92 cluster, and this mechanism plays an important role in tumor formation¹⁰¹. Furthermore, LPS treatment of human monocytes induced the expression of miR-146, -142 and -155 as determined by miRNA microarrays and that miR-146 was induced in an NF κ B-dependent manner^{97,104}. These exciting studies raise the possibility that extracellular signals via Toll like receptors (TLR) modulate the expression of key miRNAs which then regulated the levels of genes necessary for TLR dependent functions. This concept of miRNA regulation has been extended to the cytokine IFN β , which induces key miRNAs that aid in combating viral infections¹⁰⁵. More recently, miRNA profiling of TGF- β or bone morphogenic protein (BMP) treated human vascular smooth muscle cells revealed that TGF- β /BMP induces the expression of miR-21 leading to an upregulation of genes necessary for the contractile phenotype. The mechanism of miR-21 induction is quite novel, where TGF- β enhances the processing of pri-miR-21 into pre-miR-21 by regulating the miRNA processing enzyme Drosha¹⁰⁶. Accordingly,

extracellular signals can modify the levels of a miRNA and thereby its activity providing a mechanism to regulate the robustness of an integrated functional response such as an angiogenic response (Figure 2).

Recent studies investigated the regulation of miRNAs in EC in response to serum, hypoxia, VEGF and tumor-derived growth factors^{20,107-109}.

miR-130a is expressed at low levels in quiescent HUVECs and is upregulated in response to fetal bovine serum¹⁰⁷. miR-130a is a regulator of the angiogenic phenotype of EC through its ability to modulate the expression of the antiangiogenic homeobox proteins GAX (growth arrest homeobox) and HoxA5. miR-130a antagonizes the inhibitory effect of GAX on EC proliferation, migration and tube formation and the inhibitory effects of HoxA5 on tube formation¹⁰⁷. The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanisms that link vascular oxygen supply to metabolic demand⁵.

Hypoxia occurs during several pathophysiological circumstances (e.g. tumor development, chronic ischemia). In cancer cells a set of hypoxia-regulated miRNAs have been identified¹¹⁰⁻¹¹², supporting a key role of hypoxia inducible factor (HIF) as a transcription factor for miRNA expression during hypoxia¹⁰⁰, however only few miRNAs promoters have been identified experimentally^{113,114}. Hypoxia induces the expression of different growth factors including VEGF, an important angiogenic factor. For this gene, a group of candidate regulatory miRNAs has been identified recently and the miRNA-regulation of VEGF under hypoxia was investigated in cancer cells¹¹¹. Interestingly, most of the miRNAs that were predicted to target VEGF were found to respond to hypoxia, which could lead to an extra layer of complexity in the angiogenic response. miR-15 and -16 regulate VEGF expression but are downregulated by hypoxia¹¹¹. Interestingly, these miRNAs have been shown to induce apoptosis in leukemic cells by targeting the anti-apoptotic protein Bcl-2¹¹⁵, block cell cycle progression¹¹⁶ and are frequently down-regulated in chronic lymphocytic leukemia¹¹⁷. Regarding to EC, miR-210 is induced by hypoxia¹⁰⁸. Overexpression of miR-210 in normoxic EC stimulates the formation of capillary-like structures and VEGF-driven migration, whereas its blockade inhibits the formation of the capillary-like structures and decreases the migration in response to VEGF. The relevant target for miR-210 in hypoxia was Ephrin-A3 (Eph-A3). Ephrin ligands and their receptors have been shown to play a crucial role in the development of the cardiovascular system. Although the importance of EphA2 in the regulation of angiogenesis and VEGF signaling has been reported, little is known yet about the specific role of Eph-A3. However, this data suggests that down-regulation of Eph-A3 is necessary for the miR-210-mediated stimulation of capillary-like formation and EC chemotaxis in response to VEGF and may contribute to modulate the angiogenic response to ischemia.

The modulation of the expression of EC-miRNAs by VEGF has been recently investigated²⁰. VEGF treatment of HUVECs regulated the levels of several miRNAs, among them hsa-miR-191, -155, -31, -17, -18a, -20a, whose expression was increased, however little change was observed in the expression of hsa-miR-126 and -222²⁰. The first set of miRNAs are commonly overexpressed in human tumors and have been implicated in the control of tumor growth, survival and angiogenesis¹¹⁸⁻¹²². Transcription factors c-myc and E2F control the expression of the miR-17-92 cluster including miR-17, -18a, -20 and miR-19a, -19b and -92a^{101,123,124}. Interestingly, VEGF has been shown promote proliferation of cortical neurons precursors by regulating E2F expression¹²⁵. E2F1, E2F2 and E2F3 are involved in the regulation of apoptosis and cell proliferation¹²⁶. Components of this cluster target the expression of E2F1 and target the expression of E2F1 promoting proliferation by shifting the E2F transcriptional balance away from the pro-apoptotic E2F1 and toward the proliferative E2F3 transcription network^{101,124}. Since the levels of miR-17, -18a and 20a in quiescent EC were very low, VEGF induction of these miRNAs suggest that they may regulate the

proliferative actions of VEGF. In fact, overexpression of these miRNAs in Dicer knockdown EC rescues the defect in cell proliferation and cord formation²⁰ suggesting that VEGF-induced proliferation and morphogenesis are mediated in part by miR-17-92 activation. When components of this cluster are overexpressed in tumors cells (*Kras*-transformed mouse colonocytes) they specifically target anti-angiogenic proteins containing thrombospondin type 1 repeats such as Tsp1, connective tissue growth factor (CTGF) and SPARC¹²⁰. In particular, miR-18 preferentially suppresses CTGF expression, whereas miR-19 targets Tsp1. By using MiRanda algorithm both miR-18 and -19 are predicted to target Tsp1 (depending on species). In human EC, miR-18a preferentially targets Tsp1²⁰. When EC were transfected with the components of miR-17-92 cluster upregulated by VEGF, miR-18a reduces basal levels of Tsp1 expression. Moreover, increased Tsp1 levels by Dicer silencing^{16,20} were restored to control levels by expression of miR-18a²⁰. Collectively, this data indicate that VEGF modulation of miRNAs, specifically components of the miR-17-92 cluster, may participate in the control of angiogenic phenotypes in EC. Furthermore, miR-17-92 miRNAs suppress the expression of the tumor suppressor PTEN and the proapoptotic protein Bim, contributing to the lymphoproliferative disease of miR-17-92-transgenic mice and contributing to lymphoma development in patients with amplifications of the miR-17-92 region¹²⁷. Moreover, deletion of this locus in mice resulted in smaller embryos and immediate postnatal death¹²⁸.

Finally, a recent report show that glioma- or growth factor-mediated the induction of miR-296 in primary human brain microvascular EC as well as in primary tumor EC isolated from brain tumors compared to normal brain EC¹⁰⁹. Furthermore, growth factor-induced miR-296 contribution to angiogenesis is mediated by targeting hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) and thereby reducing HGS-mediated degradation of VEGFR2 and PDGFR β ¹⁰⁹.

The identification of miRNAs as regulators of both EC-mediated angiogenesis, tumor-induced angiogenesis and survival is relevant for the therapy of cancer suggesting that antagonism of these key miRNAs may be an attractive strategy.

miRNAs as potential therapeutics targets for angiogenesis

miRNAs are important players and regulators of both angiogenic processes and responses, thus making them promising targets for potential therapeutics. The fact that miRNAs bind to their target mRNAs by Watson-Crick base pairing, indicates that the usage of an oligonucleotide complementary to the miRNA that effectively competes with the mRNA target, i.e. “antimiRs”, represents an obvious and potential effective way of inactivating pathological miRNAs and thus avoiding down regulation of important targets that promote the stimulation of gene expression¹²⁹⁻¹³¹. Alternatively, miRNA mimics - double stranded oligonucleotides designed to simulate the function of endogenous mature miRNAs- may induce target down regulation and thereby diminish gene expression, however this approach has not been tested *in vivo*^{132, 133}.

The use of antimiRs in cultured cells have been successful, however, the key development was chemical modification of miRNA inhibitors for *in vivo* utility. The large body of research discovered during the development of antisense therapeutics have led to effective strategies for the pharmacological delivery of nucleic acids, facilitating the development of small interfering (si)RNA therapeutics¹³⁴, and now, also miRNA therapeutics¹³⁵. Three different chemical modifications have been carried out to fulfill the inhibition of miRNA function *in vivo*. One class of antimiRs is conjugated to cholesterol (antagomiR) to facilitate cellular uptake. Other classes use oligonucleotides with with locked nucleotides acid (LNA-antimiRs) or the 2'-O-methoxyethyl phosphorothioate (2'-MOE) modification. Antagonism of miR-122

in mouse liver using these three classes of anti-miRs in three independent studies found that miR-122 antagonism led to reduced plasma cholesterol levels¹²⁹⁻¹³¹.

An important caveat to these new therapeutic approaches is the cell-tissue specificity. Furthermore, the regulatory actions mediated by miRNAs are complex since they can act both as positive or negative modulators, bind to hundreds of different targets, while each target may be regulated by several miRNAs. Indeed, the same miRNA can cause the opposite biological effect depending on the context, as exemplified by miR-221/221, (i.e. targeting important regulators of proangiogenic endothelial cell function (c-kit, eNOS) but also the tumor suppressor p27(Kip1) in cancer cells⁸⁴. Conversely, miR-17-92 cluster components have been shown to participate in EC-mediated angiogenic functions²⁰ and oncogenic functions as indicated by their upregulation solid tumors such as colorectal cancer, non-small cell lung cancers (NSCLC)⁷⁰. Taken together, anti-miRs targeting components of this cluster is a feasible strategy for both antitumor and anti-angiogenic therapy. The relative specificity of miR-126 expression in EC and its requirement for vascular integrity and angiogenesis^{17,22}, also suggests it may be a potential target for efficient anti-miR therapy in situations of pathological vascularization, such as retinopathy and cancer. However, overexpression of miR-126 must be carefully considered as there is no direct evidence regarding introduction of miR-126 in non-endothelial cells. This underscores the critical importance of cell/tissue-specific miRNA targeting. Therefore, both the inhibition and the mimicry of a miRNA in tissues other than diseased tissue must be considered. In regards miRNA therapeutics, there are many efforts to develop a more practical and specific strategy suitable for human therapy. A promising approach for siRNA, is to use targeting antibodies that undergo internalization after binding to cell specific surface receptors. To carry siRNA, antibodies can be decorated on liposomes pre-packaged with siRNA or fused to positively charged proteins or peptides that bind nucleic acids via electrostatic interactions¹³⁶⁻¹³⁹. Since miRNA mimics constructs are analogous to siRNA molecules, similar strategies could apply for miRNA mimic cell targeting.

Concluding remarks

miRNAs are a relatively recent discovery that emerged as important regulators of gene expression, and it appears that miRNAs are implicated in most, if not all, cellular processes and many human diseases. In the present review we have summarized the role of miRNAs in the regulation of angiogenesis and examined their potential applicability for the treatment of diseases associated with aberrant pathological angiogenesis (cancer or macular degeneration) or defective angiogenesis (myocardial ischemia or peripheral vascular disease). miRNAs constitute a fundamental regulatory network for fine-tune regulation of gene expression and therefore the maintenance of cellular functions necessary for an adequate angiogenic response. The extensive number of miRNAs and the unprecedented complexity guarantee the discovery of new and unanticipated roles of miRNAs in the control of angiogenesis. Genomics efforts, such as massive parallel miRNA and mRNA expression profiling in angiogenic-associated diseases in combination with loss- or gain-of-functions screens in EC, in combination with adequate target validation and large-scale proteomics are feasible approaches to help understand the complex miRNA-mediated gene regulatory networks in angiogenesis.

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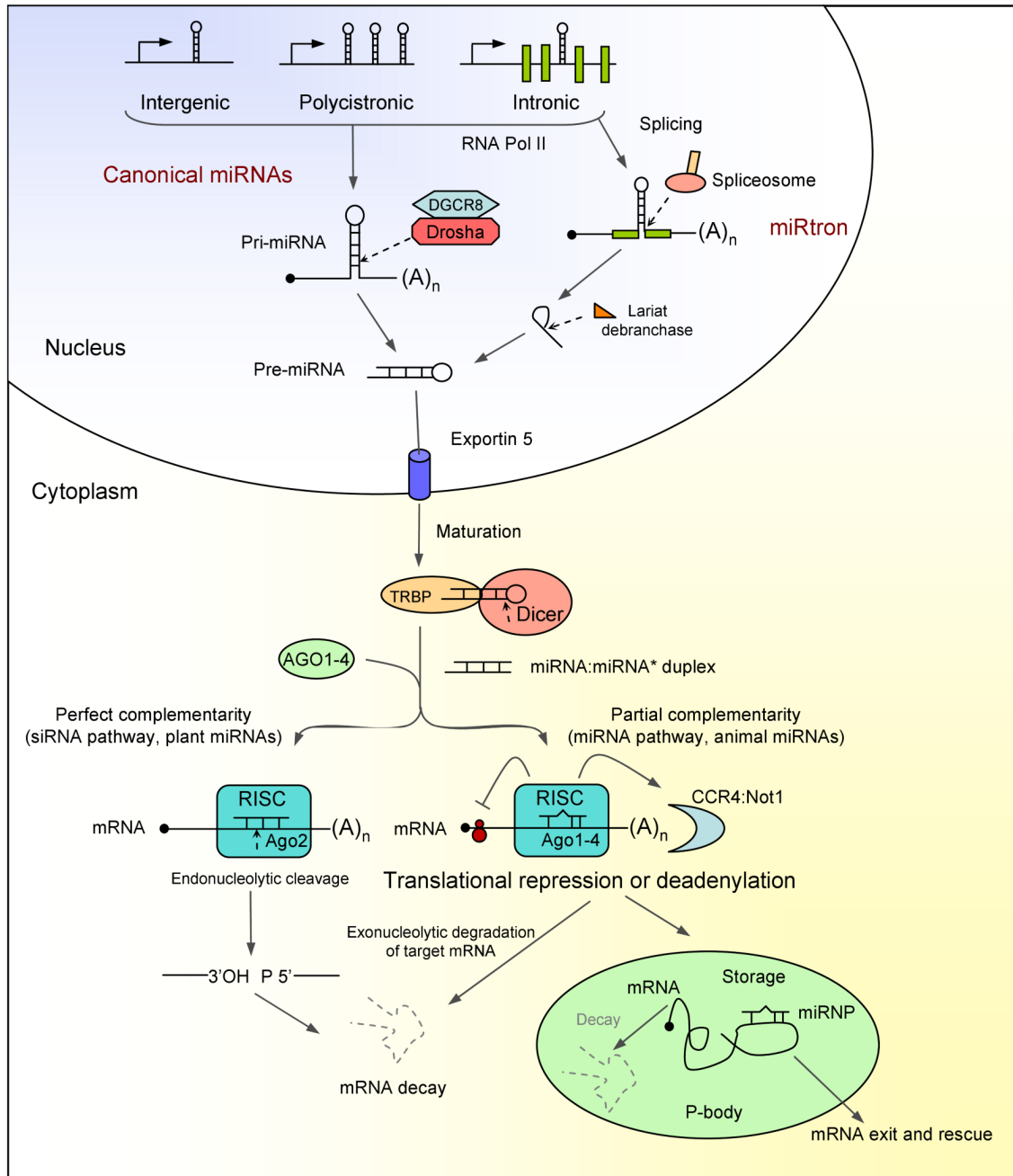


Figure 1. miRNA biogenesis and function

miRNAs originate in the nucleus as RNA polymerase II primary transcripts (pri-miRNAs), which are transcribed from independent miRNA genes, from polycistronic transcripts or from introns of protein-coding genes. Pri-miRNAs are then processed in two steps, catalyzed by the RNase III type endonuclease Drosha and Dicer. These enzymes function in complexes with dsRNA-binding domain proteins, DGCR8 and TRBP for Drosha and Dicer, respectively. Drosha-DGCR8 processes pri-miRNAs to ≈ 70 nucleotide hairpins known as pre-miRNAs. A subset of miRNAs, called miRtrons, also derived from introns, is processed into pre-miRNAs by the spliceosome and the debranching enzyme. Both canonical miRNAs and miRtrons are exported to the cytoplasm via Exportin 5, where they are further processed by Dicer-TRBP to

yield \approx 20-bp miRNA duplexes. One strand is selected to function as mature miRNA and loaded into the RISC, while the partner miRNA* strand is preferentially degraded. The mature miRNA leads to translational repression or mRNA degradation. The key components of the RISC are components of the Argonaute family (Ago 1-4). A fraction of miRNA* species can also access Ago complex and regulate targets. Perfect complementarity between miRNA and mRNA leads to an endonucleolytic cleavage, catalyzed by the human Ago2 in the RISC. This mechanism applies to siRNAs and many plant miRNAs. Animal miRNAs usually show only partial complementarity to the target mRNA promoting translational repression (initiation and post initiation steps) or deadenylation coupled to exonucleolytic degradation of target mRNA. mRNAs repressed by deadenylation or at the translation-initiation step are moved to P-bodies for either degradation or storage.

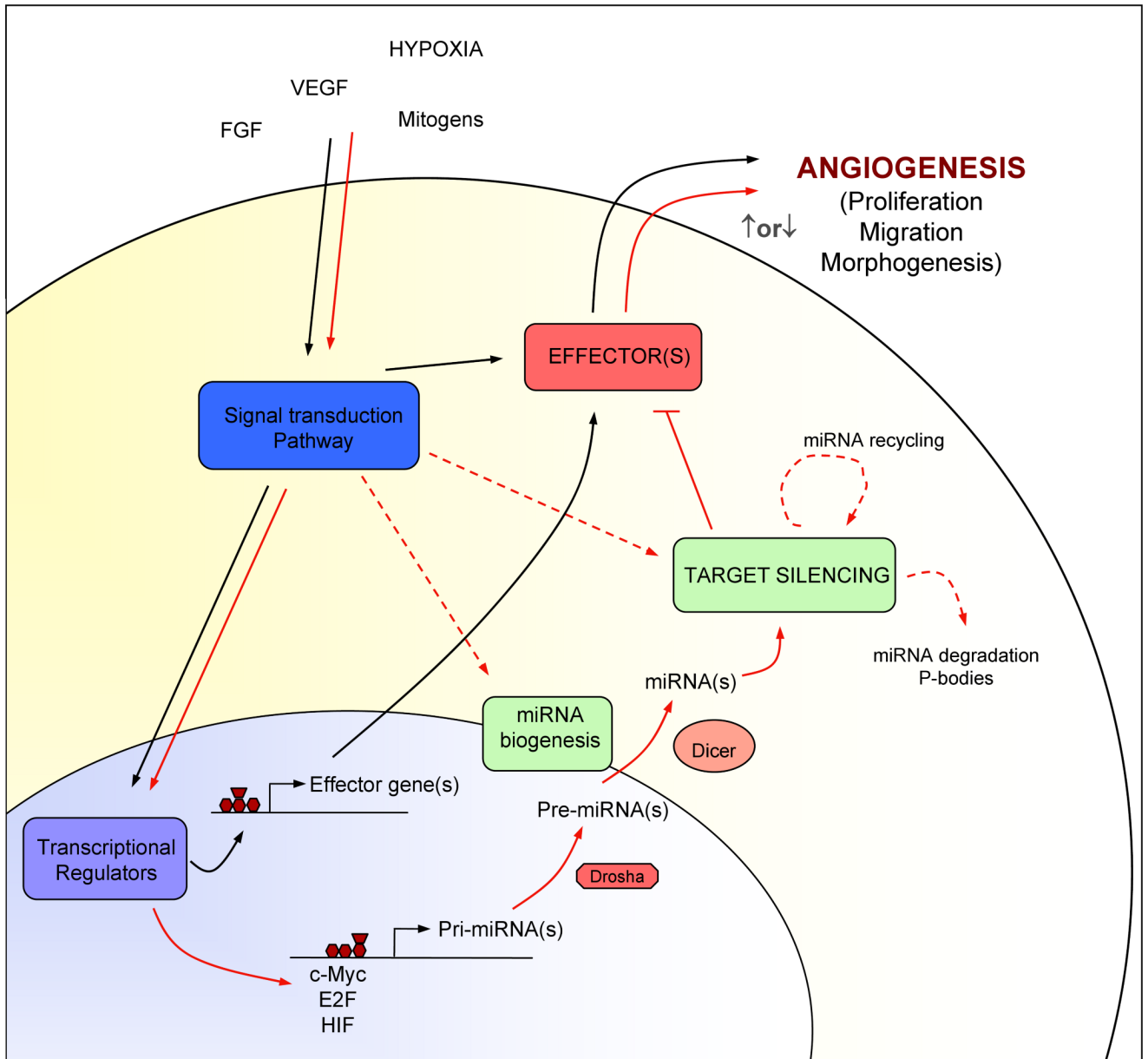


Figure 2. Function and regulation of miRNAs in angiogenesis

The schematic illustrates how stimuli such as FGF, VEGF, hypoxia or mitogens promote angiogenic phenotypes (black lines) and the potential role of how miRNAs may participate in this process (red lines). Extracellular signals activate signal transduction pathways that lead to an angiogenic response by direct activation of the specific effectors (MAPK, Akt, etc) or by induction of gene expression. In turn, the activation of the signal transduction pathways can modify the activity of a miRNA by affecting its expression, biogenesis and degradation (red dashed lines). miRNA-mediated regulation of angiogenic effectors promotes fine-tuning modulation of angiogenic responses via modulation of key effectors that promote angiogenic phenotypes such as proliferation, migration and/or morphogenesis.

Table 1
Compilation of miRNAs associated with angiogenesis

miRNA	Cell type	miRNA target(s) (direct or indirect ^a)	Function (by miRNA overexpression or inhibition) and putative role in angiogenesis	References
miR-221 miR-222	EC (HUVECs, EAhy 296) Prostate cancer cell lines	c-kit, eNOS ^b p27/Kip1	Overexpression reduces tube formation, migration and wound healing (scratch assay) in response to SCF. ↓EC-mediated angiogenesis. Induce proliferation and cell cycle progression. Inhibition reduces proliferation, increase p27 and reduces clonogenicity of cells. ↑Tumor induced angiogenesis	18· 19 84
let-7f miR-27b	EC (HUVECs)	ND	Inhibition reduces in vitro sprout formation ↑EC-mediated angiogenesis	16
miR-126	EC (HUVECs, HAEC, mouse ECs)	Spred-1, PIK3R2/p85-β, VCAM-1	Inhibition increases TNF-induced expression of VCAM-1 and leukocyte adhesion to ECs. Regulates vascular integrity and angiogenesis in miR-126 knockdown zebrafish and miR-126 ^{-/-} mice. Inhibition reduces tube formation, sprout formation, wound healing (scratch assay) and proliferation in response to VEGF and FGF. ↑EC-mediated angiogenesis	17· 22· 85
miR-130a	EC (HUVECs)	GAX, HOXA5	Overexpression antagonized the inhibitory effect of GAX on EC proliferation, migration and tube formation and the inhibitory effects of HoxA5 on tube formation ↑EC-mediated angiogenesis	107
miR-210	EC (HUVECs) Breast and colon cancer cell lines, nasopharyngeal carcinoma cell line,	EphrinA3 ND	Stimulated by hypoxia. Overexpression stimulates tube formation and migration. ↑EC-mediated angiogenesis	108 110· 111

miRNA	Cell type	miRNA target(s) (direct or indirect)	Function (by miRNA overexpression or inhibition) and putative role in angiogenesis	References
	head and squamous cell carcinoma		Stimulated by hypoxia. decrease proapoptotic signaling in a hypoxic environment. ↑Tumor induced angiogenesis	
miR-15 miR-16	Nasopharyngeal carcinoma cell line Chronic lymphocytic leukemia (CLL) Colon cancer cells	VEGF, Bcl2	Downregulated by hypoxia. Overexpression induces apoptosis in leukemic cell line model. Cell cycle regulation. Inhibition reduces number of cells G0/G1 promoting cell cycle progression. ↓Tumor induced angiogenesis	111' 115' 116
miR-378	Glioblastoma cells	Sufu, Fus-1	Overexpression promotes cell survival tumor growth and angiogenesis <i>in vivo</i> . ↑Tumor induced angiogenesis	140
miR-17-92 cluster	EC (HUVECs) Malignant lymphoma cells, Colorectal cancer cells, NSCLC Lymphocytes	Tsp-1 (miR-18a) E2F1 (miR-17-5p / miR-20a) CTGF (miR-18a, Tsp-1 (miR-19) PTEN, Bim	Stimulated by VEGF. Overexpression promotes cell proliferation and cord formation converse effects by inhibition. ↑EC-mediated angiogenesis Expression control by c-Myc and E2F. Promote cell proliferation and survival. Overexpression in colonocytes form larger and better-perfused tumors <i>in vivo</i> . ↑Tumor induced angiogenesis Overexpression contributes lymphoproliferative disease.	20 101' 120' 122' 123 127
miR-296	EC (human brain microvascular ECs)	HGS	Stimulated by glioma cells and angiogenic factors (EGF, VEGF). Inhibition reduces tube formation and migration (scratch assay) <i>in vitro</i> and angiogenesis in tumor xenografts <i>in vivo</i> . ↑EC-mediated and tumor induced angiogenesis	109

miRNA	Cell type	miRNA target(s) (direct or indirect)	Function (by miRNA overexpression or inhibition) and putative role in angiogenesis	References
miR-155	EC, VSMC, Fibroblast Breast cancer cells, malignant lymphoma cells, NSCLC Lymphocytes, macrophages	AT1R ND ND	Inhibition increases AT1R expression and Ang II-induced ERK1/2 activation. ? EC-mediated angiogenesis Required for normal immune and inflammatory responses	95, 141 97, 121, 122, 142, 143

ND= not determined