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miRNAs in endothelial cell signaling: the endomiRNAs

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Abstract

microRNAs (miRNAs) have a pivotal role during the formation and function of the cardiovascular system. More than 300 miRNAs have been currently found within the mammalian genome, however only few specific miRNAs, named endomiRNAs, showed conseved endothelial cell expression and function. In this review we present an overview of the currently know endo miRNAs, focusing on their genome localization, processing and target gene repression during vasculogenesis and angiogenesis.

Keywords

miRNA; development; signaling; endothelial cell; angiogenesis

miRNAs Biogenesis and Regulation

microRNAs (miRNAs) are highly conserved small non-coding RNAs (~22 nucleotide-nt-) that play an important role in the regulation of gene expression at the post-transcriptional level. miRNAs are located within introns and exons of protein coding genes or in intergenic regions. They are transcribed by RNA polymerase II as long primary miRNA transcripts (primiRNA) containing one or several hairpin structures with 5'CAP and polyadenylated tails[1]. In the nucleus, the pri-miRNA is cleaved by a microprocessor complex, the RNAse III enzyme Drosha and DGR8 [2]. This cleavage step results in an ~65 nt precursor miRNA (premiRNA) which is exported to the cytoplasm in association with Exportin-5 and cleaved by Dicer to a 18–24 nt duplex. Finally, this miRNA duplex is loaded into the RNA-induced silencing complex (RISC) together with the Argonaute (Ago) proteins. RISC can bind to the 3'-untranslated region (UTR) of the target mRNA on a partial miRNA-mRNA complementarity to the first 8 nt within the 5' miRNA end called the seed sequence [3]. This binding causes the degradation of the target mRNA and consequent translational inhibition [4]. miRNAs derived from intronic hairpins are called mirtrons and they bypass the cleavage step from Drosha while nuclear transport and cleavage are common to the canonical miRNA biogenesis pathway. The processing of the miRNAs is a tightly regulated mechanism involving regulatory proteins, editing of miRNA transcripts and leads to either elevated or decreased miRNA levels (Figure 1)[3]. These regulatory proteins can be subdivided in three groups: Drosha binding/associated proteins, Dicer binding proteins and proteins that bind to the terminal loop of the pri and/or pre-miRNAs. For example, the DEAD box helicase p68/

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p72 complex facilitates the processing of a set of miRNAs by binding Drosha[5]. This complex can be activated upon interaction with Smad factors after Transforming Growth Factor β (TGF-β) and Bone Morphogenesis Protein (BMP) stimulus[6]. Similarly, TAR RNA binding protein (TRBP) can interact and stabilized Dicer after phosphorylation mediated by the mitogen-activated protein kinase (MAPK) signaling pathway [7,8]. Therefore, alteration of miRNA processing by ERK may result in a pro-growth factor phenotype. Finally, many other RNA binding proteins such as LIN28 [8], hnRNP [9] and KSRP[10]can directly bind the terminal loop of different pre-miRNAs and modulate the cleavage of their mature sequence in different cell types or different stage of developments. The most recent mechanism discovered on miRNA editing and destabilization is the conversion of adenosine to inosine within the prior pre-miRNA by the adenosine deaminase ADAR11]. Splice variants of ADAR has been identified in the cardiac tissue suggesting a specific ADAR/miRNAs regulation in different cellular contexts. However, the expression and function of this protein in the cardiovascular system remains to be investigated.

Targeting miRNAs

To probe the in vitro and in vivo function of miRNAs many approaches has been developed to block the miRNAs activity. Currently, three strategies are used in miRNA loss-of function studies: genetic knockouts, miRNAs sponge, and antisense oligonucleotides. miRNA knockout allows the generation of animal systems with the whole mount or tissue specific deletion of candidate miRNA genes. The generation of knockouts in *C. Elegans* and *Drosophila* as well in mouse model has been extensively used to unravel the function of miRNAs in early embryonic development [12]. Recently, a genome-wide knockout resource covering 476 mouse miRNA genes was described and embryonic stem cells repertories has been made available to improve the studies of the miRNAs in vivo[13]. So far, the majority of the ~25 miRNAs mouse knockouts do not exhibit severe defects during embryonic development. Indeed, many miRNA families exist as duplicates or have identical seed regions, thus there remains the question of functional redundancy. In addition, genome deletion of a single miRNA encoded within a gene cluster is particularly difficult without effecting the expression of the others miRNAs. Alternative methods to perform gene knockout may overcome family complexity and redundancy of miRNA gene function. Recently improvements in artificial transcription activator-like effector nucleases (TALENs) provide a promising and powerful new approach for precise genome targeting of miRNAs genes [14]. miRNA sponges are transcripts with repeated miRNA antisense sequences that can sequester miRNAs from endogenous mRNA targets. Specific design of sponges can bind and sequestered all miRNAs seed family members or a single miRNA within a cluster offering additional advantages in the analysis of complex miRNAs activity. Sponge has been proven to induce transient, long term and tissue specific miRNAs inhibition in several animal models[15], however, the success of their activity is strongly dependent on the endogenous miRNA expression levels. Chemical modified antisense nucleotides are the most common approach to perform loss of miRNA function studies. A variety of commercial chemical modifications such as the 2’-O-Methil(2’-O-ME), 2’-O-methoxyethyl (2’-MOE) or locked nucleic acid (LNA) and morpholino oligonucleotides are now available. These modifications confer nuclease resistance and high binding affinity to target miRNAs [16]. These oligos named antimiR can target miRNAs at different stage of their biogenesis or block miRNAs activity by binding the mature miRNA sequence or by targeting the miRNA binding site within the target mRNA [17]. Several studies evaluated the efficiently of different modified antimiR mediated inhibition in both vitro and in vivo [16]. This strategy it has been used to block the activity of individual miRNAs while new strategies still have to be developed to block miRNA families. A recent publication has shown the efficiency of 8 nucleotides LNA modified phophotiorate nucleotide, named tiny LNA, complementary to the seed region of the target miRNAs. Tiny LNA inhibit individual

miRNA and/or miRNA families in culture cells and several tissues when delivered to the adult mouse[18]. AntimiR or tiny LNA oligonucleotides disadvantages are related to their transitory and partial inhibitory effect in addition to potential off-target effects.

Target prediction tools

Open-access bioinformatic databases have been developed to facilitate the analysis of miRNAs and their target prediction[19]. Prediction of miRNA–mRNA interactions is a challenging task, due to the short length of miRNAs, the requirement of only partial homology for binding, the redundancy among members of an miRNA family, and the existence of multiple putative miRNA recognition sites[20].The majority of the computational target prediction programs are based on several features, such as complementarity between the 5'-seed of the miRNA and the 3'-UTR of the target mRNA, thermodynamic stability of the miRNA–mRNA duplex, conservation among species, and the presence of several miRNA target sites. Several different *in silico* target prediction programs exist to identify miRNA-target prediction. Such prediction programs must be used carefully since i) the genome is not fully sequenced, ii) only a limited number of miRNA targets have been experimentally validated, and iii) there is low conservation among species regarding mature microRNAs sequence as well as the target prediction. Experimental high-throughput studies (such as chromatin marks and poly(A)-site mapping followed by RNA-Seq) are needed to allow for generation of precise prediction algorithms[21,22]

miRNA-target validation tools

Efficient experimental strategies are needed to validate computationally predicted miRNA target genes. To validate predicted miRNA–mRNA interactions, several experimental approaches have been used, such as biochemical methods (luciferase assays, qRT-PCR, western blot, RNA seq) "omics" approaches (SILAC, LAMP), and RISCome analysis (quantification of mRNAs in the RNA-induced sequencing complex; RISC RNA sequencing, RIP-chip)[23].Experiments performed in cultured cell lines are usually useful to validate microRNA effects on target mRNA. Recently, animal models have been also used to validate target mRNA in vivo. Novel techniques have been implemented in the zebrafish model such as fluorescent miRNA sensor and morpholino (MO) target protector. In the first case, validation of the target prediction is tested using a reporter assay based on monitoring GFP/mCherry fluorescence in zebrafish embryos microinjected with mRNA encoding the fluorescent reporter fused to the 3'-UTR of the target gene, in the presence or absence of specific miRNA duplex. In this assay, a decrease in fluorescence in the presence of miRNA duplex indicates miRNA-mediated repression and then the confirmation of the target in vivo. In the second case, MOs are designed to be complementary to specific miRNA-binding sites in target mRNAs. In this way MOs have been shown to efficiently protect the target mRNA from translational inhibition or degradation. To study the regulation of a particular target, it is important to first establish that the 3'-UTR is regulated by a particular miRNA. miRNAs may speed degradation or slow translation of their targets, but repression caused by either mechanism can be assessed by measuring protein output of a reporter. The efficiency of "target protectors" to block the interaction of miRNAs with a particular binding site in a target mRNA can also be investigated by co-injections with reporter mRNAs containing a GFP/mCherry coding sequence and a 3'-UTR region with the miRNA-binding site. Subsequently, a tested target protector can be used to study whether protection of a specific target mRNA from silencing has any biological effect also in a tissue-specific manner (by generation of a cell autonomous miRNA sensor).Recently, a proteomics approach to validate predicted miRNA targets in *C. Elegans* by using quantitative targeted proteomics via selected reaction monitoring(RIP-chip-SRM) has been reported[21]. This technique can be applied to validate candidate lists generated by computational methods or

in large-scale experiments, and the described strategy should be readily adaptable to other organisms.

miRNAs function in endothelial cells: the “endomiRNAs”

The mammalian genome encodes ~300 highly conserved miRNAs (~1–2 % of the genes) able to regulate, at least by bioinformatics prediction, over 60% of protein coding genes[24]. In the past 5 years this magnitude of gene regulation by miRNAs, has been revised based upon the numerous evidences that: 1- in animal system miRNAs are usually express in a non-correlated manner with their predicted mRNA targets [25]; 2- the majority of the miRNAs produces subtle proteins reduction (< 2 fold change) [26]; 3- many miRNAs can be deleted in vivo without causing evident phenotypes[27]. If so, what is the real inhibitory potential of miRNAs? Increasing evidence suggested that miRNAs confer robustness to complex signaling pathways, functioning as “buffers” to gene fluctuations. They can reinforce transcriptional programs and attenuate aberrant transcripts conferring accuracy and uniformity to developmental transitions, cell fate switches and stress-esponses[28]. The remodeling of the vascular system is a sophisticated result of the balance between stimulators and inhibitors pathway. These pathways are feed by a web of vascular growth factors and down stream proteins signaling tightly controlled by feedback loops and redundant components. For these properties the cardiovascular system is particularly sensitive to the regulation of miRNAs. In vitro and in vivo studies have showed that Dicer has an important function in angiogenesis. Dicer mutant mice embryos and yolk sack manifest angiogenic defects [29]. Accordingly, mice carrying a endothelial tissue specific deletion of Dicer show defects in postnatal angiogenic response to a variety of stimuli, including exogenous VEGF, tumors, limb ischemia and wound healing[30]. Finally, genetic silencing of Drosha expression in endothelial cells also results in a reduction in capillary sprouting and tube formation [31]. In this review, we will summarize the state-of-art on the major conserved families of miRNAs expressed in endothelial cells, the endo miRNAs miR-126 and themi R-17-92, miR-23-27-24 and miR-222-221 clusters (Figure 2).

miR-126

In 2008, three independent groups analyzed and reported defects in miR-126 deficient vascular cells, varying from zebrafish to human and mice[32,33]. miR-126 is the most important and possibly the only endothelial-specific miRNA in vertebrates. It is expressed in the vascular tree of vertebrate throughout development. In addition, miR-126 has been found as one of the most enriched microRNAs in cultured human endothelial cells and in endothelial cells derived from embryonic stem cells or isolated from mouse embryos. miR-126 has been clearly shown to be a master miRNA in vascular function working as a key positive regulator of angiogenic signaling in vitro and in vivo [32]. miR-126 is encoded in an intron of EGF-like domain 7 (Egfl7) gene. Egfl7 encodes a secreted matrix component that is produced by angiogenic stimuli and induces endothelial migration [34]. Since miR-126 is located within an intron of the Egfl7 gene, questions arose whether the vascular abnormalities seen in miR-126 studies are due to the loss of miR-126 or Egfl7 activity. Comparison between miR-126 and Egfl7 knock out models indicate that the severe vascular defects are only observable in animal deficient for miR-126 highlighting the requirement of miR-126 for the developing and postnatal vasculature. Remarkably, deletion of miR-126 in mice results in vascular developmental defects such as delayed angiogenic sprouting, widespread hemorrhaging, and partial embryonic lethality [33]. In addition, miR-126 mutant mice that successfully complete embryogenesis display diminished angiogenesis and increased mortality after coronary ligation, a model for myocardial infarction. Also endothelial cells deficient in miR-126 fail to respond to angiogenic factors, including VEGF, epidermal growth factor (EGF), and bFGF. In zebrafish, knockdown of miR-126 by MO

injections induced collapsed blood vessels and cranial hemorrhages in the developing organism suggesting its primary role for maintaining vascular structure during development [32]. Mechanistically, two direct targets of miR-126 that seem to explain these angiogenic defects are: Sprouty-related EVH1 domain-containing protein 1 (Spred1) and a regulatory subunit of PI3K, PIK3R2 (also known as p85 β)[33]. Because Spred1 and PIK3R2 are negative regulators of cellular signaling cascades, affecting the MAPK and PI3K signaling pathways, respectively, miR-126 promotes VEGF and other growth factor signaling by targeting multiple signaling pathways, miR-126 may fine-tune angiogenic responses. Spred1, which is a negative regulator of cell survival, was validated as a direct miR-126 target being expressed in miR-126 deficient zebrafish. Indeed, downregulation of miR-126 in endothelial progenitor cells from diabetes human patients, impairs their functional properties, via target gene Spred-1[35]. Recently, VEGF-A has been proposed as another important target of miR-126[36].More recent studies on zebrafish embryos suggested that miR-126 expression is under control of the mechano-sensitive zinc finger transcription factor klf2a. In this model, pulsatile flow induces expression of miR-126 within the endothelia cells to modulate VEGF signaling during the remodeling of the aortic arches [37]. Many endothelial miRNAs can be involved also in vascular inflammation in particular in leukocyte activation and their infiltration into the vascular wall. miR-126 is a good example. Indeed, a recent study provides first evidence that miRNAs control vascular inflammation since miR-126 inhibits the expression of vascular cell adhesion molecule 1 (VCAM-1), which mediates leukocyte adherence to endothelial cells. Thus, decreasing miR-126 in endothelial cells it is possible to increase TNFα-stimulated VCAM-1 expression and enhances leukocyte adherence to endothelial cells. Using the embryonic stem differentiation system to model primitive erythropoiesis and miR-126 null embryos, it has been found that miR-126 regulates the termination of EryP-CFC development in vivo by targeting VCAM-1[38].

miR-17-92 cluster

The miR-17-92 cluster is a polycistronic miRNA gene and encodes for 6 mature miRNAs namely miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a. All the members of this cluster are highly express in endothelial cells regulating vascular integrity and angiogenesis [39]. Interesting recent evidence showed that members of the miR-17-92 family are differentially expressed during endothelial cells differentiation of pluripotent stem cells. While miR-17, miR18a and miR-19a were increased upon induction of endothelial differentiation, miR-92a was decreased. Even so, the inhibition of the each these miRNAs did not affect endothelial cell differentiation similarly to miR-126 activity witch is required for endothelial cell function but not for endothelial differentiation [40]. Numerous studies focus on the endothelial cell function of miR-17-92 cluster revealed complex and controversial results. The entire cluster is highly up regulated in multiple human tumor cell types, however only miR-18a and miR-19a have a pro-angiogenic function during tumor angiogenesis. The overexpression of these miRNAs in tumor cell, repressed the secreted anti-angiogenic factors thrombospondin (TSP-1) and connective tissue growth factor (CTGF), inducing an increase of neovascularization in a paracrine fashion [41]. Interesting, these miRNAs showed an anti-angiogenic function when over expressed directly in endothelial cells. miR-17a, miR18a, miR19a and miR-20b inhibited endothelial cells sprouting when ectopically expressed, while individual miRNA knockdown promotes angiogenesis. In these experiments the protein kinase Jak1 was identified as the major target mRNA responsible for the pro-angiogenic effect [25]. Accordingly, the over expression of another member of the cluster, miR-92a inhibit vascular network formation in matrigel assay via targeting integrin α 5 (ITGA5) and indirectly suppressing eNOS production [42]. The anti or pro-angiogenic function of the miR-17-92 cluster seem strictly related to the cellular context. Interesting, the miR-17-92 members are also differentially regulate in endothelial

cells exposed to hemodynamic force [43]. miR-92a is down regulated in endothelial cells exposed to laminar flow while its mature sequence is up regulated by oscillatory flow consistently with its atheroprotective function in vivo [43]. The differential expression and therefore function of the single miRNAs derived from miR-17-92 cluster is mostly due to post-transcriptional regulation of the pri-miRNA [44]. Consequently, physiological and pathological conditions as well tissue dependent miRNA editing events can modulate the expression of the single miRNA belonging to the miR-17-92 cluster. Further investigations need to be performed to elucidate this aspect. Mouse targeted deletion of the miR-17-92 cluster reveals essential functions of these miRNAs during embryonic development. The functional analysis of the miR-17-92 cluster is complicated by the existence of two paralog clusters miR-106a-363 and miR-106b-25 generated by duplication event during evolution [45]. Mice lacking miR-17-92 or both miR-17-92 and miR106b-25, died shortly after birth showing lung hypoplasia and impaired B cell development due to an increased level of the pro-apoptotic Bim protein [45]. The characterization of the vascular development in these mice is currently missing. In addition, the endothelial specific deletion of miR-17-92 cluster will be necessary to elucidate the function of miR17-92 cluster during the embryonic vascular morphogenesis.

miR-23-27-24 cluster

The miR-23/27/24 cluster exist in the mammalian genome as duplicate gene located in different genomic location: the miR-23b, miR-27b and miR-24-1 are located within the intron region at the human chromosome 9q22.32 while the intergenic cluster miR-23a, miR-27a and miR-24-2 gene is localized at the chromosome 19q13.13. The mature sequence of the respective miR-23a/b, 27a/b and miR-24 1 and 2 are identical and highly conserved among vertebrates. The dissection of miR-23-27-24 gene expression and function is intrinsically complicated by their genome structure. For example, miR-24 pri-miRNA and the mature sequence is up-regulated after BMP2 stimulation in mesenchymal cells, while miR-27b and 23b are unaffected by this treatment suggesting that even if they are in close proximity within the genome the pri-miRNA of miR-24 and miR-23-27 are transcribed independently[45]. All the members of this cluster are highly enrich in vascularized organs and endothelial cells. Silencing of miR-23-27 inhibit the angiogenesis in response to VEGF by impairing the activation of MAP and PI3K-AKT kinase signaling pathway. Similarly, silencing of miR-23-27 suppresses choroid revascularization after laser injury[46,47]. miR-23 and miR-27 sequence possess different seed regions, therefore they can target diverse mRNA targets. However, in endothelia cells both miR-23-27 bind the Sprouty2 and sema6A 3’-UTR reinforcing the reciprocal inhibitory activity. The repressions of these two target genes by miR-23-27 serve to maintain an active RAC/RAF/ERK pathway after VEGF stimulation [46,47]. Intriguing, miR-24 expression is highly induced in endothelial cells after stress condition such as oxidative stress. Indeed, miR-24 activates pro-apoptotic signaling within the vasculature of the myocardium after myocardial infarction and in vivo treatment with antimiR against miR-24 improves vascularization and preserves cardiac function after myocardial infarction. GATA2 and p21 activated kinase Pak1 seem to be the major gene targets assigned to the miR-24/pro-apoptotic function during vascular remodeling [48]. Currently there are no mouse knockout models for the miR-23-27-24 cluster and therefore no data that suggests their function during embryonic vascular development. However, the down regulation of miR-27 in zebrafish embryos induced venous remodeling and angiogenesis of inter segmental vessel (ISV)defects. The miR-27 loss of function phenotype can be rescued by the repression of either Sprouty2 or Dll4 genes proposing these two genes as major miR-27 targets during zebrafish vascular development [49].

miR-222-221 cluster

miR-222 and 221 are highly conserved miRNAs transcribed from a common polyadenylated pri-miRNA located on the human chromosome X. Both miR-221 and miR-222 have a pro-proliferative effect on cancer cells [50]. miR-222 and miR-221 are also highly expressed in endothelial cells after growth factor stimulation or in the quiescent state [26]. While both miRNAs have the same seed region numerous studies identified different target genes controlled by miR-221 and miR-222. For example, compared with miR-222, miR-221 is not biological relevant for inflammation mediated by vascular growth factors [51] where during zebrafish embryonic development miR-222 is dispensable for vascular remodeling [52]. Similar observations were reported in a model of liver tumorigenesis, in which miR-221, but not miR-222 was able to accelerate tumor growth in mice [53]. The regulatory activities of this miRNA cluster seem to be cell type dependent. The inhibition of miR-222-221 decreases neointimal lesion formation but increases re-endotheliazation during hyperplasia following vascular injury. Indeed, miR-222-221 are both highly expressed in endothelial and vascular small muscle cells (VSMC). In VSMS this cluster have a pro-proliferative and pro-migratory effect by targeting two cell cycle dependent inhibitors p27 (cdkn1b/Kip1) and p57 (kip2) [54]. In contrast exogenous miR-222-221 shows anti-proliferative activity in human venous and lymphatic endothelial cells by targeting numerous target mRNAs depending on the experimental setting. These targets include ETS1 transcription factor [55], the stem cells factor receptor cKit [56] and the transcriptional repressor ZEB2 [57]. However, the molecular mechanism responsible for miR-222-221 mediated opposite cellular effects is currently unclear. Importantly, miR-221 expression varies significantly in response to both serum and VEGF treatment [25] generating discrepancies between different experimental conditions. The function of the miR-222-221 cluster during the mouse embryonic development has not been investigated yet. Recent studies using the zebrafish model showed that miR-221 is a NOTCH dependent miRNA required for endothelial tip cells proliferation and migration. miR-221 promotes endothelial cells sprouting by targeting two distinct target genes, PIK3R1 and p27. miR-221/p27 target regulation is required to coordinate proliferation while the tuning PI3K output by miR-221/PIK3R1 repression is fundamental for tip cell migration upon VEGFC-Flt4 activation[52].

Conclusion

This review summarizes current research progress and knowledge on the roles of miRNAs in regulating endothelial cell function and signaling. The discovery of miRNAs as regulators of vascular specific signaling pathways has created new options for the design of therapeutic agents that could modify gene expression in vascular-associated disease. Recently, it was discovered that extracellular miRNAs circulate in the bloodstream and that such circulating miRNAs are remarkably stable[58]. This has raised the possibility that miRNAs may be probed in the circulation and can serve as novel diagnostic markers for vascular-related diseases such as myocardial infarction, heart failure, atherosclerosis, hypertension, and type 2 diabetes. These discoveries are expected to present opportunities for clinical diagnostic and therapeutic approaches in miRNA-based vascular diseases.

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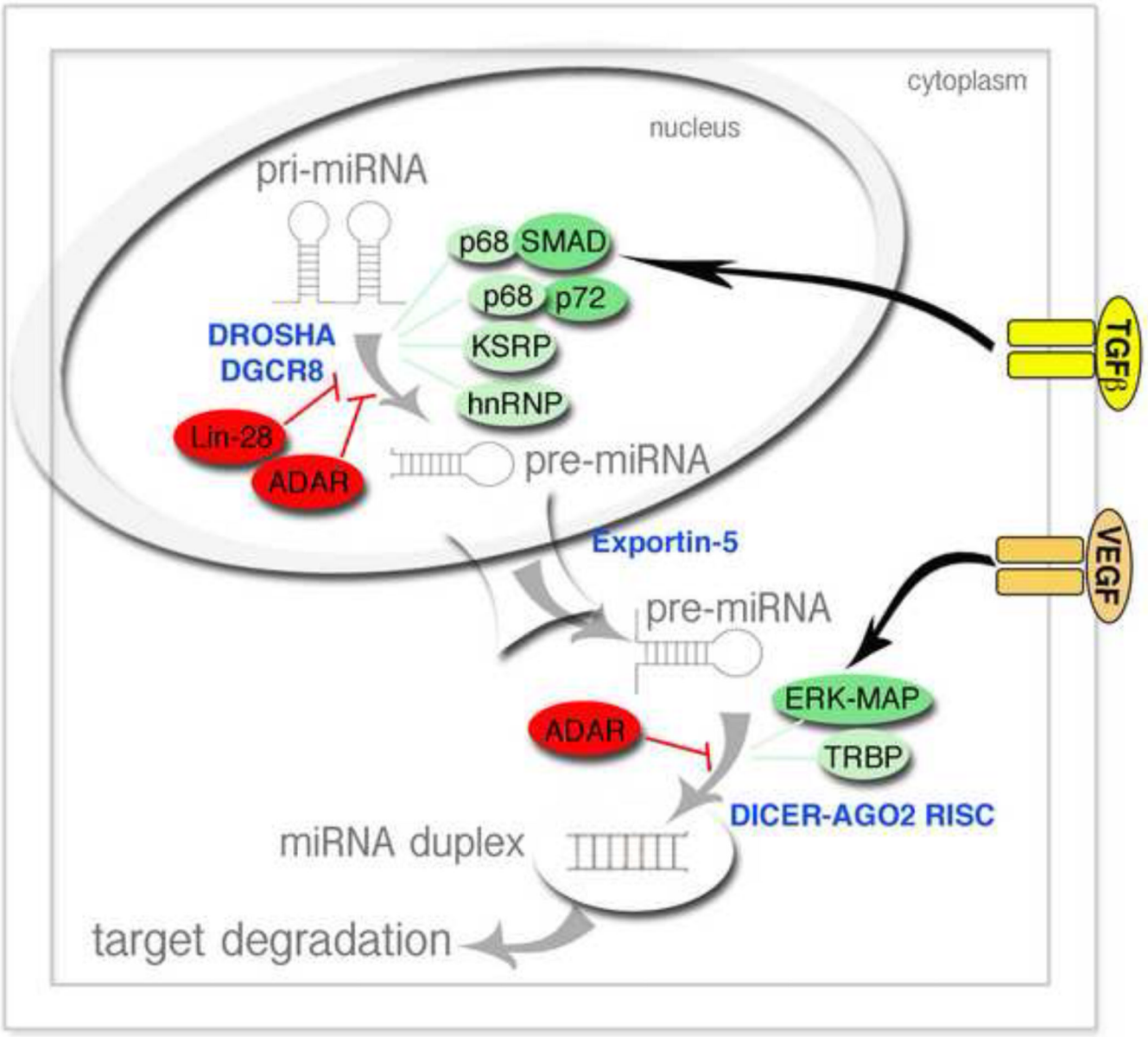

Figure 1. miRNAs processing and regulation

miRNA precursors (pri-miRNA) are processed by DROSHA and DGCR8 proteins into smaller RNA hairpins named pre-miRNAs. Protein-protein or RNA binding proteins are able to inhibit (Red) or promote (Green) pri-in to pre-miRNA processing with the nucleus. After export to the cytoplasm, pre-miRNAs are associated with the endo nuclease DICER and other regulatory protein such as TRBP. DICER cleaves the pre-miRNAs in a ~22 nt duplex miRNA which is incorporated in the RNA-Inducing–Silencing–Complex (RISC) where the mature miRNA associates with AGO2 to induce translation repression of the target mRNA. This process can be regulated by several growth factor singling pathways through ERK-MAP kinase or SMAD activation. hnRNP= heterogeneous nuclear riboprotein; KSRP= KH-type splicing regulatory protein; SMAD=mothers against decapentaplegic homolog; ADAR=adenosine deamenases acting in RNA.

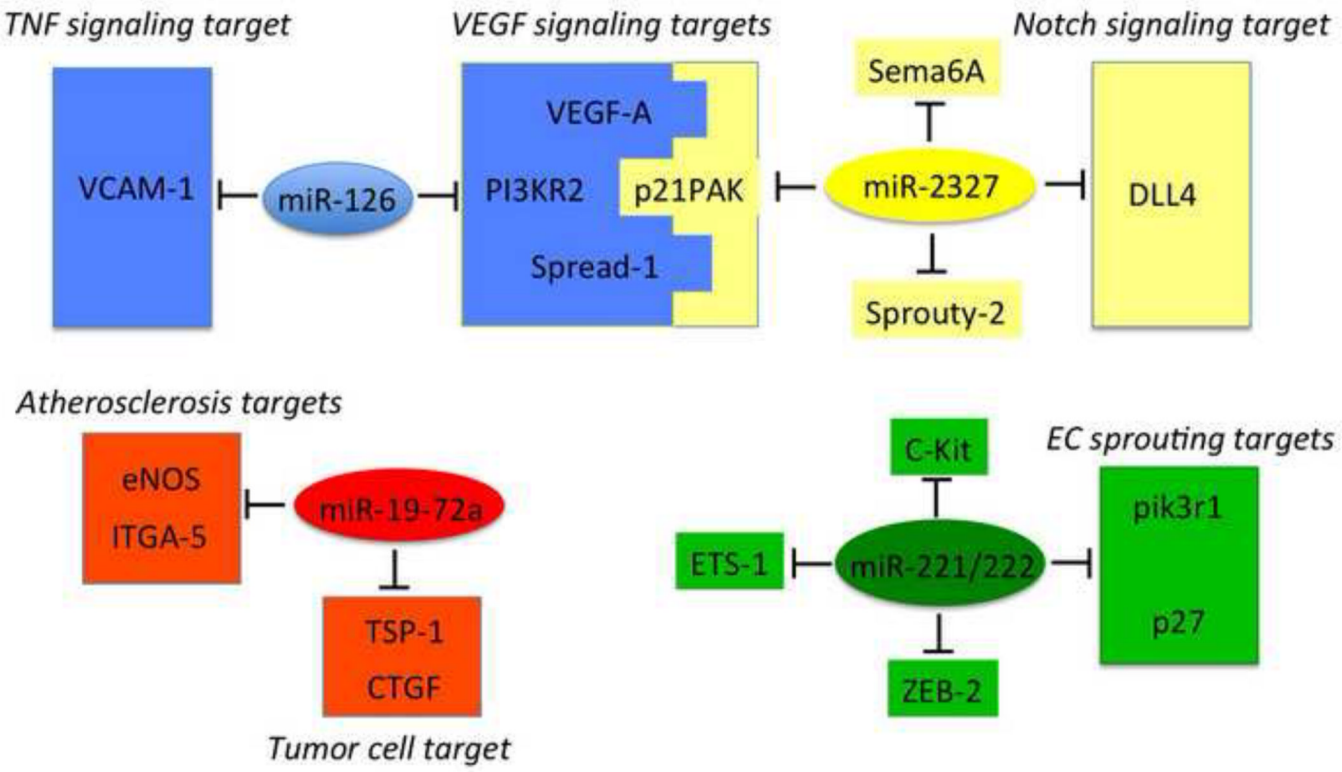

Figure 2. Schematic representation of the endomiRNAs and their targets

Four different microRNAs families have been implicated in controlling endothelial cell behavior and signaling. Endothelial microRNAs –endomiRNAs-include miR-126 and miR-23-27-24, miR-17-92 and miR-222-221 clusters. These families of microRNAs have been found to be expressed in endothelial cells (or cells that interact with the endothelium) and thus regulate endothelial cell responses. See text for detailed information.