VEGF-Induced Expression of miR-17–92 Cluster in Endothelial Cells Is Mediated by ERK/ELK1 Activation and Regulates Angiogenesis

Aránzazu Chamorro-Jorganes, Monica Y. Lee, Elisa Araldi, Shira Landskroner-Eiger, Marta Fernández-Fuertes, Mahnaz Sahraei, Maria Quiles del Rey, Coen van Solingen, Jun Yu, Carlos Fernández-Hernando, William C. Sessa, Yajaira Suárez

Rationale: Several lines of evidence indicate that the regulation of microRNA (miRNA) levels by different stimuli may contribute to the modulation of stimulus-induced responses. The miR-17–92 cluster has been linked to tumor development and angiogenesis, but its role in vascular endothelial growth factor–induced endothelial cell (EC) functions is unclear and its regulation is unknown.

Objective: The purpose of this study was to elucidate the mechanism by which VEGF regulates the expression of miR-17–92 cluster in ECs and determine its contribution to the regulation of endothelial angiogenic functions, both in vitro and in vivo. This was done by analyzing the effect of postnatal inactivation of miR-17–92 cluster in the endothelium (miR-17–92 iEC-KO mice) on developmental retinal angiogenesis, VEGF-induced ear angiogenesis, and tumor angiogenesis.

Methods and Results: Here, we show that Erk/Elk1 activation on VEGF stimulation of ECs is responsible for Elk-1-mediated transcription activation (chromatin immunoprecipitation analysis) of the miR-17–92 cluster. Furthermore, we demonstrate that VEGF-mediated upregulation of the miR-17–92 cluster in vitro is necessary for EC proliferation and angiogenic sprouting. Finally, we provide genetic evidence that miR-17–92 iEC-KO mice have blunted physiological retinal angiogenesis during development and diminished VEGF-induced ear angiogenesis and tumor angiogenesis. Computational analysis and rescue experiments show that PTEN (phosphatase and tensin homolog) is a target of the miR-17–92 cluster and is a crucial mediator of miR-17-92–induced EC proliferation. However, the angiogenic transcriptional program is reduced when miR-17–92 is inhibited.

Conclusions: Taken together, our results indicate that VEGF-induced miR-17–92 cluster expression contributes to the angiogenic switch of ECs and participates in the regulation of angiogenesis. (Circ Res. 2016;118:38-47. DOI: 10.1161/CIRCRESAHA.115.307408.)

Key Words: cell proliferation ■ endothelial cells ■ microRNAs ■ transcription activation ■ vascular endothelial growth factor A

Vascular endothelial growth factor-A (VEGF) is a major regulator of blood vessel formation and pathological processes.1 As a key proangiogenic factor for endothelial cells (ECs), VEGF regulates their proliferation, migration, and survival.1 During adulthood, the majority of ECs within vessels remain quiescent2 and only proliferate after angiogenic activation, mostly via VEGF stimulation.3

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MicroRNAs (miRNAs), important regulators of gene expression,4 modulate a wide range of biological processes, including angiogenesis.4 Although a large fraction of miRNAs is constitutively expressed, others can be dynamically regulated in ECs in
response to a variety of stimuli. However, the mechanisms by which different factors regulate the expression of specific miRNAs in ECs, in comparison with other cell systems, have not yet been studied and remain an area of considerable interest.

The miR-17–92 cluster is a polycistronic miRNA gene encoding 7 miRNAs. The cluster was initially described as an oncogene and was later demonstrated to drive key physiological responses during development and disease. Several laboratories, including ours, have shown that human ECs exhibit low baseline levels of miR-17–92 cluster members in quiescent condition that are induced on VEGF stimulation.

However, the mechanism by which VEGF stimulates the expression of the miR-17–92 cluster has not yet been examined. Members of the miR-17–92 cluster have been implicated in controlling different EC functions. However, the role of miR-17–92 cluster in regulating angiogenic EC functions is unclear and there is no genetic evidence for the role of endothelial miR-17–92 cluster in angiogenesis. Here, we report the mechanism by which VEGF stimulates the expression of the miR-17–92 cluster in ECs and the effect of endothelial postnatal genetic inactivation of this cluster on angiogenesis.

Methods

Cell culture (extended). Inhibition of miR-17–92 cluster in vitro, silencing RNA knockdown of Elk-1 or PTEN (phosphatase and tensin homolog), vector construction, transient transfection and reporter gene assay, RNA isolation, quantitative reverse transcription polymerase chain reaction, Western blot analysis, bioinformatics analysis (promoter analysis, transcription factor prediction, and miRNA target prediction analysis), chromatin immunoprecipitation assays, gene expression polymerase chain reaction array, flow cytometry analyses of apoptosis and bromodeoxyuridine incorporation, cell number assessment, fibrin gel bead assay, mice (extended), mouse lung and retina EC isolation, mouse retina vascular system analysis, tumor-induced neovascularization, ear angiogenesis, and statistical analysis are described in the online Data Supplement.

Cell Culture

Human umbilical vein ECs (HUVECs) were obtained from the tissue culture core laboratory of the Vascular Biology and Therapeutics program (Yale University, New Haven, CT). Human aortic ECs were from Lonza, Allendale, NJ.

Mice

To generate inducible vascular EC–specific miR-17–92 iEC-KO mice, we crossed miR-17–92 flox/flox mice with a tamoxifen-inducible Cre-recombinase (Cre-ERT2) under the regulation of vascular endothelial cadherin 5 promoter to achieve specific inactivation of miR-17–92 cluster in ECs.

Results

Regulation of MiR-17–92 Cluster Expression by VEGF

To analyze the effect of VEGF on miR-17–92 expression in ECs, we first measured the levels of miR-17–92 cluster primary transcript (pri-miR-17–92) in quiescent freshly isolated HUVECs and on stimulation with VEGF. Primary transcript levels were barely detectable in quiescent cells (t=0 hours), but strongly stimulated when ECs lose their quiescent state after culture in the presence of VEGF–A (t=2, 4, and 6 hours; Figure 1A). Concomitantly, the levels of all 7 components of the cluster were increased on VEGF stimulation (Figure 1B). As the miR-17–92 cluster is conserved in the mouse genome, we also established that the loss of quiescence in freshly isolated mouse ECs via stimulation with VEGF was accompanied by an induction of pri-miR-17–92 as well as its 7 mature miRNAs (Online Figure VA). Similarly, the expression of pri-miR-17–92 was also stimulated by VEGF in HUVECs that were previously serum starved (Online Figure IA). Furthermore, when HUVECs were pretreated with actinomycin D, before VEGF stimulation, the induction of pri-miR-17–92 expression was not observed (Figure 1C). These data indicate that VEGF transcriptionally induces the expression of the miR-17–92 cluster in ECs.

To ascertain the mechanism by which VEGF regulates miR-17–92 cluster expression in ECs, we transfected HUVECs with a luciferase reporter vector harboring 1353-bp upstream of the miR-17–92 gene. We observed increased promoter activity in ECs treated with VEGF (Figure 1D). In VEGF-stimulated conditions, deletions of the full-length promoter fragment revealed a reduction in promoter activity with the –805 bp fragment, suggesting the presence of cis-acting elements between –975 and –805 bp (Figure 1D). Sequence promoter analysis for cis-acting elements within this region identified Elk-1 as a potential transacting factor of miR-17–92 promoter activation by VEGF. Elk-1 belongs to the ETS (E-twenty-six) domain family proteins and plays a critical role in gene expression on mitogen stimulation and activation of the mitogen-activated protein kinase cascade. Interestingly, we observed an increase of Elk-1 phosphorylation on VEGF stimulation of ECs (Figure 1E). PD098059-mediated inhibition of MEK1/2, a direct upstream modulator of extracellular signal regulated kinase (ERK), blocked VEGF-induced activation of both ERK and Elk-1 (Figure 1F), and abrogated the VEGF-induced upregulation of pri-miR-17–92 expression (Figure 1G). Overexpression of Elk-1 also elicited a stimulatory effect on promoter activity in non-VEGF stimulated conditions (Figure 1H). In contrast, stimulation with neither VEGF nor Elk-1 overexpression increased miR-17–92 promoter activity when the Elk-1-binding site was deleted from the promoter reporter construct (Figure 1H). Furthermore, silencing of Elk-1 (Online Figure II) significantly reduced VEGF-mediated stimulation of miR-17–92 promoter activity (Figure 1I) and pri-miR-17–92 expression (Figure 1I). In line with these findings, using chromatin immunoprecipitation analysis, we demonstrated that on VEGF stimulation of ECs, Elk-1 binds to the miR-17–92 promoter region (Figure 1K). Taken together, these results indicate that the VEGF/ERK/ELK-1 pathway is involved in the transcriptional regulation of the miR-17–92 cluster in human ECs. In addition to VEGF, fibroblast growth factor 2, a well-documented angiogenic growth factor that stimulates the mitogen-activated protein
kinase pathway,\textsuperscript{21} slightly induced expression of the miR-17–92 cluster in HUVECs (Online Figure IIIA). Altogether, these data indicate that growth factor activation of the mitogen-activated protein kinase pathway is one mechanism to induce expression of miR-17–92 cluster in ECs.

Our results indicate that VEGF stimulates the expression of miR-17–92 cluster in human macrovascular venous ECs (Figure 1A and 1B; Online Figure I) as well as in mouse lung ECs (Online Figure VA), which are mostly microvascular. We also tested if the same regulatory mechanisms observed mostly in the venous context were present in arterial ECs. Interestingly, human aortic ECs stimulated with VEGF did not exhibit a significant upregulation of pri-miR-17–92 expression (Online Figure IIIB).

**Regulation of EC Angiogenic Responses In Vitro by VEGF-Induced MiR-17–92 Expression.** We next assessed if VEGF-mediated upregulation of the miR-17–92 cluster participates in regulating EC angiogenic responses in vitro. Inhibition of the miR-17–92 cluster (Figure 2A) resulted in reduced EC proliferation, evaluated by cell number (Figure 2B) and bromodeoxyuridine incorporation (Online Figure IVA). To test if induction of cell death mediates the reduction in cell number on miR-17–92 cluster inhibition,
apoptosis was blocked by preincubation with the caspase inhibitor z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone). Because z-VAD-FMK addition did not affect cell number (Online Figure IVB and IVC), we concluded that the effect on cell number was not because of cell death. By using the fibrin gel bead assay that recapitulates several crucial steps of angiogenesis22 (Figure 2C–G), we found that inhibition of the miR-17–92 cluster in VEGF-stimulated conditions decreased cumulative sprout length (Figure 2D), number of sprouts per spheroid (Figure 2E), and the early formation of branch points (Figure 2F). However, inhibition of the miR-17–92 cluster increased the number of detached cells (Figure 2G), indicative of vessel regression.22 Taken together, our results indicate that in VEGF-stimulated conditions, miR-17–92 cluster is required for proliferation and angiogenic sprouting, and therefore participates in the angiogenic switch of the quiescent endothelium.

**Role of MiR-17–92 Cluster in Angiogenesis**

Even though the miR-17–92 cluster was among the first miRNAs linked to tumor14 and corpus luteum angiogenesis,23 there is no genetic evidence for the role of endothelial miR-17–92 cluster in angiogenesis. We tested the effect of postnatal inactivation of miR-17–92 cluster in the endothelium of developing mouse retinal vasculature because this is a model highly dependent on VEGF24,25 and useful for analyzing the molecular and cellular mechanisms regulating angiogenesis.17 Interestingly, we found that miR-17–92 cluster is highly expressed in mouse retina ECs at early stages and declines as development proceeds (Online Figure VB). Consistently, several studies have shown that miR-17–92 cluster is highly expressed in mouse embryonic stem cells, with expression levels decreasing during embryonic development.26,27 Furthermore, in a human embryonic stem cell vasculogenesis model, the expression of all members of the miR-17–92 cluster is reduced after 21 days of differentiation.10 These reports support the idea that high levels of miR-17–92 cluster may promote cell proliferation and, therefore, could participate in promoting endothelial angiogenic response.

Endothelial postnatal inactivation of miR-17–92 cluster resulted in significant reduction of miR-17–92 cluster members (Online Figure VC). As such, retinal angiogenesis was impaired, as shown by reduced endothelial coverage and delayed radial outgrowth (Figure 3A and 3B, 3D–3G; Online Figure VI), without affecting growth (Figure 3C). Overall, miR-17–92 IEC-KO retinas exhibited significantly less branching, fewer segment numbers, fewer hole numbers, and shorter vessel length (Figure 3D–3G; Online Figure VI). Endothelial tip cells, localized at the retinal front during development, are characterized by specialized apical filopodia and are vital for the development of new capillaries.25 miR-17–92 deletion significantly reduced the number of filipodia projections at the leading edge of the growing vascular front (Figure 3H and 3I). Importantly, the endothelial loss of miR-17–92 decreased proliferation, quantified by nuclear phosphohistone

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Inhibition of microRNA-17–92 cluster decreases proliferation and angiogenic sprouting in endothelial cells. Human umbilical vein endothelial cells (HUVECs) were transfected with 70 nmol/L mix of miR-17–92 inhibitors (Inh-miR-17–92) or control inhibitor (Cl). A, Quantitative reverse transcription polymerase chain reaction analysis of individual miR-17–92 cluster members analyzed 36 hours post-transfection. Data are expressed as relative miRNA levels vs CI. B, Total number of cells, 24 or 48 hours post-transfection. C–G, Fibrin gel bead assay was performed 36 hours post-transfection. HUVECs were allowed to undergo morphogenesis for 2 days in the presence of EGM-2 (endothelial cell growth factor medium 2) media containing VEGF (vascular endothelial growth factor). C, Representative images of HUVECs bead spheres. D–G, Quantification of angiogenic sprouting: (D) cumulative sprout length, (E) number of sprouts, (F) number of branch, and (G) number of detached cells. Data are expressed as mean±SEM of 3 independent experiments. *P≤0.05, compared with cells transfected with CI (Scale, 84 μm).
Figure 3. Postnatal deletion of microRNA-17–92 cluster in the endothelium reduces developmental retinal angiogenesis. Effect of postnatal endothelial-specific miR-17–92 cluster deletion on retinal vasculature (mice injected with tamoxifen P1–P3, assessed at P6; control n=10; miR-17–92 iEC-KO n=17). A, Retinal whole mounts, isolectin B4 (Iso b4) immunostaining representative images. B, Radial vessel extension quantification. C, Effect of endothelial-specific miR-17–92 cluster deletion on overall growth (weight). D–G, Quantification of the effects of postnatal endothelial-specific miR-17–92 cluster deletion on retinal vasculature: (D) number of branch point, (E) number of segments, (F) vessel length, and (G) number of holes. H, Retinal vasculature front, IsoB4 immunostaining representative images (magnification ×63). I, Filipodia was quantified as number of filipodia per view. J, Retinal vasculature front, IsoB4 and collagen IV (COL IV) immunostaining representative images. K, Proliferation was quantified as a ratio of PH3-positive endothelial cells (ECs) to IsoB4-positive vessels. L, Retinal vascular plexus, IsoB4 and collagen IV (COL IV) immunostaining representative images. M, Vessel regression was quantified as number of empty sleeves COL IV–positive and IsoB4-negative. J and L, Magnification: ×20. For each retina, 4 images were quantified. Data are expressed mean±SEM.*P<0.05, compared with control littermates.

3 staining (Figure 3J and 3K) and induced vessel regression, as denoted by an increased number of collagen IV–positive, isolectin B4 (Iso b4)–negative empty sleeves (Figure 3L and 3M). Interestingly, the number of apoptotic ECs was similar in control and miR-17–92 iEC-KO mice (Online Figure VII). Therefore, premature vessel regression observed in miR-17–92 iEC-KO is likely a consequence of reduced cell proliferation rather than induced apoptosis.22,23 These data indicate that loss of miR-17–92 cluster reduces retinal vascular coverage. These results show, for the first time, that endothelial expression of miR-17–92 is an important proangiogenic signal during embryonic and retinal development, it may also play a role in the mature circulatory system. To ascertain this, we examined the importance of miR-17–92 cluster in VEGF-induced angiogenesis, a reduction of platelet/endothelial cell adhesion molecule 1–positive structures (in cross-sections; Figure 4C and 4D) in the tumor-associated vasculature was observed in miR-17–92 iEC-KO, as well as an overall reduction of tumor growth (weight; Figure 4E). Here, we show for the first time that miR-17–92 cluster participates in tumor-induced angiogenesis by regulating VEGF-induced angiogenic EC functions. Altogether, these data suggest that miR-17–92 cluster participates in the regulation of both developmental angiogenesis, as well as angiogenesis during adulthood.

Targeting Activity of MiR-17–92 Cluster

To date, several targets have been described, in ECs, for individual members of the miR-17–92 cluster.13,15 Our data indicate that, as a result of angiogenic activation of ECs, miR-17–92 is transcriptionally stimulated; therefore, the expression of individual member of the entire cluster is increased. As indicated, miR-17–92 cluster consists of 7 highly conserved miRNAs and this complicates the identification of a bona fide unique target that may explain the role of miR-17–92 as a unit.
The 7 miRNAs in the cluster can be grouped in 4 different seed families and, therefore, 4 different target specificities. To investigate the targets of miR-17–92 cluster that may explain the observed antiangiogenic phenotype after its inhibition in vitro or conditional deletion in vivo, we performed a bioinformatics analysis to identify targets common to the 4 different seeds (Figure 5A), conserved between human and mouse, and relevant to ECs (Figure 5A). From this unbiased bioinformatics analysis, we identified 4 targets expressed in ECs that are common to the 4 seed families and conserved in human and mouse. On the basis of a bibliographic search of the role of the miR-17–92 cluster, 4 candidate targets were chosen to explain the antiangiogenic phenotype observed in vivo.

Concomitant inhibition of miR-17–92 cluster and PTEN overexpression RNA considerably reduces the levels of PTEN to a similar extent than in control inhibitor condition or after miR-17–92 cluster inhibition9 (Online Figure VIIIA). Overall, by analyzing gene expression with VEGF stimulation, we found that HUVECs with inhibited expression of the miR-17–92 cluster in the endothelium reduces vascular endothelial growth factor (VEGF)-induced ear angiogenesis and tumor-induced angiogenesis. We have overexpressed or silenced PTEN together with inhibition of miR-17–92 cluster. As expected, overexpression of PTEN drastically reduced proliferation of ECs both in control inhibitor condition and on inhibition of miR-17–92 cluster. Concomitant inhibition of miR-17–92 cluster and PTEN overexpression does not further affect proliferation compared with its respective control inhibitor (Figure 5D). Therefore, these findings are in agreement with a previous work that shows that overexpression of PTEN inhibited the proliferative and angiogenic effects of VEGF50 and suggest that overexpression of PTEN overcomes the effects of miR-17–92 inhibition. Conversely, silencing of PTEN significantly increases proliferation (Figure 5E). However, although the difference in proliferation between control inhibitor and miR-17–92 inhibitor is ~20% in nonsilencing control conditions, the defect in proliferation in miR-17–92 inhibition is partially rescued when PTEN is silenced (~11% versus ~20%; Figure 5E). Because the silencing RNA considerably reduces the levels of PTEN to a similar extent than in control inhibitor condition or after miR-17–92 cluster inhibition, the differences we observe in proliferative responses are likely because of additional targeting activity of the miR-17–92 cluster. To gain a global insight on the effects of miR-17–92 cluster inhibition on angiogenic programs, we performed an expression analysis of genes implicated in the regulation of angiogenesis in HUVECs cultured in the presence of VEGF with or without miR-17–92 cluster inhibitors. As expected, the expression of the negative regulator of angiogenesis thrombospondin 1 was increased on miR-17–92 cluster inhibition5 (Online Figure VIII A). Overall, by analyzing gene expression with VEGF stimulation, we found that HUVECs with inhibited expression of the miR-17–92 cluster
Figure 5. MicroRNA-17-92 (miR-17-92) cluster targeting unit regulates phosphatase and tensin homolog (PTEN) expression and promote and angiogenic phenotype in endothelial cells (ECs). A, Schematic representation of the polycistronic miR-17–92 cluster and its host gene. The 4 seed families are indicated in different colors. Common targets for each seed sequence of the miRNA-17–92 cluster were obtained from miRNA target prediction algorithms. Targets common for all the seed sequences of the miRNA-17–92 cluster were found for mouse (16 genes) and human (82 genes). Among the targets common for all seeds, 5 were conserved among human and mouse. (Continued)
were predicted to have a less angiogenic phenotype than their noninhibited controls. In fact, the expression pattern of most genes analyzed indicates that the pathways of angiogenesis and proliferation of ECs were reduced in EC where miR-17–92 was inhibited (Online Figure VIIIB). Altogether, our present data indicate that VEGF stimulates miR-17–92 to promote angiogenic functions of ECs (Figure 5F).

Discussion

A growing body of evidence indicates that precise regulation of miRNA expression is critical for diverse physiological and pathophysiological processes. In this study, we have investigated the mechanism by which VEGF regulates the expression of miR-17–92 cluster in ECs to determine its contribution to the regulation of angiogenic functions of ECs. Our present results indicate that the VEGF/ERK/Elk-1 pathway is involved in the transcriptional regulation of the miR-17–92 cluster in ECs. Previous studies in other cell types have shown that the miR-17–92 cluster is transcriptionally regulated by different transcription factors, including c-Myc.34,35 Under physiological conditions, c-Myc expression is dependent on mitogens. This control is lost in cancer cells, resulting in elevated levels of c-Myc oncoprotein, which in turn induces the expression of the miR-17–92 cluster.35 c-Myc regulation of the miR-17–92 cluster is not likely to be operative in primary ECs because in normal cells, c-Myc activation triggers the apoptotic program.36 In the context of ECs, it has been reported that histone deacetylase 9 participates in the repression of miR-17–92 cluster,37 therefore it is possible that histone deacetylase 9 dissociates from the miR-17–92 cluster promoter in VEGF-stimulated conditions. Interestingly, fibroblast growth factor 2 also induced the expression of the miR-17–92 cluster ECs although in a less efficient manner. Given that fibroblast growth factor also stimulates the mitogen-activated protein kinase pathway21 in ECs, it is likely that Elk-1 participate in its transcriptional regulation. However, further investigation is needed to reveal if additional mechanisms are involved in fibroblast growth factor–mediated regulation of miR-17–92 cluster in ECs.

Each vascular bed has unique structural and functional properties. Indeed, ECs lining distinct blood vessels behave differently.38 We have shown that VEGF stimulates the expression of miR-17–92 cluster human macrovascular venous ECs as well as in mouse lung ECs, which are mostly microvascular, but did not affect its expression in arterial ECs. Several lines of evidence suggest a role for hemodynamics in mediating arterial/venous identity;39 however, studies in zebrafish embryos suggest that many artery- and vein-specific properties are epigenetically programmed before the onset of blood flow.40 An epigenetically repressed promoter may explain the lack of upregulation of pri-miR-17–92 on VEGF stimulation in arterial ECs, therefore, suggesting different roles of miR-17–92 depending on the vascular bed. Further investigation is warranted to ascertain the nature of these differences and their physiological relevance.

Previous reports have shown that single overexpression of pre-miR-17, pre-miR-18a, pre-miR-19a, or pre-miR-20a significantly inhibits 3-dimensional spheroid sprouting in vitro, whereas their individual inhibition has an opposite outcome.15 This is in contrast to our present and previous findings9 that indicate that in VEGF-stimulated conditions, miR-17–92 cluster is required for proliferation and angiogenic sprouting, and therefore participates in the angiogenic switch of quiescent endothelium. A possible explanation for these discrepancies is that miR-17–92 levels vary significantly in response to VEGF, suggesting that growth and culture conditions influence basal miRNA expression, and therefore, cluster function in cultured ECs. Given the potential for opposing effects of the miR-17–92 miRNAs depending on cellular compartment,9,14,15,23 we postulated that deciphering the role of this cluster in angiogenic functions in vivo might required a genetic cell type–specific deletion. We generated a mouse model of inducible deletion of the miR-17–92 cluster in the vascular endothelium and we analyzed its effects on models of angiogenesis that are highly dependent on VEGF (e.g., retinal angiogenesis, VEGF-induced ear angiogenesis, and tumor-induced neovascularization). Postnatal inducible deletion of the miR-17–92 cluster reduced normal retinal angiogenesis. Because VEGF levels are increased in patients with active proliferative diabetic retinopathy,29 one might speculate that miR-17–92 levels in ECs would increase and lead to pathological angiogenesis. However, in the murine retinopathy of prematurity model, miR-17/20 downregulation in whole retina has been linked to increased angiogenesis and increased VEGF secretion by retinoblastoma cells.41 The proposed targeting effect of VEGF is likely to occur on glial and Muller cells, which are responsible for the majority of VEGF secretion,28,42 and not directly on ECs. In fact, one could hypothesize that miR-17/20 downregulation in retinal non-ECs promotes VEGF secretion to induce angiogenic responses in ECs, in part, via miR-17–92 upregulation, as indicated by our present findings. This corroborates the notion that modulation of miR-17–92 expression is specific in different cell types and physiological situations, and governs context-specific and developmental changes in endothelial behavior. However, the miR-17–92 cluster, also known as oncomir-1, is among the most potent oncogenic miRNAs.11,12
Disclosures

None.

References

1. Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005;438:932–936. doi: 10.1038/nature04478.
2. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol. 2007;7:805–815. doi: 10.1038/nri2171.
3. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215–233. doi: 10.1016/j.cell.2009.01.002.
4. Suárez Y, Sessa WC. MicroRNAs as novel regulators of angiogenesis. Circ Res. 2009;104:442–454. doi: 10.1161/CIRCRESAHA.108.191270.
5. Brock M, Trenkmann M, Gay RE, Michel BA, Gay S, Fischler M, Ulrich S, Speich R, Huber LC. Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. Circ Res. 2009;104:1184–1191. doi: 10.1161/CIRCRESAHA.109.197941.
6. Suárez Y, Wang C, Manes TD, Pober JS. Cutting edge: TNF-induced microRNAs regulate TNF-induced expression of E-selectin and intercellular adhesion molecule-1 on human endothelial cells: feedback control of inflammation. J Immunol. 2010;184:21–25. doi: 10.4049/jimmunol.0902369.
7. Chamorro-Jorganes A, Araldi E, Penalva LO, Sandhu D, Fernández-Hernando C, Suárez Y. MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. Arterioscler Thromb Vasc Biol. 2011;31:2595–2606. doi: 10.1161/ATVBAHA.111.236521.
8. Chamorro-Jorganes A, Araldi E, Rotllan N, Cirera-Salinas D, Suárez Y. Autoregulation of gypician-1 by intronic microRNA-149 fine tunes the angiogenic response to FGF2 in human endothelial cells. J Cell Sci. 2014;127:1169–1178. doi: 10.1242/jcs.130518.
9. Suárez Y, Fernández-Hernando C, Yu J, Gerber SA, Harrison KD, Pober JS, Iruela-Arispe ML, Merkenschlager M, Sessa WC. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Nat Med. 2008;105:14082–14087. doi: 10.1073/pnas.0804457105.
10. Anand S, Majeti BK, Acevedo LM, Murphy EA, Muktahvaram R, Scheppe L, Huang M, Shields DJ, Lindquist JN, Lapinski PE, King PD, Weis SM, Chereda DA. MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. Nat Med. 2010;16:909–914. doi: 10.1038/nm.2186.
11. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell. 2008;133:217–222. doi: 10.1016/j.cell.2008.04.001.
12. Mogiljansky E, Rigoutsos I. The miR-17/92 cluster: a comprehensive up-regulate on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. Cell Death Differ. 2013;20:1603–1614. doi: 10.1038/cdd.2013.125.
13. Bonauver A, Carmona G, Iwasaki M, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science. 2009;324:1710–1713. doi: 10.1126/science.1174381.
14. Deng M, Homayouni A, Yu D, Murphy D, Savignani C, Wentzel E, Furth EE, Lee WM, Enders GH, Mendell JT, Thomas-Tikhonenko A. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat Genet. 2006;38:1060–1065. doi: 10.1038/ng.1855.
15. Doebele C, Bonauver A, Fischer A, Scholz A, Reiss Y, Urbich C, Hofmann WK, Zeiher AM, Dimmeler S. Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. Blood. 2010;115:4944–4950. doi: 10.1182/blood-2010-01-264812.
16. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, Jaenisch R, Sharp PA, Jacks T. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of microRNA clusters. Cell. 2008;132:875–886. doi: 10.1016/j.cell.2008.02.019.
17. Pitulescu ME, Schmidt I, Benedito R, Adams RH. Inducible gene target- ing in the neonatal vasculature and analysis of retinal angiogenesis in mice. Nat Protoc. 2010;5:1518–1534. doi: 10.1038/nprot.2010.113.
18. Boros J, Donaldson J, O’Donnell A, Odrowaz ZA, Zeef L, Lupien M, Meyer CA, Liu XS, Brown M, Sharrons AD. Elucidation of the ELk1 target gene network reveals a role in the coordinate regulation of core components of the gene regulation machinery. Genome Res. 2009;19:1963–1973. doi: 10.1101/gr.093047.109.
Endothelial postnatal genetic inactivation of miR-17–92 reduces angiogenesis.

What Is Known?

- Vascular endothelial growth factor (VEGF) controls angiogenesis mainly by targeting the vascular endothelium.
- The microRNA-17–92 (miR-17–92) cluster consists of 7 highly conserved miRNAs, previously shown to regulate cell proliferation and tumorigenesis.
- Individual miRNAs of the miR-17–92 cluster can exhibit antiangiogenic activity.

What New Information Does This Article Contribute?

- VEGF stimulates the expression of the miR-17–92 cluster in endothelial cells (ECs) by activating the Erk/Erk1 pathway.
- In vitro loss-of-function studies (miR-17–92 inhibition in human ECs) show that miR-17–92 cluster is required for endothelial proliferation and angiogenic sprouting.
- Endothelial postnatal genetic inactivation of miR-17–92 reduces physiological retinal angiogenesis during development, and diminishes VEGF-induced ear angiogenesis and tumor angiogenesis.

Novelty and Significance

- On angiogenic VEGF stimulation, the miR-17–92 cluster targets PTEN to promote endothelial proliferation.

The angiogenic process involves a switch from normal quiescent vasculature to an activated state, by which ECs acquire a proliferative, migratory, and morphogenic phenotype. The miR-17–92 cluster has been linked to tumorigenesis and angiogenesis, but its role in VEGF-induced EC functions is unclear, and its regulation by this key angiogenic factor remains unknown. In this report, we elucidate the mechanism by which VEGF stimulates the expression of the miR-17–92 cluster in ECs. Furthermore, we provide evidence that this stimulation is important for promoting angiogenesis. We found that the endothelial miR-17–92 cluster participates in the regulation of both developmental angiogenesis and angiogenesis during adulthood. These data and previous studies suggest functional cooperation among members of this cluster that can account for the complex biological functions of miR-17–92 in regulating angiogenesis.
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SUPPLEMENTAL MATERIAL
Supplemental Material and Methods

Cell culture
For the direct isolation of human umbilical vein endothelial cells (HUVECs), the cells were obtained by collagenase treatment of term umbilical cord veins as described\(^1\), and then incubated with red blood cell (RBC) lysis buffer (BioLegend, San Diego, CA) for 15 min at room temperature (RT). These freshly isolated quiescent HUVECs were centrifuged at 1400 rpm for 5 min and resuspended in 1 mL of 0.05% BSA PBS with FcR blocking reagent (1:200) for 30 min. HUVECs were incubated with APC anti-human CD45, FITC anti-human CD31 antibodies (BioLegend), and PE anti-human CD105 (BioLegend) for 45 min, washed twice with cold PBS and analyzed on a FACSort flow cytometer (BD Biosciences, San Jose, CA). All CD31\(^+\) CD45\(^-\)ECS were also CD105\(^-\). A portion of these cells was collected directly in Trizol and RNA was used for miR-17~92 expression analysis (freshly isolated quiescent HUVECs). The remainder of cells was directly plated on 0.1% gelatin-coated flasks in M199 10% FBS supplemented with VEGF-165 (50 ng/mL) (BD Biosciences) for different time points (stimulated freshly isolated HUVECs) to determine the effect of VEGF on miR-17~92 expression.

Regular HUVECs were obtained from the tissue culture core laboratory of the Vascular Biology and Therapeutics program (Yale University, New Haven, CT) and were cultured as previously described\(^2\). Human aortic endothelial cells (HAECs) (Lonza, Allendale, NJ) were cultured in EGM™ BulletKit™.

To determine the effect of VEGF or bFGF (Sigma, St Louis, MO) stimulation on miR-17~92 expression, HUVECs or HAECs were starved in M199 or EGM™ 0.1% BSA respectively for 12 hours prior to stimulation with VEGF-165 (50 ng/mL) or bFGF (25 ng/mL).

miR-17~92 cluster Inhibition in vitro
HUVECs were transfected with 70N mix of miR-17~92 inhibitors [10 nM each, including Inh-miR-17\(^*\), Inh-miR-17\(^+\), Inh-miR-18, Inh-miR-19a, Inh-miR-19b, Inh-miR-20 and Inh-miR-92a (Dharmacon, Lafayette, CO)] using Oligofectamine (Life Technologies, Carlsbad, CA) as previously described\(^2\);\(^3\). Control samples were treated with an equivalent concentration (70nM) of an inhibitor negative control sequence (CI).

siRNA Knockdown of Elk-1 and PTEN
HUVECs were transfected with 30 nM Elk-1 siRNA (5’GCAAGGCAAUGGCCACAU3’), 30 nM PTEN siRNA SMARTpool or 30 nM non-silencing siRNA (NS) (5’AATTCTCGAAGTGTCGTT3’) (Dharmacon) using Oligofectamine (Life Technologies) as previously described\(^2\);\(^4\).

Vector construction
The promoter region of 1353 bp (pro1353) of the human miR-17~92 cluster was kindly provided by Dr. Hammond\(^7\). Deletion constructs were generated with the following restriction endonucleases: EcoRI 975 bp construct, PstI 805 bp construct and SmaI 335 bp construct. Deletion of Elk-1 binding site within miR-17~92 promoter region (944-937) was generated using Multisite-QuikChange (Stratagene, La Jolla, CA) according to the manufacturer's protocol. For Elk-1 overexpression, Elk-1 expression vector was purchase from Life Technologies. For PTEN overexpression, human PTEN clone MGC11227 was amplified and cloned with 5’ KpnI and 3’ MfeI sites, ligated to a 5’ EcoRI and 3’ NotI T2A-EGFP-bGHpoly(A) obtained from the pSpCas9(BB)-2A-GFP plasmid (Addgene Plasmid #48138), inserted into a pENTR1A no ccDB (Addgene Plasmid #17398), then into pEZY3 (Addgene Plasmid #18672) with Clonase (Invitrogen), this vector is referred as PTEN OE and produce PTEN-T2A fusion protein. A pEZY3-EGFP plasmid was used as an empty control, referred as CT OE.
Transient transfection and reporter gene assay

**miR-17-92 promoter and deletion constructs transfection**

HUVECs (10x10^4 cells/well) plated in 12-well plate were co-transfected with 0.5 µg of the indicated miR-17-92 promoter reporter constructs (see above) and 0.01 µg of *Renilla* luciferase control reporter vector using Lipofectamine LTX (Life Technologies) for 6 hours. Then, HUVECs were washed with PBS and incubated with 20% FBS M199 for 8 hours. Finally, cells were starved overnight (O/N) and then stimulated with VEGF (50 ng/mL). Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI). Firefly luciferase activity was normalized to the corresponding *Renilla* luciferase activity.

**miR-17-92 promoter and Elk-1 construct transfection**

HUVECs were co-transfected as described above with 0.25 µg of the miR-17-92 full length promoter construct (pro1353), 0.25 µg of Elk-1 overexpression vector (Life Technologies) and 0.01 µg of *Renilla* luciferase control reporter vector.

**miR-17-92 inhibition and PTEN construct transfection**

For PTEN overexpression HUVECs were transfected with a mix of miR-17-92 inhibitors or CI and 0.25 µg of PTEN-T2A or control overexpression vector (CT OE) using lipofectamine LTX as described above.

**RNA isolation and qRT-PCR**

Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized using iScript RT supermix (Bio-Rad, Richmond, CA), following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed in triplicate using iQ SYBR green supermix (Bio-Rad) on an iCycler real-time detection system (Bio-Rad). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or 18S rRNA was used for normalization. Pri-miRNA levels were detected using the TaqMan RT kit (Life Technologies) according to manufacturer's protocol. The members of the miR-17-92 cluster are tightly grouped within an 800-bp region of human chromosome 13\. Pri-miR-17-92 was detected using two different primers, pri-miR-17-92 (1) and (2) that recognize the miR-17-18a-miR-19a stem loops and miR-20a-miR-19b-miR-92a stem loops, respectively. Mature miRNA levels of the different miR-17-92 cluster members were detected using TaqMan miRNA Assay kit (Life Technologies) according to manufacturer's protocol. Quantitative real-time PCR was performed using TaqMan Universal Master Mix (Life Technologies)^2. 18S rRNA or small RNA U6 was used for normalization, respectively.

**Primer sequences for quantitative real time PCR:**

Elk-1 forward primer 5’ CAGCCAGAGGTGTCTGTTACC 3’ and Elk-1 reverse primer 5’ GAGCCGATGTACTCGTTC 3’.

GAPDH forward primer 5’ GAAGGTGAAGGTCGGAGTC 3’ and GAPDH reverse primer 5’ GAAAGATGGTATGGGATTC 3’.

18S forward primer 5’ GCTTAAATGACTCAACACGGA 3’ and 18S reverse primer 5’ AGCTATCAATCTCTCAGATC 3’.

NR4A2 forward primer 5’ GGTTGCAATGGCCTTCTC 3’ and NR4A2 reverse primer 5’ GACCCCGTGCTGATGATGATGACC 3’.

EGR3 forward primer 5’ TGCTATGACCGGAACTC 3’ and EGR3 reverse primer 5’ AGGGAGTCGGAACAGGACT 3’.

NR4A2 and EGR3 gene expression was used as a positive control for VEGF stimulation^9  (Data not shown).

Human PTEN forward primer 5’ TGGATCGACTTTAGACTTGACC 3’ and human PTEN reverse primer 5’ TGTCTTTCAAGCACAAGTTGTAT 3’.
Mouse PTEN forward primer 5’ AGCCATCATCAAGAGATCGT 3’ and mouse PTEN reverse primer 5’ GCTTTGAATCCAAAAACCTTACTAC 3’.

**Western blot analysis**
Nuclear and cytoplasmic fractions were isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Life Technologies) according to the manufacturer’s protocol. Whole cell lysate isolation and western blot were performed as previously described. Rabbit polyclonal antibody against Phospho-Erk (Thr202/Tyr204) (1:1000), rabbit polyclonal antibody against Phospho-Elk-1 (Ser383) (1:500), rabbit polyclonal antibody against PTEN (1:1000), and mouse monoclonal antibody against Erk (1:1000) were obtained from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies against Elk-1 (1:500) and Lamin B1 (1:1000) were purchased from Abcam (Cambridge, MA). Mouse monoclonal Hsp90 (1:3000) antibody was purchased from BD Biosciences. Secondary antibodies were fluorophore-conjugated antibodies (LI-COR Biotechnology, Lincoln, NE). Bands were visualized by using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

**Bioinformatics analysis**

**Promoter analysis, transcription factor prediction**
Sequence promoter analysis to identify potential transcription factors was performed using Softberry (http://www.softberry.com), TF bind (http://tfbind.hgc.jp) and TF search (http://www.cbrc.jp/research/db/TFSEARCH.html) software.

**miRNA target prediction analysis**
Mouse miRNA target predictions were obtained from miRwalk Only targets predicted by at least 3 algorithms were chosen for further analysis. Human miRNA targets were obtained from miRanda (The microRNA.org resource: targets and expression) and TargetScan. miRNAs targets from the same seed were combined and entered as a single entry in Venny 2.0.2 [Oliveros, J.C. (2007-2015) http://bioinfogp.cnb.csic.es/tools/venny/index.html] Venny is an interactive tool for comparing lists with Venn's diagrams where the targets common for all four seeds were identified. Venny 2.0.2 was also used to find targets that were common for all four seeds in mouse and human. The targets in bold are genes that are expressed in ECs (with at least 10 fpkm in HUVEC).

**Gene expression array analysis**
HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above for 36 hours and incubated in the presence of VEGF (stimulated conditions). PCR array gene expression of human genes related to angiogenesis (Qiagen) was performed according to the manufacturer’s protocol and as described previously. Genes upregulated >1.3 or downregulated < -1.3 in Inhibitor miR-17~92 over CI and with p-value <0.1 were analyzed with the Disease and Functions feature in Ingenuity Pathway Analysis software (Qiagen) to find predicted activated or deactivated pathways. Only pathways relevant to endothelial cells with significant p-value were considered. Blue lines connecting the genes and pathways indicate predicted inhibitory effect. Green genes are downregulated, while red genes are upregulated in Inhibitor miR-17~92 compared to CI.

**Chromatin Immunoprecipitation Assay**
HUVECs starved for 12 hours and then treated with VEGF (50 ng/mL) were cross-linked in 1% formaldehyde for 15 min at RT. DNA from fixed chromatin cells were then subjected to immunoprecipitation using a ChIP assay kit (Millipore, Billerica, MA), and antibodies against Elk-1 (Abcam) or anti-rabbit IgG (Millipore). Purified DNA was analyzed by quantitative real time PCR which produced different fragments of the miR-17~92 promoter containing the Elk-1 binding site.
Primer 1 forward 5' CTGAGAATTCCGGAATTTCCT 3' and primer 1 reverse 5' CAGCTGATTAGTAAGAACTC 3'
Primer 2 forward 5' CAGTGATATGTGCTTTGCAG 3' and primer 2 reverse 5' CTCCAGTAGAAATAGCATAGCTC 3'
Primer 3' forward 5' GAATTTCCTGAACCACAATGTG 3' and primer 3 reverse 5' GCATTTAGTAAGAACTCTGGGT 3'
MCL1 primer forward 5' AGTCCCCAACTATGCCCTCT 3' and MCL1 primer reverse 5' CTCTGTGCTTCCCTGAGACC 3'
GNGT1 primer forward 5' ATTGCAAAGAGGGCAGAAGA 3' and GNGT1 primer reverse 5' TTGTGAGCGTTTTTCCAACA 3'
MCL1 and GNGT1 promoters are used as positive and negative controls respectively.

Cell number assessment
HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above. 24 or 48 hours post-transfection, cells were collected and cell number was assessed by using a hemocytometer as previously described. Viability was determined by Trypan blue dye exclusion.

Flow cytometry analyses of proliferation and apoptosis
DNA synthesis–based cell proliferation assay, BrdU incorporation, was performed described. Briefly, HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above. At 44 hours post-transfection, HUVECs were incubated with 100 µM 5-bromo-2'-deoxyuridine (BrdU) (Sigma) for 4 hours at 37°C and fixed in 70% cold ethanol. Then, cells were incubated with 2M HCl for 20 min, washed with PBS and incubated with PBS, 0.5% Tween 20 and 1% normal goat serum. Finally, cells were centrifuged and labeled with FITC anti-BrdU (BD Biosciences) for 1 hour at RT. Ten thousand events per sample were acquired in a FACS calibur flow cytometer (Becton Dickinson) for data analysis using CellQuest software by selective gating to exclude doublet cells.

Cell cycle analysis, apoptotic measurement (SubG1), was ascertained as previously described. Briefly, at 48 hours post-transfection, cells (floating and attached) were collected and fixed in 70% cold ethanol for 1 hour, washed with PBS, and stained with PI as described and analyzed by flow cytometry as described above. Apoptotic cells were determined by their hypochromic, subdiploid staining profiles (subG1 population) using WinMDI 2.9 software (J. Trotter 1993-1998). Incubation with 5 ng/mL of TNF (R&D Systems, MN) plus 5µg/mL Cycloheximide (Sigma) was used as positive control of apoptosis in ECs and incubation with pan-caspase inhibitor 50µM zVAD (R&D) was used to inhibit apoptosis.

Fibrin gel bead assay
HUVECs were transfected with a mix of miR-17~92 inhibitors or CI as described above. Fibrin gel bead assay was performed as described. Briefly, 1x10⁶ HUVECs were mixed with 2500 beads in EGM™ BulletKit™ for 4 hours at 37°C. Coated beads were transferred to T25 flask in 5mL of EGM™ BulletKit™ and left O/N. The following day, the coated beads were resuspended in fibrinogen solution (2.0 mg/mL fibrinogen, 0.15 Units/mL of aprotinin) at a concentration of ~200 beads/mL. 0.625 Units/mL of thrombin was added to each well of a 24-well plate and then 0.5 mL of the fibrinogen/bead suspension was added to each well. Finally, the plate was placed at 37°C for 10-15 min to generate a clot and 1 mL of EGM™ BulletKit™ was added to each well. HUVECs were allowed to undergo morphogenesis for 2-3 days. Angiogenic sprouting was quantified by measuring cumulative sprout length, branches, sprouts, and number of detached cells using NIH ImageJ software. Sprouting was defined as a vessel with length greater than or equal to the diameter of the bead. Branching was defined as a segment of a vessel that has branched off from the major vessel sprout. For each
experiment, at least 10 spheroids per triplicate well and condition were analyzed in each experiment.

**Mice**

All mouse experiments were approved by the Institutional Animal Care Use Committee at the Yale School of Medicine. To generate inducible vascular endothelial cell-specific miR-17–92 iEC-KO mice, we crossed miR-17–92^box/box^ mice^19^ with a Tamoxifen (TMX)-inducible expressed Cre-recombinase (Cre-ERT2) under the regulation of vascular endothelial cadherin promoter (Cdh5)^20,21^ to achieve specific inactivation of miR-17–92 cluster in ECs. For analysis at postnatal day (P) 6 neonates, 50 µg of TMX was injected via i.p. on three consecutive days, P1–P3^21,22^. Percentage of reduction of the levels of miR-17–92 cluster members in miR-17–92 iEC-KO mice compared to control littermates ranged from 33% to 77% (Supplemental Figure 5C).

**Mouse Lung and Retina Endothelial Cell Isolation**

Mouse lung endothelial cells (MLECs) were isolated, as previously described^6,22^, from three pairs of lungs dissected from 3 weeks-old wild-type (WT) mice or P6 miR-17–92 iEC-KO mice. Briefly, freshly isolated lung tissue was minced with scissors and allowed to digest at 37 °C with 2 mg/mL ≈ 175 u/mg Type I collagenase (Sigma) for 1 hour. Lung tissue was further subjected to mechanical disruption by passage ≈ 12 times through a 14-gauge needle and filtration through 70 µm steel mesh. Cells were washed once with DMEM 10% FBS, centrifuged at 1300 rpm for 8 min, and resuspended in 2 mL of cold 0.1% BSA PBS. ECs were immuno- isolated using sheep anti- rat IgG–coated magnetic beads precomplexed with 12.5 µg of rat α-platelet/endothelial cell adhesion molecule 1 (PECAM-1) antibody (Pharmingen, San Diego, CA). A portion of ECs were collected for miRNA expression analysis, the remainder plated on 0.1% gelatin-coated flasks in EGM™ BulletKit™ supplemented with VEGF-164 (50 ng/mL) (BD Biosciences).

Retinal ECs were isolated from retina dissected from WT mice at P2, P5, P12, P17, and adult mice as described above.

**Mouse retina vascular system analysis**

For retina staining, procedures were followed as described^22,23^. Briefly, retinas were dissected out and stained with Alexa-594-conjugated isoelectin B4 (1:200) (Life Technologies), mouse anti-phospho-histone H3 (1:200) (Cell Signaling) and rabbit anti-COL IV (1:200) (AbD Serotec, Richmond, CA) and rabbit anti-cleaved caspase 3 (1:200) (Cell Signaling). Slides were analyzed using a Leica TCS SP5 microscope. Branch point numbers were quantified as described^22,23^, and the retinal area and vascular density were quantified with ImageJ.

**Tumor-induced neovascularization**

LLC cells (10^6^) were injected s.c. in the dorsal flank of 6-week-old as described^2,5^, of previously treated TMX, miR-17–92 iEC-KO or CT mice. After 14 days, animals were euthanized and tumor tissues were collected and frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA). Frozen sections of LLC tumors were stained with anti-mouse PECAM-1-PE labeled antibody (BD Biosciences) and DAPI. Stained slides were imaged with a Zeiss Axiovert 200M inverted microscope and a Hamamatsu camera. For quantification, 3 sections per mouse sample were analyzed and from each sample 2 images were captured from random areas of each tissue section. Microvessel density was quantified by measuring the PECAM-1 per sample area. ImageJ was used to determine the number of positive structures or pixels per sample area.

**Ear angiogenesis**

Adenoviruses encoding murine VEGF-A164 (Ad5CMV VEGF164) (Gene Transfer Vector Core, University of Iowa) (2 x 10^6^ viral particles) were injected intradermally as described ^5 into the right ears of 6 week-old, of previously treated TMX, miR-17–92 iEC-KO or CT mice. The left ears were injected with a control virus expressing eGFP (Ad5CMV eGFP)
(Gene Transfer Vector Core, University of Iowa). After 3 days, animals were euthanized and the ears were removed and finally embedded and frozen in OCT compound (Tissue-Tek; Sakura) for staining with anti-mouse PECAM-1-PE labeled antibody (BD Biosciences) and DAPI. Stained slides were imaged using a Zeiss Axiovert 200M inverted microscope and quantification of PECAM-1 positive structures was performed as above indicated.

**Statistics**

Statistical analyses were performed with GraphPad Prism 6 software (GraphPad, San Diego, CA), using the two-tailed, unpaired Student’s t-test or one-way ANOVA, when appropriate. Data are expressed as mean ± S.E.M. P values P≤0.05 were considered statistically significant.

**Supplemental References**

1. Gimbrone MA, Jr., Cotran RS, Folkman J. Human vascular endothelial cells in culture. Growth and DNA synthesis. *J Cell Biol.* 1974;60:673-684
2. Chamorro-Jorganes A, Araldi E, Rotllan N, Cirera-Salinas D, Suarez Y. Autoregulation of glypican-1 by intronic microrna-149 fine tunes the angiogenic response to fgf2 in human endothelial cells. *J Cell Sci.* 2014;127:1169-1178
3. Chamorro-Jorganes A, Araldi E, Penalva LO, Sandhu D, Fernandez-Hernando C, Suarez Y. Microrna-16 and microrna-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. *Arterioscler Thromb Vasc Biol.* 2011;31:2595-2606
4. Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. Dicer dependent micrornas regulate gene expression and functions in human endothelial cells. *Circ Res.* 2007;100:1164-1173
5. Suarez Y, Fernandez-Hernando C, Yu J, Gerber SA, Harrison KD, Pober JS, Iruela-Arispe ML, Merkenschlager M, Sessa WC. Dicer-dependent endothelial micrornas are necessary for postnatal angiogenesis. *Proc Natl Acad Sci U S A.* 2008;105:14082-14087
6. Suarez Y, Wang C, Manes TD, Pober JS. Cutting edge: Tnf-induced micrornas regulate tnf-induced expression of e-selectin and intercellular adhesion molecule-1 on human endothelial cells: Feedback control of inflammation. *J Immunol.* 2010;184:21-25
7. Woods K, Thomson JM, Hammond SM. Direct regulation of an oncogenic microrna cluster by e2f transcription factors. *J Biol Chem.* 2007;282:2130-2134
8. Mendell JT. Miriad roles for the mir-17-92 cluster in development and disease. *Cell.* 2008;133:217-222
9. Schweighofer B, Testori J, Sturtzel C, Sattler S, Mayer H, Wagner O, Bilban M, Hofer E. The vegf-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation. *Thromb Haemost.* 2009;102:544-554
10. Dweep H, Sticht C, Pandey P, Gretz N. Mirwalk--database: Prediction of possible mirna binding sites by "walking" the genes of three genomes. *J Biomed Inform.* 2011;44:839-847
11. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microrna.Org resource: Targets and expression. *Nucleic Acids Res.* 2008;36:D149-153
12. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microrna targets. *Cell.* 2005;120:15-20
13. Zhang B, Day DS, Ho JW, Song L, Cao J, Christodoulou D, Seidman JG, Crawford GE, Park PJ, Pu WT. A dynamic h3k27ac signature identifies vegfa-stimulated endothelial enhancers and requires ep300 activity. *Genome Res*. 2013;23:917-927

14. Boros J, Donaldson IJ, O'Donnell A, OdrowazZA, Zeef L, Lupien M, Meyer CA, Liu XS, Brown M, Sharrocks AD. Elucidation of the elk1 target gene network reveals a role in the coordinate regulation of core components of the gene regulation machinery. *Genome Res*. 2009;19:1963-1973

15. Suarez Y, Gonzalez L, Cuadrado A, Berciano M, Lafarga M, Munoz A. Kahalalide f, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther*. 2003;2:863-872

16. Li JH, Pober JS. The cathepsin b death pathway contributes to tnf plus ifn-gamma-mediated human endothelial injury. *J Immunol*. 2005;175:1858-1866

17. Nakatsu MN, Hughes CC. An optimized three-dimensional in vitro model for the analysis of angiogenesis. *Methods Enzymol*. 2008;443:65-82

18. Welch-Reardon KM, Ehsan SM, Wang K, Wu N, Newman AC, Romero-Lopez M, Fong AH, George SC, Edwards RA, Hughes CC. Angiogenic sprouting is regulated by endothelial cell expression of slug. *J Cell Sci*. 2014;127:2017-2028

19. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, Jaenisch R, Sharp PA, Jacks T. Targeted deletion reveals essential and overlapping functions of the mir-17 through 92 family of mirna clusters. *Cell*. 2008;132:875-886

20. Sorensen I, Adams RH, Gossler A. Dll1-mediated notch activation regulates endothelial identity in mouse fetal arteries. *Blood*. 2009;113:5680-5688

21. Pitulescu ME, Schmidt I, Benedito R, Adams RH. Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. *Nat Protoc*. 2010;5:1518-1534

22. Lee MY, Luciano AK, Ackah E, Rodriguez-Vita J, Bancroft TA, Eichmann A, Simons M, Kyriakides TR, Morales-Ruiz M, Sessa WC. Endothelial akt1 mediates angiogenesis by phosphorylating multiple angiogenic substrates. *Proc Natl Acad Sci U S A*. 2014;111:12865-12870

23. Lee MY, Skoura A, Park EJ, Landskroner-Eiger S, Jozsef L, Luciano AK, Murata T, Pasula S, Dong Y, Bouaouina M, Calderwood DA, Ferguson SM, De Camilli P, Sessa WC. Dynamin 2 regulation of integrin endocytosis, but not vegf signaling, is crucial for developmental angiogenesis. *Development*. 2014;141:1465-1472

24. LaRusch GA, Mahdi F, Shariat-Madar Z, Adams G, Sitrin RG, Zhang WM, McCrae KR, Schmaier AH. Factor xii stimulates erk1/2 and akt through upar, integrins, and the egfr to initiate angiogenesis. *Blood*. 2010;115:5111-5120
Online Figure I. VEGF stimulates the expression of the miR-17~92 cluster in ECs. qRT-PCR analysis of pri-miRNA-17~92 levels in HUVECs starved for 12 hours and then treated with VEGF (50 ng/mL) for the indicated times. Data correspond to the mean ± S.E.M. of three independent experiments. * P≤0.05

Online Figure II. Efficiency of siRNA mediated knockdown of Elk-1 in ECs. qRT-PCR and Western blot analysis (insert) of Elk-1 mRNA and protein levels respectively, of HUVECs transfected with Elk-1 siRNA or non-silencing (NS) control siRNA for 48 hours. Data correspond to the mean ± S.E.M. of three independent experiments, * P≤0.05.

Online Figure III. FGF stimulates the expression of the miR-17~92 cluster in ECs. (A) qRT-PCR analysis of pri-miRNA-17~92 levels in HUVECs starved for 12 hours and then treated with FGF (25 ng/mL) for 3 hours. (B) qRT-PCR analysis of pri-miRNA-17~92 levels human aortic endothelial cells (HAECs) starved for 12 hours and then treated with VEGF (50 ng/mL) for 3 hours. Data correspond to the mean ± S.E.M. of three independent experiments. For (B) each experiment was performed with different batch of HAECs, * P≤0.05.

Online Figure IV. miR-17~92 cluster inhibition does not induce apoptosis in ECs. (A) HUVECs were transfected with 70nM mix of miR-17~92 inhibitors (Inh-miR-17~92) or control inhibitor (CI) for 24 hours. Cells were harvested and treated with anti-BrdU antibody and analyzed by flow cytometry as described in online Material and Methods. One representative experiment out of three with similar results is shown. HUVECs were transfected as indicated in A. After 12 hours post-transfection, HUVECs were treated with zVAD (50µM) or vehicle control (DMSO) and harvested at different time points. (B) Total number of cells. Data are expressed as mean of triplicates ± S.D. of one experiment out of three with similar results. (C) Flow cytometry analysis of DNA content at 48 hours. Percentage of apoptotic cells correspond to Sub G0/G1 population is shown. One representative experiment out of three is shown. Last row of plots shows the controls for the induction of apoptosis and ZVAD action. HUVECs were treated with TNF (5ng/mL) plus Cycloheximide (5µg/mL) ± zVAD (50µM) for 12 hours and collected for analysis of apoptosis as described above.

Online Figure V. Expression of miR-17~92 cluster in mouse ECs. (A) qRT-PCR analysis of individual miRNAs from the miR-17~92 cluster in freshly isolated mouse lung endothelial cells (MLECs) upon VEGF (50 ng/mL) stimulation. Data are expressed as relative miRNA levels vs. freshly isolated control ECs and correspond to one representative experiment out of three independent experiments with similar results. (B) qRT-PCR analysis of individual miRNAs from the miR-17~92 cluster retinal ECs isolated from wild-type (WT) mice. Data are expressed as relative miRNA levels vs. P2 retinal ECs. (C) qRT-PCR analysis of individual miRNAs from the miR-17~92 cluster in freshly MLECs from miR-17~92 iEC-KO mice (TMX P1-P3) at P6. Data are expressed as relative miRNA levels vs. control littermates.

Online Figure VI. Endothelial-specific 17~92 deletion reduces developmental retinal angiogenesis. Mice were injected with TMX P1-P3 and assessed at P6 [CT, n = 6; miR-17~92 iEC-KO, n = 7]. (A) Retinal whole mount, (B) Vascular front and (C) Vascular plexus Isob4 immunostaining representative images. Magnification: 20X.

Online Figure VII. Loss of endothelial miR-17~92 does not affect apoptosis in vivo. Mice were injected with TMX P1-P3 and assessed at P6 [CT, n = 6; miR-17~92 iEC-KO, n = 7]. Isolectin B4/Cleaved Caspase3 immunostaining representative images. Magnification: 40X.

Online Figure VIII. Inhibition of miR-17~92 in endothelial angiogenic program.
(A) Gene expression array analysis in HUVECs transfected with 70nM mix of Inh-miR-17~92 or CI and incubated in the presence of VEGF (stimulated conditions). Samples were collected 36 post-transfection. The positive regulators of angiogenesis are indicated in red, negative regulators in blue, positive/negative regulators in grey. Data are expressed as fold change compared to CI (dashed line) *, P \leq 0.05. (B) Genes upregulated >1.3 or downregulated < -1.3 in Inh-miR-17~92 over CI and with P-value <0.1 were analyzed with the Disease and Functions feature in Ingenuity Pathway Analysis software (Qiagen) to find predicted activated or deactivated pathways. Only pathways relevant to endothelial cells with significant P-value are depicted. Blue lines connecting the genes and pathways indicate predicted inhibitory effect. Green genes are downregulated, while red genes are upregulated Inh-miR-17~92 compared to CI.
Online Figure I

Relative pri-miRNAs levels (vs vehicle control)

- pri-miR-17~92 (1)
- pri-miR-17~92 (2)

Time points: 1h, 3h, 12h
Online Figure II

Relative mRNA levels (vs NS control)

- Elk-1
- Hsp90

NS siRNA
Elk-1 siRNA

*
Online Figure III

A

![Graph A](image)

- **pri-miR-17-92 (1)**
- **pri-miR-17-92 (2)**

B

![Graph B](image)

- **pri-miR-17-92 (1)**
- **pri-miR-17-92 (2)**

Legend:
- **- FGF**
- **+ FGF**
- **- VEGF**
- **+ VEGF**
Online Figure IV

A

![Graph showing BrdU positive cells](image)

- Black line: CI 21.39%
- Red line: Inh-miR-17-92 13.29%

B

![Graph showing cell numbers over time](image)

- CT
- CT + ZVAD
- Inh-miR-17-92
- Inh-miR-17-92 + ZVAD

C

**48 h**

| Condition       | FL2-A Events |
|-----------------|--------------|
| CI              | 8            |
| CI + ZVAD       | 8            |
| Inh-miR-17-92   | 12           |
| Inh-miR-17-92 + ZVAD | 12         |
| Control         | 5            |
| ZVAD            | 5            |
| TNF + CHX       | 61           |
| TNF + CHX + ZVAD| 17           |
Online Figure VI

A

CT
miR-17~92 iEC-KO

B

Vascular front

CT
miR-17~92 iEC-KO

C

Vascular plexus

CT
miR-17~92 iEC-KO
Online Figure VII

miR-17~92 iEC-KO CT

Isob4/Cleaved Casp3

Relative cleaved caspase-3+IB-4+

n.s
Online Figure VIII

A

![Bar chart showing relative expression](chart.png)

B

![Network diagram illustrating protein interactions](network.png)

### Prediction Legend

- **Red** (Upregulated)
- **Green** (Downregulated)
- **More extreme**
- **Less extreme**
- **Predicted activation**
- **Predicted inhibition**
- **Predicted relationships**
- **Effect not predicted**

**Angiogenesis**

**Proliferation of endothelial cells**