

Synchronous down-modulation of miR-17 family members is an early causative event in the retinal angiogenic switch

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Six members of the microRNA-17 (miR-17) family were mapped to three different chromosomes, although they share the same seed sequence and are predicted to target common genes, among which are those encoding hypoxia-inducible factor-1 α (HIF1A) and VEGFA. Here, we evaluated the *in vivo* expression profile of the miR-17 family in the murine retinopathy of prematurity (ROP) model, whereby Vegfa expression is highly enhanced at the early stage of retinal neovascularization, and we found simultaneous reduction of all miR-17 family members at this stage. Using gene reporter assays, we observed binding of these miRs to specific sites in the 3' UTRs of *Hif1a* and *Vegfa*. Furthermore, overexpression of these miRs decreased HIF1A and VEGFA expression *in vitro*. Our data indicate that this miR-17 family elicits a regulatory synergistic down-regulation of *Hif1a* and *Vegfa* expression in this biological model. We propose the existence of a coordinated regulatory network, in which diverse miRs are synchronously regulated to target the *Hif1a* transcription factor, which in turn, potentiates and reinforces the regulatory effects of the miRs on *Vegfa* to trigger and sustain a significant physiological response.

miRNA family | miRNA regulatory network | mouse neovascularization model | hypoxia | eye

MicroRNAs (miRs) are among the most important post-transcriptional regulators of gene expression and affect several normal biological processes, such as the cell cycle and cell fate determination during development, including angiogenesis. Recently, a large body of literature identified and confirmed a number of miRs that regulate angiogenesis (1–8).

In humans, the miR-17 family is composed of six distinct mature miRs located on three chromosomes: miR-17-5p and miR-20a are located on chromosome 13q31.3, miR-20b and miR-106a are located on chromosome Xq26.2, and miR-106b and miR-93 are located on chromosome 7q22.1. Members of this family seem to be derived from gene duplication events (9), and despite some divergences in length and nucleotide composition, their seed sequence (AAAGUG) is identical, an attribute suggestive of functional redundancy (10). Although the miR-17-92 cluster has been well-characterized, the regulatory role of the miR-17 family members has not been extensively studied (11, 12). Distinct prediction algorithms (13–16) indicate that the miR-17 miR family may target the 3' UTRs of genes encoding hypoxia-inducible factor-1 α (HIF1A) and VEGFA. Therefore, by simultaneously targeting these two key genes, miR-17 family members could, in theory, be important concerted regulators of angiogenesis. As proof of

concept, we tested this hypothesis in the experimental mouse model of retinopathy of prematurity (ROP), where *Vegfa* seems to be a pivotal factor that modulates the angiogenic switch (17–19). In this model, the expression of retinal *Vegfa* mRNA increases sharply 12 h after the animals are returned from 75% oxygen (O₂) back to room air (~21% O₂), a process that induces a relative hypoxic condition and leads to retinal neovascularization in the subsequent 9 d (17–19).

Here, we show the synchronous down-regulation of all members of the miR-17 family in the critical early steps of neovascularization in the ROP model. We propose an miR regulatory network, in which distinct and partially redundant miR-17 family members simultaneously affect the levels of the *Hif1a* transcription factor to posttranscriptionally (either directly and/or indirectly) increase the expression of one of its key downstream targets (i.e., *Vegfa*) (20–22), thereby influencing the physiological response to retinal hypoxia.

Significance

Retinal angiogenesis is a finely tuned biological phenomenon and a major cause of blindness. We studied the regulation of this phenomenon and identified cross-talk involving microRNAs (miRs) that share the same seed sequence, transcription factors, and angiogenesis effectors. In a mouse model of retinopathy of prematurity, we show the down-regulation of all miR-17 family members as an early event in the angiogenic switch, which resulted in increased levels of hypoxia-inducible factor-1 α and *Vegfa* *in vitro*. Notably, this coordinated regulation did not require the marked quantitative alterations of an individual miR but instead, relied on synchronous changes in members that share the same seed sequence. These results identify potential therapeutic targets in eye diseases with abnormal retinal angiogenesis.

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Results and Discussion

Retinal Angiogenesis and miR Expression. The O₂-induced retinal neovascularization model (17–19) has been widely used to study the molecular mechanisms of blood vessel formation during relative hypoxia. Here, we used this model to address the putative role of miR-17 family members in the early steps of the angiogenic switch (Fig. 1). This particular family was selected, because all six members were predicted to target *Hif1a* and *Vegfa* mRNA using different algorithms, albeit not experimentally confirmed. Mapping miR-17 family members to the 3' UTRs of *Hif1a* and *Vegfa* transcripts showed that the target sites were located in regions conserved in mammals, avians, and amphibians (Fig. 2), strongly indicating that these regions may, indeed, be targets for regulatory molecules.

We assumed that the time for the angiogenic switch to be triggered overlaps with maximum *Vegfa* expression. Because we intended to evaluate the regulation of *Vegfa* expression by the miR-17 family, experimental time points were selected from the established peak of *Vegfa* expression in this model (18). As expected, retinal neovascularization was observed in 21-d-old mice that were exposed to high O₂ levels for 5 d and removed to room air thereafter (Fig. 1 A–C). This vascular response was consistent with previous reports (17–19), and with the noteworthy increase in *Vegfa* expression (ANOVA, $P = 1.83 \times 10^{-15}$) that was apparent 12 h after the animals were returned to room air (Fig. 1D). To document further the mimicry of hypoxia in this model and its effects on the expression of hypoxia miR surrogates, we

evaluated the amounts of two hypoxia-induced miRs, miR-210 and miR-296, whose induction had been shown in other hypoxia models (23–27). We observed a consistent increase in both miRs (ANOVA, $P = 1.15 \times 10^{-11}$ and $P = 4.76 \times 10^{-16}$, respectively) after 12 h of induced relative hypoxia (Fig. 1E), thus confirming these miRs as general and robust molecular biomarkers of hypoxia. We investigated whether the levels of miR-17 family members were affected at two time points relative to maximal *Vegfa* expression (i.e., at 6 and 12 h after high O₂ exposure). Our real-time PCR results showed a significant reduction of all six members of the miR-17 family after 12 h of relative hypoxia (ANOVA, $P < 0.05$) (Fig. 1E).

Validation of miR Targeting. The observed down-regulation of all miRs of this family after 6 and 12 h post-O₂ exposure led us to evaluate their regulatory capabilities using luciferase reporter assays of the putative targets *Hif1a* and *Vegfa* mRNA. Luciferase expression levels were compared from WT *Hif1a* and *Vegfa* with mutated controls that contain a 7-nt deletion within the predicted 3' UTR regulatory binding sequence. Both WT and mutated 3' UTR regions were tested with or without miR transfection. Transfection of each miR resulted in a significant reduction of luciferase activity compared with the control in the presence of WT 3' UTR-targeted regions from both *Hif1a* and *Vegfa*; on the contrary, luciferase activity was increased in the presence of each miR-17 RNA when either *Hif1a* or *Vegfa* contained a 7-nt deletion within the 3' UTR region (Fig. 3) (ANOVA, $P < 0.05$).

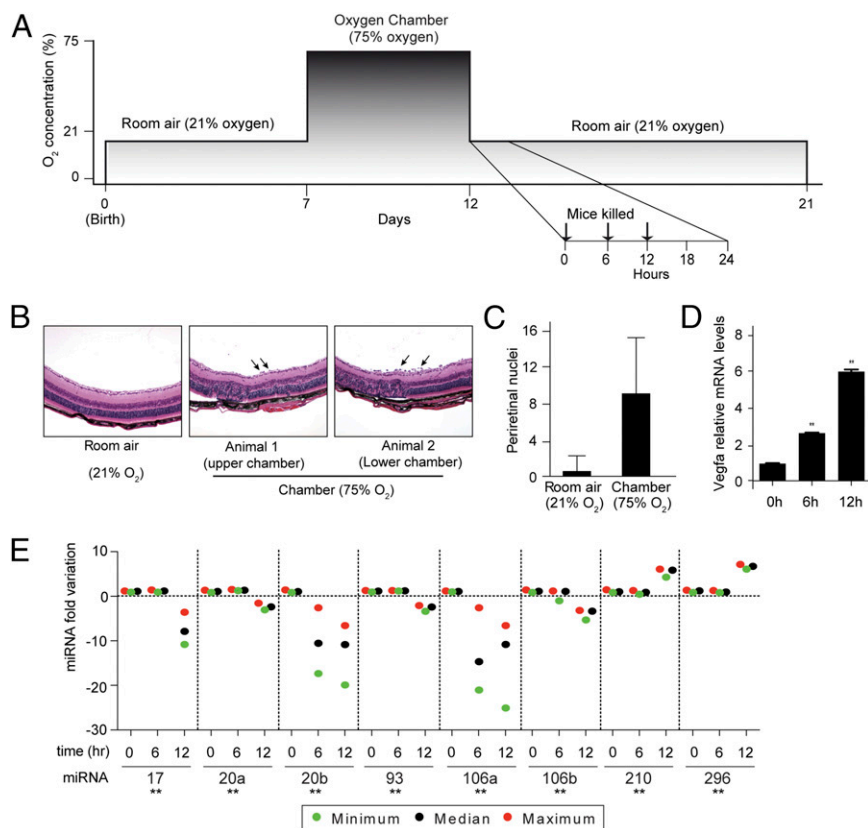


Fig. 1. Angiogenesis and *Vegfa* expression in the murine ROP model. (A) Seven-day-old pups and their mothers were transferred to 75% oxygen for 5 d. After this period, animals were returned to room air (~21% O₂) and subsequently killed at time 0, 6, or 12 h. (B and C) As a control for retinal neovascularization, some animals were kept alive until postnatal day 21, when new blood vessel sprouts (indicated by arrows) were observed in retinas of animals that were exposed to high O₂ levels. (D) Levels of *Vegfa* mRNA increased after 6 and 12 h in room air compared with *Vegfa* mRNA from animals killed immediately (deemed 0 h time point) after transfer to room air. (E) miR variations at 6 and 12 h relative to those from animals killed immediately after their return to room air O₂. At 12 h, the increase of *Vegfa* was accompanied by an augmentation of miR-210 and miR-296 (established hypoxia-induced miRs) as well as reduced expression of all miR-17 family members. The dots represent the minimum (green), median (black), and maximum (red) fold variations observed for each miR relative to control. P values were calculated by using ANOVA. miRNA, miR. ** $P < 0.01$.

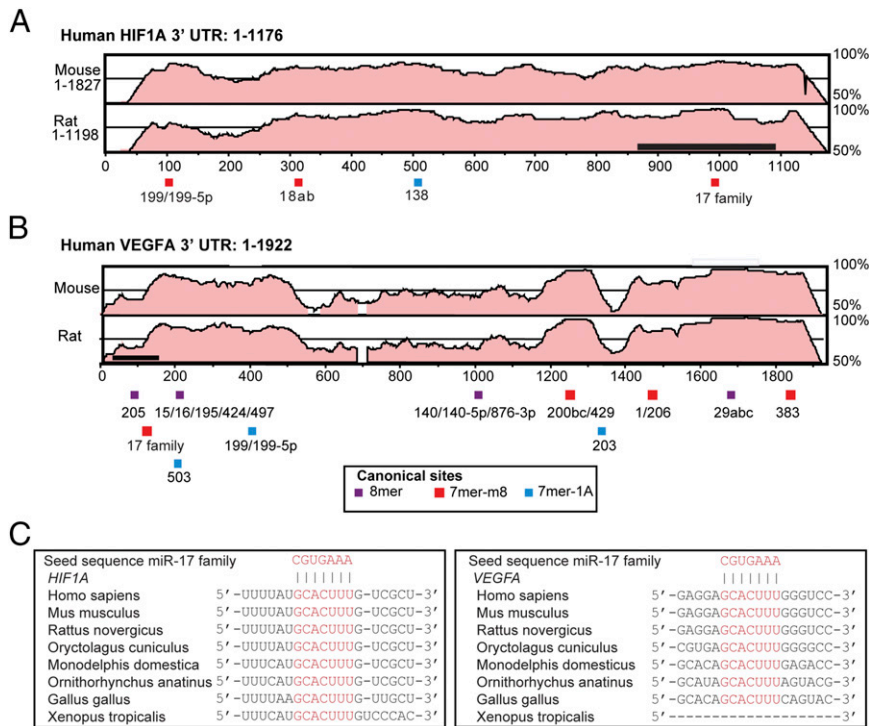


Fig. 2. Alignment of *HIF1A*, and *VEGFA* genes shows highly conserved 3' UTRs. VISTA (visualization tool for alignment) alignments of human, mouse, and rat (A) *HIF1A* and (B) *VEGFA* genes show similar 3' UTRs together with TargetScan predictions of putative miR binding sites. miR target regions used for gene reporter assays are shown as black bars for both genes. (C) The strong conservation of the putative miR binding site in this region is shown in the detailed alignment of the miR-17-5p seed sequence to compare the 3' UTR from mammals, avians, and amphibians.

These results indicate that the miR-17 family members bind to the predicted 3' UTR region, and by doing so, they regulate the expression of both *Hif1a* and *Vegfa*.

miR-17 Family Members Regulate HIF1A and VEGFA Expression. To recapitulate in vitro the regulatory events observed in vivo, we used human Y79 retinoblastoma cells, which develop from immature retinas containing amacrine interneurons and Müller glial cells (28). Y79 cells express *VEGFA*, and Müller glial cells are especially active in the mouse neovascularization model (19). We observed reduced levels of *HIF1A* by ELISA (Fig. 4A) after transfecting cells with miR-20a ($P = 0.02$), miR-93 ($P = 0.01$), miR-106a ($P = 1.04 \times 10^{-5}$), and miR-106b ($P = 0.04$). Similarly, *VEGFA* levels decreased by 30% (ANOVA, $P < 0.05$) compared with baseline levels (Fig. 4B) after cell transfection with four of six family members [namely, miR-17-5p ($P = 0.02$), miR-20b ($P = 0.005$), miR-93 ($P = 0.02$), and miR-106b ($P = 0.001$)]. These results are consistent with our working hypothesis that *Hif1a* and *Vegfa* expressions are regulated by the miR-17 family during the early stages of the angiogenic switch in the ROP model.

Low Levels of miR-17 Family in the Initial Steps of the Angiogenic Switch. The discovery of miR regulation has added another dimension to the genetic study of cancer (29). This report corroborates this concept by extending the role of the miR-17 family to early angiogenic events, an important initial step in tumor progression. Because each miR can theoretically bind to and regulate hundreds of targets, one of the most intriguing questions concerns the translational regulation of the target genes (30). In the ROP model, we observed a consistent reduction of all six miR-17 family members and a corresponding increase in *Hif1a* and *Vegfa* protein levels within hours after mice pups were returned to room air after 5 d of high O_2 exposure. Conversely, the higher intracellular concentration of these miRs in transfected Y79 cells resulted in

a substantial reduction of *VEGFA* transcript and *HIF1A* and *VEGFA* protein expression. Theoretically, the effective trigger for angiogenesis—achieved after *HIF1A* and *VEGFA* up-regulation—would require simultaneous reduction of all of its negative regulators. Because these miRs share the same seed sequence, we expected—and observed—a similar response for each family member. Although a single miR is able to repress the production of hundreds of proteins, the cumulative effect may be relatively mild (31). In this case, finely tuned and complex biological processes, such as angiogenesis, require the concerted, simultaneous silencing of all miR-17 family members to bias the biological signals that ultimately lead to blood vessel formation.

In a previous study, Shen et al. (32) investigated miR alterations by microarray analysis using the ROP model. Shen et al. (32) evaluated a later time point (3 d after high O_2 exposure on day 15 vs. 6 and 12 h on day 12 as studied here), when the events that lead to *Hif1a* and *Vegfa* up-regulation have already occurred. Shen et al. (32) identified the levels of five miR-17 family members (miR-17-5p, miR-20a, miR-20b, miR-106a, and miR-106b) increased at this later time point. We also tested the expression levels of all miR members from the miR-17 family at this same time point using a more sensitive and specific approach (quantitative RT-PCR). In our hands, only miR-20b and miR-106b levels increased at this time point, whereas the expression levels of the other four members were down-regulated (data not shown).

It should be noted that the capability of some of the miR-17 family members to regulate *VEGF* and *HIF1A* expressions has been shown. For instance, the inhibition of miR-20b led to an incremental increase of *HIF1A* and *VEGF* protein levels in normoxic tumor cells, whereas the increase of miR-20b in hypoxic tumor cells decreased the levels of these two proangiogenic proteins (33). This study is the first demonstration, to our knowledge, of the simultaneous down-regulation of all miRs of the miR-17

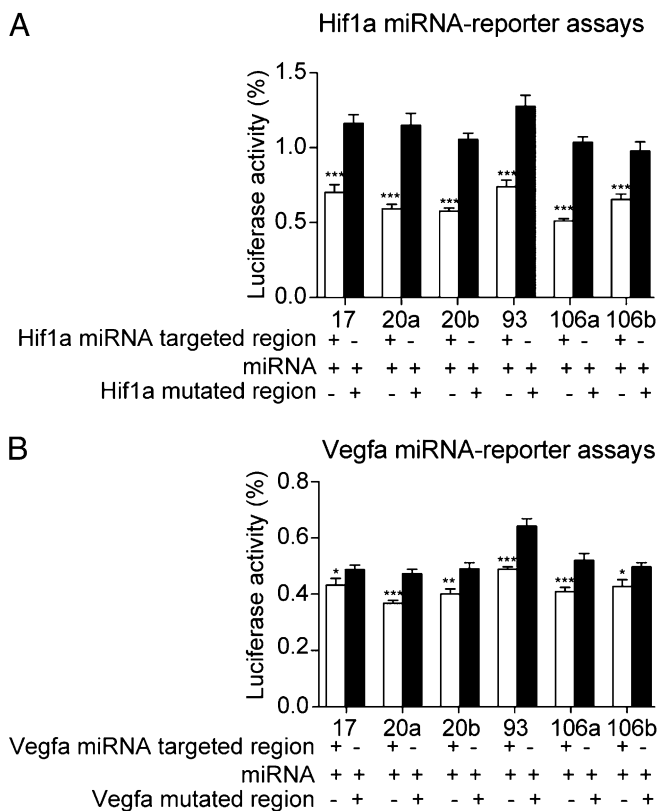


Fig. 3. Regulation of *Hif1a* and *Vegfa* mRNAs by miR-17 family members. Gene reporter assays of (A) *Hif1a* or (B) *Vegfa* expression containing WT or mutated regions of the mouse 3' UTR show that members of the miR-17 family bind to specific 3' UTRs of these genes and regulate luciferase activity. Analyses were performed after 24 h of transfection using 10 nM each miR. miRNA, miR. Statistical significances are * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

family in the early time interval that precedes up-regulation of these important proangiogenesis factors.

We propose here that the simultaneous down-regulation of all miR-17 family members stimulates a positive cumulative effect on *Hif1a* and *Vegfa* expressions. When hypoxia supervenes after 5 d of high oxygen exposure, the induction of *Hif1a* expression seemed to increase by decreased levels of the miR-17 family. Lower levels of miR regulators would positively and simultaneously affect *Hif1a* and *Vegfa* protein levels. Higher *Hif1a* levels would certainly support higher *Vegfa* levels and probably contribute to drive the early steps of the angiogenic switch. These events could initiate a cascade that leads to consistent physiological responses without requiring additional quantitative alterations of miRs. This regulation scenario may not be exclusive to this miR family and these target genes. In this sense, Martinez et al. (34) describe a miR regulatory network in *Caenorhabditis elegans* of experimentally mapped interactions among transcription factors and miRs.

Angiogenesis is a multistep process that occurs over an extended period. As a tightly regulated physiological event, angiogenesis is a central biological phenomenon and a target for therapy in diseases, such as different forms of retinopathy and cancer, among other conditions. In this study, the importance of using an established, reproducible in vivo model was crucial to assess the complexity within an actual tissue microenvironment without the interference of artificial variables from an in vitro model. The reduction of all miR-17 family members simultaneously in the hypoxic retina as well as other tissues and tumors might be an early response to contextual signals within the tissue microenvironment that triggers neovascularization. In this context,

it is interesting to note that overexpression of some of the miRs evaluated here (namely miR-17 and miR-20a) inhibits metastasis (also an angiogenesis-dependent process) in pancreatic carcinoma cells (35) as well as breast cancer (36).

The physiologic response to hypoxia is complex. The manipulation of individual miRs in arbitrary concentrations and in vitro is unlikely to recreate this complex phenomenon. The hypoxia response and regulation of neovascularization by a limited set of miRs seem to be a short-duration, context-dependent process. As shown in Fig. 1E, all miRs evaluated in this study exhibited a significant decrease 12 h after the animals were returned to normoxia (ANOVA, $P < 0.01$). Of these miRs, miR-93 and miR-106a have been described as hypoxia-regulated miRs in cells exposed to hypoxic conditions (0.2% O_2) in vitro (7), but the miRs documented in this study showed an opposite trend in vivo. Although some studies showed that the levels of some of the miR-17 family members increase in solid tumors, others have shown deletion of genomic loci encoding these same miRs (37). In this cell context-dependent manner, Doebele et al. (38) show that overexpression of some members from the miR-17 cluster (miR-17; miR-18a, miR-19a, and miR-20a) leads to a decrease of endothelial cells sprouts in vitro. Conversely, in the same study, down-regulation of these miRs in vivo, particularly miR-17/miR-20, increases neovascularization but did not affect tumor angiogenesis. This observation is in accordance with our data and indicates that miR expression can, therefore, be expected to be specific in different cell types and physiological situations and thus, is context-dependent (39).

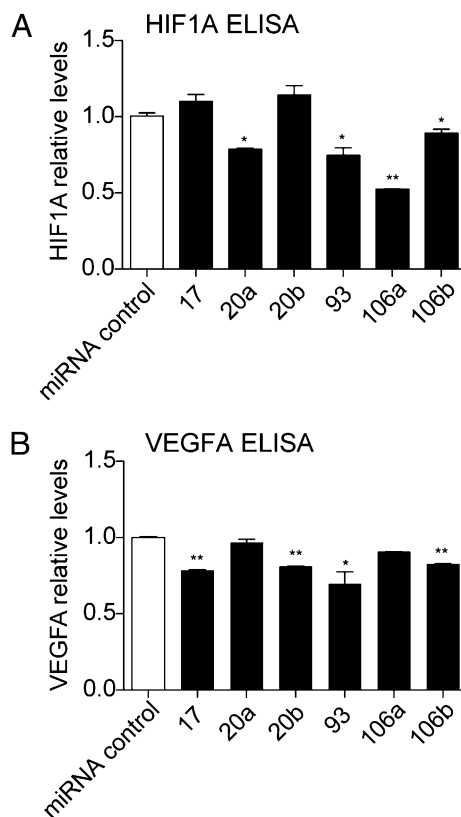


Fig. 4. miR-17 family members regulate HIF1A and VEGFA expression. Proteins derived from Y79 retinoblastoma cells transfected with each of the miR-17 RNAs show that each member of the miR-17 family decreased the levels of (A) HIF1A or (B) VEGFA expression as determined by ELISA. Analyses were performed after 24 h of transfection using 10 nM each miR. miRNA, miR. Statistical significances are indicated as * $P < 0.05$ and ** $P < 0.01$.

Another interesting analogous study showed significant down-regulation of miR-200b, an miR that targets *Vegfa*, in a mouse model of diabetic retinopathy (40). In this model, high blood glucose levels lead to down-regulation of miR-200 with subsequent up-regulation of *Vegfa* mRNA and *Vegfa* expression resulting in increased vascular permeability in vitro and in vivo.

This report puts forth a model in which the regulation of hypoxia-related genes by the miR-17 family occurs in tandem. Small reductions in the levels of miRs of all members of this family would lead to an incremental increase in Hif1a. Both higher Hif1a and lower miR levels subsequently trigger higher levels of *Vegfa* expression. This angiogenic cascade may have even broader effects, because some of these miRs have been shown to regulate *CTGF* and *TSP1* (both of which have proangiogenic properties) and are also predicted to target at least 14 additional angiogenesis-related genes (16) as well as another 5 genes recently predicted to be regulated by HIF1A (41, 42) (Fig. 5). Thus, it is conceivable that coordinated regulation of some of these genes by diminution of the miR-17 family levels might be an early step that leads to angiogenesis (Fig. 5). Within a larger context, it is interesting to note that hypoxia is also a well-known regulator of angiogenesis in cancer. Indeed, because poorly vascularized areas in solid tumors are hypoxic and low O₂ levels contribute to radiation therapy resistance (43), the miR regulation described here coupled with the hypoxic tumor microenvironment could trigger the angiogenic switch. This presumed key event occurs before blood vessel development to restore homeostasis and is required for solid tumor growth. Additionally, an even earlier event might be hypoxia-induced increase of p53 to suppress miR-17 elements at the transcriptional level by p53 binding to a specific site in the proximal region of the miR-17 promoter to competitively inhibit binding of a TATA binding protein transcription factor within an

overlapping site in the promoter region (44). Thus, a tantalizing role of the miR-17 family to regulate tumor angiogenesis remains an open question to be addressed in future studies.

In conclusion, we present three previously unrecognized findings. First, we show a decrease of all miR-17 family members in the in vivo ROP mouse model of angiogenesis. Second, we show that these miRs bind to the 3' UTRs of *Hif1a* and *Vegfa* and reduce the expression levels of these gene products after transfection into Y79 cells in vitro. Third, the miR-17 family members seem to be one of the earliest hypoxia-responsive molecular elements identified so far that triggers the angiogenic switch as part of an as yet unrecognized regulatory network of functional interactions.

Materials and Methods

ROP Model. Seven-day-old C57BL/6 pups and their mothers were kept in 75% O₂ for 5 d, after which the animals were transferred to room air and killed at 6 and 12 h thereafter (Fig. 1A). Experiments were performed in duplicate with two independent O₂ chambers. On average, five mice (i.e., 10 retinas) were used for each time point (0, 6, or 12 h) in each chamber as described (17–19). Retinas were immediately dissected under a stereomicroscope and kept in RNAlater (Ambion) at 4 °C until the RNA was extracted. Four animals from each chamber as well as four controls (i.e., not exposed to 75% O₂) were maintained until postnatal day 21 as negative controls for neovascularization. The Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center approved all experiments (Animal Protocol 119909934). This study strictly adheres to the guidelines from the Association for Research in Vision and Ophthalmology statement.

RNA Extraction and Analysis. miRs and mRNAs were extracted using reagents from the mirVana miRNA Kit (Ambion). Selected miRs and mRNAs were quantified by real-time PCR with TaqMan microRNA Qsays (Applied Biosystems) or SybrGreen (sequences of primers and probes are available on request). snoRNA-202 and snoRNA-234 were used as endogenous controls for adjusting the expression of the miRs by real-time quantitative analysis. For mRNA quantification, the β-glucuronidase gene served as the endogenous control.

Gene Reporter Assays. The predicted 3' UTR target regions of both *Vegfa* and *HIF1A* genes, which contain the putative miR binding sites for miR-17 family members, were amplified by PCR, cloned downstream of the luciferase coding sequence in the pGL3 vector (Promega), and confirmed by DNA sequencing before transfection into COS-1 cells (4 × 10⁴ cells per well in 96-microwell plates; ATCC). Plasmids containing mutated target regions were generated by deleting 7 nt in the miR binding sites using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies). Plasmid constructs (100 ng per well) were cotransfected with *Renilla* luciferase pRL-CMV vector (2 ng per well) in the absence or presence of each miR-17 family member (n = 6) at a final concentration of 30 nM. Normalized luciferase ratios were determined 24 h after transfection using the Dual-Glo Luciferase Reporter-Assay System (Promega). All experiments were performed three times and contained 10 biological replicates each.

miR Transfections. Human Y79 retinoblastoma-derived cells (4 × 10⁵ cells per well; ATCC) were transfected with Lipofectamine 2000 (Invitrogen) in 12-well plates with each of the six miRs from the miR-17 family. Distinct time points (3, 6, 12, and 24 h) as well as various miR concentrations (3, 10, and 30 nM) were tested to evaluate the effects of miR transfection on the levels of HIF1A and VEGFA expression relative to negative controls. Off-target effects and specificity of the selected miRs were further evaluated using the nontargeted, Cy5-labeled control 1 miR (Ambion) at the same time points and concentrations. Posttransfection levels of HIF1A and VEGFA expression were determined using an ELISA DuoSet Kit (R&D Systems). For HIF1A quantification, cells were lysed with RIPA buffer, and HIF1A levels were determined from 35 mg total resulting protein. Secreted VEGFA was determined from 15 mL Y79 cell-conditioned media. All experiments were repeated at least three times in triplicate.

Statistical Analysis. One-way ANOVA was used to assess the significance of the differences between groups. The criterion used for significance was P < 0.05.

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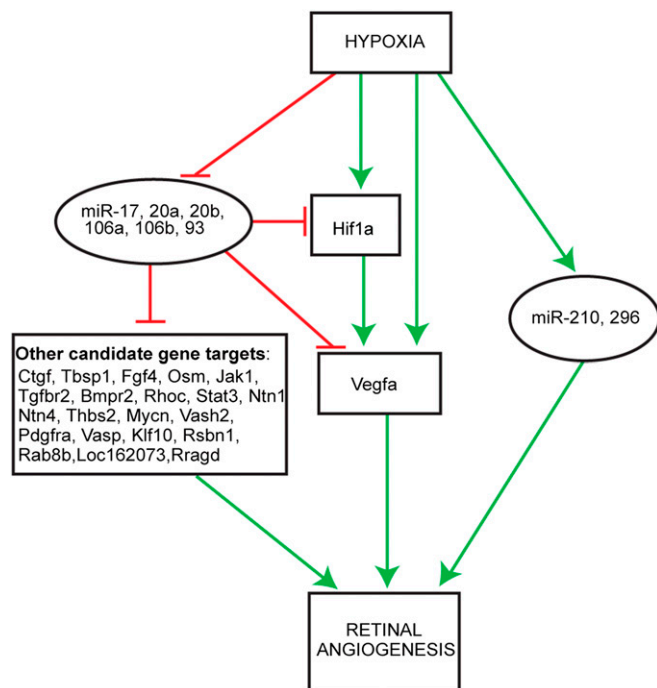


Fig. 5. Working hypothesis schematic. miR-17 family members bind to and negatively regulate *Hif1a* expression, which also affects *Vegfa* expression and other proangiogenesis genes. These miRs also simultaneously down-regulate *Vegfa* expression, which directly affects angiogenesis. Because these miRs might also regulate other proangiogenesis genes, it is likely that fluctuations in miR levels would affect angiogenesis because of its action on many different targets.

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