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miRNA-dependent cross-talk between VEGF and Ang-2 in hypoxia-induced microvascular dysfunction

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ABSTRACT

Ocular neovascularization is a vision-threatening complication of ischemic retinopathy that develops in various ocular disorders, such as retinopathy of prematurity (ROP) and diabetic retinopathy. Both Ang-2 and VEGF are implicated in this pathogenesis. However, their inter-regulation still remains elusive. Competitive endogenous RNAs (ceRNAs) are messenger RNA (mRNA) molecules that affect each other expression through the competition for the shared miRNA. Herein, we assessed whether the expression of Ang-2 and VEGF is interdependent through the sequestration of common miRNAs. Bioinformatics prediction and 3′-UTR luciferase assay revealed that Ang-2 and VEGF is commonly targeted by miR-351. Silencing either Ang-2 or VEGF increases the availabilities of shared miR-351, therefore reduces the activity of Luc-Ang-2 3′-UTR. The interdependence of VEGF and Ang-2 is miRNA- and 3′-UTR dependent, as silencing Dicer abolishes the interdependence. We also found that miR-351 dependency of VEGF-Ang-2 crosstalk occurs in retinal endothelial cells and rat retinas. miR-351 over-expression significantly reduces the level of VEGF and Ang-2 expression *in vivo* and *in vitro*. Overall, miRNA-dependent crosstalk between Ang-2 and VEGF plays a role in hypoxia-induced microvascular response. miRNA-based therapy can affect the expression of Ang-2 and VEGF, which represents a therapeutic potential for the treatment of vascular disease.

1. Introduction

The retina is one of the most metabolically active tissues in human body. It is highly sensitive to the reduction in oxygen tension. Hypoxia occurs in a series of ocular pathological processes, such as retinopathy of prematurity (ROP), Retinal vein occlusions (RVOs), or diabetic retinopathy (DR) [1]. Hypoxia is shown as a key inductor of vascular response. It could induce diseased cells to produce abnormal amounts of angiogenic factors, thereby arouse structural and functional changes in the eye [2].

Vascular endothelial growth factor (VEGF) is identified as a key angiogenic factor. It regulates angiogenic process throughout the human body and especially in the retina. VEGF expression is strongly induced upon hypoxia stress. Its inhibition with antibodies or soluble VEGF receptor is capable of blocking neovascularization [3]. Some other factors have also been identified as the potential regulators of neovascular pathologies, such as angiopoietin-2 (Ang-2), erythropoietin, insulin-like growth factor 1, its receptor or insulin-like growth factor-binding protein-3 [4]. Of them, Ang-2 is weakly expressed by the resting endothelium but strongly up-regulated following endothelial activation. Ang-2 has been identified as a vessel-destabilizing agent that plays a predominant role in controlling vessel regression. Previous studies have reported the relationship between Ang-2 and VEGF. Ang-1 is important for blood vessel maturation and stability and acts synergistically with VEGF to promote angiogenesis [5]. Ang-2 is an antagonist of Ang-1 and could promote vessel destabilization and regression in the absence of VEGF-A [6,7]. Another study reported that Ang-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival *in vivo* [8]. These evidences suggest that both Ang-2 and VEGF is involved in the regulation of angiogenesis. However, the interaction between VEGF and Ang-2 still remains controversial.

miRNAs are highly conserved, small non-coding RNA molecules that modulate gene expression at the post-transcriptional level. The role of miRNAs in endothelial cell functions and angiogenesis have been reported [9,10]. Let-7f, miR-27b, and miR-130a were identified as pro-angiogenic miRNAs [11]. miR-221 and miR-222 was found to inhibit endothelial cell migration, proliferation, and angiogenesis *in vitro* by targeting the stem cell factor receptor c-kit and indirectly regulating endothelial nitric oxide synthase expression [12]. Generally, one miRNA can target hundreds of

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mRNA transcripts, while each mRNA harbors multiple miRNA-response elements (MREs) that can be regulated by several miRNAs. Thus, different mRNAs can be regulated by a common miRNA. These RNA transcripts act as competing endogenous RNAs (ceRNAs) or natural microRNA sponges [13]. They communicate with and co-regulate each other by competing for binding to shared miRNAs [14,15]. However, it is still unknown whether such ceRNA regulatory mechanism occurs between VEGF- and Ang-2-mediated angiogenesis signaling. Here, we proposed that the expression of Ang-2 and VEGF is interdependent in response to hypoxia stress via competition for a common miRNAs.

2. Materials and methods

2.1. Cell culture and hypoxia model

RF/6A cells were obtained from American Type Culture Collection (ATCC). They were maintained at 37 °C in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO_2. For the hypoxia treatment, RF/6A cells were incubated with 200 μM $CoCl_2$ (Sigma–Aldrich) to mimic hypoxia condition. Data from cells cultured under normoxic condition was regarded as the baseline.

2.2. miRNA mimic synthesis and cell transfection

Based on the sequence of miRNA registered in miRBase database, miRNA mimics were synthesized at Ribobio Inc. (Guangzhou, China). For RF/6A cells cultured in a 6-well plate, 25 μM of miRNA mimic or control mimic was transfected using lipofectamine 2000 reagent according to the manufacturer's instruction (Life Technologies). The transfection mixture was removed 8 h post-transfection. The transfected cells were cultured for the corresponding time before they were harvested for further analysis.

2.3. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs of RF/6A cells or retinas were extracted using Trizol Reagent (Life Technologies). Small RNA enrichment was performed using mirVana miRNA Isolation Kit (Ambion). The relative miRNA expression was quantified using the mirVana™ qRT-PCR miRNA Detection Kit (Ambion). Real-time PCR was performed in 96-well plates with an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems) using SYBR® Green. The GAPDH mRNA was amplified as the internal control. The primer sequence is shown below. GAPDH: sense 5′-CCCATCACCATCTTCCAGGA-3′, anti-sense 5′-CATCGCCCCACTTGATTTTG-3′; VEGF: sense 5′-GGGCAGAATCATC ACGAAGTG-3′, anti-sense 5′-ATTGGATGG CAGTAGCTGCG-3′; Ang-2: sense 5′-GATGGCAGCGTTGATTTTCA-3′, anti-sense 5′-ACAT GCATCAAACCACCAGC-3′.

2.4. Western blot

Cell or tissue extracts were prepared by homogenizing cells or tissues in the lysis buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40) for 45 min. The soluble protein concentration was measured using Bio-Rad Protein Assay (Bio-Rad). The lysates (50 μg) were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (SDS–PAGE) and transferred to PVDF membranes for immunoblotting assay. The membrane was blocked in 5% fat-free dry milk, and probed with antibodies against the interest proteins. The blots were visualized using the enhanced ECL reagent. The levels of protein expression were semi-quantified by optical densitometry using Image J software. The fold change was shown as the ratio of experimental group against the control group.

2.5. Luciferase assay

Total cDNAs from RF/6A cells were used to amplify the 3′UTR of Ang-2 and VEGF. The 3′-UTRs of Ang-2 and VEGF were cloned to the multiple cloning sites downstream of the luciferase gene in the pMIR-REPORT vector. RF/6A cells were plated into 96-well plates at 50% confluence 24 h before transfection. Luciferase assays were conducted to investigate the effect of miRNA on its target gene [16]. In all transfections, the firefly luciferase activity was detected as a normalization control. Forty-eight hours after transfection, luciferase activities were determined using the dual luciferase reporter system.

2.6. Intraocular injection of miRNA mimic

Custom miRNA mimics were synthesized by Dharmacon based on mature miRNA sequences of miR-351 (5′-UCCCUGAGGAGCC CUUUGAGCCUGA-3′) and scramble control (5′-CACUUUGCACUG UGCUUCUGGGU-3′). For DR animal model, miR-351 mimics and negative control mimics were intravitreally injected using the lipofectin reagent (Life Technologies). Control mice were injected with the same volume of saline and lipofectin reagent. The animals were euthanized 2 month after injection, and the retinal tissues were collected. For OIR animal model, rat pups postnatal day 1 (P1) were anesthetized with halothane. miR-351 mimics and negative control mimics was administered to their right eyes using a FemtoJet microinjector (Eppendorf) with glass capillaries of approximately 60 gauge. Rats were sacrificed at P7 and P17, and retinas were dissected [17].

2.7. Statistical analysis

Data was shown as the mean ± standard error of the mean (SEM). Statistical evaluation was performed using one-way ANOVA when more than two groups were compared with a single control, and *t*-test was employed for comparison of differences between two different groups. Significant difference was assigned to $P < 0.05$.

3. Result

3.1. Ang-2 and VEGF are regulated by common miRNAs

We employed the TargetScan 6.2 (www.targetscan.org) to predict miRNAs that could regulate both Ang-2 and VEGF expression. We found that 7 miRNAs are potential common regulators, including miR-125a-5p, miR-125b-5p, miR-351, miR-670, miR-4319, miR-205, and miR-205ab (Fig. 1A). Further, the expression of these miRNAs was determined using qRT-PCR in rat retinas and RF/6A cells (derived from rhesus monkey microvascular endothelium). Of them, miR-205ab, miR-670, and miR-3419 was excluded for further analysis due to their low abundance (Fig. 1B).

Ang-2 or VEGF 3′UTR sequence containing predicted miRNA-response elements (MREs) was cloned into the luciferase vector, and then transfected with miRNA mimics. The result shows that miR-125a-5p, miR-125b-5p, and miR-351 could significantly down-regulate the activity of Luc-Ang-2-3′UTR (Fig. 1C). miR-351 and miR-205 transfection results in a marked reduction in Luc-VEGF-3′UTR activity (Fig. 1D). These data indicates that miR-351 is a common regulator of VEGF and Ang-2 expression. To further verify miR-351/VEGF and miR-351/Ang-2 interaction, we also constructed Ang-2 and VEGF 3′UTR mutants, which are deficient of miR-351 binding site. We found that miR-351 transfection could significantly reduce the activity of wild-type Luc-VEGF-3′UTR or Luc-Ang-2-3′UTR, but not alter the activity of mutant Luc-

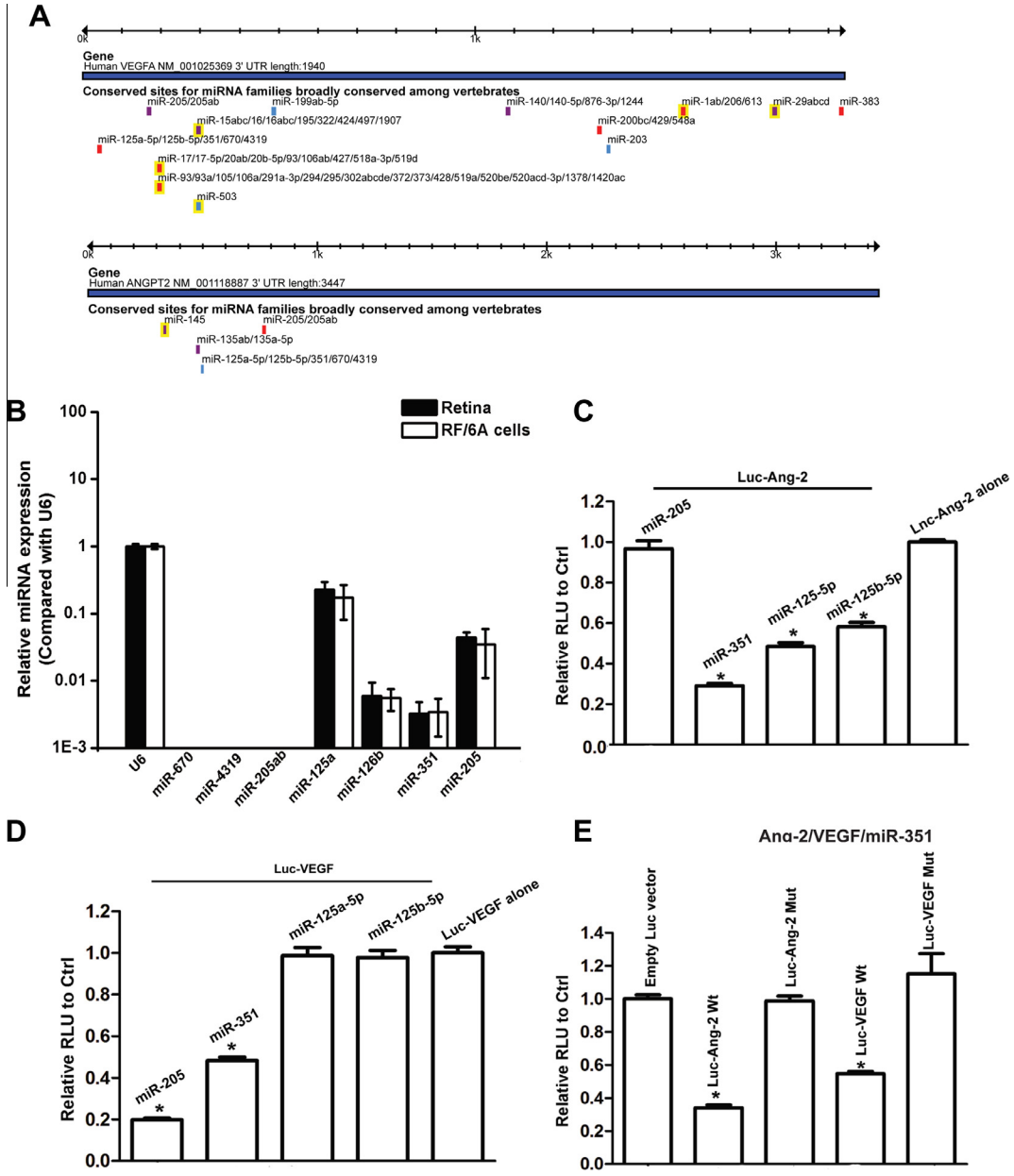

Fig. 1. Ang-2 and VEGF are regulated by the common miRNA. (A) Schematic representation of miRNA binding sites on the 3′-UTR of Ang-2 and VEGF. (B) Total RNAs were extracted from RF/6A cells or rat retinas. Real-time PCRs were performed to determine the relative expression of miRNAs. U6 was detected as the internal control. (C and D) Ang-2 or VEGF 3′UTR sequence was co-transfected with miRNA mimics. Luciferase activity was detected 48 h after transfection. The data was expressed as the relative change compared with the group transfected with Luc-Ang-2 or Luc-VEGF alone. (E) Wide-type and mutant Ang-2 or VEGF 3′UTR was co-transfected with miR-351 mimics. Luciferase activity was detected 48 h after transfection. The data was expressed as the relative change compared with the empty Luc vector. Data was shown as mean ± S.E.M. Asterisk indicates $P < 0.05$.

VEGF-3′UTR and Luc-Ang-2-3′UTR (Fig. 1E). Taken together, these results suggest that miR-351 is a potential miRNA, which could directly regulate both VEGF and Ang-2 expression.

3.2. Crosstalk between VEGF and Ang-2 expression

Retinal angiogenesis usually results from retinal ischemia due to retinal non-perfusion or a decrease in oxygen tension. Hypoxia induces the diseased cells to produce abnormal amounts of VEGF and Ang-2 [2]. To determine whether there is a crosstalk between VEGF and Ang-2 expression, we conducted siRNA-mediated gene silencing of Ang-2 or VEGF in RF/6A cells. We found that VEGF siRNA but not NC siRNA transfection results in an obvious decrease in the Ang-2 expression at mRNA and protein level under hypoxia condition (Fig. 2A–C). Inversely, Ang-2 depletion but not NC siRNA transfection could also significantly down-regulate VEGF expression at mRNA and protein under hypoxia condition (Fig. 2A–C). In addition, rat retinas were injected with negative control siRNA (NC), VEGF siRNA, or Ang-2 siRNA for 7 days. We found that VEGF siRNA results in an obvious decrease in the Ang-2 or VEGF expression. Ang-2 depletion could significantly down-regulate both Ang-2 and VEGF expression (Fig. 2D–F). Taken together, these data suggests that Ang-2 and VEGF could affect the expression of each other *in vitro* and *in vivo*.

3.3. Crosstalk between VEGF and Ang-2 is miRNA and 3′-UTR dependent

In RF/6A cells, hypoxia results in a significant increase in VEGF and Ang-2 expression at mRNA and protein level. Silencing of VEGF

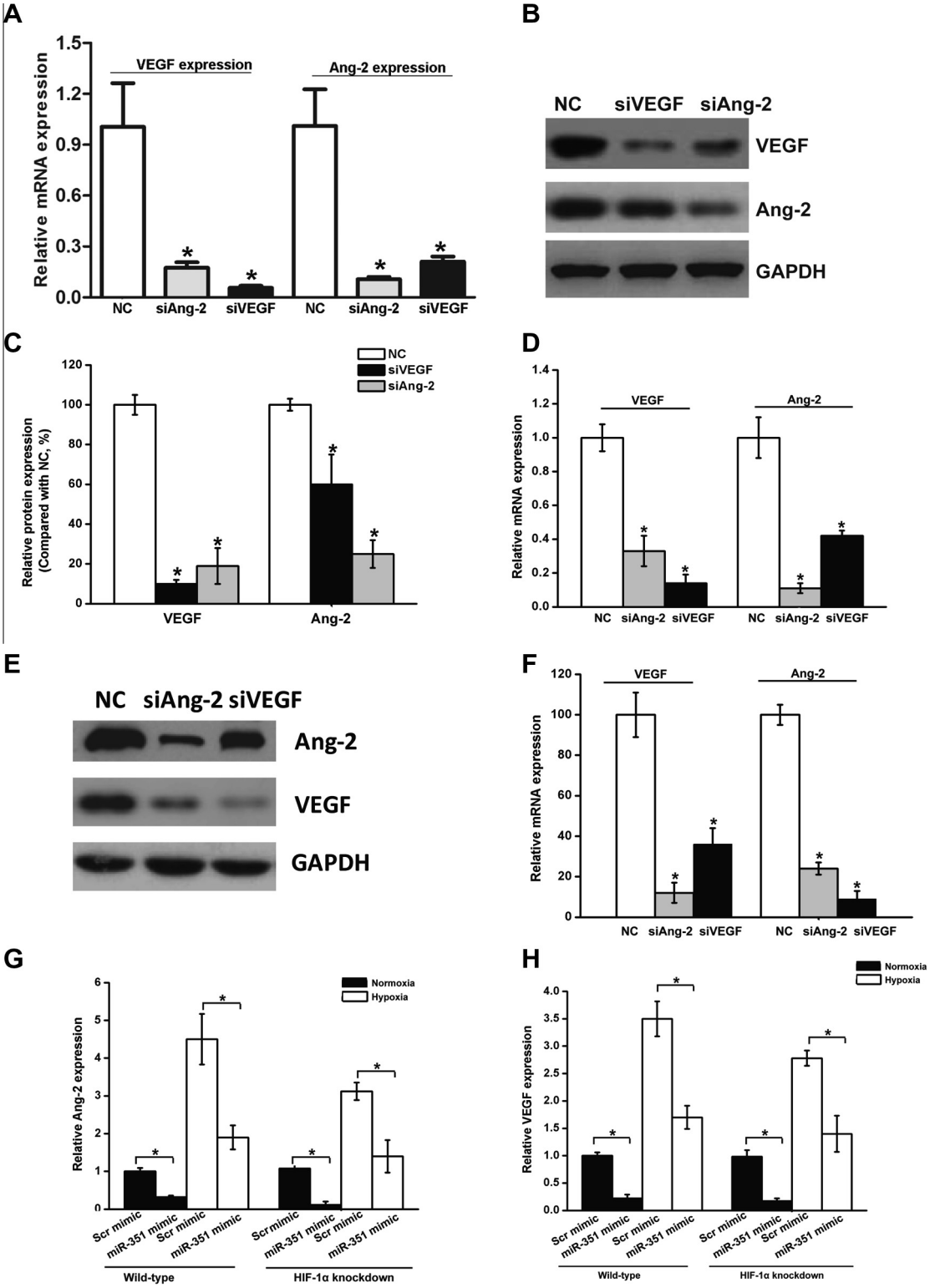

Fig. 2. Crosstalk between VEGF and Ang-2 expression. (A-C) RF/6A cells were incubated with negative control siRNA (NC), VEGF siRNA, or Ang-2 siRNA ($n = 4$ per group), respectively, and then exposed to hypoxia for 24 h. VEGF and Ang-2 mRNA or protein level was detected using real-time PCR (A) and western blot (B). Representative immunoblots (B) are shown along with quantitative data showing the mean ± S.E.M. from four separate blots (C). (D–F) Rat retinas were injected with negative control siRNA (NC), VEGF siRNA, or Ang-2 siRNA for 7 days. VEGF and Ang-2 mRNA or protein level was detected using real-time PCR (D) and western blot (E). Representative immunoblots (F) are shown along with quantitative data showing the mean ± S.E.M. from four separate blots (F). Asterisk indicates $P < 0.05$.

reduces Ang-2 expression, whereas silencing Ang-2 reduces VEGF levels (Fig. 3A–D). However, the interdependence between Ang-2 and VEGF was lost when Dicer was knockdown. VEGF expression was not altered when we added siAng-2 plus siDicer. Similarly, siVEGF plus siDicer did not affect Ang-2 expression. Dicer plays a key role on the maturation and activation of the miRNAs. Thus,

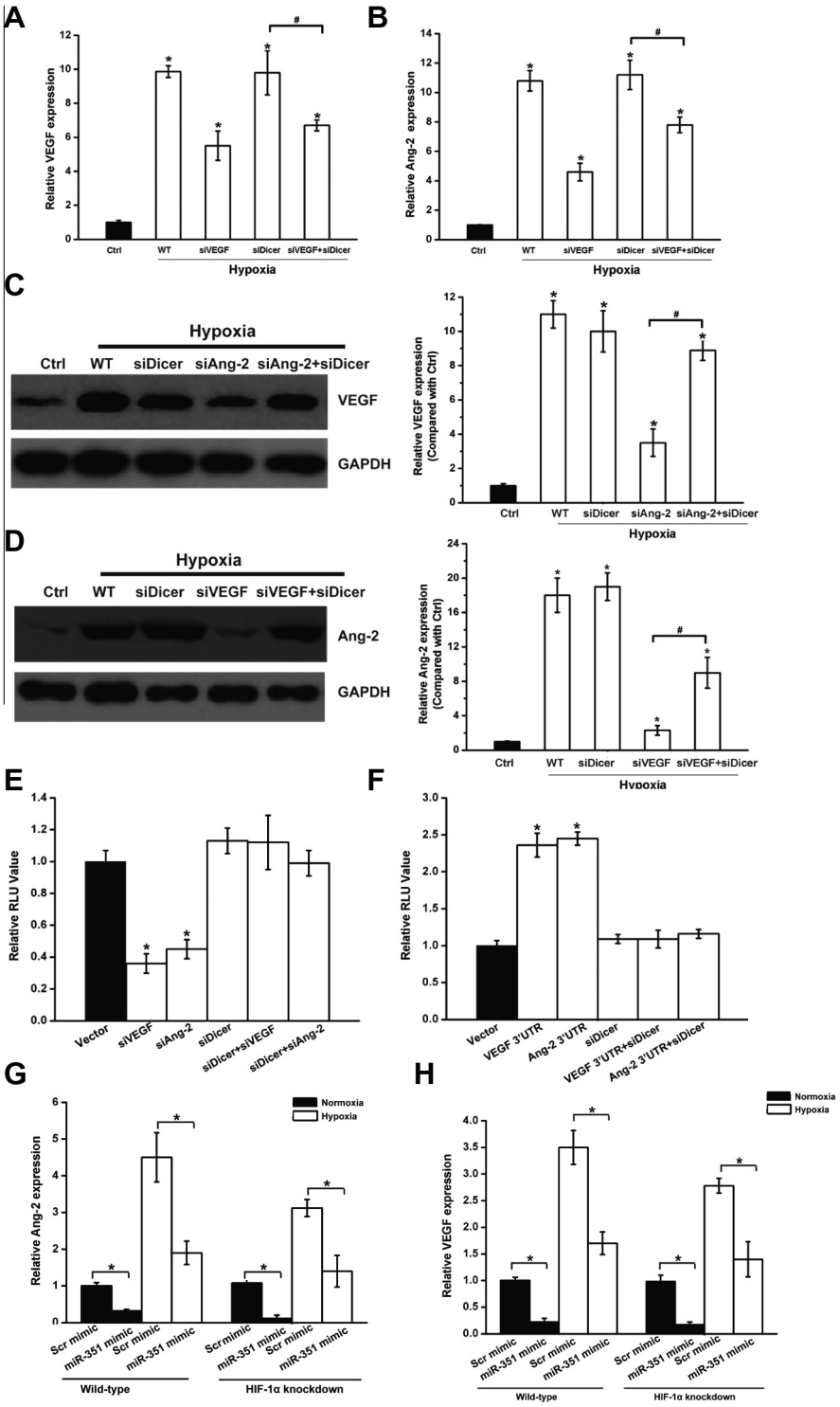

Fig. 3. Crosstalk between VEGF and Ang-2 is miRNA and 3′-UTR dependent. (A and B) RF/6A cells were treated as shown in the figure. Relative VEGF (A) and Ang-2 mRNA (B) expression was detected using real-time PCR. (C and D) Relative VEGF and Ang-2 protein expression was detected using western blot. Representative immunoblots are shown along with quantitative data showing the mean ± S.E.M. from four separate blots. Asterisk indicated $P < 0.05$. (E) RF/6A cells were treated as shown in the Fig. 3E. Luciferase activity was detected 48 h after transfection. The data was expressed as relative change compared with the group transfected with NC siRNA. Data were shown as mean ± S.E.M. Asterisk indicated $P < 0.05$. (F) Luc-VEGF 3′-UTR was transfected with negative control vector (pCMV), VEGF-3′-UTR or Ang-2-3′-UTR with or without siDicer. Luciferase activity was detected 48 h after transfection. The data was expressed as the relative change compared with pCMV vector. Data were shown as mean ± S.E.M. Asterisk indicates $P < 0.05$. (G and H) RF/6A cells were treated as shown. VEGF and Ang-2 expression was detected using real-time PCR. The data was expressed as the relative change compared with Scr mimic transfection alone. Data were shown as mean ± S.E.M. Asterisk indicates $P < 0.05$.

the fact that the crosstalk between Ang-2 and VEGF was abolished when Dicer was knocked out confirms the dependence on miRNAs (Fig. 3A–D).

miRNAs are known to post-transcriptionally regulate target mRNAs through the 3′-UTR [18]. If Ang-2 and VEGF crosstalk was miRNA-dependent, the 3′-UTR region might be involved in VEGF/

Ang-2 crosstalk. We transfected VEGF 3′-UTR luciferase reporter (Luc-VEGF 3′-UTR) in RF/6A cells, and then depleted Ang-2 or VEGF through siRNAs transfection. We found that silencing either Ang-2 or VEGF significantly decreases the activity of Luc-VEGF 3′-UTR (Fig. 3E). As silencing Ang-2 or VEGF reduces Luc-VEGF 3′-UTR activity due to increased availability of common miRNAs, we assume that overexpression of Ang-2 or VEGF 3′-UTR should have the opposite effect. Indeed, we found that overexpression of Ang-2 or VEGF-3′-UTR results in a significant increase in the activity of Luc-VEGF 3′-UTR (Fig. 3F). Moreover, silencing Dicer, the enzyme

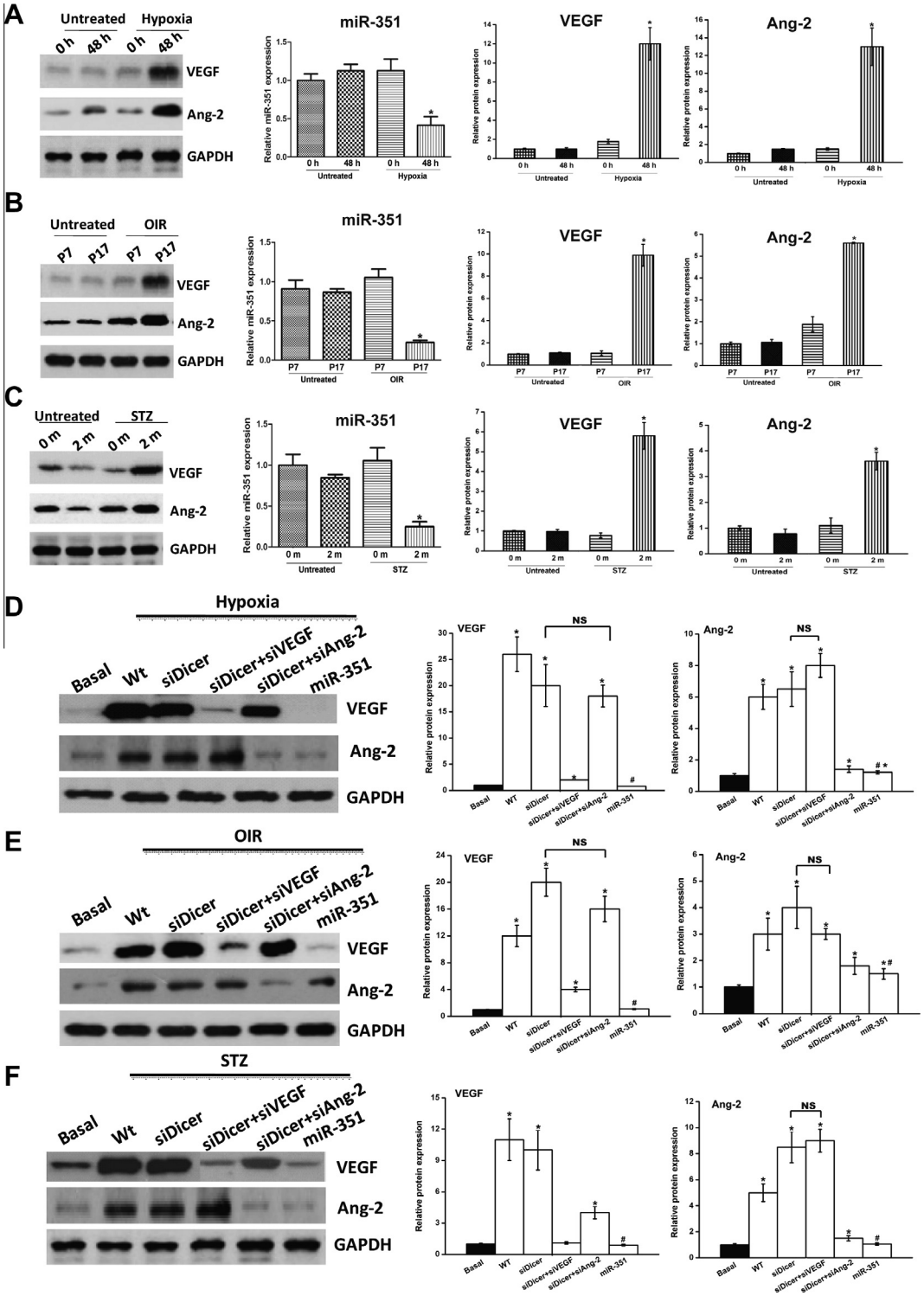

Fig. 4. miR-351 dependency of VEGF-Ang-2 crosstalk *in vitro* and *in vivo*. (A–C) RF/6A cells were collected from the hypoxia condition (1% O_2) or normoxia condition after 48 h transfection (A). Retinas were extracted from P7 and p17 of OIR and untreated animal model (B). Retinas were extracted from the rat after two months induction of diabetes and the corresponding control group (C). miR-351 expression was determined using real-time PCR. VEGF and Ang-2 expression was detected using western blot. (D) RF/6A cells were treated as shown, and then exposed to hypoxia condition. (E and F) Western blots were conducted to detect the expression of VEGF and Ang-2 levels in OIR rats and DR rats. Representative immunoblots are shown along with quantitative data showing the mean ± S.E.M. from four separate blots. Asterisk indicated $P < 0.05$.

that activates the miRNAs, abolishes the cross-talk between the 3′-UTR of VEGF and Ang-2, further supporting the fact that the crosstalk in miRNA-dependent (Fig. 3E and F), suggesting that the crosstalk between VEGF and Ang-2 is also 3′-UTR dependent.

Previous study reveals that VEGF and Ang-2 expressions are induced in hypoxia mainly through a well-known HIF1/HRE dependent mechanism. We further investigated whether miR-351 mediated VEGF-Ang-2 crosstalk is independent of HIF/HRE mechanism. We compared VEGF and Ang-2 expression difference with or without HIF expression under hypoxia or normoxia condition. We found that miR-351 could affect VEGF or Ang-2 expression in all experimental conditions (Fig. 3G and H), suggesting that miR-351 mediated VEGF-Ang-2 crosstalk is independent of HIF/HRE mechanism.

3.4. miR-351 dependency of VEGF-Ang-2 crosstalk in vitro and in vivo

To verify the existence of miR-351 dependency of VEGF-Ang-2 crosstalk, we detected its occurrence *in vitro*. We found that hypoxia results in a significant reduction in miR-351 level, whereas VEGF and Ang-2 expression is significantly up-regulated (Fig. 4A). Further, we built two different animal models of retinal vascular pathology, oxygen-induced retinopathy and diabetic retinopathy. We also found that miR-351 expression is negatively correlated with VEGF and Ang-2 expression (Fig. 4B and C).

We further investigated whether miR-351 expression change could affect VEGF and Ang-2 expression. RF/6A cells were transfected with miR-351 mimics to up-regulate miR-351 expression. We found that up-regulation of miR-351 leads to a significant reduction in Ang-2 and VEGF protein levels upon hypoxia stress *in vitro* (Fig. 4D). We also found that retinas injected with miR-351 mimics could result in a significant decrease in Ang-2 and VEGF protein expression in OIR and DR animal models (Fig. 4E and F). Moreover, Dicer expression was silenced to investigate the interdependence between Ang-2 and VEGF. We found that siVEGF knockout results in a marked reduction in VEGF level, whereas siVEGF plus siDicer transfection has no effect on Ang-2 level. Similarly, siAng-2 knockout results in a marked reduction in Ang-2 level, whereas siAng-2 plus siDicer transfection has no effect on VEGF level (Fig. 4E and F). These data also reveals miRNA-dependent interaction between Ang-2 and VEGF.

4. Discussion

Ocular diseases, such as PDR, RVOs or ROP, are usually characterized by retinal angiogenesis [19]. Retinal angiogenesis is orchestrated by the coordinate induction of a family of growth factor genes, including Ang-2 and VEGF. Hypoxia is thought to be a crucial physiologic stimulus for VEGF and Ang-2 up-regulation that precedes angiogenesis. Thus, clarifying VEGF and Ang-2 regulation during hypoxia condition is quite important to know the underlying mechanism of retinal angiogenesis [20]. In this study, we found that there is a crosstalk between VEGF and Ang-2 via the competition of miRNA binding to the 3′UTR of both genes. Reduced Ang-2 expression leads to a significant reduction in VEGF expression and vice versa. The inter-regulation of VEGF and Ang-2 and the sensitivity of this inter-regulation to miR-351 level provide a novel layer for the regulation of retinal angiogenesis.

To identify the common miRNA regulators, TargetScan prediction was conducted. Seven miRNAs were found to potentially affect both Ang-2 and VEGF expression [21]. Of them, two members are undetectable. 3′-UTR luciferase assay revealed that only one miRNA, miR-351, was found to regulate both Ang-2 and VEGF expression. Overexpression of miR-351 significantly reduces the expression of VEGF and Ang-2 protein levels *in vivo* and *in vitro*. Ang-2 can crosstalk with VEGF by competing for miR-351, and the crosstalk occurs through a ceRNA language based on MREs [22]. The ceRNA language would allow VEGF/Ang-2 to communicate. In fact, ceRNA function does not rely on the protein-encoding genetic blueprint. All MRE-containing components of the transcriptome, including mRNAs, transcribed pseudogenes, and long noncoding RNAs (lncRNA), are capable of regulating each other in this manner [22]. From this point, we speculated that VEGF or Ang-2 regulation may have other ceRNA-mediated mechanism. Thus, it is becoming an interesting topic required for further study in future.

Hypoxia is a key feature in many ocular vascular diseases. Many retinal cells can produce VEGF, including retinal pigment epithelium, endothelial cells, pericytes, glial cells, and ganglion cells. Retinal endothelial cells possess many VEGF receptors [23]. VEGF is capable of stimulating retinal endothelial cell proliferation *in vitro* [24]. We found that VEGF and Ang-2 expression is inducible in endothelial cells upon hypoxia stress. By contrast, miR-351 level is significantly reduced. During many ocular diseases, loss of retinal capillaries results in progressive retinal hypoxia, increased retinal vascular permeability, and new retinal vessel growth [25]. We also found that abnormal vascular function leads to a significant increase in VEGF and Ang-2 level, but a significant reduction in miR-351 level *in vivo*. More importantly, miR-351 alternation could alter both VEGF and Ang-2 level *in vitro* and *in vivo*. VEGF and Ang-2 plays a critical role in the development of ocular vascular disease, it is not surprised that miR-351 is a promising therapeutic target for ocular angiogenesis.

miRNA-based therapy has been reported in the prevention of ocular angiogenesis [26]. miR-200b mimic transfection down-regulates VEGF level and decreases ocular angiogenesis permeability in STZ-induced diabetic rats [17]. miR-126 is significantly decreased in retina from OIR mice. miR-126 up-regulation overcomes the high levels of VEGF, IGF-2 and HIF-1α, thereby reducing retinal neovascularization [27]. Intraocular injection of pre-miR-31, -150, or -184 significantly reduces ischemia-induced retinal neovascularization, while injection of pre-miR-31 or -150 could significantly reduce choroidal neovascularization [26]. Angiogenesis is a complex process in which several gene products participate [28]. miR-351 not only affects VEGF expression, but also regulates Ang-2 expression. VEGF and Ang-2 are emerged as the critical regulators of ocular angiogenesis at different stages. Thus, miR-351-based therapeutics could target different stages of ocular angiogenesis. In addition, miRNAs regulation also provides a post-transcriptional mechanism that complements transcriptional regulation to change the balance of pro-angiogenic and anti-angiogenic factors during the development of ocular angiogenesis.

In conclusion, we revealed that there is a cross-talk between VEGF and Ang-2 mediated via the competition for miR-351 binding. Reduced Ang-2 expression leads to a significant down-regulation of VEGF and vice versa. Both VEGF and Ang-2 have an important role in the development of ROP, DR and other retinal diseases such as age-related macular degeneration. miR-351-based intervention not only affects VEGF expression, but also alters Ang-2 expression. Thus, VEGF/Ang-2/miR-351 cross-talk may have a significant therapeutic implication for the treatment of vascular disease.

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