Cell Biology/Signaling

Evidence for the Involvement of miRNA in Redox Regulated Angiogenic Response of Human Microvascular Endothelial Cells

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Objective—A Dicer knockdown approach was used to test the significance of miRNA in regulating the redox state and angiogenic response of human microvascular endothelial cells (HMECs).

Methods and Results—Lowering of miRNA content by Dicer knockdown induced vascular endothelial growth factor expression but diminished the angiogenic response of HMECs as determined by cell migration and Matrigel tube formation. Such impairment of angiogenic response in the Matrigel was rescued by exogenous low micromolar H$_2$O$_2$. Dicer knockdown HMECs demonstrated lower inducible production of reactive oxygen species (ROS) when activated with either phorbol ester, tumor necrosis factor-α, or vascular endothelial growth factor. Limiting the production of ROS by antioxidant treatment or NADPH oxidase knockdown approaches impaired angiogenic responses. Experiments to identify how ROS production is limited by Dicer knockdown identified lower expression of p47phox protein in these cells. This lowering of cellular miRNA content induced expression of the transcription factor HBP1, a suppressor transcription factor that negatively regulates p47phox expression. Knockdown of HBP1 restored the angiogenic response of miRNA-deficient HMECs.

Conclusion—This study provides the first evidence that redox signaling in cells is subject to regulation by miRNA. Specifically, p47phox of the NADPH oxidase complex has been identified as one target that regulates the angiogenic properties of endothelial cells. (Arterioscler Thromb Vasc Biol. 2008;28:471-477)

Key Words: free radicals/free-radical scavengers ▪ gene expression ▪ hypoxia ▪ oxygen ▪ vascular biology ▪ redox ▪ angiogenesis ▪ endothelial function

miRNA represent a class of endogenous small (~22nt) RNA molecules that can repress protein synthesis. It is estimated that there are more than 600 miRNAs in mammalian cells, and that about 30% of all genes are regulated by miRNA. The key protein responsible for miRNA maturation is the cytosolic enzyme Dicer. Arrest of Dicer activity represents a productive approach to evaluate the overall functional significance of miRNA in any specific biological paradigm.

Several studies have demonstrated a central role of NADPH oxidase–derived reactive oxygen species (ROS) as signaling messengers in driving angiogenesis. Whether such redox control of angiogenesis is subject to regulation by miRNA remains unknown. In this study, we used a Dicer knockdown approach to test the significance of miRNA in regulating the redox state and angiogenic response of human microvascular endothelial cells (HMECs).

Materials and Methods

Cells and Cell Culture

HMECs were grown under standard culture conditions (at 37°C in a humidified atmosphere consisting of 95% air and 5% CO$_2$) in MCDB-131 growth medium supplemented with 10% FBS, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 mmol/L L-glutamine (GIBCO-BRL) as described previously.

siRNA Delivery to Cells

HMECs (0.2×10$^6$ cells per well in a 12-well plate) were seeded in antibiotic-free medium 24 hours before transfection. Dharmacine FECTITM 1 transfection reagent (Dharmacon RNA Technologies) was used to transfect cells with 100 nmol/L siRNA smart pool, for human Dicer, p47phox, or HBP1 as required. Transfection of nontargeting siRNA (Dharmacon RNA Technologies) was performed for the control group. The first transfection was performed for 72 hours as described previously (single transfection). In addition, a second transfection was performed for lowering cellular miRNA content. After 48 hours of the first transfection, cells were reseeded in 12-well plates and retransfected the following day for an additional 72 hours. After transfection, cells were collected or assayed for miRNA content. siRNA transfections for the knockdown of HBP1 were performed during the process of second siDicer transfection.

Quantification of mRNA Transcription Levels

Total RNA was isolated from cells using the Absolutely RNA Miniprep kit (Stratagene). RNA (1 μg) was reverse transcribed into

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cDNA using a Superscript III First-Strand synthesis system (Invitrogen). The transcription levels of miRNA for Dicer, vascular endothelial growth factor (VEGF)-A, HBPI, and β-Actin were quantified using real-time polymerase chain reaction (PCR) using SYBR green-I (Applied Biosystems). See details of primer sets in supplemental Table I (available online at http://atvb.ahajournals.org). The relative quantification approach was used to analyze the results.

**Quantification of miRNA Expression Level**

Total RNA including the miRNA fraction was isolated using mirVana miRNA isolation kit, according to the manufactures protocol (Ambion), miRNA levels were then quantified using specific Taqman assays for miRNA (Applied Biosystems) and mirVana qRT-PCR miRNA RT Kit (Applied Biosystems) using real-time PCR system and Taqman universal master mix. miRNA levels were quantified with the relative quantification method using β-actin as control.

**Nuclear Protein Extraction**

Nuclear protein extracts of HMECs were prepared using Nuclear Extraction Kit (Active Motif) according to the manufacturer’s protocol.

**Protein Detection**

For details of Western Blot and ELISA procedures used see supplemental Table II.

**Cell Proliferation**

Transfected cells were assayed for cell proliferation using CyQUANT cell proliferation assay kit (Invitrogen).

**Cell Viability**

Transfected cells were washed with PBS, centrifuged (500g, 5 minutes), resuspended in PBS, and cell membrane integrity was detected using a flow cytometer (FACSort, BD). For this assay, the nonpermeant DNA-intercalating dye propidium iodide (PI), which is excluded by viable cells, was used.15

**In Vitro Angiogenic Response**

For the methods used for Matrigel endothelial tube formation assay and endothelial cell migration assay see supplemental Table III.

**Determination of Intracellular ROS**

See supplemental Table IV.

**Immunocytochemistry**

See supplemental Table V.

**Statistical Analyses**

Data reported represent means±SD of at least 3 independent experiments. Difference between 2 means was tested by Student t test. P<0.05 was considered statistically significant.

**Results**

**Dicer siRNA Double Transfection in HMECs Silences Dicer Expression and Effectively Decreases miRNA Expression Levels**

Our goal was to lower miRNA content in HMECs by arresting the function of Dicer. This approach would enable us to examine the significance of miRNA in the angiogenic properties of HMECs. We noted that standard siRNA transfection procedure (single transfection, 72 hour) to knockdown Dicer resulted in lower abundance of Dicer mRNA and protein (supplemental Figure IA and IB). However, the protocol was insufficient to significantly lower the content of abundant16 endothelial cell miRNAs such as Hsa-miR-222 and Hsa-miR-18a (supplemental Figure IC and ID). The single transfection procedure did not significantly influence cell proliferation as well (supplemental Figure IE). To obtain significant lowering of miRNA content in the HMECs, we performed a double transfection procedure. This procedure included a second transfection of the same cells after 72 hours of the first transfection. After the second transfection, cells were maintained for an additional 72 hours. This additional time was allowed for the miRNA pool to be depleted whereas maturation of new miRNA was arrested by Dicer knockdown. Using this approach we were able to obtain not only significant lowering of Dicer mRNA and protein in HMECs but also significant lowering of both Has-miR-222 and Has-miR-18a (supplemental Figure IF through II). The double transfection approach also significantly lowered the rate of cell proliferation (supplemental Figure IJ). For all subsequent experiments, the double transfection approach was used.

**Lowering of miRNA Content by Dicer Knockdown Induced VEGF Expression**

To determine the general effect of miRNA depletion in HMECs on its angiogenic pathways, the expression of VEGF-A mRNA and proteins was determined in Dicer knockdown HMECs. Compared with siControl transfected cells, we noted induction of VEGF-A transcript as well as protein in miRNA-deficient HMECs (supplemental Figure II). These observations suggest the presence of a repressive control of VEGF by miRNA in HMECs.

**Lowering of miRNA Content by Dicer Knockdown Diminished the Angiogenic Response of HMECs as Determined by Tube Formation in Matrigel**

The ability to form tubes in a Matrigel is one of the major angiogenic characteristics of endothelial cells. After double transfection with siDicer or siControl, cells were counted and reseeded on Matrigel for 24 hours. Although miRNA-deficient Dicer knockdown cells produced more VEGF-A (supplemental Figure II), interestingly these cells demonstrated limited ability for tube formation in Matrigel compared with the corresponding siControl treated cells (Figure IA). Objective analysis of this observation was performed using standardized quantitation of tube length based on specific criteria described previously17 (Figure 1B).

**Lowering of miRNA Content by Dicer Knockdown Limited Angiogenic Response of HMECs as Determined by the Analysis of Cell Migration**

Endothelial cell migration represents an integral component of the angiogenic response and is driven by inducible NADPH oxidase derived ROS.18,19 To further elucidate the role of miRNA in regulating the angiogenic properties of HMECs, the migratory response of Dicer knockdown HMECs to scratch was investigated. This represents a standard model to investigate endothelial cell migration.20 Results of HMEC migration were recorded every 2 hours (Supplemental Figure III). Dicer knockdown HMECs, with lower miRNA content, demonstrated compromised migratory properties (Figure 1C). Compared with siControl transfected cells,
the rate of migration of siDicer transfected cells was cut down by half (Figure 1D).

Lowering of miRNA Content by Dicer Knockdown did not Influence Cell Viability
Compared with their corresponding controls, the viability of Dicer knockdown HMECs was not statistically different (Figure 1E). This result excludes the possibility that the limited angiogenic response of miRNA depleted cells was because of compromised cell viability of Dicer knockdown HMECs.

Lowering of miRNA Content by Dicer Knockdown Limited ROS Production in HMECs
ROS-dependent signaling is required for the angiogenic behavior of endothelial cells. NADPH-oxidase derived ROS are required for endothelial cell migration and tube formation. We sought to test the effect of miRNA depletion in HMECs on inducible ROS production. The phorbol ester PMA (phorbol myristate acetate) is known to induce ROS production and facilitate angiogenic response. When activated with PMA for 30 minutes, Dicer knockdown HMECs demonstrated lower DHE fluorescence indicating lower superoxide production in response to PMA activation (Figure 2A). Consistent results were obtained when DCF was used as the fluorescent probe for the detection of cellular ROS. Additionally, we noted that miRNA depletion does not only dampen inducible ROS production but also lowers resting production of ROS by HMECs (Figure 2B). Additional study of cellular ROS levels using a microscopic approach produced consistent results demonstrating lower ROS levels in Dicer knockdown HMECs (supplemental Figure IVA). To test whether the observed effects of miRNA depletion on lower cellular ROS was limited to the phorbol ester pathway we tested the effects of tumor necrosis factor (TNF) as well as that of VEGF on HMECs. TNFα is known to induce ROS in endothelial cells and stimulate angiogenic behavior. miRNA depletion in Dicer knockdown HMECs significantly lowered TNFα induced ROS production (Figure 2C). VEGF induces oxidant production in endothelial cells and drives angiogenic responses via redox signaling. miRNA depletion in Dicer knockdown HMECs significantly lowered VEGF-induced ROS production (Figure 2D and supplemental Figure IVB). Thus, results from phorbol ester, TNFα, as well as VEGF-activated HMECs consistently demonstrate that miRNA depletion impaired both resting as well as inducible cellular ROS production.

Significance of Cellular ROS in the Angiogenic Response of HMECs
Decomposition of cellular ROS by the treatment of HMECs with the antioxidant N-acetylcysteine (NAC) significantly slowed cell migration (supplemental Figure VA) and impaired tube formation on the Matrigel (supplemental Figure VB). Of note, compromise in Matrigel tube formation of

![Figure 1](https://www.ahajournals.org/doi/fig/10.1161/01.CIR.0000390204.27898.a5)

**Figure 1.** Dicer knockdown induced miRNA deficiency impaired the angiogenic response of human microvascular endothelial cells. A-B, Matrigel® tube formation assay; A, Matrigel® tube formation visualized by phase contrast microscopy. Representative image of at least 3 independent experiments; B, measurement of tube curve length (µM); Results are mean ± S.D. *p<0.05 compared with control. C, cell migration, quantitative data of the observation shown in Supplementary figure III; D, Cell migration, as determined by the slope of the line between the 4h and 6h time points (see supplementary figure III), was diminished by 50% in response to Dicer knockdown; E, Cell viability as measured by propidium iodide exclusion following double transfection for Dicer knockdown. Results are mean ± S.D. *p<0.05 compared with control.

![Figure 2](https://www.ahajournals.org/doi/fig/10.1161/01.CIR.0000390204.27898.a5)

**Figure 2.** Dicer knockdown induced miRNA deficiency limited ROS production under basal and activation conditions. A, HMEC double transfected with siDicer or siControl were activated with 100 nM PMA for 30 min. DHE fluorescence was measured using a flow cytometer; B, Dicer knockdown HMEC were activated with 100 nM PMA for 4 h. DCF fluorescence was measured using a flow cytometer; C, Dicer knockdown HMEC were activated with 50 ng TNFα for 90 min. DCF fluorescence was measured as in B; D, HMEC double-transfected with siDicer or siControl were activated with 50 ng/ml VEGF for 1 h. Fluorescence microscopic images (see supplementary figure IV) were collected. Quantitation of DCF green fluorescence. Results are mean ± S.D. *p<0.05 compared with corresponding siControl.
Dicer knockdown HMECs could be significantly corrected by administration of exogenous H2O2 to cells. Low micromolar H2O2 (0.1 and 1 μM) restored the tube forming property of miRNA depleted cells in a time dependent manner (Figure 3; for images see supplemental Figure VI). These observations underscore the critical importance of ROS, especially H2O2, in driving the angiogenic properties of HMECs.

Lowering of miRNA Content by Dicer Knockdown Specifically Lowered p47phox Expression in HMECs

The superoxide-generating NADPH oxidase is converted to an active state by the assembly of a membrane-localized cytochrome b559 (gp91) with 3 cytosolic components: p47(phox), p67(phox), and GTPase Rac1 or Rac2. In our attempt to characterize the mechanisms which underlie lower inducible ROS production in Dicer knockdown miRNA-deficient HMECs, we investigated the expression of the components of the NADPH oxidase complex. Whereas miRNA depletion caused by Dicer knockdown did not influence the expression of gp91, Rac1 and Rac2, the expression of p47phox was significantly lowered (Figure 4). In support on the Western blot data shown in Figure 4C and 4D, the study of p47phox localization during tube formation on the Matrigel demonstrated lower p47phox levels in miRNA-deficient HMECs (Figure 4E).

p47phox Is a Central Component That Supports NADPH Oxidase Activity and the Angiogenesis Property of HMECs

HMECs were transfected with sip47phox or siControl. Knockdown of p47phox compromised PMA-induced ROS production and cell migration (Figure 5A through 5C).

Lowering of miRNA Content by Dicer Knockdown Upregulated Expression of the Transcription Factor HBP1

miRNA are expected to negatively regulate gene expression.28 Thus, lowering of miRNA is expected to upregulate miRNA target genes. In contrast, we noted a lowering of p47phox expression in miRNA-deficient cells (Figure 4). Thus, we tested the hypothesis that a negative regulator of p47phox expression represents a direct target of miRNA. HBP1 is a suppressor transcription factor that negatively regulates p47phox expression.29 To visualize the nuclear localization and abundance of the transcription factor HBP1 we adopted an immunocytochemical approach. In addition, the abundance of HBP1 protein in nuclear extracts was examined by immunoblotting. Dicer knockdown miRNA-deficient HMECs demonstrated significantly higher levels of nuclear HBP1 (Figure 6A through 6D). These results demonstrate that in HMECs, HBP1 expression is directly regulated by miRNA. Global lowering of miRNA levels caused by Dicer knockdown withdraws the negative control of miRNA on HBP1 expression resulting in higher levels of HBP1. Elevated HBP1, in turn, downregulates p47phox expression and limits ROS-dependent angiogenic signaling.

Figure 3. Exogenously administered H2O2 rescued Dicer knockdown HMEC from impaired Matrigel® tube formation. Dicer knockdown and control HMEC were seeded on Matrigel® and treated or not with 0.1 or 1 μM H2O2. Images were taken after 5h, 10h and 20h of H2O2 treatment. H2O2 induced tube formation in Dicer knockdown HMEC. Representative images (20h time-point) of 4 independent experiments are shown in supplementary figure VI. Scoring of tube curve length (μm). Results are mean ± S.D. *, compared to control, p <0.05.

Figure 4. Dicer knockdown induced miRNA deficiency specifically downregulated p47phox expression while not affecting the expression of gp91, Rac1 and Rac2 components of the NADPH oxidase complex. Following double transfection of HMEC with siDicer or siControl, whole cell lysates were extracted and subjected to Western blot analysis. A, representative images of Dicer, gp91, Rac1, Rac2 protein levels; B, densitometry analysis of A; C, p47phox expression was decreased in Dicer knockdown cells; D, densitometry analysis of C; E, following transfection with siDicer or siControl, cells were seeded on Matrigel® for 2h to enable tube formation. Cells were fixed and stained with anti-p47phox. Compared to siControl transfected cells, Dicer knockdown cells showing lower abundance of p47phox in the tubes (top panel shows DIC image for reference). Results are mean ± S.D. * p<0.05 compared with corresponding siControl.
Such conclusion is supported by the observation that HBP1 knockdown improved the angiogenic outcome in HMECs subjected to Dicer knockdown (Figure 6E through 6G).

**Discussion**

Dicer is the key enzyme controlling miRNA biogenesis. Silencing Dicer in vivo causes embryonic lethality because of impairments in angiogenesis. Silencing Dicer in vitro impairs the angiogenic response of human umbilical vein endothelial cells (HUVECs) and EA.hy.926 endothelial cells. Although these observations indicate the possible regulation of angiogenesis by miRNA, the underlying mechanisms remain unknown. Majority of human miRNA loci are located within intronic regions and are transcribed by RNA polymerase II as part of their hosting transcription units. The primary transcripts are cleaved by Drosha to release approximately 70-nt pre-miRNAs that are subsequently processed by Dicer to generate mature approximately 22-nt miRNAs. Endogenous miRNAs have a long half-life. As such, it is understandable why the long double-transfection approach was more effective in lowering endogenous miRNA levels.

Our observation that Dicer knockdown miRNA-deficient HMECs show impaired tube formation on the Matrigel is consistent with a recent report demonstrating that adding VEGF to the cell culture media of Dicer knockdown cells do not restore the ability of HUVECs to form tubes on Matrigel. Results of the current study display that impaired tube formation in Dicer knockdown HMECs is noted despite elevated VEGF expression. This finding is consistent with the observation that Dicer-deficient embryos contain higher levels of VEGF yet impaired angiogenesis. These lines of evidence lead to the notion that Dicer-dependent miRNAs regulate the angiogenic response of endothelial cells downstream of VEGF.

ROS have been implicated to serve as signaling molecules in numerous mechanisms including angiogenesis. VEGF stimulates proliferation, migration, and tube formation of endothelial cells primarily through the VEGF receptor type 2 (VEGFR2). Ligation of VEGF to VEGFR2 activates NADPH oxidase which in turn produces ROS to support the angiogenic response of endothelial cells. Activation of VEGF through ROS mediates 20-hydroxyeicosatetraenoic acid-
induced endothelial cell proliferation. ROS seems to be a common denominator signaling mediator for angiogenesis beyond that caused by VEGF. Hydrogen peroxide produced by angiopoietin-1 mediates angiogenesis. Endothelial cell migration is regulated by localized production of ROS at the leading edge and at the site of injury. Decomposition of ROS stalls angiogenesis both in vivo as well as in vitro. The current finding that Dicer knockdown miRNA-deficient HMECs produce lower levels of basal ROS demonstrates that nonphagocytic oxidases and other sources of ROS in cells under standard culture conditions are subject to regulation by endogenous miRNAs. Two lines of evidence in the current study support a central role of ROS in facilitating the angiogenic behavior of HMECs. First, that downregulation of cellular ROS by the antioxidant NAC impairs Matrigel tube formation. Second, that impaired Matrigel tube formation in miRNA-deficient HMECs can be corrected by exogenous H2O2. The latter line of evidence also indicates that the effect of miRNA on cellular redox state is functionally significant with respect to determining angiogenic outcomes. NADPH oxidase activity represents a major pathway that contributes to cellular ROS in response to activation by phorbol ester, TNFα, and VEGF. Our observation that miRNA-deficient HMECs show lower NADPH oxidase activity, both basal as well as inducible, provide first pointer indicating that miRNA are critically important in regulating the redox signaling network that are now known to be implicated in numerous physiological as well pathophysiological processes.

NADPH oxidase is one of the major sources of ROS in vasculature. It consists of a catalytic subunit (Nox1, Nox2, Nox3, Nox4, or Nox5), p22phox, p47phox, and the small GTPase Rac1 and Rac2. The phagocytic NADPH oxidase consists of a membrane-localized glycosylated, catalytic subunit, gp91phox (also known as Nox2), along with a second membrane-associated subunit, p22phox. gp91phox and p22phox stabilize one another in a tightly associated heterodimer which mediates the transfer of electrons from NADPH to molecular oxygen to generate superoxide anion radicals. The interaction of the membrane components with cytosolic regulatory subunits is important for the activation of electron flow. p22phox plays a central role in this process, via interaction with p47phox. Cytosolic small GTPase proteins Rac1 and Rac2 support optimal activity of the NADPH oxidase complex. Findings of the current study demonstrate that whereas miRNA deficiency caused by Dicer knockdown does not affect the expression of gp91phox, Rac1, or Rac2, it specifically downregulates both p47phox expression as well as ROS production. Serine phosphorylation of p47phox and its enhanced binding to p22phox is involved in NADPH oxidase function in endothelial cells. p47phox-deficient mice exhibit impaired wound angiogenesis. Consistently, results of this study demonstrate that knockdown of p47phox impaired ROS production and cell migration.

Given that the primary function of miRNA is to interfere with the expression of gene products, downregulation of p47 expression in miRNA-deficient cells indicated secondary regulation of p47phox by one or more repressors which may have been upregulated in response to the withdrawal of the negative control of genes by miRNA. Upregulation of HBPI (3-hydroxy-3-methylglutaryl [HMG] box-containing protein 1) in miRNA-deficient HMECs proved that hypothesis. The transcriptional repressor HBPI regulates the gene for the p47phox regulatory subunit of the NADPH oxidase. HBPI represses growth regulatory genes (eg, N-Myc, c-Myc, and cyclin D1) and is an inhibitor of G1 progression. The promoter of the p47phox gene contains 6 tandem high-affinity HBPI DNA-binding elements at positions −1243 to −1318 bp from the transcriptional start site which were required for repression. Furthermore, HBPI represses the expression of the endogenous p47phox gene through sequence-specific binding. Thus, HBPI downregulates NADPH oxidase-dependent superoxide production through transcriptional repression of the p47phox gene. The observation that HBPI is upregulated in miRNA-deficient HMECs is consistent with computational predictions of TargetScan and miRbase. These databases list HBPI as being highly susceptible to miRNA regulation. The family of hsa-miR-29 (a, b, and c) seem likely to regulate HBPI expression for 2 reasons. First, they were predicted with the highest scores in both computational models. Second, hsa-miR-29c and hsa-miR-29a have been recently known to be abundant in endothelial cells.

In summary, this study provides the first evidence that redox signaling in cells is subject to regulation by miRNA. Specifically, p47phox of the NADPH oxidase complex has been identified as one functional target. Although the focus of the current study has been on angiogenesis, it is plausible that other redox-sensitive aspects of human cell biology are subject to control of endogenous miRNA.

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Disclosures

None.

References


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SUPPLEMENTARY INFORMATION (SI): Legends for Figures I to VI.

**Fig. I.** *Double, but not single, transfection of HMEC with Dicer siRNA successfully down-regulated miRNA content.* Single transfection of HMEC with Dicer siRNA (72h) decreased Dicer mRNA transcription (A), lowered Dicer protein expression (western blot), C+D. did not reduce significantly hsa-miR-222 and hsa-miR-18a expression levels (real-time PCR), E. cell proliferation was not effected in 24 h and 48 h, F. double Dicer siRNA transfection (48+72h) decreased Dicer mRNA transcription (real-time PCR), G. silenced Dicer protein expression (western blot), H+I. significant reduction of hsa-miR-222 and hsa-miR-18a expression (real-time PCR); J. cell proliferation is decreased; Results are mean ± S.D. * p<0.05 compared with control.

**Fig. II.** *Dicer knockdown induced miRNA deficiency increased VEGF expression.* Dicer knockdown increased VEGF gene (A) and protein (B) expression. VEGF protein was measured from cell culture media. Results are mean ± S.D. * p<0.05 compared with control.

**Fig. III.** *Dicer knockdown induced miRNA deficiency impaired human microvascular endothelial cell migration.* Following double transfection with control and Dicer siRNA, cells were re-seeded, scratched the next day and cell migration was measured every 2 h using phase contrast microscopy. miRNA deficient HMEC showed impaired cell migration. Representative image of at least 3 independent experiments. For quantitative results see Fig. 1D.
**Fig. IV.** Dicer knockdown induced miRNA deficiency limited ROS production under basal and activation conditions. Dicer knockdown HMEC were activated with 100 nM PMA for 4 h. 

*Fig. IV A.* Fluorescence microscopic images of DCF treated HMEC. DAPI staining shows nuclei, image representative of 3 independent experiments;  

*Fig. IV B.* HMEC double-transfected with siDicer or siControl were activated with 50 ng/ml VEGF for 1 h. Fluorescence microscopic images of DCF (*green*, ROS) and DAPI (*blue*, nuclei) were collected. Images shown are representative of three independent experiments. For quantitative results see Fig. 2D.

**Fig. V.** Significance of ROS in the angiogenic response of HMEC.  

*Fig. V A.* Cells were seeded and scratched the following day. Percentage of the gap covered by migrating cells was monitored by time lapse microscopy. Treatment of HMEC with the antioxidant NAC limited cell migration.  

*Fig. V B.* Cells were seeded on Matrigel® and tube formation was determined. Results are mean ± S.D. *p*<0.05 compared with control.

**Fig. VI.** Exogenously administered H$_2$O$_2$ rescued Dicer knockdown HMEC from impaired Matrigel® tube formation.  

*Fig. VI* Dicer knockdown and control HMEC were seeded on Matrigel® and treated or not with 0.1 or 1 µM H$_2$O$_2$. Images were taken after 5h, 10h and 20h of H$_2$O$_2$ treatment. H$_2$O$_2$ induced tube formation in Dicer knockdown HMEC. Representative images (20h time-point) of 4 independent experiments are shown. For quantitative scoring see Figure 3.
Supplementary Table III. Methods to assay angiogenic responses in vitro

*In vitro angiogenic response: Matrigel® endothelial tube formation assay*

Four well plates were coated with 100 µl Matrigel® (Cultrex® Basement Membrane Extract reduced growth factor; R&D Systems, Minneapolis, MN) and let to solidify for 30 min at 37°C. Next, transfected HMEC were seeded (10,000 cells/well) on top of the solidified Matrigel® and maintained in a cell culture incubator. Endothelial tube formation was observed and digitally photographed 24h post seeding under an inverted light microscope at 10 X magnification (Axiovert 200M; Zeiss, Oberkochen, Germany) as described previously 1.

*In vitro angiogenic response: Endothelial cell migration assay*

After transfection, HMEC were reseeded in 4-well plates (0.2 x 10⁶/500µl in each well). The next day, the monolayers were scratched with a 1-10 µl size pipette tip. The distance of the gap caused was measured under a 10x phase objective of a light microscope and recorded every 2 h (Axiovert 200M; Zeiss, Oberkochen, Germany) 2.

References

Supplementary Table IV. Methods to determine intracellular reactive oxygen species (ROS)

Detection of ROS was performed using dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) (Molecular Probes, Invitrogen, Carlsbad, CA). This probe has high reactivity to hydrogen peroxide and low reactivity to superoxide anions. After transfection, the cells were washed with PBS, centrifuged (500g, 5 min), resuspended in PBS and incubated with 10 µM H$_2$DCF-DA for 20 min at 37°C. To detect cellular fluorescence, the fluorochrome-loaded cells were excited using a 488-nm argon-ion laser in a flow cytometer. The dichlorofluorocein (DCF) emission was recorded at 530 nm. Data were collected using a flow-cytometer from at least 5000 cells. Alternatively dihydroethidium (DHE) (Molecular Probes, Invitrogen, Carlsbad, CA) fluorescence was recorded using the same protocol with excitation at 488 nm and emission at 575 nm. As additional approach, the Image-iT live green ROS detection system (Molecular Probes, Invitrogen, Carlsbad, CA) was used to visualize ROS in live cells. Fluorescent carboxy-H$_2$DCF-DA permeates live cells and is deacetylated by non-specific intracellular esterases. In the presence of ROS, the reduced fluorescein compound is oxidized and emits bright green fluorescence. Fluorescence microscopy (10x, Axiovert 200M; Zeiss, Oberkochen, Germany) was performed to capture images of nuclei (blue fluorescence; Hoechst 33342) and oxidized fluorescein. Transfected cells were activated with either PMA (Sigma-Aldrich, St. Louis, MO), TNFα or VEGF$_{165}$ (R&D Systems, Minneapolis, MN). Cells were serum-starved for 16 h with 0.5 % FBS prior to treatment with VEGF.

References

**Supplementary Table V. Immunohistochemical methods used.**

Following transfection of HMEC, cells (0.5x10^6/well) were seeded on cover slips in 6-well plates. The following day, cells were washed twice with ice-cold PBS and then fixed in 10% buffered formalin for 20 min. Next, the cells were washed thrice with PBS followed by permeabilization using 0.1% Triton X-100/PBS for 15 min. The cells were then washed 3 times with PBS and incubated with 10% goat or horse serum (Vector Laboratories) for 1h at room temperature. Next, the cells were incubated with anti-Dicer (1:100, in 10% goat serum) or anti-HBP1, (Santa Cruz Biotechnologies, Santa Cruz, CA; 1:75, in 10% horse serum) overnight in 4ºC. After incubation with primary antibodies, cells were washed thrice with PBS and incubated with an Alexa-fluor mouse (green) for Dicer and Alexa-fluor goat (red) for HBP1 for 1h at room temperature. After three washes with PBS and incubation with DAPI (1:10,000) for 2 min, the endothelial cells were washed with PBS and mounted in gelmount (aqueous mount, Vector Laboratories) for microscopic imaging. For immunocytochemistry during tube formation, cells were seeded on Matrigel® for tube formation assay and fixed after 2 h. Next, the same staining assay was performed using goat serum, anti-p47phox and Alexa-fluor mouse (red).
**Supplementary Table VI.** Detailed Figure Legends for Illustrations Appearing in Hard-Print

**Fig. 1.** Dicer knockdown induced miRNA deficiency impaired the angiogenic response of human microvascular endothelial cells. *A-B*, Matrigel® tube formation assay: *A*, Matrigel® tube formation visualized by phase contrast microscopy. Representative image of at least 3 independent experiments; *B*, measurement of tube curve length (µM); Results are mean ± S.D. *p*<0.05 compared with control. *C*, cell migration, quantitative data of the observation shown in *Supplementary figure III*; *D*, Cell migration, as determined by the slope of the line between the 4h and 6h time points (*see Supplementary Fig. III*), was diminished by 50% in response to Dicer knockdown; *E*, Cell viability as measured by propidium iodide exclusion following double transfection for Dicer knock-down. Results are mean ± S.D. *p*<0.05 compared with control.

**Fig. 2.** Dicer knockdown induced miRNA deficiency limited ROS production under basal and activation conditions. *A*, HMEC double transfected with siDicer or siControl were activated with 100 nM PMA for 30 min. DHE fluorescence was measured using a flow cytometer; *B*, Dicer knockdown HMEC were activated with 100 nM PMA for 4 h. DCF fluorescence was measured using a flow cytometer; *C*, Dicer knockdown HMEC were activated with 50 ng TNFα for 90 min. DCF fluorescence was measured as in *B*; *D*, HMEC double-transfected with siDicer or siControl were activated with 50 ng/ml VEGF for 1 h. Fluorescence microscopic images (*see Supplementary Fig. IV*) were collected. Quantitation of DCF green fluorescence. Results are mean ± S.D. *p*<0.05 compared with corresponding siControl.
Fig. 3. Exogenously administered H$_2$O$_2$ rescued Dicer knockdown HMEC from impaired Matrigel® tube formation. Dicer knockdown and control HMEC were seeded on Matrigel® and treated or not with 0.1 or 1 µM H$_2$O$_2$. Images were taken after 5h, 10h and 20h of H$_2$O$_2$ treatment. H$_2$O$_2$ induced tube formation in Dicer knockdown HMEC. Representative images (20h time-point) of 4 independent experiments are shown in supplementary Fig. VI. Scoring of tube curve length (µm). Results are mean ± S.D. *, compared to control, p <0.05.

Fig. 4. Dicer knockdown induced miRNA deficiency specifically down-regulated p47phox expression while not affecting the expression of gp91, Rac1 and Rac2 components of the NADPH oxidase complex. Following double transfection of HMEC with siDicer or siControl, whole cell lysates were extracted and subjected to Western blot analysis. A, representative images of Dicer, gp91, Rac1, Rac2 protein levels; B, densitometry analysis of A; C, p47phox expression was decreased in Dicer knockdown cells; D, densitometry analysis of C; E, following transfection with siDicer or siControl, cells were seeded on Matrigel® for 2h to enable tube formation. Cells were fixed and stained with anti-p47phox. Compared to siControl transfected cells, Dicer knockdown cells showing lower abundance of p47phox in the tubes (top panel shows DIC image for reference). Results are mean ± S.D. * p<0.05 compared with corresponding siControl.

Fig. 5. Knockdown of p47phox impaired cell migration and decreased inducible ROS production. HMEC were transfected with siRNA against p47phox for 72h. A, Western blot; B, following activation with 100 nM PMA for 4h, p47phox knockdown cells showed decreased levels of ROS production as detected by DCF staining and flow cytometry; C, following transfection with sip47phox, cells were seeded and scratched the following day. Cell migration (2h) was impaired in p47phox knockdown cells; Results are mean ± S.D. * p<0.05 compared with corresponding siControl.
Fig. 6. Dicer silencing up-regulated the expression of the transcription factor HBP1 which played a central role in impairing the angiogenic response of HMEC. Following siDicer or siControl double transfection, HMEC were re-seeded on cover slips and stained with antibodies against Dicer and HBP1. DAPI was used for staining of nuclei. A, Dicer knockdown lowered the abundance of cellular Dicer protein levels; B, Dicer knockdown elevated the expression of HBP1; C, Quantitation of HBP1 red fluorescence shown in B; D, Western blot of nuclear extracts; E, co-transfection of siDicer with siRNA against HBP1 decreased HBP1 mRNA levels; F-G, co-transfection of siDicer with siRNA against HBP1 significantly released the inhibitory effects of Dicer knockdown on Matrigel® tube formation. increased the cells angiogenic response shown by tube formation on Matrigel; G, Quantitation of tube length. * p<0.05 compared with corresponding siDicer transfected controls.
Figure I
Figure II

Panel A: VEGF mRNA (fold change)

Panel B: VEGF (ng/µg protein)
Figure IV

A  siControl  siDicer

DCF

DAPI

PMA 4 h (100 nM)

B  siControl  siDicer

VEGF (50 ng/ml) no treatment

Figure IV
Figure V

A

% cell migration

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* denotes significant difference.
Figure VI

siControl

no treatment 0.1 μM H₂O₂ 1 μM H₂O₂

siDicer
Supplementary Table I. Primer sets used for real-time PCR

h Dicer,
F: 5’- TGC CAG TTG GGA AAG AGA CTG TTA A-3’
R: 5’- TAT GGG TTT GGC CGT CAG TAT T-3’;

h VEGF A,
F: 5’- TGC CCA CTG AGG AGT CCA ACA T-3’
R: 5’- CAC GTC TGC GGA TCT TGT ACA AAC A-3’

h HBP1,
F: 5’- GAATTTGCCATCTTCACCTGGATAT-3’
R: 5’- CTGAGGTATTTTGGTTCCAAGATGA-3’

h β-Actin,
F: 5’- TCA ACT CCA TCA TGA AGT GTG ACG T-3’
R: 5’- TTC TGC ATC CGT TCG GCA AT-3’.
Supplementary Table II. Methods for the detection of proteins

Western blot

After protein extraction, protein concentrations were determined using BCA protein reagents (Pierce, Rockford, IL). Samples (20-35 µg of protein/lane for whole cell lysates; 15 µg of protein/lane for nuclear extracts) were separated on 8%-12.5% SDS-polyacrylamide gel electrophoresis, and probed with the following antibodies: anti-Dicer (Abcam, Cambridge, MA), anti-gp91, anti-RAC1, anti-RAC2 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-p47phox (BD transduction laboratories, San Jose, CA) or anti-HMG box containing protein-1 (anti-HBP1, Santa Cruz Biotechnologies, Santa Cruz, CA). The secondary antibodies used were as follows: anti-mouse and anti-rabbit (Amersham Biosciences, Buckinghamshire, UK) and anti-goat (Sigma-Aldrich, St. Louis, MO). To evaluate the loading efficiency, membranes were probed with anti-GAPDH-HRP (Sigma-Aldrich, St. Louis, MO) or with anti-Lamin A (Sigma-Aldrich, St. Louis, MO) antibody.

Determination of VEGF Protein

Following transfection, cell media was collected. VEGF level in the medium was determined using commercially available ELISA kit (R&D Systems, Minneapolis, MN). Results were adjusted against total protein concentration in the samples.