Dicer Dependent MicroRNAs Regulate Gene Expression and Functions in Human Endothelial Cells

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Abstract—Dicer is a key enzyme involved in the maturation of microRNAS (miRNAs). miRNAs have been shown to be regulators of gene expression participating in the control of a wide range of physiological pathways. To assess the role of Dicer and consequently the importance of miRNAs in the biology and functions of human endothelial cells (EC) during angiogenesis, we globally reduced miRNAs in ECs by specific silencing Dicer using siRNA and examined the effects on EC phenotypes in vitro. The knockdown of Dicer in ECs altered the expression (mRNA and/or protein) of several key regulators of endothelial biology and angiogenesis, such as TEK/Tie-2, KDR/VEGFR2, Tie-1, endothelial nitric oxide synthase and IL-8. Although, Dicer knockdown increased activation of the endothelial nitric oxide synthase pathway it reduced proliferation and cord formation of EC in vitro. The miRNA expression profile of EC revealed 25 highly expressed miRNAs in human EC and using miRNA mimicry, miR-222/221 regulates endothelial nitric oxide synthase protein levels after Dicer silencing. Collectively, these results indicate that maintenance and regulation of endogenous miRNA levels via Dicer mediated processing is critical for EC gene expression and functions in vitro. **(*Circ Res*. 2007;100:1164-1173.)**

Key Words: endothelium ■ Dicer ■ miRNA ■ angiogenesis

MicroRNAs (miRNAs) are short noncoding RNAs that have been identified in a variety of organisms and have been shown to regulate gene expression.[1,2] In mammalian cells, these small RNAs (≈22 nt) are transcribed as parts of longer molecules that are processed in the nucleus into hairpins RNAs by the protein Drosha.[3,4] These premiRNAs are then transported to the cytoplasm, via an exportin 5-dependent mechanism, where they are digested by a second, doubled-stranded specific ribonuclease called Dicer.[5,6] The mature miRNAs are incorporated into a ribonucleoprotein complex[7,8] or RISC complex,[9] that mediates the down-regulation of target gene activity by translational inhibition or target mRNA degradation, resulting in reduced levels of the corresponding protein or transcript, respectively.[7,10,11]

miRNAs have been implicated in the control of a wide range of physiological pathways[12,13] such as development, differentiation, growth and metabolism.[14–17] Moreover, tissue-specific patterns of miRNAs are providing insights into their possible functions. Many miRNAs exhibit striking organ specific expression patterns, or even expression restricted to single tissue layer within an organ[18] and different miRNAs have been specifically cloned from heart, brain, embryonic stem cells and pancreatic islet cells.[19–23]

To dissect the significance of miRNAs in mammalian biology, several groups have disrupted the *Dicer* gene in mice[24,25] and the loss of *Dicer* resulted in embryonic lethality, demonstrating that Dicer is necessary for normal mouse development. Other reports using conditional knockout approaches have demonstrated that Dicer plays essential roles in the maintenance of hair follicles,[26] lung epithelium morphogenesis,[27] T cell differentiation,[28] whereas it is dispensable for some T cell lineage-specific gene expression programs.[29] One report suggested the embryonic lethality observed in $Dicer^{ex1/2}$ mice was because of defective blood vessel formation and maintenance. These anatomical defects were associated with altered expression of vascular endothelial growth factor (VEGF), its receptors KDR (VEGFR2) and FLT-1 (VEGFR1) and the angiopoietin receptor, Tie-1. These data suggest that Dicer exerts its function in the processing of miRNAs during embryonic angiogenesis and regulates the expression levels of critical angiogenic regulators.[24] More recently, the expression of miRNAs in cultured human endothelial cells (EC) has recently been explored and the miRNA, miR-221/2 is necessary for the expression of c-Kit and Stem Cell Factor induced migration of EC.[30]

Angiogenesis is the development of new blood vessels from existing vascular structures and is a highly coordinated, multistep process. It is believed that EC migration, proliferation, differentiation and structural rearrangement of cells into patent vessels are crucial events for this process.[31–33]

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However, the role of miRNAs in the biology and responses of EC during angiogenesis are incompletely understood. In the present work, we have globally reduced miRNAs in ECs by specifically reducing Dicer levels using siRNA and have examined several phenotypic responses in vitro. The knockdown of Dicer in ECs alters the expression of several important regulators of endothelial biology and angiogenesis, such as TEK/Tie-2, KDR/VEGFR2, endothelial nitric oxide synthase (eNOS) and IL-8. Moreover, Dicer knockdown increases activation of the eNOS pathway but reduces proliferation and cord formation of EC in vitro. Collectively, these results indicate that the reduction in miRNA levels via Dicer silencing strongly impacts EC functions suggesting a critical role for miRNAs in angiogenesis and EC remodeling.

Materials and Methods

Quantitative real-time PCR, gene expression analysis by PCR array, Quantitative RT-PCR for miRNA, miRNA-array analysis, Western blot analysis, NO release, flow cytometry analyses, cell number assessment, crystal violet staining method, immunofluorescence microscopy, cord formation assay, migration experiments and statistical analysis are described in the online data supplement available at http://circres.ahajournals.org.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from discarded umbilical veins by collagenase digestion, under protocols approved by Yale, cultured onto tissue culture dishes with 0.1% gelatin in M199 (Invitrogen) containing 20% FBS (Hyclone), 50 μg/mL endothelial cell growth supplement (ECGS with heparin, BD Biosciences), penicillin-streptomycin; and L-glutamine (Invitrogen). HUVECs were used in passage 1 or 2. EA.hy.926 cells (endothelial cell line) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FBS, penicillin-streptomycin, L-glutamine and HAT (Sigma) at 37°C in a humidified atmosphere of 5% CO_2.

RNA Interference

To silence *Dicer*, we used the published sequences of siRNA[34] (siRNA Dicer 2 and 3) plus an additional one whose target sequence was 5′-AAGGGCACCCATCTCTAATTA-3′ (siRNA Dicer 1). A scrambled siRNA was used as a negative nonsilencing control (NS) (5′-AATTCTCCGAACGTGTCACGT-3′). All the siRNA sequences were purchased from Qiagen. For cell transfection, EA.hy.926 cells and HUVECs were plated before transfection at a density of 25,000 to 35,000 cells/cm2, respectively. Individual siRNAs for Dicer 25 nM each (75 nM total) or nonsilencing siRNA (75 nM) were mixed with Oligofectamine and Opti-MEM (Invitrogen) and added to the cells. The cells were incubated for 8 hours at 37°C, and then HUVEC or EA.hy.926 growth medium, respectively, was added. In other experiments, cells were cotransfected with mi*RIDIAN* miRNA mimics (Dharmacon). In all these expreriments control samples were treated with and equal concentration of a control mimic negative control.

Northern Blot Analysis for miRNA

Total RNA (10 μg) was resolved in a 15% polyacrylamide gel and blotted to a Nylon membrane Hybond-N+ (Amersham Biosciences). DNA oligonucleotides for hsa-miR-16, hsa-let-7a, hsa-miR-222, hsa-miR-31, hsa-miR-499 and hsa-miR-107 were 5′-C G C C A A T A T T T A C G T G C T G C T A-3′, 5′-A A C T A T A C A A C C T A C T A C C T C A 3′, 5′-G A G A C C C A G T A G C C A G A T G T A G C T-3′, 5′-C A G C T A T G C C A G C A T C T T G C C-3′, 5′-T T A A A C A T C A C T G C A A G T C T T A A-3′ and 5′-T G A T A G C C C T G T A C A A T G C T G C T-3′ respectively, complementary to the mature miRNA were end-labeled with [α-^{32}p] ATP and T_4 polynucleotide kinase (New England Biolabs) to generate high-specific activity probes. The oligonucleotide sequence for 5SrRNA was 5′-C A G G C C C G A C C C T G C T T A G C T T C C G A G A T C A G A C G A G A T-3′. Hybridization was carried according Express Hyb (Biorad) protocol. Probes were washed twice for 10 minutes at 25°C in 4 ×SSC/0.5% SDS.

Results

We initially assessed the expression of Dicer protein in EC via Western blotting. Dicer levels were readily detectable in HUVECs and were not changed after treatment (for up to 8 hours) with vascular endothelial growth factor (VEGF) a well known regulator of angiogenesis (Figure 1A). VEGF induced the phosphorylation of eNOS on serine 1179 as a positive control for VEGF action. To investigate the role of endogenous Dicer on EC functions, we developed a siRNA approach to reduce levels of Dicer in EC. As shown in Figure 1B, transfection of ECs (EA.hy.926 cells and HUVECs) with Dicer siRNA duplex (at 75 nM) reduced the expression of Dicer (via qRT-PCR) in both cell types in a time-dependent manner, with a maximal reduction at 48 hour (Figure 1B and 1C). The expression of Drosha, a ribonuclease implicated in the biogenesis of miRNAs was not affected (data not shown). Western blotting of cell extracts confirmed the reduction of Dicer expression was still evident at the protein level after 72 hour in both EC cultures (Figure 1D and 1E). Given that Dicer is the main regulator of miRNA biogenesis, we analyzed the expression of two miRNAs expressed in the majority of the cells including HUVECs ([30] namely, hsa-miR-16 and hsa-let-7) before and after 60 hour of knockdown of Dicer. Analysis of these endogenous miRNA levels after Dicer silencing resulted in a substantial decrease in the levels of both miRNAs when analyzed by Northern blotting and qRT-PCR (Figure 1F, left panel for Northern and right panel in Figure 1G for qRT-PCR). The knockdown of Dicer in HUVECs did not affect cell morphology (by crystal violet staining), vWF localization in discrete cytoplasmic granules or VE-cadherin localization at the cell junctions, indicating no overt signs of endothelial cell injury or damage (Figure 1H).

To examine potential functions attributed to miRNAs in ECs, we analyzed the effects of Dicer knockdown on the expression profile of several genes by PCR Arrays. EA.hy.926 cells were transfected with Dicer-specific siRNA or the nonsilencing siRNA for 60 hour and the levels of several angiogenesis and vascular remodeling genes were assessed. As seen in Figure 2A, the expression levels of several growth factors receptors were upregulated such as TEK (Tie-2), KDR (VEGFR2), adhesion molecules and proteins implicated in matrix remodeling including COL18A1 (Collagen, typeXVIII, α1), EDG_1 (the endothelial differentiation sphingolipid G protein-coupled receptor 1) for the bioactive lipid, sphingosine 1- phosphate (S1P), ANPEP (Aminopeptidase N), ENG (Endoglin), and SERPINF1 (α-2 antiplasmin) were upregulated, whereas PLAU (Urokinase Plasminogen activator) was downregulated. The expression of distinct chemokines and cytokines, IL-8, IL1β, CXCL1, and CXCL3, implicated in inflammation and angiogenesis were also downregulated. Likewise several other angiogenesis related genes such as the protein kinase AKT1, the transcription factor Id3 (inhibitor of DNA binding 3) and the

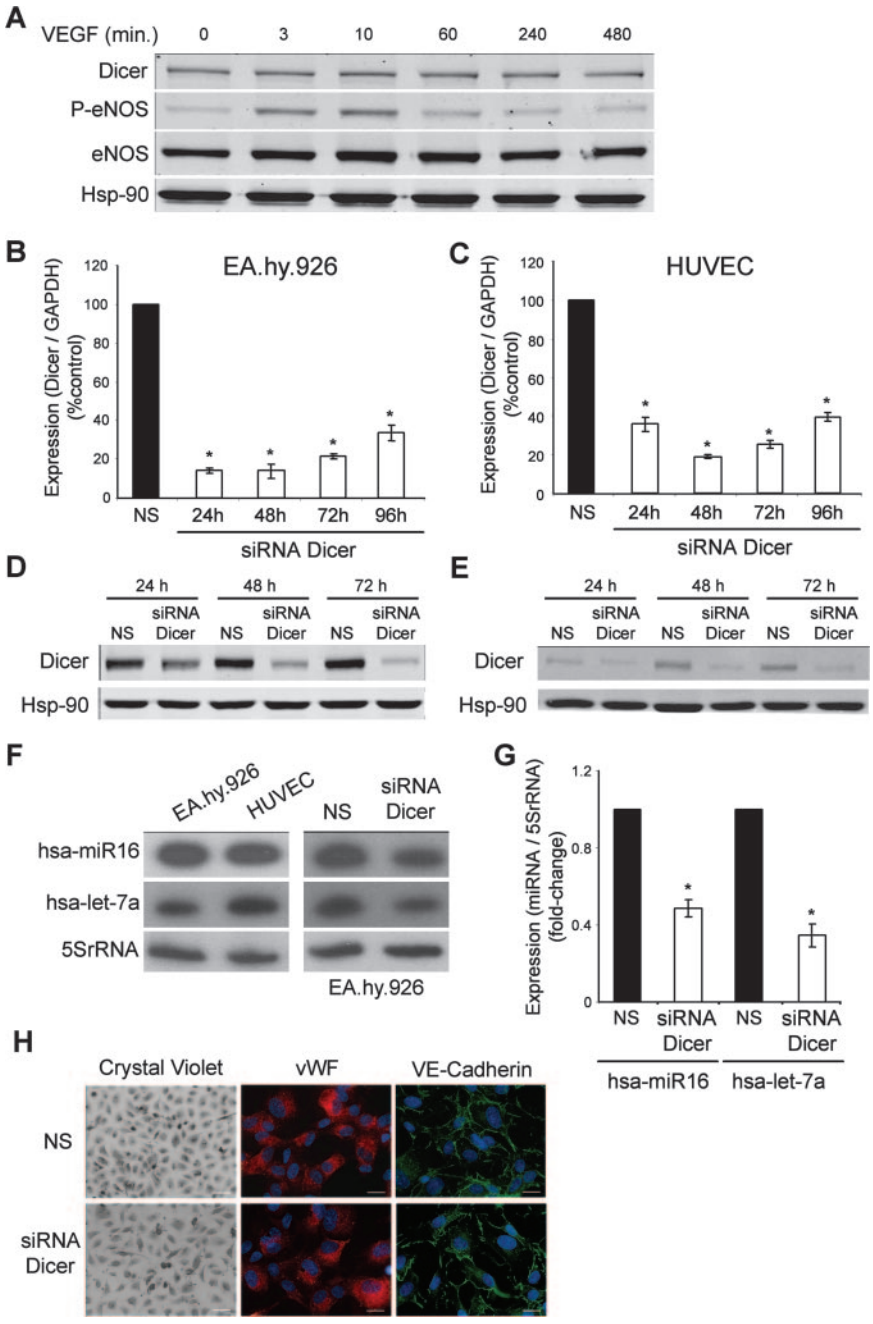

Figure 1. Dicer expression and siRNA mediated Dicer knockdown in endothelial cells. A, Endogenous levels of Dicer are not affected by VEGF treatment. HUVECs were serum-starved for 18 hour and then incubated with VEGF (60 ng/mL) for the indicated times. Dicer, eNOS, phospho-eNOS and Hsp-90 (loading control) were detected by Western blot. B–H, Analysis of Dicer knockdown in ECs. EA.hy.926 cells (B) and HUVECs (C) were transfected with Dicer siRNA (siRNA Dicer) or nonsilencing (NS) siRNA for the indicated times and the mRNA expression of Dicer was analyzed by qRT–PCR. Data are presented as mean±SEM from 3 independent experiments performed in triplicate. *, $P<0.05$ versus control. EA.hy.926 and HUVECs (D and E, respectively) were transfected as previously indicated the protein expression of Dicer was by Western blot. Representative blots of 3 independent experiments with similar results are shown. F, Northern blot analysis of hsa-miR-16 and hsa-let-7a expression in EA.hy.926 and HUVECs (left panel) and after 60 hour treatment of EA.hy.926 cells with the Dicer siRNA (right panel). Expression of 5SrRNA served as a loading control. Representative experiments of 3 with similar results, G, qRT-PCR analysis of hsa-miR-16 and hsa-let-7a miRNAs in EA.hy.926 after Dicer silencing for 60 hour. (mean±SEM of 3 independent experiments performed in duplicate). Statistical comparisons vs control (NS): *$P<0.05$. H, HUVECs were transfected with nonsilencing (NS) or Dicer siRNA for 48 hour fixed and stained with crystal violet (micrographs on the left) or fixed and permeabilized and stained for vWF (red) and nuclei (DAPI; blue) (middle), VE-Cadherin (green) and nuclei (DAPI; blue) (right) and analyzed by immunofluorescence microscopy. Bars, 20 μm.

growth factors, ANGPT2 (Angiopoietin 2) and ANGPTL4 (Angiopoietin-like 4) were also regulated after the silencing of Dicer. The upregulation of gene expression in response to Dicer silencing were additionally confirmed by semi-quantitative Western blotting. As seen in Figure 2B in both EA.hy926 cells (left) and HUVECs (right), the loss of Dicer increased the protein levels of Tie-2, eNOS and AKT proteins, but did not change the levels of ERK or Hsp-90 (supplemental Figure IA). The increased protein levels of Tie-2, eNOS and AKT were also observed at different time points after Dicer silencing (Figure 2C). The expression of other proteins was assessed by flow cytometry. As seen in Figure 2D, the expression of KDR/VEGFR2 (first panel) was increased, whereas as the levels of VEGF (second panel) and PECAM-1 (third panel) were unchanged consistent with the mRNA data. Next, we examined the levels of the angiopoietin receptor, Tie-1, altered in *Dicer*$^{1/2}$ embryos.[24] The protein levels were increased without a significant increase in mRNA levels (supplemental Figure IA and IB available at http://circres.ahajournals.org). Collectively, our data indicate that the reduced expression of miRNAs by means of the knockdown of Dicer altered the expression of several critical regulators of EC function.

Although the mRNA levels for Tie-1 and eNOS did not significantly increase after Dicer knockdown (supplemental Figure IA and Figure IIA available in the online data supplement), the levels of both proteins were consistently elevated (see Figure 3A, using different amounts of protein loaded with densitometric evaluation below the blots). To examine whether the increase in eNOS protein influenced NO

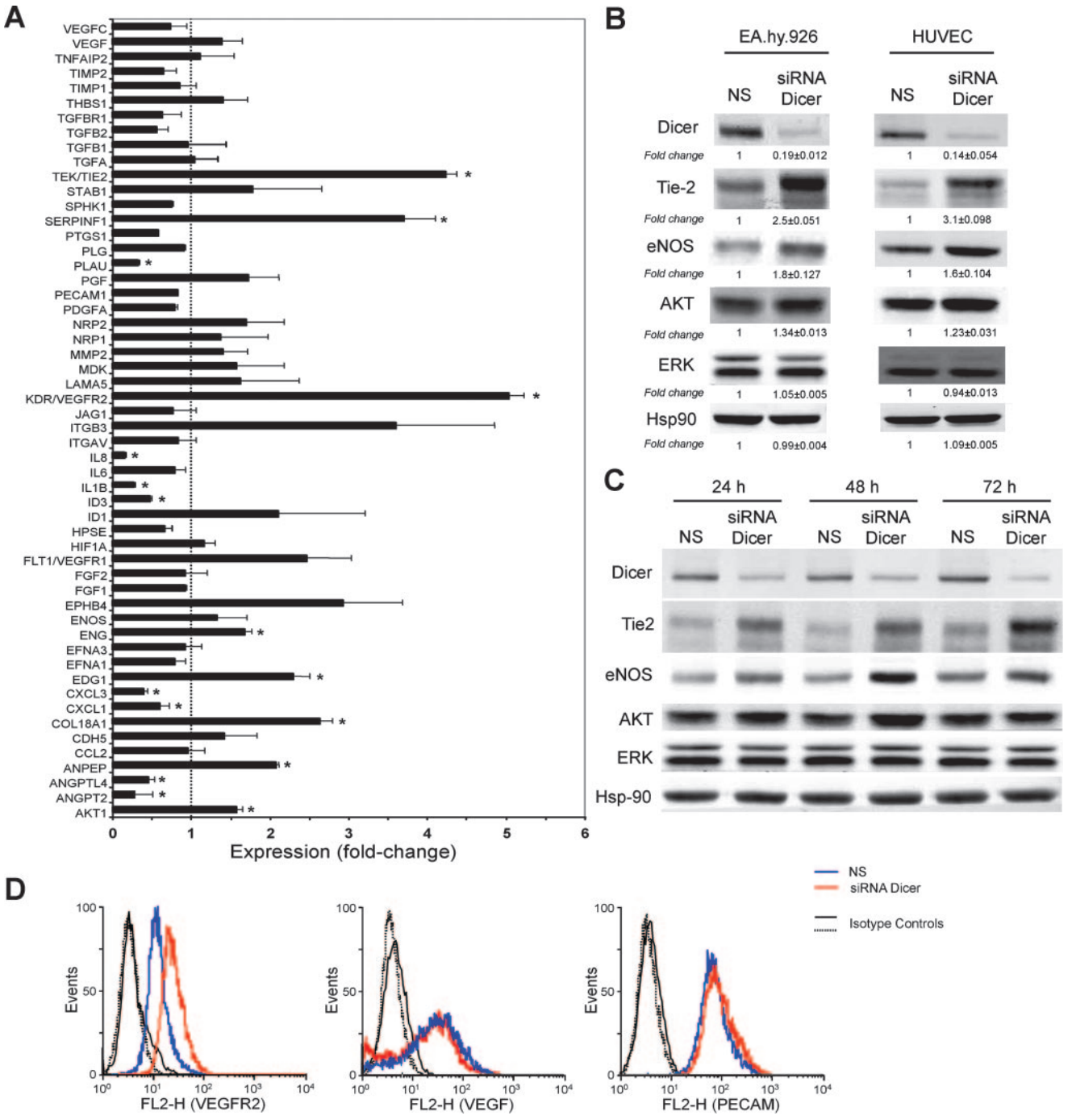

Figure 2. Dicer knockdown alters the expression profile of angiogenesis related genes. A, EA.hy.926 cells were transfected with Dicer siRNA or nonsilencing (NS) siRNA. Total RNA was isolated after 60 hours and gene expression profiles were assessed with PCR Array technology. Data were first expressed as fold-change from control to Dicer silencing as indicated previously, and shown as mean±SEM. Statistical comparisons versus control (NS): *$P<0.05$. B and C, Validation of changes in protein levels of some genes identified in the PCR Array. ECs were transfected as above for 60 hours (B and D) and for the indicated times (C). Proteins levels of Dicer (control of the knockdown) and the indicated proteins were determined by Western blot in both EC types (B) or EA.hy.926 cells (C). Numbers refer to fold-change over control and are shown as mean±SEM of 3 independent experiments. D, FACS analysis of cell protein expression of VEGFR2, VEGF, and PECAM on EA.hy.926 cells under the indicated conditions. Data correspond to one representative experiment of 2 with similar results.

production, the levels of NO (quantified by the stable breakdown product nitrite NO_2^-) were measured. As seen in Figure 3B (top panels), basal (10 hour accumulation) and stimulated (ATP; 10 μmol/L or S1P; 400 nM treatment for 30 minutes) NO release was increased following the knockdown of Dicer. Thus, the loss of Dicer dependent miRNAs does not significantly change eNOS mRNA levels but increases the levels of eNOS protein and NO production suggesting miRNA regulation of eNOS translational efficiency.

To characterize the importance of miRNAs for additional EC functions in vitro, we examined the effects of Dicer silencing on three angiogenic phenotypes of EC, namely

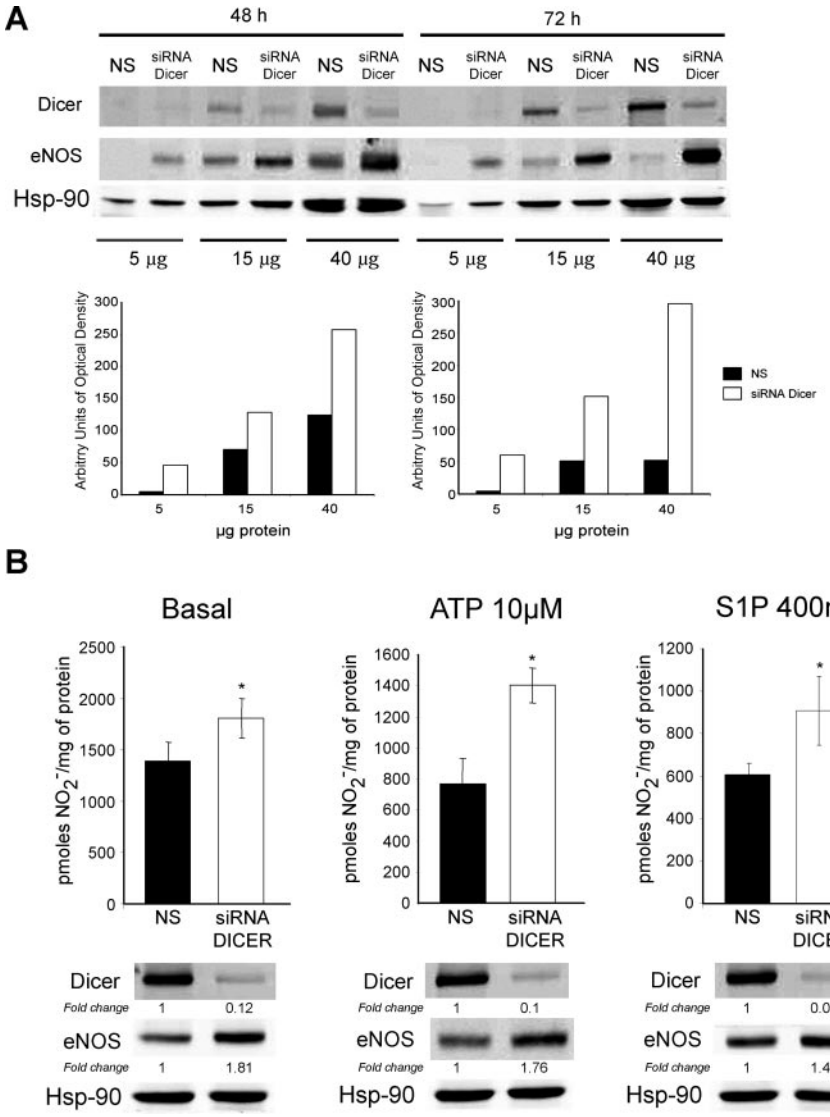

Figure 3. Dicer knockdown increases eNOS protein levels and NO release. A, EA.hy.926 cells were transfected with nonsilencing (NS) or siRNA Dicer for the indicated times. Different amounts of protein were loaded and subjected to Western blotting. Antibodies against Dicer, eNOS and Hsp-90 (loading control) were used. Lower panels show the quantification analysis of eNOS protein levels. B, NO release from EA.hy.926 cells transfected with nonsilencing (NS) or Dicer siRNA for 48 hour. The nitrite accumulation was quantified for 8 hour (basal; left) and after stimulation with ATP (middle) or S1P (right) for 30 minutes. The data represent the mean±SEM of samples in 3 separate experiments. *$P<0.05$ vs control (NS).

proliferation, migration and cord formation on reduced growth factor containing Matrigel. In both EC types, silencing of Dicer had a negative effect on cell proliferation. As seen in Figure 4A, knockdown of Dicer inhibited cell proliferation after 48 to 72 hour treatment with Dicer siRNA and similar results were obtained using the crystal violet staining for quantification (not shown). The effects of Dicer knockdown on cell proliferation was not associated with diminished cell viability, as indicated by absence of the subG$_1$ population, or because of cell cycle arrest (Figure 4C). Instead, it was a result of a cell cycle delay from G$_1$ to S phase indicated by a diminished incorporation of BrdUrd[35] (Figure 4B inserts). In addition, the decrease in cell proliferation was not associated with activation of a dsRNA-interferon response (data not shown). Next, we examined the effects of Dicer silencing on EC migration in both EC types. Knockdown of Dicer reduced basal migration in EA.hy.926 cells, but did not significantly reduce VEGF or S1P induced migration in either EC type (see supplemental Figure II). However, knockdown of Dicer resulted in significant impairment of the cord formation under basal conditions and after stimulation with serum or VEGF (Figure 5A and quantified in graphs) in both EC types.

Next, we determined the miRNA expression profile in both EC types. (Table; see also supplemental Table I in the online data supplement). In brief, miRNAs were isolated from exponentially growing HUVECs (3 cords pulled together for one sample repeated with an additional 3 cords) and EA.hy.926 cells (experimental duplicates collected at different time points) and duplicates of each sample run on miRNA microarrays (see Material and Methods). Background was subtracted from all samples and data normalized to the level of let-7A (which was 1 to 1.5-fold above background). The Table shows the 25 miRNAs giving the strongest hybridization signals in HUVECs compared with the same miRNAs in EA.hy.926 cells. The levels of certain miRNA identified in the arrays, were validated by Northern blotting using 5Sr-RNA as a loading control. Specifically, the expression of hsa-miR-222, hsa-miR-31, hsa-miR-107 and "pred00211" (predicted miRNA) recently described as hsa-miR-499,[36] was confirmed by Northern blotting and compared with the expression of these miRNAs in other human cells (Figure 6).

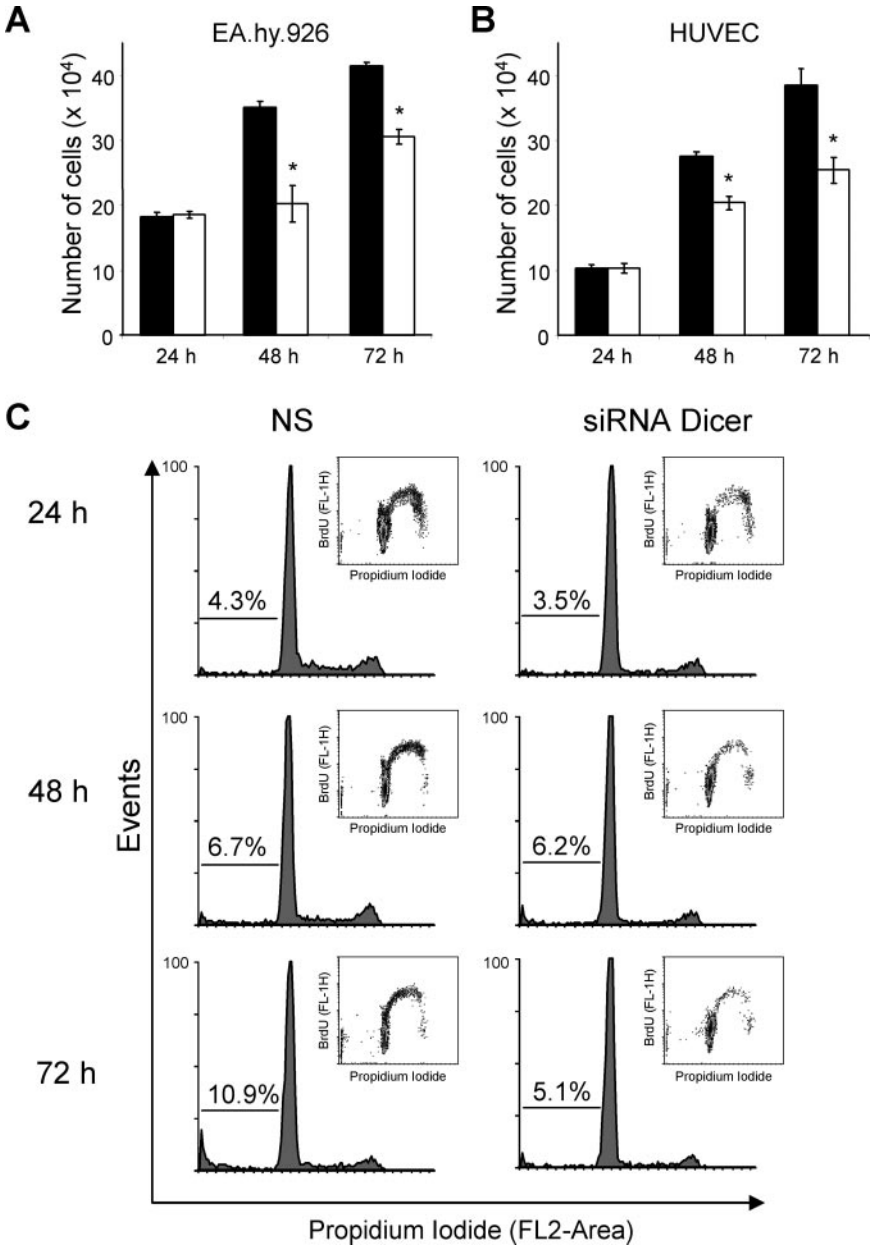

Figure 4. Silencing of Dicer reduces EC proliferation. A and B, ECs, were transfected with Dicer siRNA or nonsilencing (NS) siRNA. At the indicated times, cells were harvested and counted. Data correspond to the means±SEM of 3 experiments performed in triplicate. Statistical comparisons vs control (NS): $*P<0.05$. C, Flow cytometry analysis of DNA content of EA.hy.926 cells transfected with nonsilencing (NS) or Dicer siRNA for the indicated times. Percentages of apoptotic cells corresponding to the subG$_1$ population are shown. Inserts correspond to dot plots that represent DNA content vs incorporation of BrdUrd (active phase S) into DNA as indicated by the inverted U-shape BrdUrd incorporation. In both cases, data correspond to one representative experiment of 3 with similar results.

These results indicate that hsa-miR-222 and hsa-miR-499 are highly expressed in EC and VSMC in comparison to hsa-miR-31 (preferentially expressed in VSMC) and hsa-miR-107 (highly expressed in monocytes) suggesting cell specific expression patterns of miRNAs in vascular cells.

Because hsa-miR-222 and hsa-miR-221 were highly expressed miRNAs in EC, we examined whether specific mimics for these miRNAs could rescue/restore some of the effects produced by Dicer silencing (Figure 7). As seen in Figure 7A, Dicer silencing increased the protein levels of Tie-2 and eNOS as shown above (see Figure 2 and 3). Transfection with hsa-miR-222 or hsa-miR-221 mimics slightly reduced the increased eNOS protein levels without affecting the levels of Tie-2. However, these mimics could not rescue the defect in EC proliferation because of Dicer silencing (Figure 7C).

Discussion

In this study, the levels of Dicer were reduced using siRNA to ascertain the global role of miRNA function in cultured human ECs. The RNAi-mediated knockdown of Dicer in ECs, resulted in a significant reduction, but not complete loss of mature miRNAs. The incomplete loss of miRNAs likely reflects the long half-life of endogenous miRNAs[37] over the relatively short time period of these experiments.[34] Under these circumstances, the reduction in Dicer levels changed the pattern of gene expression in ECs presumably via miRNA modulation of steady-state mRNA levels and/or mRNA translation of many gene products.[11,38] After silencing Dicer in ECs, we found that different pathways implicated in regulating EC functions were either increased or decreased. The net phenotype of Dicer knockdown in ECs was an increase in NO release, and decrease in both EC growth and

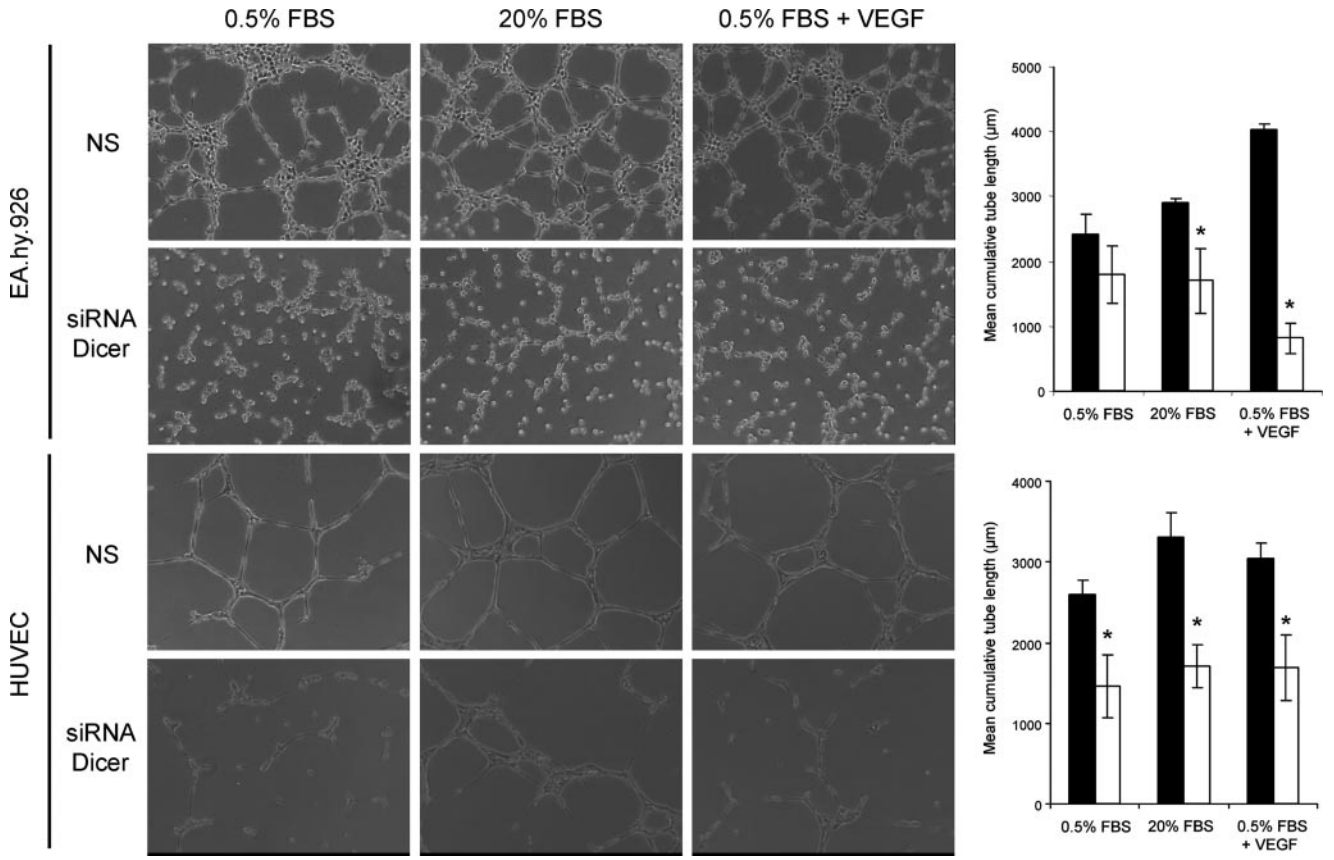

Figure 5. Dicer knockdown impairs endothelial cell cord formation. ECs were transfected with nonsilencing (NS) or Dicer siRNA. After 72 hours, cells were seeded on a Growth Factor Reduced Matrigel in the presence of 0.5% FBS (basal; left panels), 20% FBS or 0.5% FBS + VEGF (50 ng/mL) (stimulated; middle and right panels, respectively). Cumulative sprout length of capillary-like structures was measured by light microscopy after 24 hours. Representative micrographs and statistical summary are show. Data are presented as mean±SEM; n=3). *$P<0.05$ vs control (NS).

cord formation in Matrigel, but a negligible effect on EC migration. The decrease in growth and morphogenesis in ECs with reduced levels of Dicer, is consistent with a recent report showing that Dicer is required for embryonic and yolk sac angiogenesis in mice,[24] suggesting that some of the molecular signals and mechanisms that govern the activity of miRNAs in embryonic vascular development are also functional in cultured human EC.

Work in hypomorphic *Dicer*$^{ex1/2}$ mice has shown that the defects in embryonic angiogenesis were associated with an upregulation of FLK/VEGFR2, FLT/VEGFR1 and downregulation of Tie-1 suggesting that the VEGF signaling pathway was upregulated to compensate for failed angiogenesis and remodeling. In cultured ECs, we also detected a marked upregulation of KDR/VEGFR-2, FLT/VEGFR1 but little changes in VEGF-A or VEGF-C suggesting that upregulation of VEGF receptors is likely a direct effect of the loss of Dicer via miRNA regulation of mRNA stability or translational interference. Accordingly, Tie-2 and Tie-1, were also upregulated, suggesting that the VEGF and angiopoietin pathways are targets for miRNA regulation.[39,40] Paradoxically, in cultured ECs the protein levels of Tie-1 were increased whereas in the hypomorphic *Dicer*$^{ex1/2}$ embryos were diminished.[24] Several angiogenesis- and vascular remodeling- related genes such as ANGPT2, ANPEP, SERPINF1, COL18A1, IL-8 and ANGPTL4 were also regulated. However, no consistent pattern of pro- versus antiangiogenic profiles, of the observed genes, was apparent at the time points examined after Dicer knockdown in cultured ECs.

Although both Tie-1 and eNOS mRNA levels were not significantly elevated after Dicer siRNA treatment, the levels of Tie-1 and to a greater extent eNOS protein were consistently higher suggesting miRNA control of protein translational efficiency. eNOS was initially defined as a constitutively expressed enzyme, however recent studies highlight the contribution of both transcription and mRNA stabilization to overall expression levels of eNOS mRNA and protein[41,42] and our data supports a translational or posttranslational mechanism of eNOS and Tie-1 regulation by miRNAs. Functionally, the increased levels of eNOS correlated with an increased NO release. NO synthesized by eNOS is necessary for endothelial cell survival, migration and angiogenesis[43] in post-natal mice,[44] whereas it is dispensable for vascular development.[44] Paradoxically, although eNOS and other proangiogenic genes were upregulated by reducing the expression of miRNAs after Dicer silencing, EC proliferation and cord forming activity were reduced. This is consistent with an impaired vascular development in Dicer^{ex½} mice[24] despite compensatory upregulation of FLK/VEGFR2 and FLT/VEGFR1.

miRNAs Detected With High Signal Intensity in miRNA Arrays of EA.hy.926 Cells and HUVECs

Name	EA.hy0.926	HUVEC
CAND853_ZF	172	628
hsa-let-7bL	207	509
hsa-let-7eL	196	426
hsa-miR-100	824	1156
hsa-miR-103–1,2	1256	1403
hsa-miR-106	143	451
hsa-miR-107	1431	1662
hsa-miR-181a	1498	2550
hsa-miR-181b	81	1551
hsa-miR-213	358	875
hsa-miR-22	110	311
hsa-miR-221	7295	8444
hsa-miR-222	13237	10923
hsa-miR-23[a]	2012	3269
hsa-miR-23b	638	1432
hsa-miR-24–1,2	1104	1658
hsa-miR-27	252	526
hsa-miR-29	505	1223
hsa-miR-31	875	1494
hsa-miR-320	755	1090
hsa-miR-321	2194	3689
hsa-miR-91	80	263
hsa-miR-92–1,2	269	510
miR-21	1153	2287
pred00209	313	757
pred00211	5723	8447

The 25 miRNAs giving the highest average signal intensities in HUVEC along with the comparative intensities of EA.hy.926 cells. For more detail see supplemental Table I available at http://circres.ahajournals.org. Background signals were subtracted before calculation of averages.

Based on miRNA microarrays, we detected 40 miRNAs commonly expressed in HUVECs and EA.hy.926 cells (supplemental Table I) and the most abundant 25 shown in Table. Twelve of the 25 highly expressed miRNAs were consistent with a recent study,[30] however, our analysis detected an additional 13 miRNAs namely, CAND853_ZF, hsa-miR-107,

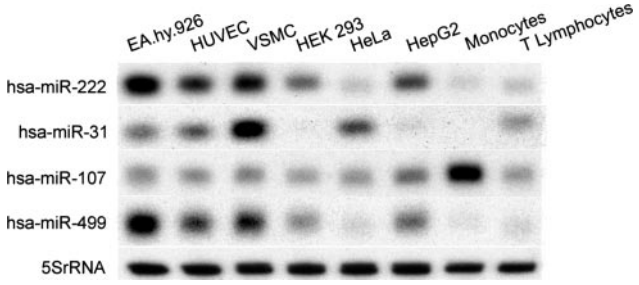

Figure 6. Validation and expression profiles of miRNAs. Northern blot analysis of hsa-miR-222, hsa-miR-31, hsa-miR-107 and hsa-miR-499 expression in various primary human cells including HUVECs, VSMCs, monocytes and T lymphocytes ($CD4^+$) and cells lines, EA.hy.926, HEK293, HeLa, and HepG2. Expression of 5S rRNA served as a loading control.

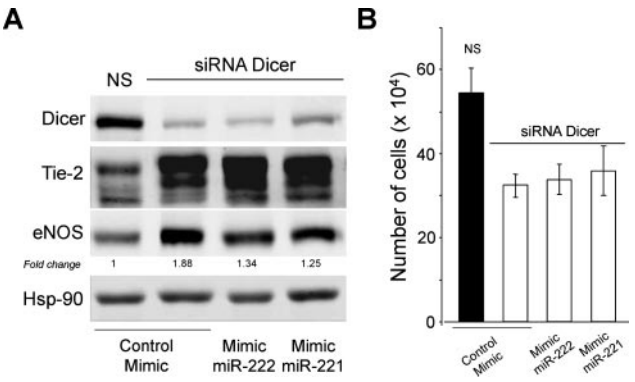

Figure 7. Dicer knockdown mediated increase of eNOS levels are partially restored by hsa-miR-222 and hsa-miR-221 mimics, but cellular growth is not restored. EA.hy.926 cells were transfected with control nonsilencing (NS) or Dicer siRNA in the presence or absence of a control mimic sequence or specific mimic-miR-222 or -221. A, After 60 hour cells were lysed of Dicer, Tie-2, eNOS, Hsp-90 protein levels were determined by Western blot. Panels correspond to 1 experiment of 2 with similar results. Numbers refer to ratio of eNOS/Hsp-90 expressed as fold change over control. B, After 60 hour of transfection cells were harvested and counted. Data correspond to the means±SEM of 2 experiments in triplicate.

-181a, -181b, -213, -22, -27, -320, -321, -91, -92 to 1,2, -499 (aka pred 00211) and pred 00209 (see Table and supplemental Table I). The expression of 6 different miRNAs were confirmed by Northern blotting with miR-222 and -499 being highly expressed in EC to a greater extent than in VSMC, whereas, miR-31 was more robustly expressed in VSMC and miR-107 in monocytes. Because miR-221 and 222 were among the highest expressed in ECs, we examined two potential targets regulated by Dicer silencing, namely eNOS and Tie-2, in EC by transfecting with miRNA mimics. In theory, the miRNA mimic should recapitulate the effects of the endogenous miRNA on a given gene product. Interestingly, transfection of miR-221and 222 mimics partially restored the elevated eNOS protein levels, but not Tie-2 levels, after Dicer silencing. However, using different predictive algorithms, miR-221/222 were not found to target the 3′ UTR of eNOS and Tie-2; similar results for Tie-2 were found by Poliseno et al.[30] This suggests that the partial rescue with the miR-221 and -222 mimics could be indirect via gene expression, translational efficiency or post-translational mechanisms. Indeed, overexpression and knockdown of individual miRNAs should be performed to gain further insight into miRNA regulation of targets reported here. The only example of a cell specific miRNA regulating a specific target in EC has been recently shown. Felli and Poliseno[45,30] found high expression of miR-222 and miR-221 in human cord blood-derived $CD34^+$ hematopoietic progenitor cells (HPCs) and HUVECs, respectively. Bioinformatic analysis suggested c-kit as a possible target whose levels correlate inversely with those of hsa-miR-222 and hsa-miR-221 in cultured cells. Forced expression of these miRNAs mimics slowed cell growth concomitant with reduced c-kit protein levels in HPCs,[45] or diminished stem cell factor mediated cell migration.[30] Although target disruption of individual miRNAs and targeted mutation of putative miRNA-binding sites of candidate genes are necessary to prove specific targets of miRNAs,

the redundancy among miRNA regulators may limit the power of such experiments and a thorough understanding of miRNA functions will only emerge from integrated consideration of complementary results.[46]

The possibility that a single miRNA may target multiple transcripts and that individual transcripts may be subject to regulation by multiple miRNAs amplifies the scope of putative miRNA regulation of mRNA and protein levels in cells. Additionally, it is likely that not all miRNAs function similarly: some may indeed regulate a single gene tightly, whereas others may have a wider influence over gene expression programs associated with selected developmental or physiological states.[46] In line with this concept, the antiproliferative effect of Dicer silencing was not rescued by transfection of the miR-222 and 221 mimics suggesting that other individual or multiple miRNAs clusters may regulate specifically EC growth, similar to the lack of the effects of these mimics on growth in HUVECs,[30] but not in HPCs, TF-1 cells (Erythroleukemic cell line).[45] Because the role of miRNA regulation and function in vascular cells is a new emerging area, additional investigation is needed to study the contribution of individual miRNAs or miRNA clusters in controlling gene expression that governs the angiogenic program in EC.

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Disclosures

None.

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References

1. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004;5:522–531.
2. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–297.
3. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003;425:415–419.
4. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. *Nature*. 2004;432:231–235.
5. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science*. 2001;293:834–838.
6. Cullen BR. Transcription and processing of human microRNA precursors. *Mol Cell*. 2004;16:861–865.
7. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. *Science*. 2002;297:2056–2060.
8. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev*. 2002;16:720–728.
9. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*. 2000;404:293–296.
10. Saxena S, Jonsson ZO, Dutta A. Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J Biol Chem*. 2003;278:44312–44319.
11. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev*. 2006;20:515–524.
12. Ambros V. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell*. 2003;113:673–676.
13. Krutzfeldt J, Stoffel M. MicroRNAs: a new class of regulatory genes affecting metabolism. *Cell Metab*. 2006;4:9–12.
14. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell*. 2003;113:25–36.
15. Xu P, Vernooy SY, Guo M, Hay BA. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr Biol*. 2003;13:790–795.
16. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004;303:83–86.
17. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. *Nature*. 2005;438:685–689.
18. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature*. 2005;436:214–220.
19. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RH. MicroRNA expression in zebrafish embryonic development. *Science*. 2005;309:310–311.
20. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature*. 2004;432:226–230.
21. Krichevsky AM, Sonntag KC, Isacson O, Kosik KS. Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells*. 2006;24:857–864.
22. Houbaviy HB, Murray MF, Sharp PA. Embryonic stem cell-specific MicroRNAs. *Dev Cell*. 2003;5:351–358.
23. Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, Baskerville S, Hammond SM, Bartel DP, Schier AF. MicroRNAs regulate brain morphogenesis in zebrafish. *Science*. 2005;308:833–838.
24. Yang WJ, Yang DD, Na S, Sandusky GE, Zhang Q, Zhao G. Dicer is required for embryonic angiogenesis during mouse development. *J Biol Chem*. 2005;280:9330–9335.
25. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. *Nat Genet*. 2003;35:215–217.
26. Andl T, Murchison EP, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, Andl CD, Seykora JT, Hannon GJ, Millar SE. The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr Biol*. 2006;16:1041–1049.
27. Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X. Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci U S A*. 2006;103:2208–2213.
28. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med*. 2005;202:261–269.
29. Cobb BS, Nesterova TB, Thompson E, Hertweck A, O'Connor E, Godwin J, Wilson CB, Brockdorff N, Fisher AG, Smale ST, Merkenschlager M. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med*. 2005;201:1367–1373.
30. Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, Mercatanti A, Hammond S, Rainaldi G. MicroRNAs modulate the angiogenic properties of HUVEC. *Blood*. 2006;109:3068–3071.
31. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9:653–660.
32. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005;438:932–936.
33. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature*. 2005;438:967–974.

34. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*. 2005;436:740–744.
35. Michea L, Ferguson DR, Peters EM, Andrews PM, Kirby MR, Burg MB. Cell cycle delay and apoptosis are induced by high salt and urea in renal medullary cells. *Am J Physiol Renal Physiol*. 2000;278:F209–F218.
36. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet*. 2005;37:766–770.
37. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol*. 2005;6:376–385.
38. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005; 433:769–773.
39. Peters KG, Kontos CD, Lin PC, Wong AL, Rao P, Huang L, Dewhirst MW, Sankar S. Functional significance of Tie2 signaling in the adult vasculature. *Recent Prog Horm Res*. 2004;59:51–71.
40. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol*. 2006;7:359–371.
41. SenBanerjee S, Lin Z, Atkins GB, Greif DM, Rao RM, Kumar A, Feinberg MW, Chen Z, Simon DI, Luscinskas FW, Michel TM, Gimbrone MA, Jr., Garcia-Cardena G, Jain MK. KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. *J Exp Med*. 2004;199:1305–1315.
42. Tai SC, Robb GB, Marsden PA. Endothelial nitric oxide synthase: a new paradigm for gene regulation in the injured blood vessel. *Arterioscler Thromb Vasc Biol*. 2004;24:405–412.
43. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest*. 1997;100: 3131–3139.
44. Yu J, deMuinck ED, Zhuang Z, Drinane M, Kauser K, Rubanyi GM, Qian HS, Murata T, Escalante B, Sessa WC. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc Natl Acad Sci U S A*. 2005;102:10999–11004.
45. Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, Valtieri M, Calin GA, Liu CG, Sorrentino A, Croce CM, Peschle C. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci U S A*. 2005;102:18081–18086.
46. Shivdasani RA. MicroRNAs: regulators of gene expression and cell differentiation. *Blood*. 2006;108:3646–3653.