Role of Dicer and Drosha for Endothelial MicroRNA Expression and Angiogenesis

Angelika Kuehbacher, Carmen Urbich, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by binding to the cellular transcript leading to translational repression or degradation of the target mRNA. Dicer and Drosha are the miRNA processing enzymes that are required for the maturation of miRNAs. Here, we investigated the role of Dicer and Drosha for angiogenesis. Endothelial cells were transfected with siRNA against Dicer and Drosha to inhibit miRNA biogenesis. Genetic silencing of Dicer and Drosha significantly reduced capillary sprouting of endothelial cells and tube forming activity. Migration of endothelial cells was significantly decreased in Dicer siRNA–transfected cells, whereas Drosha siRNA had no effect. Silencing of Dicer but not of Drosha reduced angiogenesis in vivo. Next, we attempted to identify miRNAs expressed in endothelial cells. A screening analysis of 168 human miRNAs using real-time PCR revealed that members of the let-7 family, mir-21, mir-126, mir-221, and mir-222 are highly expressed in endothelial cells. Dicer and Drosha siRNA reduced let-7f and mir-27b expression. Inhibitors against let-7f and mir-27b also reduced sprout formation indicating that let-7f and mir-27b promote angiogenesis by targeting antiangiogenic genes. In silico analysis of predicted targets for let-7 cluster identified the endogenous angiogenesis inhibitor thrombospondin-1. Indeed, Dicer and Drosha siRNA significantly increased the expression of thrombospondin-1. Taken together, transient reduction of the miRNA-regulating enzyme Dicer impairs angiogenesis in vitro and in vivo, whereas Drosha siRNA induced a minor antiangiogenic effect in vitro and was not effective in vivo. The let-7 family and mir-27b appear to be attractive targets for modulating angiogenesis. (Circ Res. 2007;101:59-68.)

Key Words: angiogenesis ■ endothelial cells ■ gene expression

MicroRNAs (miRNAs) are a new class of highly conserved, noncoding small RNAs that regulate gene expression on the posttranscriptional level by binding to the transcript, leading to translational repression or degradation of the target mRNA.1 There is increasing evidence that miRNAs are involved in various biological processes, such as cardiogenesis, skeletal muscle proliferation and differentiation, brain morphogenesis, oncogenesis, and hematopoietic lineage differentiation.2–6 miRNAs are generated in a 2-step processing pathway mediated by 2 major enzymes, Dicer and Drosha, which belong to the class of RNase III endonucleases. Drosha is part of a multiprotein complex, the microprocessor, which mediates the nuclear processing of the primary miRNAs into stem–loop precursors of approximately 60 to 70 nucleotides (pre-miRNA).7 The treatment of Hela cells with RNA interference against Drosha results in the strong accumulation of pre-miRNAs and the reduction of pre-miRNAs and mature miRNAs.7 Exportin-5 mediates the nuclear export of correctly processed miRNA precursors in a Ran-GTP–dependent manner.8 In the cytoplasm, the pre-miRNA is cleaved by Dicer into the mature 22 nucleotide miRNA.1 Dicer was originally recognized for its role in generating small interfering (si)RNAs9 and was later shown to be also involved in miRNA maturation.10 The mature miRNA incorporates as single-stranded RNAs into a ribonucleoprotein complex, known as the RNA-induced silencing complex.11 This complex directs the miRNA to the target mRNA, which leads either to translational repression or degradation of the target mRNA.1

Although the function of Drosha in vivo is so far unknown, Dicer plays a crucial role in vertebrate development. Dicer-deficient mice die early in embryonic development, between embryonic day 12.5 and 14.5, showing an impaired blood vessel and yolk sac formation.12 Zebrafish Dicer mutant embryos display abnormal morphogenesis during gastrulation, brain formation, somitogenesis, and heart development.4 One recent study indicated that mir-221 and mir-222 modulate the angiogenic properties of human umbilical vein endothelial cells (HUVECs).13 However, the function of miRNAs in endothelial cell biology is unclear. Therefore, we investigated the role of the major miRNA-regulating enzymes Dicer and Drosha for angiogenic functions of endothelial cells. Our results demonstrate that Dicer and Drosha are critical regulators of endothelial sprout and network formation in vitro, whereas only Dicer siRNA significantly blocked angiogenesis in vivo.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.107.153916
Materials and Methods

Cell Culture

Pooled HUVECs were purchased from Cambrex and cultured in endothelial basal medium (EBM; Cambrex) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor, and 10% FCS (GIBCO) until the third passage. After detachment with trypsin, cells were grown in 6-cm culture dishes for at least 40 hours.

RNA Interference

For siRNA-mediated gene knockdown, HUVECs were grown to 60% to 70% confluence and transfected with GeneTrans II (MoBiTec) according to the protocol of the manufacturer. Two different siRNA duplexes for Dicer and Drosha have previously been described.7,14,15 If not stated otherwise, cells were transfected with Dicer and Drosha siRNA I (Dicer I: 5'-UUGCUUGAAAGCG-CUCUGGA-3'; Dicer II: 5'-UUGGUUGAGGCGUCAGUAUC-3'; Drosha I: 5'-AACAGGUAGGCUUCGACUCU-3'; Drosha II: 5'-AAGGACAAAGAUUUCGCAAG-3'). A nontargeted scrambled siRNA was used as a control (5'-UCAGAGGAGCAAGGAAUU-3' or 5'-CAACUAUCAUAAGGAA-3'). For inhibition of specific miRNAs, HUVECs were grown to 60% to 70% confluence before transfection with the specific inhibitors. 2'-O-Methyl antisense oligoribonucleotides against mir-27b (5'-GCAGAACUUAG-CACUGGUA-3') and let-7i (5'-AACAUAAUCAACUACCUC-3') were synthesized by VBC Biotech, and a concentration of 200 nmol/L was transfected with GeneTrans II (MoBiTec) according to the protocol of the manufacturer.

Western Blot Analysis

For Western blot analysis, HUVECs were lysed in a lysis buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride) for 20 minutes on ice. After centrifugation for 15 minutes at 20 000g (4°C), the protein content of the samples was determined according to the Bradford method. Nuclear and cytoplasmic extracts from HUVECs were prepared using NE-PER nuclear and cytoplasmic kit (Pierce) according to the protocol of the manufacturer. Equal amounts of protein were loaded onto sodium dodecyl sulfate–polyacrylamide gels and blotted onto poly(vinylidene difluoride) membranes. Western blots were performed by using antibodies directed against Dicer (mouse monoclonal anti-Dicer, 1:500; Abcam), Drosha (rabbit polyclonal anti-Drosha, 1:1000; Upstate), Topo I (rabbit polyclonal anti-Topo I, 1:250; Santa Cruz Biotechnology), Hsp70 (rabbit polyclonal anti-Hsp70, 1:500; Upstate), thrombospondin (TSP-1) (mouse monoclonal anti-TSP, 1:250; Laboratory Vision), or tubulin (mouse monoclonal anti-tubulin, 1:1500; DiaNovo).

Migration Assay

To determine the migration of endothelial cells, HUVECs were detached with trypsin, harvested by centrifugation, resuspended in 0.1% BSA and counted manually in 5 random microscopic fields.

Tube Formation Assay

HUVECs (7×10^4) were cultured in a 12-well plate (Greiner) coated with 200 μL of Matrigel Basement Membrane Matrix (BD Biosciences). Tube length was quantified after 24 hours by measuring the cumulative tube length in three random microscopic fields with a computer-assisted microscope using the program KS300 3.0 (Zeiss).

Spheroid-Based Angiogenesis Assay

Endothelial cell spheroids of defined cell number were generated as described previously.16,17 In vitro angiogenesis was quantified by measuring the cumulative length of the sprouts that had grown out of each spheroid with or without human basic fibroblast growth factor (30 ng/mL) using a digital imaging software (Axioplan, Zeiss) analyzing 10 spheroids per experimental group and experiment.

Reverse-Transcription PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Afterward, 1 μg of RNA from each sample was reverse transcribed into cDNA and subjected to conventional PCR. Primer sequences for PCR were: Dicer, 5'-CAAGTTGCGACTGTCGCAAGACTC-3' and 5'-CAATCCACCCCAATCTCATCAGT-3'; Drosha, 5'-CACCCTGT-CTTACGCTCAGAC-3' and 5'-CTCTCTCCACGTAGACGTTTTATT-3'. To assess the differential miRNA expression in scrambled, Dicer siRNA–transfected, and Drosha siRNA–transfected HUVECs, we isolated total RNA using TRIzol (Invitrogen) according to the protocol of the manufacturer. RT-PCR was performed using the mirVana quantitative RT-PCR miRNA detection kit (Ambion) and primer sets specific for amplification of hsa-mir-27b and hsa-let-7i (Ambion) (1 cycle: 3 minutes at 95°C; 20 cycles: 15 seconds at 95°C, 30 seconds at 60°C).

MTT Viability Assay

Assessment of cell viability was performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide] assay. Twenty-four and 48 hours after transfection, 0.5 mg/mL MTT was added to each well and cells were incubated for 4 hours at 37°C. Cells were washed with PBS and lysed 30 minutes at room temperature with lysis buffer (40 mmol/L HCl in isopropanol). Absorbance was photometrically measured at 550 nm.

Immunocytochemistry

HUVECs grown on a cover slip were fixed in 4% paraformaldehyde for 15 minutes. After permeabilization with 0.25% Triton X-100, the cells were blocked in 2% BSA for 30 minutes at room temperature and incubated with antibodies against Dicer (mouse monoclonal anti-Dicer, 1:50; Abcam), Drosha (rabbit polyclonal anti-Drosha, 1:1000; Upstate), Topo I (rabbit polyclonal anti-Topo I, 1:250; Santa Cruz Biotechnology), Hsp70 (rabbit polyclonal anti-Hsp70, 1:500; Upstate), thrombospondin (TSP-1) (mouse monoclonal anti-TSP, 1:250; Laboratory Vision), or tubulin (mouse monoclonal anti-tubulin, 1:1500; Diavano).

In Vivo Matrigel Plug Assay

This assay was performed as described previously18 with the following modifications: HUVECs were transfected with siRNA against Dicer and Drosha or scrambled oligonucleotides as described above. Eighteen hours after transfection, cells were labeled with cell tracker CM-Dil (Invitrogen), were detached, washed, and counted. Cells (1×10^6) were reseeded in 30 μL of PBS and mixed with 500 μL of Matrigel Basement Membrane Matrix (BD Biosciences) containing 15 U of heparin (Sigma-Aldrich). The cell–Matrigel mixture was injected subcutaneously into 6- to 8-week-old female athymic nude mice (Harlan) along the abdominal midline. After 7 days, blood vessel infiltration in Matrigel plugs was quantified by analysis of lectin or CD31-stained sections using microscopy. For hemoglobin analysis, the Matrigel plug was removed after 7 days and homogenized in 130 μL of deionized water. After centrifugation, the supernatant was used in the Drabkin assay (Sigma-Aldrich) to measure hemoglobin concentration. Stock solutions of hemoglobin are used to generate a standard curve. Results are expressed relative to total protein in the supernatant.
MiRNA Expression Analysis

For determination of global baseline miRNA expression in HUVECs, total RNA was isolated using TRIzol. The expression of 168 mature human miRNAs in HUVECs was profiled using real-time PCR (DNAvision, Gosselies, Belgium). Gene expression data were normalized to let-7a. The relative expression was determined for each of the 168 miRNAs using the formula $2^{-\Delta\Delta Ct}$.

To assess the differential miRNA expression in scrambled, Dicer siRNA–transfected, and Drosha siRNA–transfected HUVECs, we isolated total RNA using TRIzol. The expression analysis of 344 miRNA was performed by a miRNA-profiling service (Exiqon) using miRCURY LNA arrays.

Statistical Analysis

Data are expressed as mean±SEM. Three or more treatment groups were compared by 1-way ANOVA followed by post hoc analysis adjusted with a least-significant-difference correction for multiple comparisons (SPSS Inc). Results were considered statistically significant when $P<0.05$.

Results

Expression and Localization of Dicer and Drosha in Endothelial Cells

Because little is known about Dicer and Drosha in endothelial cells, we first assessed the expression and localization of the 2 enzymes using immunocytochemistry and Western blot analysis. Dicer was predominantly localized in the cytoplasm, whereas the localization of Drosha was restricted to the nucleus of endothelial cells (Figure 1A and 1B). To determine whether Dicer and Drosha localization might be different in...
proliferating cells, we additionally identified proliferating cells by phospho–histone-H3 staining. However, the proliferation status had no influence on the localization of the 2 enzymes (Figure 1A and 1B).

**Role of Dicer and Drosha for Sprouting, Tube Formation, and Migration of Endothelial Cells**

To investigate the role of Dicer and Drosha for angiogenesis, we measured endothelial sprouting and network formation after silencing of Dicer and Drosha expression with siRNA (Figure 1C and 1D). Western blotting confirmed the efficient and specific suppression of Dicer and Drosha by the respective siRNA oligonucleotides (Figure 1D). The reduction of Dicer and Drosha significantly inhibited basal and basic fibroblast growth factor–stimulated endothelial cell sprout formation, as measured by capillary sprouting in a 3D collagen-embedded spheroid culture assay (Figure 2A and 2B), whereas VEGF-induced sprout formation was selectively blocked by Dicer siRNA (Figure 1 in the online data supplement, available at http://circres.ahajournals.org). Next, we tested whether the combined silencing of Dicer and Drosha further suppressed sprout formation. However, no additive effect was detected compared with the single reduction of Dicer or Drosha gene expression (supplemental Figure IIA and IIB). As a control for the specificity of Dicer and Drosha silencing, a second Dicer and Drosha siRNA was generated that reduced capillary sprout formation to a similar extent as compared with Dicer and Drosha siRNA I (data not shown). Moreover, suppression of Dicer and Drosha led to a significant impairment of network-forming activity using a Matrigel assay (Figure 3A and 3B). To address whether the reduction of Dicer and Drosha contributes to reduced endothelial cell migration, we performed a Boyden chamber migration assay. Whereas Dicer siRNA–mediated knockdown significantly decreased the migratory capacity, Drosha knockdown had no effect on cell migration (Figure 3C). To test whether the reduction of endothelial cell sprouting and tube formation was secondary to a nonspecific effect on cell growth or apoptosis, we measured cell viability using a MTT assay. As shown in Figure 3D, Dicer and Drosha knockdown did not impair cell viability after 24 hours. After 48 hours, Dicer siRNA transfection slightly reduced viability, whereas Drosha siRNA transfection did not affect viability (Figure 3E). To test whether an induction of cell death mediates the antiangiogenic effect of Dicer siRNA, apoptosis was blocked by the addition of the caspase inhibitor zVAD. However, the reduced sprout-forming activity of Dicer siRNA–transfected endothelial cells was not improved by zVAD addition (data not shown), indicating that the inhibition of sprout formation is independent of the induction of cell death.

**Dicer Is Required for In Vivo Angiogenesis**

Next, we assessed the effects of Dicer on in vivo angiogenesis. Because Dicer-deficient mice are embryonic lethal and thus not available for the study, we subcutaneously injected siRNA-transfected HUVECs in a Matrigel plug into nude mice and assessed the sprout formation. Vessel-like structures were significantly reduced in Matrigel plugs with Dicer
Figure 3. Effect of Dicer and Drosha on tube formation, migration, and cell viability. A through G, HUVECs were transfected with Dicer and Drosha siRNA or scrambled oligonucleotides. A and B, HUVECs were seeded on a "growth factor–enriched" Matrigel Basement Membrane Matrix (BD Biosciences) 24 hours after transfection. Representative micrographs and statistical summary of the tube-forming activity. Length of capillary-like structures was measured by light microscopy after 24 hours in a blinded fashion. Data are means±SEM (percentage scrambled); n=4. C, Cell migration was measured using a modified Boyden chamber. Cells were seeded in the upper chamber of a modified Boyden chamber 24 hours after transfection. Endothelial cell migration was assessed using VEGF (50 ng/mL) as a chemoattractant. Data are means±SEM (percentage scrambled); n=3. D and E, Cell viability was measured using a MTT viability assay after 24 (D) or 48 (E) hours. Data are means±SEM (percentage scrambled); n=3. F and G, The in vivo Matrigel plug assay was performed as described in Materials and Methods. Data are means±SEM (percentage scrambled); n=6 (F) and n=3 (G) per group.
miRNA expression profile in HUVECs. The expression of 168 human miRNAs was profiled using real-time PCR. Comparative analysis was performed for 92 miRNAs, detected in all 3 samples. Raw data were normalized to let-7a and converted using the formula $2^{-\Delta Ct} \times 1000$. Data represent miRNAs that are highly expressed in HUVECs (relative expression $\geq 100$). Data are means $\pm$ SEM; n=3.

Figure 4. miRNA expression profile in HUVECs. The expression of 168 human miRNAs was profiled using real-time PCR. Comparative analysis was performed for 92 miRNAs, detected in all 3 samples. Raw data were normalized to let-7a and converted using the formula $2^{-\Delta Ct} \times 1000$. Data represent miRNAs that are highly expressed in HUVECs (relative expression $\geq 100$). Data are means $\pm$ SEM; n=3.
siRNA–transfected HUVECs (Figure 3F). Silencing of Dicer additionally reduced Matrigel plug hemoglobin concentrations indicating that the blood supply is reduced (Figure 3G). In contrast, Drosha siRNA transfection did not significantly affect sprouting angiogenesis and hemoglobin concentrations of Matrigel plugs in vivo (Figure 3F and 3G).

**Dicer and Drosha Regulate the Expression of miRNAs in Endothelial Cells**

To assess the specific involvement of miRNAs in endothelial cell function, we analyzed the expression of 168 human miRNAs in HUVECs. miRNAs with a relative expression (2^-ΔΔCt) of ≥100 are listed in Figure 4. In particular, miR-21, miR-126, mir-221, and mir-222 are highly expressed in endothelial cells. In addition, several members of the let-7 family revealed a high abundance in HUVECs. To investigate the role of Dicer and Drosha in miRNA expression in endothelial cells, we analyzed the expression of 344 human miRNAs after knockdown with siRNA targeting Dicer and Drosha using miRNA array (Figure 5 and the Table). The expression of 202 miRNAs was below the detection limit of the assay. The selective analysis of the effect of Dicer and Drosha downregulation revealed that the expression of 2 miRNAs was reduced to more than 30% by Dicer knockdown, 29 miRNAs were reduced to more than 30% by Drosha knockdown, and 31 miRNAs were regulated by both enzymes. The reduction in miRNA expression after Dicer and Drosha knockdown was confirmed by RT-PCR for mir-27b and let-7f (Figure 6A and 6B). To test the functional effect of mir-27b and let-7f, the miRNA were blocked by specific inhibitors. Inhibition of mir-27b and let-7f significantly reduced in vitro sprout formation (Figure 6C).

**Dicer and Drosha Silencing induces the Upregulation of TSP-1 Expression**

In silico prediction of potential target genes using miRanda software revealed that the let-7 miRNA family may regulate the expression of TSP-1, which was recently identified as an endogenous angiogenesis inhibitor. Therefore, we assessed the expression of TSP-1 after Dicer and Drosha siRNA transfection. As shown in Figure 6D and 6E, the reduction of Dicer and Drosha by siRNA transfection significantly increased the expression of TSP-1 to 263±68% in Dicer siRNA–transfected cells and to 358±95% in Drosha siRNA–transfected cells, respectively. However, depletion of TSP-1 expression by siRNA did not rescue the impaired sprout formation induced by Dicer or Drosha deficiency (supplemental Figure III), indicating that additional targets are involved to fully explain the angiogenesis inhibition. Because let-7f in comparison to other let-7 family members was most profoundly downregulated by Dicer and Drosha, we investigated the effect of a let-7f inhibitor on TSP-1 expression. However, inhibition of let-7f induced only a minor, not significant, increase in TSP-1 expression to 115±18% (n=6), suggesting that additional miRNAs are involved in the regulation of TSP-1.

**Effect of Dicer and Drosha Silencing on Cytokine Expression and Akt Signaling**

Finally, we elucidated the effect of Dicer and Drosha on the expression of angiogenic growth factors by using a human cytokine array. Although Dicer siRNA significantly suppressed angiogenesis, most of the proangiogenic cytokines were increased in Dicer-silenced endothelial cells (supplemental Figure IVA). Moreover, no significant difference in the expression of the VEGF receptor 2 was detected (supplemental Figure IVB). In contrast, phosphorylation of Akt was selectively inhibited in Dicer siRNA compared with Drosha siRNA–treated cells (supplemental Figure IVC), indicating an interference of Dicer with Akt activation.

**Discussion**

Although several studies have dissected the role of miRNAs in controlling embryonic development, myogenesis and cardiogenesis, the involvement of miRNAs in vascular signaling and function is less clear. Using transient silencing of the miRNA processing enzymes, the present study demonstrates that Dicer and Drosha are involved in the regulation of angiogenesis in vitro. Silencing of Dicer expression additionally impaired in vivo angiogenesis, whereas depletion of Drosha did not exert significant effects. Profiling of highly expressed miRNAs in endothelial cells provides evidence that miRNA let-7f and hsa-mir-27b are significantly downregulated by Drosha and Dicer siRNA. In accordance with in silico prediction of putative let-7 targets, our data suggest that Dicer and Drosha regulate the angiogenesis-inhibitor TSP-1 via let-7 family members.

Interestingly, the knockdown of Dicer exerted more profound effects on endothelial sprout formation in vitro and in vivo compared with Drosha. To exclude that the distinct biological response is caused by a methodological problem, control experiments were performed to confirm a similar efficiency of the downregulation on protein and mRNA level. Moreover, the findings were reproduced using a second siRNA sequence excluding an nonspecific effect of one of the sequences. The genetic downregulation of Drosha efficiently reduced the expression of ~42% of the detected miRNAs, indicating that the minor phenotypic alteration in Drosha...
Examples of miRNAs Regulated by Dicer and Drosha

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<th>Dicer siRNA (% vs Scrambled)</th>
<th>Drosha siRNA (% vs Scrambled)</th>
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HUVECs were transfected with Dicer and Drosha siRNA or scrambled oligonucleotides. The expression analysis of 344 human miRNAs was performed using miRCURY LNA arrays. The table summarizes some miRNAs regulated by Dicer and Drosha. ND indicates not detected; expression in Dicer and Drosha siRNA-transfected cells under the detection limit of the assay. Data are shown as median ratios (%) vs scrambled.

siRNA–transfected cells is not related to an insufficient reduction of Drosha enzymatic activity. An explanation for the distinct effects of Dicer versus Drosha may be related to other biological properties of Dicer, namely its role in the formation and maintenance of heterochromatin, which might also affect endothelial cell functions independent on the reduction of miRNA processing. The minor role of Drosha compared with Dicer on sprouting might alternatively be explained by a concomitant effect on antiapoptotic or proangiogenic genes, which are less affected by Dicer siRNA. A downregulation of miRNAs targeting antiapoptotic or proangiogenic genes might lead to an upregulation of protective and proangiogenic proteins in Drosha-silenced endothelial cells and may partially compensate for the increase in angiogenesis inhibitors. It is well established that angiogenesis is regulated by a balance of pro- or antiangiogenic genes. A disturbance of this critical balance by interfering with various miRNAs, each of which inhibiting translation or transcription of multiple target miRNAs, may well explain differences in the phenotype.

Profiling of miRNA expression in endothelial cells, as it has been done in the present study, additionally provides a tool to identify potentially important miRNAs in endothelial cell biology. Strikingly, the relative expression of ~60% of the miRNAs was below the detection limit, indicating that the majority of the miRNAs are downregulated in endothelial cells. These data were selectively confirmed by demonstrating that several miRNAs, such as miR-142-3p, were not detected by RT-PCR (data not shown). This is consistent with the concept that miRNAs are differentially expressed during development and control lineage commitment. A cell type–specific regulation of miRNA expression may contribute to tissue and cell type–specific patterning of gene expression. Therefore, the data of the present miRNA array analysis allow for selectively evaluating the function and regulation of endothelial cell–enriched miRNAs in future studies. Indeed, the high expression of miR-221 is in accordance with a recent study demonstrating that miR-221 may affect the angiogenic properties of endothelial cells by regulating c-kit, the receptor for the cytokine stem cell factor.

Surprisingly, the downregulation of Drosha and Dicer only affected a subset of miRNAs. Based on the assumption that these 2 enzymes are essential for miRNA processing, one would have expected to see a downregulation of the majority of miRNAs. However, in accordance with our data, others also reported that only a subset of miRNAs is reduced after Dicer downregulation (55% to 59%4). The incomplete inhibition of miRNAs might be explained by an insufficient downregulation of the enzymes or a higher stability of some of the miRNAs exceeding the transient effect of Drosha or Dicer downregulation by siRNA. Finally, the data should be interpreted with caution because of the limitation of the miRNA array technology. Although the assays preferentially detect mature miRNAs, the discrimination of primary, pre-, and mature miRNAs requires confirmation by Northern blotting. The low sensitivity of Northern blot, however, limits its general use.

By using in silico prediction of targets for the highly expressed and Drosha/Dicer downregulated miRNA let-7 cluster, we identified several potentially interesting genes including TSP-1. TSP-1 is a potent endogenous inhibitor of angiogenesis. Upregulation of TSP-1 in endothelial progenitor cells contributes to impaired vasculogenesis in diabetic mice. Consistent with a predicted role of the let-7 cluster for TSP-1 expression, Drosha and Dicer silencing augmented TSP-1. Given that TSP-1 is a potent angiogenesis inhibitor, its upregulation might contribute to the impairment of angiogenesis after Drosha or Dicer downregulation. A let-7f inhibitor, however, increased TSP-1 expression only ~15%, indicating that other factors (eg, other let-7 family members) may, in part, compensate for the loss of let-7f. Indeed, other let-7 family members target TSP-1 as predicted by in silico analysis. Moreover, TSP-1 is also repressed by the miR-17 to 92 miRNA cluster.

In summary, our data provide evidence for an important role of Dicer and Drosha for endothelial cell functions. The profound impairment of in vitro angiogenesis in Dicer-deficient endothelial cells is consistent with findings that were recently published during the revision of the present
The angiogenesis suppressive effect of Dicer and Drosha downregulation may in part be related to the augmentation of the angiogenesis inhibitor TSP-1. However, the profound difference between Dicer and Drosha silenced cells is more likely attributable to the selective impairment of Akt signaling in Dicer siRNA–treated cells. Because a variety of miRNAs are highly expressed and dysregulated by Drosha and/or Dicer siRNA and each miRNA has multiple pro- and antiangiogenic targets, further studies are required to dissect the complex process of posttranscriptional regulation of gene expression during angiogenesis.

**Acknowledgments**

We thank Andrea Knau, Nicole Konecny, and Tino Roexe for excellent technical help.

**Sources of Funding**

This study was supported by the Deutsche Forschungsgemeinschaft (FOR501 [Di6006/3-1]). A.K. is supported by the Alfried Krupp Stiftung (S.D.). C.U., A.M.Z., and S.D. belong to the European Vascular Genomics Network, a Network of Excellence supported by the European Community’s sixth Framework Programme for Research Priority 1 “Life sciences, genomics and biotechnology for health” (contract no. LSHM-CT-2003-503254).
Disclosures

None.

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Circ Res. 2007;101:59-68; originally published online May 31, 2007;
doi: 10.1161/CIRCRESAHA.107.153916
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
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Supplementary Figure 1: HUVEC were transfected with siRNA against Dicer and Drosha or scrambled oligonucleotides. Quantitative analysis of endothelial sprouting in response to VEGF stimulation. Data are mean±SEM, n=4.
Supplementary Figure 2: HUVEC were transfected with siRNA against Dicer and Drosha or a combination of both siRNAs. Scrambled siRNA was used as control. A) RT-PCR analysis of Dicer and Drosha mRNA expression 18 h after transfection. A representative gel is shown. GAPDH serves as loading control. B) Quantitative analysis of endothelial sprouting capacity in response to bFGF-stimulation. Data are mean ± SEM, n=4.
**Supplementary Figure 3**

**A**

![Western blot analysis of thrombospondin-1](image1)

**B**

![Quantitative analysis of endothelial sprouting](image2)

**Supplementary Figure 3**: HUVEC were transfected with siRNA against Dicer, Drosha or TSP-1 or a combination of two different siRNAs. Scrambled siRNA was used as control. A) 18 hours after transfection, cells were lysed and subjected to western blot analysis with an antibody against thrombospondin-1. Tubulin was used as loading control. A representative western blot is shown. B) Quantitative analysis of endothelial sprouting in response to bFGF stimulation. Data are mean ± SEM, n=3.
Supplementary Figure 4: HUVEC were transfected with siRNA against Dicer and Drosha or scrambled oligonucleotides. A) 24 h after transfection, cells were starved for 20 h in EBM + 0.05 % BSA. Supernatants were collected and concentrated 10-fold using Vivaspin columns. 10x supernatants were subjected to a human cytokine antibody array (Ray Bio) according to the instructions of the manufacturer. Quantitative analysis of cytokine expression is shown. Data are mean±SEM, n=3. B) KDR expression was assessed by FACS staining. Quantitative analysis is shown. Data are mean±SEM, n=3. C) To assess Akt phosphorylation, cells were lysed and subjected to western blot analysis using an antibody against phospho-Akt. Total Akt was used as loading control. A representative western blot and quantitative analysis is shown. Data are mean±SEM, n=3.
Supplementary Figure 5: HUVEC were transfected with siRNA against Dicer and Drosha or scrambled oligonucleotides. The expression of c-kit was detected by Western blot analysis. Tubulin was used as loading control. Quantitative analysis is shown. Data are mean±SEM, n=3.