Dicer Is Required for Embryonic Angiogenesis during Mouse Development*

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Dicer is a multi-domain protein responsible for the generation of short interfering RNAs (siRNAs) from long double-stranded RNAs during RNA interference. It is also involved in the maturation of microRNAs, some of which are transcriptional regulators of developmental timing in nematodes. To assess the role of Dicer in mammals, we generated Dicer<sup>ex1/2</sup> mice with a deletion of the amino acid sequences corresponding to the first and second exons of the dicer gene via homologous recombination. We found that Dicer<sup>ex1/2</sup> homozygous embryos displayed a retarded phenotype and died between days 12.5 and 14.5 of gestation. Thus, these results show that dicer<sup>ex1/2</sup> is severely hypomorphic and that Dicer is essential for normal mouse development. Interestingly, we also found that blood vessel formation/maintenance in dicer<sup>ex1/2</sup> embryos and yolk sacs were severely compromised, suggesting a possible role for Dicer in angiogenesis. This finding is consistent with the altered expression of vegf, flt1, kdr, and tiet in the mutant embryos. Taken together, the results of this study indicate that Dicer exerts its function on mouse embryonic angiogenesis probably through its role in the processing of microRNAs that regulate the expression levels of some critical angiogenic regulators in the cell.

RNA interference (RNAi) is a post-transcriptional gene regulation process that is conserved in organisms ranging from fungi to humans (1–5). When cells encounter long double-stranded RNA molecules, Dicer, a ribonuclease III type enzyme, cleaves them into small interfering RNAs (siRNAs) of 21–23 nucleotides. These siRNAs are incorporated into a multicomponent protein complex known as RISC (RNA-induced silencing complex). The antisense strand of siRNAs is believed to guide the RISC to locate its cognate mRNA molecule. As a result, the mRNAs are degraded (6–8).

As one of the critical enzymes of the RNAi pathway, Dicer was first identified from Drosophila embryo and S2 cell extracts as the initiation enzyme for RNAi (7). It is a large (~220 kDa), multi-domain protein that consists of an amino-terminal helicase domain followed by a PAZ domain, two RNase III domains, and a C-terminal double-stranded RNA-binding domain (dsRBD). The dsRBD and RNase III domains are involved in the binding to and cleavage of long double-stranded RNAs, but the functions of other domains remain unclear (9). Genetic and biochemical studies have established that Dicer is required for the production of siRNAs from double-stranded RNAs in Caenorhabditis elegans, Drosophila, and humans.

In addition to its important role in the RNAi pathway, Dicer also plays pivotal roles in development. In C. elegans, inactivation of the dcr-1 (homolog of Drosophila Dicer) gene results in defects in the RNAi pathway as well as developmental abnormalities similar to those caused by the loss of function of let-7, a gene encoding a microRNA (miRNA) essential for the control of developmental timing in this organism (10, 11). This finding suggests that Dicer is also responsible for processing miRNA precursors, which often form a stem-loop structure creating a double-stranded RNA molecule (12). In addition, human Dicer was shown to be responsible for the generation of mature let-7 in HeLa cells (13). Therefore, the function of Dicer in development may be well conserved during evolution.

Currently, there is an emerging interest in miRNAs because, under normal conditions, miRNAs rather than siRNAs appear to have a major role in small RNA-mediated gene regulation. Therefore, the principle role of Dicer in vivo may be involved in the biogenesis of miRNAs (14). Recently, ~90 miRNAs have been identified from C. elegans, Drosophila, mouse tissues, and human cell cultures. Among these, nine are found in more than one phylum (15–18). This finding suggests that miRNA-mediated post-transcriptional regulation may function in a wide variety of organisms. Furthermore, the large numbers of miRNAs as well as the source from where they have been identified also suggest that miRNAs may be involved in the regulation of multiple physiological pathways (14).

To assess the function of Dicer in a mammalian system, we sought to generate Dicer-deficient mice via homologous recombination in embryonic stem cells. We show that dicer<sup>ex1/2</sup> embryos are growth and developmentally retarded and die between embryonic day (E) 12.5 and 14.5. Therefore, Dicer is essential for normal development. In addition, the mutant embryos and their yolk sacs are found to display defects in the blood vessel formation/maintenance, suggesting a role for Dicer in the regulation of embryonic angiogenesis via its function in the processing of miRNAs.

**EXPERIMENTAL PROCEDURES**

**Construction of a dicer Targeting Vector**—A DNA primer corresponding to the 5′-region of the mouse dicer genomic sequence was used to screen a mouse CITB BAC library by Research Genetics (Carlsbad, CA). One positive clone (474 E 20) was identified and confirmed by colony PCR using two primers, wyj1 and wyj3, corresponding to mouse dicer cDNA sequences (wyj1, 5′-AGCATGGCCGGCCTGCAG-3′; wyj3, 5′-TGATGCGCACGCTTTGG-3′). An ~10-kb BamHI-Xhol fragment from this BAC clone was ligated into a plusscriptor plasmid (Stratagene). The 1.2-kb HindIII-PmlI fragment containing exons 1 and 2 of dicer was then replaced by a neo expression cassette.

**Generation of dicer<sup>ex1/2</sup> Mice**—The targeting construct was linearized at the XhoI site and introduced into mouse embryonic stem cell E14 via

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The abbreviations used are: RNAi, RNA interference; siRNA, small interfering RNA; miRNA, microRNA; E, embryonic day; PECAM, platelet endothelial cell adhesion molecule; RT, reverse transcription; VEGF, vascular endothelial growth factor.
Electroporation. G418-resistant colonies selected were screened for the homologous recombination by Southern blotting analysis using a 3′-flanking probe, a 5′-flanking probe, and a neo probe. Both the 3′- and 5′-probes were generated by PCR reaction off the BAC clone. The primers used for the 3′-probe were 5′-CTCTGGTGGAAAAAGGTAACG-3′ and 5′-TGGAAAAAGCCCTCATTTCAAG-3′. The primers for amplifying the 5′-probe were 5′-GTTATGGCTATATACGGCG-3′ and 5′-GCCAAGGCTTGGTTTACC-3′. The neo probe was a 259-bp PstI fragment from a plasmid containing the neo cassette. Three of six positive clones identified were microinjected into blastocysts and implanted into pseudopregnant C57BL/6 (B6) females. Chimeric males were mated with B6 females (Harland) to yield heterozygous F1 offspring, which were then intercrossed to produce +/+ , +/− , −/− offspring. Tail DNA from mice was isolated using genomic isolation kit from Lambda Biotech, and genotyping was carried out by PCR reaction using the primers KO (5′-AGGCATUTCCCGAGAATGTC-3′), which was the forward primer common for both the wild-type and the targeted allele, and KOR2 (5′-CCGAGACA-3′), and KOR1 (5′-CCGAGACA-3′), which were designed for the amplification of the wild type and the targeted alleles, respectively.

Analysis of Embryos—Detection of vaginal plugs in the morning was taken as gestation day 0.5 (E0.5). Embryos were staged and dissected from the pregnant females. Yolk sacs from different stage embryos were taken as gestation day 0.5 (E0.5). Embryos were staged and dissected to the wild type and the targeted alleles, respectively.

TAGGGTTCAGTCATTCGT-3′

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were then intercrossed to produce +/+ , +/− , −/− offspring. Tail DNA from mice was isolated using genomic isolation kit from Lambda Biotech, and genotyping was carried out by PCR reaction using the primers KO (5′-AGGCATUTCCCGAGAATGTC-3′), which was the forward primer common for both the wild-type and the targeted allele, and KOR2 (5′-CCGAGACA-3′), and KOR1 (5′-CCGAGACA-3′), which were designed for the amplification of the wild type and the targeted alleles, respectively.

Northern Blotting Analysis—Total RNA was isolated from wild type, heterozygous, and mutant mouse embryonic fibroblasts. Messenger RNA was purified using an Oligotex mRNA isolation kit (Qiagen). Each mRNA sample (5 μg) was separated in a glyoxal-agarose gel, transferred to nylon membranes, and hybridized with a 32P-labeled cDNA probe generated by PCR reaction using wjy1 and wjy3 under the conditions described earlier (19). The same probe was also used to hybridize a membrane containing RNAs derived from different mice tissues obtained at different developmental stages (Clontech).

Antibody Generation and Western Blotting Analysis—A Dicer-specific antibody was generated against a peptide corresponding to the C terminus of the mouse Dicer protein with an amino acid sequence of H-Cys-Leu-Arg-Ser-Leu-Lys-Ala-Aan-Gin-Pro-Gin-Val-Pro-Pro-Ser-OH. Proteins (100 μg) from whole embryos of wild type or dicer+/- mouse embryos at E14.5 were separated on a SDS-polyacrylamide gel (4–20%) and transferred onto polyvinylidene difluoride membranes. The membranes were probed with a rabbit antibody (Research Diagnostics, Inc.) at 2 μg/ml, a rabbit anti-Glut1 antibody (Alpha Diagnostic) at 2 μg/ml, a mouse anti-actin antibody (Sigma) at 1:10,000, a rabbit anti-Dicer antisera (Anaspec) at 1:500, or a rabbit anti-PTEN antibody (Upstate) at 1:1000 and, subsequently, with appropriate peroxidase-conjugated secondary antibodies. The signals were detected with a Supersignal West Femto maximum sensitivity substrate (Pierce).

TaqMan RT-PCR Analysis—Total RNA was isolated from wild type and mutant embryos using TRIzol (Invitrogen) according to the manufacturer’s protocol. Messenger RNA was purified using an Oligotex mRNA isolation kit (Qiagen). The mRNA (10 ng) was subjected to quantitative RT-PCR analysis using 300 nM primers and 200 nM probe in 25-μl reaction mixtures using Taq Platinum (Invitrogen) according to the manufacturer’s protocol. Messenger RNA was purified using an Oligotex mRNA isolation kit (Qiagen). The mRNA (10 ng) was subjected to quantitative RT-PCR analysis using 300 nM primers and 200 nM probe in 25-μl reaction mixtures using Taq Platinum (Invitrogen) according to the manufacturer’s suggestions. For histology, embryos were fixed overnight in 3% paraformaldehyde in phosphate-buffered saline, dehydrated and embedded in paraffin. Ten-micron sections were stained with hematoxylin and eosin.

Results

The dicer Gene Is Expressed in Mouse Embryos and Adult Tissues—to determine the relative levels of dicer expression during development, a dicer 3′-cdna fragment was generated by PCR reactions from an E11.5 limb cDNA library (Stratagene) using the primers 5′-GACTTGCACAAGGAGCAAGT-3′ and 5′-GACTTCCAGGAGGAATTCAAG-3′. This fragment was cloned into a pGEM-T easy vector (Promega). Sense and antisense digoxigenin-labeled riboprobes were synthesized using T7 and SP6 RNA polymerases, respectively. E10.5 embryos from white Swiss mice (Harland) were collected and processed for in situ hybridization as described (20) and photographed. PECAM immunohistochemistry of yolk sacs were performed as described (21).

RESULTS

The expression of dicer in several adult tissues was also examined.

The expression of dicer in several adult tissues was also examined. Interestingly, dicer was strongly expressed in the brain, spleen, lung, skeletal muscle, and testis (Fig. 1B).

Generation of dicer+/- via Gene Targeting—to determine the in vivo function of Dicer during development, a targeting vector was designed to disrupt the dicer by replacing the first primer sets were obtained as a 20% mixture from Applied Biosystems.

In Situ Hybridization and Immunohistochemistry—An ~400-bp dicer 3′-cdna fragment was generated by PCR reactions from an E11.5 limb cDNA library (Stratagene) using the primers 5′-GACTTGCACAAGGAGCAAGT-3′ and 5′-GACTTCCAGGAGGAATTCAAG-3′. This fragment was cloned into a pGEM-T easy vector (Promega). Sense and antisense digoxigenin-labeled riboprobes were synthesized using T7 and SP6 RNA polymerases, respectively. E10.5 embryos from white Swiss mice (Harland) were collected and processed for in situ hybridization as described (20) and photographed. PECAM immunohistochemistry of yolk sacs were performed as described (21).

FIG. 1. Expression of dicer mRNA during development and in adult tissues. A and B, polyA mRNA from embryonic days 7, 11, 15, and 17 (A) and adult mouse tissues (MTN blots; Clontech) (B) were hybridized with a dicer 3′ cDNA probe as described under “Experimental Procedures.” An ~6.5-kb dicer transcript is indicated by the arrow. C, E10.5 embryos were hybridized with a dicer sense probe. D, E10.5 embryos were hybridized with a dicer antisense probe. The dicer mRNA was detected at similar levels throughout the embryos.
Targeted Disruption of Dicer in Mice

Developmental Defects in dicer<sup>e<sup>x</sup>1/2</sup> Embryos—Germline chimeras were mated to B6 females to generate heterozygous carriers of the mutant allele. Heterozygous animals were viable, healthy, and did not exhibit any overt abnormalities. To obtain homzygous mutants, heterozygous carriers were intercrossed, and the resulting offspring were genotyped. Of 59 mice born, none was a homozygous mutant, indicating that dicer<sup>e<sup>x</sup>1/2</sup> animals were not viable. To further determine the timing of the lethality, embryos from heterozygous intercrosses were analyzed at various developmental stages. At E10.5, dicer<sup>e<sup>x</sup>1/2</sup> embryos appeared morphologically normal (data not shown). However, starting from E11.5, virtually all dicer<sup>e<sup>x</sup>1/2</sup> embryos were growth and developmentally retarded as compared with their wild type or heterozygous litter mates (Fig. 4, A–D and F–I). Histological analyses of E12.5 mutant embryos revealed that they had overtly normal structures, except that they were smaller and slightly underdeveloped (data not shown). The number of dicer<sup>e<sup>x</sup>1/2</sup> embryos obtained was within normal Mendelian distribution up to this stage, although resorbed embryos were found at these stages as well (Table I). Most of the dicer<sup>e<sup>x</sup>1/2</sup> embryos recovered at E13.5 and E14.5 were morphologically abnormal and nonviable. The few viable ones all had various degrees of edema (Fig. 4, H and I). Noticeably, these mutant embryos appeared to be very pale with smaller livers. The blood vessels in these mutants were thin and suboptimally developed (arrowheads in Fig. 4, D and I). No viable embryos were retrieved beyond E14.5, indicating that dicer<sup>e<sup>x</sup>1/2</sup> embryos died between E12.5 and 14.5.

Defects in the Yolk Sacs of dicer<sup>e<sup>x</sup>1/2</sup> Embryos—In addition to the embryonic phenotypes, yolk sacs from mutant embryos were abnormal as well. As early as E10.5 a subset of dicer<sup>e<sup>x</sup>1/2</sup> yolk sacs started to display phenotypes different from those of wild type or heterozygous controls. In general, they appeared pale (Fig. 5, A and D), and this phenomenon became more pronounced in yolk sacs from older embryos. Microscopic examination revealed that there were fewer blood vessels in the dicer<sup>e<sup>x</sup>1/2</sup> yolk sacs and that these vessels were thin, small, and less organized than those of control yolk sacs (Fig. 5, B, C, E, and F). In some extreme cases, there appeared to be no yolk sac blood vessels at all (data not shown). Together, these observations indicate that Dicer may be required for blood vessel development during embryogenesis.

Impaired Angiogenesis in dicer<sup>e<sup>x</sup>1/2</sup> Embryos and Their Yolk Sacs—To analyze vascular network formation, yolk sacs from E11.5 embryos were stained with anti-PECAM antibodies specific to the endothelial cell. The blood vessels in yolk sacs from wild type embryos at this stage were well into vascular re-modeling and formed an orderly arranged network of blood vessels with large vitelline arteries and veins branching in a fractal pattern to smaller vessels (Fig. 5G). In contrast, the blood vessels in dicer<sup>e<sup>x</sup>1/2</sup> yolk sacs were thin and disorganized (Fig. 5H). However, the presence of a honeycomb-like primary vascular structure indicates that vasculogenesis and the initial steps of angiogenesis proceeded normally in the mutants. Hence, these observations suggested that in the absence of a fully functional Dicer, either angiogenesis failed to go to completion and/or that the formed vessels could not be maintained or stabilized. Whole mount PECAM staining of E14.5 embryos also revealed defects in the angiogenesis of mutant embryos proper. The tree-like blood vessel structures observed in the wild type embryos (Fig. 4E) were absent in mutant embryos (Fig. 4I). Instead, there were many disorganized vascular structures. Again, the presence of vascular bedding suggests that Dicer is not required for vasculogenesis and early differentiation of the endothelial cells. These immunohistochemical data suggest that Dicer is required for the proper angiogenesis.
A receptor tyrosine kinase gene, was lower in the mutant em-

**TABLE I**

<table>
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<tr>
<th>Age/Stage</th>
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Asterisk indicates the liver, and the black arrowhead indicates the blood vessels. Panel E shows PECAM staining of wild type embryos at E14.5. Panel J shows PECAM staining of dicer<sup>ex1/2</sup> embryos at E14.5. The stage and genotype of each embryo at the top and bottom of each panel. Scale bar, 1 mm.

**FIG. 4.** Retarded development and defective angiogenesis in dicer<sup>ex1/2</sup> embryos. Dicer deficient embryos (F-I) and wild type litter mates (A-D) at the same stage. Asterisk indicates the liver, and the black arrowhead indicates the blood vessels. Panel E shows PECAM staining of wild type embryos at E14.5. Panel J shows PECAM staining of dicer<sup>ex1/2</sup> embryos at E14.5. The stage and genotype of each embryo at the top and bottom of each panel. Scale bar, 1 mm.

of both the embryos and their yolk sacs.

**Altered Expression of vegf, flt1, kdr, and tie1 in dicer<sup>ex1/2</sup> Embryos**—The vascular defects observed in the dicer<sup>ex1/2</sup> embryos prompted us to examine the expression of several crucial genes involved in embryonic angiogenesis. Messenger RNA was isolated from pooled E14.5 wild type and dicer<sup>ex1/2</sup> embryos and subjected to Taqman RT-PCR analysis. Interestingly, the mRNA levels of vegf and the genes of its receptors, flt1 and kdr, in dicer<sup>ex1/2</sup> embryos were significantly higher than those in wild type embryos. On the contrary, the mRNA level of tie-1, a receptor tyrosine kinase gene, was lower in the mutant embryos (Fig. 6A). These results were confirmed by quantitative RT-PCR analysis of individual embryos at the same stage (data not shown). The reduction of the Tie-1 protein level in dicer<sup>ex1/2</sup> embryos was further confirmed by Western blotting analysis (Fig. 6B). Thus, these results indicate that the impaired angiogenesis in dicer<sup>ex1/2</sup> embryos is probably due to the altered expression levels of angiogenesis regulators in the cell. These results also indicate that Dicer probably exerts its regulatory function on embryonic angiogenesis through the processing of miRNA molecules that control the expression levels of angiogenesis regulators.

**DISCUSSION**

We have attempted to disrupt the dicer gene via homologous recombination in embryonic stem cells. Because exon 1 contains the putative translation initiation codon AUG, we reasoned that an elimination of the first two exons of the mouse dicer gene with a neo expression cassette should abolish the expression of a functional Dicer protein. However, to our surprise we detected a protein with a size of ~220 kDa in dicer<sup>ex1/2</sup> embryos. This protein is probably synthesized by using the in-frame AUG in exon 4, although this codon is not preceded with a consensus Kozak sequence. As one of the primary roles for Dicer is to generate miRNAs, and because hundreds of miRNA targets have recently been identified (22), we sought to investigate whether the truncated Dicer protein still retains function by examining the RNA and protein levels of the putative miRNA target genes, pten and ang-1, in the mutant and wild type embryos. Our findings that the levels of both gene products were significantly higher in the mutant embryos than in the wild type embryos but that their transcript levels were similar clearly indicate that Dicer function is severely compromised in mutant embryos. Recently, Bernstein et al. also reported the embryonic lethality for their dicer knock-out animals (23). In their study, however, the dicer<sup>−/−</sup> embryos died around E7.5, which is much earlier than the time for dicer<sup>ex1/2</sup> embryos obtained in this study. Therefore, these results show that dicer<sup>ex1/2</sup> is a severe hypomorphic allele.

The absence of live offspring of dicer<sup>ex1/2</sup> demonstrates that Dicer is essential for normal development. This finding is consistent with the hypothesis that Dicer has a developmental role, probably through its ability to process miRNAs. Further analyses of embryos between E10.5 to 16.5 revealed that the dicer<sup>ex1/2</sup> embryos die at E12.5–E14.5, with notable defects in vascular structures. The formation of blood vessels during embryonic development consists of two distinct processes: 1) vasculogenesis, in which angioblasts differentiate and proliferate in situ to form the primary vascular network (24, 25); and 2) angiogenesis, a process that modifies the primitive vessel structures through sprouting and vessel enlargement to form the branching patterns of the mature vasculature (26). In the absence of Dicer, vasculogenesis in both the yolk sacs and the embryos seems to proceed normally. Therefore, Dicer may not be required for the differentiation and proliferation of endothelial cells. The initiation of angiogenesis is probably not affected.
by the absence of Dicer either, because the first sign of yolk sac defects arises at E10.5, and only a subset of dicer<sup>ex1/2</sup> yolk sacs appeared to be abnormal. Branching vessels can also be found in yolk sacs of some E14.5 dicer<sup>ex1/2</sup> embryos. Also, in the mutant embryos proper the dorsal aortae, the intersomitic arteries, and the limb bud vasculature all appeared to be present. These observations indicate that Dicer is specifically required for the maintenance of blood vessel integrity during late embryogenesis.

Analyses of the expression of several important regulators for embryonic vasculogenesis and angiogenesis revealed an alteration in their expression levels. Interestingly, the phenotypes of our dicer<sup>ex1/2</sup> embryos are similar to those of the Tie-1 deficient embryos. Both mutant embryos exhibit vascular defects, moderate edema, and die around days 13.5 and 14.5 of gestation. The receptor tyrosine kinase Tie1 has been shown to be required cell autonomously for the integrity and survival of vascular endothelial cells during late embryogenesis (27). Chimeric analyses indicate that Tie1 is not required for the early differentiation of endothelial cells, nor is it required for the early phase of angiogenic vessel growth. Rather, Tie-1 functions to support capillary endothelial cell proliferation (28). Recently, Tie-1 was also shown to activate phosphatidylinositol 3-kinase and Akt to inhibit apoptosis (29). In E14.5 dicer<sup>ex1/2</sup> embryos, the mRNA level of tie1 was decreased to ~28% of that in the wild type embryos. As a result, the TIE-1 protein also decreased dramatically in the mutant embryos. Therefore, this decreased expression of Tie-1 could lead to the apoptosis of endothelial cells, thereby causing the collapse of blood vessels in the mutant embryos.

VEGF is one of the most important growth factors for blood vessel formation, as it is required for both vasculogenesis and angiogenic sprouting. Developing mouse embryos are very sensitive to the level of VEGF. A slight variation in the VEGF level during vessel formation can cause severe vascular defects, eventually leading to embryonic lethality. For example, disruption of even a single allele of vegf in mice results in severe vascular abnormalities and embryonic lethality (30, 31), whereas an inappropriate induction of VEGF results in the formation of immature and leaky vessels (32, 33). VEGF

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**FIG. 5.** Morphology of dicer<sup>ex1/2</sup> yolk sacs. Wild type (A–C) and dicer<sup>ex1/2</sup> yolk sacs (D–F) at various stages. Panel G shows PECAM staining of E11.5 yolk sac from wild type embryos. Panel H depicts PECAM staining of E11.5 yolk sac from dicer<sup>ex1/2</sup> embryos. Arrows indicates the blood vessels. The stage and genotypes are indicated at the bottom and top of each panel. Scale bar, 1 mm.

**FIG. 6.** Altered expression of vegf, fltl, kdr, and tie-1 in dicer<sup>−/−</sup> embryos. A, TaqMan RT-PCR analysis of mRNA from pooled E14.5 wild type (wt) (white bar) and dicer<sup>ex1/2</sup> embryos (mut, mutant) (black bar) was performed as described under “Experimental Procedures.” Data were normalized according to the levels of β-actin. B, Western blotting analysis of proteins (100 μg) extracted from whole embryos of wild type (+/+) and dicer<sup>−/−</sup> at E14.5. Blots were probed with anti-Tie1 (top), anti-Glut1 (middle), and anti-actin (bottom).
mainly functions through its binding to the receptors Flt-1 and Kdr. It has been shown that oxygen homeostasis plays an important role in the regulation of angiogenesis. The transcription of the VEGF gene is up-regulated via hypoxia-inducible factor-1 under hypoxic conditions. Although increased VEGF expression in a hypoxic environment can promote vessel outgrowth, the formed vessels tend to be leaky, exhibiting disorganized patterns (34). Hypoxia also up-regulates Flt-1 and Kdr in cultured cells (35, 36). In E14.5 embryos, vegf, flt-1, and kdr levels are significantly higher than those in wild type embryos. This increased expression most likely results from the disrupted vessel structures, which subsequently cause compromised circulation and hypoxic condition in these mutant embryos. Consistent with this hypothesis, a dramatic increase in Glut1, a known hypoxia-inducible factor-1 target gene and a hypoxia marker, was observed in the dicerex/2 embryos (Fig. 6B).

Because the primary function of Dicer in vivo is to process miRNAs, our results suggest that embryonic angiogenesis might also be regulated by miRNAs. Because all miRNAs studied so far seem to act as post-transcriptional repressors of their targets, a defective Dicer is expected to cause a defect in the processing of miRNAs, which leads to overexpression of their target genes in the mutant embryos. This prediction has been confirmed, because the expression levels of Ang-1 and PTEN were significantly higher in the mutant embryos than those in the wild type embryos. Thus, the dicerex/2 embryos can be a very valuable system for the validation of miRNA targets in nature. Finally, miRNAs from the mir-80 family have been implicated in the control of developmental cell death and/or cell proliferation (37). It is possible that miRNAs related to the mir-80 family also regulate endothelial cell proliferation/death in mice during development.

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