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Biochem Biophys Res Commun. Author manuscript; available in PMC 2013 March 13.

Published in final edited form as:
Biochem Biophys Res Commun. 2011 May 13; 408(3): 369–374. doi:10.1016/j.bbrc.2011.02.119.

Conditional deletion of Dicer in vascular smooth muscle cells leads to the developmental delay and embryonic mortality

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Abstract

Dicer is a RNAase III enzyme that cleaves double stranded RNA and generates small interfering RNA (siRNA) and microRNA (miRNA). The goal of this study is to examine the role of Dicer and miRNAs in vascular smooth muscle cells (VSMCs). We deleted Dicer in VSMCs of mice, which caused a developmental delay that manifested as early as embryonic day E12.5, leading to embryonic death between E14.5 and E15.5 due to extensive hemorrhage in the liver, brain, and skin. Dicer KO embryos showed dilated blood vessels and a disarray of vascular architecture between E14.5 and E15.5. VSMC proliferation was significantly inhibited in Dicer KOs. The expression of VSMC marker genes were significantly downregulated in Dicer cKO embryos. The vascular structure of the yolk sac and embryo in Dicer KOs was lost to an extent that no blood vessels could be identified after E15.5. Expression of most miRNAs examined was compromised in VSMCs of Dicer KO. Our results indicate that Dicer is required for vascular development and regulates vascular remodeling by modulating VSMC proliferation and differentiation.

Keywords

Dicer; miRNA; Vascular smooth muscle cells; Embryonic mortality

1. Introduction

Dicer is an endoribonuclease in the RNase III family that cleaves double-stranded RNA and pre-microRNA (pre-miRNA) into two classes of tiny regulatory RNAs: small interfering RNA (siRNA) and miRNA [1–3]. SiRNA and miRNA are incorporated into RNA induced silencing complex (RISC) to regulate gene expression by either degrading mRNA or inhibiting protein translation. Dicer is evolutionarily conserved between worms, flies, plants, fungi, and mammals, although there are different isoforms in some of species [2]. siRNA

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Conflicts of interest
None declared.

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.119.

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plays fundamental roles in the defense against viral infections, inhibition of retroposon movement, and development [4]. miRNA regulates cell proliferation, apoptosis and differentiation. Thus, Dicer plays a dual role by producing functional siRNA and also miRNA. siRNA and miRNA play important roles not only in development but also in various human diseases including viral diseases, cancer, and cardiovascular disorders.

Dicer plays an essential role in mouse development shown by experiments that disruption of exon 21 of the Dicer led to early embryonic mortality at E7.5 [5]. In a similar study, the Dicer knockout mice generated by deleting exons 1 and 2 also showed embryonic mortality but died between E12.5 and E14.5 [6]. Several conditional knockouts of Dicer have already been generated, all of which showed that Dicer was important for cell proliferation, apoptosis, and differentiation [7–14]. However, the role of Dicer in the vascular system has not been well elucidated. Suarez et al. showed that Dicer is required for postnatal angiogenesis by deleting Dicer in the endothelial cells [15]. One of the major roles of Dicer is to facilitate miRNA maturation. Several miRNAs have been investigated in the context of vascular biology. miR-126 is important in the maintenance of vascular integrity and angiogenesis [16,17]. miR-143/145 cluster was found to regulate VSMC phenotype by regulating the expression of vascular smooth muscle marker genes. Deletion of miR-143/145 cluster leads to reduced vascular tone and blood pressure [18–20]. Here we report that conditional deletion of exon 23 of Dicer in mouse VSMCs that disrupts its RNAase IIIb motif, leads to developmental delay and embryonic mortality.

2. Methods

2.1. Generation of VSMC conditional Dicer knockout mice

All mouse experiments were approved by the University of Tennessee Animal Care and Use Committee. VSMC cKO mice were generated by crossing SM22-cre mice with $Dicer^{loxp/loxp}$ mice [21]. SM22-cre and $Dicer^{loxp/loxp}$ mice were obtained from The Jackson Laboratory. $Dicer^{loxp/loxp}$; SM22-cre were obtained by intercrossing $Dicer^{loxp/+}$; SM22-cre or breeding $Dicer^{loxp/loxp}$ mice with Dicer $Dicer^{loxp/+}$; SM22-cre mice. $Dicer^{loxp/loxp}$ littermates were used as control for all experiments.

2.2. Genotyping Dicer knockout mice and embryos

Mouse tails clippings or yolk sacs were collected into 200 μl 50 mM NaOH and heated at 95 °C for 50 min, followed by addition of 20 μl 1 M Tris–HCL and centrifugation for 5 min. Two μl of the supernatant was used for PCR. The cre-specific primers were 5′-GCTGCCACGACCAAGTGACAGCAATG (forward), GTAGTTATTCGGATCATCAGCTACAC (reverse). The Dicer specific primers were: 5′ CCT GAC AGT GAC GGT CCA AAG (forward), and 5′ CAT GAC TCT TCA ACT CAA ACT (reverse). The PCR products were separated by agarose gel electrophoresis. The product specific for the wild type Dicer is 350 bp whereas, it is 420 bp in the cKO genotype.

2.3. Histological analysis

To analyze the phenotype of Dicer knockout embryos, the time-mated pregnant mothers were sacrificed and the embryos were dissected. The harvested embryos were weighed and fixed overnight in 4% paraformaldehyde and embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

2.4. Detection of miRNA and mRNA expression using real-time PCR

Total RNA was extracted from two pooled umbiblical cord of Dicer cKO mutants and littermate controls at E14.5. Three samples were collected from six Dicer cKO mutants or six littermate controls. The specific method was described in our previous publication [22].

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To detect SMA, CNN1, Myh11 and SM22 VSMC differentiation marker gene expression we used previously published primer sequences [23]. The SYBR Green-based real-time PCR was performed using a LightCycler 4800 real-time PCR instrument (Roche Applied Science; Indianapolis, IN). The melting curve was performed to examine the PCR product specificity. The relative expression was normalized to U6 small nuclear RNA in ΔΔCt method. Data was expressed using mean ± SD ($n = 3$).

2.5. Immunofluorescence

Deparafinized sections were rehydrated and the antigen was retrieved by incubation of the slides for 30 min at 95–100 °C in 10 mM sodium citrate, 0.05% Tween 20 (pH6.0). The sections were treated with blocking buffer (5% normal goat serum, 3% bovine serum albumin, and 0.1% Triton-X 100 in PBS) for 1 h, followed by incubation with primary antibody to proliferating cell nuclear antigen (PCNA) at 4 °C overnight. After three rinses for 5 min with 0.05% Tween 20 in PBS (TPBS) the sections were incubated with Alexa 488 or Alexa 594 conjugated goat anti-rabbit or mouse (InVitrogen, 1:200 in TPBS) secondary antibody for 1 h at room temperature. After three washes the sections were mounted with Vectashield medium containing DAPI or PI (Vector Laboratories, Inc., Burlingame, CA). The images were taken with a NIKON TiE inverted fluorescence microscope using the elements image analysis software package (NIKON).

2.6. Western blot

Umbilical cords were collected at E14.5 and sonicated in RIPA buffer (Thermo Scientific, Rockford, IL) with Halt Proteinase inhibitor Cocktail(Thermo Scientific, Rockford, IL). An equal amount of 40 μg of protein was loaded to 8% SDS–PAGE gels and transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat milk for 1 h and incubated with the primary antibodies against Dicer (Santa Cruz, CA), β-actin (Sigma St. Louis, MO), p-AKT, AKT, pERK, or ERK (Cell Signaling, Danvers, MA).

2.7. Statistical analysis

Data are shown are the mean plus standard deviation from at least 3 different experiments. The differences were analyzed using Student's *t*-test. P values < 0.05 were considered significant.

3. Results

3.1. Conditional deletion of Dicer in VSMC leads to developmental delay and embryonic mortality

To investigate the role of Dicer in vascular smooth muscle cells, we disrupted Dicer by deleting the floxed exon 23 through crossing $Dicer^{loxp/loxp}$ mice with the SM22-cre transgenic line (Fig. 1A). Exon 23 encodes the RNase IIIb motif of Dicer (Fig. 1B). The SM22-cre transgenic mouse line has been widely used to delete target genes in VSMCs due to the restricted expression of cre recombinase in vascular smooth muscle cells of the aorta, cerebral vessels, bladder, uterus and the intestine [24–27]. Dicer cKOs were generated by intercrossing $Dicer^{loxp/+}$; SM22-cre or crossing between $Dicer^{loxp/loxp}$ and $Dicer^{loxp/+}$; SM22-cre. Heterozygous $Dicer^{loxp/+}$; SM22-cre mice were viable and did not show any noticeable abnormalities. Of 43 mice born, none was found to be homozygous for lack of Dicer, which suggested that $Dicer^{loxp/loxp}$; SM22-cre mice could have died during embryonic development. To further determine the timing of death, timed mating was set up. Embryos were dissected and genotyped at various developmental stages by PCR (Fig. 1C). To detect Dicer expression, umbilical arteries were collected at E14.5 embryos for protein extraction and Dicer was detected by western blotting. Dicer levels were significantly

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reduced in Dicer$^{loxp/loxp}$, SM22-cre compared to Dicer$^{loxp/loxp}$ mice (Fig. 1D). We found homozygous Dicer$^{loxp/loxp}$; SM22-cre embryos were all alive before E14.5 and appeared morphologically normal except a slight developmental delay compared to their wild type or heterozygous littermates. After E15.5, no surviving Dicer cKO embryos could be retrieved. Dicer homozygous embryos showed red skin in their head and trunk at E15.5 and E16.5 (Fig. 2A). The body weight of embryos between control (Dicer$^{loxp/loxp}$) and Dicer mutant showed a significant difference at the various stages of sampling between E12.5 and E16.5 (Fig. 2B). The homozygous cKO Dicer embryos were absorbed and could not be retrieved beyond E17.5.

3.2. Deletion of Dicer leads to dilation of blood vessels and vasculature defects in the yolk sac

As early as E12.5, Dicer knockout mutants already exhibited underdeveloped blood vessel walls in the yolk sac and the embryo, which were thinner than that of littermate controls. The yolk sac and embryos appeared pale and anemic before E14.5. The blood vessels disappeared after E15.5 in the yolk sacs and the embryos of Dicer cKOs. Blood pools accumulated under the skin (Fig. 2C). To further examine the pathological alterations of the blood vessels, we dissected the Dicer cKOs and littermate controls embryos at different developmental stages. Dicer cKOs showed dilated blood vessels with a media layer thinner than that of E13.5 controls. The layers of the vessel wall were irregular and some segments were abnormally thin at E14.5 in Dicer cKOs. At E15.5, the blood vessel already showed a disarrayed structure with the intima, media and adventitia layers disorganized and the vascular barrier integrity was severely impaired(Fig. 3A–C).

3.3. Dicer cKOs display extensive organ hemorrhage between E15.5 and E16.5

Although we found that blood vessels were dilated before E14.5, Dicer cKO embryos survived before E14.5. However, Dicer cKO embryos harvested between E15.5 and E16.5 displayed extensive blood accumulation under the skin indicating subcutaneous hemorrhage. To analyze this bleeding problem, we dissected E15.5 embryos and found that cerebrovascular hemorrhage was present leading to the accumulation of red blood cells in the subarachnoid space. No bleeding was found in the brain parenchyma. Similarly, hemorrhage was found in the dermis of the skin and subcutaneous layer that red blood cells were spread across all this area of the body. The vascular structures of dermis and subcutis were extremely dilated and a number of red blood cells extravasated into the dermis and subcutaneous connective tissue and caused diffuse hemorrhage. In liver, the branches of hepatic artery and portal vein as well as the sinusoids were congested with red blood cells. The liver hemorrhage in most of the embryos appeared very localized and found only in some areas. In some embryos, the hemorrhage was found diffusely in the liver and the hepatic structure was completely disrupted that red blood cells occupied the sinusoid space without the hepatic cell plates. The hemorrhages were also found in skeletal muscle (Fig. 4A).

3.4. Disruption of Dicer inhibits VSMC proliferation

Our histological results showed that arterial walls were dilated between E13.5 and E14.5 and severely impaired by E15.5. To examine whether abnormalities in the Dicer cKO arteries is due to defects in VSMC proliferation we examined proliferating cell nuclear marker (PCNA). The number of proliferating VSMCs was significantly reduced in the thoracic aorta in Dicer cKO embryos compared with littermate controls (Fig. 4B).

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3.5. Dicer cKO mutants show reduced VSMC marker gene expression

VSMC contractile phenotype is modulated by the expression of differentiation marker genes. To examine whether Dicer plays a role in VSMC differentiation, using real-time RT-PCR we examined a panel of marker genes that included αSMA, CNN1, SM22 and Myh11 in samples collected from unbiblical arteries at E14.5. The expression of SMA, SM22, CNN1 and Myh22 were all significantly down-regulated in the Dicer cKOs compared to control mice, which indicates that Dicer plays a role in the phenotypic modulation of VSMCs (Fig. S1A).

3.6. miRNA expression profiles are dysregulated in Dicer cKO mice

Dicer is a key regulator of miRNA maturation, consequently, disruption of Dicer may lead to dysregulation of miRNA expression. To test this hypothesis, we examined the expression of miRNA in umbilical arteries of E14.5 embryos from control and Dicer KO mutants. Several miRNAs species were selected based on previous reports that implicated their role in vascular biology. These miRNAs included the miR-143/145 and miR-17/92 clusters. We also selected miR-451 as a Dicer independent miRNA for control purposes. We found that the VSMC-specific miR-143 and miR-145 were significantly downregulated. Interestingly, miR-451, a Dicer independent miRNA was also significantly downregulated. Several other miRNAs such as Let-7a, miR-16, miR-21, miR-19a, 19b and miR-92b, the expressions were all significantly reduced, whereas miR-15a was not significantly altered in the cKOs compared with littermate controls. However, two miRNAs, miR-17 and miR-20a, were significantly upregulated, which was in contrast to the majority of miRNAs in VSMCs of Dicer cKO mice (Fig. S1B). Our data indicate that Dicer is a key regulator in miRNA maturation in VSMCs.

3.7. Cell survival signaling pathways are compromised in Dicer cKO vessels

We found that Dicer KO mutant displayed a developmental delay accompanied by reduced VSMC proliferation. To understand the molecular mechanism underlying this phenotype, we examined the activation of the PI3K/AKT and ERK1/2 signaling pathways, which have been shown to play a major role in VSMC proliferation previously [28–30]. Western blots performed using proteins extracted from umbilical cords of E14.5 embryos showed reduced levels of phospho-AKT and phospho-ERK in Dicer cKOs compared to the appropriate controls whereas, the expression level of total AKT and ERK were not different (Fig. S1C). These observations suggest that Dicer through the controlling miRNA maturation regulates the ERK1/2 and PI3K/AKT pathways, which play an essential role in cell growth and survival.

4. Discussion

Dicer is a key regulator in siRNA and miRNA biogenesis. We revealed the role of Dicer in VSMCs using conditional knockout approach by deleting Dicer in VSMCs. Our data clearly showed that loss of Dicer significantly inhibited VSMC proliferation and differentiation without enhancing apoptosis in vivo. The Dicer KO mutant embryos displayed a growth delay phenotype. Our finding differed from those recently reported by Albinsson and colleagues [31] who found no growth delay phenotype in their DICER KOs. Moreover, the phenotype we found is considerably more severe than that they reported. Our Dicer KO mutants died between E14.5 and E15.5, whereas the Dicer mutants died between E16.5 and E17.5 in their studies. We found hemorrhage in multiple organs, including the brain, liver, skeletal muscle and small intestine. This contrasts the hemorrhage these authors found in the liver of their KOs. In our Dicer cKO mutants all organs were smaller than in littermate controls. The difference between the KO strategy used by Albinson et al. and us is that they deleted exon 21 and exon 22 whereas, in our study, only exon 23 of Dicer was deleted. The

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exon 21/22 encodes the first motif of RNAase III whereas, exon 23 encodes the second motif known as RNase IIIb. It is not clear whether the RNase IIIa and b domains have any difference in function, but both of them are coordinately responsible for the cleavage of double stranded RNA. The same SM22-cre mouse line was used in both studies and for this reason it is unlikely to be the source of the difference between our findings. In an earlier report on Dicer KO mice [6] deletion of exons 1 and 2 has led to embryonic death between E12.5 and E14.5. In another report on Dicer KO with exon 21 ubiquitously deleted embryonic death occurred at E7.5 [5]. It is not clear whether in vascular smooth muscle cells a truncated Dicer protein was expressed following the deletion of exon 21, which could have rescued and mitigated of the phenotype. In both studies, VSMC proliferation and differentiation were found to be compromised. The AKT and ERK survival pathways were not altered in their studies, whereas both pathways were inhibited in our Dicer KO. Both AKT and ERK pathways have been shown to play a pivotal role in VSMC proliferation [28,32,33]. One of the main functions of Dicer is to cleave double stranded RNA and generate siRNA and miRNA. Dicer regulates cell proliferation, apoptosis and differentiation by controlling small RNA maturation. We detected several changes in VSMC-related miRNAs in Dicer KO mutants. Our data suggest that loss of Dicer leads to miRNA dysregulation. We found that two VSMC-specific miRNAs, miR-143 and miR-145, were downregulated in Dicer KO mutants. miR-145 has been shown to regulate VSMC plasticity and play a pivotal role in maintaining the contractile phenotype of VSMC [18–20]. Clearly, disruption of Dicer expression compromised most but not all miRNA expression we examined indicating the role of other mechanisms involved in miRNA maturation. Therefore, miRNA dysregulation might be responsible in part for the phenotype caused by loss of Dicer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by awards HL095957 and HD061420 to J. Yue from National Heart, Lung, and Blood Institute and the Eunice Kennedy Shriver National Institute of Child Health & Human Development, respectively. The authors thank Dr. Chuanhe Yang' help in analyzing real-time PCR data. We also appreciate the assistance of Jin Emerson-Cobb of the Physiology Department in editing the manuscript.

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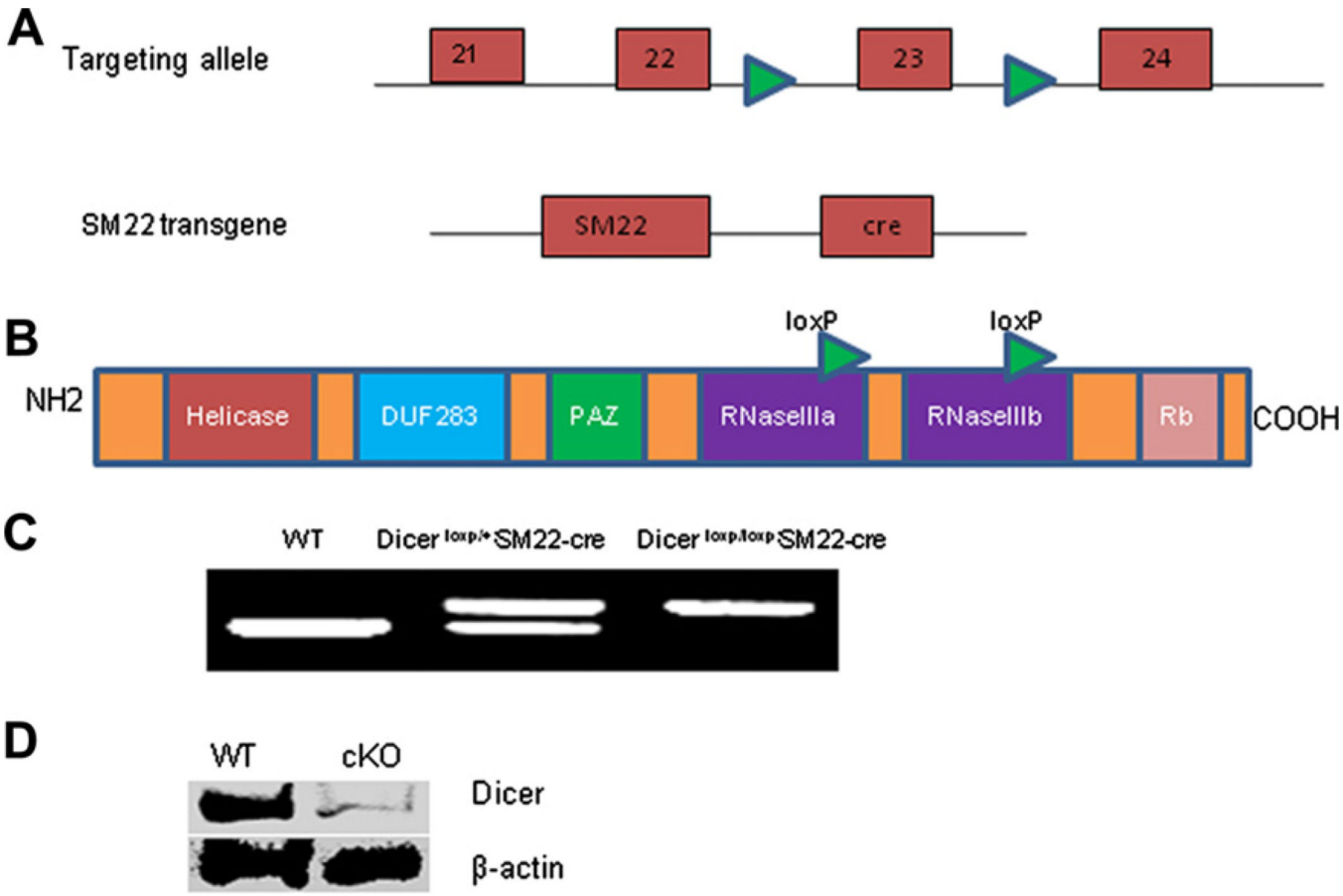

Fig. 1. Conditional inactivation of Dicer in VSMCs of mouse by gene targeting

(A) Dicer conditional allele contains loxP sites flanking exon 23. The VSMC-specific Cre transgenic mouse line is SM22-Cre, in which Cre recombinase is driven by VSMC specific promoter SM22. (B) The second RNase IIIb domain, which is encoded by exon 23, was deleted in the Dicer conditional mouse line. (C) Genotyping Dicer cKO mice by PCR showed three different genotypes: wild type, heterozygote, and homozygote. (D) The expression of Dicer in umbilical cord at E14.5 was detected by Western blot.

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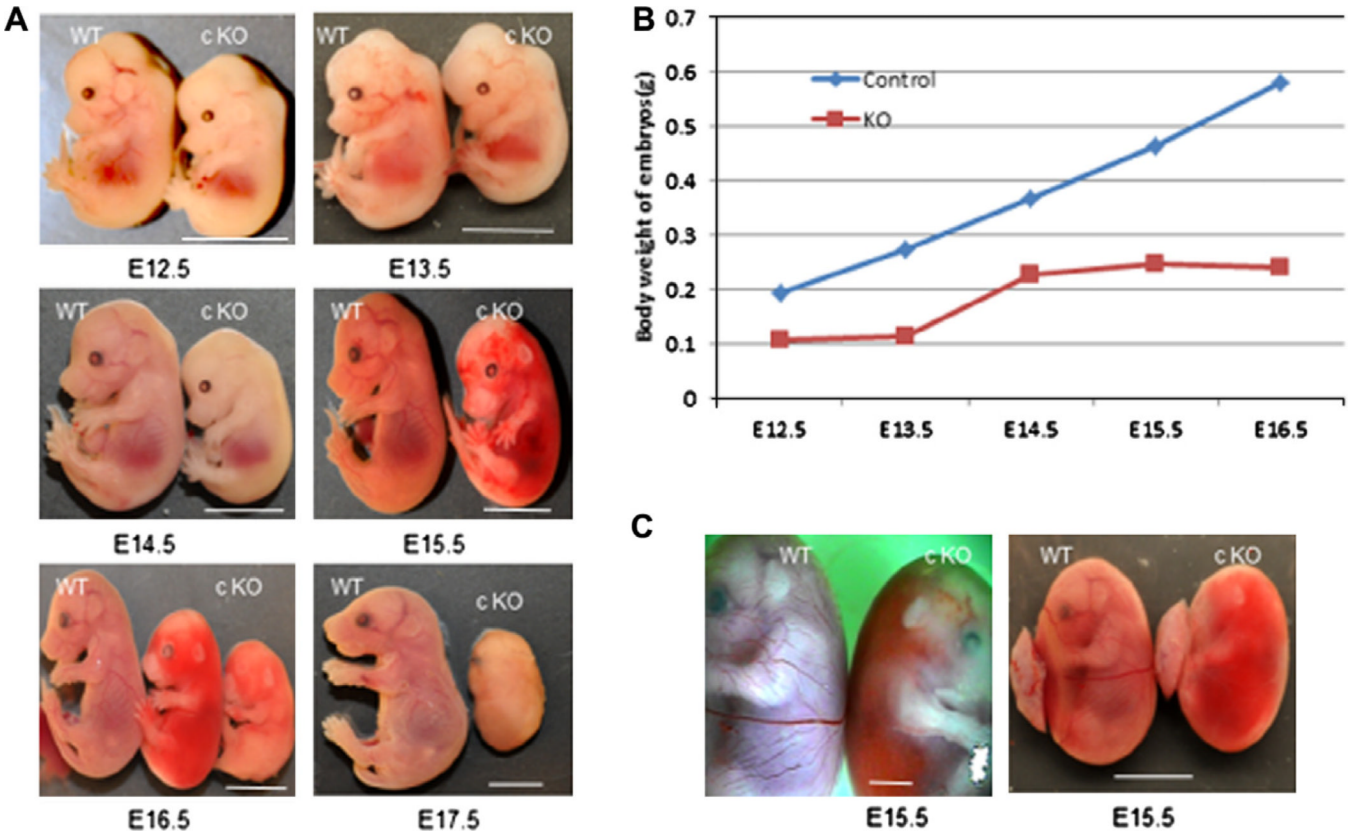

Fig. 2. Dicer$^{loxp/loxp/SM22\text{-}cre}$ mice display growth delay and embryonic mortality

(A) Morphology of embryos at different embryonic developmental stages in wild type controls and Dicer cKO mutants, which, as early as E12.5, showed a growth retardation that became more pronounced at E13.5 and E14.5 compared with littermate controls. The Dicer cKO mutants at E15.5 and E16.5 displayed extensive hemorrhage in brain and trunk, with more severe developmental delay. At E17.5 the Dicer KO mutants were already absorbed. (B) Vascular structure was not observed in yolk sacs of Dicer cKO mutants at E15.5, and blood was accumulated in the subcutis. (C) The differences in body weight were observed at different embryonic developmental stages, indicating the developmental delay in Dicer cKO mutants ($n = 5$).

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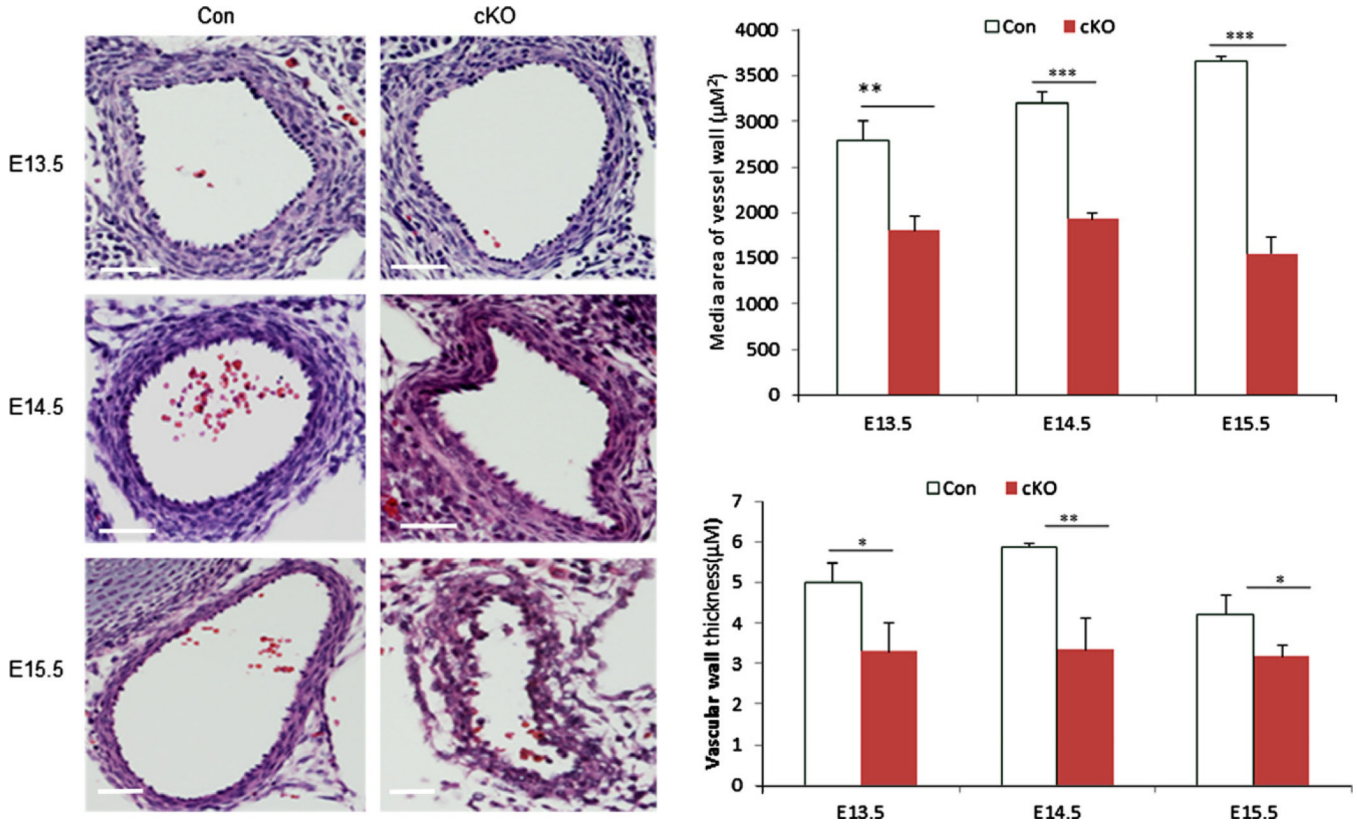

Fig. 3. Blood vessel development and vascular remodeling in Dicer cKO mutants

(A) Paraffin-embedded abdominal aorta of Dicer cKO and control embryos at E13.5, E14.5, and E15.5 were sectioned and stained, respectively, with hematoxylin and eosin. (B and C) The medial area and thickness of blood vessel walls of Dicer cKO mutants and littermate controls were measured using a Nikon microscopy program in three sections from each embryo. The medial area of the vessel and wall thickness were calculated from the inner and outer medial area or circumference of vessel walls. Error bar indicates standard deviation. Scale Bars indicate 100 μm (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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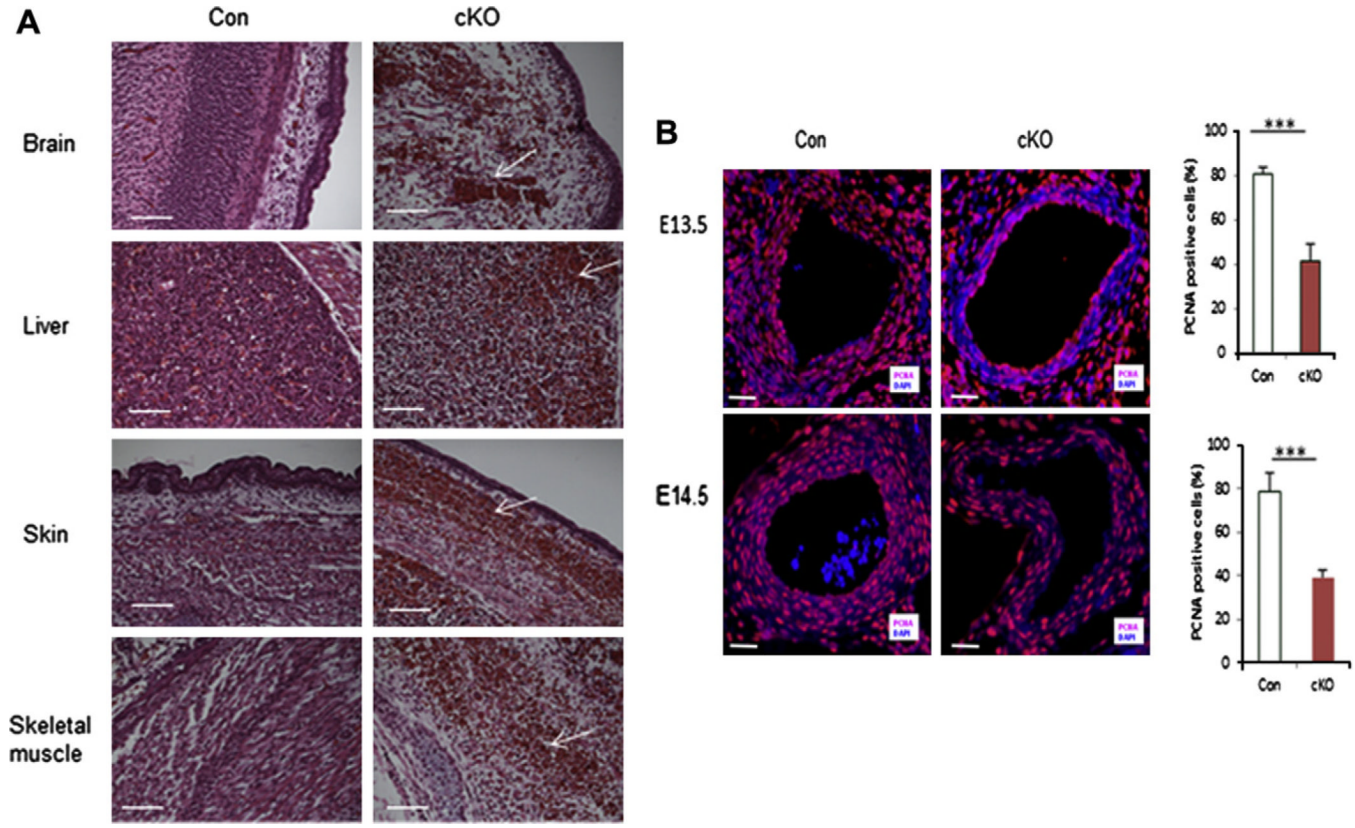

Fig. 4. Histological analysis of hemorrhage of several organs in Dicer cKO embryos

(A) Sections of paraffin-embedded embryos of Dicer cKO and littermate controls at E15.5 were stained with hematoxylin and eosin. These tissues, including brain, liver, skin, and skeletal muscle, were photographed using inverted Nikon microscopy (200× magnification). Red blood cells were accumulated in those tissues, as indicated by arrow. Scale bars indicate 100 μm. (B) Dicer cKO mutants showed reduced proliferation. Paraffin-embedded sections of abdominal aorta at E13.5 and E14.5 were immunostained with proliferating cell nuclear marker PCNA; cell nuclei were counterstained with DAPI. The proliferating cells were counted and divided by the total number of nuclei as the proliferating index. Five different embryos were analyzed. Error bar indicates standard deviation, scale bars indicate 100 μm (***$p < 0.001$).