DiGeorge Syndrome Critical Region 8 (DGCR8) Protein-mediated microRNA Biogenesis Is Essential for Vascular Smooth Muscle Cell Development in Mice*

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Background: The role of DGCR8 in VSMCs is not known.

Results: Loss of DGCR8 in VSMCs results in embryonic mortality by inhibiting cell proliferation and differentiation and promoting apoptosis.

Conclusion: DGCR8 is required for vascular development.

Significance: Elucidation of the role of DGCR8 in VSMCs will reveal the significance of DGCR8-mediated miRNA maturation in vascular diseases.

DiGeorge Critical Region 8 (DGCR8) is a double-stranded RNA-binding protein that interacts with Drosha and facilitates microRNA (miRNA) maturation. However, the role of DGCR8 in vascular smooth muscle cells (VSMCs) is not well understood. To investigate whether DGCR8 contributes to miRNA maturation in VSMCs, we generated DGCR8 conditional knockout (cKO) mice by crossing VSMC-specific Cre mice (SM22-Cre) with DGCR8loxP/loxP mice. We found that loss of DGCR8 in VSMCs resulted in extensive liver hemorrhage and embryonic mortality between embryonic days (E) 12.5 and E13.5. DGCR8 cKO embryos displayed dilated blood vessels and disarrayed vascular architecture. Blood vessels were absent in the yolk sac of DGCR8 KOs after E12.5. Disruption of DGCR8 in VSMCs reduced VSMC proliferation and promoted apoptosis in vitro and in vivo. In DGCR8 cKO embryos and knockout VSMCs, differentiation marker genes, including αSMA, SM22, and CNN1, were significantly down-regulated, and the survival pathways of ERK1/2 mitogen-activated protein kinase and the phosphatidylinositol 3-kinase/AKT were attenuated. Knockout of DGCR8 in VSMCs has led to down-regulation of the miR-17/92 and miR-143/145 clusters. We further demonstrated that the miR-17/92 cluster promotes VSMC proliferation and enhances VSMC marker gene expression, which may contribute to the defects of DGCR8 cKO mutants. Our results indicate that the DGCR8 gene is required for vascular development through the regulation of VSMC proliferation, apoptosis, and differentiation.

DiGeorge syndrome is often accompanied by developmental defects that include congenital heart disease, tetralogy of Fallot, interrupted aortic arch, ventricular septal defect, and persistent truncus arteriosus (1). DGCR8 is monoallelic deleted on the human chromosome 22q11.2 region that contributes to DiGeorge syndrome. DGCR8 is a double-stranded RNA binding protein that interacts with the RNase III enzyme Drosha and forms a microcomplex in the nucleus to process primary microRNA (pri-miRNA) into precursor miRNA. The precursor miRNA is transported into the cytoplasm, where the RNase III enzyme Dicer processes the precursor miRNA into mature miRNA through the RNA-induced silencing complex (2–4). DGCR8 contains two double-stranded RNA binding domains and one WW domain (5). The N-terminal region of DGCR8, including the WW domain, is not required for pri-miRNA processing but plays a role in nuclear localization (5). The C terminus of DGCR8, including two tandem RNA binding domains, binds pri-miRNA and Drosha (5). DGCR8 forms a self-regulatory feedback loop with Drosha, thus controlling the conversion of pri-miRNA into precursor miRNA (3). Although Dicer plays a role in the processing of miRNA and other small RNAs such as siRNA or shRNA, DGCR8 specifically targets miRNA (6–8). To investigate the role of DGCR8 and miRNAs in VSMCs, we generated DGCR8 conditional knockout (cKO) mice in which the DGCR8 gene was deleted in VSMCs using VSMC-specific Cre mice, SM22-cre. Loss of DGCR8 in VSMCs caused marked developmental delay, extensive hemorrhage in the liver, and vascular wall hypoplasia. DGCR8 cKO embryos died between E12.5 and E13.5. Our findings indicate that DGCR8 is required for vascular development.
EXPERIMENTAL PROCEDURES

Primary VSMCs were isolated from 2-month-old DGCR8loxp/loxp mice and immortalized using SV40 large T antigen and then transduced with Ade-cre and Ade-con adenoviruses to generate DGCR8 knockout and control VSMCs, respectively. Primary VSMCs were characterized by immunostaining with αSMA antibody. Mouse VSMCs were obtained from the ATCC and cultured in DMEM supplemented with 10% FBS (Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). HEK293 FT cells were cultured in DMEM with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% glutamine, 1% nonessential amino acid, and 1 μg/ml geneticin.

Generation of VSMC DGCR8 cKO Mice—All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996) and approved by the University of Tennessee Animal Care and Use Committee. Mice were anesthetized by intraperitoneal injection of the Avertin (0.75 mg/g body weight) and euthanized by cervical dislocation prior to tissue collection. VSMC DGCR8 cKO mice were generated by crossing SM22-Cre mice (The Jackson Laboratory, stock no. 004746) with DGCR8loxp/loxp mice (obtained from Dr. Elaine Fuchs, Rockefeller University). DGCR8 loxp/loxp /SM22-Cre mice were obtained by intercrossing DGCR8loxp/loxp mice with DGCR8loxp/loxp/SM22-Cre mice. DGCR8loxp/loxp littermates were used as controls for all experiments. All mice used in this study were of the B6/129 SvJ genetic background.

Genotyping DGCR8 KO Mice and Embryos—Mouse tail clipplings or yolk sacs were collected into 200 μl of 50 mM NaOH and heated at 95 °C for 50 min, followed by addition of 20 μl of 1 M Tris-HCl and centrifugation for 5 min. 2 μl of the supernatant were used for PCR. The Cre-specific primers we used have been described previously (9). The DGCR8-specific primers are described in supplemental Table 1. The 244-bp WT and 370 bp for the DGCR8 cKO PCR products were separated by agarose gel electrophoresis.

Histological Analysis—To analyze the phenotype of DGCR8 KO embryos, time-mated pregnant females were sacrificed, and the embryos were collected. The harvested embryos were weighed and fixed overnight in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with H&E.

miRNA Array—Total RNA was isolated from umbilical arteries using TRIzol reagent (Invitrogen), and RNA was further purified with the RNeasy MinElute cleanup kit (Qiagen, Valencia, CA). The quality and integrity of the total RNA was determined with an Agilent bioanalyzer. The miRNA microarray profiling was performed using Affymetrix GeneChip miRNA arrays (Santa Clara, CA). 1 μg of total RNA was labeled by poly(A) polymerase addition using the Genisphere FlashTag HSR kit following the instructions of the manufacturer instructions (Genisphere, Hatfield, PA). Labeled RNA was hybridized to the Affymetrix miRNA array 1.0. Chips were washed, stained in a Fluidic Station 450, and image scanning was performed with an Affymetrix scanner 3000 7G.

Lentiviral Vector Production—Lentiviral vectors were constructed by inserting mouse miR-17/92 or miR-145 genes using standard molecular cloning methods. The primer sequences used for cloning are listed in supplemental Table 1. The lentiviruses were produced as described previously (10).

Collagen Gel Contraction Assay—The collagen gel contraction assay was performed as described previously (11). Briefly, control and DGCR8 knockout VSMCs were washed, trypsinized, and collected by centrifugation for 3 min at 1500 x g. The rat tail collagen solution (BD Biosciences) was
diluted to a final concentration of 2.5 mg/ml using DMEM (pH 7.3–7.4), and mixed with the harvested cells (2.5/10^5 cells/ml). Then, 250 μl of gel/cell suspension was added to each well of a 48-well plate and left at 37 °C for 30 min to facilitate collagen polymerization. A pipette tip was used to loosen the adhering gel from the well plate so that it would not impede contraction. After 48 h of incubation (37 °C in a 5% CO2 incubator), the contraction index was calculated using the following formula: contraction index = (well area − gel area)/well area and normalized to controls.

Detection of miRNA and mRNA Expression Using Real-time PCR—Total RNA was extracted from pooled umbilical cords of DGCR8 cKO mice or littermate controls at E12.5. Three samples were collected from six DGCR8 cKO mice or six littermate controls. Poly(A) tailing real-time PCR was performed as described previously (9). The pri-miR-21 primer is listed in supplemental Table 1. The SYBR Green-based real-time PCR was performed on a LightCycler 4800 real-time PCR instrument (Roche). A melting curve analysis was performed to examine the PCR product specificity. The relative expression was normalized to U6 small nuclear RNA by the 2^-ΔΔCt method and expressed as mean ± S.D.

Immunofluorescence—Deparaffinized 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 (pH 6.0). 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. To detect the VSMC proliferation and differentiation, sections were incubated with primary antibody to proliferating cell nuclear antigen (PCNA) or α-smooth muscle actin (αSMA) at 4 °C overnight, respectively. After three rinses for 5 min with 0.05% Tween 20 in PBS, the sections were incubated with Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-rabbit or mouse secondary antibody (Invitrogen, 1:200 in Tween 20 in PBS) for 1 h at room temperature. After three washes, the sections were mounted with Vectashield medium containing DAPI or propidium iodide (Vector Laboratories, Inc., Burlingame, CA). To detect apoptosis in VSMCs in DGCR8 E12.5 cKO embryos, sections of the thoracic aorta were subjected to TUNEL staining (Roche). To detect apoptosis in DGCR8 KO VSMCs, annexin-V staining was performed (BD Biosciences) and analyzed by flow cytometry.

Western Blotting—Umbilical cords or VSMCs were collected in radioimmune precipitation assay buffer (Thermo Scientific, Rockford, IL) containing 1% Halt protease inhibitor mixture (Thermo Scientific). An equal amount of protein (40 μg/lane) was loaded on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. The membrane was blocked with 5% nonfat milk for 1 h and incubated with primary antibodies against DGCR8, SM22 (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin, CNN, αSMA, and GAPDH (Sigma), phospho(p)-
ERK1/2, phospho(p)-AKT, ERK1/2, AKT (Cell Signaling Technology, Danvers, MA), or PCNA, Vector Laboratories, Inc., Burlingame, CA).

**Statistical Analysis**—Data shown are the mean ± S.D. from at least three different experiments. The differences were analyzed using Student’s t test. p values < 0.05 were considered significant.

**RESULTS**

**Conditional Deletion of DGCR8 in VSMCs to Embryonic Mortality**—The SM22-Cre transgenic mouse line has been widely used to delete target genes in VSMCs because of the restricted expression of Cre recombinase in VSMCs of the aorta, cerebral vessels, bladder, intestine, and uterus (12–15).

To determine the role of DGCR8 in VSMCs, we generated DGCR8 cKO mice by crossing DGCR8loxP/loxP mice with SM22-Cre transgenic mice. Exon 3 of DGCR8 was floxed in the gene targeting vector. Hence, the cross-breeding results in the deletion of exon 3 of the DGCR8 gene, causing a frameshift that leads to disruption of DGCR8 expression (Fig. 1A) (6). DGCR8 homozygotes were obtained by crossing DGCR8loxP/loxP mice with DGCR8loxP/+ /SM22-Cre mice. The offspring were genotyped by PCR (Fig. 1B). Of 78 live pups genotyped, no homozygote DGCR8 mice were found, suggesting that DGCR8 homozygotes did not survive embryonic development (Fig. 1C).

Deletion of DGCR8 in VSMCs leads to blood vessel dilation—DGCR8 cKO embryos contained hypoplastic blood vessel walls that were markedly thinner in their tunica media than those in controls before E12.5. The area and thickness of the tunica media of the thoracic aorta of DGCR8 cKOs was significantly reduced compared with controls (Fig. 3A).

**Deletion of DGCR8 in VSMCs Leads to Blood Vessel Dilation**—DGCR8 cKO mice were viable and did not show any noticeable abnormalities. To further define the time of embryonic death, timed matings were performed, and vaginal mucus plugs were checked daily. Embryos were dissected and genotyped at various developmental stages by PCR. DGCR8 expression was detected using Western blot analyses in extracts from the pooled umbilical arteries. In the homozygous DGCR8 cKO embryos, a significant reduction in DGCR8 expression was observed (Fig. 1D). DGCR8 homozygotes were alive prior to E11.5 and appeared morphologically normal except for a slight developmental delay compared with WT or heterozygous embryos (Fig. 2A). The majority of the DGCR8 homozygous cKO embryos died between E12.5 and E13.5. In addition, the heart and liver were larger, whereas the lung and stomach were smaller in DGCR8 cKOs compared with control embryos (supplemental Fig. 1A). The left and right ventricles of the heart were enlarged, and the ventricular wall was thinner in DGCR8 cKO mice (supplemental Fig. 1, B–D), which are similar to the findings described in mice with cardiac-specific knockout of DGCR8 (16). These observations are similar to what is clinically observed in end-stage heart failure, suggesting that the cKO embryos developed a cardiomyopathy-like condition. No vasculature was found in the yolk sacs of DGCR8 cKO mice after E12.5 (Fig. 2B). No viable DGCR8 cKO embryos were found beyond E14.5 (Fig. 2C).

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Significantly reduced compared with controls, which indicated that a dysregulation in the development of VSMCs led to vascular wall hypoplasia (Fig. 3A).

DGCR8 cKOs Display Extensive Liver Hemorrhage—Extensive hemorrhage was found in the liver of cKO embryos harvested between E12.5 and E13.5. The branches of the hepatic artery, the portal vein, and the sinusoids were filled with congested red blood cells. The hemorrhage in the liver of DGCR8 cKOs was diffuse, and the hepatic structure was completely disarrayed, with red blood cells occupying the sinusoid space, leading to the disruption of the hepatic cell plates (Fig. 3B and supplemental Fig. 1B).

Disruption of DGCR8 in VSMCs Reduced Cell Proliferation and Promotes Apoptosis—To determine whether abnormalities in DGCR8 cKO arteries were due to a defect in VSMC proliferation or apoptosis, we stained sections of thoracic aorta harvested between E12.5 and E13.5 with anti-PCNA antibody, which showed that VSMC proliferation was reduced significantly in DGCR8 cKO embryos compared with controls (Fig. 4A). Western blot analyses of PCNA expression in umbilical arteries of E12.5 DGCR8 cKO embryos also showed significant reduction compared with that of control mice (Fig. 4B). In addition, we examined cell proliferation in DGCR8 KO VSMC cells, which we generated by transducing Ade-Cre and Ade-control adenoviruses, and found that the VSMC proliferation was significantly reduced in DGCR8 KO as compared with control VSMCs. The expression of PCNA was also examined by Western blot analysis, which further confirmed a significant reduction in DGCR8 KO compared with control VSMCs (Fig. 4C).

To examine whether loss of DGCR8 led to apoptosis in VSMCs of DGCR8 cKO embryos, sections from the thoracic aorta of E12.5 embryos were examined by TUNEL staining. Apoptosis in VSMCs was significantly increased in the medial area of thoracic aorta of DGCR8 cKO embryos as compared with controls (Fig. 5A). We also examined apoptosis in DGCR8 KO VSMCs transduced with Ade-Cre and Ade-Con adenoviruses by annexin V staining and flow cytometry. DGCR8 KO VSMCs showed a significant increase in apoptosis. Similarly, a significant increase of cleaved caspase 3 was found in DGCR8 KO compared with control cells (Fig. 5B). Taken together, these data indicate that the loss of DGCR8 in VSMCs reduced proliferation and enhanced apoptosis in vitro and in vivo.

DGCR8 cKO Mice Show Reduced VSMC Marker Gene Expression—The contractile phenotype of VSMC is regulated by the expression of differentiation marker genes, including

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**FIGURE 4.** DGCR8 cKO mice showed reduced VSMC proliferation. A, paraffin-embedded sections of thoracic aorta at E12.5 and 13.5 were immunostained with the proliferating cell marker PCNA (indicated by the arrow), and cell nuclei were counterstained with DAPI. The proliferating cells were counted and divided by the total number of nuclei as the proliferating index. Four different embryos were analyzed. Error bar represents mean ± S.D. Scale bars = 100 μm. ***, p < 0.01. Con, control. B, VSMC proliferation rate in the umbilical arteries was examined using Western blot analysis (n = 6). C, VSMC proliferation rates at different time points were examined by cell counts in DGCR8 cKO and control VSMCs, which were established by transducing Ade-Cre and Ade-Con viruses, respectively. The proliferative marker gene PCNA was detected by Western blot analysis. The band intensity was normalized to B-actin, and the ratios were used for the analysis of significant difference. *, p < 0.05; **, p < 0.01.
aSMA, SM22, and CNN1. To determine whether DGCR8 plays a role in VSMC differentiation, we stained sections of the thoracic aorta of DGCR8 cKO embryos at E12.5 and DGCR8 knockout VSMCs using aSMA antibody. Our results indicated that loss of DGCR8 led to significant down-regulation of aSMA in DGCR8 cKOs (Fig. 6A) or KO VSMCs (supplemental Fig. 2A) compared with controls. We also detected significant down-regulation in the expression of the VSMC differentiation marker genes aSMA, SM22, and CNN1 in umbilical arteries of E12.5 embryos using Western blot analyses (Fig. 6B). In addition, their expressions were also examined in DGCR8 KO VSMCs. Similarly, we found that loss of DGCR8 significantly reduced the expression of aSMA, SM22, and CNN1 in DGCR8 KO VSMCs compared with controls (Fig. 6C). To examine whether loss of DGCR8 leads to the reduced contraction of VSMC, we performed a collagen gel contraction assay, which is widely used to determine VSMC contractile ability (11, 17–20). We found that the contractile capacity in DGCR8 KO cells was slightly impaired compared with controls (supplemental Fig. 2B). In this regard, we also found that loss of Dicer reduced the contractile capacity of VSMCs (data not shown).

miRNA Expression Profile Is Dysregulated in DGCR8 cKO Mice—Because DGCR8 interacts with Drosha, thus controlling miRNA biogenesis, disruption of DGCR8 leads to dysregulation of miRNA expression. To determine the impact of DGCR8 KO on miRNA expression in VSMCs, we performed a miRNA array by extracting RNA from umbilical arteries pooled from E13.5 embryos of control and DGCR8 cKO mice. Of 670 mouse miRNA probes in the array, after filtering out miRNAs with low signals, 218 miRNAs had detectable signals. 171 showed significant down-regulation, 16 were not significantly altered, and 31 were up-regulated specifically in DGCR8 cKO umbilical arteries. The array results indicated that the loss of DGCR8 leads to a global down-regulation of miRNA expression. miR-143/145 and miR-17/92 clusters were the most significantly down-regulated in the umbilical arteries of DGCR8 cKO embryos (supplemental Table 2). The miR-143/145 cluster modulates the VSMC phenotypic switch between proliferation and differentiation (21–24). We found that in the umbilical arteries of DGCR8 cKO embryos, the expression of miR-143 and 145 decreased ~10- and 7-fold, respectively (supplemental Fig. 3A). To validate the role of miR-145, we transduced WT VSMCs using a lentiviral miR-145 expression vector and immunostained the cells for aSMA expression. Overexpression of miR-145 resulted in a significant increase in aSMA gene expression (supplemental Fig. 3B). The role of miR-17/92 cluster had not been investigated previously in VSMCs. The miR-17/92 cluster contains six miRNAs (miR-17, miR-18a, miR-19a, miR-19b,
miR-20a, and miR-92a), which were found to be modulated by interleukin 6. These miRNAs have also been linked to the pathogenesis of pulmonary hypertension and coronary artery disease (25, 26). We found that loss of DGCR8 in VSMCs led to significant down-regulation of miRNAs in this cluster, so that miR-17, 18a, 19a, 19b, 20a, and 92a were reduced 7-, 7-, 5-, 6-, 3-, and 4-fold, respectively (Fig. 7A). To examine whether the down-regulation of miR-17/92 cluster contributes to the phenotypic defects in DGCR8 cKO mice, we overexpressed the miR-17/92 cluster using lentiviral vector in WT VSMC and stained these cells for the PCNA marker. We found that overexpression of the miR-17/92 cluster significantly increased VSMC proliferation (Fig. 7B). The expression of PCNA was also significantly up-regulated, as shown by Western blotting (Fig. 7C).

We further examined two survival pathways, ERK1/2 and AKT, in miR-17/92 transduced VSMCs by Western blotting and found that both pathways were activated as a result of overexpression of the miR-17/92 cluster (Fig. 7C). We also examined the rate of cell growth and found that the cell numbers were significantly increased in miR-17/92-transduced VSMCs compared with miR-con transduced cells (Fig. 7D). In addition, we stimulated serum-deprived VSMCs stably transduced with miR-17/92 with PDGF or fibroblast growth factor 2 (FGF2). Similarly, we found that overexpression of miR-17/92 enhanced PDGF- or FGF2-induced cell growth (supplemental Fig. 4, A and B). To examine whether the miR-17/92 cluster plays a role in VSMC differentiation, we determined the expression of SMA, SM22, and CNN1 genes in VSMC transduced with miR-17/92 or control vector. We found that SMA and SM22 were significantly up-regulated, whereas CNN1 was not significantly altered following the overexpression of miR-17/92 compared with lentiviral control vector-transduced cells (Fig. 7E). Our results indicate that the miR-17/92 cluster promotes VSMC proliferation and differentiation, which may contribute to the phenotypic and functional defects we observed in the DGCR8 cKO mutants.

Disruption of DGCR8 in VSMCs Attenuates Activations of ERK1/2 and PI3K/AKT—Previous studies have shown a cross-talk between the miRNA biogenesis and the TGFβ cellular sig-

FIGURE 6. Expressions of VSMC differentiation marker genes in DGCR8 cKO mice. A, sections of thoracic aorta at E12.5 were stained with αSMA antibody. Data represent mean ± S.D. n = 6. B and C, the expressions of αSMA, SM22, CNN1, DGCR8, and GAPDH in umbilical arteries (B) and KO VSMCs (C) were detected by Western blot analysis, respectively. Protein bands were quantified by densitometry for analysis of significant differences. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
naling pathways in VSMCs (27). The miRNA biogenesis pathway has been shown to be regulated by ERK1/2 (28). To determine whether disruption of the DGCR8-mediated miRNA biogenesis pathway affects cellular survival pathways in DGCR8 cKO mice, we examined the levels of activated ERK1/2 and AKT in umbilical arteries of DGCR8 KO in E12.5 embryos by Western blot analysis. We found that both pathways were attenuated in umbilical arteries of DGCR8 cKO as compared with control embryos (Fig. 8A). We also examined the two pathways in knockout VSMCs generated by transducing AdeCre and Ade-Con adenoviruses. Similarly, phosphorylation of ERK1/2 and AKT was attenuated in DGCR8 KO VSMCs compared with controls (Fig. 8B). Our data demonstrate that loss of DGCR8 resulted in the significant down-regulation of the miR-17/92 and miR-143/145 clusters, which subsequently reduced VSMC proliferation and differentiation marker gene expression (Fig. 8C).

DISCUSSION

Loss of DGCR8 in VSMCs Results in Multiple Phenotypic Defects—Loss of DGCR8 in VSMCs resulted in multiple embryonic defects, including severe liver hemorrhage, vascular wall dilation, and developmental delay. The DGCR8 cKO mutants died between E12.5 and E13.5. We reported previously that loss of Dicer in VSMCs has led to a similar phenotype but that Dicer cKO mutant mice died at a later stage between E14.5 and E15.5 (9). Similar to our findings, deletion of exons 21 and 22 of Dicer in VSMCs led to liver hemorrhage and embryonic death at E17.5, although no obvious growth delay was observed in this study (29). Both DGCR8 and Dicer play a role in miRNA biogenesis, but Dicer also functions in the processing of other small RNAs, including siRNA and shRNA. A recent report (8) showed that Dicer cKO mice showed a more severe phenotype than that of DGCR8 when both genes were deleted individually in post mitotic neurons. This difference
was attributed to the fact that loss of Dicer in neurons resulted in the Dicer-dependent loss of mirtrons and H/ACA small non-coding RNA (snoRNA)-derived small RNAs, which are DGCR8 independent (8). Therefore, the role of Dicer and DGCR8 appears to be highly cell- or tissue type-specific. In VSMCs, it appears that DGCR8 plays a more important role than Dicer because DGCR8 cKO mice died 2 days earlier than Dicer cKO mice. It is possible that miRNAs might play a more important role than other small RNAs in VSMC function. For this reason, the biological role of siRNAs or other small RNAs responsible for the phenotypic difference between Dicer and DGCR8 VSMC-restricted KO mice remains to be elucidated in future studies. We found that loss of DGCR8 in VSMCs inhibits cell proliferation and promotes apoptosis. In contrast, loss of Dicer in VSMCs only inhibits cell proliferation but plays no detectable role in apoptosis (data not shown). These findings suggest a functional difference between these genes in VSMC and mouse development because DGCR8 cKO mice die earlier than Dicer cKO mice. It is possible that miRNAs might play a more important role than other small RNAs in VSMC function. For this reason, the biological role of siRNAs or other small RNAs responsible for the phenotypic difference between Dicer and DGCR8 VSMC-restricted KO mice remains to be elucidated in future studies. We found that loss of DGCR8 in VSMCs inhibits cell proliferation and promotes apoptosis. In contrast, loss of Dicer in VSMCs only inhibits cell proliferation but plays no detectable role in apoptosis (data not shown). These findings suggest a functional difference between these genes in VSMC and mouse development because DGCR8 cKO mice die earlier than Dicer cKO mice. It is possible that miRNAs might play a more important role than other small RNAs in VSMC function. For this reason, the biological role of siRNAs or other small RNAs responsible for the phenotypic difference between Dicer and DGCR8 VSMC-restricted KO mice remains to be elucidated in future studies.

**VSMC-linked miRNAs Are the Most Affected by Disruption of DGCR8**—We found that the miR-143/145 and miR-17/92 clusters were among the most down-regulated miRNAs in DGCR8 cKO mice. In a previous study, Dicer was deleted in VSMCs by crossing Myh11-Cre with Dicer loxp/loxp mice (31). Similarly to our findings, the majority of miRNAs were down-regulated in VSMCs or the small intestine of Dicer KO mice, including the miR-143/145 and miR-17/92 clusters (31). However, some miRNAs were up-regulated in VSMC-specific DGCR8 or Dicer cKO mice. In our previous study, we found that miR-17 and miR-20a were up-regulated in VSMC specific Dicer cKO mice, although they were down-regulated in DGCR8 cKO mice (9). The maturation of up-regulated miRNAs may be independent of the Dicer process. For example, Dicer is not essential for miR-451 maturation but requires Ago2 (32). Therefore, DGCR8 or Dicer may regulate the maturation of some specific miRNAs in a cell type- or tissue-specific manner. The up-regulated miRNAs we observed in DGCR8 cKO mice are distinct from those found in Dicer cKO mice (31). Moreover, future studies will provide insight on the role in VSMC proliferation and differentiation of individual miRNAs from the miR-17/92 cluster.

The miR-143/145 cluster has been shown to regulate VSMC plasticity and maintain the contractile phenotype of VSMCs (22–24). Down-regulation of the miR-143/145 cluster leads to reduced VSMC differentiation marker gene expression, thus weakening the contractile capacity of VSMCs. The function of the miR-17/92 cluster in VSMCs has not been investigated before. Our data showed that miR-17/92 cluster promoted VSMC proliferation and differentiation. This cluster has been shown to promote cell proliferation in vivo using transgenic and KO mice (33, 34). Other miRNAs are also involved in the phenotypic defects of DGCR8 KO mutants. For example, we found that miR-221/222 cluster and miR-21 were also signifi-
cantly down-regulated in DGCR8 cKO mice, although they are not the most down-regulated miRNAs (supplemental Fig. 3, C and D). It is known that the miR-221/222 cluster and miR-21 promote VSMC proliferation (35). We have recently found that in transgenic rats overexpressing miR-21, VSMC proliferation and differentiation were enhanced, although the majority of miRNAs were down-regulated severalfold but not depleted completely in DGCR8 cKO mice, which suggests that miRNA production is a complex process regulated by a variety of molecular mechanisms, including epigenetic regulation, miRNA editing, microprocessor self-regulation, and RNA binding proteins (36). In addition, other mechanisms may also be involved in miRNA maturation, including miRNA turnover and stability (31, 37).

**Loss of DGCR8 Attenuates the Cell Survival Pathways**—DGCR8 participates in the canonical miRNA biogenesis pathway, whereas Dicer plays an additional role beyond miRNA biogenesis. Therefore, the DGCR8 cKO may represent a better model than the Dicer cKO for addressing miRNA function during vascular development. Further analyses of DGCR8 cKO mice will not only reveal the role of DGCR8 but also shed light on the global function of miRNAs in VSMCs. We showed that disruption of DGCR8 in VSMCs leads to attenuation of the ERK1/2 and AKT cellular survival pathways, suggesting that the miRNA biogenesis pathway cross-talks with the cellular signaling pathways. We found that the miR-17/92 cluster activates ERK1/2 and AKT in VSMCs, which indicates that loss of the miR-17/92 cluster might contribute to the attenuation of these pathways in our DGCR8 cKO mice. Further investigation of the mechanistic link between miRNA and cellular signaling pathways will help us understand the miRNA and gene regulatory network and thus pave the way to new approaches for the treatment of cardiovascular disease.

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