SIRT1 Deletion Impairs Retinal Endothelial Cell Migration Through Downregulation of VEGF-A/VEGFR-2 and MMP14

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PURPOSE. Silent information regulator protein 1 (SIRT1) is a nicotinamide adenine dinucleotide-dependent deacetylase that is abundantly expressed in vascular endothelial cells (VECs), and it has an essential role in angiogenesis. However, its contribution to retinal vascular development remains unclear. Here we characterize its involvement in regulating this process under both physiological and pathologic conditions.

METHODS. Endothelium-specific Sirt1 knockout mice were established using the Cre-lox system. VECs were isolated using magnetic beads and identified by immunostaining. Retinal whole-mount staining analyzed the retinal vascular patterns. SIRT1 was knocked down or overexpressed in human retinal microvascular endothelial cells (HRMECs) using small interfering RNA (siRNA) or lentivirus infection, respectively. Scratch assay, Transwell, and Matrigel angiogenesis assay evaluated cell migration and tube formation, respectively. Quantitative RT-PCR analyzed genes regulating VEC migration. Western blotting determined protein expression. Coimmunoprecipitation detected the interaction of hypoxia-inducible factor 1α (HIF-1α) and SIRT1 as well as acetylation status of HIF-1α.

RESULTS. Specific deletion of Sirt1 in VECs dramatically delayed retinal vessel expansion and reduced vessel density. In the oxygen-induced retinopathy (OIR) mouse model, Sirt1 ablation markedly suppressed retinal revascularization and consequently increased retinal avascularity. SIRT1 downregulation in HRMECs inhibited cell migration and tube formation, while overexpression of SIRT1 had the opposite effects. Vascular endothelial growth factor-A (VEGF-A)/VEGF receptor-2 (VEGFR-2), and matrix metalloproteinases 14 (MMP14) expression significantly declined in Sirt1-null VECs, as well as SIRT1 siRNA-transfected HRMECs. SIRT1 downregulation upregulated the HIF-1α acetylation status. Conversely, SIRT1 overexpression decreased this response.

CONCLUSIONS. SIRT1 contributes to both physiological and pathologic angiogenesis through promoting retinal VEC migration. Its underlying molecular mechanism involves SIRT1-mediated deacetylation of HIF-1α and subsequent upregulation of VEGF-A/VEGFR-2 and MMP14 expression.

Keywords: SIRT1, retinal endothelial cell, migration, VEGF-A, MMP14
SIRT1 Regulates Retinal Angiogenesis

EC migration and invasion, and formation of capillary tubes, as well as maintenance and homeostasis of the vascular bed.15

Previous studies have shown that SIRT1 plays an essential role in vasculature of multiple organs. Sirt1 deletion in endothelium impairs angiogenesis in ischemia hindlimb and kidney.16,17 Sirt1 can regulate ET-1 and TGF-β1 expression levels in mouse kidney and retina, whereas decline in its function aggravates renal and retinal injury in diabetes.18 In the cerebral vasculature, Sirt1 has an essential role in mediating endothelium-dependent relaxation.19 SIRT1 also acts as an intrinsic negative modulator of Notch signaling in ECs, and its activity depletion promoted a nonsprouting, stalk-cell-like phenotype.20

Although SIRT1 plays a critical role in the regulation of angiogenesis, its precise role in retinal vascular development and recovery in the oxygen-induced retinopathy (OIR) mouse model. Such control is mediated through modulating the hypoxia-inducible factor 1α (HIF-1α) acetylation status. Increased activity of HIF-1α induces increases in VEGF-A/VEGFR-2 and MMP14, expression which in turn stimulates EC migration. Our study indicates that SIRT1 may be a potential drug target to treat aberrant retinal angiogenesis.

**Materials and Methods**

**Endothelial Cell–Specific Sirt1 Conditional Knockout Mice**

Sirt1flox/flox mice (B6;129-Sirt1tm1Yiu/J, containing a loxp-flanked neomycin cassette upstream and downstream of exon 4 of the targeted gene) and Kdr-Cre transgenic mice (Kdrtm1(cre)Sato/J, expressing Cre-recombimase in VECs) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The endothelial Sirt1-deleted mouse model was created by cross-breeding Sirt1flox/flox and Kdr-Cre mice. The resulting Sirt1+/- mice were mated with Sirt1flox/flox mice to obtain EC-specific Sirt1 conditional knockout mice (Sirt1 cKO mice; Sirt1+/-) and the corresponding Sirt1flox/flox mice (Sirt1+/-) as the knockout control. Genotyping was performed by PCR analysis of tail DNA. The primer sequences used to detect Kdr-Cre transgene were as follows: Kdr-common, 5'-CAG GAC TGA AAG CCC AGA CT-3'; Kdr-WT, 5'-AAG TCA CAG AGG CGG TAT GC-3'; Kdr-cre, 5'-CCG TTA TTC AAC TTG CAC CA-3'. Primer sequences used for genotyping floxed Sirt1 allele were as follows: forward, 5'-GGT GTA CTT AGG TCT TGT CTG-3'; reverse, 5'-GGT GCC TTG TAA GTG TTC CC-3'. The PCR products were analyzed on 1% agarose gels.

All animal treatments were performed in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with approval of the Wenzhou Medical University Animal Care and Use Committee.

**Mouse Model of OIR**

OIR was achieved in the Sirt1 cKO mouse pups and the control mouse pups according to the protocol previously reported.21 Briefly, at postnatal day 7 (P7), mouse pups along with their mother were placed in an airtight incubator and exposed to an atmosphere of 75% ± 0.5% oxygen for 5 days. Incubator temperature was maintained at 23 ± 2°C and oxygen was continuously monitored with an oxygen controller (Biospherix Ltd, Lacona, NY, USA). At P12, the mice were removed from the incubator and returned to room air. Pups were killed for retinal whole-mount staining as described below.

**Retinal Whole-Mount Staining**

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and then killed via cervical dislocation. Eyes were enucleated and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 minutes at room temperature (RT). Retinas were dissected under a stereo microscope (SZM745; OPLENIC, Beijing, China), and then postfixed in ice-cold methanol for 30 minutes. After 1 hour of blocking in PBST (PBS plus 3% Triton X-100; Sigma-Aldrich Corp., St. Louis, MO, USA) containing 5% goat serum, the retinas were incubated in Isolectin GS-IB4 (Alexa Fluor 594 Conjugate; Life Technologies, Carlsbad, CA, USA) at 4°C overnight. After four 1-hour washes in PBST, retinas were flat mounted on microscope slides using SlowFade antifade reagent (Life Technologies). Mounted retinas were imaged at ×5 magnification using a Zeiss Imager Z1 microscope (Zeiss, Jena, Germany). Avascular and neovascularized areas of the retinas were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

**Isolation of Mouse Vascular Endothelial Cells**

Mouse vascular endothelial cells (mVECs) were isolated and purified as previously described.22 For mVEC isolation from brain, 6- to 8-day-old Sirt1 cKO mouse pups and the control Sirt1+/- mice from one litter were anesthetized and killed via cervical dislocation. Brains were dissected, finely minced, and incubated in 1 mg/mL warm collagenase/dispase (Roche, Mannheim, Germany) for 45 minutes at 37°C on a rotator. The digested homogenate was passed through a 40-μm syringe Filcon filter (BD Biosciences, San Jose, CA, USA). It was neutralized with 20% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM), centrifuged at 400 g for 10 minutes. The cell pellet was recovered and then incubated with anti-CD31 antibody-conjugated magnetic beads (Sheep anti rat IgG Dynabeads; Life Technologies) at RT. After five washes in wash buffer (Miltenyi Biotec, San Diego, CA, USA), the cell suspensions were plated on a fibronectin (5 μg/cm²; Sigma-Aldrich Corp.) precoated petri dish and cultured in endothelial cell complete medium (Vascutive EnGs-Mv complete kit, containing 50 μg/mL ascorbic acid, 1 μg/mL hydrocortisone, 10 mM L-glutamine, 5 ng/mL epidermal growth factor, 0.2% endothelial cell growth supplement [EGS], 5% FBS; Lifeline Cell Technology, San Diego, CA, USA).

For mouse retinal microvascular endothelial cell (mREMC) isolation, retinas were dissected from the Sirt1 cKO pups and the control animals (3–5 pups per group from one litter), minced into small pieces, and digested in 1 mg/mL collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) for 30 minutes at 37°C. As described above, mREMCs were acquired using anti-CD31 antibody-conjugated magnetic beads.

**Immunostaining**

Cells were fixed in 4% PFA at RT for 30 minutes and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. After washing with PBS, they were blocked in PBS containing 5% goat serum for 1 hour, and then incubated with the primary antibody (1:100 dilution) in the blocking solution overnight at 4°C. The following primary antibodies were used: Cd31 (rat anti-mouse; BD Biosciences), Icam2 (rabbit anti-mouse; BD Biosciences), VE-cadherin (rabbit anti-mouse; Cell Signaling Technology, Beverly, MA, USA), Vegfr2 (rabbit anti-mouse; Cell Signaling Technology), Sirt1 (rabbit anti-mouse; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIF-1α (rabbit anti-human; Abcam, Cambridge, MA, USA). After washing with PBS, cells were incubated with the secondary antibody (Alexa Fluor
488 goat anti-rat, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 594 goat anti-rabbit; 1:800 dilution; Life Technologies) for 1 hour at RT in dark. Cells were washed and then stained for 15 minutes with 4′6-diamidino-2-phenylindole (DAPI) to display the nuclei. Stained cells were mounted in a fluorescent mounting medium, and images were captured using a confocal laser scanning microscope (LSM 710; Zeiss).

Cell Culture
Primary HRMECs were purchased from Angio-Protemie (Boston, MA, USA). Cells were cultured in endothelial cell medium (ECM; ScienCell, San Diego, CA, USA) containing 2.5% FBS and ECGs, and maintained in a humidified 5% CO₂ incubator at 37°C. HRMECs were used for experiments before passage 8.

SIRNA Transfection
When HRMECs reached 60% to 70% confluence, the cells were transfected with either SIRT1-specific small interfering RNA (siRNA) (50 nM; Ambion, Austin, TX, USA) or negative control siRNA (50 nM; Ambion) mixed with Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s instructions. Specific knockdown of SIRT1 was confirmed by Western blot analysis.

Lentivirus Infection
Human SIRT1 lentiviral vector construction and subsequent lentivirus production were completed by Genechem Co., Ltd (Shanghai, China). For lentivirus infection, 5 × 10⁴ HRMECs were seeded in 12-well culture plates (Corning, Inc., Corning, NY, USA) and cultured in a complete medium. After the cells attached, lentivirus encoding SIRT1 (Lv-SIRT1) or lentivirus containing empty vector (negative control lentivirus, Lv-NC) was diluted in complete medium containing polybrene (10 μg/mL) at a final multiplicity of infection (MOI) of 10. The medium was replaced after 12 hours of cell culture. Overexpression of SIRT1 was confirmed by Western blot analysis.

Scratch Assay
When mVECs reached 100% confluence in 24-well tissue culture plates (Corning, Inc.), a sterile 100-μL micropipette tip detached cells from the monolayer across the center of the well. Floating cells were flushed out by gently rinsing with PBS and replaced with 0.1% PBS medium. Photographs were taken using a camera attached to a phase-contrast microscope (×100, Axiovert 200; Zeiss) immediately and at 24 hours after scratching. The migration capacity of cells was expressed as percentage of wound closure: Relative Migrated Distance = (A₀h−A₉₀h)/A₀h × 100%. A₀h and A₉₀h representing the area of wound measured immediately and 24 hours after scratching, respectively. The areas of the wounds were quantitatively evaluated using Image-Pro Plus 6.0 software.

Transwell Assay
HRMECs (2 × 10⁴) were added to the upper chambers of a Transwell plate (8 μm; Corning, Inc.). After 18 hours of culture, HRMECs that had migrated into the lower chamber were fixed in 4% PFA, and then stained with crystal violet. Cells in the upper chamber were wiped off; photographs were taken under a phase-contrast microscope (Axiovert 200, Zeiss). The average number of cells was counted from five randomly chosen fields (100%), and the experiment was repeated three times.

Tube Formation Assay
According to the previously reported protocol, HRMECs (3 × 10⁵) were cultured in a 48-well plate (Corning, Inc.) precoated with 100 μL Matrigel Basement Membrane Matrix (BD Biosciences). HRMECs were cultured at 37°C in a 5% CO₂ atmosphere for another 16 hours and photographs were taken with a camera attached to a microscope (Axiovert 200, Zeiss). Tube numbers were quantified in three random microscopic fields, and each assay was repeated at least three times.

Quantitative Reverse Transcription Polymerase Chain Reaction
Total RNA was isolated from the mRMECs or HRMECs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed using an ABI ViiA7 Real-time PCR system (Thermo Scientific, Waltham, MA, USA). The Reverse Transcription System (Promega, Madison, WI, USA) was used to generate cDNA from 1 μg total RNA with random primers. Quantitative PCR was carried out using Power SYBR Green PCR master mix (Thermo Scientific) according to the manufacturer’s instructions. TaqMan Gene Expression Assays (Thermo Scientific) were used to analyze gene expression in mRMECs according to the manufacturer’s instructions. TaqMan probes (Thermo Scientific) used in RT-qPCR were Gapdh (Mm00444237_m1), Sirt1 (Mm00440085_m1), VEGF (Mm00477582_m1), VEGFR2 (Mm00440085_m1), and Mmp14 (Mm00485054_m1). Gapdh expression level was used as a reference for gene expression normalization. The data were analyzed and expressed as relative gene expression using the 2⁻ΔΔCT method. The PCR primer sequences are provided in Supplementary data.

Western Blot Analysis
Western blot analysis was performed according to the standard protocol. Total cell lysate was prepared by RIPA lysis buffer with protease inhibitor cocktail (Merck, Darmstadt, Germany). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose blotting membranes (0.2 μm; GE Healthcare Life Sciences, Uppsala, Sweden). Membranes were blocked in 5% nonfat milk for 3 hours and incubated with primary antibody overnight at 4°C. Fluorescence-conjugated secondary antibody (goat anti-rabbit/mouse IRDye; LI-COR Biosciences, Lincoln, NE, USA) diluted in blocking buffer (1:5000) was with the membrane incubated for 1 hour at RT, and protein bands were detected using Odyssey infrared Imaging System (LI-COR Biosciences). Primary antibodies were SIRT1 (1:1000; Santa Cruz Biotechnology), acetylated lysine (1:1000; Cell Signaling Technology), HIF-1α (1:1000; Abcam), MMP14 (1:1000; Abcam), VEGFA (1:1000; Abcam), VEGFR-2 (1:1000; Cell Signaling Technology), and GAPDH (1:5000; Cell Signaling Technology). GAPDH was used as a loading control.

Coimmunoprecipitation
Coimmunoprecipitation (co-IP) was performed according to the standard procedures in Pierce Classic IP Kit (Thermo Scientific). Briefly, cell lysates were precleared using the control agarose resin, and then incubated overnight at 4°C with primary antibody to form the immune complexes. Targeted immune complexes were captured using Protein A/G agarose, and then eluted with elution buffer containing...
**EC-Specific Deletion of Sirt1 Delays Retinal Vascular Development**

To determine whether loss of endothelial Sirt1 affects postnatal retinal vascular development, we examined the retinal vascular pattern using lectin-based angiography. At P5, the retinal vessel coverage in the Sirt1 cKO mice was 35.8% ± 6.7% whereas that in the control mice was 47.2% ± 6.7%, indicating that loss of Sirt1 remarkably reduced retinal vascularization (n = 10 per group, P = 0.0038, Fig. 2A, a, b). Meanwhile, the retinal vessel density was dramatically reduced in the Sirt1 cKO mice (i.e., 51.63 ± 8.2% per view of the Sirt1 cKO retina versus 75.05 ± 15.05% per view of the control retina, n = 10 per group, P = 0.0224, Fig. 2A, a, c, d). At P8, vessels almost reached the retinal periphery (83% ± 2.5% of the total retinal area) in the control mice, whereas in the Sirt1 cKO mice, the retinal vessel elaboration only extended outward to reach 60.5% ± 9.6% of the total retinal area (Fig. 2A, e, f), and the vascular density was only 57.6 ± 7.6% per view in the Sirt1 cKO retina versus 88.1 ± 12.1% per view in the control retina (n = 10 per group, P = 6.25E-06, Fig. 2A, g, h). Therefore, although the retinal vasculature developed in the Sirt1 cKO mice, its extension into the retinal periphery was markedly inhibited compared with the expansion displayed in the control mice.

**EC-Specific Deletion of Sirt1 Retards Central Retinal Revascularization in the OIR Mouse Model**

To further investigate the endothelial Sirt1 contribution to pathologic retinal vascularization, we examined the vascular response to retinal hyperxia-hypoxia using the OIR mouse model. 

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20mM dithiothreitol (DTT). The immunoprecipitates were subjected to Western blot analysis described above.

**Statistical Analysis**

Results are presented as mean ± standard error of mean (SEM) and analyzed using the 2-tailed Student’s t-test. A P value < 0.05 was considered to be statistically significant.

**RESULTS**

**Generation of Sirt1 EC-Specific Deletion Mice and Sirt1-Null Vascular Endothelial Cells**

In order to determine the role of Sirt1 in retinal vascular development, we first generated mice with the EC-specific deletion of Sirt1 by crossing Sirt1^{floxed} mice with Kdr-Cre transgenic mice expressing Cre-recombinase under control of the EC-specific Kdr promoter (Fig. 1A). Figure 1B shows the results of genotyping. The Sirt1 cKO mice are viable and fertile, with a normal appearance. To further confirm that Sirt1 was deleted, we isolated mVECs from the brain and retina using anti-Cd31–coated magnetic beads. Primary cultured mVECs exhibited a cobblestone morphology under brightfield microscopy (Fig. 1C, a), and strongly expressed EC-specific markers (Cd31, VE-cadherin, Icam2, and Vegfr2), as confirmed by immunostaining (Fig. 1C, b–e). Furthermore, both immunostaining and Western blot analysis show a complete deletion of Sirt1 in mVECs from Sirt1 cKO mice (Figs. 1D, 1E).
model. Our results indicated that there was no difference between the central avascular area in the Sirt1 cKO mice and the control mice counterpart at P12 (25.2% ± 1.9% of retinal area versus 26.3% ± 2.6% of retinal area, n = 10 per group, P = 0.349, Fig. 3A, a, b). At P17, the retinal vaso-obliterated (VO) area in the Sirt1 cKO mice was significantly larger than that in the control mice (22.2% ± 1.0% of retinal area versus 13.73% ± 2.9%, n = 10 per group, P = 1.7E-06, Fig. 3A, c, d), but there was no difference in the neovascular (NV) area. At P21, the VO area in the Sirt1 cKO mice was 16.8% ± 0.5% of the retinal area as compared with 3.8% ± 0.5% of retinal area in the control mice (Fig. 3A, e, f), and the NV area was larger than that of the control group (11.2% ± 2.8% of the retinal area versus 4.2% ± 0.8%). At P26, the VO and NV area was fully revascularized in the control retinas, but in Sirt1 cKO retinas, the remaining VO area was 2.4% ± 1.0% of the retinal area, and the NV area was still 6.8% ± 2.3% (n = 10, P = 3.42E-07, Fig. 3A, g, h). All this taken together, EC-specific ablation of Sirt1 delays central retinal revascularization in the OIR mouse model.

SIRT1 Promotes Retinal Microvascular Endothelial Cell Migration and Tube Formation

Since EC-specific deletion of Sirt1 delayed both retinal vascular development and revascularization in the OIR mouse model, the underlying mechanism was evaluated in vitro using VECs. We first used primary cultured VECs from either the Sirt1 cKO mice or the control mice to determine if Sirt1 deletion contributes to cell migration. The scratch assay showed that Sirt1-null mVECs migrated significantly more slowly by 40% than the control counterpart (Figs. 4A, 4B). We then examined the effects of SIRT1 on primary HRMECs. Target-specific siRNA was employed to knock down SIRT1 expression in HRMECs (Supplementary Fig. S1A). Based on the results of Transwell and tube formation assays, it is apparent that SIRT1 knock-down significantly decreased HRMEC migration by 35% and tube formation by 33% (Figs. 5A, 5B). To determine if SIRT1 upregulation results in corresponding opposite effects, SIRT1 was overexpressed in HRMECs with lentivirus-mediated SIRT1 infection (Supplementary Fig. S1B). As indicated, overexpression of SIRT1 promoted HRMEC migration and tube formation by 30% and 40%, respectively (Figs. 5C, 5D). Taken together, these results indicate that SIRT1 promotes retinal EC migration as well as tube formation.

SIRT1 Regulates VEGF-A, VEGFR-2, and MMP14 Expression in Retinal Microvascular Endothelial Cells

To explore the underlying molecular mechanism accounting for how SIRT1 regulates retinal VEC migration, we first screened for EC migration-associated genes that were differentially expressed in mVECs of the Sirt1 cKO mice. Among these genes, RT-qPCR results indicated that Vegfa, Vegfr2, and Mmp14 mRNA levels were significantly downregulated in Sirt1-null mVECs compared with the control (Fig. 6A), as further confirmed based on Western blot analysis showing an agreement with corresponding changes in protein expression levels (Fig. 6B). We also evaluated the changes in Vegfa, Vegfr2, and Mmp14 mRNA expression levels in isolated mRMECs. In agreement with the aforementioned results, the expression levels of these genes declined as expected in Sirt1-null mRMECs from P8 mice compared with the control mice (Fig. 6C).
SIRT1, as well as the levels in the isolated mRMECs from the OIR P17 mice (Fig. 6D). In HRMECs, specific siRNA-mediated SIRT1 downregulation had the same profound inhibitory effects on VEGF-A, VEGFR-2, and MMP14 (Figs. 7A, 7C). In contrast, lentiviral-mediated SIRT1 overexpression correspondingly upregulated the expression of VEGF-A, VEGFR-2, and MMP14 on both mRNA and protein levels in HRMECs (Figs. 7B, 7D). Our data showed that SIRT1 regulates VEGF-A, VEGFR-2, and MMP14 expression in retinal microvascular ECs both in vivo and in vitro.

**SIRT1 Modulates HIF-1α Acetylation Status in HRMECs**

The mouse retina is exposed to a relatively hypoxic condition at P5 and P17 of the OIR model, which results in an increase of HIF-1α. In addition, hypoxia-inducible migration was critically dependent on HIF-1α activity in VECs. In contrast, lentiviral-mediated SIRT1 overexpression correspondingly upregulated the expression of VEGF-A, VEGFR-2, and MMP14 on both mRNA and protein levels in HRMECs (Figs. 7B, 7D). Our data showed that SIRT1 regulates VEGF-A, VEGFR-2, and MMP14 expression in retinal microvascular ECs both in vivo and in vitro.

**DISCUSSION**

Emerging evidence indicates the importance of SIRT1 in regulating vascular development. In this study, we address its specific role in retinal vascularization. Our approach to identifying the physiological and pathologic role of SIRT1 in controlling retinal vascularization involved generating Sirt1 cKO mice. In the EC-specific Sirt1 deletion mice, retinal vascular development was dramatically delayed compared with the control mice. Such a difference in vascular patterns is consistent with other reports in which it was shown that loss of SIRT1 function resulted in defective vascular growth and patterning in zebrafish. Considering the fact that Kdr is also expressed in nonvascular cells of the retina, we showed that there is no significant difference in Sirt1 expression between the retinas from cKO mice and the control counterpart (Supplementary Figs. S2A, S2B). Furthermore, histologic analysis indicates no difference in retinal structure (Supplementary Fig. S2C). Therefore, a potential impact of deleting Sirt1 in the retinal cells can be excluded. These findings clearly document that Sirt1 expression is essential for normal pattern formation and expansion in the early stage of retinal vascular development.

SIRT1 is a metabolic sensor whose expression and activity increase during hypoxia, while mouse retina is in a relatively hypoxic status at an early developmental stage. Its involvement in a pathologic condition was evaluated using an OIR mouse model.
model. This animal model of retinopathy of prematurity (ROP) has two phases: vaso-oblitration and consequent hypoxia-induced pathologic retinal neovascularization. Although it was reported that loss of neuronal Sirt1 function decreased retinal vascular regeneration into the avascular zone and increased hypoxia-induced pathologic vascular growth, we investigated instead the possible role of Sirt1 in regulating retinal angiogenesis. 

The OIR mouse model at P17 is analogous to high-energy conservation. Under this condition, the mice are in a metabolic state associated with avascular zone, which removes a hindrance to cell migration. Therefore, knockdown of Sirt1 led to a significant decline of relative migrated distance of mVECs compared to control. This result supports the hypothesis that Sirt1 plays a crucial role in regulating retinal angiogenesis.

In order to confirm if the insights gained from the Sirt1 KO mice can be extrapolated to humans, we further investigated the impact of changes in Sirt1 expression using HRMECs. As expected, SIRT1 downregulation significantly inhibited HRMEC migration and tube formation, which is consistent with the result in normal development of retinal vasculature in vivo. It is also consistent with our results obtained with mVECs isolated from Sirt1 cKO mice. Furthermore, SIRT1 upregulation promoted migration and tube formation in HRMECs. Taken together, the results from in vitro experiments are in good agreement with those from in vivo.

To gain further insight into the retinal angiogenic actions of SIRT1, we used RT-qPCR analysis to screen for differentially expressed genes in the Sirt1 cKO mVECs. Among these genes, Vegfa, Vegfr2, and Mmp14 decreased significantly in Sirt1-null mVECs, which can explain the impaired capillary expansion observed in Sirt1 cKO retinas. Given that MMP14 is a key enzyme in the regulation of extracellular matrix remodeling, we tested the MMP-14 activity in si-SIRT1-transfected HRMECs. As a result, knockdown of SIRT1 led to a significant decline of MMP2 activity. These findings support the hypothesis that Sirt1 contributes to both physiological and pathological retinal angiogenesis.
FIGURE 6. In vivo deletion of Sirt1 leads to a significant downregulation of Mmp14, Vegfa, and Vegfr2 in mRMECs. (A) Quantitative RT-PCR was performed to analyze the expression of candidate genes associated with endothelial cell migration. The expression of Mmp14, Vegfa, and Vegfr2 was decreased significantly in Sirt1-null mRMECs as compared with the control mRMECs (*P < 0.05). (B) Western blot analysis showed that Mmp14, Vegfa, and Vegfr2 were significantly reduced in Sirt1-null mRMECs. Gapdh was used as an internal control. (C) Quantitative RT-PCR analysis showed that the expression of Mmp14, Vegfa, and Vegfr2 declined significantly in mRMECs isolated from P8 Sirt1-null retinas (*P < 0.05). (D) The expression of the three genes was also significantly decreased in mRMECs from P17 Sirt1-null OIR mice (*P < 0.05), as determined by RT-qPCR analysis.

FIGURE 7. SIRT1 regulates the expression of MMP14, VEGF-A, and VEGFR-2 in HRMECs. (A) Quantitative RT-PCR analysis indicated that the expression of MMP14, VEGF-A, and VEGFR-2 was significantly downregulated in si-SIRT1-transfected HRMECs, as compared with negative control (*P < 0.05). (B) Western blot analysis showed that the expression of these genes was also significantly reduced in si-SIRT1-transfected HRMECs. GAPDH was used as an internal control. (C) RT-qPCR analysis showed that the expression of MMP14, VEGF-A, and VEGFR-2 was significantly upregulated in Lv-SIRT1-infected HRMECs, as compared with the negative control (*P < 0.05). (D) Western blot analysis also showed upregulation of these genes in Lv-SIRT1-infected HRMECs.
of the developing vasculature. Deletion of Mmp14 in mouse ECs compromised cell migration and tube formation both in vitro and in vivo. All these results show here that SIRT1 contributes to the control of retinal VEC migration by regulating VEGF-A, VEGFR-2, and MMP14 expression both in vitro and in vivo.

Retinal vascularization reaches maturity 3 weeks after birth in mice, and therefore, the retinal ECs are in a relatively hypoxic condition at P5, as well as at OIR P17. Since HIF-1α is a key transcription factor under hypoxic conditions, we determined the effects of HIF-1α on VEGF-A, VEGFR-2, and MMP14 expression both in vitro and in vivo.

In our study, we found that SIRT1 can colocalize and bind to HIF-1α in the nucleus of HRMECs. We also showed that inhibiting SIRT1 expression increased HIF-1α acetylation status. An increase in HIF-1α acetylation level was reported to decrease its stability in previous findings, which may directly blunt VEGF-A, VEGFR-2, and MMP14 expression in HRMECs. Taken together, our results definitively show that SIRT1 deacetylase activity contributes to determining retinal vascularization patterns by modulating HIF-1α acetylation status. Variations in its acetylation status in turn affect angiogenic factor expression (VEGF-A, VEGFR-2, and MMP14) and retinal vascularization. However, we do not exclude the possibility that SIRT1 can regulate retinal vascularization via other targets such as endothelial nitric oxide synthase (eNOS). We feel that eNOS is a potential candidate and should be further evaluated in future studies to clarify if it actually contributes to SIRT1-mediated retinal vascularization.

Taken together, this study shows that SIRT1 makes an essential contribution to controlling retinal vascularization under both the physiological and pathologic conditions. We also found that SIRT1 deletion dramatically inhibits retinal VEC migration through the inhibition of VEGF-A, VEGFR-2, and MMP14 expression, which may result from sustaining the effect of hyperacetylated HIF-1α. These results provide additional substantiation that SIRT1 may be a potential drug target to prevent maladaptive vascularization responses in diseases whose progression is sensitive to the modulation of vascular oxygen levels.

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