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Endothelial sirtuin 1 inactivation enhances capillary rarefaction and fibrosis following kidney injury through Notch activation

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ABSTRACT

Peritubular capillary (PTC) rarefaction along with tissue fibrosis is a hallmark of chronic kidney disease (CKD). However, molecular mechanisms of PTC loss have been poorly understood. Previous studies have demonstrated that functional loss of endothelial sirtuin 1 (SIRT1) impairs angiogenesis during development and tissue damage. Here, we found that endothelial SIRT1 dysfunction causes activation of endothelial Notch1 signaling, which leads to PTC rarefaction and fibrosis following kidney injury. In mice lacking functional SIRT1 in the endothelium (Sirt1 mutant), kidney injury enhanced apoptosis and senescence of PTC endothelial cells with impaired endothelial proliferation and expanded myofibroblast population and collagen deposition. Compared to wild-type kidneys, Sirt1 mutant kidneys up-regulated expression of Delta-like 4 (DLL4, a potent Notch1 ligand), Hey1 and Hes1 (Notch target genes), and Notch intracellular domain-1 (NICD1, active form of Notch1) in microvascular endothelial cells (MVECs) post-injury. Sirt1 mutant primary kidney MVECs reduced motility and vascular assembly and enhanced senescence compared to wild-type kidney MVECs. This difference in the phenotype was negated with Notch inhibition. Concurrent stimulation of DLL4 and transforming growth factor (TGF)-β1 increased *trans*-differentiation of primary kidney pericytes into myofibroblast more than TGF-β1 treatment alone. Collectively, these results indicate that endothelial SIRT1 counteracts PTC rarefaction by repression of Notch1 signaling and antagonizes fibrosis via suppression of endothelial DLL4 expression.

1. Introduction

The kidney has considerable capacity to repair and regenerate. Central to nephron repair and regeneration is repair of peritubular capillaries (PTCs), but kidney PTCs are particularly susceptible to permanent loss after injury [1]. Rarefaction of PTCs, together with interstitial fibrosis, is one of the major hallmarks of chronic kidney disease (CKD) [2]. Animal models of CKD have shown a negative correlation between PTC density and the severity of kidney fibrosis [2]. Moreover, in patients with CKD, the extent of PTC loss predicts the severity of interstitial fibrosis and the decline in renal function [3,4]. Thus, PTC could be a novel therapeutic target to mitigate CKD progression to end stage kidney disease. However, the molecular mechanisms of PTC rarefaction after kidney injury remain poorly understood.

Sirtuin 1 (SIRT1) is an NAD+-dependent deacetylase that is highly expressed in endothelial cells [5]. SIRT1 maintains angiogenic potential of endothelial cells by regulating signaling pathways via its deacetylase activity [6]. Roles of SIRT1 in endothelial cells *in vivo* have been studied using mutant mice, in which SIRT1 lacks its deacetylase activity in the endothelium by deletion of exon 4 (encoding SIRT1 catalytic domain) [7]. These mutant mice (hereafter called Sirt1 mutant mice) demonstrated capillary rarefaction in the microvasculature of neonatal retina and in the hindlimb ischemia model due to impaired angiogenesis [5,8]. In the kidney of Sirt1 mutant mice, impairment of renal function and fibrosis were enhanced following folic acid-induced injury [9], indicating that endothelial SIRT1 protects against kidney injury. However, the mechanisms that afford renal protection are not completely elucidated. SIRT1 represses Notch1 signaling in retinal endothelial cells, which contributes to retinal angiogenesis [8]. We therefore investigated how endothelial SIRT1 counteracts PTC loss

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and fibrosis during kidney injury through Notch1 signaling.

2. Materials and methods

2.1. In Vivo studies

To delete catalytic domain of SIRT1 protein in endothelial cells, we crossed *Tie2-Cre* mice (the Jackson Laboratory, stock No: 008863, strain: B6) with *Sirt1*$^{F/F}$ mice (exon 4 of Sirt1 gene is flanked by two loxP sites) (the Jackson Laboratory, stock No: 008041, strain: B6; 129S). Genotyping was performed according to instructions by the company [9]. Resultant Sirt1 mutant mice were subjected to UUO surgery. The surgical procedures were performed as previously described [10]. Mice were euthanized 10 days post-UUO. Contralateral kidneys without injury were served as controls. αSMA-GFP mice (gifted from Ivo Kalajzic) [11] were used for kidney pericyte isolation. Animal protocols were conducted in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at New York Medical College.

2.2. Tissue preparation and histology

Mouse tissues were prepared and stained as previously described [10]. Briefly, PLP-fixed kidneys were cryo-sectioned and stained with antibodies against primary antibodies. These antibodies include CD31 (1:200; BD Biosciences, #550274), αSMA (1:200; abcam, #ab5694), NICD1 (1:100; abcam, #ab8925), H3K9me3 (1:400; abcam, #ab8898), Ki67 (1:200; abcam, #ab15580), F4/80 (1:200; eBioscience, #14-4801-81) and PDGFRβ (1:400; a gift from William Stallcup). Primary antibodies were detected with either Alexa488- or Alexa-594 conjugated secondary antibodies (1:400; Molecular Probes). PTC density was quantified using CD31-stained sections and grid methods. Fibrosis was assessed using Picrosirius red stained sections and a polarized light. TUNEL reaction was performed by In Situ Cell Death Detection Kit (Roche).

2.3. Quantitative PCR (qPCR)

Total RNA was extracted using TRIzol (Invitrogen). Purity of RNA was determined by A260 to A280 ratio. After cDNA was synthesized, qPCR reaction was performed by Mx3000P qPCR system (Stratagene) and PerfeCta SYBR Green FastMix (Quanta bioscience). Primer sequences were listed in Supplemental Table 1.

2.4. Isolation and culture of cells from kidney

Primary MVECs and pericytes were isolated from adult mouse kidneys using magetic beads based methods. To prepare kidney single cell suspension, kidney was decapsulated, minced, and digested with Collagenase H (1 mg/mL; Roche) and Collagenase/Dispase (1 mg/mL; Roche) in DMEM/F12 medium (Gibco). Single cell suspension was filtered through a cell strainer (40 μm; Fisher Scientific) to remove glomeruli and arterioles, centrifuged, and re-suspended in Isolation Buffer (1× PBS, 1% BSA). Purified anti-CD31 antibodies (BD Biosciences, #553369) were incubated with Dynabeads anti-rat IgG (Invitrogen) (4 °C, overnight) prior to cell isolation procedure. Cells were incubated with CD31 antibody-beads complex (4 °C, 1 h). Positive selection was performed by Magnetic Particle Concentrator (Invitrogen). Selected cells were directly applied to RNA isolation or cultured in endothelial medium (IMDM [Gibco], 15% FBS, 1% BSA, 0.01 mg/mL recombinant human insulin, 0.2 mg/mL human transferrin, 100 ng/mL recombinant mouse VEGF, 100 ng/mL recombinant mouse basic FGF, 100 ng/mL recombinant mouse EGF, 1× penicillin/streptomycin) in 6 cm dish pre-coated with 1% gelatin. High concentration of VEGF (>40 ng/mL) supports selective MVEC growth with high purity [12]. Purity of primary cultures was confirmed by staining for CD31 or VE-cadherin. If its purity was less than 85%, second positive selection was performed. Cells were used between passages 1 and 3.

Primary pericytes were isolated from kidneys of αSMA-GFP mice using abovementioned methods. Kidney single cell suspension was incubated with PDGFRβ antibody-beads complex in Isolation Buffer (4 °C, 1 h). PDGFRβ antibodies (gifted from William Stallcup) were pre-incubated with Dynabeads M-280 anti-rabbit IgG (Invitrogen). Isolated cells were directly applied to RNA isolation or cultured in DMEM/F12 with 10% FBS and 1× penicillin/streptomycin in 6 cm dish pre-coated with 1% gelatin. High purity of primary cultures (>93%) was confirmed by staining for PDGFRβ. Cells were used between passages 1 and 2.

2.5. Staining of isolated cells

Cells were fixed with cold methanol (4 °C, 15 min) and blocked with 5% BSA/PBS (room temperature, 30 min). Primary antibodies (CD31 [1:200; BD Biosciences], VE-cadherin [1:200; BD Biosciences, #550548], F4/80 [1:200; eBioscience]) were incubated with cells (4 °C, overnight). Primary antibodies were detected with either Alexa488- or Alexa-594 conjugated secondary antibodies (1:400; Molecular Probes). For senescence-associated β-galactosidase (SA-β-Gal) staining, kidney MVECs were seeded to 24-well plates pre-coated with 1% gelatin and human DLL4 (1 μg/mL; PeproTech) and cultured until 50% confluent. After serum deprivation, MVECs were cultured in IMDM with 0.5% FBS including mouse VEGF (100 ng/mL) for 48 h with DAPT (*N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) (γ-secretase inhibitor to suppress NICD generation) (2 μM; abcam, #120633) or its vehicle, DMSO. Cells were fixed with 3% paraformaldehyde (PFA) in PBS (room temperature, 5 min) and stained for SA-β-Gal. Images (×100) were captured and the number of SA-β-Gal+ cells was normalized with total number of nuclei. To assess NICD1 expression, WT or mutant MVECs were seeded to gelatin/DLL4 coated wells. MVECs (50% confluent) were treated with DAPT or DMSO for 48 h after serum deprivation. Cells were fixed with 4% PFA, permeabilized with 0.5% Triton/PBS, and incubated with NICD1 antibodies (1:100). NICD1+ cells were identified by nuclear NICD1 staining.

2.6. Migration assays

Migration of MVECs was assessed using a wound healing assay as described [10] with some modifications. In gelatin/human DLL4-coated 24-well plates, MVECs were cultured until 100% confluent. After serum deprivation, the monolayer of cells was wounded using a 200-μL micropipette tip. After wells were washed with PBS, cells were cultured in IMDM including 0.5% FBS and 100 ng/mL VEGF with DAPT or its vehicle, DMSO for 48 h. Cell free areas were measured using ImageJ software. A percentage of wound closure was calculated by the following formula: [(wound area at time 0 − wound area at 48 h)/wound area at time 0] × 100.

2.7. 2-Dimensional angiogenesis assay

In 24-well plate, WT or Sirt1 mutant kidney MVECs (5×10^4 cells/well) were seeded onto Matrigel (200 μl/well; Corning, #356231) and cultured in IMDM including 2% FBS, 100 ng/mL VEGF, and 1 μg/mL human DLL4 with DAPT (3 μM) or DMSO. After 2 h, the number of endothelial branch point was quantified under ×100.

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