Original Paper

Melatonin Attenuates Endothelial-to-Mesenchymal Transition of Glomerular Endothelial Cells via Regulating miR-497/ROCK in Diabetic Nephropathy

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Key Words

Melatonin • Endothelial-to-mesenchymal transition • Glomerular endothelial cells • miR-497/ROCK • Diabetic nephropathy

Abstract

Background/Aims: Endothelial-to-mesenchymal transition (EndMT) of glomerular endothelial cells (GEnCs) can induce albuminuria in diabetic nephropathy. Melatonin attenuates diabetic nephropathy, but its role and mechanism in EndMT of GEnCs in diabetic nephropathy remain unknown. **Methods:** The effect of melatonin on EndMT induced by transforming growth factor (TGF)- β 2 in human renal GEnCs was determined by assaying the expression of endothelial marker cells (VE-cadherin and CD31) and mesenchymal cells (α -SMA and Snail), as well as monolayer permeability. The molecular mechanism of melatonin in these processes was focused on miR-497/ROCK signaling. Furthermore, the effect and mechanism of melatonin in EndMT were confirmed in glomeruli of rats with streptozotocin-induced diabetes. **Results:** Melatonin increased expression of VE-cadherin and CD31 and inhibited α -SMA and Snail levels that were altered by TGF- β 2 in GEnCs. Melatonin treatment reduced expression and activity of ROCK1 and ROCK2, which suppressed TGF- β 2-induced hyperpermeability of GEnCs and EndMT of GEnCs. Melatonin reduced ROCK1 and ROCK2 expression and activity in TGF- β 2-stimulated GEnCs by enhancing expression of miR-497, which targets ROCK1 and ROCK2. Furthermore, we found that melatonin inhibited EndMT in glomeruli and albuminuria in rats with streptozotocin-induced diabetes. MiR-497 expression increased, whereas ROCK1 and ROCK2 expression and activity decreased in melatonin-treated diabetic rats. **Conclusion:** Melatonin attenuated EndMT of GEnCs via regulating miR-497/ROCK signaling in diabetic

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nephropathy. This study improves understanding of EndMT and the role of melatonin in diabetic nephropathy.

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Introduction

Diabetic nephropathy, a serious microvascular complication of diabetes, is characterized by persistent albuminuria and progressive decline of the glomerular filtration rate. It is the leading cause of end-stage renal disease [1]. Fibrosis in the kidney is the most common pathway for the pathogenesis of diabetic nephropathy. Kidney fibroblasts play vital roles in renal fibrosis, and myofibroblasts reportedly are derived from resident renal fibroblasts, mesangial cells, tubular epithelial cells, and bone marrow-derived cells [2]. In the last decade, studies have found that endothelial-to-mesenchymal transition (EndMT), a specific form of epithelial-to-mesenchymal transition, is another important mechanism that generates myofibroblasts in diabetic nephropathy [3, 4]. EndMT of glomerular endothelial cells (GEnCs) thus likely plays a vital role in this disease [2, 5].

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized in the pineal gland and many other organs [6]. Melatonin possesses multiple pathophysiological properties, including circadian rhythm regulation, antioxidant activity, immune defense, and inflammation and cancer prevention [7, 8]. Accumulating evidence has suggested that melatonin's antioxidant activity might be useful in treating diabetes and attenuating diabetic nephropathy [9, 10]. However, the effect of melatonin on EndMT of GEnCs in diabetic nephropathy remains unknown.

Rho-associated kinases (ROCKs) belong to serine-threonine protein kinases and were originally identified as effector proteins of the small GTPase Rho. The ROCK family includes two members, ROCK1 and ROCK2, which share 64% overall amino acid identity and 92% of kinase domains, indicating that they perform many of the same functions [11]. ROCKs are ubiquitously expressed, though ROCK1 is not expressed in the brain and muscle [11]. They mediate a wide array of cellular processes in cytoskeleton organization and assembly, energy metabolism, cell proliferation, and epithelial-to-mesenchymal transition [12, 13]. ROCKs are activated in the kidneys and in cultured cells of diabetic models [14, 15]. The ROCK inhibitor fasudil slows progression of diabetic nephropathy [16].

Notably, Peng et al. [17] have shown increased ROCK1 expression and decreased CD31 in the glomerular endothelia of diabetic mice. *In vitro* studies have shown suppressed EndMT in GEnCs with upregulated ROCK1. Inhibition of ROCK1 significantly suppresses EndMT in GEnCs stimulated by high glucose and in glomeruli of diabetic mice, leading to reduced endothelial permeability and albuminuria [17]. Suppressing ROCK2 expression reduces high-glucose-induced hyperpermeability in renal glomerular endothelia [18]. Previous studies also have shown that melatonin modulates ROCK1 and ROCK2 activation to inhibit cancer cell progression [19, 20]. Yet, the relationship between melatonin and ROCK activation in diabetic nephropathy remains unknown. We speculated that melatonin might suppress EndMT in diabetic nephropathy by regulating ROCK activation. We first evaluated the role of melatonin in EndMT and monolayer permeability of GEnCs and then the role of ROCK signaling in the mechanism of melatonin in diabetic nephropathy.

MicroRNAs (miRNAs) are a class of small (20–22 nucleotides), non-coding, endogenous RNAs that modulate gene expression at posttranscriptional levels by binding to the 3' untranslated region (UTR) of target mRNAs [21]. Melatonin ameliorates alcohol-induced bile acid synthesis by enhancing miR-497 expression [21]. Hence, we also investigated whether melatonin suppressed ROCK signaling by modulating miR-497 expression.

Materials and Methods

Cell culture and treatment

Human renal GEnCs were purchased from ScienCell (Carlsbad, CA, USA) and cultured in endothelial cell medium containing 10% fetal bovine serum, 1% endothelial cell growth supplement, and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. We used cells within the five passages in this study. After culturing overnight, GEnCs were stimulated with a 5 ng/mL concentration of recombinant human transforming growth factor (TGF)-β2 for 48 hours.

MTT assay

The viability of GEnCs was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay (Beyotime, Shanghai, China). Briefly, GEnCs were seeded into 96-well plates at a density of 6×10^3 cells per well. After adhesion overnight, GEnCs were pre-treated with 0 μM, 1 μM, 10 μM, 50 μM, 100 μM, or 200 μM of melatonin (Sigma, St. Louis, MO, USA) for 2 hours, followed by stimulation with TGF-β2 (5 ng/mL) for 48 hours. Then, the medium was changed to fresh medium containing a 500 μg/mL concentration of MTT. Cells were cultured for another 4 hours at 37 °C. After adding 100 μl of formazan-dissolving solution, optical density was measured at 570 nm using a microplate reader.

Transfection

The miR-497 mimic and anti-miR-497 antagomir were synthesized by Genechem Co., Ltd (Shanghai, China) and used to upregulate or downregulate miR-497 expression, respectively. GEnCs were placed in a 6-well plate. After reaching about 70% confluence, the cells were transfected with miR-497 mimic, anti-miR-497, or constructed plasmids pcDNA3.1-ROCK1, pcDNA3.1-ROCK2, or their negative controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 24 hours after transfection, the cells were treated with melatonin and TGF-β2, as previously described.

Dual Luciferase assay

To construct luciferase reporter vectors, the predicted 3' UTR or mutant 3' UTR of ROCK1 or ROCK2 was cloned into the pmirGLO luciferase reporter vector (Promega, Madison, WI, USA). GEnCs were transfected with 500 ng of constructed luciferase vector and 40 nM of miR-497 mimic or control mimic using Lipofectamine 2000 (Invitrogen) for 48 hours. Then, luciferase activities were assayed with a dual-luciferase assay kit (Promega).

Endothelial permeability assay

GEnCs were transfected with or without pcDNA3.1-ROCK1 and pcDNA3.1-ROCK2 for 24 hours and then dissociated into single-cell suspensions. Next, 3×10^5 cells were seeded into the upper chamber of 24-well Transwell plates with cell culture inserts (pore size 0.4 μm, 12-mm diameter). Then, 500 μl of medium was added to the lower chamber. Cells were pre-treated with 50 μM of melatonin for 2 hours and stimulated with a 5 ng/mL concentration of TGF-β2 for another 48 hours. Fluorescein isothiocyanate dextran (1 mg/mL) was added to the upper chamber and incubated for 2 hours. Fluorescence (excitation, 492 nm; emission, 530 nm) in the lower chamber was measured with an LS45 spectrofluorometer (Perkin Elmer Life Sciences, Waltham, MA, USA).

Animal study

We obtained 8-week-old male Sprague-Dawley from SLAC laboratory Animal Co. Ltd. (Shanghai, China). All rats were housed in a 12-hour light and dark cycle with free access to food and water. Animal studies were carried out in accordance with the Institutional Animal Care and Use Committee of China-Japan Union Hospital of Jilin University. After fasting for 12 hours, 12 rats received a single, intraperitoneal injection of streptozotocin (150 mg/kg, dissolved in 0.1 M sodium citrate buffer at pH 4.5; Sigma) to induce diabetes. A normal control (NC) group ($n = 6$) received the same volume of sodium citrate buffer only.

At 2 weeks after the streptozotocin injection, diabetes was confirmed if blood glucose levels exceeded 16 mM. Diabetic rats were randomly separated into an untreated (DM) group ($n = 6$) and a melatonin-treated (DM + Mel) group ($n = 6$). To mimic physiological circadian changes in blood concentrations of melatonin,

rats received water treated with melatonin (20 mg/L) only during the 12-hour dark cycle [10] and untreated distilled water during the 12-hour light cycle. Rats in the NC and DM groups received distilled water in light and dark periods. After 4 weeks of melatonin treatment, 24-hour urine was collected from all animals in metabolic cages. Then, the animals were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg). Both kidneys were collected, the left for molecular analysis and the right for histological study. Albuminuria was measured as the ratio of urinary albumin (μg) to urinary creatinine (mg), which was determined, respectively, by Exocell Albuwell M and Creatinine companion kits (Exocell, Inc., Philadelphia, PA, USA).

Table 1. Primer sequences. h: Human; r: Rat

Gene	Forward (5'-3')	Reverse (5'-3')
hVE-cadherin	CTTCACCCAGACCAAGTACACA	AGGGCTCATGTATCGGAGGT
hCD31	CCAAGCCCGAAGTGAATCT	CACTGTCCGACTTTGAGGCT
h α -SMA	AAATGAACGTTTCCGCTGCC	CTTCATGGTGCTGGGTGCTA
hSnail	GCGAGCTGCAGGACTCTAAT	TCCCAGATGAGCATTGGCAG
hROCK1	TGTCGACTGGGGACAGTTT	AGCATCCAATCCATCCAGCA
hROCK2	GCCGCCGTTGCCATATTAAG	CTGGAGCTGGGGGCTTTT
hGAPDH	GGTCACCAGGGCTGCTTTTA	TTCCCGTTCTCAGCCTTGAC
rVE-cadherin	CAACTGGCCTGTGTTTACGC	ATCTGCATCCGTTGCTGTCA
rCD31	AAATGGTGCTTCGGTGCTCT	GGTGCCAAACACTTGGGTG
r α -SMA	ACCATCGGGAATGAACGCTT	CTGTCAGCAATGCCTGGGTA
rSnail	GAAGATGCACATCCGAAGCC	TTCACATCCGAGTGGGTCTG
rROCK1	CATGCAAGCGCAATTGGTAGA	TAAGGAATGCAGGCAGAACCA
rROCK2	GAGATCAGTGCAGCGCTAT	CACTACCACGCTTGACAGGT
rGAPDH	TGTGAACGGATTGGCCGTA	GATGGTGATGGGTTTCCCGT

Periodic acid-Schiff (PAS) staining and immunofluorescence

5 μm frozen sections of kidney tissues were performed PAS staining as previously described [17]. Also, frozen sections of rat kidney were used for EndMT analysis by double-labeling them with antibodies against CD31 and α -SMA. After antigen retrieval in proteinase K, sections were incubated with primary antibodies against CD31 (1:100; Abcam, Cambridge, MA, USA) and α -SMA (1:200; Cell Signaling Technology, Danvers, MA, USA), followed by incubation with fluorescent-conjugated secondary antibodies (Invitrogen). The immunolabeled sections were analyzed by fluorescence microscopy (Biozero, Keyence, Osaka, Japan). For each rat, images of six different fields of view were obtained in a blinded manner.

Real-time PCR

Total RNA from GEnCs and kidney tissues was extracted using TRIzol reagent (Invitrogen). One μg of total RNA was reverse-transcribed into cDNA using SuperScriptVILO MasterMix reagent (Invitrogen). Real-time PCR was performed on a Fast Real-time PCR 7500 System (Applied Biosystems, Carlsbad, CA, USA) using SYBR Green Master Mix (Takara, Japan). GAPDH was used as a housekeeping gene. To test miR-497 expression, reverse transcription of RNA was conducted using a TaqMan MicroRNA Reverse Transcription Kit (ABI, Foster City, CA, USA). Real-time PCR reaction was performed using a TaqMan MicroRNA PCR Kit (ABI). U6 was used as an internal control of miR-497. Relative expression of the target gene was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, with normalization to GAPDH or U6. Table 1 shows the primer sequences.

Western blot

Cells and tissues were lysed in RIPA buffer containing 1 mM of phenylmethylsulfonyl fluoride. Protein concentrations were estimated using a BCA kit (Biotechnology Co., Ltd, Shanghai, China). Proteins (40 μg per lane) were separated with SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% nonfat milk, membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were detected with enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA). Primary antibodies used in this study were as follows: anti-VE-cadherin (1:1000, Abcam); anti-CD31 (1:800, Abcam); anti- α -SMA (1:1000, Cell Signaling Technology); anti-Snail (1:1000, Cell Signaling Technology); anti-ROCK1 (1:1000, Cell Signaling Technology); anti-ROCK2 (1:1000, Cell Signaling Technology); and anti-phospho-MYPT1 (pMYPT1; 1:1000, Cell Signaling Technology).

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) and analyzed using GraphPad Prim5 software. The statistical significance of differences between groups was examined by one-way ANOVA followed by a Bonferroni post-test. $P < .05$ was considered statistically significant.

Results

Melatonin and GENC proliferation

We first determined the effect of different concentrations of melatonin on GENCs stimulated by TGF- β 2. As shown in Fig. 1, compared with the control group, TGF- β 2 significantly suppressed GENC viability, and this effect was abrogated by 10 μ M and 50 μ M doses of melatonin in a dose-dependent manner. Therefore, we used 50 μ M of melatonin.

Melatonin inhibited endothelial-to-mesenchymal transition in glomerular endothelia

We analyzed melatonin's effect on EndMT in GENCs by determining EndMT-related gene expression. VE-cadherin and CD31 are important endothelial markers, and α -SMA and Snail are markers of mesenchymal cells. Compared with the normal control group, TGF- β 2 remarkably decreased the mRNA expression of VE-cadherin and CD31 but increased α -SMA and Snail. Compared with the TGF- β 2 group, the melatonin-treated groups showed higher mRNA levels of CD31 and VE-cadherin and lower expression of α -SMA and Snail (Fig. 2A). Similarly, the results of western blot demonstrated that the protein levels of EndMT markers were consistent with their mRNA expression (Fig. 2B and 2C). These results suggest that melatonin inhibited TGF- β 2-induced EndMT in GENCs.

Fig. 1. The effects of melatonin on the proliferation of glomerular endothelial cells. Glomerular endothelial cells were pre-treated with different concentrations (0 μ M, 1 μ M, 10 μ M, 50 μ M, 100 μ M, or 200 μ M) of melatonin for 2 hours, followed by stimulation with a 5 ng/mL concentration of TGF- β 2 for 48 hours. Then, cell viability was determined by MTT assay. N = 3, data are presented as mean $\pm$ SD, **P < .01 versus the control group; #P < .05, ##P < .01 versus the TGF- β 2 group.

Fig. 2. Melatonin inhibited TGF- β 2-induced endothelial-to-mesenchymal transition and monolayer permeability in glomerular endothelial cells. (A) Glomerular endothelial cells were pre-treated with 50 μ M of melatonin for 2 hours and then stimulated with TGF- β 2 (5 ng/mL) for 48 hours. Real-time PCR was used to detect the mRNA expression of VE-cadherin, CD31, α -SMA, and Snail. (B) and (C) Protein levels of VE-cadherin, CD31, α -SMA, and Snail were analyzed by western blot. (D) The permeability of glomerular endothelial cells

was assayed by the amount of fluorescein isothiocyanate dextran in the cell monolayer. N = 3, data are presented as mean $\pm$ SD, **P < .01 versus the control group; #P < .05, ##P < .01 versus the TGF- β 2 group.

Melatonin suppressed TGF- β 2-induced hyperpermeability of glomerular endothelia

We further evaluated the effect of melatonin on monolayer permeability of GEnCs and found that, compared with the normal control group, TGF- β 2 treatment significantly increased monolayer permeability of GEnCs, the effect of which was reduced by melatonin treatment (Fig. 2D).

Melatonin prevented TGF- β 2-induced endothelial-to-mesenchymal transition and hyperpermeability by modulating ROCK signaling

We explored how ROCK signaling influenced the molecular mechanism of melatonin in TGF- β 2-induced EndMT and hyperpermeability. As shown in Fig. 3A and 3B, the mRNA and protein levels of ROCK1 and ROCK2 were significantly higher in the TGF- β 2 group than in the normal control group. Compared with the TGF- β 2 group, melatonin treatment remarkably suppressed the expression of ROCK1 and ROCK2 at the transcriptional and translational levels. Furthermore, TGF- β 2 increased phosphorylation of MYPT1, a downstream effector of ROCK, the effect of which was abrogated by melatonin treatment.

Fig. 3. Melatonin prevented TGF- β 2-induced endothelial-to-mesenchymal transition and monolayer permeability by suppressing ROCK signaling. (A) The mRNA expression of ROCK1 and ROCK2 was determined by real-time PCR. (B) The protein levels of ROCK1, ROCK2, and pMYPT1 were measured by western blot. (C) Glomerular endothelial cells were transfected with pcDNA3.1 (empty) or pcDNA3.1-ROCK1 (ROCK1) plasmid for 72 hours, and the protein levels of ROCK1 and pMYPT1 were analyzed. (D) Glomerular endothelial cells were transfected with pcDNA3.1 (empty) or pcDNA3.1-ROCK2 (ROCK2) plasmid for 72 hours, and the protein levels of ROCK2 and pMYPT1 were assayed. (E) Glomerular endothelial cells were transfected with pcDNA3.1-ROCK1 (ROCK1) or pcDNA3.1-ROCK2 (ROCK2) plasmid for 24 hours, pre-treated with 50 μ M of melatonin for 2 hours, followed by stimulation with a 5 ng/mL concentration of TGF- β 2 for 48 hours. Western blot was used to detect the expression of CD31 and α -SMA. (F) The permeability of glomerular endothelia was assayed under different treatments. N = 3, data are presented as mean $\pm$ SD, **P < .01 versus the control group; #P < .05, ##P < .01 versus the TGF- β 2 group; &&P < .01 versus the TGF- β 2 + Mel group.

To confirm whether melatonin regulates ROCK signaling, GEnCs were transfected with pcDNA3.1-ROCK1/ROCK2 plasmid. As revealed in Fig. 3C, the pcDNA3.1-ROCK1 plasmid but not the empty plasmid (pcDNA3.1) significantly enhanced ROCK1 expression. Similarly, the pcDNA3.1-ROCK2 plasmid markedly upregulated ROCK2 expression (Fig. 3D). The pcDNA3.1-ROCK1 and pcDNA3.1-ROCK2 plasmids also increased pMYPT1 levels (Fig. 3C and 3D). Compared with the TGF- β 2-only treated group, the melatonin-treated groups showed significantly increased CD31 expression and reduced α -SMA and monolayer permeability, the effects of which were partially reversed by upregulation of ROCK1 or ROCK2 (Fig. 3E and 3F). Collectively, these results demonstrate that melatonin prevented TGF- β 2-induced EndMT and hyperpermeability by inhibiting ROCK expression and activity.

MiR-497 targeted ROCK1 and ROCK2

ROCK1 and ROCK2 reportedly have a post-transcriptional regulation mechanism [22], and miR-497 inhibits epithelial-to-mesenchymal transition [23, 24]. Hence, we analyzed the relationship between miR-497 and ROCK1/ROCK2. A TargetScan analysis indicated that miR-497 targeted ROCK1 and ROCK2 (Fig. 4A). Dual-luciferase reporter assays demonstrated that the miR-497 mimic but not the control mimic inhibited luciferase activities of the ROCK1 wild-type and ROCK2 wild-type plasmids. However, the miR-497 mimic did not alter luciferase activities of the ROCK1 mutant or ROCK2 mutant plasmids (Fig. 4B and 4C). Additionally, compared with the control mimic group, the miR-497 mimic group showed decreased mRNA and protein levels of ROCK1 and ROCK2 (Fig. 4D and 4E). These results indicate that miR-497 targeted ROCK1 and ROCK2 in GEnCs.

Fig. 4. ROCK1 and ROCK2

are targets of miR-497. (A) Conserved miR-497 binding sites in the 3' untranslated regions of ROCK1 and ROCK2 and the sequences of ROCK1 and ROCK2 3' untranslated region mutants. (B) The miR-497 mimic or control mimic was co-transfected with pmirGLO-ROCK1-WT or pmirGLO-ROCK1-Mut luciferase plasmid in glomerular endothelial cells. Luciferase activity was assayed using a dual-luciferase reporter assay kit. (C) The miR-497 mimic or control mimic was co-transfected with pmirGLO-ROCK2-WT or pmirGLO-ROCK2-Mut luciferase

plasmid in glomerular endothelial cells, and luciferase activity was determined. Glomerular endothelia were transfected with the miR-497 mimic or control mimic. The (D) mRNA and (E) protein levels of ROCK1 and ROCK2 were detected by real-time PCR and western blot, respectively. N = 3, data are presented as mean $\pm$ SD, **P < .01 versus the control mimic group.

Fig. 5. Melatonin regulated ROCK signaling by elevating miR-497 expression. (A) MiR-497 expression was determined in glomerular endothelia under different treatments. (B) Glomerular endothelial cells were transfected with anti-NC or anti-miR-497 for 72 hours, and miR-497 expression was measured by real-time PCR. (C) Glomerular endothelia were transfected with anti-miR-497 for 24 hours, treated with 50 μ M of melatonin for 2 hours, and then exposed to a 5 ng/mL concentration of TGF- β 2 for 48 hours. ROCK1, ROCK2, and pMYPT1 were measured by western blot. N = 3, data are presented as mean $\pm$ SD, **P < .01 versus the control group; ##P < .01 versus the TGF- β 2 group; &&P < .01 versus the TGF- β 2 + Mel group.

Melatonin regulated ROCK signaling by elevating miR-497 expression

We investigated whether melatonin-suppressed activation of ROCK signaling depended on regulation of miR-497 and found that, compared with the normal control group, TGF- β 2 notably decreased miR-497 expression and this effect was reversed by melatonin treatment (Fig. 5A). Anti-miR-497 significantly reduced miR-497 expression in these GENCs that were transfected with anti-miR-497 for 24 hours and then exposed to TGF- β 2 stimulation with melatonin pre-treatment for 2 hours (Fig. 5B). Downregulation of miR-497 attenuated the melatonin-induced inhibition of ROCK1, ROCK2, and pMYPT1 (Fig. 5C). Taken together, these data demonstrate that melatonin suppressed ROCK signaling via elevating miR-497 expression.

The miR-497 and ROCK pathway influenced melatonin-attenuated endothelial-to-mesenchymal transition in glomeruli of diabetic rats

The effect of melatonin on EndMT was confirmed in the glomeruli of diabetic rats. Compared with the NC group, rats in the DM group had lower expression of VE-cadherin and CD31 in kidney tissues and higher expression of α -SMA and Snail. Compared with the DM group, the melatonin-treated groups showed elevated VE-cadherin and CD31 expression but decreased α -SMA and Snail expression (Fig. 6A and 6B). PAS staining revealed that diabetic rats exhibited glomerular hypertrophy and extracellular matrix deposition, and melatonin treatment attenuated these changes (Fig. 6C). We used double-labeling immunofluorescence to exhibit colocalization of CD31 and α -SMA, where double-positive cells indicate EndMT. The DM group had more double-positive cells than the NC group, whereas the DM + Mel group had significantly lower double-positive cells than the DM group (Fig. 6C). Moreover, compared with the NC group, the DM group had higher albuminuria, which notably decreased after melatonin treatment (Fig. 6D).

We verified the molecular mechanism of melatonin in EndMT *in vivo*. Compared with the NC group, miR-497 expression decreased and expression of ROCK1, ROCK2, and pMYPT1 increased in the DM group. Melatonin-treated rats in the DM group showed elevated miR-497 expression and reduced ROCK1, ROCK2, and pMYPT1 expression, compared with the DM group (Fig. 6A, 6E, and 6F). These data suggest that melatonin attenuated EndMT in glomeruli of diabetic rats and that this effect was related to the miR-497/ROCK pathway.

Fig. 6. Melatonin attenuated endothelial-to-mesenchymal transition in the glomeruli of diabetic rats, which also involved the miR-497 and ROCK pathway. (A) Protein levels of VE-cadherin, CD31, α -SMA, Snail, ROCK1, ROCK2, and pMYPT1 in rat kidney tissues were measured by western blot. (B) Real-time PCR was used to analyze the mRNA expression of VE-cadherin, CD31, α -SMA, and Snail in rat kidney tissues. (C) Kidney sections were treated with PAS staining and double-label immunofluorescence was used to show colocalization of CD31 and α -SMA in glomeruli of rats (arrow pointed out). (D) Albuminuria was measured as the ratio of urinary albumin (μ g) to urinary creatinine (mg). (E) MiR-497 expression was detected in kidney tissues of rats. (F) Real-time PCR assay for the mRNA expression of ROCK1 and ROCK2 in rat kidney tissues. N = 6, data are presented as mean $\pm$ SD, **P < .01 versus the NC group; ##P < .01 versus the DM group.

Discussion

Impairment of the glomerular filtration membrane leads to diabetic albuminuria or proteinuria. Podocytes are terminally differentiated epithelial cells of the glomerulus that attach to the outside of the glomerular filtration barrier. Damage to or loss of podocytes can cause proteinuria and diabetic nephropathy [25]. GEnCs form the inner layer of the glomerular filtration membrane, and studies have shown that GEnCs play important roles in diabetic nephropathy. Decreased GEnCs causes glomerular filtration dysfunction and proteinuria [26]. More important, EndMT plays a vital role in endothelial dysfunction, which

causes diabetic nephropathy [2, 27]. In our study, we found that melatonin suppressed EndMT of GEnCs in diabetic nephropathy. Further investigation revealed that melatonin inhibited EndMT of GEnCs by modulating miR-497 and ROCK signaling.

Melatonin can ameliorate many complications of diabetes mellitus, including retinopathy [28] and nephropathy [29]. *In vivo* studies have demonstrated that melatonin has antioxidant and anti-inflammation activities in diabetic nephropathy [10, 29, 30]. In an *in vitro* diabetic nephropathy model, Ji et al. [31] showed that melatonin has anti-apoptotic effects in angiotensin II-induced podocyte injury. However, the roles of melatonin in GEnCs and EndMT in diabetic nephropathy remain unclear.

Although abnormal angiogenesis plays a pathological role in diabetic nephropathy, EndMT of GEnCs leads to increased permeability, resulting in proteinuria [17, 32]. In the current study, we found that melatonin significantly inhibited EndMT in GEnCs exposed to TGF- β 2. In previous literatures the staining for endothelial markers in diabetic glomeruli seems to be discrepancy [17, 32, 33]. Our results showed that CD31 staining was reduced in glomeruli of diabetic rats. Melatonin suppressed the EndMT in glomeruli of diabetic rats. Also, melatonin inhibited TGF- β 2-induced hyperpermeability in GEnCs and attenuated streptozotocin-elevated albuminuria levels. These results suggest that melatonin suppressed EndMT of GEnCs in diabetic nephropathy.

ROCK activation plays vital roles in diabetic nephropathy [16-18]. Melatonin reportedly suppresses ROCK activation, which abrogates tumor growth [19, 20]. Hence, we found that TGF- β 2 elevated expression of ROCK1, ROCK2, and pMYPT1 in GEnCs, the effects of which were abrogated by melatonin. In parallel, melatonin suppressed ROCK1, ROCK2, and pMYPT1 levels in the kidneys of diabetic rats. Activation of ROCK1 or ROCK2 partially reversed the effects of melatonin on TGF- β 2-induced EndMT and hyperpermeability in GEnCs. Collectively, these data indicate that melatonin inhibited EndMT of GEnCs by suppressing ROCK activation.

ROCK1 and ROCK2 can be regulated post-transcriptionally by miRNAs [22]. MiR-497 is a newly identified miRNA that inhibits epithelial-to-mesenchymal transition of cancer cells, keratinocytes, and alveolar epithelial cells [23, 24, 34]. A TargetScan analysis revealed that both ROCK1 and ROCK2 are potential targets of miR-497. Luciferase assays confirmed that the miR-497 mimic notably reduced luciferase activities of ROCK1 and ROCK2 wild-type plasmids but not mutant ones. Also, miR-497 overexpression notably reduced the mRNA and protein levels of ROCK1 and ROCK2 in GEnCs. These results indicate that miR-497 targeted ROCK1 and ROCK2 in GEnCs.

Kim et al. [21] have demonstrated that melatonin ameliorates alcohol-induced bile acid synthesis by augmenting miR-497 expression. We investigated whether melatonin regulates ROCK activation by modulating miR-497 expression and found decreased miR-497 in TGF- β 2-exposed GEnCs and in kidney tissues of diabetic rats. Melatonin treatment significantly enhanced miR-497 expression in these diabetic nephropathy models. Additionally, downregulation of miR-497 abrogated the effects of melatonin on TGF- β 2-induced expression and activity of ROCK1 and ROCK2. These data demonstrate that melatonin suppressed ROCK activity by elevating miR-497 expression in GEnCs.

Conclusion

For the first time, we found that melatonin inhibited EndMT in GEnCs exposed to TGF- β 2 and in the glomeruli of diabetic rats. We also found that miR-497 targeted ROCK1 and ROCK2. Melatonin elevated miR-497 and suppressed ROCK activity. This study therefore further elucidates the mechanism of EndMT in diabetic nephropathy and highlights the role of melatonin in this disease.

Disclosure Statement

All authors claim no conflicts of interest.

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