Am J Transl Res 2019;11(11):7000-7008
www.ajtr.org /ISSN:1943-8141/AJTR0099736

Original Article

miR-1 and miR-802 regulate mesenchymal-epithelial transition during kidney development by regulating Wnt-4/β-catenin signaling

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Received July 16, 2019; Accepted October 12, 2019; Epub November 15, 2019; Published November 30, 2019

Abstract: Objective: Mesenchymal-epithelial transition (MET) is an important part of kidney development. However, the role of microRNA (miRNA) in MET and the regulating mechanism is still not well known. Materials and methods: qRT-PCR and western blot were performed to detect the expression of miR-1 and miR-802 and related protein expression. Luciferase reporter assay and western blot were used to identify the target of miR-1 and miR-802. Confocal microscopy was used to analyze the MET process. Results: We demonstrated that miR-1 expression was downregulated and miR-802 expression was upregulated during kidney development. And during the process, proteins levels of Wnt-4 and β-catenin changed significantly. In MDCK cells, overexpression of Wnt-4 inhibited the expression of β-catenin, and promote the MET, and overexpression of β-catenin inhibited MET. Further studies suggested that miR-1 and miR-802 regulated MET by binding to Wnt-4 and β-catenin mRNA, regulated the expression of Wnt-4 and β-catenin. In conclusion, miR-1 and miR-802 regulate MET during kidney development by regulating Wnt-4/β-catenin signaling.

Keywords: Mesenchymal-epithelial transition, microRNAs, miR-1, miR-802, Wnt-4/β-catenin signaling

Introduction

There are nearly 30% kidney congenital anomalies among all the prenatally diagnosed malformations [1]. Mesenchymal-epithelial transition (MET) is an important part of kidney development [2]. Development of metanephros, the permanent mammalian kidney, relies on the interactions of the ureteric bud and the metanephric mesenchyme, which begins with the invasion of metanephric mesenchyme by the ureteric bud [3]. The metanephric mesenchyme is a mixed population of cells that includes a subset of nephron progenitor cells including cap mesenchyme cells which surround the tips of the ureteric tree during the kidney development to form the cap domains [4]. These cap mesenchyme cells then undergo a mesenchymal-epithelial transition (MET) process to sequentially form the mature nephron [5]. So, it is important to clarify the mechanism of MET process for understanding the etiology of congenital renal diseases.

Wnt4 is a member of the Wnt family of secreted molecules that were originally identified as mammalian homologues of the *Drosophila* wingless gene [6]. Wnt family regulate various developmental changes, such as angiogenesis, and kidney development, and Wnt-4 was reported to involve in the regulation of kidney development [7]. However, the regulation of Wnt-4 in kidney development is still not clear. MicroRNAs (miRNA) are small, endogenous, noncoding RNA molecules of 21-25 nucleotides which play important roles in various processes, including tissue development [8, 9]. Here, we investigated the role of miRNAs in the regulation of kidney development, and reported that miR-1 and miR-802 were involved in the regulation of MET and kidney development.

Materials and methods

Isolation of embryonic kidneys and tissues processing

24 adult mice (Swiss-Webster) were bred according to two genders at the ratio of 1:1. Day 0 of gestation coincided with appearance of the vaginal plug. Embryonic kidneys isolated from day 5, 10, 15 mouse embryos were homoge-

nized in RIPA lysis buffer (Thermo) containing 1% protease cocktail inhibitor and 1% phosphatase inhibitor cocktail (Sigma-Aldrich) for 30 min at 4°C, centrifuged at 10,000 g for 20 min, and the supernatants collected. Sample containing 40 μg of proteins were separated for Western blot analysis. Animal care and euthanasia were carried out with the approval of the Institutional Animal Care and Use Committee (IACUC) of the Affiliated Hospital of Zunyi Medical University.

Western blot analysis

Samples from embryonic kidneys tissues lysate was then electrophoresed, and transferred onto PVDF membranes, blocked with 5% milk and incubated with primary antibodies against Wnt-4 (1:1000, abcam, Shanghai, China), β-catenin (1:1000, abcam, Shanghai, China), and β-actin (1:1000, abcam, Shanghai, China). Following primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibodies (1:5000, abcam, Shanghai, China). Protein bands were visualized using a HiSignal™ ECL WB Detection Kit (Synthgene Biotech, Nanjing, China) according to the manufacturer's protocol.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from embryonic tissues from mice with different treatments by using a Total RNA Extraction Kit (Synthgene Biotech, Nanjing, China) according to the manufacturer's protocol. The quantity and purity of total RNA were measured with a NanoDrop spectrophotometer (Thermo Fisher, Wilmington, DE, USA). To detect the expression level of miR-21, 1 μg total RNA was reverse transcribed into cDNA using specific Taqman® RT primers (Thermo Fisher, Wilmington, DE, USA) and PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Biotech, Dalian, China) following the manufacturer's protocol. qRT-PCR was then performed using TaqMan™ Fast Advanced Master Mix (Thermo Fisher, Wilmington, DE, USA). Thermocycling conditions: 95°C 5 min, 95°C 15 s and 60°C 1 min for 40 cycles. U6 was served as an internal control. miRNA expression levels were finally normalized to the U6 snRNA with the $2^{-\Delta\Delta Cq}$ method.

Cell culture and transfection

Madin-Daby canine kidney cells (MDCK cells) were purchased from ATCC. For plasmids transfection, cells were cultured in 6 well plates (Corning-Star) in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 1% antibiotics (Penicillin-Streptomycin solution Sigma) at 37°C with 5% CO_2. After the cells reached 60% confluence, the transfection was performed. The mouse β-catenin expression vector and Wnt-4 expression vector was purchased from Synthgene Biotech. Each well was transfect with 1 μg β-catenin plasmid and Wnt-4 plasmid, respectively or simultaneously. Transient transfection was performed using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. For microRNA knockdown or overexpression, a miR-1 overexpressing and miR-802 knockdown lentiviruses (Synthgene Biotech) were used to infect the MDCK cells. Cells were treated with miR-802 KD lentiviruses and miR-1 OE lentiviruses seperately, or miR-1 OE lentiviruses and Wnt-4 plasmid simultaneously.

Dual-luciferase reporter assay

pMIR-Wnt-4-3'-UTR-WT, pMIR-Wnt-4-3'-UTR-Mut, Pmir-β-catenin-3'-UTR-WT, and pMIR-β-catenin-3'-UTR-Mut luciferase reporter plasmids were constructed by Synthgene Biotech (Nanjing, China). MDCK cells were seeded in a 24 well plate until reaching 60% confluence. Each well was co-transfected with 1 μg luciferase reporter plasmids and 100 pmol RNA mimics using HiTrans™ LipoPlus Reagent (Synthgene Biotech, Nanjing, China) according to the manufacturer's protocol. Following a 48-h transfection, cells were collected and dual-luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega, Shanghai, China) according to the manufacturer's instructions and normalized to Renilla signals.

Confocal microscopy

After a 48-h transfection, the cells were washed twice with PBS. After the cells were fixation with 4% paraformaldehyde, permeabilized using 0.5% Triton X-100 for 10 minutes and blocked with 10% rabbit serum for 1 hour at room temperature. Then, the cells were allowed to react overnight with the primary antibod-

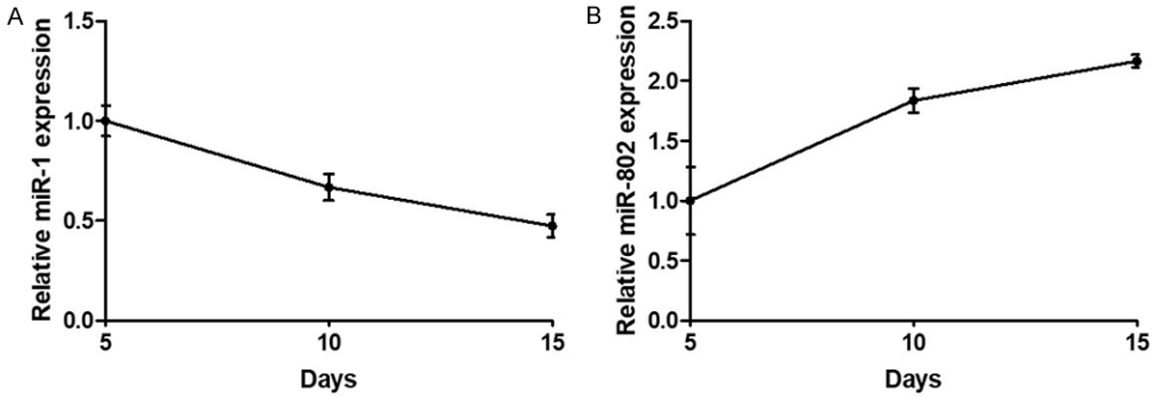

Figure 1. The expression of miR-1 was downregulated and the expression of miR-802 was up regulated during the kidney development. A: The expression of miR-1 was determined in the embryonic kidney using qRT-PCR. B: The expression of miR-1 was determined in the mouse embryonic kidney using qRT-PCR. n=3. Data are presented as the mean ± sem.

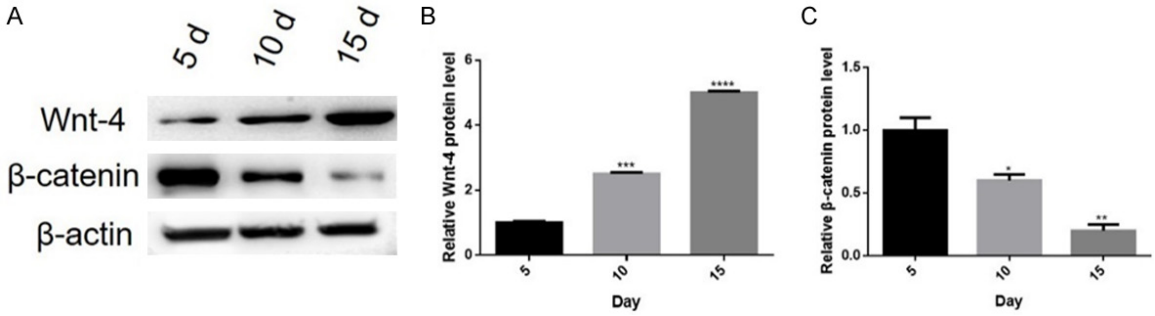

Figure 2. The expression of Wnt-4 and β-catenin in the mouse embryonic kidney. A: Protein expression level was determined by Western blot. B, C: ImageJ densitometric analysis of the expression levels of indicated proteins. n=3. Data are presented as the mean ± sem. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

ies at 4°C. The primary antibodies consisted of Mouse Anti-E-Cadherin antibody (abcam) and Rabbit Anti-Wnt4 antibody (abcam). The cells were hybridized with the corresponding secondary antibodies (Alexa Fluor 488 or 594, 1:800, Jackson Immuno Research) the following day at 37°C for 2 hours. Hoechst 33258 was utilized for identification of nuclei. Immunofluorescence images were observed on a confocal microscope (Fluoview FV1000, Olympus). Five randomly-selected fields from one coverslip were included to get an average, and experiments were repeated independently at least three times.

Statistical analysis

Statistical assay was performed using GraphPad Prism 7.0. All data in this study were shown as the mean ± Standard Error of Mean (SEM) of three independent experiments. The significance of the differences in mean values between and within multiple groups was examined by one-way ANOVA followed by Duncan's multiple range test. *P* value < 0.05 was considered as statistically significant.

Results

The expression of miR-1 was downregulated and the expression of miR-802 was up regulated during the kidney development

qRT-PCR showed that miR-1 expression level gradually decreased in the kidney of the mouse embryonic kidney at the day 5, 10, and 15 (**Figure 1A**). On the contrary, miR-802 expression level gradually increased in the kidney of the mouse embryonic kidney at the day 5, 10, and 15 (**Figure 1B**).

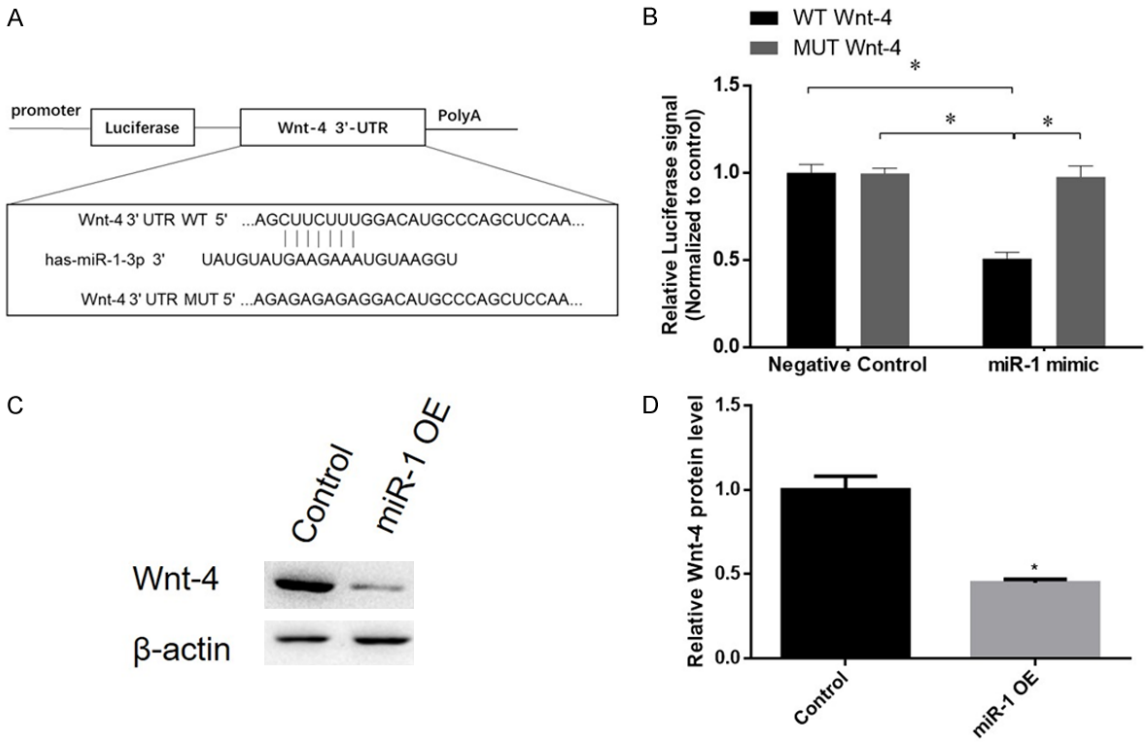

Figure 3. Wnt-4 was identified as a direct target gene of miR-1. A: The putative binding sequence for miR-1 in the 3'UTR of the Wnt-4 mRNA. B: Relative luciferase signals from reporter MDCK cells. C: Protein expression level was determined by Western blot. D: ImageJ densitometric analysis of the expression levels of indicated proteins. n=3. Data are presented as the mean ± sem. *P < 0.05.

The expression of Wnt-4 was upregulated during kidney development

As shown in **Figure 2**, Wnt-4 expression level gradually increased in the kidney of the mouse embryonic kidney at the day 5, 10, and 15. β-catenin expression level gradually decreased in the kidney of the mouse embryonic kidney at the day 5, 10, and 15.

Wnt-4 was identified as a direct target gene of miR-1 in the kidney

We then performed bioinformatics analysis with RNAhybird and TargetScan (www.targetscan.org). The analysis revealed that Wnt-4 was a likely target gene of miR-1 (**Figure 3A**). To confirm whether miR-1 regulated Wnt-4 expression, the luciferase reporter assay was performed. The luciferase activity significantly decreased following co-transfection with pMIR-Wnt-4-3'-UTR-WT and miR-1 mimic, compared with co-transfection with pMIR-Wnt-4-3'-UTR-Mut and miR-1 mimic, indicating that miR-1 specifically binds to the 3'-UTR of Wnt-4, and regulated the Wnt-4 expression negatively (**Figure 3B**). Western blot further showed that overexpression of miR-1 inhibited the expression of Wnt-4 (**Figure 3C**, **3D**).

β-catenin was identified as a direct target gene of miR-802 in the kidney

We also found that that β-catenin was a likely target gene of miR-802 by using bioinformatics analysis with RNAhybird and TargetScan (www.targetscan.org) (**Figure 4A**). We then performed the luciferase reporter assay to confirm the prediction. The luciferase activity significantly decreased following co-transfection with pMIR-β-catenin-3'-UTR-WT and miR-802 mimic, compared with co-transfection with pMIR-β-catenin-3'-UTR-Mut and miR-802 mimic, indicating that miR-802 specifically binds to the 3'-UTR of β-catenin, and regulated the β-catenin expression negatively (**Figure 4B**). Western blot further showed that overexpression of miR-802 inhibited the protein expression of β-catenin (**Figure 4C**, **4D**).

Wnt-4 inhibited β-catenin during MET

β-catenin is the key molecule of Wnt-4 signaling [10]. In MDCK cells, overexpression of Wnt-4

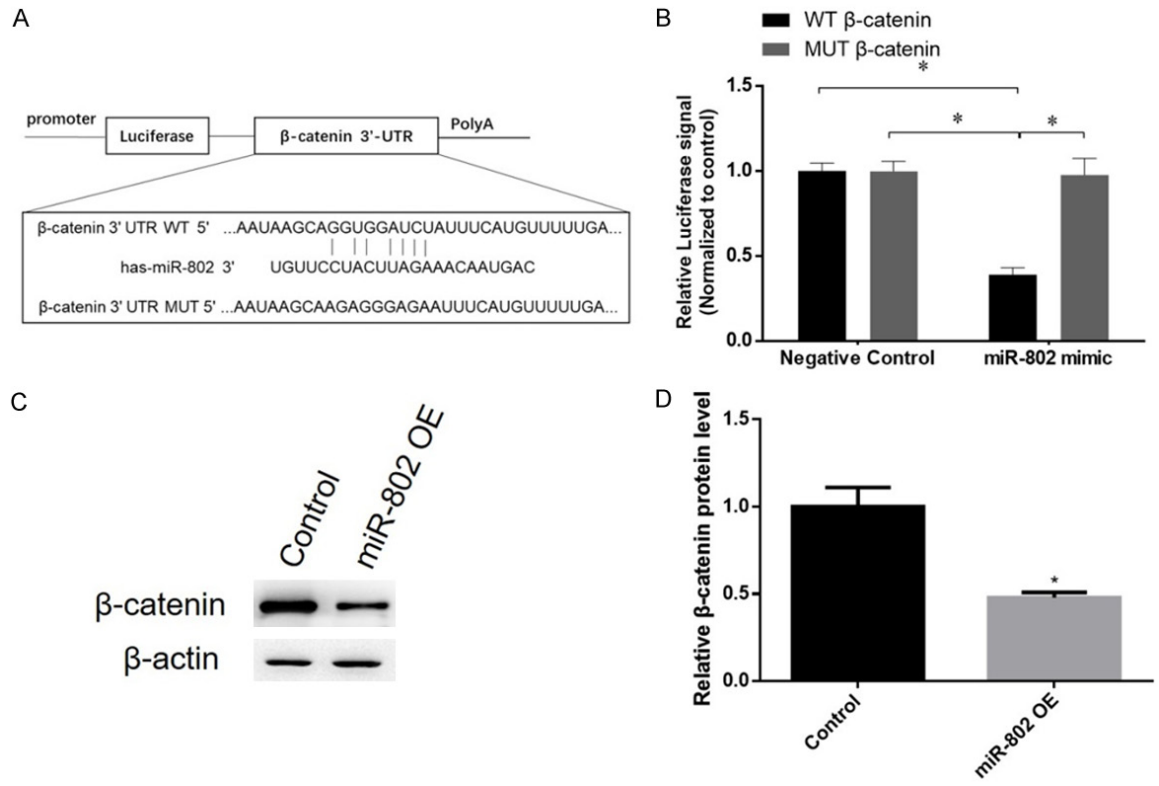

Figure 4. β-catenin was identified as a direct target gene of miR-802. A: The putative binding sequence for miR-802 in the 3'UTR of the β-catenin mRNA. B: Relative luciferase signals from reporter MDCK cells. C: Protein expression level was determined by Western blot. D: ImageJ densitometric analysis of the expression levels of indicated proteins. n=3. Data are presented as the mean ± sem. *P < 0.05.

significantly decreased the expression of β-catenin (**Figure 5A-C**). Confocal microscopy showed that overexpression of Wnt-4 rescued the downregulated epithelial biomarker, E-cadherin induced by overexpression of β-catenin, and decreased the expression of mesenchymal biomarker, Vimentin (**Figure 5D**).

miR-1 and miR-802 targeted Wnt-4 and β-catenin to regulate MET

To further confirm the relationship among miR-1, miR-802, Wnt-4/β-catenin signaling and MET, we treated MDCK cells with miR-802 KD lentiviruses and miR-1 OE lentiviruses seperately, or miR-1 OE lentiviruses and Wnt-4 plasmid simultaneously. Western blot showed that knockdown of miR-802 increased the expression of β-catenin (**Figure 6A**, **6C**), and overexpression of miR-1 decreased the expression of Wnt-4, and increased the expression of β-catenin, which could be reversed by co-overexpression of Wnt-4 (**Figure 6A**, **6B**). Confocal microscopy showed that knockdown of miR-802 downregulated the expression of E-cadherin, and overexpression of miR-1 upregulated the expression of Vimentin and downregulated E-cadherin, which could be rescued by co-overexpression of Wnt-4 (**Figure 6D**).

Discussion

In this study, we demonstrated that miR-1 expression was downregulated and miR-802 expression was upregulated during kidney development. And during the process, proteins level of Wnt-4/β-catenin signaling changed significantly. In MDCK cells, overexpression of Wnt-4 inhibited the expression of β-catenin, and promote the MET, and overexpression of β-catenin inhibited MET. Furthermore, we identified the Wnt-4 as the direct target gene of miR-1, and β-catenin as the direct target gene of miR-802. Knockdown of miR-802 or overexpression of miR-1 inhibited MET, and restoration of Wnt-4 partially rescued this inhibitory effect. Taken together, our data suggested that miR-1 and miR-802 played important role in

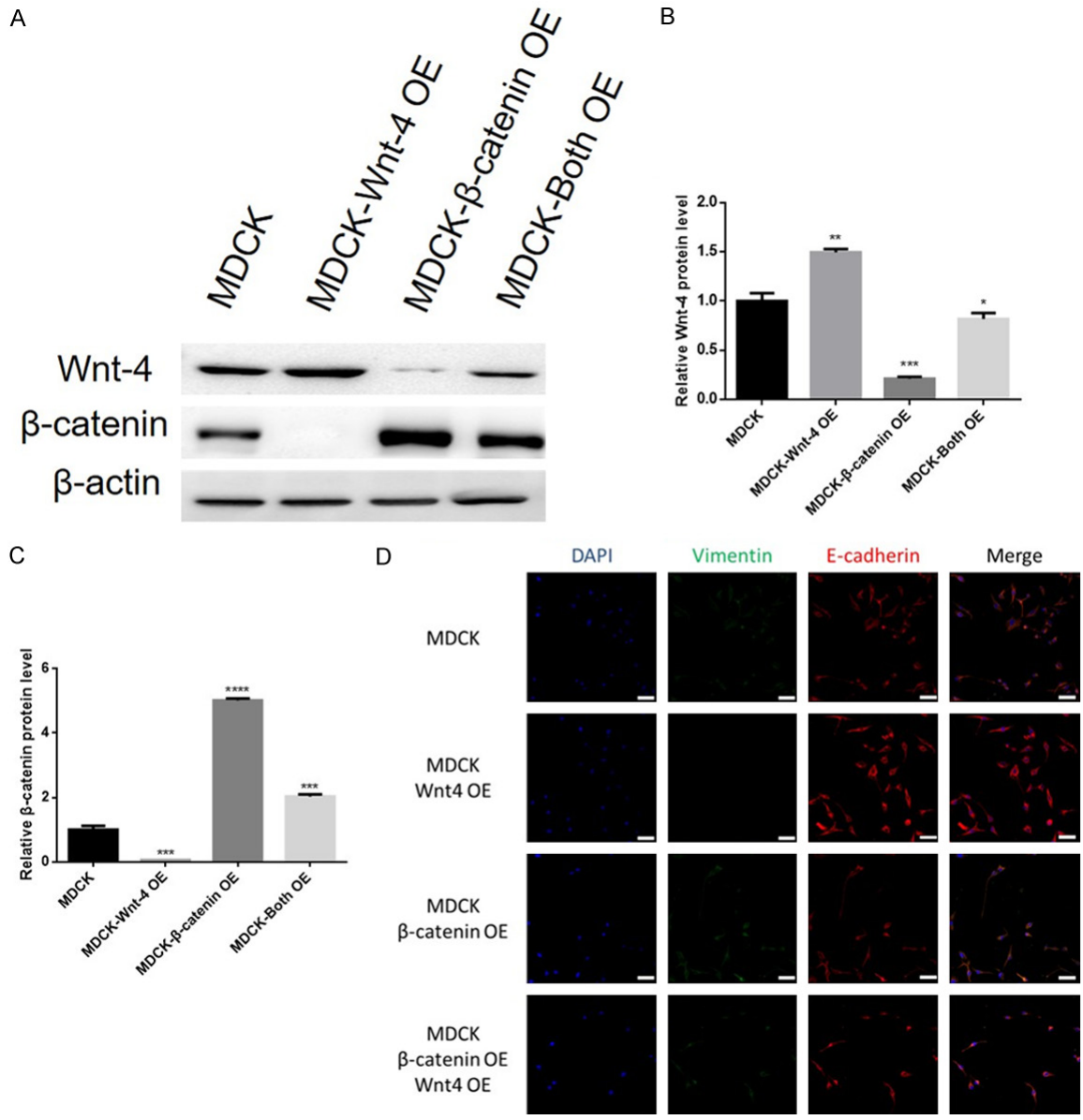

Figure 5. Wnt-4 inhibited β-catenin during MET. A: Protein expression level was determined by Western blot. B, C: ImageJ densitometric analysis of the expression levels of indicated proteins. D: Confocal images of MDCK cells of indicated treatment. n=3. Data are presented as the mean ± sem. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001.

the regulation of MET in the kidney development by regulating Wnt-4 and β-catenin.

Emerging evidences showed that miRNA participated in many physiological and pathological processes. Previous studies demonstrated that miRNA acts as regulator of epithelial mesenchymal transition to regulate the cancer proliferation and migration (EMT) [11-13]. However, little research on the role of miRNA in the regulation of MET has been done. In our studies, we showed a change of miR-1 and miR-802 expression in MET during kidney development, indicating they may play some roles in the MET process. miR-1 was reported to regulate cardiac arrhythmogenic potential, cell apoptosis, and cell differentiation [14-16]. Recently its role in the tissue developing has been noticed. Zheng et al. [17] showed that miR-1 regulated neural stem cell differentiation. MiR-1 was also reported to regulate the differentiation programme of muscle cells [18]. miR-802 was reported to play roles in glucose metabolism, cell proliferation, and inflammation [19-21]. The role of

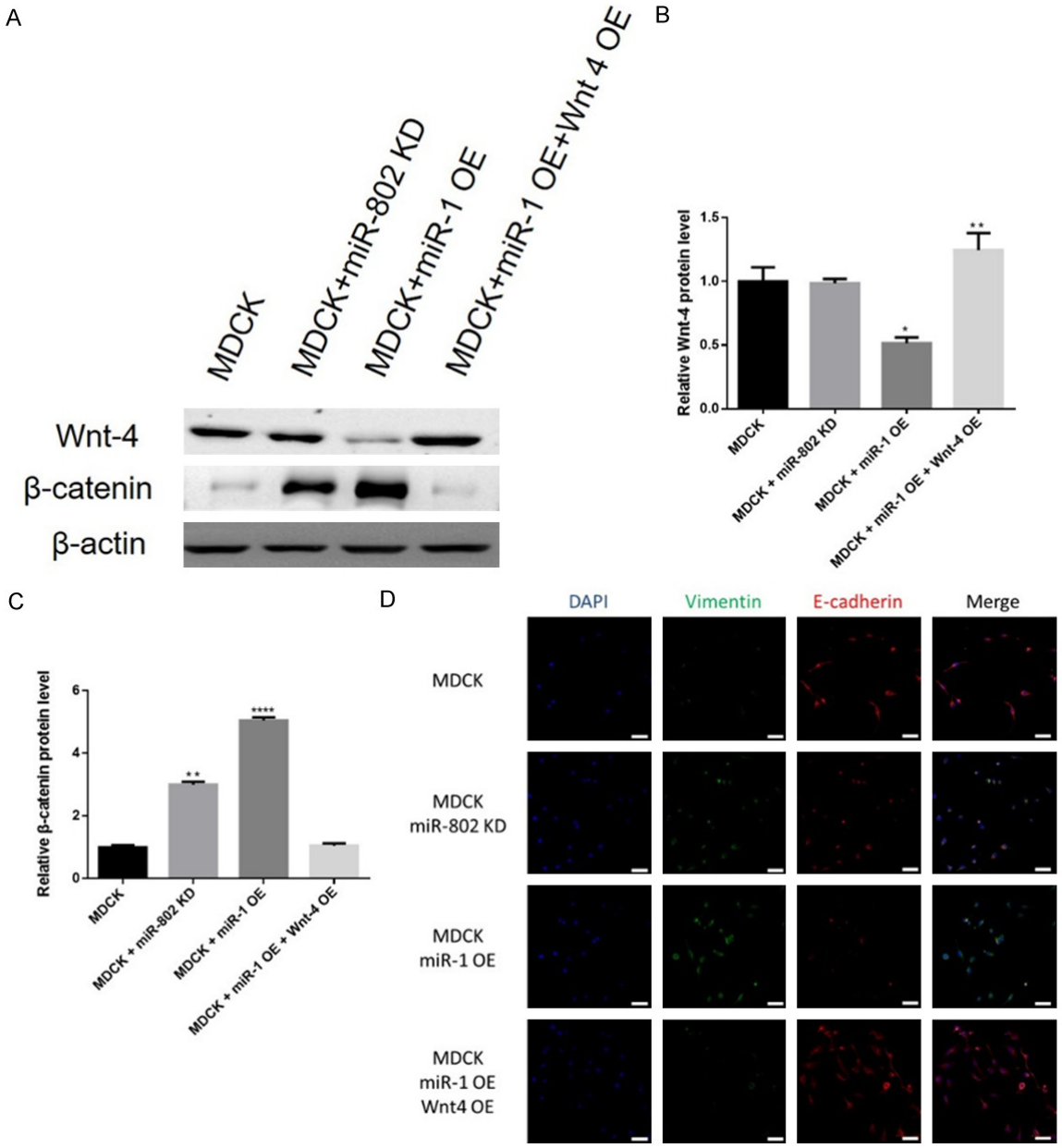

Figure 6. miR-1 and miR-802 targeted Wnt-4 and β-catenin to regulate MET. A: Protein expression level was determined by Western blot. B, C: ImageJ densitometric analysis of the expression levels of indicated proteins. D: Confocal images of MDCK cells of indicated treatment. n=3. Data are presented as the mean ± sem. *P<0.05, **P<0.01, ****P<0.0001.

miR-1 and miR-802 in MET has not been studied well. In the present studies, we found that Wnt-4 is a direct functional effector of miR-1, and β-catenin is a direct functional effector of miR-802. Wnt-4 is a member of the Wnt family, which functions most in the regulation of organism development and growth [22-24]. It was reported that wnt5a-Ror2 signaling play important roles in morphogenesis of the metanephric mesenchyme during ureteric budding [25]. β-catenin is the key factor of Wnt signaling regulation. Perantoni et al. [26] reported that β-catenin is necessary for MET in nephron formation. Consistently, our studies showed that overexpression of Wnt-4 inhibited the expression of β-catenin and promote the MET, while overexpression of β-catenin inhibited MET. Overexpression of miR-1 inhibited MET by negatively regulating Wnt-4. It is reported that Wnt-4 regulated β-catenin expression negative-

ly [27]. So it's possible that during MET, both Wnt-4 and miR-802 may work in concert to regulate β-catenin.

In summary, we demonstrated that miR-1 expression was downregulated and miR-802 expression was upregulated during kidney development and regulate MET through directly targeting Wnt-4 and β-catenin. The newly identified miR-1-Wnt-4/miR-802-β-catenin axis provides a new mechanism of MET and kidney development.

Acknowledgements

This work was supported by the grants from the National Natural Science Foundation of China (31560327 and 31560325).

Disclosure of conflict of interest

None.

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