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LncRNA MEG3 inhibits the growth, invasion and migration of Wilms' tumor *via* Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Long non-coding RNA (lncRNA) maternally expressed 3 (MEG3) has been identified to participate in the progression of malignant tumors. However, the role and function of MEG3 in Wilms' tumor (WT) remain unknown. Therefore, the aim of this study was to detect the role of MEG3 in the development of Wilms' tumor, and to explore the underlying mechanism.

PATIENTS AND METHODS: Expression of MEG3 in WT tissues and blood samples were detected using quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between MEG3 level and clinicopathological character and histogenesis was analyzed. WT-CLS1 and WiT49 cells were cultured *in vitro*, and the influence of ectopic MEG3 expression was determined. Colony formation assay and Edu assay were employed to measure cell proliferation, while transwell assay and Matrigel assay were adopted to detect cell metastasis. Furthermore, Western blot was applied to explore the mechanism of MEG3 in WT.

RESULTS: MEG3 was lowly expressed in WT tissues and blood samples (p<0.05). Over-expression of MEG3 significantly reduced the proliferation, invasion and migration of CLS1cells than control cells (p<0.05). However, inhibition of MEG3 in WiT49 cells significantly promoted cell growth and metastasis compared with cells in negative control group (p<0.05). In addition, MEG3 influenced the protein expression of β-catenin by regulating the Wnt/β-catenin pathway.

CONCLUSIONS: MEG3 was low-expressed in WT tissues and blood samples. Meanwhile, it could inhibit the proliferation and metastasis of WT cells *via* wt/β-catenin pathways. All our findings indicated that MEG3 served as a potential target for the diagnosis, treatment and prognosis prediction of WT.

Key Words:
MEG3, Wilms' tumor (WT), Growth, Metastasis, Wnt/β-catenin.

Introduction

Wilms' tumor (WT), a kind of mixed embryo tumor, is one of the most common malignancy in children. Currently, WT accounts for the vast majority of pediatric diseases requiring surgery, accounting for about 8% of all solid tumors in children[1]. The combination of surgery, chemotherapy and radiation therapy has greatly improved the prognosis of patients with WT[2]. However, the overall cure rate of WT is still not optimistic. Meanwhile, the mechanism of WT tumorigenesis and progression remains unknown.

With the application of high-throughput sequencing technology, more and more long non-coding RNAs (long non-coding RNAs, lncRNAs) have been discovered. LncRNAs are widely involved in cell biological processes, in the form of RNA regulating gene expression at multiple levels including pre- and post-transcription. LncRNA is a type of RNA longer than 200 nucleotides, with no protein-coding function[3,4]. Abnormal expression of lncRNA is closely related to embryo development and malignancies, including WT. LINC00473 mediates the pathogenesis of WT through IKKα expression *via* antagonizing the tumor suppressor miR-195[5]. LncRNA LINP1 regulates Wnt/β-catenin signaling pathway to induce tumorigenesis of WT[6]. Inhibition of lncRNA SNHG6 reduces the proliferation, invasion and migration of WT cells *via* binding miR-15a[7]. Furthermore, three lncRNAs, including DLGAP1 antisense RNA 2 (DLGAP1-AS2), RP11-93B14.6 and RP11554F20.1, can be used to predict the survival of patients with WT[8]. LncRNA maternally expressed 3 (MEG3) is abnormally expressed in many tumors. It acts

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as a tumor suppressor gene in tongue squamous cell carcinoma, gallbladder cancer, liver cancer, and ovarian carcinoma. However, the expression and role of MEG3 in WT have not been fully elucidated[9-12].

Here, in this study, the expression of MEG3 in WT tissues and adjacent normal tissues was first detected. The level of MEG3 in blood samples of WT patients and health subjects was measured as well. By using established WT-CLS1 and WiT49 cells, functional experiments were employed to elucidate the effects of ectopic expression of MEG3 *in vitro*. Furthermore, we found that MEG3 acted as a tumor suppressor gene in WT by regulating the Wnt/β-catenin pathway.

Patients and Methods

Clinical Tissues and Blood Samples

A total of 54 pairs of WT tissues and adjacent normal tissues (at least 2 cm away from the lesion) were collected from The Second Children and Women's Healthcare of Jinan City from December 2016 to June 2018. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). None of the subjects received radiotherapy or chemotherapy before surgery. Blood samples were collected from 54 patients and 54 adjacent healthy volunteers. Informed consent was obtained from patients, volunteers and their families before the study. This investigation was approved by the Ethics Committee of The Second Children and Women's Healthcare of Jinan City.

Cell Culture and Transfection

WT-derived cell lines WT-CLS1 and WiT49 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in McCoy's 5A (Modified) medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 1% Penicillin and 1% Streptomycin (Gibco, Rockville, MD, USA) in an incubator with humidified air containing 5% CO_2. Lentivirus (LV-MEG3, LV-NC) and short harpin RNA (shRNA) (shRNA-MEG3, shRNA-NC) used for cell transfection were purchased from GeneWiz Co., Ltd. (Suzhou, China). The shRNA sequence for MEG3 was: GCTGGTGGTTACCGTGCTAAT-3. WT-CLS1 cells were transfected with LV-MEG3 or LV-NC, while WiT49 cells were treated with shRNA-MEG3 or shRNA-NC according to the instructions of polybrene (GeneWiz, Suzhou, China). Transfection efficiency was verified using quantitative real-time polymerase chain reaction (qRT-PCR).

RNA Extraction and qRT-PCR

Total RNA in tissues, blood samples and cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentration of RNA were measured using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was completed with ABI Prism 7900HT (ABI, Applied Biosystems, Foster City, CA, USA) using SYBR Green Real-Time PCR Mix (Thermo Fisher Scientific, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalizing MEG3 expression. All primers used in this study were as follows: MEG3: 5'-CTCGTTGCTCCCTTGAGTGT-3' (forward), 5'-ACACTACAGCTGTTCCCGTG-3' (reverse); GAPDH: 5'-ACAACTTTGGTATCGTGGAAGG-3' (forward), 5'-GCCATCACGCCACAGTTTC-3' (reverse).

Colony Formation Assay

Colony formation assay was employed to analyze the changes in the ability of WT cells to form clones after intervention of MEG3 expression. Briefly, transfected WT-CLS1 and WiT49 cells in the logarithmic growth phase were first seeded in 60 mm petri dishes at a density of 1,000 cells per dish. After 2 weeks of culture, the cells were fixed with 5 ml of methanol for 15 minutes and stained with crystal violet solution for 20 minutes. Number of colonies containing more than 50 cells was counted. This experiment was repeated for 3 times.

5-Ethynyl-2'- Deoxyuridine (EdU) Assay

Cell proliferation was measured in strict accordance with the EdU Cell Proliferation Assay Kit (Ruibo, Guangzhou, China). Briefly, transfected WT-CLS1 and WiT49 cells were stained with 50 μmol/L of EdU reagent for 2 h, followed by fixation and permeabilization. Next, stained WT-CLS1 and WiT49 cells were analyzed using flow cytometry (BDLSR Fortessa, Becton Dickinson,

Franklin Lakes, NJ, USA). The proportion (%) of EdU-positive WT-CLS1 and WiT49 cells was analyzed by FACS Diva software.

Transwell and Matrigel Assay

Transwell and Matrigel assays were utilized to detect the migration and invasion of WT-CLS1 and WiT49 cells, respectively. The 8-μm insert (Corning, Corning, NY, USA) was bought for the detection. For transwell assay, the chamber was placed in a 24-well plate prepared with Matrigel and incubated in an incubator under the above conditions overnight. A total of 500 μL of 10% FBS McCoy's 5A (Modified) medium was added to the lower chamber of the transwell insert. WT-CLS1 or WiT49 cells in each group were resuspended in serum-free FBS McCoy's 5A (Modified) medium, and 100 μL of cell suspension (about 1×10^5 cells in total) was added to the upper chamber. After 48 h culture, the medium in the lower chamber was removed and the insert was fixed in 4% paraformaldehyde for 15 minutes. Cells on the top surface of chamber membrane were wiped off using cotton swabs. Next, the cells were stained with crystal violet solution (0.1%) for 10 minutes, followed by rinsing with phosphate-buffered saline (PBS). Migrating cells were observed under a microscope, and 5 fields of vision were randomly selected for each sample.

Western Blot

Total protein in WT-CLS1 or WiT49 cells was extracted using radioimmunoprecipitation assay (RIPA) reagent containing protease inhibitor cocktail (Roche, Basel, Switzerland). The concentration of extracted protein was measured by the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). A total of 50 μg protein samples were separated by sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk powder in Tris-Buffered Saline and Tween-20 (TBST) at room temperature for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were washed with TBST for 3 times (with 10 minutes for each), the membranes were immersed in horseradish peroxidase-labeled secondary antibody (1:3000) at room temperature for 1 h. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA). All primer antibodies used in this study were obtained from Cell Signaling Technology, lnc (CST; Danvers, MA, USA), including: anti-β-catenin (1:2000), anti-Cyclin D1 (1:1000), anti-C-Jun (1:1000), anti-MMP7 (1:1000) and anti-GAPDH (1:2000). GAPDH was used as an internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., IBM, Armonk, NY, USA) was used for statistical analysis. GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA) was applied for image editing. Differences between two groups were analyzed by using the Student's *t*-test. Comparison among multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

MEG3 was Down-Regulated in WT Tissues and Blood Samples

To detect the expression of MEG3 in WT, we first collected 54 pairs of WT tissues and adjacent normal tissues. As shown in Figure 1A, qRT-PCR results indicated that the expression of MEG3 in WT tissues was significantly lower than that in adjacent normal tissues ($p<0.05$). This indicated that MEG3 acted as a tumor suppressor gene in WT. Blood samples were collected from 54 WT patients and 54 healthy volunteers as well. The results demonstrated that MEG3 was lowly expressed in the blood samples of WT patients compared with healthy volunteers ($p<0.05$, Figure 1B). These findings suggested that MEG3 might act as an anti-tumor biomarker in WT.

MEG3 was Associated with Clinicopathological Characteristics of WT

Clinicopathological features were collected from 54 WT patients, including age, gender, laterality, histological type, lymph node metastasis and NWTS-5 stage. The relationship between the characteristics of WT patients and MEG3 expression was analyzed. As shown in Table I, no statistically significant difference was observed between high MEG3 expression group and low MEG3 expression in terms of

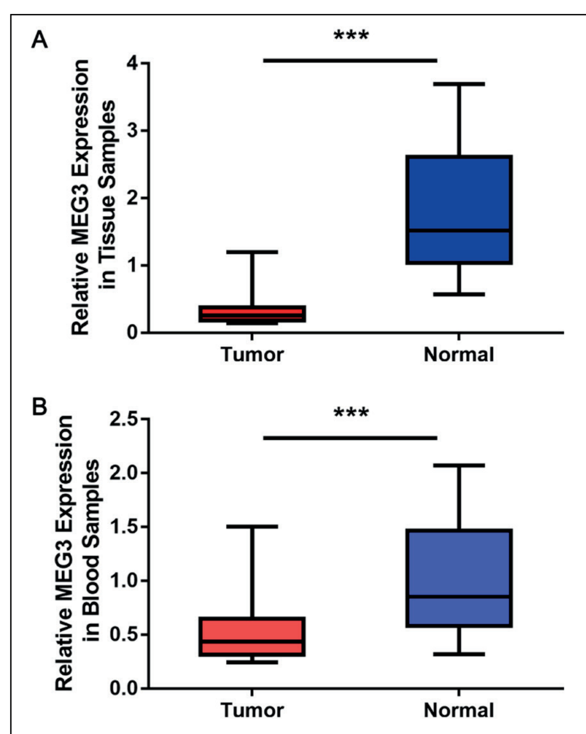

Figure 1. MEG3 was low-expressed in WT tissues and blood samples. **A,** QRT-PCR showed the expression of MEG3 in 54 pairs of WT tissues and normal tissues. **B,** MEG3 expression in blood samples of WT patients and health volunteers was detected using qRT-PCR. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ compared to control group.

age, gender and laterality ($p>0.05$). However, there were significant differences in histological type, lymph node metastasis and NWTS-5 stage between high MEG3 expression group and low MEG3 expression group ($p<0.05$). WT patients with lower MEG3 expression exhibited significantly more malignant histological types and lymph node metastases, and worse NWTS-5 stage ($p<0.05$). These findings indicated that MEG3 was associated with WT progression.

MEG3 Affected Proliferation of WT Cells

To explore the function of MEG3 in WT, WT-CLS1 cells and WiT49 cells were cultured *in vitro*. Using LV-MEG3 or shRNA-MEG3, we over- or down-expressed MEG3 in WT-CLS1 cells or WiT49 cells, respectively. MEG3 was highly expressed in LV-MEG3 treated WT-CLS1 cells, while was lowly expressed in shRNA-MEG3 treated WiT49 cells compared with LV-NC or shRNA-NC group, respectively ($p<0.05$, Figure 2A, 2B). Subsequently, the influence of MEG3 on cell proliferation ability was detected by colony formation assay. The number of formed colonies decreased significantly in WT-CLS1 cells with MEG3 over-expression ($p<0.05$, Figure 2C. In contrast, WiT49 cells showed mark-

Table I. Correlation between MEG3 level and clinicopathological characteristics in Wilms' tumor.

Characteristics	Total	MEG3 expression		*p*-value
		High	Low	
Age				
< 24 months	33	18	15	0.4023
> 24 months	21	9	12	
Gender				
Male	29	14	15	0.7849
Female	25	13	12	
Laterality				
Left	26	14	12	0.8519
Right	24	11	13	
Both	4	2	2	
Histological type				
FH	40	25	15	0.0019*
UH	14	2	12	
Lymph node metastasis				
Yes	19	2	17	0.0000*
No	35	25	10	
NWTS-5 stage				
I-II	33	23	10	0.0003*
III-IV	21	4	17	

FH, Favorable histology; UH, Unfavorable histology; NWTS, National Wilms Tumor Study. The expression level of MEG3 was cut off by median expression level and *indicated $p<0.05$.

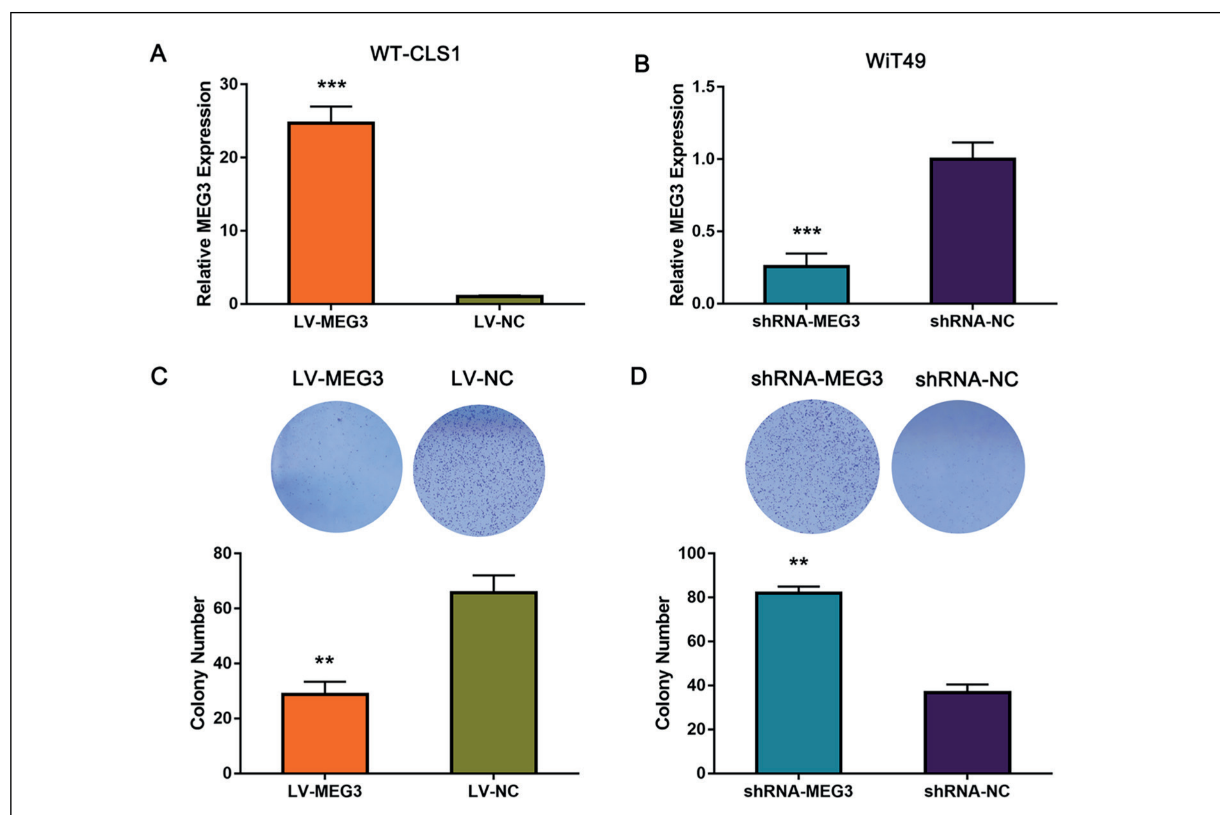

Figure 2. MEG3 affected the colony formation ability of WT cells. **A,** LV-MEG3 and LV-NC were transfected into WT-CLS1 cells. **B,** Oligonucleotides targeting MEG3 (shRNA-MEG3) and negative controls (shRNA-NC) were transfected into WiT49 cells. **C, D,** Clone formation assay showed the proliferation ability of WT-CLS1 cells transfected with LV-MEG3 and LV-NC or WiT49 cells transfected with shRNA-MEG3 and shRNA-NC (magnification: 40×).

edly enhanced colony formation ability after MEG3 inhibition ($p<0.05$, Figure 2D). Similarly, we employed EdU assay to verify the effect of MEG3 on cell growth. Up-regulation of MEG3 remarkably reduced the proliferation of WT-CLS1 cells, while down-regulation of MEG3 significantly promoted the growth of WiT49 cells ($p<0.05$, Figure 3A, 3B). These results indicated that MEG3 could reduce the proliferation of WT cells.

MEG3 Influenced Invasion and Migration of WT Cells

We investigated the effect of MEG3 on the invasion and migration of WT cells using transwell and Matrigel assays. As predicted, LV-MEG3 transfection significantly inhibited the invasion of WT-CLS1 cells compared with LV-NC group ($p<0.05$, Figure 4A). However, shRNA-MEG3 treatment promoted the invasion of WiT49 cells compared with shRNA-NC group ($p<0.05$, Figure 4B). Homoplastically, Matrigel assay elucidated that over-expression or down-expression of MEG3 significantly reduced or promoted the invasion of WT cells, respectively ($p<0.05$, Figure 4C, 4D). These results suggested MEG3 inhibited the invasion and migration of WT.

MEG3 Regulated the Function of Wnt/β-Catenin Pathway

The above findings demonstrated that MEG3 could inhibit the proliferation and metastasis of WT cells. Subsequently, we explored the possible underlying mechanism of MEG3. Several studies have proved that MEG3 inhibits the canonial Wnt pathway to influence the expression of downstream molecules. Here, we detected the expression of several downstream proteins of Wnt pathway, including: β-catenin, MMP7, Cyclin D1, and C-Jun. The results indicated that β-catenin decreased significantly in MEG3

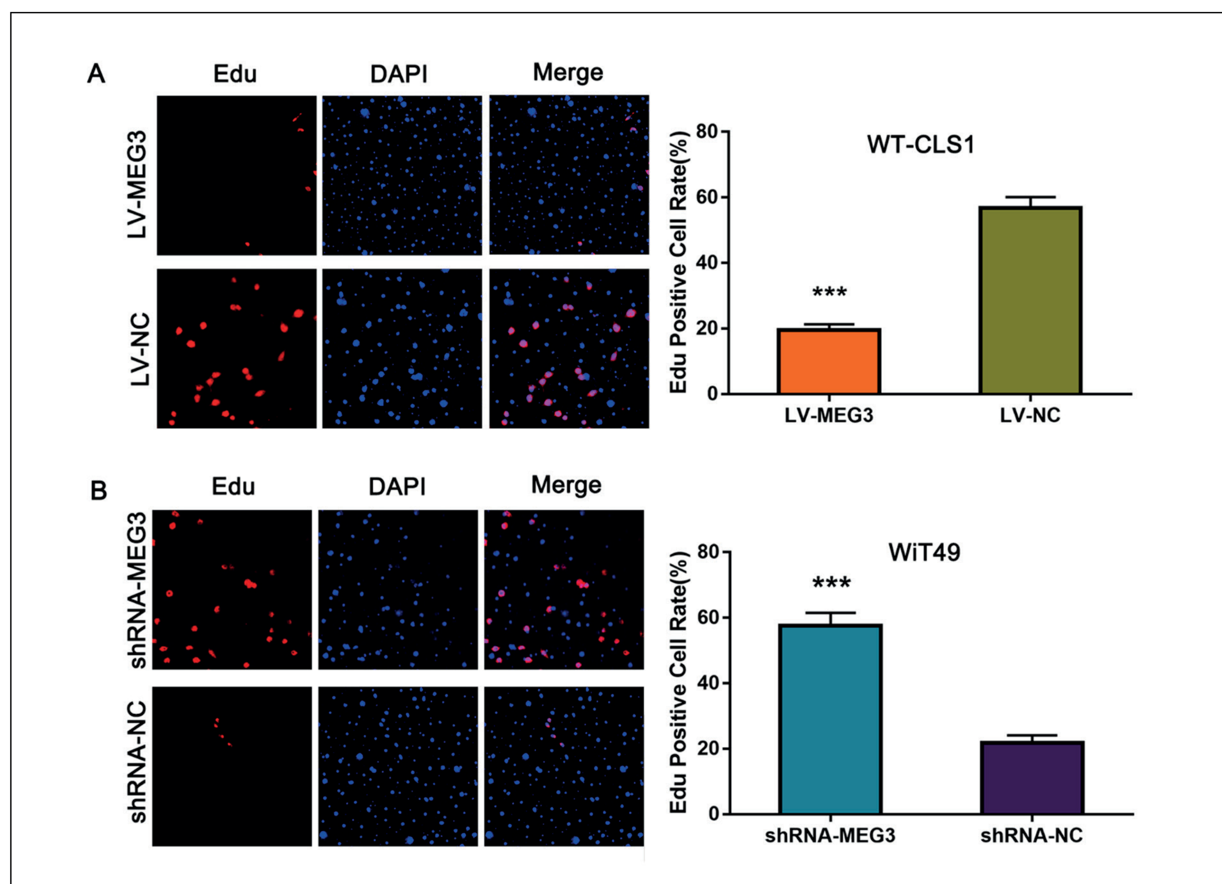

Figure 3. MEG3 affected the proliferation of WT cells. **A, B,** EdU assay showed the proliferation ability of WT-CLS1 cells transfected with LV-MEG3 and LV-NC or WiT49 cells transfected with shRNA-MEG3 and shRNA-NC, (magnification: 400×). $^{**}p < 0.01$, $^{**}p < 0.05$ compared to control group.

over-expressed WT-CLS1 cells but increased significantly in MEG3 down-expressed WiT49 cells ($p<0.05$, Figure 5A-5C). This indicated that MEG3 might affect canonial Wnt pathway in WT as well. MEG3 up-regulation or down-regulation remarkably inhibited or elevated the expressions of MMP7, Cyclin D1 and C-Jun, respectively ($p<0.05$, Figure 5A-5C). These results showed that MEG3 regulated Wnt/β-catenin pathway to inhibit the progression of WT.

Discussions

WT is the most common malignant tumor in children's kidney, accounting for about 95% of children's kidney tumors. The occurrence and progression of WT are a complicated biological process, involving multiple factors and genes[13]. Non-coding RNAs, including miRNA and lncRNA, have been identified to participate in the regulation of WT development and progression[14]. In the present study, we demonstrated that MEG3 acted as a tumor suppressor gene in WT. All our findings might provide a novel sight for the exploration of WT tumorigenesis and metastasis. As a tumor suppressor gene, MEG3 has been proved to regulate the development of prostate cancer through facilitating H3K27 trimethylation of EN2 *via* binding to EZH2[15]. Meanwhile, it influences the growth and migration of colorectal cancer cells by inhibiting miR-376/PRKD1 signaling pathway[16]. In glioma, MEG3 suppresses cell proliferation *via* miR-96-5p/MTSS1 axis[17]. Moreover, MEG3 induces cell apoptosis and reduces cell proliferation in laryngeal cancer by regulating miR-23a/APAF-1 pathway[18].

In this study, we first detected the expression of MEG3 in 54 pairs of WT tissues and adjacent

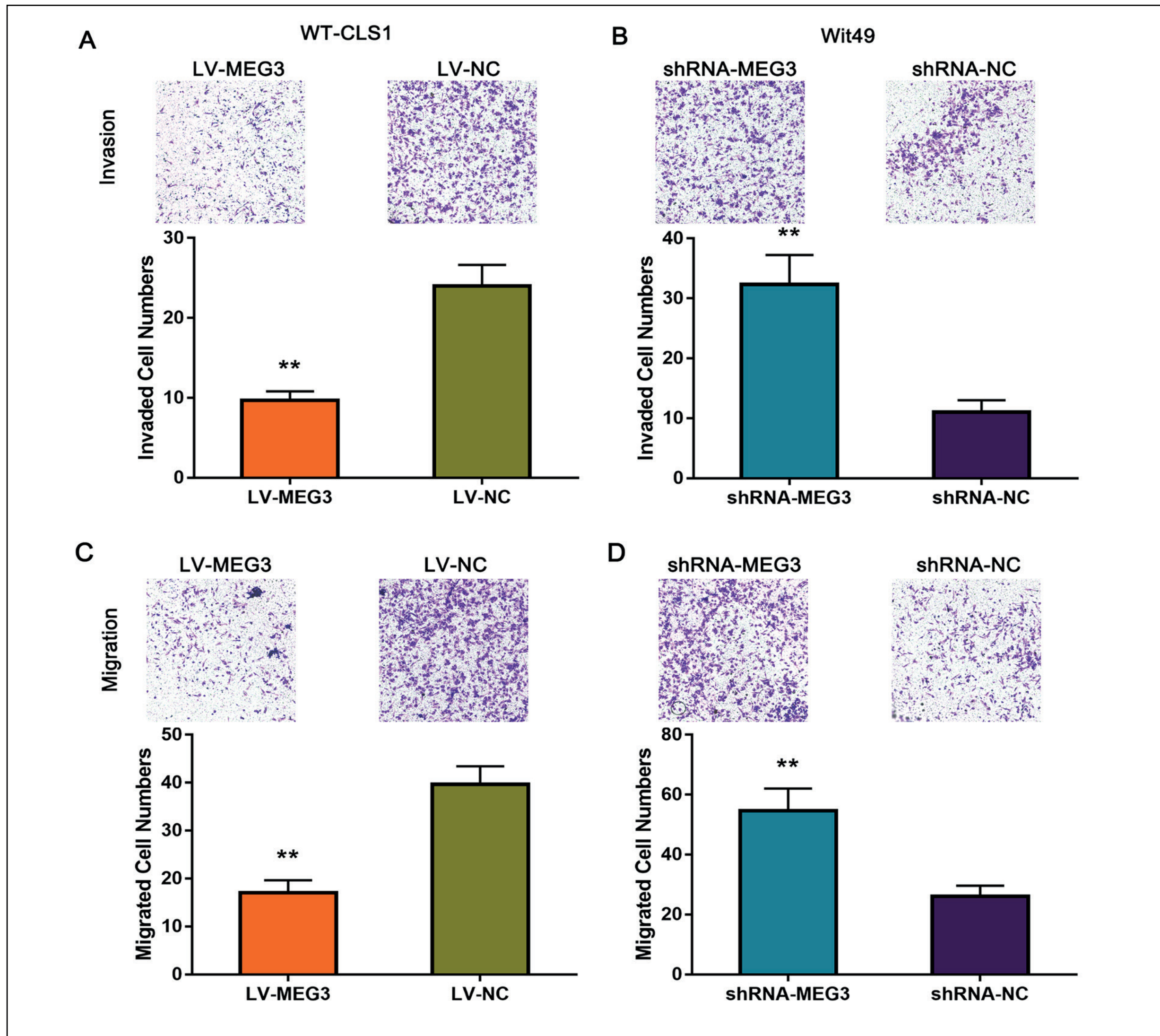

Figure 4. MEG3 influenced the invasion and migration of WT cells. **A, B,** Transwell invasion assay indicated the number of invasive WT-CLS1 and WiT49 cells (magnification: 40×). **C, D,** Matrigel assay showed the number of migrated WT-CLS1 cells and WiT49 cells, (magnification: 40×). $^{**}p < 0.01$, $^{*}p < 0.05$ compared to control group.

normal tissues. MEG3 was highly expressed in WT tissues compared with adjacent normal tissues. Therefore, we suggested that MEG3 acted as a tumor suppressor gene in WT. Similarly, the expression of MEG3 in WT patients' blood samples was obviously higher than paired healthy volunteers. Subsequently, the relationship of MEG3 level with clinicopathological characteristics was analyzed. The results demonstrated that lower MEG expression indicated poor histological types, more lymph node metastases, and worse NWTS-5 stage. These results all indicated that MEG3 served as a suppressed gene in WT. Next, we confirmed that overexpression of significantly inhibited the proliferation, invasion, and metastasis of WT cells. In contrast, knockdown of MEG3 significantly promoted the growth and metastasis of WiT49 cells. These results show that MEG3 plays the role of tumor suppressor in WT. Previous studies have shown that MEG3 regulates the Wnt pathway to play its role in other malignant tumors. In the present study, we verified the expressions of Wnt pathway markers in WT[19-21]. The results demonstrated that the protein level of β-catenin was significantly inhibited in WT-CLS1 cells over-expressing MEG3, leading to decreased expressions of MMP-7, C-Jun and Cyclin D1. Conversely, in WiT49 cells, expression

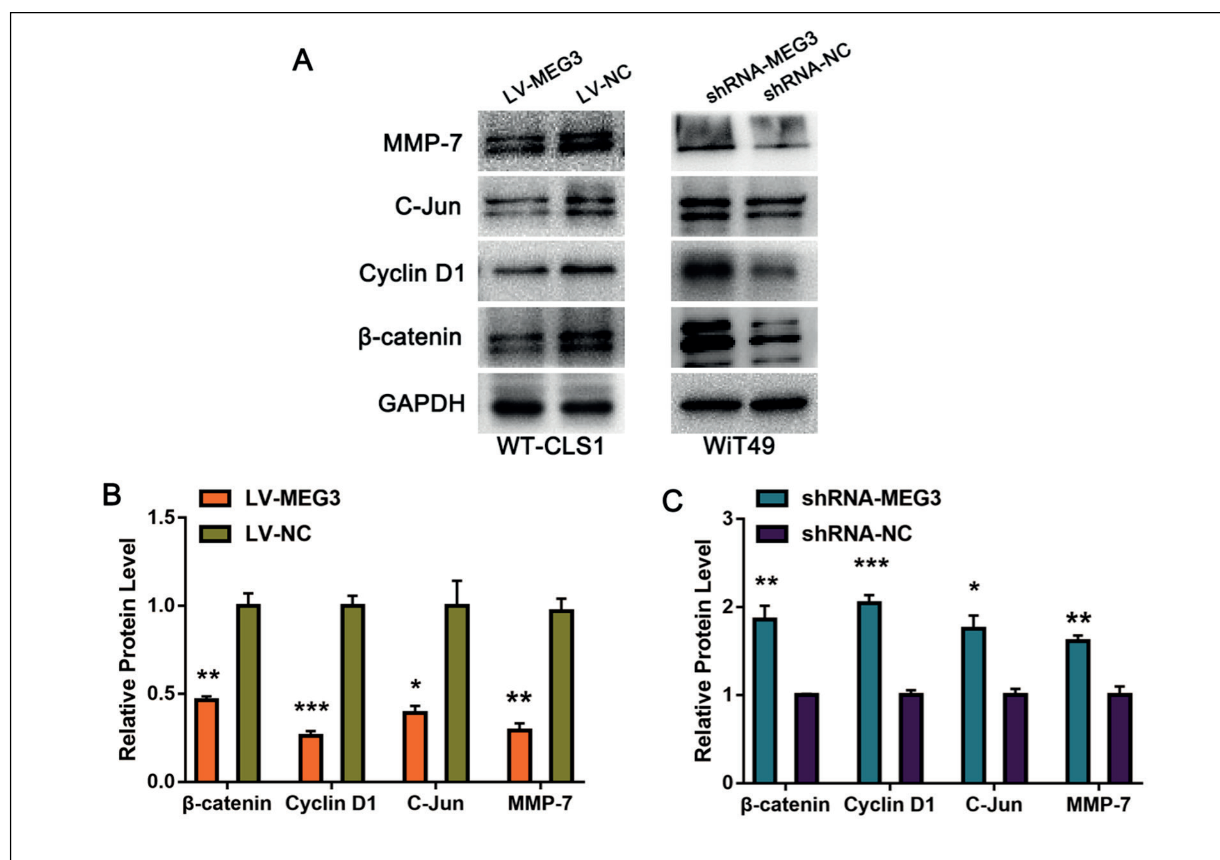

Figure 5. MEG3 inhibited Wnt/β-catenin axis. **A,** The sequences of SOCS5 mRNA 3'-UTR and miR-18a, including wild type and mutant binding site. **B,** Luciferase reporter assay indicated the molecular bound within SOCS5 and miR-18a. **C,** Western blot assay indicated the protein expressions of SOCS5 and EMT markers in WT-CLS1 cells and WiT49 cells. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to control group.

of β-catenin was obviously elevated by MEG3 inhibition, resulting in the up-regulation of MMP-7, C-Jun and Cyclin D1. Numerous studies have shown that the canonical Wnt pathway plays a crucial role in the occurrence and development of WT[22,23]. Meanwhile, it determines lineage specificity in WT[24]. Studies have also shown that WT can be used as candidate neoplasia for treatment with Wnt/β-catenin pathway modulators[25]. Therefore, our results might dedicate a potential therapeutic target for the treatment of WT, especially for the regulation of Wnt/β-catenin.

Conclusions

Taken together, our results demonstrated for the first time that MEG3 was lowly expressed in WT tissues and blood samples. Over-expression of MEG3 significantly decreased the proliferation, invasion and migration of WT cells *via* regulating Wnt/β-catenin pathway. The novelty of this study was that all our findings might provide a novel target for the treatment of WT.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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