FOXO3a inhibits nephroblastoma cell proliferation, migration and invasion, and induces apoptosis through downregulating the Wnt/β-catenin signaling pathway

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Abstract. Forkhead transcription factor O subfamily 3A (FOXO3a) is an important tumor suppressor gene that is expressed in renal tissue and has been reported to be downregulated in clear cell renal cell carcinoma (CCRCC). Notably, the overexpression of FOXO3a was previously discovered to inhibit the progression of CCRCC. However, the expression levels of FOXO3a in nephroblastoma cell lines remain unknown. The present study aimed to investigate the expression levels of FOXO3a in nephroblastoma cell lines and to determine the mechanism of action of the biological functions of FOXO3a. Western blotting and reverse transcription-quantitative PCR were used to analyze the expression levels of FOXO3a in nephroblastoma cell lines. Subsequently, the effects of the overexpression of FOXO3a and the genetic knockdown of the Wnt/β-catenin signaling protein Axin-2 on the biological functions were determined through Cell Counting Kit-8, cell colony formation assays, scratch and Transwell assay and flow cytometric analysis experiments. The expression levels of FOXO3a were discovered to be downregulated in nephroblastoma cell lines. The overexpression of FOXO3a inhibited the proliferation, invasion and migration of nephroblastoma cells, while inducing apoptosis. Furthermore, the overexpression of FOXO3a downregulated the expression levels of β-catenin and Cyclin-D1 proteins involved in the Wnt/β-catenin signaling pathway. Cell proliferation and the migration and invasion ability of 17-94 cells in shRNA-Axin2-2 group were promoted. Cell apoptosis was predominantly increased by overexpressed FOXO3a, which was reversed by shRNA-Axin2-1. The biological effects of overexpressing FOXO3a on nephroblastoma were reversed after activation of Wnt/β-catenin. In conclusion, the findings of the present study suggested that FOXO3a may inhibit nephroblastoma cell proliferation, migration and invasion, while inducing apoptosis, by downregulating the Wnt/β-catenin signaling pathway. These results may provide a novel method for the early diagnosis and precise treatment of nephroblastoma.

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Introduction

Wilms tumor, also known as nephroblastoma, is the most common type of renal malignancy diagnosed in children (1-3). In the past few years, the treatment of nephroblastoma has significantly improved following the application of various treatment regimens and techniques, including surgery, radiotherapy, chemotherapy and autologous stem cell transplantation (4). Thus, nephroblastoma is one of the most successful types of pediatric malignant tumor in terms of prognosis (5). At present, the 5-year survival rate of patients with nephroblastoma has reached 90%, but there remains 10% of children who die due to recurrence, metastasis and insensitivity to chemotherapy drugs (6-7). However, the current understanding of the pathogenesis and metastatic mechanism of action of nephroblastoma is insufficient, and corresponding effective targeted treatments are lacking. Therefore, current clinical research is focused on investigating more effective targeted treatments for nephroblastoma to reduce its metastatic and recurrence rate.

The forkhead transcription factor O (FOXO) family, also named FKHR, consists of four subtypes: FOXO1/FKHR/FOXO subfamily 1a (FOXO1a), FOXO3/FKHRL1/FOXO3a, FOXO4/AFX and FOXO6, which have similar structures, functions and regulatory mechanisms (8). FOXOs are an essential protein family that have been discovered to regulate cell apoptosis, the cell cycle, DNA damage repair, oxidative stress, energy metabolism and longevity, and cancer development, amongst other cell functions (9-11). In particular, FOXO3a is a tumor suppressor gene belonging to the FOXO family (12), which has been reported to control various signaling pathways and biological processes of tumor cells. FOXO3a can inhibit the invasion of breast cancer cells by activating ER-α signaling pathway (13). The low expression of FOXO3a may promote

the invasion and migration of non-small cell lung cancer cells through PI3K/Akt signaling pathway (14). It was found that FOXO3a can induce EMT and promote the metastasis of renal cancer cell (15).

Downregulated expression levels of FOXO3a have been discovered to be associated with the occurrence, progression and drug resistance of breast, pancreatic, liver and bladder cancer, amongst other types of tumor (16-18). Notably, it has been reported that FOXO3a may exert an antitumor effect in breast cancer, non-small cell lung cancer and renal cancer (19).

The Wnt and PI3K/AKT signaling pathways are reported to be closely related to the development of nephroblastoma (20). AKT serves an important role in a variety of interrelated cellular signaling mechanisms involved in cellular metabolism, growth and division, the inhibition of apoptosis and angiogenesis (21,22). The activation of Wnt/β-Catenin has been identified to have a crucial role in tumor development (23,24). β-catenin is a multifunctional protein, which has not only been discovered to serve as the main structural component of cell adhesion, but also to participate in embryogenesis and tumor formation (25). However, to the best of our knowledge, the relationship between nephroblastoma and the Wnt signaling pathway remains poorly understood, and the biological role and pathogenic mechanism of the Wnt signaling pathway in the development of nephroblastoma has not been reported. Since nephroblastoma is an embryonic renal tumor, abnormalities during renal development have been identified to be closely associated with its occurrence (26). As the Wnt signaling pathway is an important signaling transduction pathway involved in the process of renal development (27-29), it may be of great significance to investigate the influence of the abnormalities in the Wnt signaling pathway in association with the occurrence of nephroblastoma.

The present study aimed to analyze the expression levels of FOXO3a in nephroblastoma and to determine the role of FOXO3a in the proliferation, migration and invasion of nephroblastoma. The results suggested that FOXO3a may regulate the Wnt/β-catenin signaling pathway to inhibit nephroblastoma progression, thus providing a potential, novel therapeutic target for the treatment of the disease.

Materials and methods

Cell culture and transfection. The normal human renal cell line (HK-2) and nephroblastoma cell lines (17-94, AG01615, HFWT, WILTU-1 and WIT49) were obtained from the American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin, and maintained at 37˚C in an humidified atmosphere of 5% CO_2.

For cell transfection, $1x10^5$ 17-94 cells/well were plated into six-well plates and transfected with overexpression (OE)-FOXO3a, overexpression (OE)-NC, short hairpin RNA (shRNA)-targeting Axin-2 (shRNA-Axin-2-1 and shRNA-Axin-2-2) and shRNA-NC (100 nM) were using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's protocol. All plasmids were supplied from Guangzhou RiboBio Co., Ltd. Subsequent experiments were performed 48 h after transfection.

Cell Counting Kit-8 (CCK-8) assay. A total of $5x10^3$ 17-94 cells/well were plated into 96-well plates. Following 24, 48 or 72 h of incubation at 37˚C, a CCK-8 kit (Dojindo Molecular Technologies, Inc.) was used to analyze the proliferative capacity/cell viability of transfected 17-94 cells, according to manufacturer's protocol. Briefly, 10 μl CCK-8 reagent was added/well and incubated for 1 h at 37˚C. The absorbance was subsequently measured at a wavelength of 450 nm using a microplate reader.

Colony formation assay. Following 48 h of transfection at 37˚C, 800 cells were seeded into 6-well plates and incubated at 37˚C in complete medium for 21 days with the medium changed every 3 days. Following the incubation at 37˚C, these cells were fixed with the 70% ethanol solution and the plates were stained with 0.5% crystal violet at room temperature for 20 min. (Santa Cruz Biotechnology, Inc.).

Wound healing assay. A total of $5x10^5$ 17-94 cells/well were plated into six-well plates and cultured until 100% confluence in complete medium at 37˚C. Subsequently, the monolayer of cells was scratched by a sterilized pipette tip (20 μl) and the cells were incubated in serum-free DMEM for 24 h at 37˚C. The migratory distance of the cells was observed under a light microscope (magnification, x200; Olympus Corporation) at 0 h (and the scratch width is recorded as W0.) and 24 h (the scratch width was recorded as W24), and analyzed using ImageJ version 1.49 software (National Institutes of Health). The migration rate was calculated as Migration rate=$(W_{24}-W_0)/W_0x100\%$.

Transwell Matrigel assay. A total of $1x10^4$ cells/well were plated into the upper chambers of 24-well Transwell plates (8.0-μm PET membrane; Corning, Inc.) in 400 μl serum-free DMEM. The membranes were precoated with Matrigel at 37˚C for 2 h. The lower chambers were filled with 600 μl DMEM supplemented with 10% FBS. Following incubation for 24 h at 37˚C, the cells on the bottom of the lower chamber were fixed with 90% ethanol solution for 30 min at 37˚C and stained with 0.1% crystal violet for 10 min at room temperature. The invasive cells were visualized using a light microscope (Olympus FV500; Olympus Corporation, magnification, x200) and analyzed using ImageJ version 1.49 software (National Institutes of health).

Flow cytometric analysis of apoptosis. The rate of cell apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) flow cytometry assay kit (BD Biosciences), according to the manufacturer's protocol. Briefly, cells were centrifuged at ~200 x g for 3-5 min for precipitation and collection, then fixed in precooled 70% ethanol at 4˚C overnight. The cells were then resuspended in 300 μl cold binding buffer and incubated with 5 μl Annexin V-FITC for 10 min in the dark at room temperature. Following the addition of 5 μl PI and 200 μl binding buffer, the samples were incubated in the dark at room temperature for a further 5 min. Apoptotic cells were subsequently analyzed using the FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using BD Accuri C6 (BD Biosciences). The percentage of early and late apoptotic cells was calculated. The experiments were independently repeated 3 times.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from 17-94 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at -40˚C. Total RNA was reverse transcribed into cDNA using the TaqMan Reverse Transcription kit (Takara Bio, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed using a SYBR Taq kit (Roche Diagnostics) to detect the relative expression of FOXO3a and AXIN2 on an a Bi 7500 real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Primers were FOXO3a, forward, 5'-CGGACAAACGGCTCACTCT-3' and reverse, 5'-GGACCCGCATGAATCGACTAT-3'; β-actin, forward, 5'-ATCACCATTGGCAATGAGCG-3' and reverse, 5'-TTGAAGGTAGTTTCGTGGAT-3'; AXIN2, forward, 5'-CACGGAAACTGTTGACAGTGGATAC-3' and reverse, 5'-GGTGGCTGGTGCAAAGACATAG-3'; GAPDH, forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-GTGA AGACGCCAGTGGA-3'. Gene expression levels were then normalized to that of GAPDH, whereas fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (30).

Western blotting. Total protein was extracted from 17-94 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) with protease inhibitors (Roche Diagnostics). Total protein was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) and 30 μg protein/lane was separated using 10% SDS-PAGE. The proteins were subsequently transferred onto PVDF membranes (EMD Millipore) and blocked with 5% non-fat milk for 2 h at room temperature. The membranes were then incubated overnight at 4˚C with the following primary antibodies: Anti-FOXO3A (1:5,000; cat. no. ab109629; Abcam); anti-MMP2 (1:4,000; cat. no. ab92536; Abcam); anti-MMP9 (1:10,000; cat. no. ab76003; Abcam); anti-MMP13 (1:1,000; cat. no. ab51072; Abcam); anti-Caspase-3 (1:500; cat. no. ab13847; Abcam); anti-Bim (1:1,000; cat. no. ab32158; Abcam); anti-β Catenin (1:1,000; cat. no. ab16051; Abcam); Rabbit anti-Cyclin D1 (1:200; cat. no. ab16663; Abcam); anti-Axin 2 (1:500; cat. no. ab32197; Abcam); Anti-Bcl-2 (1:1,000; cat. no. 3498; Cell Signaling Technology, Inc.), anti-Bax (1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), anti-cleaved-caspase-3 (1:500; cat. no. 9661; Cell Signaling Technology, Inc.) and anti-GAPDH (1:2,000; cat. no. MAB374; EMD Millipore). Following the primary antibody incubation, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:10,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) secondary antibodies at room temperature for 2 h. Densitometric analysis was performed using ImageJ software (version 1.49; National Institutes of Health). Protein bands were detected using an ECL detection reagent (EMD Millipore) and protein expression levels were normalized to GAPDH. The experiments were performed in triplicate.

Statistical analysis. All data are presented as the mean ± SEM and all experiments were repeated three times. Statistical analysis was performed using GraphPad 6.0 software (GraphPad Software, Inc). Statistical differences among groups were determined using a one-way ANOVA followed by a Tukey's or Dunnett's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FOXO3a expression levels are significantly downregulated in nephroblastoma cells. To investigate the expression levels of FOXO3a in nephroblastoma cells, 17-94, AG01615, HFWT, WILTU-1 and WIT49, and the normal kidney cell HK-2, RT-qPCR and western blotting were performed. The expression levels of FOXO3a were significantly downregulated at both the mRNA and protein level in nephroblastoma cells compared with the normal kidney HK-2 cells (Fig. 1A-C). The expression levels of FOXO3a in 17-94 cells were downregulated the most, thus, 17-94 cells were used for subsequent experiments.

Overexpression of FOXO3a inhibits nephroblastoma cell proliferation, cell invasion and migration. To determine the role of FOXO3a in nephroblastoma cells, OE-FOXO3a and OE-NC plasmids were transfected into 17-94 cells. The results of the RT-qPCR and western blotting demonstrated that the OE-FOXO3a plasmid was successfully transfected into 17-94 cells; the expression levels of FOXO3a were significantly upregulated in the OE-FOXO3a group compared with the control and OE-NC groups (Fig. 1D-F). The results of the CCK-8 assay indicated that the cell proliferation rate was significantly reduced in 17-94 cells transfected with the OE-FOXO3a plasmid compared with the control and OE-NC groups (Fig. 1G). Similar results were observed in the colony formation abilities of 17-94 cells (Fig. 1H). Furthermore, the overexpression of FOXO3a significantly reduced the invasive and migratory abilities of 17-94 cells compared with the OE-NC and control groups (Fig. 2A-D). Western blotting results also indicated that the overexpression of FOXO3a significantly downregulated the expression levels of the invasion and migration-related proteins, matrix metalloproteinase (MMP)2, MMP9 and MMP13 in 17-94 cells compared with the control and OE-NC groups (Fig. 2E).

Overexpression of FOXO3a promotes nephroblastoma cell apoptosis. Flow cytometric analysis was used to detect the rate of cell apoptosis. The apoptotic rate was significantly increased in the 17-94 cells overexpressing FOXO3a compared with the control and OE-NC groups (Fig. 3A and B). To further verify the role of FOXO3a in cell apoptosis, western blotting was used to detect the expression levels of the apoptosis-related proteins, Bax, Bcl-2, Bim, cleaved caspase-3 and caspase-3. The results revealed that the protein expression levels of Bax, Bim and cleaved caspase-3 were significantly upregulated, while those of the Bcl-2 protein were significantly downregulated in the OE-FOXO3a group compared with the control and OE-NC groups (Fig. 3C).

FOXO3a-induced suppression over cell proliferation, migration and invasion is regulated by Wnt/β-catenin signaling. To further investigate whether FOXO3a regulated cell viability, migration and invasion by activating Wnt/β-catenin signaling, western blotting was used to

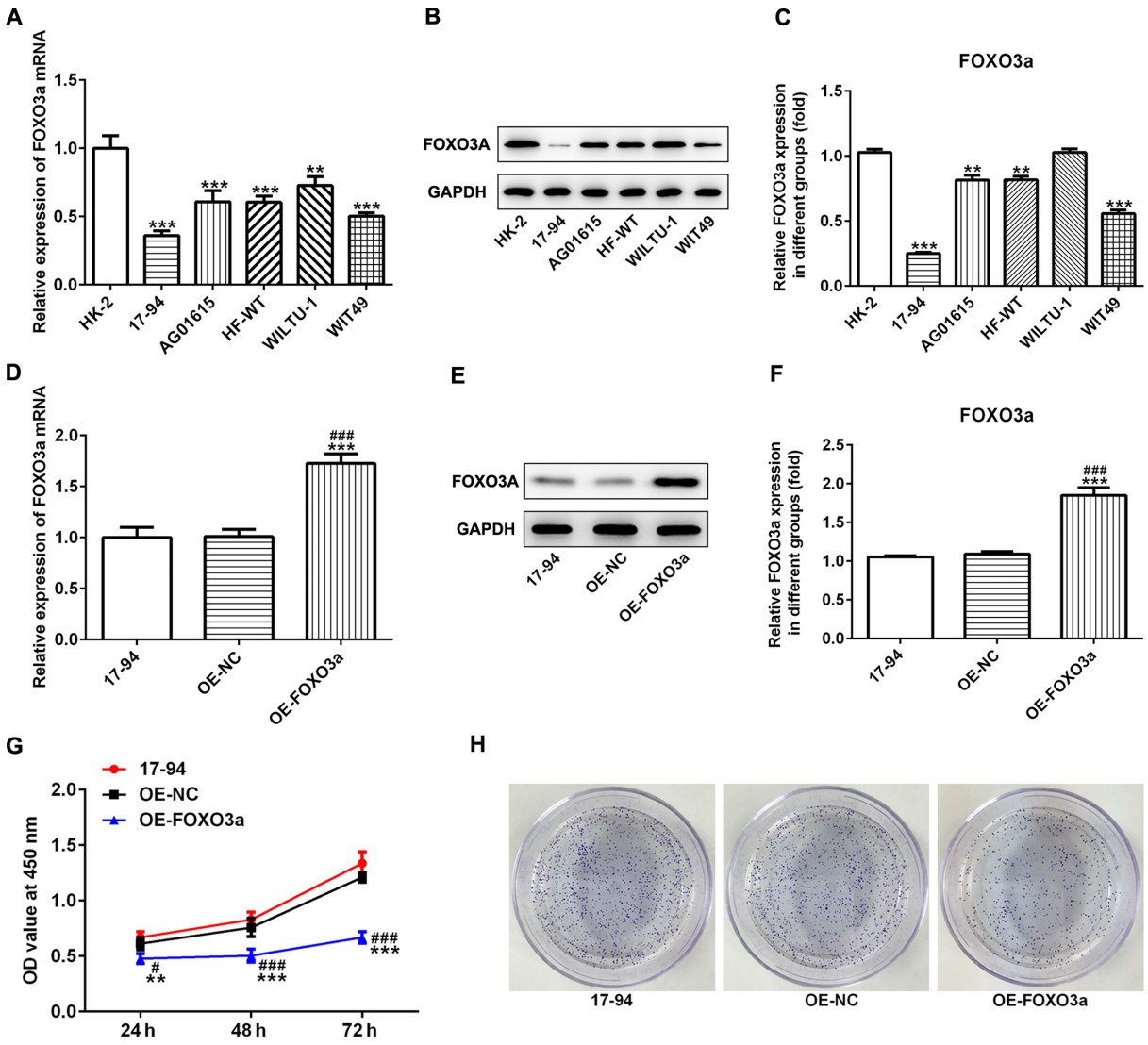

Figure 1. Overexpression of FOXO3a inhibits the proliferation of nephroblastoma cells. Expression levels of FOXO3a in nephroblastoma cell lines (17-94, AG01615, HFWT, WILTU-1 and WIT49) and the normal kidney cell line HK-2 were analyzed using (A) RT-qPCR and (B) western blotting. (C) Semi-quantification of the expression levels of FOXO3a presented in part (B). **P<0.01, ***P<0.001 vs. HK-2. Effect of OE-FOXO3a on the expression levels of FOXO3a in 17-94 cells was determined using (D) RT-qPCR and (E) western blotting. (F) Semi-quantification of the expression levels of FOXO3a from part (E). (G) Cell Counting Kit-8 assay was used to determine that OE-FOXO3a-transfected 17-94 cells had significantly decreased rates of cell proliferation. (H) OE-FOXO3a-transfected 17-94 cells demonstrated decreased rates of colony formation. **P<0.01, ***P<0.001 vs. control; $^{\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. OE-NC. n=3. FOXO3a, forkhead transcription factor O subfamily 3A; OE, overexpression; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; OD, optical density.

analyze the expression levels of the Wnt/β-catenin signaling pathway-related proteins, Axin-2, β-catenin and cyclin D1. The results identified that the overexpression of FOXO3a significantly upregulated the protein expression levels of Axin-2, while the protein expression levels of β-catenin and cyclin D1 were significantly downregulated, compared with the control and OE-NC groups (Fig. 4A). The transfection efficiency of shRNA-Axin-2-1 and shRNA-Axin-2-2 was analyzed using western blotting and RT-qPCR; the results demonstrated that the inhibitory effect of shRNA-Axin-2-1 on the Axin-2 expression levels was superior compared with shRNA-Axin-2-2 in 17-94 cells, thus shRNA-Axin-2-1 was chosen for subsequent experiments (Fig. 4B and C).

Subsequently, OE-FOXO3a and shRNA-Axin-2 were co-transfected into 17-94 cell lines. The CCK-8 assay revealed that the cell viability of 17-94 cells was significantly increased in the OE-FOXO3a + shRNA-Axin-2 group compared with the OE-FOXO3a and OE-FOXO3a + shRNA-NC groups (Fig. 4D). Furthermore, similar results were recorded in the colony formation ability of 17-94 cells (Fig. 4E). Taken together, these findings suggested that the activation of Wnt/β-catenin signaling may reverse the FOXO3a overexpression-induced inhibition of cell viability and proliferation in nephroblastoma.

The migratory and invasive abilities of 17-94 cells in the OE-FOXO3a + shRNA-Axin-2 group were significantly increased compared with the OE-FOXO3a and OE-FOXO3a + shRNA-NC groups (Fig. 5A-D). In addition, western blotting was used to analyze the expression levels of the invasion and migration-related proteins, MMP2, MMP9 and MMP13. Compared with the OE-FOXO3a and OE-FOXO3a + shRNA-NC groups, significantly upregulated expression levels of MMP2, MMP9 and MMP13 were observed in the OE-FOXO3a + shRNA-Axin-2 group (Fig. 5E).

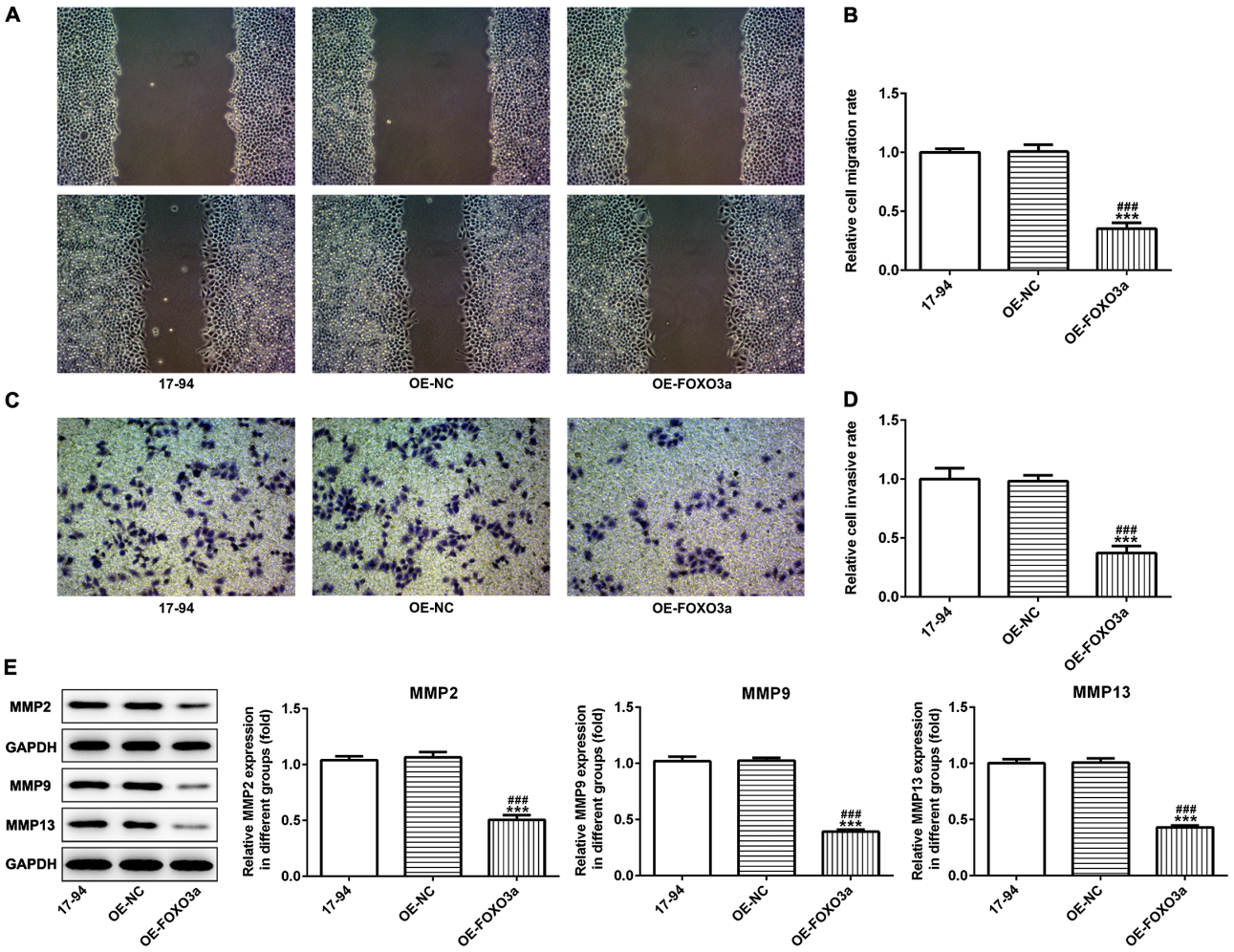

Figure 2. Overexpression of FOXO3a inhibits the invasion and migration of nephroblastoma cells. (A) Wound healing assay was used to determine the migratory ability of OE-FOXO3a-transfected cells (magnification, x200). (B) Semi-quantification of the cell migration rate in part (A). (C) Transwell Matrigel assays were used to determine the invasive ability of OE-FOXO3a-transfected cells (magnification, x200). (D) Semi-quantification of the cell invasive rate from part (C). (E) Western blotting was performed to analyze the protein expression levels of MMP2, MMP9 and MMP13 in OE-FOXO3a-transfected cells. ***P<0.001 vs. control; $^{###}$P<0.001 vs. OE-NC; n=3. FOXO3a, forkhead transcription factor O subfamily 3A; OE, overexpression; NC, negative control; MMP, matrix metalloproteinase.

FOXO3a-induced cell apoptosis is regulated by Wnt/β-catenin signaling. 17-94 cells were co-transfected with OE-FOXO3a and shRNA-Axin-2 and flow cytometry was used to determine the rate of cell apoptosis. Compared with the OE-FOXO3a + shRNA-NC and OE-FOXO3a groups, the cell apoptotic rate was significantly decreased in the OE-FOXO3a + shRNA-Axin-2 group (Fig. 6A and B). Furthermore, western blotting was performed to analyze the expression levels of the apoptosis-related proteins, Bax, Bcl-2, Bim, cleaved caspase-3 and caspase-3. The results revealed that the upregulated expression levels of Bax, Bim and cleaved caspase-3, and down-regulated expression levels of Bcl-2, induced by OE-FOXO3a were significantly reversed following the co-transfection with OE-FOXO3a and shRNA-Axin-2 (Fig. 6C).

Discussion

Over the past few years, the incidence of childhood tumors has increased significantly (31). Renal cancer is the most type of common malignant tumor to occur in children, accounting for 7% of childhood cancers (32). The most common pathological type of renal cell carcinoma in children is nephroblastoma (2). The rare pathological types include rhabdomyoma sarcoma, clear cell sarcoma, congenital mesodermal nephroma, renal Ewing's sarcoma, primary renal myoepithelial carcinoma, cystic partially differentiated nephroblastoma, multilocular cystic nephroma, primary synovial sarcoma and anaplastic sarcoma (2,31-33). Nephroblastoma has been discovered to affect the function of unilateral or bilateral kidneys (33,34). Although the diagnosis and treatment of nephroblastoma has significantly improved, the mortality rate of nephroblastoma in children remains high (10.7%) (35,36). Thus, studying the effects of factors related to early nephroblastoma may provide a novel target and treatment method for nephroblastoma.

FOXO3a is a member of the FOXO family; it enters the nucleus after binding with β-catenin, where it was reported to accelerate the self-renewal of cancer cells, thus promoting the formation of tumors (37,38). FOXO3a has also been discovered to combine with the transcription regulatory machinery of different target genes in the nucleus to serve a role in transcriptional regulation, in addition to participating in the proliferation, migration, invasion and apoptosis of cells (12,39). FOXO3a was also discovered to serve a role in abnormal activity, stress tolerance and the metabolic homeostasis of

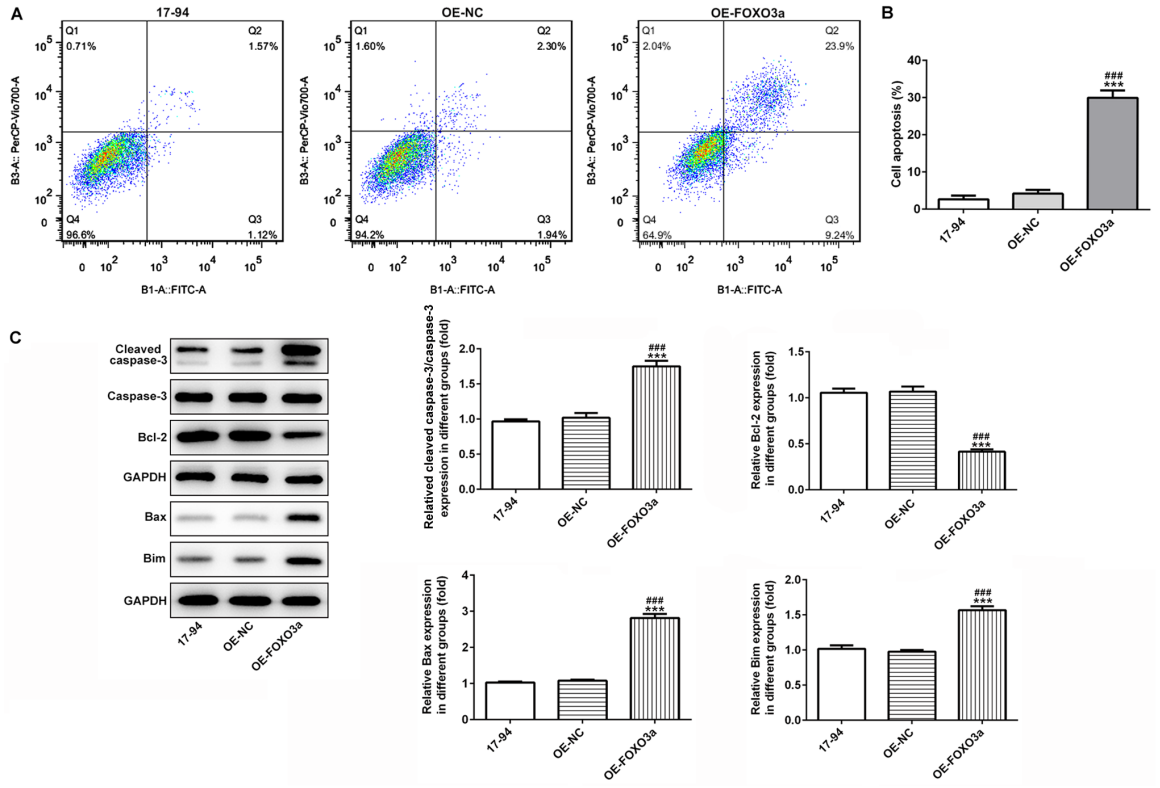

Figure 3. Overexpression of FOXO3a promotes cell apoptosis in nephroblastoma cells. (A) Flow cytometric analysis of cell apoptosis in OE-FOXO3a-tranfected cells. (B) Quantification of cell apoptosis from part (A). (C) Western blotting was used to analyze the protein expression levels of apoptosis-related molecules, Bcl-2, Bax, Bim, caspase-3/cleaved caspase-3 in OE-FOXO3a-transfected cells. ***P<0.001 vs. control; ###P<0.001 vs. OE-NC; n=3. FOXO3a, forkhead transcription factor O subfamily 3A; OE, overexpression; NC, negative control; Bim, Bcl-2-like protein 2.

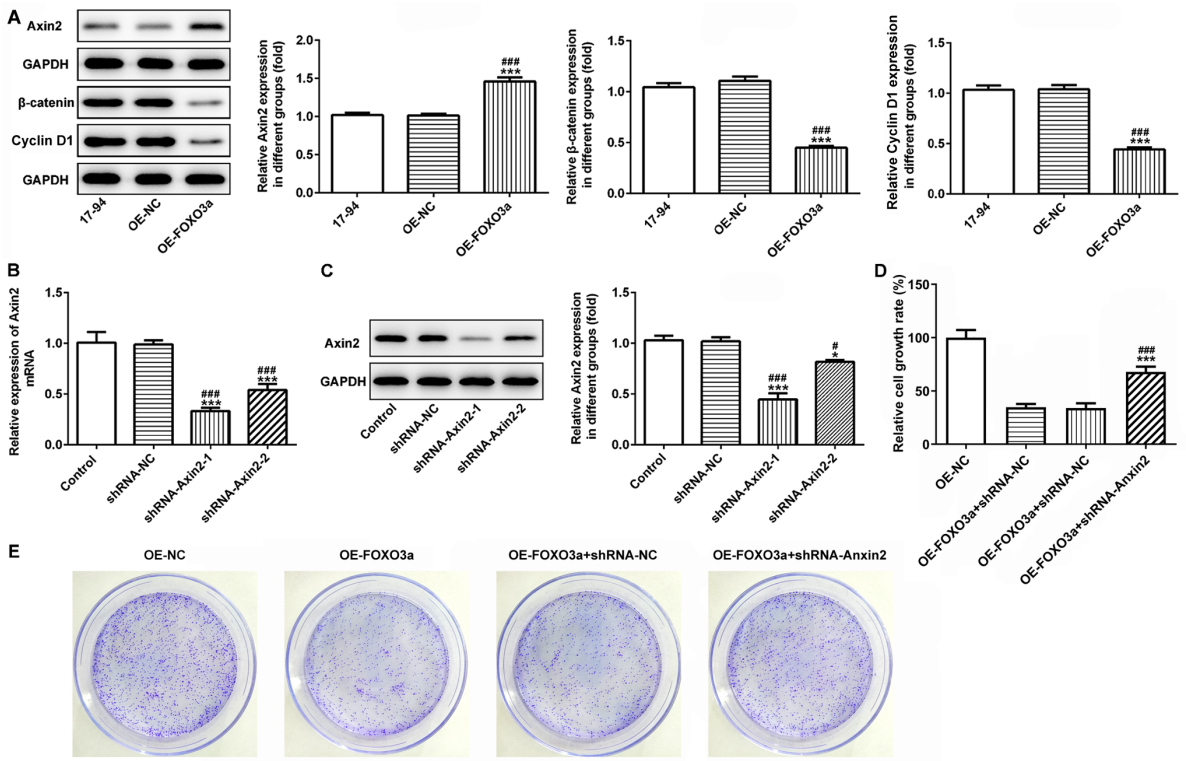

Figure 4. Activation of Wnt/β-catenin signaling reverses FOXO3a overexpression-induced inhibition of the proliferation of nephroblastoma cells. (A) Western blotting was used to analyze the expression levels of the Wnt/β-catenin signaling-related proteins, Axin-2, β-catenin and Cyclin D1. ***P<0.001 vs. control; ###P<0.001 vs. OE-NC; n=3. The transfection efficiency of shRNA-Axin-2-1 and shRNA-Axin-2-2 were detected using (B) reverse transcription-quantitative PCR and (C) western blotting. OE-FOXO3a and shRNA-Axin-2 were co-transfected into 17-94 cell lines and the cell viability and proliferation of 17-94 cells was detected using a (D) Cell Counting Kit-8 assay and (E) colony formation assay, respectively. *P<0.05, ***P<0.001 vs. control; #P<0.05, ###P<0.001 vs. shRNA-NC; n=3. FOXO3a, forkhead transcription factor O subfamily 3A; OE, overexpression; NC, negative control; OD, optical density; shRNA, short hairpin RNA.

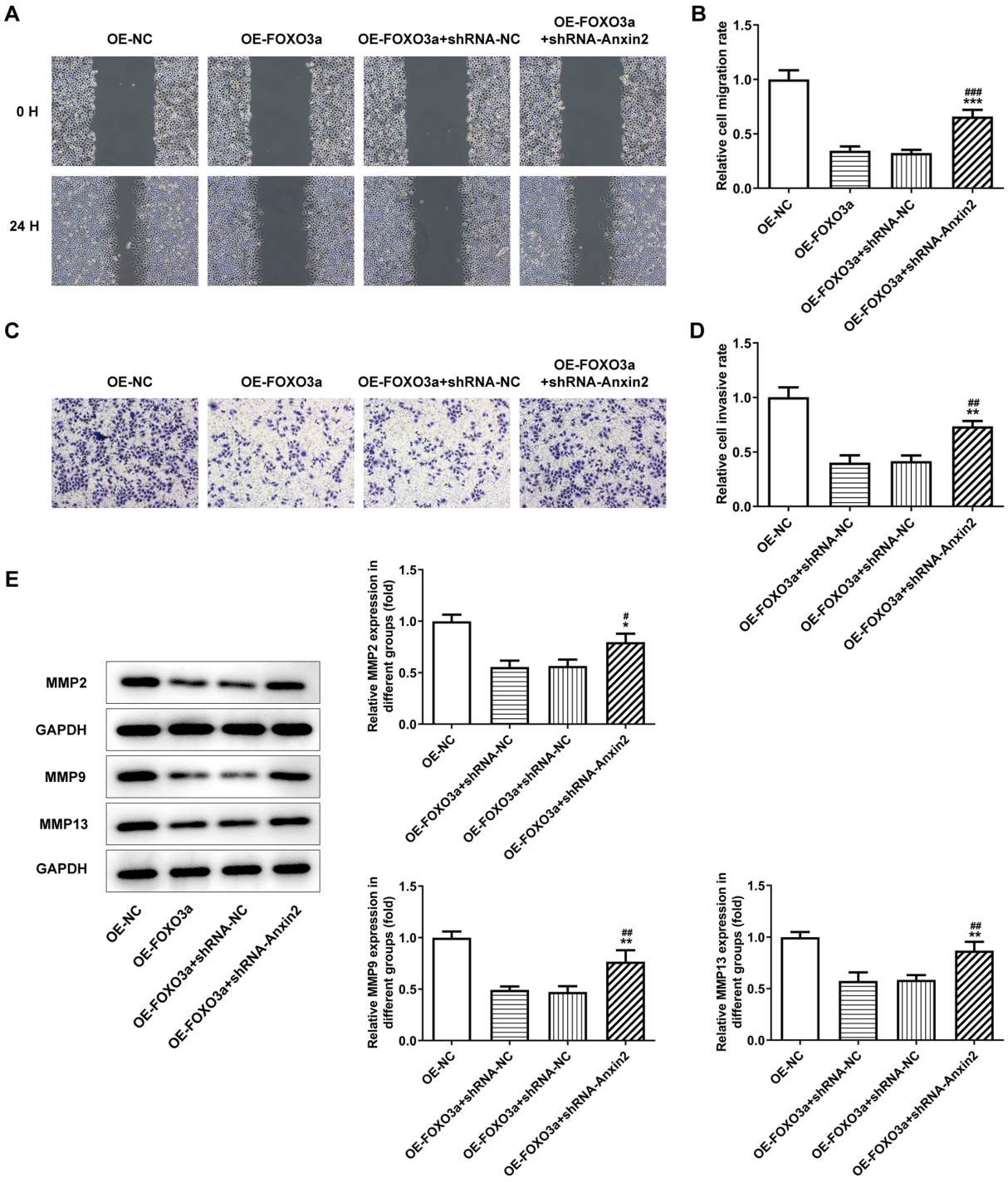

Figure 5. Activation of Wnt/β-catenin signaling reverses FOXO3a overexpression-induced inhibition of the invasion and migration of nephroblastoma cells. (A) Wound healing assay was used to analyze the migratory ability of cells transfected with OE-FOXO3a + shRNA-Axin-2 (magnification, x200). (B) Semi-quantification of the cell migration rate from part (A). (C) Transwell Matrigel assay was used to analyze the invasive ability of cells transfected with OE-FOXO3a + shRNA-Axin-2 (magnification, x200). (D) Semi-quantification of the cell invasive rate from part (C). (E) Western blotting was performed to analyze the protein expression levels of MMP2, MMP9 and MMP13 in cells transfected with OE-FOXO3a + shRNA-Axin-2. *P<0.05, **P<0.01, ***P<0.001 vs. OE-FOXO3a; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. OE-FOXO3a + shRNA-NC; n=3. FOXO3a, forkhead transcription factor O subfamily 3A; OE, overexpression; NC, negative control; shRNA, short hairpin RNA; MMP, matrix metalloproteinase.

tumor cells by regulating the expression of cell cycle-related factors and apoptosis-related factors effect (40,41). In the present study, FOXO3a expression levels were discovered to be downregulated in nephroblastoma cell lines, suggesting that FOXO3a may serve an essential role in nephroblastoma. Furthermore, the results revealed that the overexpression of FOXO3a significantly attenuated nephroblastoma cell proliferative, migratory and invasive abilities. In addition, the overexpression of FOXO3a promoted the apoptosis of nephroblastoma cells. A previous study suggested that FOXO3a may be a potential biomarker for the diagnosis, prognosis and treatment of a variety of types of malignant tumor, including ovarian, prostate and pancreatic cancers (42,43). Similarly, previous studies also reported that FOXO3a expression

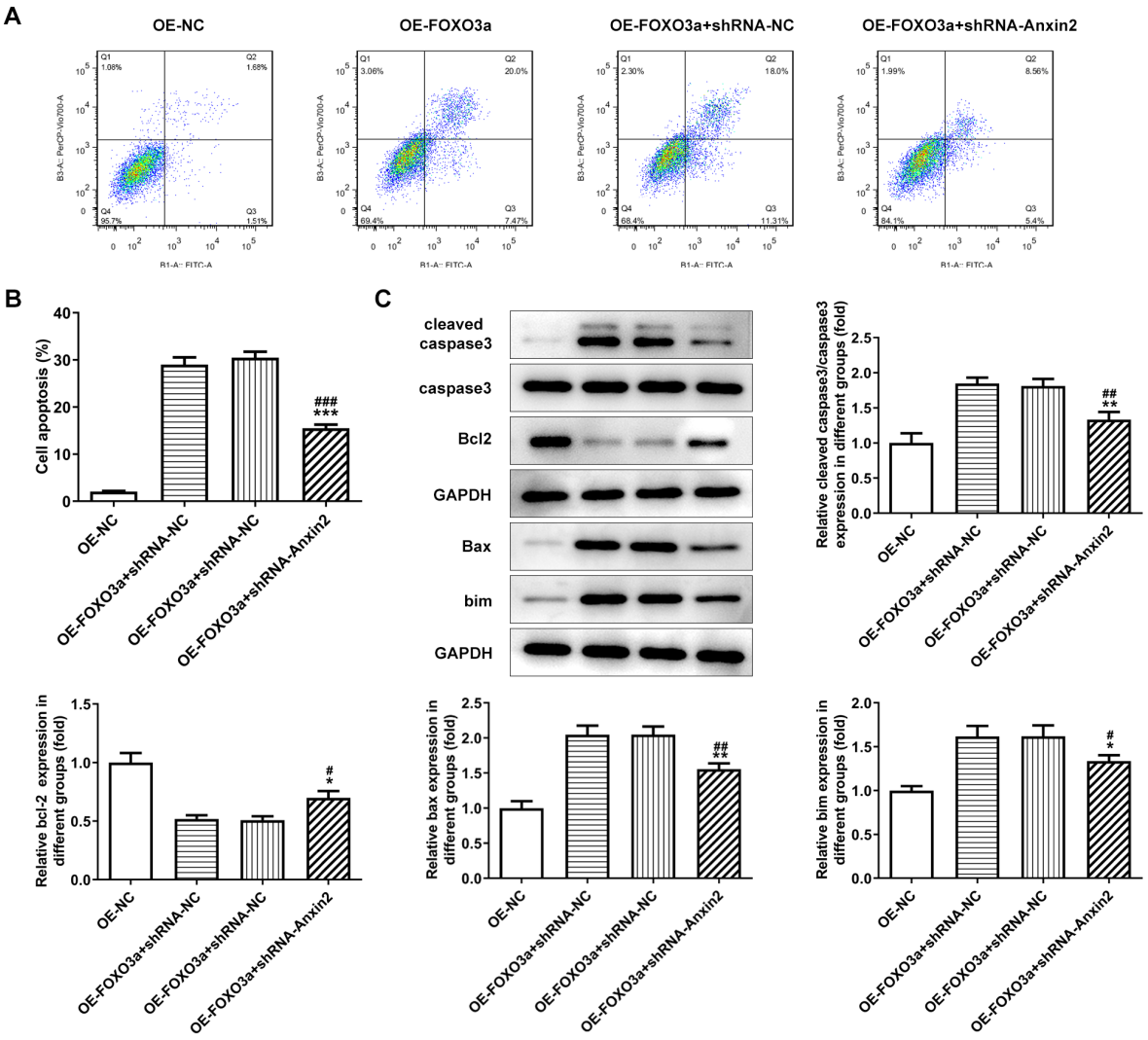

Figure 6. Activation of Wnt/β-catenin signaling reverses the FOXO3a overexpression-induced apoptosis of nephroblastoma cells. (A) Flow cytometric analysis of cell apoptosis in cells transfected with OE-FOXO3a + shRNA-Axin-2. (B) Quantification of cell apoptosis from part (A). (C) Western blotting was used to analyze the protein expression levels of the apoptosis-related molecules, Bcl-2, Bax, Bim, caspase-3/ cleaved caspase-3. *P<0.05, **P<0.01, ***P<0.001 vs. OE-FOXO3a; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. OE-FOXO3a + shRNA-NC, n=3. FOXO3a, forkhead transcription factor O subfamily 3A; OE, overexpression; NC, negative control; shRNA, short hairpin RNA; Bim, Bcl-2-like protein 2.

levels could be used as a prognostic biomarker for clear cell renal cell carcinoma, cervical carcinoma and colorectal cancer (14,29,42). Interestingly, the overexpression of FOXO3a has been associated with the poor prognosis of patients with triple negative breast cancer, glioblastoma and gastric cancer, while downregulated expression levels of FOXO3a were associated with the poor prognosis of patients with glioma and ovarian cancer (44-45). Therefore, these studies suggested that FOXO3a may be a potential biomarker for the diagnosis of nephroblastoma, and may potentially serve as an oncogene or suppressor factor to be investigated in relation to nephroblastoma progression, which requires further investigations. Based on these previous studies (46-50) and the present data, FOXO3a was hypothesized to be a potential molecular target for nephroblastoma therapy and diagnosis.

The Wnt/β-catenin signaling pathway is an important signaling pathway involved in regulating cell proliferation and differentiation, and serves a crucial role in tumor development, metastasis and embryonic development (23,24). In addition, it was reported that the Wnt/β-catenin signaling pathway was highly activated in various types of cancer, such as ovarian epithelial cancer and prostate cancer (51,52), thus, the inhibition of this signaling pathway has become a research hotspot. A previous study revealed that the inhibition of the Wnt/β-catenin signaling pathway inhibited growth and promoted apoptosis in ovarian cancer cells (53), while microRNA-218 promoted the apoptosis of ovarian cancer cells by inhibiting the Wnt/β-catenin signaling pathway (53). In addition, lysyl oxidase homolog 2, a member of the lysyl oxidase family, was suggested to affect the growth of ovarian cancer cells by inhibiting the Wnt/β-catenin signaling pathway (54). Thus, to further determine the possible mechanisms by which FOXO3a participated in nephroblastoma, the relationship between FOXO3a and the Wnt/β-catenin signaling pathway in nephroblastoma cells was investigated. The present study detected the expression of WNT/β-catenin signaling proteins Axin2, β-catenin and cyclin D1. Following overexpression of FOXO3a, the expression of Axin2 increased significantly, and the expression of β-catenin and cyclin D1

decreased significantly. Following co-transfection with shRNA-Axin-2, WNT signaling was activated, the activation of the Wnt/β-catenin signaling pathway reversed the FOXO3a overexpression-induced suppression of proliferation, invasion and migration in nephroblastoma cells, in addition to reversing the FOXO3a-induced apoptosis of nephroblastoma cells.

In conclusion, the findings of the present study suggested that FOXO3a may inhibit nephroblastoma cell proliferation, invasion and migration, while inducing apoptosis through downregulating the Wnt/β-catenin signaling pathway. These results may provide a novel method for the early diagnosis and treatment of nephroblastoma. A limitation of the present study was that FOXO3a was only studied in a nephroblastoma cell line; its application *in vivo* and in clinic was not studied but will be investigated in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QL designed the study, collected the data, performed the data analysis and wrote the manuscript. CQ conceived the study, participated in designing the study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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