• Basic Research •

Promotion of human choroidal melanoma cell metastases by FOXP3 *via* Wnt5a/CaMKII signaling pathway

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Abstract

● **AIM:** To investigate the role of Forkhead box protein P3 (FOXP3) in choroidal melanoma (CM) metastases and elucidate its underlying mechanisms.

● **METHODS:** FOXP3 protein expression was analyzed in CM clinical specimens and cell lines. A stable FOXP3 knockout cell line and a transient FOXP3-overexpressing cell line were established, with transfection efficiencies confirmed by Western blotting (WB). Functional assays, including monoclonal formation, cell counting kit-8 (CCK-8) proliferation, migration, invasion, and *in vivo* tumorigenesis assays in nude mice, were performed to assess the biological effects of FOXP3. Additionally, WB was employed to evaluate epithelial-mesenchymal transition (EMT) markers and the activation of the Wnt5a/CaMKII signaling pathway.

● **RESULTS:** FOXP3 expression was significantly elevated in both CM clinical specimens and cell lines. Functional analyses revealed that FOXP3 enhanced CM cell proliferation, migration, and invasion *in vitro* and promoted tumorigenesis *in vivo*. Mechanistically, FOXP3 upregulated EMT-related proteins and activated the Wnt5a/CaMKII signaling pathway. Rescue experiments further confirmed that the oncogenic effects of FOXP3 were mediated *via* modulation of the Wnt5a/CaMKII axis.

● **CONCLUSION:** This study identifies FOXP3 as an oncogenic driver in CM, promoting tumor progression through the Wnt5a/CaMKII signaling pathway. These findings provide new insights into the molecular mechanisms of CM pathogenesis and highlight FOXP3 as a potential therapeutic target.

● **KEYWORDS:** Forkhead box protein P3; choroidal melanoma; epithelial-mesenchymal transition; Wnt5a/CaMKII; metastasis

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INTRODUCTION

Choroidal melanoma (CM) accounts for approximately 70% of uveal melanomas, the second most common intraocular malignancy[1]. Despite advances in diagnostics and treatment, more than 50% of CM patients succumb to metastatic disease, with a median survival of less than one year following the onset of metastases[2]. The liver is the most common site of metastasis, occurring in 55%-90% of cases, followed by the lungs (46%) and brain (8%). Current therapeutic options for CM remain limited, highlighting an urgent need for the development of novel treatment strategies to improve patient outcomes.

Forkhead box protein P3 (FOXP3) protein belongs to the forkhead/wing helix family with encoded gene on the human X chromosome, which is a transcription factor. It is well known that FOXP3 is essential for the regulatory T cells development, but more and more researchers indicate that FOXP3 can also act as a carcinogen to enhance the growth and metastasis abilities of lung cancer[3]. Conversely, the protein can also inhibit the metastasis and growth of tumors in prostate cancer, breast cancer and ovarian cancer[4]. Previous study found that $FOXP3^+$ T cells were a predictor of poor prognosis in patients with cyclooxygenase (COX)-2^+ uveal melanoma. Besides, FOXP3 was found to be higher level in metastatic uveal melanoma patients than that of primary uveal melanoma patients *in vivo*[5]. These combined researches indicate that

FOXP3 could be a key factor in CM development. However, no study has reported the specific mechanism of FOXP3 on CM. The main purpose of this study was to investigate the role of FOXP3 in CM cells and its possible mechanism, which could provide potential therapeutic targets for the treatment of CM.

The Wnt signaling pathway has been widely studied for its crucial effects in cell proliferation and metastasis[6-7]. Among the molecules involved in this signaling pathway, Wnt5a has increasingly been revealed to play an essential role in tumor growth and metastasis. Wnt5a acts as an oncogenic factor, promoting the tumor growth of ovarian cancer, pancreatic cancer, and malignant melanoma[8]. The expression level of Wnt5a is upregulated in uveal melanoma, which indicates that Wnt5a may be closely related to its occurrence[9]. In addition, FOXP3 can decrease the metastasis ability of non-small cell lung cancer by modulation of Wnt signal[10]. The Wnt5a/CaMKII pathway has been poorly studied in tumors, and our previous study demonstrated that artesunate could resist the malignant phenotype of CM by inhibiting the Wnt5a/CaMKII pathway[11]. Since the regulatory role of FOXP3 on Wnt5a was unknown, which led us to speculate whether FOXP3 could affect CM tumorigenesis by regulating Wnt5a.

MATERIALS AND METHODS

Ethical Approval The animal study was reviewed and approved by the Ethic Committee of the Affiliated Hospital of Qingdao University (permit number: QYFYWZLL26381).

CM Clinical Specimens and Cell Culture CM clinical specimens of 20 were gained from the Affiliated Hospital of Qingdao University. Adult retinal pigment epithelial-19 (ARPE-19) cell and ocular choroidal melanoma-1 (OCM-1) cell lines (the human CM cell) were purchased from BeNa (Beijing, China). C918 cell lines (the human CM cell) was purchased from Procell life Science & Technology Co.ltd. (Wuhan, China). ARPE-19 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), OCM-1 and C918 cell lines were incubated in Roswell Park Memorial Institute (RPMI)-1640 with 10% FBS. The signifcance of FOXP3 in CM was analyzed using tumor immune estimation resources (TIMER v2.0; https://cistrome.shinyapps.io/timer/) and the GEPIA database (http://gepia.cancer-pku.cn/). RNA sequencing and clinical data of 80 uveal melanoma patients were downloaded from The Cancer Genome Atlas Program (TCGA).

FOXP3-shRNA CRISPR Cas9 Lentivirus Transfection We used CRISPR Cas9 to knockdown the FOXP3 gene. Both CRISPR Cas9-short hairpin lentivirus-FOXP3 (sh-FOXP3) and the CRISPR Cas9-short hairpin lentivirus-negative control (sh-NC) were purchased in Genechem company (Shanghai, China). The sense sequence of the FOXP3-shRNA-cas9 was 5'-CACCGAGCTCTGGGGCACAGCCGAA-3', while the antisense sequence was 5'-AAACTTCGGCTGTGCCCCAGAGCTC-3'. OCM-1 and C918 cells were spread into six-well plates. When the cell density approaches 80%, lentivirus was added into the plates accordingly. After 24h, fresh medium was used to replace the medium containing lentivirus. After a further 3d, culture fluid with 4 μg/mL puromycin (Invivogen, San Diego, CA, USA) was used to incubate the cells. After 7 more days, substitute medium containing 1 μg/mL puromycin for the prior medium. The cell proteins from the sh-NC and sh-FOXP3 groups were extracted, then Western blotting was used to confirm that FOXP3 was successfully downregulated in the sh-FOXP3 group.

Plasmid and Small Interference RNA Transfection The plasmids used for the overexpression- plasmid CMV DRV-HIV (pCDH)-negative control (pCDH-NC) and overexpression-pCDH-FOXP3 (pCDH-FOXP3) were obtained from the team of Prof. He Ren in the Affiliated Hospital of Qingdao University. Negative control siRNA (si-NC) and Wnt5a-siRNA (si-wnt5a) were purchased from Gene Pharma Company (Shanghai, China). The sense sequence of si-Wnt5a was 5'-GCUACGUCAAGUGCAAGAATT-3', while the antisense sequence was 5'-UUCUUGCACUUGACGUAGCTT-3'. The OCM-1 and C918 cells were spread into six-well plates. When the cell density is close to 90%, transfection was carried out using lipofectamine 3000 reagent and P3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48h, the cell proteins were extracted by Western blotting.

Monoclonal Formation Assay A total of 150 OCM-1 and C918 cells, respectively, were inoculated into 6-well plates and left for 14d. Then, the cells were hold using formaldehyde containing 0.5% crystal violet to observe the number and size of the resulting cell clusters under an inverted microscope (≥50 cells/colony).

Cell Counting Kit-8 Assay Totally 2000 of cells were seed into 96-well plates. Serum-free medium and cell counting kit (CCK)-8 reagent (Solaobio, Beijing, China) were prepared into a mixture at a ratio of 9:1, and 100 μL of the mixture was added to the wells. Totally 60min later, the optical density was recorded at 450 nm. We carried out CCK-8 kit at 0, 24, 48, and 72h after the cell static adherence.

Cell Migration and Invasion Assays Approximately 5×10^4 cells were injected into the upper chambers (8 μm pore size, corning, New York, USA) for the migration assays. Approximately 8×10^4 cells were put into the upper chambers containing polymerized Matrigel for the invasion assays. The cells were cultured by serum-free 1640 medium. A 600 μL volume of culture fluid with 10% FBS was added to the lower chambers. After 48h later, the migrated and invaded cells under the membrane were dyed and hold using formaldehyde

containing 0.5% crystal violet. Subsequently, the number of cells in 5 non-overlapped fields was counted under a microscope.

Western Blotting Assay The logarithmic phase cells were collected and lysed by RIPA (Solarbio, Beijing, China) with 1% phosphatase inhibitors and 1% protease inhibitors (Solarbio, Beijing, China). The lysates were centrifuged at 4℃ for 20min at 13 000 g to extract total protein. The protein concentration was measured by BCA kit (Solarbio, Beijing, China). Proteins were separated by SDS-PAGE and were transferred onto PVDF membranes (Merck Millipore, Billerica, MA, USA) for 120min at 300 mA. Then, we used phosphate-buffered saline with Tween (PBST; Solarbio, Beijing, China) with 5% non-fat milk to block the membranes for 90min. Finally, the membranes were incubated with primary antibodies: FOXP3 (Novus, NB100-39002, 1:1000), E-cadherin (CST, 14472S, 1:1000), N-cadherin (CST, 13116T, 1:1000), Snail (CST, 3879T, 1:1000), matrix metalloproteinase-9 (MMP9; CST, 13667T, 1:1000), Wnt5a (CST, 2530T, 1:1000), β-catenin (CST, 8480S,1:1000), CaMKII (CST, 4436S, 1:1000), p-CaMKII (CST, 12716T, 1:1000), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Proteintech Chicago, 60004-1-Ig, 1:5000). The membranes were washed three times using PBST and were incubated in goat anti-rabbit/mouse IgG (H+L; CST, 1:5000). The bands were analyzed by 1D image analysis program and the gray value was analyzed using Image J.

Immunohistochemistry The expression of FOXP3 in the CM clinical specimens, E-cadherin, and Snail levels in the tissues were examined using immunohistochemistry (IHC). Briefly, 3.5 μm paraffin-embedded sections were deparaffinized and rehydrated. Then, 3% hydrogen peroxide was used to block the endogenous peroxidase. The antigen was retrieved, and then the sections were incubated with primary antibodies against FOXP3 (Novus, NB100-39002, 1:400), E-cadherin (Proteintech, 20874-1-AP, 1:400), and Snail (Elabscience, E-AB-63597, 1:200) at 4℃ overnight. The following day, secondary antibodies (Zhongshan Jinqiao Biotechnology Co., Ltd from Beijing) were used to incubate the tissue sections. The sections were subsequently counterstained with hematoxylin. Immunoreactivity was visualized with diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA). Image J was used to calculate the positive area, and the integrated optical density (IOD) value was obtained by analysis. Meanwhile, the number of positive cells were counted and the positive cell rate was calculated, sequentially, the IHC score was obtained 0 (no staining), 1 (<10% of malignant cells staining), 2 (10%-50% of malignant cells staining), or 3 (>50% of malignant cells staining).

***In vivo* Tumor Xenograft Assays** Ten female BALB/c nude mice, each 6 weeks old, were procured from Jinan Pengyue. Five nude mice each from sh-NC and sh-FOXP3 groups were used for *in vivo* experiments. The total number of 2×10^6 C918 cells were subcutaneously injected into the left armpit of nude mouse (Vital River Laboratory Animal Technology Co., Ltd, Beijing) for the *in vivo* xenograft assay. Approximately 6d later, palpable tumors appeared. Gross tumor volume was observed diebus tertius. Totally 21d later, the tumors were removed from mouse. Tumor volume was calculated as: tumor volume=$a^2\times b\times 0.5$. where "a" is the minimum diameter, and "b" is the diameter perpendicular to "a".

Statistical Analysis All assays in this research were repeated more than three times. One-way analysis of variance and the unpaired *t*-test in Prism 8.0 (GraphPad, USA) were used to conduct data analysis, which were shown as the mean±standard error of the mean (SEM). $P<0.05$ was identified as statistically significant.

RESULTS

FOXP3 Upregulation in CM and the Associated Poor Prognosis of Patients Analysis of the TIMER database revealed that FOXP3 is upregulated in various tumor types (Figure 1A). To investigate its role in CM, we examined FOXP3 expression in CM cell lines using Western blotting. The results showed significantly higher FOXP3 levels in OCM-1 and C918 CM cells compared to ARPE-19 control cells (Figure 1B). Consistently, IHC analysis of clinical specimens demonstrated that FOXP3 protein levels were markedly elevated in CM tissues compared to adjacent non-tumor uveal tissues (Figure 1C).

We next analyzed the correlation between FOXP3 expression and prognosis of CM patients. Survival analyze was performed by GEPIA database; the results showed that higher overall survival rate in low FOXP3 group compared with high FOXP3 group (Figure 1D). In order to explore other clinical features, RNA sequencing and clinical data of 80 uveal melanoma were downloaded from TCGA, then divided into high FOXP3 group and low FOXP3 group according to FOXP3 level. There was significant difference of new tumor events between two groups ($P=0.005$; Table 1).

Ectopic Expression of FOXP3 Promoted Cell Activity and Proliferation in CM Cells Based on the above findings, the effects of FOXP3 on cell function were further detected in CM cells (OCM-1 and C918). The pCDH-FOXP3 plasmid and CRISPR Cas9-FOXP3 shRNA (sh-FOXP3) were transfected into the two cell lines, and Western blotting analysis confirmed that the level of FOXP3 in the sh-FOXP3 group was lower than that in the sh-NC group, while the expression of FOXP3 in the pCDH-FOXP3 group was higher than that in the pCDH-NC group (Figure 2A). The CCK-8 assay showed that the overexpression of FOXP3 significantly increased cell proliferative activity in the CM cells compared with the NC

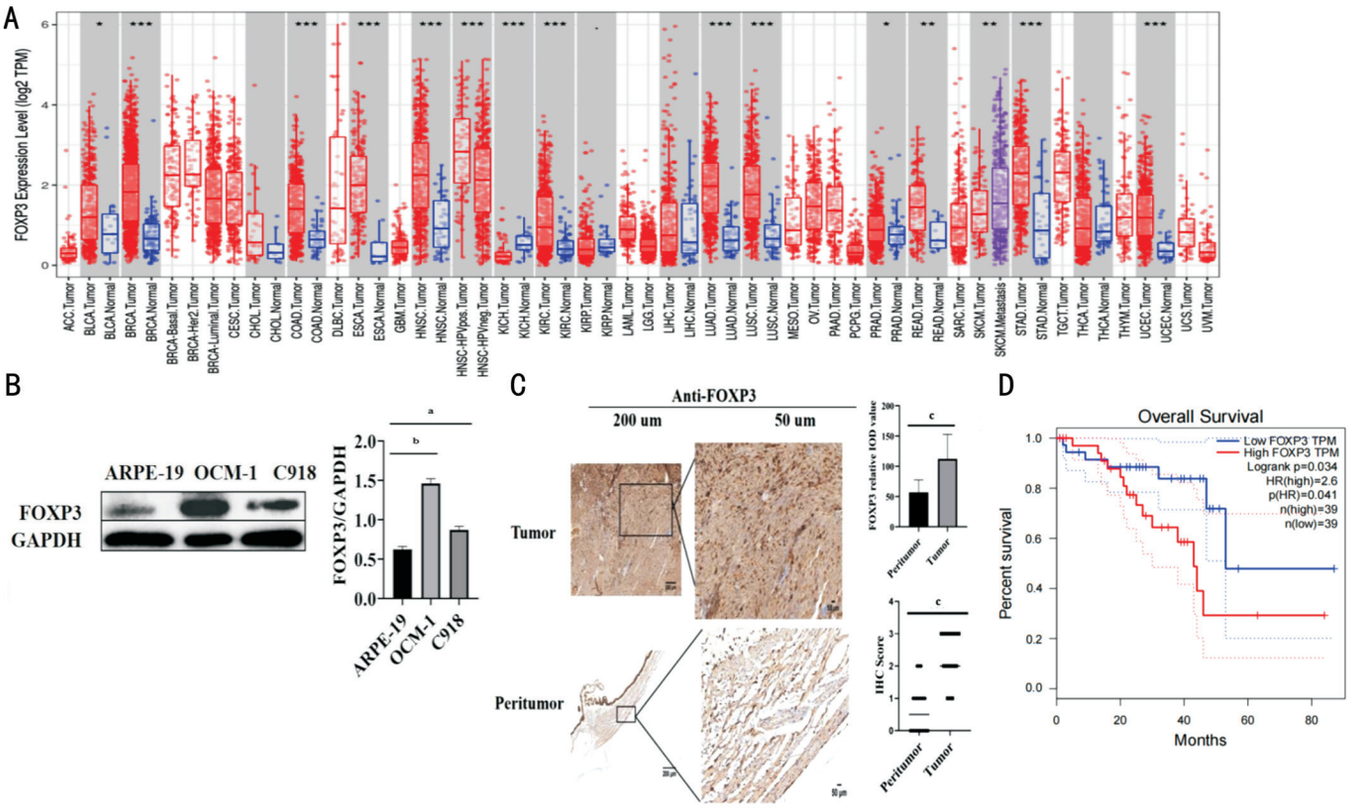

Figure 1 FOXP3 was upregulated in CM and correlated with poor prognosis of patients A: FOXP3 expression in various tumors was analyzed by TIMER database. B: WB results of FOXP3 protein level in OCM-1 and C918 cell lines and ARPE-19 cell line. C: Immunohistochemistry result of FOXP3 expression in CM clinical specimens. Scale bar represents 200 μm and 50 μm; *n*=20. D: Kaplan-Merier curve of CM patients between high and low FOXP3 group (*P*<0.05). FOXP3: Forkhead box protein P3; WB: Western blotting; OCM-1: Ocular choroidal melanoma-1; CM: Choroidal melanoma; ARPE-19: Adult retinal pigment epithelial-19. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001.

Table 1 Correlation between FOXP3 expression and clinical features of CM

Parameters	All (*n*=80)	High FOXP3 (*n*=27)	Low FOXP3 (*n*=53)	*P*
Age	61.6±13.9	61.1±12.4	61.9±14.8	0.810
Gender				0.270
Female	35 (43.8%)	9 (33.3%)	26 (49.1%)	
Male	45 (56.2%)	18 (66.7%)	27 (50.9%)	
Person neoplasm cancer status				0.058
Tumor free	61 (77.2%)	17 (63.0%)	44 (84.6%)	
With tumor	18 (22.8%)	10 (37.0%)	8 (15.4%)	
New tumor events				0.005
No	51 (63.7%)	11 (40.7%)	40 (75.5%)	
Yes	29 (36.2%)	16 (59.3%)	13 (24.5%)	
Clinicial stage				0.570
Stage II	36 (45.0%)	10 (37.0%)	26 (49.1%)	
Stage III	40 (50.0%)	16 (59.3%)	24 (45.3%)	
Stage IV	4 (5.00%)	1 (3.70%)	3 (5.66%)	
Tumor basal diameter	16.9±3.45	17.3±3.23	16.7±3.57	0.506
Tumor thickness	10.4±2.81	10.5±3.07	10.4±2.70	0.884

FOXP3: Forkhead box protein P3; CM: Choroidal melanoma.

cells (Figure 2B) while the knockdown of FOXP3 expression resulted in a significant decrease in cell proliferation activity compared with the NC cells (Figure 2C). Moreover, the monoclonal formation assay showed that the low level of FOXP3 reduced the number of clonal cell clusters in the CM cells compared with control group while the overexpression of FOXP3 increased the number of clonal cell clusters (Figure 2D). These findings suggest that FOXP3 promotes CM cell proliferation capacity, underscoring its potential role in tumor progression.

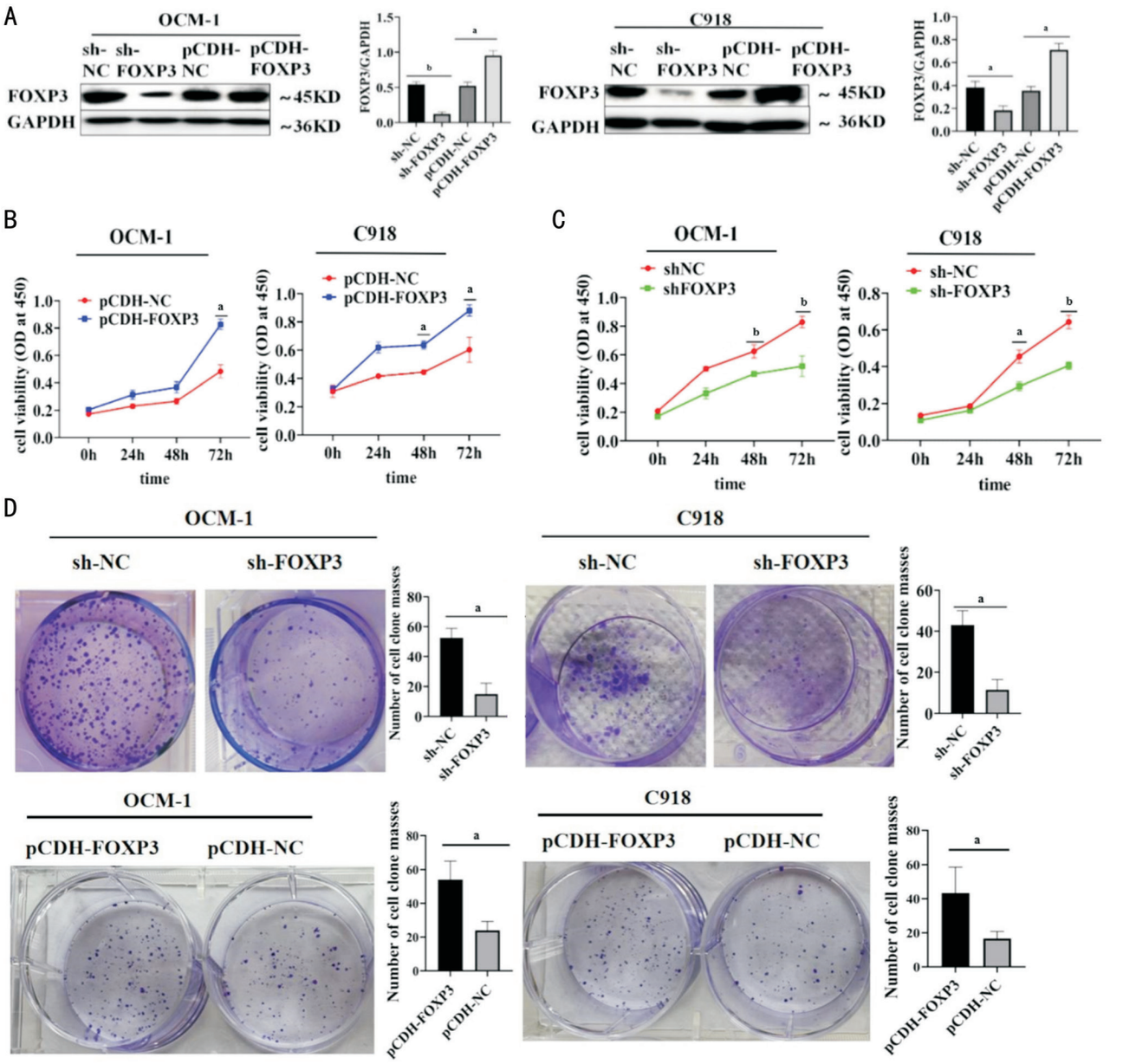

Figure 2 FOXP3 promoted proliferation of CM cells A: Knockdown and overexpression of FOXP3 in CM cells; B: FOXP3 overexpression promoted proliferation of CM cells according to CCK-8 assay, *n*=6; C: FOXP3 knockdown inhibited proliferation of CM cells according to CCK-8 assay, *n*=6; D: FOXP3 knockdown inhibited CM cell clonogenicity and overexpression of FOXP3 promoted cell clonogenicity by monoclonal formation experiment. sh: Short hairpin lentivirus; NC: Negative control; pCDH: Plasmid CMV DRV-HIV; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; FOXP3: Forkhead box protein P3; OCM-1: Ocular choroidal melanoma-1; CM: Choroidal melanoma. [a]*P*<0.05, [b]*P*<0.01.

Ectopic Expression of FOXP3 Promoted Both Migration and Invasion in CM Cells Cell migration and invasion assays revealed that FOXP3 promotes metastatic potential in CM cells. Overexpression of FOXP3 significantly increased the number of CM cells migrating and invading through the transwell chamber compared to the control group (Figure 3A). In contrast, FOXP3 knockdown markedly reduced the number of migrated and invaded cells compared to the NC group (Figure 3B). These results highlight the role of FOXP3 in enhancing the migratory and invasive capabilities of CM cells.

Ectopic Expression of FOXP3-Induced Epithelial-Mesenchymal Transition in CM Cells Transfection of pCDH-FOXP3 plasmids into CM cells induced notable morphological changes associated with epithelial-mesenchymal transition (EMT). Specifically, cells exhibited elongated pseudopodia and reduced cell-cell contact, along with other EMT characteristics, such as altered cell morphology and diminished intercellular adhesion (Figure 4A). To further investigate, Western blotting was performed to analyze EMT-related markers. FOXP3 overexpression resulted in a significant decrease in E-cadherin levels, a key epithelial marker, while increasing the expression of mesenchymal markers, including N-cadherin and Snail (Figure 4B). In contrast, FOXP3 knockdown led to elevated E-cadherin levels

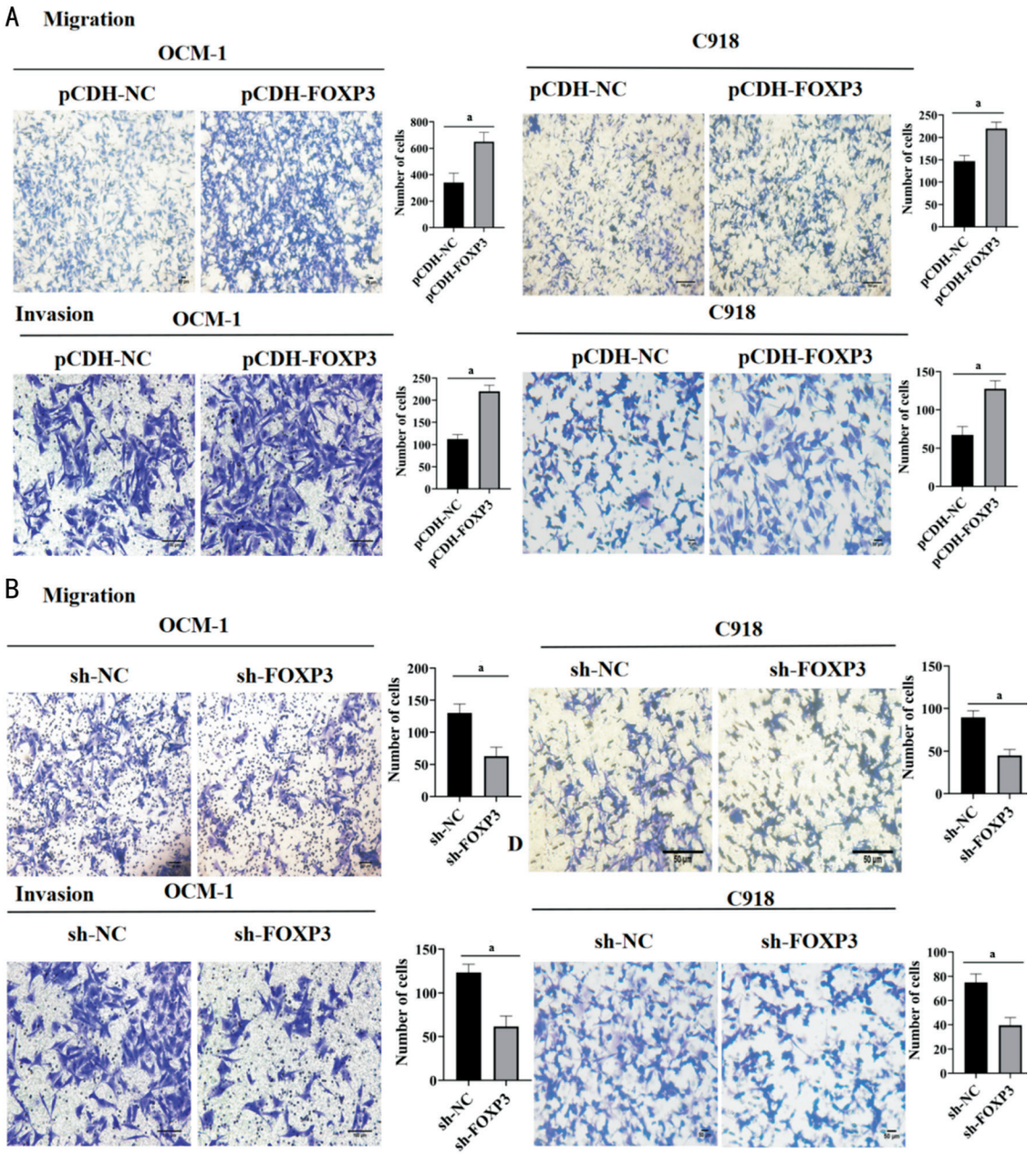

Figure 3 FOXP3 promoted migration and invasion of CM cells A: FOXP3 overexpression promoted migration and invasion of CM cells; B: FOXP3 knockdown inhibited migration and invasion of CM cells. Scale bar represents 50 μm. sh: Short hairpin lentivirus; NC: Negative control; pCDH: Plasmid CMV DRV-HIV; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; FOXP3: Forkhead box protein P3; OCM-1: Ocular choroidal melanoma-1; CM: Choroidal melanoma. [a]*P*<0.05.

and a reduction in N-cadherin, Snail, and MMP9 expression (Figure 4C). These findings suggest that FOXP3 promotes EMT in CM cells, facilitating a more invasive and metastatic phenotype.

Ectopic Expression of FOXP3 Upregulated Wnt5a/CaMKII in CM cells To further explore the mechanism of FOXP3 promoting EMT process in CM, we analyzed the expression of Wnt5a/CaMKII, which are essential in the process of EMT. The results indicated that FOXP3 knockdown inhibited expression of Wnt5a, CaMKII, p-CaMKII and β-catenin, while its overexpression was the opposite (Figure 5).

FOXP3-induced C918 Cell Metastasis, Invasion, EMT Markers *via* Wnt5a/CaMKII Signaling Upregulation To confirm the role of FOXP3 in promoting EMT through the Wnt5a/CaMKII signaling pathway, we conducted replication experiments by specifically silencing Wnt5a. Western blot analysis verified that Wnt5a silencing effectively reduced its expression (Figure 6A). Functional assays demonstrated that Wnt5a knockdown attenuated the pro-migratory and pro-invasive effects of FOXP3. In cell migration and invasion assays, the number of migrated and invaded C918 cells was significantly lower in the pCDH-FOXP3/si-Wnt5a group compared to the pCDH-FOXP3 group. Similarly, the number

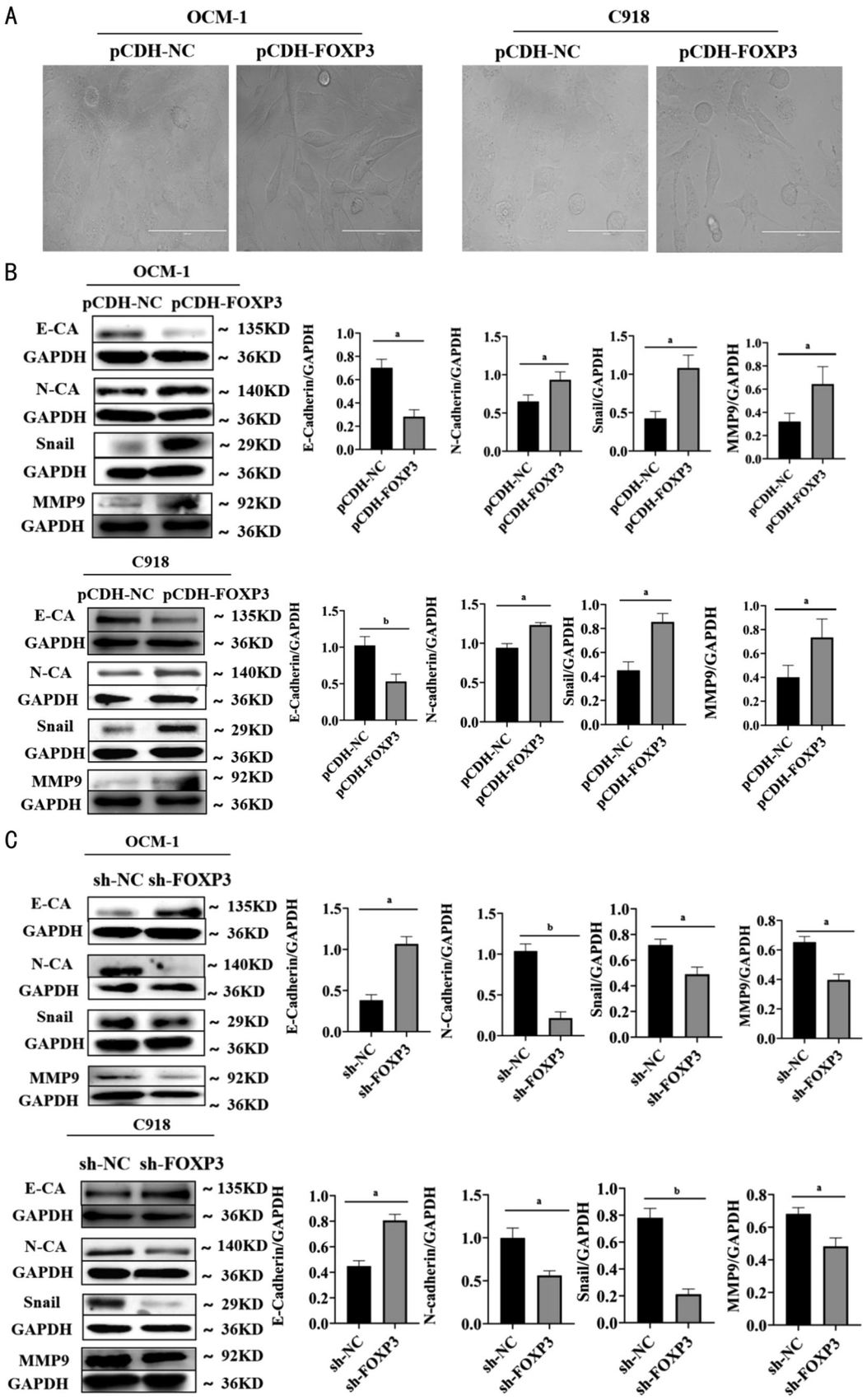

Figure 4 FOXP3 promoted EMT transition and degratation of MMP9 in CM cells A: FOXP3 overexpression induced changes in mesenchymal morphology in OCM-1 and C918 cells: cell pseudopodia lengthening and loss of intercellular contact, Scale bar represents 100 μm; B: FOXP3 overexpression promoted EMT process and MMP9 expression; C: FOXP3 knockdown inhibited EMT process and MMP9 expression. E-CA: E-cadherin; N-CA: N-cadherin; MMP9: Matrix metalloproteinase 9; sh: Short hairpin lentivirus; NC: Negative control; pCDH: Plasmid CMV DRV-HIV; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; FOXP3: Forkhead box protein P3; OCM-1: Ocular choroidal melanoma-1; CM: Choroidal melanoma; EMT: Epithelial-mesenchymal transition. $^{a}P<0.05$, $^{b}P<0.01$.

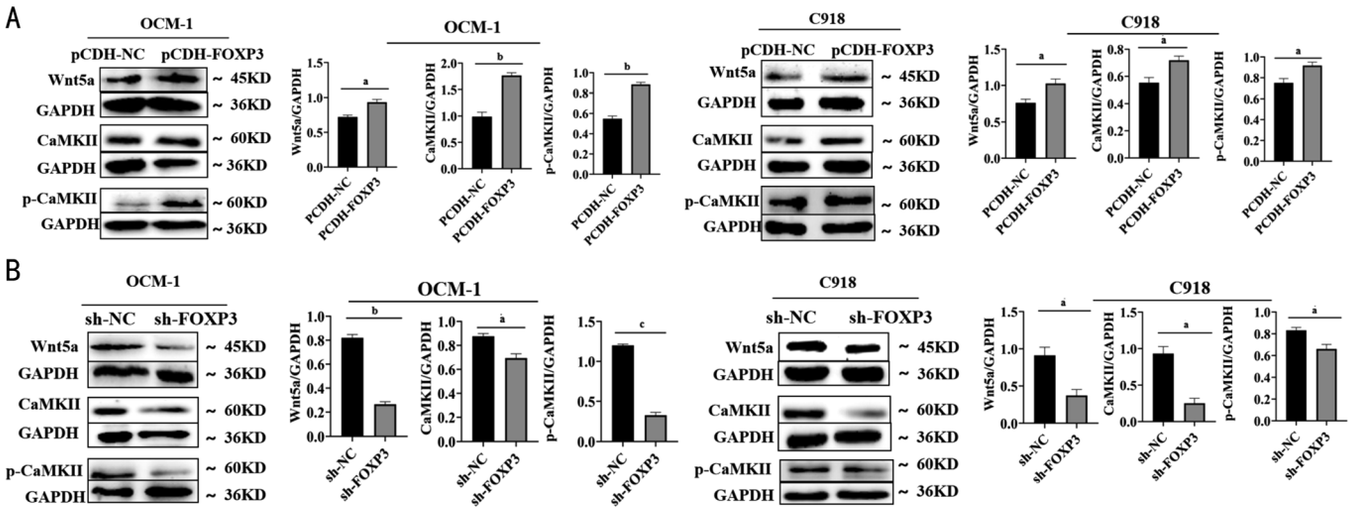

Figure 5 FOXP3 promoted Wnt5a/CaMKII in CM cells A: FOXP3 overexpression up-regulated expression of Wnt5a, CaMKII and p-CaMKII; B: FOXP3 knockdown inhibited expression of Wnt5a, CaMKII and p-CaMKII. sh: Short hairpin lentivirus; NC: Negative control; pCDH: Plasmid CMV DRV-HIV; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; FOXP3: Forkhead box protein P3; OCM-1: Ocular choroidal melanoma-1; CM: Choroidal melanoma. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001.

of migrated and invaded cells was reduced in the sh-FOXP3/si-Wnt5a group compared to the sh-FOXP3 group (Figure 6B). Further analysis of EMT markers revealed that Wnt5a silencing suppressed FOXP3-driven EMT. In the pCDH-FOXP3/si-Wnt5a group, Snail expression was significantly reduced compared to the pCDH-FOXP3 group. In the sh-FOXP3/si-Wnt5a group, both N-cadherin and Snail levels were markedly downregulated compared to the sh-FOXP3 group (Figure 6C). These results indicate that Wnt5a downregulation mitigates the FOXP3-mediated promotion of cell migration, invasion, and EMT in C918 cells, underscoring the importance of the Wnt5a/CaMKII signaling pathway in FOXP3-driven tumor progression.

FOXP3 Knockdown Suppressed Tumor Growth in a Nude Mouse Model To evaluate the role of FOXP3 in CM growth, we utilized a murine xenograft model. Subcutaneous tumors derived from FOXP3-knockdown cells exhibited significantly smaller volumes compared to those from controls, indicating a suppression of tumor growth (Figure 7A). Further molecular analysis supported these findings. Western blotting revealed that FOXP3 knockdown upregulated E-cadherin, a key epithelial marker, while reducing p-CaMKII expression (Figure 7B). Consistently, IHC demonstrated increased E-cadherin levels and decreased expression of the EMT-related transcription factor Snail in FOXP3-knockdown tumors (Figure 7C). These results suggest that FOXP3 knockdown inhibits CM growth *in vivo* by suppressing EMT, highlighting its potential as a therapeutic target in CM.

DISCUSSION

As a specific factor, FOXP3 is known to be important in the process of immune escape mechanism[12]. Recently, it has been reported that FOXP3 not only acts on regulatory T cells but also affects the growth and invasion of tumors[13]. It is worth noting that tumor FOXP3 functions inconsistently or even reversely in different tumors[14], indicating that the role of FOXP3 in the tumor may be more important and complex than previously thought. FOXP3 inhibits breast cancer cells invasion and metastasis *in vitro* and *in vivo* by directly inhibiting MTA1 promoter activity and downregulating MTA1 expression[15]. Moreover, high expression of FOXP3 was significantly associated with better clinical prognosis in gastric adenocarcinoma patients[16]. In cholangiocarcinoma, FOXP3 promotes tumor metastasis by upregulating both MMP9 and MMP2[17]. In non-small cell lung cancer, FOXP3 can induce EMT and regulate vascular endothelial growth factor (VEGF), thereby promoting tumor growth and metastasis[18]. A previous study reported that FOXP3 was positively expressed in uveal melanoma[5], but the exact effect of FOXP3 on uveal melanoma as well as its relevant underlying molecular mechanism have both remained unclear. Therefore, we hypothesized that FOXP3 may facilitate the progression of CM. The study demonstrated for the first time the carcinogenic effect of FOXP3 on CM through Wnt5a/CaMKII pathway in cytological and *in vivo* animal experiments.

We first explored FOXP3 expression in CM and its correlation with clinical features. Western blotting results showed higher FOXP3 level in CM cells. Meanwhile, IHC results also showed that higher FOXP3 expression in CM than that in normal uveal tissue specimens. These results suggests that FOXP3 was upregulated in CM cells. In additional, higher FOXP3 expression was correlated with shorter overall survival time and tumor recurrence of CM patients.

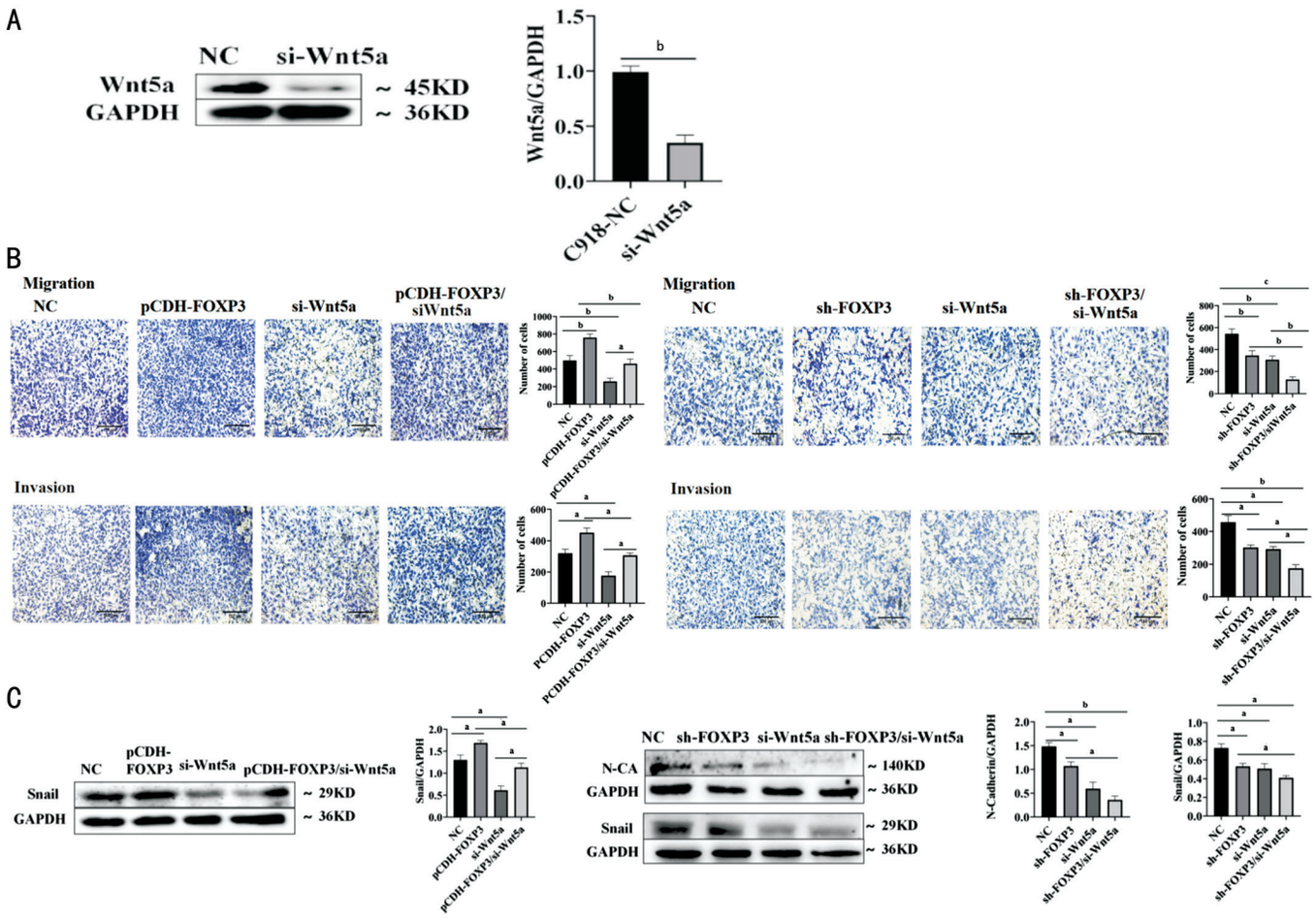

Figure 6 FOXP3 promoted cell metastasis, invasion, and EMT process in C918 cells through the Wnt5a/CaMKII signaling pathway A: Knockdown of wnt5a in C918 cells; B: Wnt5a knockdown inhibited migration and invasion of C918 cells according to transwell assay, scale bar represents 50 μm; C: Wnt5a knockdown inhibited EMT process. EMT: Epithelial-mesenchymal transition; sh-: Short hairpin lentivirus; NC: Negative control; pCDH: Plasmid CMV DRV-HIV; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; FOXP3: Forkhead box protein P3; OCM-1: Ocular choroidal melanoma-1; CM: Choroidal melanoma. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001.

To investigate the effects of FOXP3 on CM, we transfected pCDH-FOXP3 plasmids to overexpress FOXP3 or used sh-FOXP3 CRISPR-Cas9 lentivirus to knock down its expression in CM cells. Functional assays revealed that FOXP3 significantly influences tumor behavior. The CCK-8 assay and monoclonal colony formation assay demonstrated that FOXP3 overexpression enhanced cell proliferation in CM cells, while FOXP3 knockdown inhibited proliferation. Similarly, transwell assays showed that FOXP3 upregulation promoted cell migration and invasion, whereas FOXP3 downregulation suppressed these malignant behaviors. To confirm these findings *in vivo*, we utilized a nude mouse xenograft model. Tumor growth was significantly reduced in mice injected with FOXP3-knockdown (sh-FOXP3) C918 cells compared to the control group, demonstrating the tumor-promoting role of FOXP3. Collectively, these results suggest that FOXP3 upregulation drives the malignant progression of CM by promoting proliferation, migration, and invasion, whereas its downregulation inhibits tumor growth and progression. These findings highlight FOXP3 as a potential therapeutic target for CM.

EMT is a key element in tumor metastasis[19]. This transition is characterized by the acquisition of mesenchymal cell marker N-cadherin and Snail protein and the loss of epithelial cell marker E-cadherin protein[20]. The downregulation of E-cadherin can lead to decreased cell-cell contact and cell mobility[21]. Tumor cells with EMT mesenchymal characteristics show higher metastasis and invasion abilities, thereby resulting in both an increase in the occurrence of distant metastasis and a poorer prognosis in colorectal cancer, breast cancer, lung cancer, and other tumors[22]. In this study, the expression of E-cadherin was found to be downregulated after CM cells were transfected with the pCDH-FOXP3 plasmid, while the N-cadherin, Snail and MMP9 levels were increased in converse. In the same time, mesenchymal morphological characteristics appeared in transfected cells. Furthermore, FOXP3 knockdown promoted the expression of E-cadherin and inhibited the expressions of N-cadherin, Snail, and MMP9.

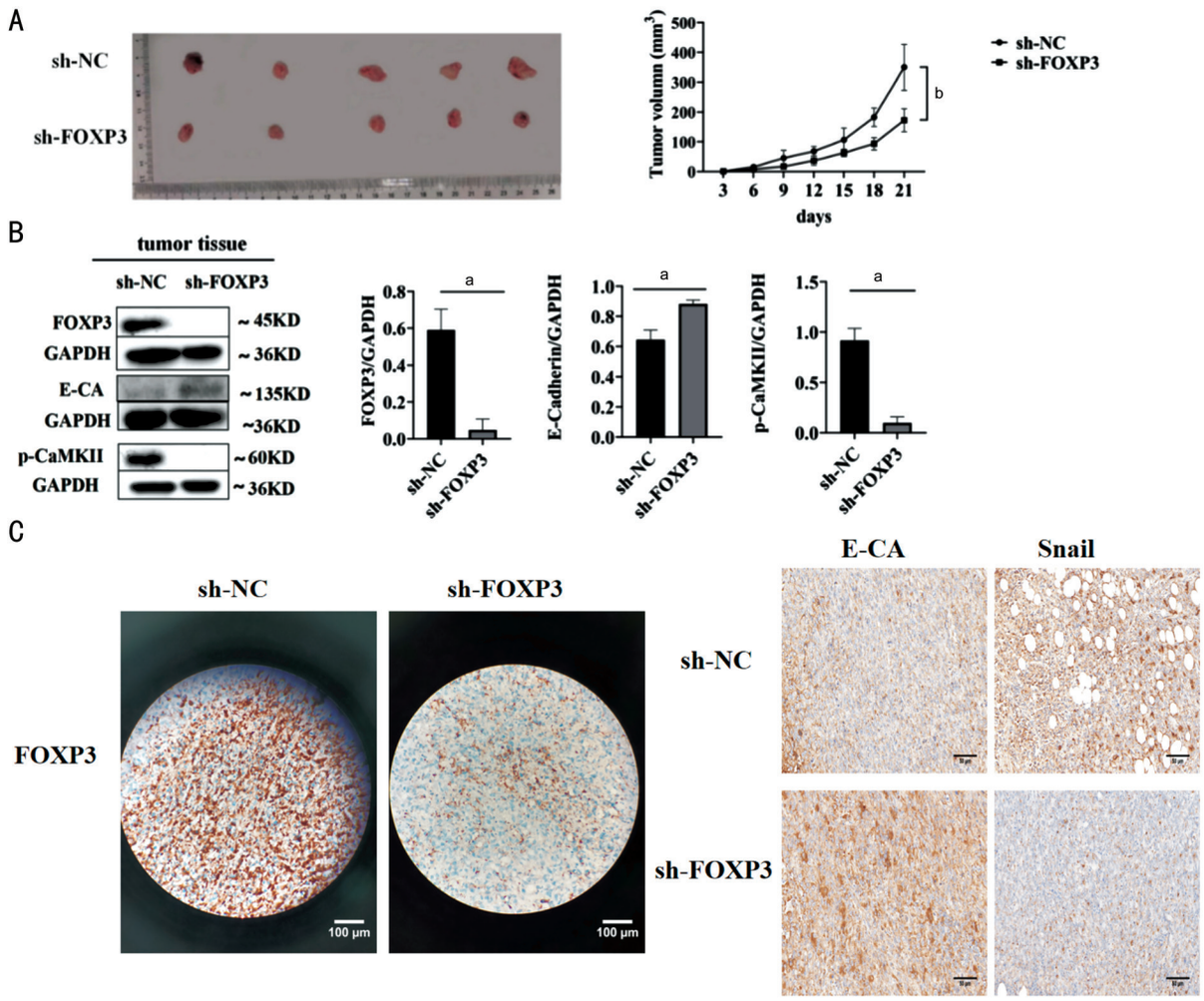

Figure 7 FOXP3 knockdown inhibited CM growth and EMT process in a murine xenograft model A: FOXP3 knockdown inhibited the growth of xenograft tumors; B: FOXP3 knockdown increased E-cadherin level and decrease p-CaMKII level in xenograft tumor; C: Immunohistochemical assay showed that FOXP3 knockdown promoted the expression of E-cadherin and inhibited the expression of Snail in xenograft tumor. EMT: Epithelial-mesenchymal transition; sh-: Short hairpin lentivirus; NC: Negative control; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; FOXP3: Forkhead box protein P3; E-CA: E-cadherin; CM: Choroidal melanoma. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

The changes in EMT-related markers and cell morphology indicated that FOXP3 promoted the transformation of CM cells from epithelial to mesenchymal property.

The Wnt signaling pathway can induce EMT process, facilitate the separation of cells from the primary tumor, and infiltrate into surrounding tissues[23]. Li *et al*[24] found that the protein expression of Wnt was related to FOXP3 protein expression, thereby affecting the immunosuppressive activity of Treg cells in tumors. Among the Wnt signaling pathway-related markers, Wnt5a can induce EMT by regulation of endocrine, paracrine, and downstream pathways and factors, thus promoting the growth and metastasis of non-small cell lung cancer, gastric cancer, and prostate cancer[25-28]. In addition, some researches have shown that CaMKII is closely related to the development of EMT[29]. Wnt-5a induces EMT by activating CaMKII, reducing the adhesion between cells[30]. Wnt5a has been shown to regulate CaMKII in a variety of cells, thereby activating TGFβ-activated kinase to regulate cell function and biological processes[31-32]. In periodontitis study, Wnt5a significantly increased the expression of total CaMKII protein and p-CaMKII level[33]. Because of the elevated expression of Wnt5a in uveal melanoma and the fact that FOXP3 affects the malignant phenotype of breast cancer by regulating the Wnt/β-catenin signaling pathway[34], we hypothesized that FOXP3 may affect the Wnt5a/CaMKII signaling pathway in CM. It has been shown that tumor-associated macrophages promote colorectal cancer progression through the Wnt5a/CaMKII/ERK/CCL2 signaling pathway. There are few studies on the role of Wnt5a/CaMKII on tumors, and this will be the first time to explore the effect of FOXP3 on the Wnt5a/CaMKII pathway.

In this study, we demonstrated that FOXP3 regulates the Wnt5a/CaMKII signaling pathway in CM cells. Overexpression of FOXP3 upregulated the pathway, whereas FOXP3 knockdown downregulated it. To further elucidate the mechanism, we analyzed EMT and Wnt5a/CaMKII markers

in xenografts derived from nude mouse models using Western blotting. Consistent with *in vitro* results, FOXP3 knockdown in xenografts reduced p-CaMKII activity and increased E-cadherin expression, indicating suppression of EMT progression. These findings confirm that FOXP3 promotes the malignant phenotype of CM by activating the Wnt5a/CaMKII pathway and driving EMT. To verify whether FOXP3 mediates its effects *via* the Wnt5a pathway, we employed siRNA technology to specifically silence the Wnt5a gene. Wnt5a downregulation attenuated the pro-migratory, pro-invasive, and EMT-inducing effects of FOXP3 overexpression in C918 cells. Collectively, these results confirm that FOXP3 facilitates CM cell metastasis, invasion, and EMT progression by activating the Wnt5a/CaMKII signaling pathway, underscoring its potential as a therapeutic target in CM.

However, this study has some limitations. Notably, we utilized a subcutaneous xenograft model in nude mice, which may not fully recapitulate the tumor microenvironment of UVM. Future research will focus on developing orthotopic UVM xenograft models and metastatic models to better investigate the *in vivo* mechanisms of FOXP3. Additionally, we aim to explore whether FOXP3 facilitates immune evasion by influencing regulatory T cells within the tumor microenvironment. These approaches will provide deeper insights into the role of FOXP3 in CM progression and its potential as a therapeutic target.

In conclusion, the study demonstrated that FOXP3 promoted the growth, metastasis, invasion, and EMT transition of CM cells through Wnt5a/CaMKII signaling at both cellular and *in vivo* levels, thereby revealing the role of FOXP3 in the development of CM as well as its underlying molecular mechanism. This study, therefore, provides a new potential target for the research and diagnosis of CM, which is of great significance for its clinical control.

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