FOXC1 silencing inhibits the epithelial-to-mesenchymal transition of glioma cells: Involvement of β-catenin signaling

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Abstract. Glioma is a type of malignant brain tumor. Forkhead box C1 (FOXC1) is a conserved transcription factor that is involved in tumorigenesis; however, the function of FOXC1 in glioma remains unclear. The present study aimed to investigate the effects of FOXC1 silencing on the epithelial-to-mesenchymal transition (EMT) of glioma cells. FOXC1-specific small interfering RNAs were employed to downregulate the expression levels of FOXC1 in glioma cells. The proliferation, migration and invasion of glioma cells were assessed by MTT assay, wound healing assay and Transwell assay. Western blot analysis was performed to reveal the effects of FOXC1 on EMT-associated proteins and β-catenin signaling. The results revealed that, following FOXC1 silencing, the proliferation, migration and invasion of glioma cells were decreased. The expression levels of EMT-associated proteins were also affected. Further examination demonstrated that β-catenin signaling was involved in the effects of FOXC1 on glioma cells. Previous results suggested that overexpression of β-catenin reversed the effects of FOXC1 silencing on glioma cells. The present study demonstrated that FOXC1 may regulate the EMT of glioma cells, potentially via β-catenin signaling. Therefore, FOXC1 may be a potential therapeutic target for the treatment of glioma.

Introduction

Glioma is one of the most common types of malignant tumor in the central nervous system, which accounts for ~29% of all types of brain tumor and contributes to cancer-associated mortality rates (1). Characteristics of glioma are high incidence and mortality rates, high recurrence and low cure rates. It is difficult to diagnose and treat glioma at an early stage due to the lack of auxiliary examination indexes. Although advances have been made in the diagnostic and therapeutic strategies for glioma, the prognoses of patients with glioma remain poor, particularly for those with glioblastoma, with an estimated 2-year survival rate of 15% (2).

Epithelial-to-mesenchymal transition (EMT) is a biological process in which epithelial cells are transformed to mesenchymal-like cells. Throughout the EMT process, epithelial cells lose their connectivity to basement membranes and acquire improved migratory and invasive capabilities. EMT, which is regulated by complex networks, is an important process in development. It also serves important roles in the tumorigenic process, resulting in improvement of the migration and invasion of tumor cells (3-5).

Forkhead box (FOX) proteins are a class of conserved transcription factors, which are implicated in various biological and physiological processes. The dysregulation of FOX proteins is also implicated in tumor progression; therefore, FOX proteins are considered potential diagnostic markers or therapeutic targets for cancer (6,7). As a member of the FOX protein family, FOXC1 is located on chromosome 6p25. FOXC1 contains a conserved forkhead domain, which binds upstream of target genes, thus promoting gene activation. FOXC1 has critical roles in physiological processes, including growth, development and differentiation (8-11). FOXC1 also serves important roles in pathological conditions and the dysregulation of FOXC1 additionally contributes to tumorigenesis. Previous evidence has revealed that FOXC1 is involved in tumor development (7). Notably, FOXC1 has been demonstrated to be overexpressed in breast cancer and lung cancer, and is correlated with the poor survival rates of these cancer types (12-15). Furthermore, FOXC1 has been reported to regulate the growth, metastasis and differentiation of several types of cancer (16-20). Therefore, FOXC1 is regarded as a potential biomarker for certain cancer types.

FOXC1 is associated with the proliferation and migration of various types of cancer (15,21) and is regarded as an EMT inducer. It has previously been reported that FOXC1...
is highly expressed in glioma (22); however, to the best of our knowledge, there have been no previous studies on the association between FOXC1 and glioma. The present study aimed to examine the function of FOXC1 in glioma cells and to investigate the underlying mechanism.

Materials and methods

Materials. FOXC1 small interfering (si)RNA1 (target sequence: 5'-CCACTGCAACCTGCGAGCCCAT-3'), FOXC1 siRNA2 (target sequence: 5'-GCGGCACTACCTGCGGGTT-3') and negative control (NC) siRNA (5'-TTTCTCGAAGTGTTACCAG TT-3') were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). β-catenin overexpression (OE) plasmid and empty vector were obtained from Addgene, Inc. (cat. no. 19286; Cambridge, MA, USA). Antibodies against FOXC1 (cat. no. 55365-1-AP), N-cadherin (cat. no. 22018-1-AP), E-cadherin (cat. no. 20874-1-AP), Vimentin (cat. no. 10366-1-AP), Snail (cat. no. 13099-1-AP), Twist (cat. no. 25465-1-AP), β-catenin (cat. no. 17565-1-AP) and c-myc (cat. no. 10828-1-AP) were purchased from Wuhan Sanying Biotechnology, (Wuhan, China). Antibodies against phosphorylated (p)-β-catenin (cat. no. 4176) and GAPDH (cat. no. 2118) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. U251 cells and SHG44 cells were obtained from Procell Life Science and Technology Co., Ltd. (Wuhan, China). U251 cells were grown in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel). SHG44 cells were grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. All cells were cultured in a cell incubator at 37°C in an atmosphere containing 5% CO₂.

Transfection. Cells were seeded into a 6-well plate (4x10⁵ cells/well). A total of 1 h prior to transfection, the medium was replaced with fresh serum-free medium. A total of 100 pmol FOXC1 siRNA1, FOXC1 siRNA2 or negative control (NC) siRNA were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 4 h at 37°C, the cell medium was replaced with fresh cell medium containing 10% FBS. Subsequently, the cells were subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR) 24 h post-transfection or western blotting 48 h post-transfection. For co-transfection, cells were co-transfected with 1 μg empty vector or β-catenin OE plasmid alongside 50 pmol NC siRNA or FOXC1 siRNA2 using Lipofectamine® 2000, as aforementioned.

RT-qPCR. TRIpure lysis buffer (BioTeke Corporation, Beijing, China) was used to extract total RNA, according to the manufacturer’s protocol. Subsequently, total RNA was reverse transcribed to cDNA using Super M-MLV reverse transcriptase (BioTeke Corporation) according to the manufacturer’s protocol. FOXC1 mRNA expression levels were detected using the SYBR-Green method. SYBR Green was visualized using an enhanced chemiluminescent kit (Beyotime Institute of Biotechnology, Shanghai, China). After culturing for 4 h at 37°C, 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a final concentration of 0.5 mg/ml was added to each well at 12, 24, 36, 48 and 72 h. After culturing for 4 h at 37°C, 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well following the removal of cell medium. Absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

MTT assay. Cells were seeded in 96-well plates (4x10³ cells in each well) and were transfected with NC siRNA, FOXC1 siRNA1 or FOXC1 siRNA2. MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a final concentration of 0.5 mg/ml was added to each well at 12, 24, 36, 48 and 72 h. After culturing for 4 h at 37°C, 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well following the removal of cell medium. Absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Wound healing assay. Cells were seeded into a 6-well plate (4x10³ cells/well) and were transfected with negative control siRNA, FOXC1 siRNA1 or FOXC1 siRNA2. After 24 h, when the cell confluence reached 80%, cells were treated with mitomycin C (1 μg/ml; Sigma-Aldrich; Merck KGaA) for 1 h at 37°C. Subsequently, wounds were created on the surface of the cell monolayer with a 200-μl pipette tip. Cells were cultured in a cell incubator and the images were captured under an inverted microscope with magnification, x100 at 0 h and 24 h. The relative migration ratio was calculated as follows: Relative migration ratio=(incipient gap between two edges-migrated gap between two edges)/incipient gap between the two edges.

Transwell assay. Transwell inserts were purchased from Corning Incorporated (Corning, NY, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). A total of 4x10⁵ cells in 200 μl cell medium were added into Transwell inserts ( precoated with Matrigel) and 800 μl medium
supplemented with 30% FBS was added into the lower chambers. Then the cells were transfected with the negative control siRNA, FOXC1 siRNA1 or FOXC1 siRNA2, or co-transfection with empty vector or β-catenin OE plasmid and negative control siRNA or FOXC1 siRNA2. Thereafter, the cells were cultured in a cell incubator and allowed to invade for 24 h. After rinsing, cells on top of the membranes were removed. Cells that had invaded through the membranes were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 0.5% crystal violet for 5 min. Images were captured under an inverted microscope (Motic Instruments, Richmond, BC, Canada) with a magnification, x200.

Immunofluorescence. Cells were seeded onto slides in 12-well plates (5x10^4 cells/well) and were transfected with negative control siRNA, FOXC1 siRNA1 or FOXC1 siRNA2. A total of 24 h post-transfection, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 30 min at room temperature. The cells were blocked with goat serum (Beijing Solarbio Science and Technology Co., Ltd.) for 15 min at room temperature and then incubated with a primary antibody against N-cadherin (cat. no. 13116; 1:200; Cell Signaling Technology, Inc.) overnight at 4°C. After rinsing, the cells were incubated with a Cy3-labeled secondary antibody (cat. no. A0516; 1:400; Beyotime Institute of Biotechnology) for 60 min at room temperature. Subsequently, the cells were rinsed, stained with DAPI (Sigma-Aldrich; Merck KGaA), and observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan) with magnification, x400.

Statistical analysis. Each experiment was repeated three times and the results are presented as the means ± standard deviation. Differences between groups were analyzed using a one-way or two-way analysis of variance, followed by Bonferroni’s multiple comparison as a post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Silencing FOXC1 inhibits the proliferation, migration and invasion of glioma cells. To examine the function of FOXC1 in glioma, FOXC1-specific siRNAs were used in the present study, and the expression levels of FOXC1 were detected in U251 and SHG44 cells. The RT-qPCR results revealed that, post-transfection with FOXC1 siRNA1 or FOXC1 siRNA2, the mRNA expression levels of FOXC1 were significantly

Figure 1. FOXC1 siRNAs effectively decrease the mRNA and protein expression levels of FOXC1. (A and B) Post-transfection with FOXC1 siRNA1 or FOXC1 siRNA2, the mRNA expression levels of FOXC1 were detected by reverse transcription-quantitative polymerase chain reaction. Relative mRNA expression levels of FOXC1 were normalized to GAPDH and calculated using the 2^−ΔΔCq method. (C and D) Protein expression levels of FOXC1 in SHG44 and U251 cells were detected by western blot analysis post-transfection with FOXC1 siRNA1 or FOXC1 siRNA2. GAPDH served as the internal control. All experiments were repeated three times and the results are presented as the means ± standard deviation. ***P<0.001 vs. the negative control siRNA group. FOXC1, forkhead box C1; siRNA, small interfering RNA.
decreased (Fig. 1A and B) \((P<0.001)\). The results of western blot analysis also revealed a significant decrease in the protein expression levels of FOXC1 in U251 cells and SHG44 cells in response to FOXC1 siRNA compared with the NC siRNA group (Fig. 1C and D) \((P<0.001)\). These results indicated that FOXC1 siRNA1 and FOXC1 siRNA2 effectively decreased FOXC1 expression.

The effects of FOXC1 silencing were also detected on the proliferation, migration and invasion of glioma cells. An MTT assay revealed that, post-transfection with FOXC1 siRNA1 or FOXC1 siRNA2, the proliferation of SHG44 and U251 cells was reduced compared with those transfected with the negative control siRNA (Fig. 2A and B). These results revealed that silencing FOXC1 inhibited the proliferation of glioma cells.
A wound healing assay demonstrated that, compared with the negative control siRNA group, the relative migration ratio of glioma cells was decreased by FOXC1 siRNA1 and FOXC1 siRNA2 (Fig. 2C). In addition, a Transwell assay revealed that the ratio of invasive cells was decreased by FOXC1 siRNA1 and FOXC1 siRNA2 compared with the negative control siRNA (Fig. 2D). These results revealed that silencing FOXC1 inhibited the migration and invasion of glioma cells.

**FOXC1 modulates the expression of EMT-associated proteins.** Since EMT contributes to the migration and invasion of cancer cells, the effects of FOXC1 silencing on the expression of EMT-related proteins were examined. Western blot analysis showed that the protein levels of N-cadherin, E-cadherin, Vimentin, Snail, and Twist were upregulated in the negative control siRNA group compared with the FOXC1 siRNA groups (Fig. 3). The expression of these proteins was significantly downregulated in the FOXC1 siRNA groups compared with the negative control siRNA group. These findings suggest that FOXC1 silencing modulates the expression of EMT-associated proteins, thereby inhibiting the migration and invasion of glioma cells.
of EMT-associated proteins were detected by western blot analysis. As presented in Fig. 3A and B, in SHG44 and U251 cells, the expression levels of N-cadherin were decreased by FOXC1 siRNAs, whereas the expression levels of E-cadherin were increased by FOXC1 silencing. In addition, the expression levels of Vimentin were decreased post-transfection with FOXC1 siRNAs (Fig. 3C), and the protein expression levels of Snail and Twist were also decreased (Fig. 3D and E). Furthermore, the expression and distribution of N-cadherin were detected by immunofluorescence. Consistent with the results of western blot analysis, immunofluorescence analysis revealed that, following silencing of FOXC1, N-cadherin expression was reduced, particularly in the cell membranes (Fig. 4). These results revealed that FOXC1 silencing modulated the expression of EMT-associated proteins.

**FOX1 silencing affects β-catenin signaling.** The expression and phosphorylation levels of β-catenin were detected by western blot analysis. Post-transfection with FOXC1 siRNA1 and FOXC1 siRNA2, the expression levels of p-β-catenin were increased in SHG44 and U251 cells, whereas the protein
expression levels of total β-catenin were significantly decreased compared with the negative control siRNA group (Fig. 5A). The protein expression levels of c-myc, which is a downstream target of β-catenin signaling, were also detected by western blotting. After silencing FOXC1, the expression levels of c-myc in SHG44 and U251 cells were decreased compared with the negative control siRNA group (Fig. 5B). These results revealed that silencing FOXC1 affected β-catenin signaling.

β-catenin signaling is involved in the effects of FOXC1 silencing. Since FOXC1 silencing inhibited β-catenin signaling, a β-catenin OE plasmid was used in the present study. β-catenin OE plasmid and FOXC1 siRNA were co-transfected into cells, and the protein expression levels of β-catenin, p-β-catenin and c-myc were detected by western blot analysis. As presented in Fig. 6, following OE of β-catenin, the protein expression levels of β-catenin were increased, whereas the expression levels of p-β-catenin were decreased compared with the negative control group. Co-transfection with β-catenin OE plasmid and FOXC1 siRNA increased the expression levels of β-catenin, but decreased the levels of p-β-catenin compared with the empty vector and negative control siRNA group (Fig. 6A). In addition, co-transfection with β-catenin OE plasmid and FOXC1 siRNA increased the protein expression levels of c-myc compared with the empty vector and negative control siRNA group (Fig. 6B), indicating that co-transfection with β-catenin OE and FOXC1 siRNA enhanced the activation of β-catenin signaling. However, our previous data (Fig. 5) demonstrated that FOXC1 silencing alone suppressed β-catenin signaling, which was distinctly different from the effects of co-transfection with β-catenin OE and FOXC1 siRNA, suggesting that β-catenin OE may reverse the effects of FOXC1 silencing on β-catenin signaling.

Following co-transfection with β-catenin OE plasmid and FOXC1 siRNA, the invasion of SHG44 cells and U251 cells was assessed using a Transwell assay. β-catenin OE enhanced the invasive capability of SHG44 and U251 cells. However, following co-transfection with β-catenin OE and FOXC1 siRNA, the invasive capability of SHG44 and U251 cells demonstrated no significant difference compared with cells co-transfected with the empty vector and negative control siRNA (Fig. 7A). Our previous data in Fig. 2 showed that FOXC1 silencing alone inhibited the invasion of glioma cells. These results indicated that β-catenin OE may abolish the effects of FOXC1 silencing on the invasion of glioma cells. Additionally, compared with the empty vector and negative control siRNA group, the expression levels of N-cadherin were increased and the expression levels of E-cadherin were decreased.
following co-transfection with β-catenin OE and FOXC1 siRNA (Fig. 7B and C). However, our previous data (Fig. 3) demonstrated that FOXC1 silencing alone decreased that levels of N-cadherin and increased the levels of E-cadherin. These results provide further evidence supporting the hypothesis that β-catenin signaling is involved in the inhibitory effects of FOXC1 silencing on the EMT of glioma cells.

**Discussion**

Glioma is a type of malignant brain cancer, which is difficult to diagnose and cure at an early stage. FOXC1 has been revealed to be highly expressed in glioma (22,24). In the present study, it was demonstrated that silencing FOXC1 inhibited the proliferation, migration and invasion of glioma cells. Further experiments revealed that β-catenin signaling was involved in the inhibitory effects of FOXC1 silencing.

Growth of cancer cells is necessary for tumorigenesis. In numerous cancer types, FOXC1 functions as an oncogene; silencing FOXC1 inhibits the proliferation of cancer cells (15,21,25,26), whereas OE of FOXC1 promotes the proliferation of cancer cells (19,27). In the present study, silencing FOXC1 was revealed to suppress the proliferation of glioma cells, indicating that FOXC1 may function as an oncogene and promote the growth of glioma. The cell cycle is a crucial factor impacting cancer cell growth. FOXC1 silencing has been revealed to arrest the cell cycle of non-small-cell lung carcinoma cells at the G0/G1 phase and regulate cyclin D1 expression levels (15). FOXC1 also exerts effects on cancer cell apoptosis; knockdown of FOXC1 induces apoptosis in cervical cancer and endometrial cancer (25,26). These findings indicated that the effects of FOXC1 silencing on glioma cell growth may be associated with its effects on the cell cycle and apoptosis of glioma. However, future studies investigating this implication are required.
Migration and invasion additionally contribute to tumor development. The invasion of glioblastoma into adjacent normal tissues often results in incomplete resection. Liu et al (22) revealed that, through targeting FOXC1, microRNA-133 is able to inhibit the proliferation and invasion of glioma, indicating that FOXC1 may function as an oncogene in glioma. In the present study, silencing FOXC1 was revealed to inhibit the migration and invasion of glioma cells, indicating that FOXC1 may regulate the metastasis of glioma. The present study provided direct evidence to suggest that FOXC1 performs as an oncogene in glioma cells. Consistently, the expression of FOXC1 is positively correlated with lymph node metastasis and the distant metastasis of nasopharyngeal cancer (18). FOXC1 also affects the migration and invasion of cervical cancer, endometrial cancer, osteosarcoma and melanoma (19,21,25‑27). The results of the present study verified the hypothesis that FOXC1 functions as an oncogene in glioma at the cellular level; however, a lack of in vivo data is a limitation of this study.

EMT is a process that enables epithelial cells to lose their cell-cell adhesion, and gain migratory and invasive properties. EMT also serves a crucial role in tumor metastasis and is regarded
as a crucial function for cancer cells to escape from primary sites (28). N-cadherin is a cell-cell adhesion glycoprotein. It is able to facilitate transendothelial migration and is regarded as a marker of mesenchymal cells. E-cadherin is a component of an adhesion complex located in adherens junctions, and is regarded as a marker of epithelial cells. Dysregulation of E-cadherin results in the disintegration of adherens junctions (29). Vimentin is responsible for stabilization of the cytoskeleton, and Snail and Twist are regulators of E-cadherin and N-cadherin. All of these proteins serve important roles in the EMT process. The results of the present study revealed that these proteins, which are closely associated with EMT, were modulated by FOXC1 silencing, indicating that FOXC1 exerts effects on the EMT of glioma cells, which may contribute to its effects on the metastasis of glioma. Consistent with the present study, in other types of cancer, FOXC1 also contributes to the process of EMT (17,20,21). In addition, throughout the EMT process, FOXC1 regulates the microvascular invasion of hepatocellular carcinoma (30).

The Wnt/β-catenin pathway is involved in the regulation of numerous biological processes (31) and has been implicated in various malignancies (32,33). The accumulation and nuclear translocation of β-catenin is a vital event in the activation of Wnt signaling. Nuclear β-catenin serves an important role in tumorigenesis; it has been reported that the expression levels of nuclear β-catenin, and its downstream targets cyclin D1 and c-Myc, are increased in glioma tissues and cell lines (34), and are associated with the proliferation and apoptosis of glioma cells (35,36). In the present study, it was revealed that FOXC1 silencing alone suppressed β-catenin signaling, however, co-transfection of β-catenin OE and FOXC1 siRNA enhanced the activation of β-catenin signaling, indicating that the OE of β-catenin abolished the effects of FOXC1 silencing, thus it was concluded that β-catenin signaling may be implicated in the effects of FOXC1 on glioma. FOXC1 mutations may also result in a reduction of endothelial Wnt signaling (37). Furthermore, it has been demonstrated that there is a β-catenin binding site near the transcriptional start site of FOXC1, and β-catenin is able to directly regulate the transcription of FOXC1 (38). Therefore, the regulatory association between FOXC1 and β-catenin requires further exploration. In addition to β-catenin signaling, alternative signaling pathways, including phosphoinositide 3-kinase/protein kinase B and nuclear factor-κB, are also involved in the effects of FOXC1 in cancer. Whether these signaling pathways are additionally implicated in the effects of FOXC1 on glioma remains unclear and requires further investigation (16,19,21).

In conclusion, silencing FOXC1 was demonstrated to suppress the proliferation, migration and invasion of glioma cells. Further study revealed that β-catenin signaling was implicated in the function of FOXC1. The present study is the first, to the best of our knowledge, to provide direct evidence to suggest that FOXC1 functions as an oncogene in glioma cells. The present study indicated that silencing FOXC1 may be considered a potential therapeutic method for glioma; however, further investigation is required.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

QC, MZ and YS contributed to the study design, experiment performance and manuscript writing. XW, JY, MD, YM, ZZ, KL, LJ, NW and PW contributed to the performance of the experiments.

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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