MicroRNA-182 promotes epithelial-mesenchymal transition by targeting FOXN3 in gallbladder cancer

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Abstract. Increasing evidence has suggested an association between the expression profiles of microRNAs (miRs) and gallbladder cancer (GBC). Recently, miR-182 has been demonstrated to exert tumor-promoting effects. However, the biological activity and molecular mechanisms of miR-182 in GBC remain unclear. The results of the present study demonstrated that miR-182 expression was significantly upregulated in GBC tissues and cell lines (GBC-SD and SGC-996). In addition, miR-182-knockdown attenuated epithelial-mesenchymal transition (EMT) in GBC cells, as indicated by decreased cell migratory and invasive abilities, decreased vimentin expression, and increased E-cadherin expression. The activities of β-catenin and its downstream factors, Cyclin D1 and c-Myc, were also demonstrated to decrease following miR-182-knockdown. Forkhead box N3 (FOXN3) was identified as the direct target of miR-182. Overexpression of FOXN3 ameliorated EMT and the β-catenin pathway. Taken together, the results of the present study suggested that miR-182 promotes EMT in GBC cells by targeting FOXN3, which suppresses the Wnt/β-catenin pathway.

Introduction

Gallbladder cancer (GBC) is the most common biliary malignancy and is characterized by an advanced stage diagnosis, high recurrence rate and poor prognosis due to no specific clinical signs, symptoms or reliable sensitive markers (1). Patients with GBC have a poor outcome, with a 5-year survival rate of only 10-20% (2). Therefore, identifying novel biomarkers remains essential for the early diagnosis and effective treatment of patients with GBC (3).

MicroRNAs (miRNAs/miRs) regulate biological processes, including cell proliferation, differentiation and migration, by binding to the 3'-untranlsated region (3'-UTR) and degrading the mRNA of target genes (4). For example, miR-182 has been reported to promote breast cancer cell motility and invasiveness by targeting Missing in Metastasis (5). Furthermore, miR-182 expression is upregulated in colorectal cancer, which significantly promotes epithelial-mesenchymal transition (EMT), cell proliferation, invasion and migration by targeting Special AT-rich Sequence-Binding protein 2 (6). Aberrant miR profiles in GBC have been reported (7); however, the role of miR-182 in the pathogenesis of GBC remains unclear.

The EMT event of cancer cells is associated with cancer invasion, metastasis and recurrence (8). The Wnt/β-catenin pathway has been implicated in EMT (9). Activation of β-catenin leads to β-catenin stabilization and translocation to the nucleus, where it associates with transcription factors, lymphoid enhancer factor/T-cell (LEF/TCF), to activate the expression of target genes (10). It has been demonstrated that the EMT factor, ZEB1, is transcriptionally regulated by the β-catenin/TCF4 complex pathway (11). Recently, it was reported that Forkhead box N3 (FOXN3) may interact with β-catenin and suppress the activity of the Wnt/β-catenin pathway (12). Prediction analysis using TargetScan 7.2 software (http://www.targetscan.org/vert_72) demonstrates that miR-182 may bind to the 3'-UTR of FOXN3. However, whether miR-182 promotes EMT by negatively regulating FOXN3 remains unclear. Therefore, the present study aimed to investigate the role of miR-182 in GBC.

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Key words: gallbladder carcinoma, microRNA-182, epithelial-mesenchymal transition, Forkhead box N3, Wnt/β-catenin pathway

Materials and methods

Tissue collection. A total of 18 GBC tissues and adjacent normal tissues were collected from patients with GBC (8 males and 10 females; mean age, 70.2±3.6 years; age range, 63-79 years) who underwent surgical resection at The First Affiliated Hospital of Gannan Medical University between March 2017 and May 2019. Inclusion criteria were as follows: i) patients who were diagnosed by histopathological examinations for the first time and had not received any therapies and ii) patients who had provided written informed consent. Exclusion criteria were as follows: i) patients complicated with other clinical disorders and ii) patients with a history of malignancies. Tissue samples were collected from all patients prior to biopsy, which was performed using a fine needle under the guidance of Magnetic Resonance Imaging. Tissue samples were stored at -80˚C until subsequent experimentation. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Gannan Medical University, Ganzhou, China (GMU38972623) and was performed in accordance with the Declaration of Helsinki (13). Written informed consent was provided by all patients prior to the study.

Cell culture. The non-tumorigenic human intrahepatic biliary epithelial cell line (H69) and the human GBC cell lines (GBC-SD and SGC-996) were purchased from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2.

Cell transfection. miR-182 inhibitors (5'-TTCTACCATTGCCAA-3') and miRNA negative control (miR-NC; 5'-ACGTCTATACGCCCA-3') were purchased from Guangzhou RiboBio Co., Ltd. To the best of our knowledge, no effects of miR-NC on cell viability have been confirmed. GBC-SD and SGC-996 cells were seeded onto 6-well plates at a density of $1x10^5$ cells/well. Once they reached 60% confluence, the cells were transfected with 50 nM miR-182 inhibitors and miR-NC using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Inc.) for 24 h at 37˚C, according to the manufacturer's protocol.

The pcDNA3.1-FOXN3 vector (Guangzhou RiboBio Co., Ltd) was prepared by cloning the open reading frame of FOXN3 into the vector. GBC-SD and SGC-996 cells were transfected with the pcDNA3.1-FOXN3 vectors for 24 h at 37˚C, using Lipofectamine 3000 reagent.

MTT assay. GBC-SD and SGC-996 cells transfected with miR-182 inhibitors and miR-NC were seeded onto 96-well plates at a density of $5x10^3$ cells/well and cultured at 37˚C for 48 h. Cells were subsequently incubated with 0.5 mg/ml MTT reagent at 37˚C for 4 h. Following the MTT incubation, the purple formazan crystals were dissolved using 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) in the dark and viability was subsequently analyzed at a wavelength of 490 nm, using a microplate reader (Thermo Fisher Scientific, Inc.).

Transwell migration and invasion assays. GBC-SD and SGC-996 cells were collected 24 h post-transfection and prepared for single-cell suspensions using serum-free medium, at a density of $3x10^4$ cells/ml. The single-cell suspensions were transferred into the upper chambers of Transwell plates, while culture medium supplemented with 20% FBS was plated into the lower chambers. For the invasion assay, Transwell membranes were precoated with Matrigel (EMD Millipore) overnight at room temperature. Following incubation for 2 h at 37˚C, the migratory and invasive cells were collected, washed and stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. Stained cells were counted in five randomly selected fields under an inverted microscope (Olympus CK-40; Olympus Corporation) at a magnification of x100.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from GBC tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Total RNA (2 μg) was reverse transcribed into cDNA using M-MLV (Promega Corporation) for 60 min at 42˚C. qPCR was subsequently performed using an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) to detect the mRNA expression levels of FOXN3, β-catenin, E-cadherin and vimentin. The procedure was performed as follows: 95˚C for 6 min, followed by 40 cycles at 95˚C for 40 sec, 65˚C for 30 sec, and finally at 75˚C for 8 min. miR-182 primers (Biomics) and EzOmics SYBR qPCR kits (cat. no. BK2200) (Biomics) were used to detect the expression levels of miRNAs. The following primer sequences (Biomics) were used for the qPCR: FOXN3 forward, 5'-TGCCAATCACTCCCATTGGG-3' and reverse, 5'-CCGCATCCGGCAGCTGG-3'; Cyclin D1 forward, 5'-TGTTTGCAAGCAGGACTTTG-3' and reverse, 5'-ACGTCAGCCTCCACACTCTT-3'; and c-Myc forward, 5'-CTCCTGGCAAAAGGTCAGAG-3' and reverse, 5'-TCGGTTGTTGCTGATCTGTC-3'. The expression levels of miRNA and mRNA were normalized to the endogenous reference, U6 (forward, 5'-AGACAATTGATGCGTGCGATC-3' and reverse, 5'-GCTGCAACTGCACTACCAAC-3') and 18S rRNA (forward, 5'-GTAACCCGTTGAACCCCATT-3' and reverse, 5'-CCATCCAATCGGTAGTAGCG-3'), respectively. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (14).

Western blotting. Cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a BCA protein assay kit (cat. no. 23250; Pierce Biotechnology; Themo Fisher Scientific, Inc.) and 25 μg protein/lane was separated via SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and blocked with Tris-buffered saline supplemented with 5% skimmed milk for 1 h at room temperature. The membranes were incubated with primary antibodies against FOXN3 (cat. no. 95568), β-catenin (cat. no. 8480), TCF4 (cat. no. 2569), E-cadherin (cat. no. 3195), Vimentin (cat. no. 5741), Cyclin D1 (cat. no. 55506), c-Myc (cat. no. 18583) and GAPDH (cat. no. 5174S) overnight at 4˚C (all 1:1,000 dilutions and purchased from Cell Signaling Technology, Inc.). Following the primary incubation, the membranes were incubated with a horseradish peroxidase-labelled secondary

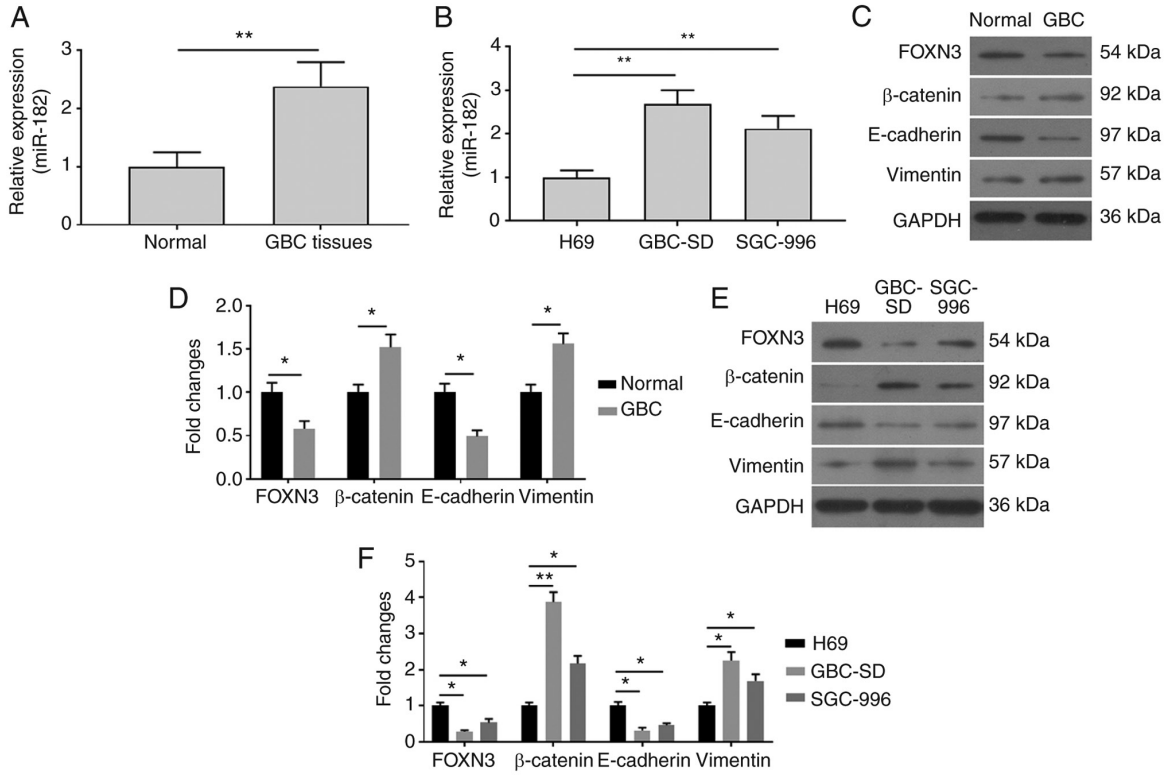

Figure 1. miR-182 expression is upregulated in GBC tissues and cell lines. (A) RT-qPCR analysis was performed to detect miR-182 expression in GBC tissues and adjacent normal tissues. (B) RT-qPCR analysis was performed to detect the expression levels of miR-182 in GBC-SD, SGC-996 and H69 cells. (C) Western blot analysis was performed to detect the protein expression of FOXN3, β-catenin, E-cadherin and vimentin in GBC tissues and adjacent normal tissues. (D) The summarized protein expression levels for (C). (E) Western blot analysis was performed to detect the protein expression levels of FOXN3, β-catenin, E-cadherin and vimentin in GBC-SD, SGC-996 and H69 cells. (F) The summarized protein expression levels for (E). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. miR, microRNA; GBC, gallbladder cancer; RT-qPCR, reverse transcription-quantitative PCR.

antibody (1:2,000; cat. no. 32935; Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were detected using the enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc.) and Quantity One software v4.6.2 (Bio-Rad Laboratories, Inc.).

Dual-luciferase reporter assay. Prediction analysis was conducted using TargetScan 7.2 software (http://www.targetscan.org/vert_72) (15). After inputting miR-182 and clicking the button 'submit', the prediction results were obtained. It indicated that the position 6109-6116 of FOXN3 3'-UTR was paired with miR-182. To validate the prediction, the 3'-UTR of FOXN3 mRNA (both the wild-type and mutant) was cloned into the psiCHECK™-2 vector (Promega Corporation) at the *Xho*I/*Not*I sites, according to the manufacturer's protocol (In-Fusion Advantage PCR cloning kit; cat. no. 630489; Clontech Laboratories, Inc.). Cells were cultured for 24 h at 37˚C. Subsequently, the psiCHECK-FOXN3 3'-UTR reporter vector (the wild-type or the mutant) was co-transfected into cells with miR-182 mimics or miR-NC, using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation for 48 h at 37˚C, firefly and *Renilla* luciferase activities were detected using the Glomax 96 luminometer (Promega Corporation). Firefly luciferase reporter was normalized to *Renilla* luciferase activity.

Co-immunoprecipitation (Co-IP) assay. Cells were harvested using IP lysis buffer (Pierce Biotechnology; Thermo Fisher Scientific, Inc.), of which, 10% was used as the input sample. The remaining amount (90% of IP lysate) was used for IP assays through co-incubation with the goat IgG (cat. no. 6990) or β-catenin (cat. no. 8480) antibodies (both 1:50 dilutions and purchased from Cell Signaling Technology, Inc.). Following the primary incubation, the samples were incubated with protein A-sepharose beads at 4˚C for 2 h. IP washing buffer was used, and the eluted samples (25 μg protein) after centrifugation at 3,000 x g for 4 min at 4˚C were subjected to western blotting.

Statistical analysis. All experiments were performed in triplicate and data are presented as the mean ± standard error of the mean. SPSS 20.0 software (IBM Corp.) was used for statistical analysis. Differences between GBC and normal tissues were analyzed using a paired t-test. One-way analysis of variance, followed by Tukey's post hoc test, was used to compare differences between multiple groups. An unpaired Student's t-test was used to make statistical comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-182 expression is upregulated in GBC tissues and cells. To investigate the role of miR-182 in GBC, RT-qPCR analysis was performed to detect relative miR-182 expression in GBC tissues and cells. As presented in Fig. 1A, miR-182 expression

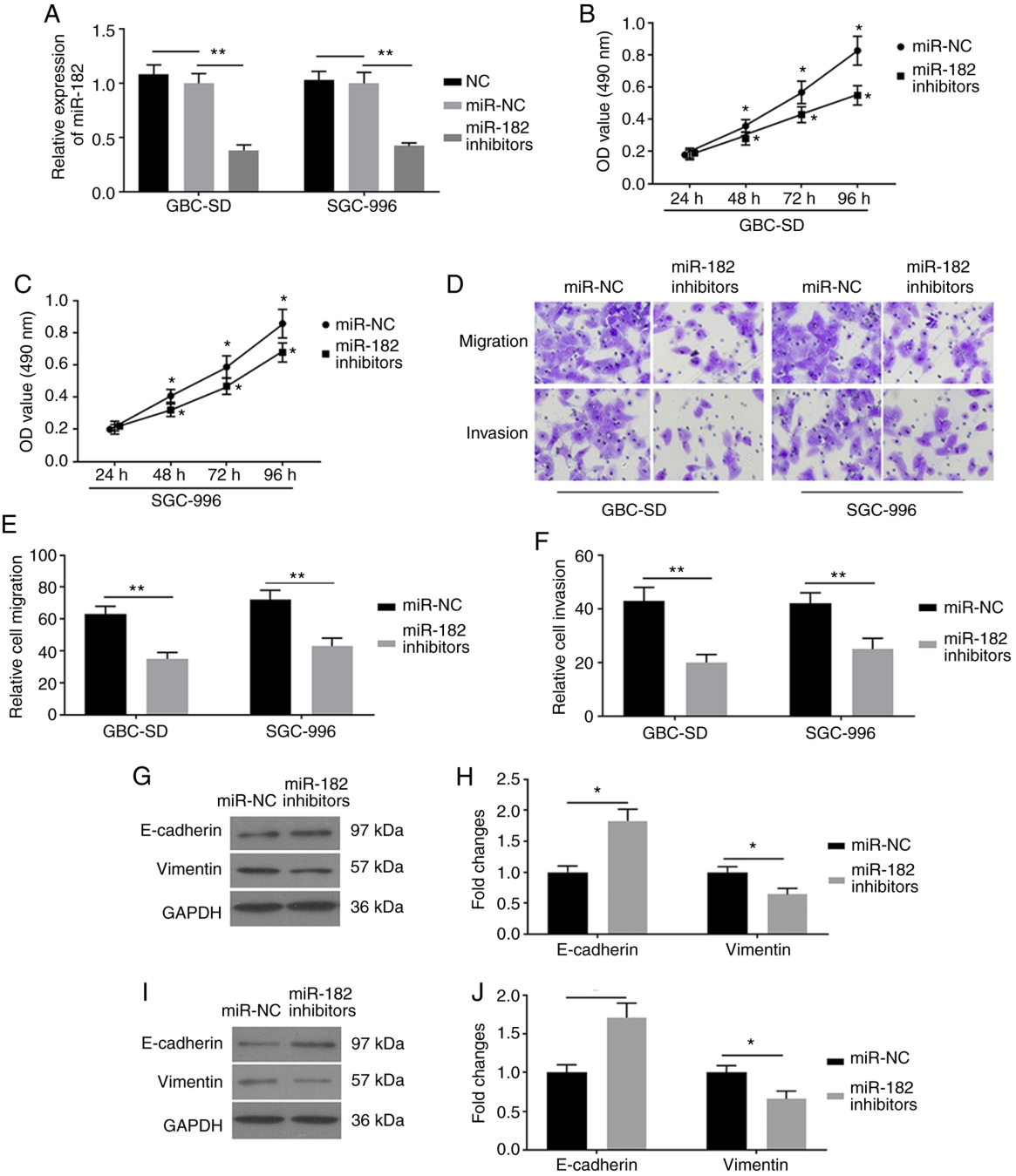

Figure 2. miR-182-knockdown attenuates epithelial-mesenchymal transition in GBC-SD and SGC-996 cells. (A) Reverse transcription-quantitative PCR analysis was performed to detect miR-182 expression in GBC cells transfected with miR-182 inhibitors and miR-NC, respectively. NC groups indicate the untreated GBC-SD or SGC-996 cells without any transfection. The viability of (B) GBC-SD and (C) SGC-996 cells was assessed using an MTT assay. (D) The cell migratory and invasive abilities were assessed via the Transwell assays. (E) The summarized staining levels for migration. (F) The summarized staining levels for invasion. (G) Western blot analysis was performed to detect protein expression levels of E-cadherin and vimentin in GBC-SD cells transfected with miR-182 inhibitors and miR-NC, respectively. (H) The summarized protein expression levels for (G). (I) Western blot analysis was performed to detect protein expression levels of E-cadherin and vimentin in SGC-996 cells transfected with miR-182 inhibitors and miR-NC, respectively. (J) The summarized protein expression levels for (I). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. miR, microRNA; GBC, gallbladder cancer; NC, negative control.

was significantly upregulated in GBC tissues, compared with adjacent normal tissues. miR-182 expression was also assessed in GBC cells. As presented in Fig. 1B, miR-182 expression markedly increased in GBC-SD and SGC-996 cells, compared with H69 cells. To further investigate the association between miR-182 and EMT mediated by β-catenin signaling in GBC cells, western blot analysis was performed to detect protein expression levels of FOXN3/β-catenin and EMT-related factors, E-cadherin and vimentin. As presented in Fig. 1C-F, the protein expression levels of β-catenin and vimentin significantly increased, while FOXN3 and E-cadherin expression levels were attenuated in GBC tissues (Fig. 1C and D) and GBC cells (Fig. 1E and F), compared with adjacent normal tissues and H69 cells, respectively. Taken together, these

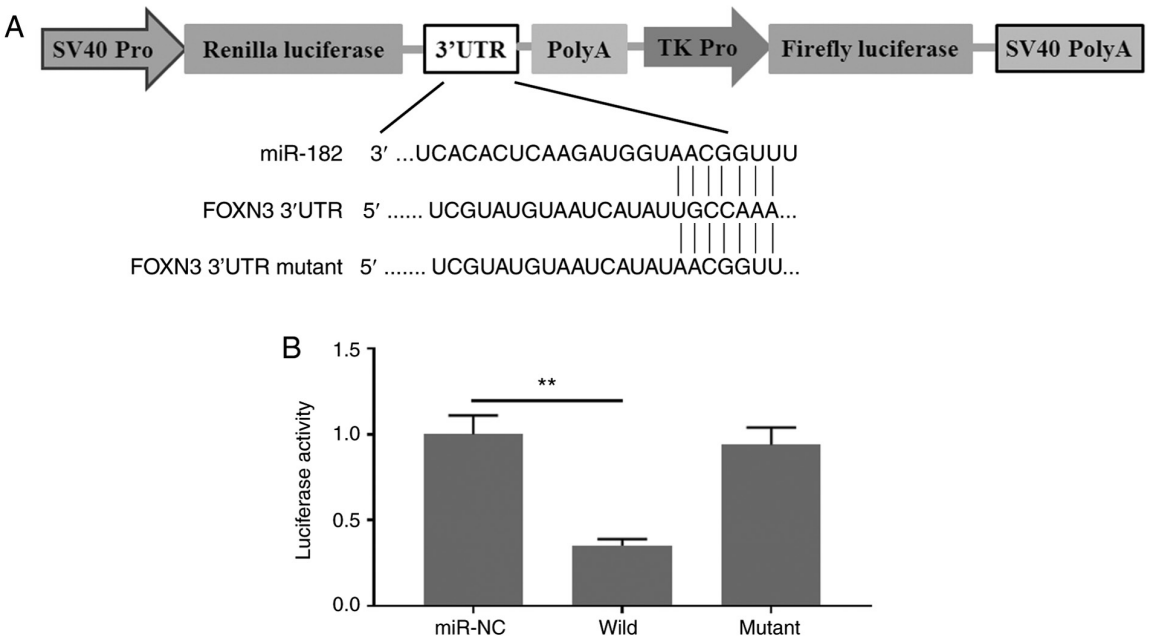

Figure 3. FOXN3 is a direct target of miR-182. (A) Prediction analysis was performed using TargetScan 7.2 software to predict the complementary sequence of 3'-UTR in FOXN3 with miR-182. The results were verified via the dual-luciferase reporter assay. (B) H69 cells were co-transfected with luciferase constructs and miR-182-mimics. Firefly luciferase reporter activity was normalized to *Renilla* luciferase activity. All experiments were performed in triplicate and data are presented as the mean ± standard deviation. **P<0.01. FOXN3, Forkhead box N3; miR, microRNA; 3'-UTR, 3'-untranslated region.

results suggested that increased miR-182 expression may be involved in the EMT of GBC cells.

miR-182-knockdown inhibits EMT in GBC cells. To investigate the role of miR-182 in EMT, GBC-SD and SGC-996 cells were transfected with miR-182 inhibitors and miR-NC, respectively. RT-qPCR analysis was performed to detect miR-182 and the results demonstrated that miR-182 expression was significantly decreased in cells transfected with miR-182 inhibitors, compared with cells transfected with miR-NC (Fig. 2A). These results demonstrated that transfection of GBC cells with miR-182 inhibitors was successful. The results of the MTT assay indicated that miR-182 inhibitors decreased the viability of GBC cells (Fig. 2B and C). To determine the biological functions of miR-182 inhibitors on EMT, cell migration and invasion assays were performed. The results demonstrated that miR-182 inhibitors significantly attenuated the activity of migration and invasion in GBC-SD and SGC-996 cells (Fig. 2D-F). To further investigate the potential molecular mechanism of miR-182 inhibitors on EMT, western blot analysis was performed to detect protein expression levels of the EMT-related factors, E-cadherin and vimentin. miR-182-knockdown effectively decreased vimentin expression and increased E-cadherin expression in GBC-SD (Fig. 2G and H) and SGC-996 (Fig. 2I and J) cells. Taken together, these results suggested that miR-182-knockdown may inhibit EMT in GBC cells.

FOXN3 is a direct target of miR-182. To investigate the potential molecular mechanism of miR-182 in promoting EMT in GBC cells, it was hypothesized that miR-182 targets and degrades FOXN3, which may form a complex with β-catenin and negatively regulate its nuclear transcriptional activity in GBC cells. Prediction analysis demonstrated that FOXN3 is a direct target of miR-182. These results were verified via the dual-luciferase reporter assay. As presented in Fig. 3A, the 3'-UTR of FOXN3 containing a wild-type or mutant binding site for miR-182 was artificially cloned into the firefly luciferase reporter system. No significant differences were observed in the relative luciferase activities between the NC reporter and the reporter containing the mutant binding site of FOXN3. By contrast, the reporter containing the wild-type binding site of FOXN3 exhibited decreased luciferase activity for >60% (Fig. 3B). Taken together, these results suggested that miR-182 specifically aims to degrade FOXN3 by binding to its 3'-UTR.

miR-182/FOXN3 promotes EMT by regulating the β-catenin pathway. The effects of miR-182/FOXN3 on EMT by mediating the β-catenin pathway were assessed. FOXN3 has been reported to be associated with β-catenin, and negatively regulates the expression of its downstream factors (16). The present study assessed the expression levels of Cyclin D1 and c-Myc, which are the downstream factors of the Wnt/β-catenin signaling pathway (17). As presented in Fig. 4A-F, miR-182-knockdown downregulated the mRNA and protein expression levels of Cyclin D1 and c-Myc in GBC cells transfected with miR-182 inhibitors.

To further investigate the effects of miR-182 on EMT, FOXN3 was overexpressed by transfection with pcDNA3.1-FOXN3 into GBC cells, which were also co-transfected with miR-182 inhibitors. The mRNA (Fig. 5A and B) and protein expression (Fig. 5C-F) levels of FOXN3 were significantly upregulated, indicating successful transfection of FOXN3 into GBC-SD and SGC-996 cells. Furthermore, successful co-transfection of FOXN3 and miR-182 inhibitors is

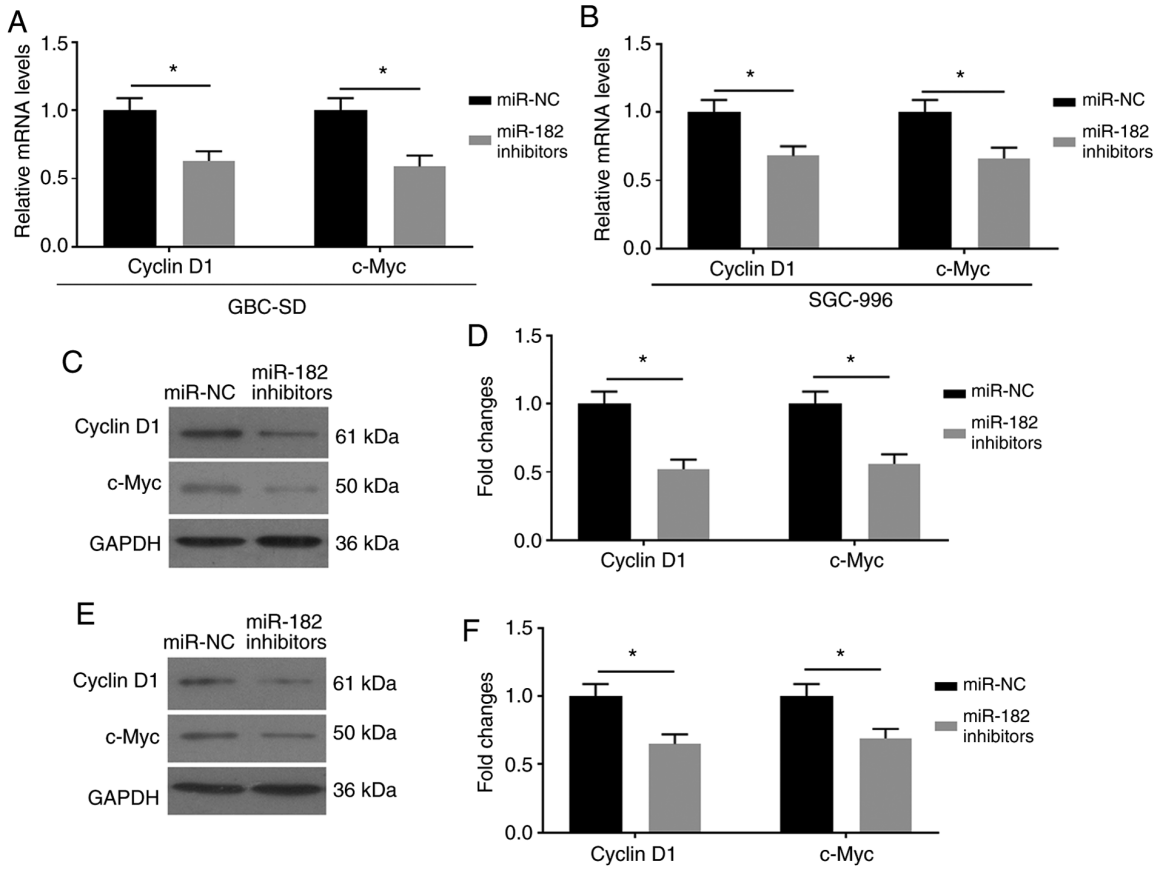

Figure 4. mRNA and protein expression levels of Cyclin D1 and c-Myc are downregulated in GBC cells transfected with miR-182 inhibitors. Reverse transcription-quantitative PCR analysis was performed to detect mRNA expression levels of Cyclin D1 and c-Myc in (A) GBC-SD and (B) SGC-996 (B) cells transfected with miR-182 inhibitors. (C) Western blot analysis was performed to detect protein expression levels of Cyclin D1 and c-Myc in GBC-SD cells transfected with miR-182 inhibitors. (D) The summarized protein expression levels for (C). (E) Western blot analysis was performed to detect protein expression levels of Cyclin D1 and c-Myc in SGC-996 cells transfected with miR-182 inhibitors. (F) The summarized protein expression levels for (E). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. *P<0.05. GBC, gallbladder cancer; miR, microRNA.

indicated in Fig. 5G-L. Notably, the overexpression of FOXN3 was demonstrated to compromise the promoting activity of miR-182 on EMT, as indicated by the increased migration and invasion (Fig. 6A-C), increased E-cadherin expression, and decreased vimentin expression (Fig. 6D-G). In addition, overexpression of FOXN3 ameliorated the promoting activity of miR-182 on the expression levels of Cyclin D1 and c-Myc. The results of the Co-IP assay demonstrated that FOXN3 may form a complex with β-catenin in GBC cells by competing with TCF4 (Fig. 6H and I). Taken together, these results suggested that miR-182 may promote EMT by targeting FOXN3 and negatively regulating the Wnt/β-catenin pathway in GBC cells.

Discussion

GBC is the most common type of cancer in the biliary tract, and only 20% of patients with GBC are diagnosed with non-metastatic GBC (18). Studies of the molecular mechanisms of GBC pathogenesis are required to identify and develop effective therapeutic strategies. EMT is associated with the invasion and metastasis of GBC (19). The results of the present study demonstrated that miR-182 was significantly upregulated in GBC tissues and cell lines. miR-182-knockdown was demonstrated to inhibit EMT. This may be due to the fact that miR-182 targets and degrades FOXN3, which suppressed the activity of the β-catenin pathway by competing with TCF4. The rescue assays from pcDNA3.1-FOXN3 transfection supported that miR-182/FOXN3 promoted EMT by mediating the β-catenin pathway in GBC cells.

Increasing evidence has demonstrated an association between miRNA expression profiles and cancer development. Recently, the pathogenesis of GBC has been indicated to be associated with the dysregulation of miRNAs (7,20). miR-181b expression is increased in GBC tissues, and it may promote cell proliferation and autophagy, and attenuate cell apoptosis in GBC cells by targeting CREBRF, which is a suppressor of autophagy (21). miR-7 and miR-29c have been demonstrated to be downregulated in GBC, while the overexpression of these two miRNAs may effectively reverse EMT and decrease the metastatic activity of GBC cells (22). Overexpression of miR-182 promotes motility and the invasive ability of hepatocellular carcinoma cells by targeting FOXO3A, which represses the activity of Wnt/β-catenin signaling (23). Recently, miR-182 has been reported to promote transforming growth factor (TGF)-β-mediated migration and invasion in GBC cells by targeting the cell adhesion molecule 1 (24). Consistent with these findings, the results of the present study demonstrated the aberrant expression of miR-182 in GBC tissues. In addition, miR-182-knockdown in GBC cells may effectively attenuate EMT, as demonstrated by decreased migration and invasion,

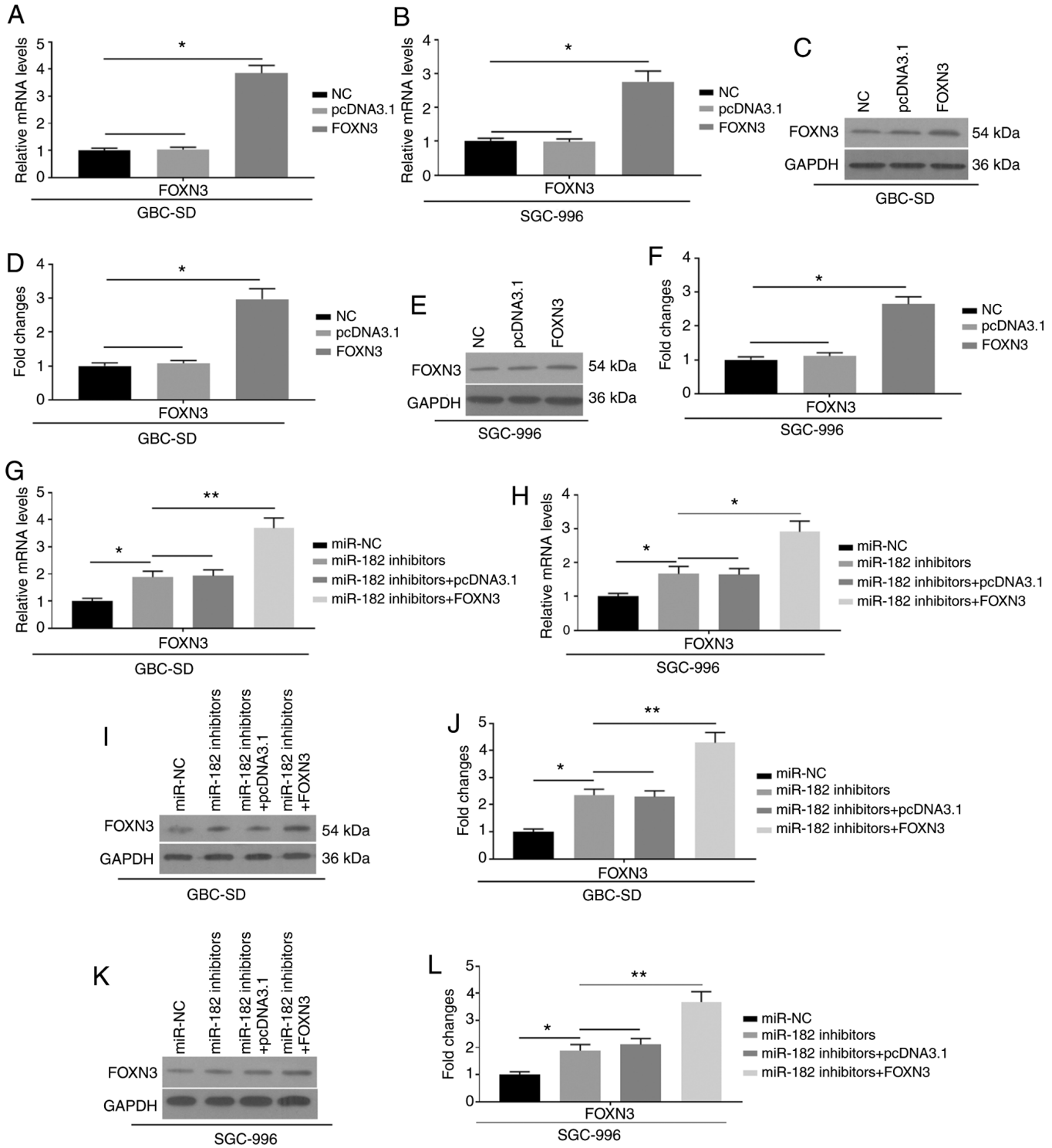

Figure 5. Co-transfection with FOXN3 and miR-182 inhibitors into GBC cells. Reverse transcription-quantitative PCR analysis was performed to detect mRNA FOXN3 expression in (A) GBC-SD and (B) SGC-996 cells transfected with pcDNA3.1-FOXN3. Western blot analysis was performed to detect protein FOXN3 expression in (C and D) GBC-SD and (E and F) SGC-996 cells transfected with pcDNA3.1-FOXN3. Similarly, the mRNA FOXN3 expression in (G) GBC-SD and (H) SGC-996 cells co-transfected with pcDNA3.1-FOXN3 and miR-182 inhibitors. The protein FOXN3 expression was also determined in (I and J) GBC-SD and (K and L) SGC-996 cells co-transfected with pcDNA3.1-FOXN3 and miR-182 inhibitors. All experiments were performed in triplicate and data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. FOXN3, Forkhead box N3; miR, microRNA; GBC, gallbladder cancer; NC, negative control.

decreased vimentin expression and increased E-cadherin expression.

Several studies have reported that activation of the Wnt/β-catenin signaling pathway may promote the transcription of EMT-related genes (25-27). β-catenin interacts with SMAD3 and promotes TGF-β-regulated EMT in lens epithelial cells (25). In addition, β-catenin is required for TGF-β to induce EMT in lens epithelial cells by interacting with SMAD3 and translocation into the nucleus (27). The expression of β-catenin and its downstream factors, Cyclin D1 and

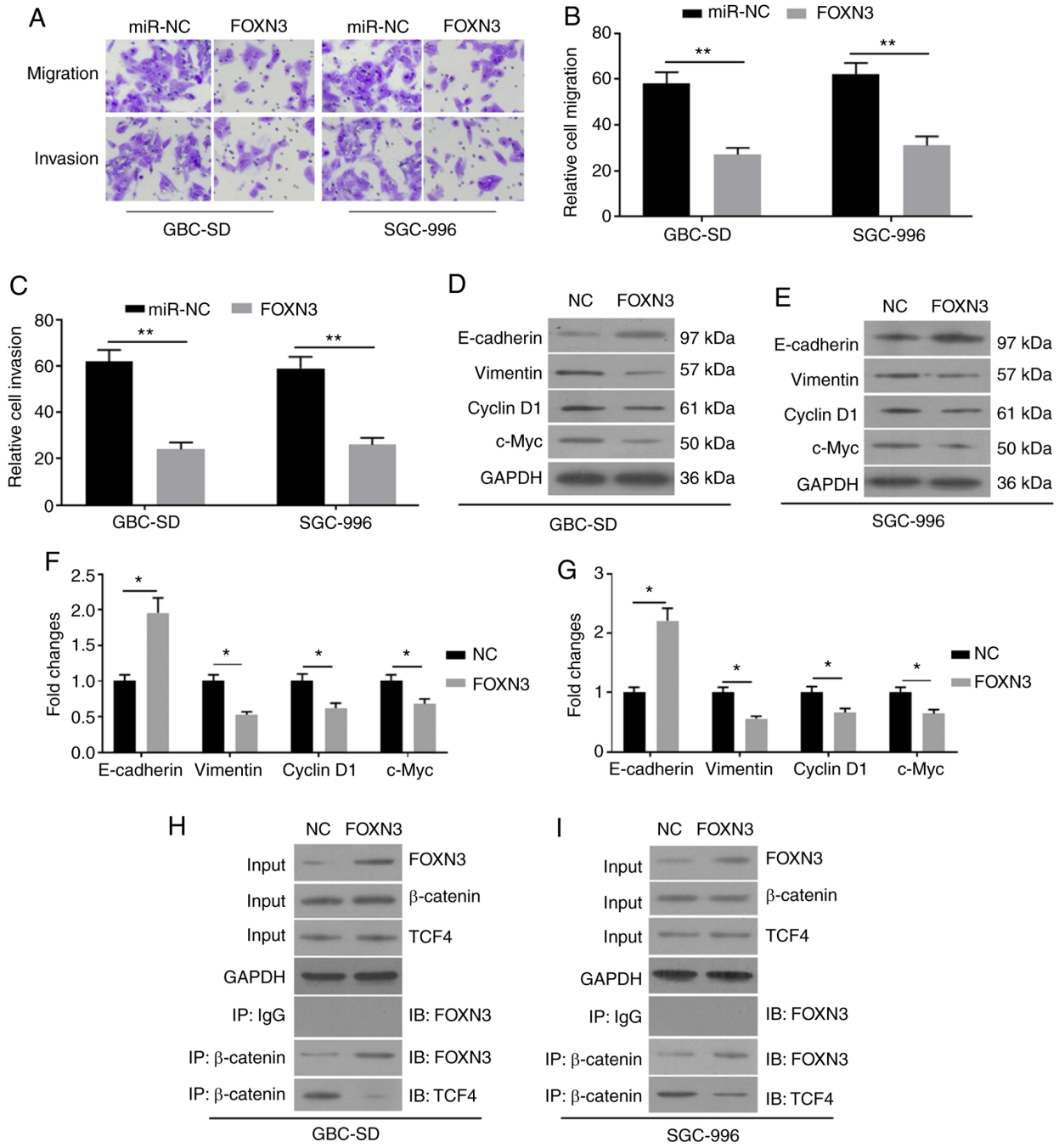

Figure 6. Overexpression of FOXN3 compromises the effects of miR-182 on epithelial-mesenchymal transition in GBC cells co-transfected with miR-182 inhibitors and pcDNA3.1-FOXN3. (A) The cell migratory and invasive abilities were detected via the Transwell assays following transfection with miR-NC and FOXN3, respectively. (B) The summarized staining levels for migration. (C) The summarized staining levels for invasion. Western blot analysis was performed to detect the protein expression levels of E-cadherin, Vimentin, Cyclin D1 and c-Myc in (D) GBC-SD and (E) SGC-996 cells. (F) The summarized data of (D). (G) The summarized data of (E). (H and I) The Co-IP assay was performed to assess the association between FOXN3 and β-catenin. Cell protein extracts (10%) were used as the input sample, which was subjected to Western blot analysis. The remaining protein extracts were subjected to IP using control goat IgG or β-catenin antibodies, followed by IB with anti-FOXN3 or anti-TCF4. All experiments were performed in triplicate and data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. FOXN3, Forkhead box N3; miR, microRNA; GBC, gallbladder cancer; NC, negative control; IP, immunoprecipitation; IB, immunoblotting.

c-Myc, are augmented by H_2O_2, accompanied by an increase of EMT in human lens epithelial cells (28). miR-425 has been demonstrated to promote cell proliferation, migration, invasion and EMT by activating the β-catenin pathway (29). miR-23b is involved in tumor-promoting effects, which are associated with the activation of the JAK/STAT and Wnt/β-catenin pathways in A549 cells (30). In addition, the key EMT factor, ZEB1, is transcriptionally regulated by the β-catenin/TCF4 complex (11). These findings suggested that activation of the Wnt/β-catenin pathways may promote EMT. The overexpression of miR-182 has been demonstrated to target SMAD7, leading to an increase in TGFβ-induced EMT and osteoclastogenesis for bone metastasis in cancer cells (31). The results of the present study demonstrated that the transfection with

miR-182 inhibitors decreased migration and invasion, and decreased the expression levels of Cyclin D1 and c-Myc, which are the two downstream factors of the Wnt/β-catenin pathway.

FOXN3 is a tumor suppressor, and alternative FOXN3 expression profiles are often observed in different types of cancer, including melanoma, osteosarcoma and hepatocellular carcinoma (32). The association between FOXN3 and tumor development has been reviewed comprehensively (32). FOXN3 has been demonstrated to regulate the TGF-β/SMAD signaling pathway, which modulates the proliferation and differentiation of cancer cells (33). Overexpression of FOXN3 significantly inhibits tumor growth of papillary thyroid carcinoma, accompanied by decreased expression of the β-catenin pathway (12). Furthermore, FOXN3 may interact with β-catenin and block the interaction between β-catenin and TCF4 in colon cancer (16). The present study on the prediction from the system on the website indicated that miR-182 may bind to the 3'-UTR of FOXN3. The results of the present study demonstrated that the overexpression of FOXN3, similar to miR-182 inhibitors transfection, may effectively attenuate EMT in GBC cells, as indicated by decreased migration and invasion, decreased vimentin expression, and increased E-cadherin expression. The present study also demonstrated that FOXN3 may co-precipitate with β-catenin, interrupting the interaction between β-catenin and TCF4.

Further studies were still required. The biological effects of FOXN3 in the pathological development of GBC were not fully understood. In addition, the positive effects of miR-182 on the regulation of cellular metabolism in the gallbladder were still absent, although the deficiency of miR-182 may ameliorate EMT. However, the miR-182-knockout mice may be further used to confirm the effects of miR-182 on EMT in GBC. Taken together, the results of the present study demonstrated that miR-182 expression was increased in GBC tissues and cells, and miR-182-knockdown ameliorated EMT. The potential molecular mechanisms may be that miR-182 targets and degrades FOXN3, which competes with TCF4 for binding to β-catenin and suppresses the Wnt/β-catenin pathway.

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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

YX designed the study and wrote the manuscript. JZ, ZH, CW, QL, BH, JP, XT and ZC performed the experiments and analyzed the data. QL, BH, JP, XT and ZC revised and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Gannan Medical University, Ganzhou, China (GMU38972623) and was performed in accordance with the Declaration of Helsinki (13). Written informed consent was provided by all patients prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Wennmacker SZ, Lamberts MP, Di Martino M, Drenth JP, Gurusamy KS and van Laarhoven CJ: Transabdominal ultrasound and endoscopic ultrasound for diagnosis of gallbladder polyps. Cochrane Database Syst Rev 8: CD012233, 2018.
2. Cubertafond P, Gainant A and Cucchiaro G: Surgical treatment of 724 carcinomas of the gallbladder. Results of the French Surgical Association Survey. Ann Surg 219: 275-280, 1994.
3. Zali MR, Zamanian Azodi M, Razzaghi Z and Heydari MH: Gallbladder cancer integrated bioinformatics analysis of protein profile data. Gastroenterol Hepatol Bed Bench 12 (Suppl 1): S66-S73, 2019.
4. Lee HE, Huh JW and Kim HS: Bioinformatics analysis of evolution and human disease related transposable element-derived microRNAs. Life (Basel) 10: 95, 2020.
5. Lei R, Tang J, Zhuang X, Deng R, Li G, Yu J, Liang Y, Xiao J, Wang HY, Yang Q and Hu G: Suppression of MIM by microRNA-182 activates RhoA and promotes breast cancer metastasis. Oncogene 33: 1287-1296, 2014.
6. Yang MH, Yu J, Jiang DM, Li WL, Wang S and Ding YQ: microRNA-182 targets special AT-rich sequence-binding protein 2 to promote colorectal cancer proliferation and metastasis. J Transl Med 12: 109, 2014.
7. Chandra V, Kim JJ, Mittal B and Rai R: MicroRNA aberrations: An emerging field for gallbladder cancer management. World J Gastroenterol 22: 1787-1799, 2016.
8. Zhao M, Ang L, Huang J and Wang J: MicroRNAs regulate the epithelial-mesenchymal transition and influence breast cancer invasion and metastasis. Tumour Biol 39: 1010428317691682, 2017.
9. Li S, Liu F, Xu L, Li C, Yang X, Guo B, Gu J and Wang L: Wnt/β-catenin signaling axis is required for TFEB-mediated gastric cancer metastasis and epithelial-mesenchymal transition. Mol Cancer Res 18: 1650-1659, 2020.
10. Wu L, Huang X, Li L, Huang H, Xu R and Luyten W: Insights on biology and pathology of HIF-1α/-2α, TGFβ/BMP, Wnt/β-catenin, and NF-κB pathways in osteoarthritis. Curr Pharm Des 18: 3293-3312, 2012.
11. Sánchez-Tilló E, de Barrios O, Siles L, Cuatrecasas M, Castells A and Postigo A: β-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. Proc Natl Acad Sci USA 108: 19204-19209, 2011.
12. Zhao C, Mo L, Li C, Han S, Zhao W and Liu L: FOXN3 suppresses the growth and invasion of papillary thyroid cancer through the inactivation of Wnt/β-catenin pathway. Mol Cell Endocrinol 515: 110925, 2020.

13. Chen J, Yu Y, Li H, Hu Q, Chen X, He Y, Xue C, Ren F, Ren Z, Li J, *et al*: Long non-coding RNA PVT1 promotes tumor progression by regulating the miR-143/HK2 axis in gallbladder cancer. Mol Cancer 18: 33, 2019.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
15. Agarwal V, Bell GW, Nam JW and Bartel DP: Predicting effective microRNA target sites in mammalian mRNAs. Elife 4: e05005, 2015.
16. Dai Y, Wang M, Wu H, Xiao M, Liu H and Zhang D: Loss of FOXN3 in colon cancer activates beta-catenin/TCF signaling and promotes the growth and migration of cancer cells. Oncotarget 8: 9783-9793, 2017.
17. Kim GH, Fang XQ, Lim WJ, Park J, Kang TB, Kim JH and Lim JH: Cinobufagin suppresses melanoma cell growth by inhibiting LEF1. Int J Mol Sci 21: 6706, 2020.
18. Mishra SK, Kumari N and Krishnani N: Molecular pathogenesis of gallbladder cancer: An update. Mutat Res 816-818: 111674, 2019.
19. Nakada S, Kuboki S, Nojima H, Yoshitomi H, Furukawa K, Takayashiki T, Takano S, Miyazaki M and Ohtsuka M: Roles of Pin1 as a key molecule for EMT induction by activation of STAT3 and NF-κB in human gallbladder cancer. Ann Surg Oncol 26: 907-917, 2019.
20. Wang J, Jin Y, Li S, Song Q and Tang P: Identification of microRNAs associated with the survival of patients with gallbladder carcinoma. J Int Med Res 48: 300060520918061, 2020.
21. Wu K, Huang J, Xu T, Ye Z, Jin F, Li N and Lv B: MicroRNA-181b blocks gensenoside Rg3-mediated tumor suppression of gallbladder carcinoma by promoting autophagy flux via CREBRF/CREB3 pathway. Am J Transl Res 11: 5776-5787, 2019.
22. Lu K, Feng F, Yang Y, Liu K, Duan J, Liu H, Yang J, Wu M, Liu C and Chang Y: High-throughput screening identified miR-7-2-3p and miR-29c-3p as metastasis suppressors in gallbladder carcinoma. J Gastroenterol 55: 51-66, 2020.
23. Cao MQ, You AB, Zhu XD, Zhang W, Zhang YY, Zhang SZ, Zhang KW, Cai H, Shi WK, Li XL, *et al*: miR-182-5p promotes hepatocellular carcinoma progression by repressing FOXO3a. J Hematol Oncol 11: 12, 2018.
24. Qiu Y, Luo X, Kan T, Zhang Y, Yu W, Wei Y, Shen N, Yi B and Jiang X: TGF-β upregulates miR-182 expression to promote gallbladder cancer metastasis by targeting CADM1. Mol Biosyst 10: 679-685, 2014.
25. Charbonney E, Speight P, Masszi A, Nakano H and Kapus A: β-catenin and Smad3 regulate the activity and stability of myocardin-related transcription factor during epithelial-myofibroblast transition. Mol Biol Cell 22: 4472-4485, 2011.
26. Zhou B, Liu Y, Kahn M, Ann DK, Han A, Wang H, Nguyen C, Flodby P, Zhong Q, Krishnaveni MS, *et al*: Interactions between β-catenin and transforming growth factor-β signaling pathways mediate epithelial-mesenchymal transition and are dependent on the transcriptional co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP). J Biol Chem 287: 7026-7038, 2012.
27. Taiyab A, Holms J and West-Mays JA: β-catenin/Smad3 interaction regulates transforming growth factor-β-induced epithelial to mesenchymal transition in the lens. Int J Mol Sci 20: 2078, 2019.
28. Li J, Chen Y, Han C, Huang S, Chen S, Luo L and Liu Y: JNK1/β-catenin axis regulates H_2O_2-induced epithelial-to-mesenchymal transition in human lens epithelial cells. Biochem Biophys Res Commun 511: 336-342, 2019.
29. Liu D, Zhang H, Cui M, Chen C and Feng Y: Hsa-miR-425-5p promotes tumor growth and metastasis by activating the CTNND1-mediated β-catenin pathway and EMT in colorectal cancer. Cell Cycle 19: 1917-1927, 2020.
30. Wang L, Hu Z, Guo Q, Yang L, Pang Y and Wang W: miR-23b functions as an oncogenic miRNA by downregulating Mcl-1S in lung cancer cell line A549. J Biochem Mol Toxicol 34: e22494, 2020.
31. Yu J, Lei R, Zhuang X, Li X, Li G, Lev S, Segura MF, Zhang X and Hu G: MicroRNA-182 targets SMAD7 to potentiate TGFβ-induced epithelial-mesenchymal transition and metastasis of cancer cells. Nat Commun 7: 13884, 2016.
32. Kong X, Zhai J, Yan C, Song Y, Wang J, Bai X, Brown JAL and Fang Y: Recent advances in understanding FOXN3 in breast cancer, and other malignancies. Front Oncol 9: 234, 2019.
33. Kirmizitas A, Meiklejohn S, Ciau-Uitz A, Stephenson R and Patient R: Dissecting BMP signaling input into the gene regulatory networks driving specification of the blood stem cell lineage. Proc Natl Acad Sci USA 114: 5814-5821, 2017.