IGF2BP3-stabilized SIX4 promotes the proliferation, migration, invasion and tube formation of ovarian cancer cells

JINBIAO HAN[1,2] and XIA HU[1,2]

[1]Department of Gynecology Nursing, West China Second University Hospital, Sichuan University/West China School of Nursing, Sichuan University; [2]Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Chengdu, Sichuan 610041, P.R. China

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Abstract. The mortality rate of ovarian cancer (OC) is the highest among the different types of female reproductive system cancers. SIX homeobox 4 (SIX4), a member of the homeobox family, subfamily SIX, fulfills an important role in metastasis and angiogenesis in a variety of types of cancer. The aim of the present study was to investigate both the effects and the underlying mechanism of SIX4 on angiogenesis in OC. The Gene Expression Profiling Interactive Analysis and Encyclopedia of RNA Interactomes databases were employed to predict the expression levels of SIX4 in OC tissues, and its association with the overall survival (OS) rate of patients with OC. The expression levels of SIX4 in OC cell lines were detected by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. Following silencing of SIX4, the proliferation, invasion, migration and angiogenesis of OC cells were investigated via Cell Counting Kit-8, colony formation, wound healing, Transwell and tube formation assays. Subsequently, the levels of insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) in OC cell lines were detected by RT-qPCR and western blot analysis. The ability of IGF2BP3 to bind to SIX4 mRNA was detected via an RNA immunoprecipitation assay, and the stability of SIX4 mRNA was assessed by RT-qPCR following Actinomycin D treatment. Finally, the effects of transfection of sh-SIX4 and overexpression of IGF2BP3 simultaneously were examined to further delineate the mechanism involved. It was revealed that SIX4 was highly expressed in OC tissues and cells, and its expression was associated with low OS rates in patients with OC. SIX4 knockdown with short hairpin RNA inhibited the proliferation, migration and invasion of cells, as well as angiogenesis. In addition, IGF2BP3 overexpression led to an improvement in the stability of SIX4 mRNA. Overexpression of IGF2BP3 also reversed the inhibitory effect of SIX4 interference on the malignant phenotypes of OC cells. Taken together, the results of the present study demonstrated that IGF2BP3-stabilized SIX4 promoted the proliferation, metastasis and angiogenesis of SKOV3 cells.

Correspondence to: Mrs Xia Hu, Department of Gynecology Nursing, West China Second University Hospital, Sichuan University/West China School of Nursing, Sichuan University, 20 Renmin South Road, Chengdu, Sichuan 610041, P.R. China
E-mail: huxiahx06@163.com

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Introduction

Ovarian cancer (OC) is a common malignant tumor of the female reproductive system. The pathological classification of OC can be divided into four types: Epithelial tumor, germ cell tumor, sex cord stromal tumor and other types (1). Among them, epithelial OC accounts for 90% of OC, and serous OC is the most common subtype with the highest malignant degree among epithelial OC subtypes (2). Serous OC accounts for 70-80% of OC deaths, and overall survival (OS) rates have not changed significantly over the past few decades, remaining at ~30% (3). In the present study, OC refers to serous OC. OC is insidious, and often displays no obvious symptoms or signs before metastasis (4). Furthermore, ~70% of patients with OC are already at an advanced stage when diagnosed with OC, and consequently the mortality rate of OC is the highest among the various types of cancer of the female reproductive system (5). The recurrence and metastasis of OC present great obstacles to its effective treatment, and greatly contribute towards the high mortality rate of OC (6). Therefore, it is imperative to fully elucidate the mechanisms underlying the invasion and metastasis in OC, and to develop specific targeted drugs for the treatment of OC.

SIX homeobox 4 (SIX4), a member of the homeobox family, subfamily SIX, was first found to regulate the expression of Na^+/K^+-ATPase subunit (7). Previous studies have demonstrated that the gene expression levels of SIX4 are closely associated with the occurrence, development, invasion, metastasis and prognosis of esophageal squamous cell carcinoma (8), breast cancer (9), bladder cancer (10), non-small cell lung cancer (11) and other tumor types, suggesting that the SIX4 gene may function as an oncogene in the development of a wide variety of tumors. In addition, a previous study has revealed that SIX4 is involved in

the differentiation of epithelial follicular stem cells in the ovaries of *Drosophila* (12). Therefore, it was possible to hypothesize that the role of SIX4 in OC may also be that of an oncogene.

Insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) has been reported to promote the invasion and metastasis of tumor cells through the local translation of IGF2BP3-bound transcripts (13). In addition, a previous study revealed that high expression levels of IGF2BP3 may serve as an oncogenic marker in clear cell carcinoma of the ovary (14). Upregulation of the RNA-binding proteins lin-28 homolog B and IGF2BP3 is associated with chemoresistance and adverse disease outcomes in OC (15). Therefore, it may be hypothesized that IGF2BP3 exerts a role in SKOV3 cells by binding to the protein SIX4.

The aim of the present study was to investigate the role of SIX4 in OC, and the mechanism through which it is involved in the regulation of OC cell proliferation, metastasis and angiogenesis, in order to provide a theoretical basis for targeted treatment of OC.

Materials and methods

Databases. The Gene Expression Profiling Interactive Analysis (GEPIA) database (gepia.cancer-pku.cn) was used to detect the expression levels of SIX4 in OC tissues (16). In addition, the ENCORI (starbase.sysu.edu.cn) database was used to detect the association between the expression levels of SIX4 and the OS rate in patients with OC (17).

Cell culture. IOSE-80, A2780, OVCAR3 and SKOV3 cells were obtained from BeNa Culture Collection; Beijing Beina Chunglian Institute of Biotechnology while human umbilical vein endothelial cells (HUVECs) were acquired from Procell Life Science & Technology Co., Ltd. All cells were cultured in HyClone® DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% HyClone® FBS and 1% penicillin and streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere of 5% CO_2.

Reverse transcription-quantitative PCR (RT-qPCR) assay. Total RNA was extracted from cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Then, reverse transcription was conducted according to the manufacturer's instructions of the HIFiscript cDNA Synthesis kit (CoWin Biosciences) and the system for RT-qPCR was established using SYBR Green Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control. The thermocycling conditions for RT-qPCR were as follows: 95˚C in a 20-μl reaction volume for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (18). The primer sequences used were as follows: SIX4 forward, 5'-CGAGCT CTACAGCATCCTCG-3' and reverse, 5'-CGGTACTTG TCTACGGCTCC-3'; IGF2BP3 forward, 5'-CAAGCAGAA ACCATGTGATTTG-3' and reverse, 5'-AGAGGTGCCTTC AGGAGTAGAG-3'; and GAPDH forward, 5'-GAGCCC GCAGCCTCCCGCTT-3' and reverse, 5'-CCCGCGGCC ATCACGCCACAG-3'.

Western blot analysis. Total protein was extracted from SKOV3 cells in RIPA lysis buffer (MilliporeSigma) and the protein concentration was detected using a BCA protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Equal amounts of protein (30 μg per lane) were separated by 12% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). Subsequently, the membranes were blocked with 5% skimmed milk powder for 1.5 h at room temperature, and then incubated with primary antibodies, including anti-SIX4 (1:500 dilution; cat. no. LS-C101744), anti-IGF2BP3 (1:1,000 dilution; cat. no. 57145S), anti-E-cadherin (1:1,000 dilution; cat. no. 14472S), anti-N-cadherin (1:1,000 dilution; cat. no. 13116S), anti-Snail (1:1,000 dilution; cat. no. 3879S), anti-VEGF (1:1,000 dilution; cat. no. 65373S) and anti-GAPDH (1:1,000 dilution; cat.no. 5174S) at 4˚C overnight (anti-SIX4 antibody was purchased from LifeSpan BioSciences, Inc.; all the other primary antibodies were purchased from Cell Signaling Technology, Inc.). The next day, after washing with PBS with 0.1% Tween-20, the membranes were incubated with a secondary antibody conjugated to HRP (1:1,000 dilution; cat. no. BS13278; Bioworld Technology, Inc.) for 1 h at room temperature. The expression levels of the different proteins were detected using enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.). Proteins bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). The data were analyzed using ImageJ 1.52 k software (version 1.46; National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assay. Cells were seeded into 96-well plates (density, $5x10^4$ cells/ml) and a CCK-8 kit (cat. no. G021-1-1; Beyotime Institute of Biotechnology) was used to detect the cell viability. Following incubation, 10 μl CCK-8 solution was added to the cells in each well at 37˚C for 3 h. Cell viability was measured at 450 nm absorbance (optical density) with a microplate reader (Bio-Rad Laboratories, Inc.).

Cell transfection. Short hairpin RNAs (shRNAs/sh) were cloned into the lentiviral vector pLKO.01 from Sangon Biotech Co., Ltd using the restriction enzyme pair *Age*I/*Eco*RI using the 3rd generation system. The molar ratio used for the lentivirus, packaging and envelope plasmids was 1:1:1. A total of 2 μg pLKO-sh-SIX4 (#1 and 2) and pLKO-sh-IGF2BP3 (#1 and 2), or pLKO-scramble (sh-NC), pCMV-DR8.9 and pCMV-VSVG plasmids, were transfected into 293T cells (ATCC) to produce lentiviruses. Cell culture media containing lentiviral particles were collected 48 h after transfection and the lentiviruses were harvested and used to infect SKOV3 cells at a multiplicity of infection of 40 with Invitrogen® Lipofectamine™ 3000 reagent (Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. At 48 h post-infection, puromycin (2 μg/ml) was used to select stably infected cells for 3-5 days. RT-qPCR and western blot analyses were employed to determine the cell transfection efficiency. The sequences of primers were as follows: sh-SIX4#1, 5'-CCTCCTCATTAGTTAATGTAT-3'; sh-SIX4#2, 5'-CCT CAGCCTTTCCAGTCATAT-3'; sh-IGF2BP3#1, 5'-GCCTCA TTCTTATTTCAAGAT-3'; sh-IGF2BP3#2, 5'-CGGTGAATG AACTTCAGAATT-3'; and sh-NC, 5'-CAACAAGATGAA GAGCACCAA-3'.

For the transient IGF2BP3 overexpression experiments, the cells were transfected either with 2 μg pCMV-N-Flag-IGF2BP3 (Ov-IGF2BP3) or with pCMV-N-Flag control vector (Ov-NC) from Beyotime Institute of Biotechnology using Invitrogen® Lipofectamine™ 3000 reagent (Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. RT-qPCR and western blot analyses were subsequently used to assess the cell transfection efficiency at 48 h post-infection.

Colony formation assay. Following cell transfection, SKOV3 cells were seeded ($1.5x10^3$ cells/well) into a 6-well plate. After 14 days, the medium was discarded, and the clones were fixed with methanol for 15 min at room temperature. Subsequently, crystal violet was used to stain the cells for 20 min at room temperature. The numbers of clones with >10 cells were counted manually under a light microscope. The number of clones: (cell number >10 cells/colony) x100%.

Wound healing assay. SKOV3 cells were seeded onto 6-well plates (density, $1x10^6$ cells/ml) and cultured to 80% confluence. After cell transfection, a scratch-wound assay was performed using a 10-μl pipette tip. The medium was replaced by DMEM without serum, and 1 μM 5-fluouracil (MilliporeSigma) was added to block cell proliferation after wounding. The widths of the wounds were measured after 0 and 24 h with a light microscope (magnification, x200). The cell migration rate was calculated as follows: (Initial width-final width)/Initial width.

Transwell assay. The upper compartment surface of the bottom membrane of the Transwell chamber was coated with Matrigel™ (50 mg/l; 1:8 diluted solution; BD Biosciences) at room temperature for 24 h and air-dried at 4˚C. The culture in the culture plate was removed, and serum-free DMEM containing 10 mg/ml BSA (Shanghai Aladdin Biochemical Technology Co., Ltd.) was added to each well at 37˚C for 30 min. Subsequently, the treated cells suspended in 200 μl serum-free DMEM were seeded onto the upper chamber membranes of 24-well 8-μm pore Transwell insert (Costar; Corning, Inc.) at a density of $1x10^4$ cells/ml. DMEM supplemented with 10% FBS was added to the lower chamber. After incubation for 24 h at 37˚C, the membrane was fixed with 4% paraformaldehyde for 15 min and sequentially stained with 0.1% crystal violet solution for 30 min (all at room temperature). The inside of the membrane was gently wiped with a cotton swab. Finally, the numbers of cells were counted under a light microscope.

Tube formation assay. Matrigel™ (BD Biosciences) was used to detect the tube formation of human umbilical vein endothelial cells (HUVECs). A total of $5x10^4$ HUVECs were plated and co-cultured with the same amount of SKOV3 cells transfected with sh-SIX4#2 and Ov-IGF2BP3 on a 96-well plate precoated with Matrigel™ (50 μl/well) for 30 min at 37˚C. After 12 h, the enclosed networks of tubes from six random high-power microscope fields were examined under a light microscope. The mean value of 10 cumulative total lengths per well represented an experimental point. The number of tubes was assessed using ImageJ 1.52 k software (version 1.46; National Institutes of Health).

RNA immunoprecipitation (RIP) assay. A RIP RNA-Binding Protein Immunoprecipitation Kit (MilliporeSigma) was used to conduct RIP assays. Cells were lysed in Invitrogen RIP buffer (Thermo Fisher Scientific, Inc.) after being collected by centrifugation at 200 x g for 5 min at 4˚C. Then, 100 μl cell lysate was pre-cleared with 50 ul protein A/G magnetic beads (MilliporeSigma) which were conjugated to 5 μg anti-SIX4 antibody (1:500 dilution; cat. no LS-C101744; LifeSpan BioSciences, Inc.) or 5 μg anti-IgG antibody (1:50 dilution; cat. no ab172730; Abcam). A protein-RNA complex was captured and digested with 0.5 mg/ml proteinase K containing 0.1% SDS to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption. Finally, the expression levels of SIX4 were determined using an RT-qPCR assay as described above.

Actinomycin D treatment. Following transfection, the cells were treated with 2 mg/ml actinomycin D (MedChemExpress) for 0, 3, 6, 9 or 12 h at 37˚C. The mRNA levels of SIX4 following actinomycin treatment were subsequently measured using an RT-qPCR assay as described above.

Statistical analysis. All experiments were repeated independently three times. SPSS 21.0 (IBM Corp.) was employed for statistical analysis. The data are presented as the mean ± SD. Comparisons between two groups were performed using unpaired Student's t-test, whereas comparisons among multiple groups were performed with one-way ANOVA followed by a Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SIX4 is highly expressed in OC. The GEPIA database analysis revealed that SIX4 was highly expressed in OC tissues compared with tissues from healthy subjects (Fig. 1A). Subsequently, the ENCORI database was employed to reveal that high expression levels of SIX4 were associated with low OS rates in patients with OC (Fig. 1B). The human normal ovarian epithelial cell line (IOSE-80) and the OC cell lines (A2780, OVCAR3 and SKOV3) were selected for subsequent experiments as previously described (19). Western blot and RT-qPCR analyses were used to detect the expression levels of SIX4 in cells. The results obtained revealed that, compared with those in the IOSE-80 cells, the expression levels of SIX4 were significantly increased in the A2780, OVCAR3 and SKOV3 cells (Fig. 1C and D). Furthermore, SIX4 displayed the highest expression in SKOV3 cells among all selected OC cells; therefore, SKOV3 cells were selected for the follow-up experiments.

Interference with SIX4 inhibits the proliferation of SKOV3 cells. Transfection was used to interfere with SIX4 expression in SKOV3 cells, and the transfection efficiency was assessed by RT-qPCR and western blot analyses. The results demonstrated that, compared with those in the sh-NC control group, the expression levels of SIX4 in the sh-SIX4#1 and sh-SIX4#2 groups were significantly decreased, indicating successful transfection (Fig. 2A and B). The sh-SIX4#2 group

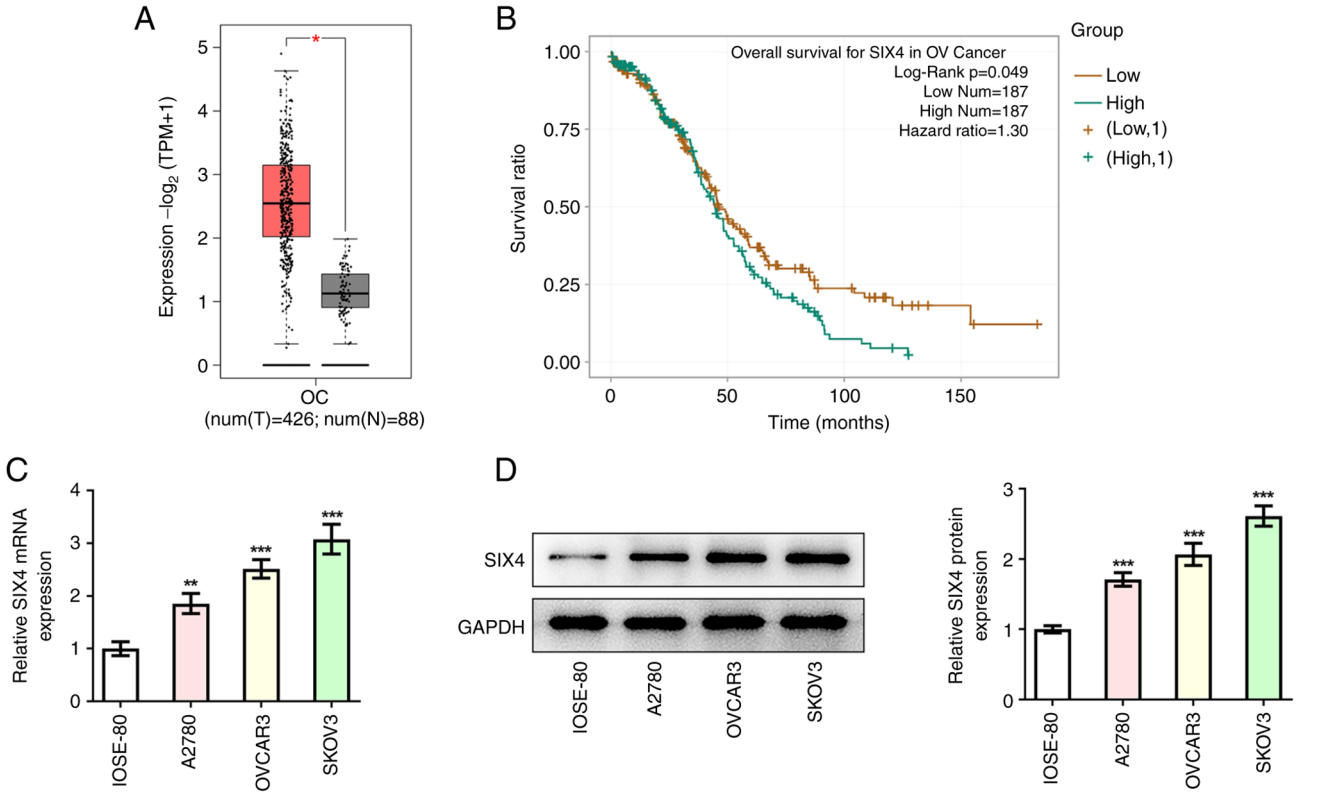

Figure 1. SIX4 is highly expressed in OC. (A) Gene Expression Profiling Interactive Analysis database analysis predicted the high expression levels of SIX4 in patients with OC. Orange box corresponds to OC. Grey box corresponds to healthy samples. *P<0.05 vs. N. (B) Encyclopedia of RNA Interactomes database analysis predicted that SIX4 expression was associated with the overall survival rate of patients with OC. (C) Reverse transcription-quantitative PCR was used to detect the expression levels of SIX4. (D) Western blotting was used to detect the expression levels of SIX4. **P<0.01, ***P<0.001 vs. IOSE-80. OC, ovarian cancer; SIX4, SIX homeobox 4; TPM, transcripts per million; N, normal; T, tumor.

was demonstrated to exhibit a better interference efficiency. Therefore, this group was selected for follow-up experiments.

A CCK-8 assay was then used to assess the viability of the cells following interference with SIX4. The results revealed that, compared with the sh-NC control group, the cell survival rate of the sh-SIX4#2 group was significantly decreased at 48 and 72 h (Fig. 2C). A colony formation assay subsequently demonstrated that cell proliferation was significantly decreased in the sh-SIX4#2 group compared with the sh-NC group (Fig. 2D).

Interference with SIX4 expression inhibits the migration, invasion and tube formation of SKOV3 cells. Subsequently, the effects of interference with SIX4 expression on the migration, invasion and tube formation of SKOV3 cells were examined, and it was found that the invasion and migration of the sh-SIX4#2 group were significantly reduced compared with those of the sh-NC control group (Fig. 3A-D). Subsequently tube formation assay was performed to detect the tube formation of the HUVECs. The results obtained revealed that, compared with the sh-NC group, the tube formation of the sh-SIX4#2 group was significantly decreased (Fig. 3E and F). Western blot analysis was then used to investigate the expression levels of the metastasis- and angiogenesis-associated proteins E-cadherin, N-cadherin and Snail, as well as VEGF. The results demonstrated that, the expression levels of E-cadherin in the sh-SIX4#2 group were significantly increased, whereas the expression levels of N-cadherin, Snail and VEGF were significantly decreased compared with the sh-NC group (Fig. 3G), suggesting that interference with SIX4 expression could lead to an inhibition of the metastasis and angiogenesis of SKOV3 cells.

IGF2BP3 increases the stability of SIX4 mRNA. The GEPIA database analysis revealed that IGF2BP3 was highly expressed in OC tissues (Fig. 4A). In addition, a statistically significant positive correlation between the expression levels of IGF2BP3 and SIX4 in OC tissues was also identified (Fig. 4B). Western blot and RT-qPCR analyses revealed that IGF2BP3 expression in SKOV3 cells was abnormally elevated compared with that in IOSE-80 cells (Fig. 4C and D). Subsequently, a RIP assay was used to assess the binding ability of IGF2BP3 and SIX4 mRNA, and the results revealed that, compared with the control group, the expression levels of SIX4 in the IGF2BP3 group were significantly increased, indicating the binding of IGF2BP3 and SIX4 (Fig. 4E). Subsequently, transfection was used to interfere with IGF2BP3 expression in SKOV3 cells, and the transfection efficiency was determined by western blot and RT-qPCR analyses (Fig. 4F and G). Since the shRNA sh-IGF2BP3#2 was demonstrated to have the best interference efficiency, sh-IGF2BP3#2 was selected for subsequent experiments. Following actinomycin D treatment, the stability of SIX4 mRNA was detected by an RT-qPCR assay. The results demonstrated that, compared with the sh-NC group, the stability of SIX4 decreased sharply upon inhibiting IGF2BP3 expression (Fig. 4H), indicating that IGF2BP3 could stabilize

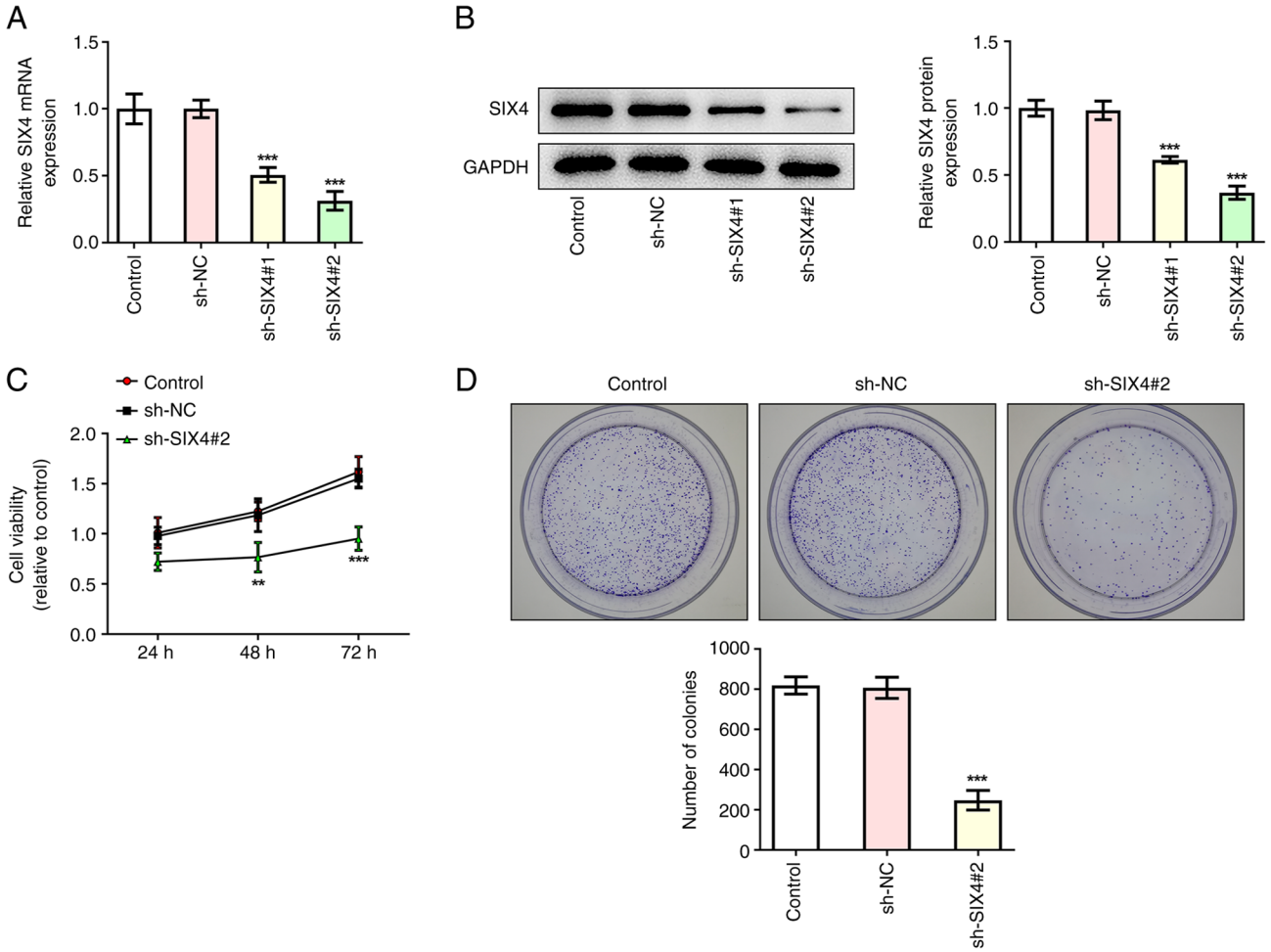

Figure 2. Interference with SIX4 inhibits proliferation of SKOV3 cells. (A) Reverse transcription-quantitative PCR was used to detect the expression levels of SIX4. (B) Detection and semi-quantification of the expression levels of SIX4 by western blotting. (C) Cell Counting Kit-8 assay of the viability of SKOV3 cells. (D) Colony formation assay detecting the proliferation of SKOV3 cells. **P<0.01, ***P<0.001 vs. sh-NC. NC, negative control; sh, short hairpin RNA; SIX4, SIX homeobox 4.

SIX4 expression. In addition, it was found that the expression levels of SIX4 were significantly decreased following the inhibition of IGF2BP3 expression in comparison with the sh-NC group, with lowest SIX4 expression when treated with Actinomycin D at 12 h (Fig. 4I and J). These results suggested that IGF2BP3 could improve the stability of SIX4 mRNA in SKOV3 cells.

IGF2BP3-stabilized SIX4 promotes the proliferation, migration, invasion and tube formation of SKOV3 cells. To further explore the regulatory role of IGF2BP3/SIX4 in SKOV3 cells, IGF2BP3 was overexpressed (Fig. 5A and B), and the cells were divided into the control, sh-SIX4#2, sh-SIX4#2+Ov-NC and sh-SIX4#2+Ov-IGF2BP3 treatment groups. The results obtained from the CCK-8 and colony formation assays demonstrated that, compared with the sh-SIX4#2+Ov-NC group, the cell viability of the sh-SIX4#2+Ov-IGF2BP3 group was significantly increased at 72 h (Fig. 5C), and the impeded cell proliferation caused by SIX4 silencing was restored by IGF2BP3 overexpression (Fig. 5D). In addition, the wound healing and Transwell assay results revealed that, compared with the sh-SIX4#2+Ov-NC group, the invasion and migration rates of the cells in the sh-SIX4#2+Ov-IGF2BP3 group were significantly increased (Fig. 6A-D). Compared with the sh-SIX4#2+Ov-NC group, the tube formation of the sh-SIX4#2+Ov-IGF2BP3 group was significantly increased (Fig. 6E and F). Western blotting was subsequently used to detect the protein expression levels of E-cadherin, N-cadherin, Snail and VEGF, and the results obtained demonstrated that overexpression of IGF2BP3 could reverse the regulation of these metastasis- and angiogenesis-associated proteins following the inhibition of SIX4 (Fig. 6G). Collectively, these results suggested that IGF2BP3-stabilized SIX4 could promote the proliferation, migration, invasion and tube formation of SKOV3 cells.

Discussion

The pathogenesis and treatment of OC have for a long time provided a focus for biomedical research. In the present study, the effects of SIX4 on OC cell proliferation, metastasis and angiogenesis were studied, and the underlying mechanism was discussed.

Solid tumor growth is dependent upon continuous and extensive tumor angiogenesis, which provides nutrients and oxygen to tumor tissue and also the means for tumor tissue

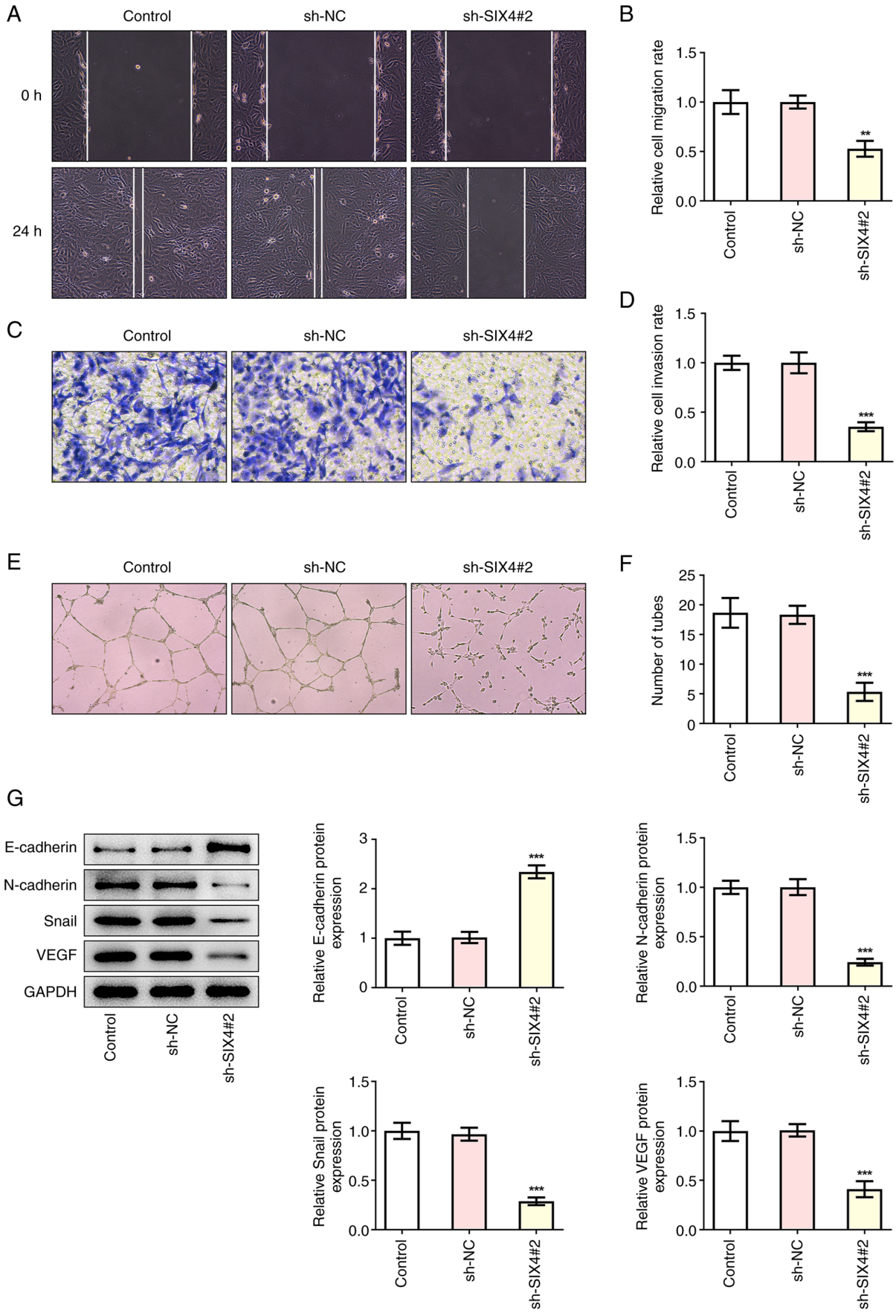

Figure 3. Interference with SIX4 inhibits the migration, invasion and tube formation of SKOV3 cells. (A) Wound healing assay (magnification, x100) detecting the cell migration. (B) Statistical analysis of cell migration. (C) Transwell assay (magnification, x100) detecting the cell invasion. (D) Statistical analysis of cell invasion. (E) A tube formation assay (magnification, x40) was used to detect the tube formation of human umbilical vein endothelial cells. (F) Statistical analysis of tube formation. (G) Western blotting was used to detect metastasis- and angiogenesis-related proteins. **P<0.01, ***P<0.001 vs. sh-NC. NC, negative control; sh, short hairpin RNA; SIX4, SIX homeobox 4.

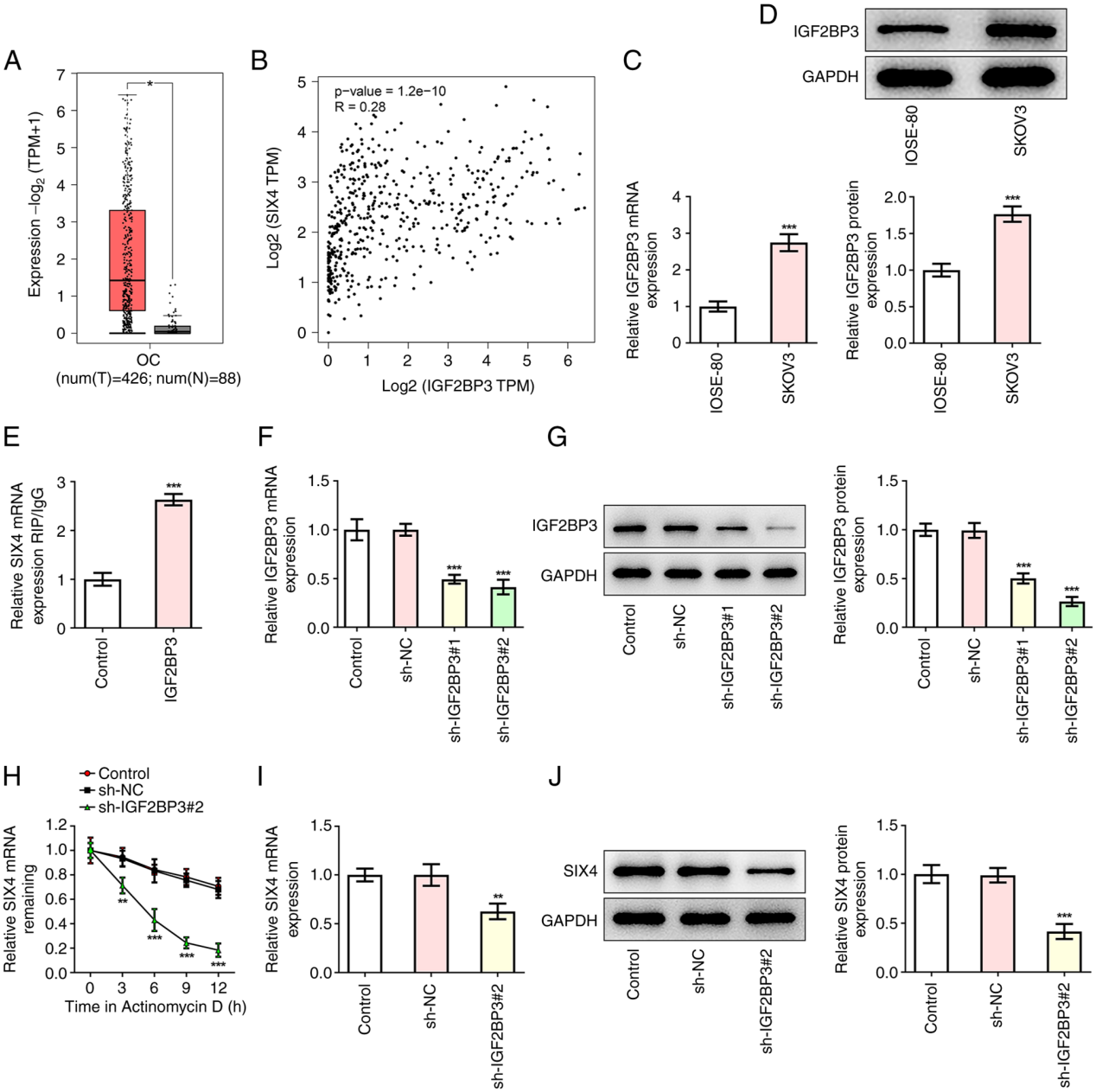

Figure 4. IGF2BP3 increases the stability of SIX4 mRNA. (A) GEPIA database analysis predicted the high expression levels of IGF2BP3 in samples from patients with OC. Orange box corresponds to OC. Grey box corresponds to healthy samples. *P<0.05 vs. normal. (B) GEPIA database analysis predicted that SIX4 expression was correlated with IGF2BP3 expression in patients with OC. (C) RT-qPCR was used to detect the mRNA levels of IGF2BP3. ***P<0.001 vs. IOSE-80. (D) Western blotting was used to detect the protein levels of IGF2BP3. ***P<0.001 vs. IOSE-80. (E) RIP assay detecting the binding ability of IGF2BP3 to SIX4 mRNA. ***P<0.001 vs. Control. (F) RT-qPCR was used to detect the mRNA levels of IGF2BP3 after inhibition of IGF2BP3. (G) Western blotting was used to detect the protein levels of IGF2BP3 after inhibition of IGF2BP3. ***P<0.001 vs. sh-NC. (H) After actinomycin D treatment, the stability of SIX4 mRNA was detected by RT-qPCR. (I) RT-qPCR was preformed to detect the mRNA levels of SIX4 after inhibition of IGF2BP3. (J) Western blotting was performed to detect the protein levels of SIX4 after inhibition of IGF2BP3. ***P<0.001 vs. sh-NC. GEPIA, Gene Expression Profiling Interactive Analysis; IGF2BP3, insulin-like growth factor 2 mRNA binding protein 3; NC, negative control; OC, ovarian cancer; RIP, RNA immunoprecipitation; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin RNA; SIX4, SIX homeobox 4; TPM, transcripts per million.

metastasis (20,21). OC has the highest mortality rate among the different types of gynecological tumors, and exhibits clear characteristics of easy metastasis (22,23). The invasion, growth and metastasis of OC are largely dependent on the formation of an abundant blood supply (24). Folkman (25) first proposed treating tumors via inhibiting tumor angiogenesis in 1971. In recent years, several studies have demonstrated that inhibition of tumor angiogenesis can inhibit the proliferation and metastasis of tumor cells, thereby inhibiting the occurrence and development of tumors (21,26,27). Therefore, the focus of the present study was to explore the possibility of a novel type of OC therapy via inhibiting the angiogenesis of OC.

SIX4 fulfills an important role in the development of multiple tumors. A previous study indicated that the upregulation of SIX4 is an indicator of poor clinical prognosis of esophageal squamous cell carcinoma, and that this leads to the promotion of tumor growth and cell metastasis (8). SIX4 is able

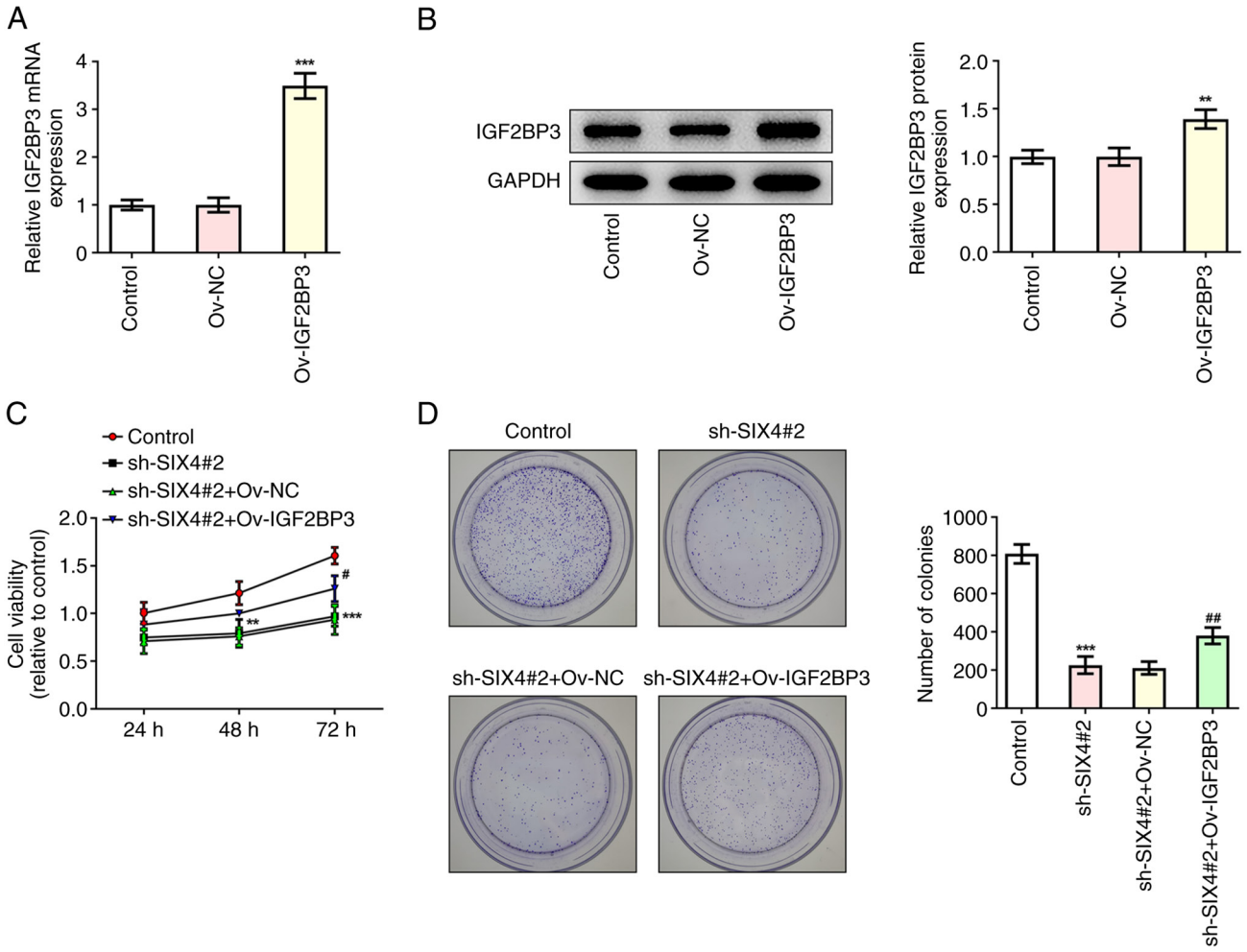

Figure 5. IGF2BP3-stabilized SIX4 promotes proliferation of SKOV3 cells. (A) Reverse transcription-quantitative PCR was performed to detect the mRNA expression levels of IGF2BP3 after overexpression of IGF2BP3. ***P<0.001 vs.Ov-NC. (B) Western blotting was performed to detect the protein levels of IGF2BP3 after overexpression of IGF2BP3. **P<0.01, ***P<0.001 vs. Ov-NC. (C) Cell Counting Kit-8 assay detecting the viability of SKOV3 cells. **P<0.01, ***P<0.001 vs. control. #P<0.05 vs. sh-SIX4+Ov-NC. (D) Colony formation assay detecting the proliferation of SKOV3 cells. **P<0.01, ***P<0.001 vs. control. #P<0.05, ##P<0.01 vs. sh-SIX4+Ov-NC. IGF2BP3, insulin-like growth factor 2 mRNA binding protein 3; NC, negative control; Ov, overexpression; sh, short hairpin RNA; SIX4, SIX homeobox 4.

to promote hepatocellular carcinoma metastasis via upregulation of the proteins Yes associated transcriptional regulator 1 and C-Met (28). Notably, in colorectal cancer cells, SIX4 increases the expression levels of VEGF-A in conjunction with hypoxia-inducible factor-1α and upregulation of SIX4 has been demonstrated to promote tumor growth and angiogenesis (29). These results suggested that SIX4 has an important role in cancer metastasis and angiogenesis. The GEPIA database analysis revealed that SIX4 is highly expressed in OC tissues. High expression levels of SIX4 were associated with low OS rates in patients with OC. Cell experiments in the present study also demonstrated that SIX4 expression is abnormally elevated in OC cells. Interference with the expression of SIX4 can therefore lead to inhibition of OC cell proliferation, migration, invasion and tube formation.

Subsequently, the present study provided novel insights into the regulatory mechanism of SIX4. The expression levels of IGF2BP3 and SIX4 were positively correlated according to GEPIA database analysis. The m6A reader IGF2BP3 has an important role in the regulation of angiogenesis in gastric cancer cells. In colon cancer cells, IGF2BP3 has been demonstrated to bind to the m6A-modified region of VEGF mRNA to regulate the expression and stability of VEGF mRNA, thereby inhibiting the angiogenesis of colon cancer (30). In addition, studies have reported that high expression level of IGF2BP3 is a marker of poor prognosis of clear cell OC (31,32). In the present study, GEPIA database analysis revealed that IGF2BP3 was highly expressed in tissues from patients with OC. IGF2BP3 was also highly expressed in OC cell lines. Furthermore, the experiments performed in the present study demonstrated that IGF2BP3 can improve the stability of SIX4 mRNA. Subsequently, the associated mechanism was further investigated by inhibiting the expression of SIX4 and overexpressing IGF2BP3. These experiments revealed that the overexpression of IGF2BP3 could reverse the inhibitory effect of SIX4 on the malignant progression of SKOV3 cells, suggesting that IGF2BP3 regulates the function of SIX4 during the malignant progression of OC.

To the best of our knowledge, the present study was the first to investigate the role of SIX4 and IGF2BP3 in SKOV3 OC cells and the regulatory relationship between them. In the

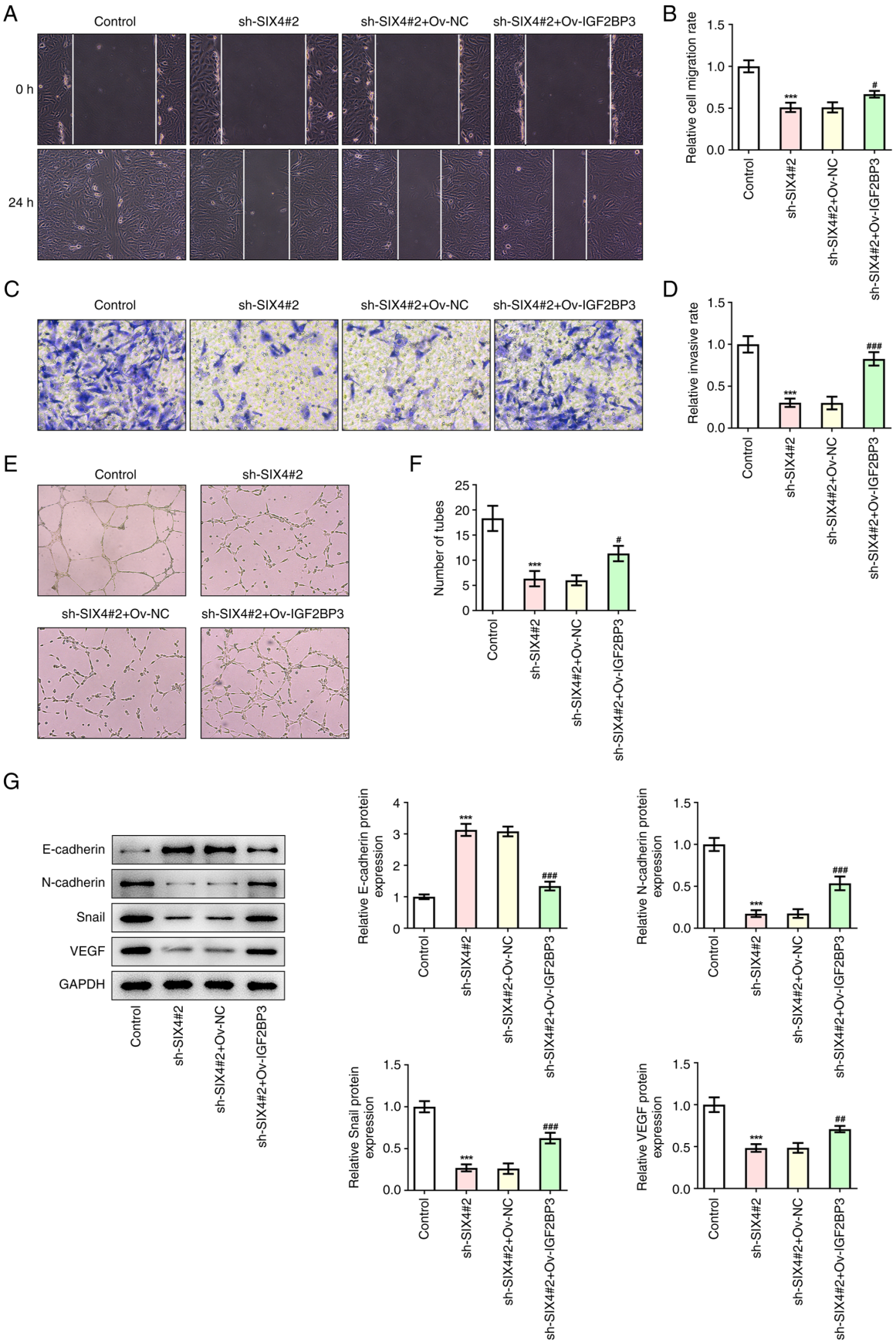

Figure 6. IGF2BP3-stabilized SIX4 promotes the migration, invasion and tube formation of SKOV3 cells. (A) Wound healing assay (magnification, x100) detecting the cell migration after cell transfection. (B) Statistical analysis of cell migration. (C) Transwell assay (magnification, x100) detecting the cell invasion. (D) Statistical analysis of cell invasion. (E) A tube formation assay (magnification, x400) was used to detect the tube formation of human umbilical vein endothelial cells. (F) Statistical analysis of tube formation. (G) Western blotting was used to detect the metastasis- and angiogenesis-associated proteins after cell transfection. ***P<0.001 vs. control. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. sh-SIX4+Ov-NC. IGF2BP3, insulin-like growth factor 2 mRNA binding protein 3; NC, negative control; Ov, overexpression; sh, short hairpin RNA; SIX4, SIX homeobox 4.

present study, the mechanism of OC was explored through the combination of bioinformatics and experiments. However, the present study also had certain limitations. First of all, the existing conclusions were not further verified in clinical samples and animal experiments, and our research group will further verify them in future experiments. In addition, in the mechanism experiments, only experiments on interference of SIX4 were conducted, while no experiments on overexpression of SIX4 in cells were conducted. The mechanism will be further investigated through overexpression of SIX4 in future experiments.

In conclusion, it may be determined from the present study that IGF2BP3-stabilized SIX4 is able to promote the proliferation, migration, invasion and tube formation of SKOV3 cells. Furthermore, the present study also provides a theoretical basis for the improved understanding of the mechanism of OC, which will hopefully facilitate the treatment of OC.

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Availability of data and materials

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

Authors' contributions

JH designed and performed experiments. XH analyzed the data and wrote the manuscript. JH edited the manuscript. JH and XH confirm the authenticity of all the raw data. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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