OTX1 promotes tumorigenesis and progression of cervical cancer by regulating the Wnt signaling pathway

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Abstract. Cervical cancer is a common malignant tumor in females. Orthodenticle homolog 1 (OTX1) serves a key role in the occurrence and progression of tumors. The present study aimed to investigate the role and potential mechanism of OTX1 in cervical cancer. OTX1 expression was analyzed by western blotting, reverse transcription-quantitative PCR and immunohistochemistry. MTT assay was performed to assess cell viability. EdU and colony formation assay were used to measure cell proliferation. Wound healing and Transwell assays were performed to measure cell migration and invasion. Western blot assay was performed for the assessment of protein expression. Gene set enrichment analysis (GSEA) was performed to analyze signaling pathways regulated by OTX1. Co-Immunoprecipitation assay was performed to confirm the interaction between OTX1 and Wnt9b. In cervical cancer tissue and cells, OTX1 was significantly upregulated. OTX1 overexpression promoted proliferation, migration and invasion of cervical cancer cells. OTX1 silencing significantly decreased cell proliferation, migration and invasion of cervical cancer. GSEA showed that OTX1 activated the Wnt signaling pathway. OTX1 silencing inhibited the increased levels of adenomatous polyposis coli (APC), glycogen synthase kinase (GSK)-3β and axis inhibition protein (AXIN)2 and decreased levels of Wnt9b and β-catenin. OTX1 overexpression decreased the levels of APC, GSK-3β and AXIN2 and increased levels of Wnt9b and β-catenin. However, XAV939 (a Wnt signaling inhibitor) and β-catenin silencing partly eliminated the effect of OTX1 overexpression on cervical cancer cells. OTX1 promoted the progression of cervical cancer by activating the Wnt signaling pathway.

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Introduction

Cervical cancer is a prevalent type of malignant tumor in the female reproductive system worldwide with ~570,000 new cases and 311,000 deaths annually (1-3). Although developments in chemotherapy, radiotherapy and clinical treatment have improved the prognosis of cervical cancer, it remains a life-threatening malignancy worldwide (4,5). Therefore, further study of the developmental mechanisms of cervical cancer is crucial.

Orthodenticle homolog 1 (OTX1) is a member of OTX family (6) and serves an important role in development of early human fetal retina, mammary gland, sensory organs and brain (7). Studies have shown that OTX1 is frequently overexpressed in numerous types of cancer, including pancreatic (8) and breast cancer (6), laryngeal squamous cell carcinoma (LSCC) (9), gastric and (10) colorectal cancer (11) and hepatocellular carcinoma (12), indicating that OTX1 may be a key regulator in the development and progression of human cancer. Previous studies have found that OTX1 promotes tumor progression of colorectal cancer and hepatocellular carcinoma (11,12). OTX1 promotes the proliferation and increased motility of cancer cells by promoting cell cycle progression in bladder cancer (13). OTX1 promotes cell proliferation, invasion and migration by inducing activation of JAK/STAT signaling in lung squamous cell carcinoma (14). Moreover, OTX1 overexpression significantly increases migration and proliferation of triple-negative breast cancer cells (6). However, the role and mechanism of OTX1 in cervical cancer remains unclear.

The present study aimed to explore the biological function and potential mechanism of OTX1 in cervical cancer, thus identifying potential novel therapeutic targets for cervical cancer.

Materials and methods

Tumor specimens. The present study was a retrospective study of cervical cancer. A total of 51 female patients with cervical cancer (32~70 years old) and 35 with non-cancer disease who underwent hysterectomy in Maternal and Child Health Hospital of Hubei Province (Wuhan, China) from May 2020 to December 2021 were enrolled in present study. The present study was approved by the Ethics Committee of Maternal and

Child Health Hospital of Hubei Province [approval no. (2021) IEC (xm017); Tongji Medical College, Huazhong University of Science and Technology]. All participants signed written informed consent. The cervical cancer and normal cervical tissue was collected for reverse transcription-quantitative (RT-q)PCR and immunohistochemistry (IHC).

Cell culture. The human cervical cancer cell lines C-33A (HPV -), SiHa (HPV +), CaSki (HPV +) and ME-180 (HPV +) (Procell Life Science & Technology Co., Ltd.) and cervical epithelial cell line H8 (BeNa Culture Collection) were grown in RPMI-1640 supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 atmosphere. CaSki cells ($1x10^5$ cells/ml) were treated with XAV939 (20 μM; MedChemExpress), a Wnt signaling inhibitor, at 37˚C for 12 h to inhibit Wnt signaling pathway *in vitro*.

Cell transfection. Small interfering (si)RNAs for OTX1 (si-OTX1#1 forward 5'-CCGACUGUCUGGACUAUA AGG-3' and reverse 5'-UUAUAGUCCAGACAGUCG GGG-3'; si-OTX1#2 forward 5'-GCAUGAUGUCUUACC UCAAAC-3' and reverse 5'-UUGAGGUAAGACAUCAUG CUA-3'), β-catenin (si-β-catenin#1 forward 5'-GAUGGUGUC UGCUAUUGUACG-3' and reverse 5'-UACAAUAGCAGA CACCAUCUG-3'; si-β-catenin#2 forward 5'-GCACAAGAA UGGAUCACAAGA-3' and reverse 5'-UUGUGAUCCAUU CUUGUGCAU-3') and negative control (NC forward 5'-UUC UCCGAACGUGUCACGUTT-3' and reverse 5'-ACGUGA CACGUUCGGAGAATT-3') were obtained from Guangzhou RiboBio Co., Ltd. OTX1 overexpression plasmid pcDNA3.1 (pc-OTX1) and pc-NC were obtained from Shanghai GenePharma Co., Ltd.

OTX1 siRNAs (siRNA#1 and siRNA#2; 10 nM) and si-NC (10 nM) were transfected into C-33A cells. OTX1 overexpression plasmid pcDNA3.1 (pc-OTX1; 1 μg) and pc-NC (1 μg) were transfected into CaSki cells. β-catenin siRNAs (si-β-catenin#1, si-β-catenin#2, 10 nM) and si-NC (10 nM) were transfected into CaSki cells. All the transfection was performed using Lipofectamine™ 2000 (Thermo Fisher Scientific) at 37˚C for 8 h. At 48 h after transfection, subsequent experiments were performed.

MTT assay. C-33A and CaSki cells were seeded in 96-well plates ($1x10^4$ cells/well) and incubated at 37˚C for 24, 48 and 72 h. Following incubation, MTT solution (10 μl) was added to cells and incubated for 4 h at 37˚C. DMSO was used to dissolve formazan crystals. The absorbance at 450 nm was detected using a microplate reader (Thermo Fisher Scientific, Inc.).

EdU assay. BeyoClick™ EdU Cell Proliferation kit (Beyotime Institute of Biotechnology) was used to detect the proliferation of cervical cancer cells. C-33A and CaSki cancer cells ($1x10^4$ cells/well) were seeded in 24-well plates and incubated at 37˚C for 24 h. Then, EdU regent was added and incubated 37˚C for 2 h. Subsequently, cells were fixed with 4% paraformaldehyde for 15 min and then stained by DAPI for 15 min at 25˚C. Images were captured under a fluorescent microscope at 200x magnification (Olympus Corporation) to calculate cell proliferation using ImageJ software (Version 1.45s; National Institutes of Health).

Colony formation assay. Following transfection for 48 h, C-33A and CaSki cells ($1x10^4$ cells/well) were seeded in 6-well plates and cultured at 37˚C in a 5% CO_2 atmosphere. Following 10-14 days culture, cells were fixed with 4% paraformaldehyde at 37˚C for 15 min and stained with 0.5% crystal violet at 37˚C for 1 h. The colonies (>50 cells) was counted using ImageJ software (Version 1.45s; National Institutes of Health).

Transwell assay. For invasion assay, C-33A and CaSki cells ($1x10^5$ cells/well) were seeded in a 24-well Transwell upper chamber (8-μm pore) pre-coated with Matrigel (BD Biosciences) 37˚C for 24 h and cultured in DMEM without FBS. For the migration assay, C-33A and CaSki cells ($1x10^5$ cells/well) were seeded in the upper chamber without pre-coated Matrigel and cultured in DMEM without FBS. A total of 600 μl DMEM supplemented with 10% FBS (Thermo Fisher Scientific) was added to the lower chamber. Following 24 h incubation at 37˚C, the migrated or invaded cells were fixed with 4% paraformaldehyde for 15 min at 37˚C and stained with 0.1% crystal violet for 3 min at room temperature. The images were captured using a light microscope at 200x magnification (Olympus Corporation) and analyzed using ImageJ software (Version 1.45s; National Institutes of Health).

Wound healing assay. C-33A and CaSki cells ($1x10^5$ cells/well) were inoculated into 6-well plates with DMEM without FBS and cultured at 37˚C until 90% confluence. Then, cells were scratched to create a wound using a 200-μl pipette tip. Following 48 h incubation at 37˚C, images were captured using an light microscope (Nikon Corporation) at 200x magnification and analyzed using ImageJ software (Version 1.45s; National Institutes of Health).

RT-qPCR. Total RNA was extracted from cervical cancer tissue and cells (C-33A and CaSki cells) using TRIzol (Thermo Fisher Scientific). RT was performed using the Prime Script RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. A total of 1 μg cDNA and SYBR Green (Takara Biotechnology Co., Ltd.) was used for qPCR analysis. The thermocycling conditions were as follows: 40 cycles at 95˚C for 5 min, 95˚C for 30 sec, 60˚C for 45 sec and 72˚C for 30 min. GAPDH was used as an internal control. Data were analyzed using the comparative $2^{-\Delta\Delta Cq}$ method (15) for relative quantification. Primers were as follows: OTX1 forward, 5'-GCCACTCCGACAAGGTTGG-3' and reverse, 5'-TCATGCTAACAGCTGGGTGG-3' and GAPDH forward, 5'-GCATCTTCTTTTGCGTCGCC-3' and reverse, 5'-CCC AATACGACCAAATCCGT-3'.

Western blotting. The total protein was extracted from C-33A and CaSki cells by RIPA buffer (Beyotime Institute of Biotechnology). The concentration of protein was detected by BCA kit (Beyotime Institute of Biotechnology). Subsequently, protein samples (20 μg/per lane) were separated with 10% SDS-PAGE and transferred onto PVDF membranes. Following blocking with non-fat milk at room temperature for 2 h, membranes were incubated with primary antibodies (all 1:1,000) as follows: OTX1 (cat. no. ab25985; Abcam), matrix metalloproteinase (MMP)2 (cat. no. ab181286; Abcam), MMP9 (cat. no. ab76003; Abcam), tissue inhibitor of MMP

(TIMP)2 (cat. no. ab180630; Abcam), Wnt9 (cat. no. ER60346; Huabio), β-catenin (cat. no. ab223075, Abcam), adenomatous polyposis coli (APC; cat. no. ab239828; Abcam), Glycogen synthase kinase (GSK)-3β (cat. no. ab32391; Abcam) and Axis inhibition protein (AXIN)2 (cat. no. ab109307; Abcam) at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated goat Anti-Rabbit IgG H&L secondary antibody (1:10,000; cat. no. ab205718, Abcam) for 1 h at room temperature. β-actin (1:1,000; cat. no. ab8226; Abcam) was used as an internal reference protein. The bands were visualized using a Novex™ ECL Chemiluminescent Substrate Reagent kit (Thermo Fisher Scientific, Inc.). The grey value was analyzed using ImageJ software (Version 1.45s; National Institutes of Health).

IHC. Cervical tumor and normal tissue samples were fixed with 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin and cut into 5 μm-thick sections. Then, the sections were dewaxed with xylene for 15 min and rehydrated conventionally using an ethanol gradient (from 99 to 70%, then demineralized water). The sections were heated in a microwave at 92-98˚C for 15 min for antigen retrieval. The endogenous peroxidase activity was blocked at room temperature using 3% H2O2 in methanol for 15 min. After blocking the non-specific protein binding with 5% skimmed milk at room temperature (21-26˚C) for 10 min, sections were incubated with anti-OTX1 (1:200; cat. no. PA5-62556, Thermo Fisher Scientific, Inc.) at 4˚C overnight. Following incubation with AP-conjugated Goat anti-Rabbit IgG (H + L) secondary antibody (1:500; cat. no. 31340; Thermo Fisher Scientific, Inc.) for 1 h at room temperature, sections were stained with diaminobenzidine and counterstained with hematoxylin for 1 min at room temperature. Stained sections were examined under a light microscope (Nikon Corporation) at 400x magnification, five fields of view were randomly selected and OTX1-positive cells were counted using ImageJ software (Version 1.45s; National Institutes of Health).

Co-immunoprecipitation (Co-IP) assay. For Co-IP, cells were lysed in 200 μl Western/IP lysis buffer (Beyotime Institute of Biotechnology) incubated with primary antibodies at 4˚C overnight. Subsequently, cell lysates (0.4 ml) were incubated with antibody-conjugated protein A/G magnetic beads (30 μl, Thermo Fisher Scientific, Inc.) at 4˚C for 3 h. After centrifugation (800 x g) at 4˚C for 5 min, immunoprecipitate isolated with magnetic beads were washed five times with Western/IP lysis buffer and boiled with sample loading buffer. The resulting immunoprecipitate was analyzed using western blotting, as aforementioned. The primary antibodies used for Co-IP were as follows: OTX1 (1:1,500; cat. no. sc-517000, Santa Cruz Biotechnology, Inc.), Wnt9b (1:1,000; cat. no. abx178928; CiteAb, Ltd.) and GAPDH (1:1,000; cat. no. ab181602; Abcam). The secondary antibody used for Co-IP was Goat anti-Rabbit IgG (H + L) secondary antibody (1:500; cat. no. 31340; Thermo Fisher Scientific, Inc.).

Bioinformatics analysis. UALCAN (ualcan.path.uab.edu/) was employed to analyze the expression of OTX1 in pan-cancer based on The Cancer Genome Atlas (TCGA) database. GeneExpressionProfilingInteractiveAnalysis) GEPIA2 (gepia.cancer-pku.cn/index.html) was used to analyze the expression of OTX1 in cervical cancer based on TCGA database. Kaplan-Meier plotter (kmplot.com/analysis/) was employed to examine the prognosis of OTX1 in cervical cancer based on TCGA database (Auto select best cutoff was chosen in the analysis). LinkedOmics (linkedomics.org/login.php) was used to perform Gene set enrichment analysis (GSEA), co-expression analysis, and target prediction based on RNA-seq data derived from TCGA database of Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) (16). GSEA was used to analyze the DataSet 'pathway_Wikipathway_cancer' (wikipathways.org/index.php/WikiPathways) to search potential signaling pathways regulated by OTX1. GeneMANIA (www.genemania.org/) was used to construct the protein-protein interaction network of OTX1 and Wnt protein.

Statistical analysis. Data are expressed as the mean ± SD. Statistical analysis was determined using GraphPad Prism 7.0 software (GraphPad Software, Inc.). All experiments were repeated in triplicate. The differences between two groups were analyzed by unpaired Student's t test. The differences between >2 groups were analyzed by one-way analysis of variance followed by Tukey's post hoc test. Kaplan-Meier Plotter (kmplot.com/analysis/) (17) was used for survival analysis of patients with cervical cancer. The correlation of OTX1 and co-expressed genes was analyzed by Pearson's coefficient. Fisher's exact test was performed to assess the association between OTX1 and clinicopathological factors. $P<0.05$ was considered to indicate a statistically significant difference.

Results

OTX1 is upregulated in cervical cancer tissue and cells. TCGA database was used to analyze OTX1 expression in cancer. UALCAN was used to analyze OTX1 expression in pan-cancer. The results showed that OTX1 was upregulated in numerous types of cancer, including cervical cancer (Fig. 1A). Additionally, GEPIA2 was used to analyze OTX1 expression in cervical cancer based on TCGA database. OTX1 was upregulated in tumor compared with normal samples (Fig. 1B). Additionally, Kaplan-Meier plotter analysis based on TCGA database was performed to analyze the effect of OTX1 on survival of patients with cervical cancer. The results indicated that patients with high OTX1 showed lower survival probability than patients with low levels of OTX1 (Fig. 1C). IHC analysis and RT-qPCR showed that OTX1 was significantly upregulated in cervical tumor compared with normal samples (Fig. 1D and E). The association between OTX1 expression and clinical characteristics of cervical cancer patients was evaluated. OTX1 upregulation in cervical cancer tissue was significantly associated with clinicopathological factors, including tumor size, International Federation of Gynecology and Obstetrics (FIGO) stage, histological differentiation and lymph node metastasis (Table I). Furthermore, OTX1 expression in cervical cancer (C-33A, SiHa, CaSki and ME-180) and normal cervical epithelial cells (H8) was detected by RT-qPCR and western blotting. OTX1 expression was significantly higher in both HPV (+) and HPV (-) cervical cancer cells than in H8 cells (Fig. 1F).

Table I. Association between OTX1 expression and clinical characteristics of patients with cervical cancer.

Characteristic	n	OTX1 expression		P-value
		Low	High	
Age, years				
≤45	16	6	10	0.7619
>45	35	16	19	
Histological type				
Squamous cell carcinoma	42	17	25	0.4740
Adenocarcinoma	9	5	4	
Tumor size, cm				
≤4	26	16	10	0.0107[a]
>4	25	6	19	
FIGO stage				
I-II	22	13	9	0.0432[a]
III-IV	29	8	21	
Histological differentiation				
Well/moderate	28	18	10	0.0015[a]
Poor	23	4	19	
Lymph node metastasis				
No	31	18	13	0.0098[a]
Yes	20	4	16	

[a]P<0.05, analyzed by Fisher's exact test. OTX1, orthodenticle homolog 1; FIGO, International Federation of Gynecology and Obstetrics.

OTX1 promotes proliferation of cervical cancer cells. To evaluate the effect of OTX1 on cervical cancer cells, OTX1 siRNAs were transfected into cervical cancer cell lines. OTX1 expression was highest in C-33A and lowest in CaSki cells (Fig. 1F). Thus, the effect of OTX1 silencing was examined in C-33A cells and the effect of OTX1 overexpression was examined in CaSki cells. RT-qPCR and western blotting showed that OTX1 siRNAs significantly decreased OTX1 expression (Fig. 2A) and OTX1 overexpression significantly increased OTX1 expression in cervical cancer cells (Fig. 2B). The effect of OTX1 on viability of cervical cancer cells was assessed. MTT results showed that OTX1 silencing significantly decreased viability of C-33A cells while OTX1 overexpression significantly increased viability of CaSki cells (Fig. 2C). Furthermore, EdU and colony formation assay showed that proliferation was significantly decreased by OTX1 silencing in C-33A cells and significantly increased by OTX1 overexpression in CaSki cells (Fig. 2D and E).

OTX1 increases migration and invasion of cervical cancer cells. To assess the effect of OTX1 on migration and invasion of cervical cancer cells, western blot, wound healing and Transwell assays were performed. The migration and invasion of cancer cells involves MMPs (18,19), specifically MMP2 and MMP9 (20). TIMP2, a specific inhibitor of MMP2, is reported to serve a key role in tumorigenesis and progression (21). Expression levels of MMP2, MMP9 and TIMP2 were analyzed by western blotting. Levels of MMP2, MMP9 and TIMP2 were significantly downregulated in si-OTX1#1 and si-OTX1#2 compared with si-NC (Fig. 3A). The expression levels of MMP2, MMP9 and TIMP2 in pc-OTX1 group were significantly higher than those in pc-NC group. Wound healing showed that OTX1 silencing significantly decreased migration rate while OTX1 overexpression significantly enhanced the migration of cervical cancer cells (Fig. 3B). Consistent with these findings, Transwell assay showed that OTX1 silencing significantly decreased migration and invasion of C-33A cells and overexpression of OTX1 increased the migration and invasion of CaSki cells (Fig. 3C and D).

OTX1 activates the Wnt signaling pathway. To determine the biological function of OTX1 in cervical cancer, GSEA analysis was performed on RNA-sequencing data derived from TCGA of Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) using the online tool LinkedOmics. A total of 10,356 genes was significantly positively associated with OTX1 (red), while 9,548 genes showed significant negative correlation (green; Fig. 4A). The top 50 genes significantly associated with OTX1 were visualized as a heat map (Fig. 4B). Enrichment of these genes in 'pathway-wikipathway_cancer dataset was analyzed and 'Wnt signaling pathway (Netpath)' was selected for further investigation (Fig. 4C). 'Wnt signaling pathway (Netpath)' (enrichment score, 0.41; normalized enrichment score, 1.50) was positively associated with OTX1 expression (Fig. 4D). To determine whether Wnt signaling pathway was modulated by OTX1, GeneMANIA was used to confirm the interaction between OTX1 and Wnt protein. The GeneMANIA analysis showed that OTX1 interacted with Wnt9b (Fig. 4E). To confirm the GeneMANIA result, Co-IP assay was performed to validate the interaction between OTX1 and Wnt9b. OTX1 interacted with Wnt9b in both C-33A and CaSki cells (Fig. 4F). Expression levels of genes enriched in 'Wnt signaling pathway (Netpath)' were determined by western blotting. OTX1 silencing significantly decreased levels of Wnt9b and β-catenin and increased levels of APC, GSK-3β and AXIN2 in C-33A cells (Fig. 4G). Overexpression of OTX1 significantly increased levels of Wnt9b and β-catenin and decreased the levels of APC, GSK-3β and AXIN2 in CaSki cells (Fig. 4H).

Inhibition of Wnt signaling pathway eliminates the effect of OTX1 on cervical cancer cells. To investigate whether the effects of OTX1 cervical cancer were achieved by regulating Wnt signaling pathway, XAV939, a Wnt inhibitor, was used to treat cervical cancer cells. The results of western blotting showed that XAV939 treatment significantly promoted the levels of APC, GSK-3β and AXIN2 and decreased β-catenin. (Fig. 5A). Additionally, XAV939 treatment partly eliminated the effect of OTX1 overexpression on genes associated with the Wnt signaling pathway (Fig. 5A). EdU results showed that XAV939 significantly inhibited proliferation of cervical cells and eliminated the promotive effect of OTX1 overexpression on proliferation of cervical cancer cells (Fig. 5B). Wound healing showed

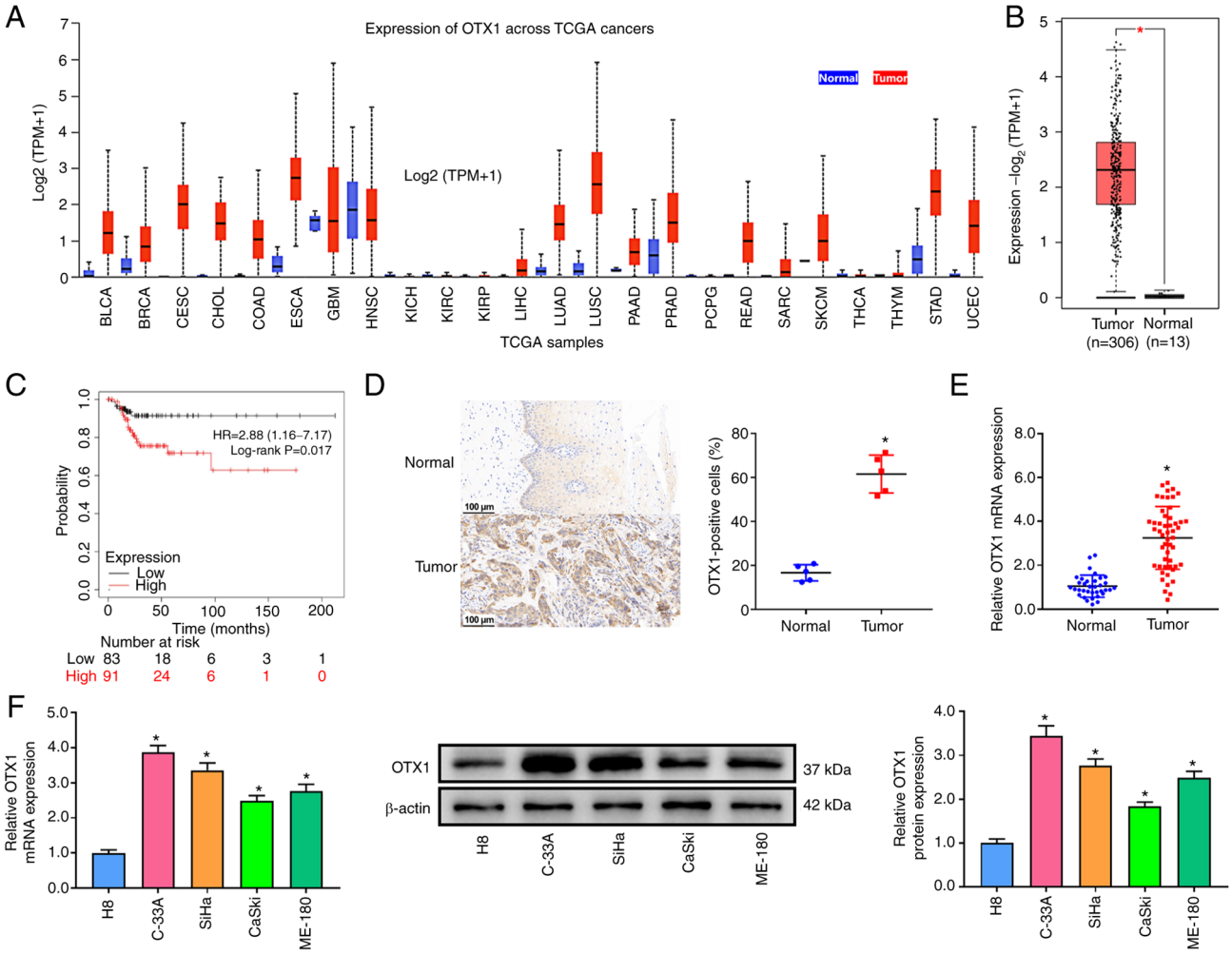

Figure 1. OTX1 is upregulated in cervical cancer tissue and cells. (A) OTX1 expression in cancer was analyzed by TCGA. (B) TCGA analysis showed OTX1 was upregulated in cervical cancer tissues. *P<0.05 vs. Normal group. (C) Kaplan-Meier plotter analysis based on TCGA database showed that survival probability was worse in patients with high OTX1 expression. (D) Immunohistochemical staining and (E) RT-qPCR showed that OTX1 was highly expressed in cervical cancer tissue. *P<0.05 vs. Normal group. (F) RT-qPCR and western blot analysis showed that OTX1 was highly expressed in cervical cancer cells. All experiments were performed in triplicate. *P<0.05 vs. H8 cells. OTX1, orthodenticle homolog 1; TCGA, The Cancer Genome Atlas; RT-q, reverse transcription-quantitative; TPM, Transcripts Per Kilobase of exon model per Million mapped reads.

that XAV939 significantly decreased migration and eliminated the promotive effect of OTX1 overexpression on cell migration (Fig. 5C). The similar results were obtained in Transwell assay. XAV939 treatment significantly decreased invasion of cervical cancer cells and partly eliminated the promotive effect of OTX1 overexpression on invasion of cervical cancer cells (Fig. 5D).

Silencing of Wnt signaling pathway eliminates the effect of OTX1 on cervical cancer cells. Changes in Wnt signaling pathway in cervical cancer cells following β-catenin silencing were determined. β-catenin was significantly decreased by β-catenin siRNAs, particularly si-β-catenin#1 (Fig. 6A). β-catenin silencing significantly enhanced the levels of APC, GSK-3β and AXIN2 and suppressed β-catenin (Fig. 6B). Additionally, β-catenin silencing significantly inhibited proliferation, migration and invasion of cervical cells (Fig. 6C-E). Compared with pc-OTX1 + si-NC, EdU-positive cells, migration and invasion were significantly decreased in pc-OTX1 + si-β-catenin#1 group (Fig. 6C-E).

Discussion

Cervical cancer is a tumor that endangers fertility and quality of life for patients (3). Determining the mechanisms of cervical cancer is useful to develop novel improved therapeutic strategies to increase survival rate of patients with cervical cancer. The present results indicated that OTX1 promoted cell proliferation, migration, and invasion of cervical cancer.

OTX1 is a homeobox gene that belongs to the OTX family, which is responsible for early human mammary gland and fetal retina development (22). OTX1 is highly expressed in breast cancer (6,23). OTX1 overexpression partly reverses the inhibitory effect of ADP dependent glucokinase antisense RNA 1 (ADPGK-AS1) silencing on proliferation and migration of breast cancer cells (23). Patients with LSCC with low OTX1 expression exhibit lower survival rate than those with high OTX1 expression (9). OTX1 silencing significantly inhibits migration of pancreatic cancer cells (8). Here, TCGA analysis showed upregulation of OTX1 by TCGA analysis with 306 cervical cancer and 13 normal samples. OTX1 was

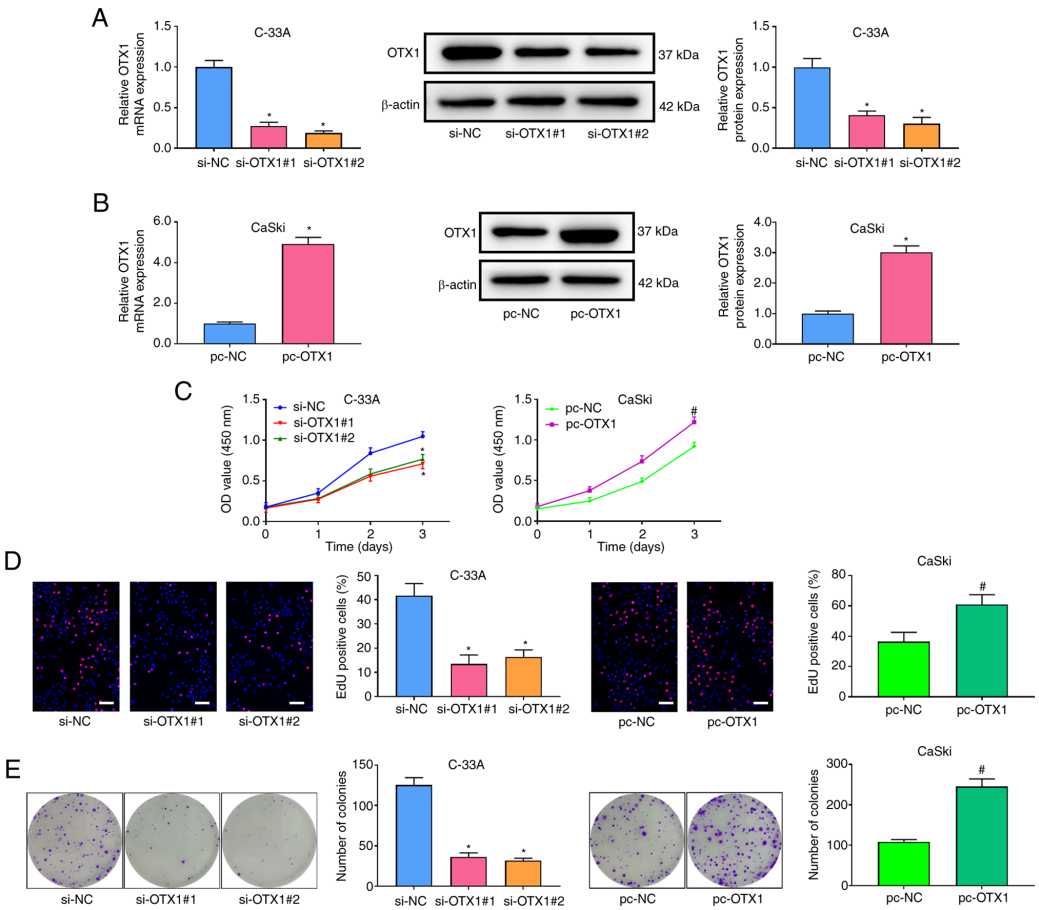

Figure 2. OTX1 promotes proliferation of cervical cancer cells. (A) Reverse transcription-quantitative PCR and western blotting showed that OTX1 expression was decreased following OTX1 siRNAs transfection and (B) increased following OTX1 pcDNA3.1 transfection. (C) MTT assay showed cell viability following transfection with OTX1 siRNAs and pcDNA3.1. (D) EdU assay for proliferation following transfection with OTX1 siRNAs and pcDNA3.1 (scale bar, 100 μm). (E) Colony formation assay following transfection with OTX1 siRNAs and pcDNA3.1 (magnification, x40). All experiments were performed in triplicate. *P<0.05 vs. si-NC; #P<0.05 vs. pc-NC. OTX1, orthodenticle homolog 1; si, small interfering; NC, negative control; OD, optical density.

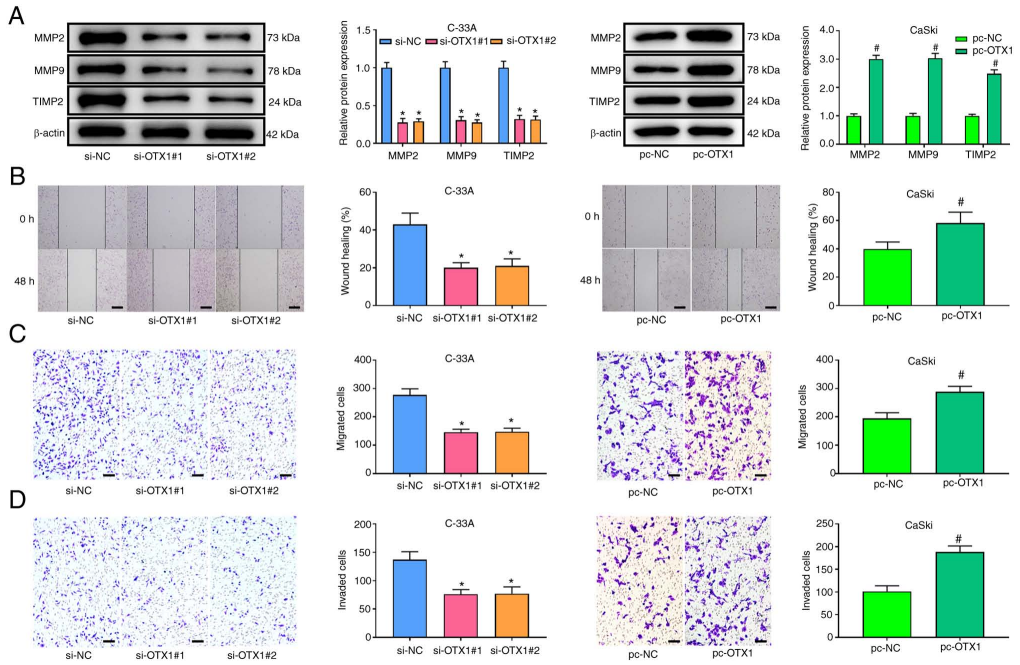

Figure 3. OTX1 increases migration and invasion of cervical cancer cells. (A) Western blotting of protein expression of MMP2, MMP9 and TIMP2 in cervical cancer cells. (B) Wound healing (scale bar, 100 μm) and (C) Transwell assay of migration of cervical cancer cells (scale bar, 200 μm). (D) Transwell assay of invasion of cervical cancer cells (scale bar, 200 μm). All experiments were performed in triplicate. *P<0.05 vs. si-NC; #P<0.05 vs. pc-NC. OTX1, orthodenticle homolog 1; si, small interfering; NC, negative control; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase.

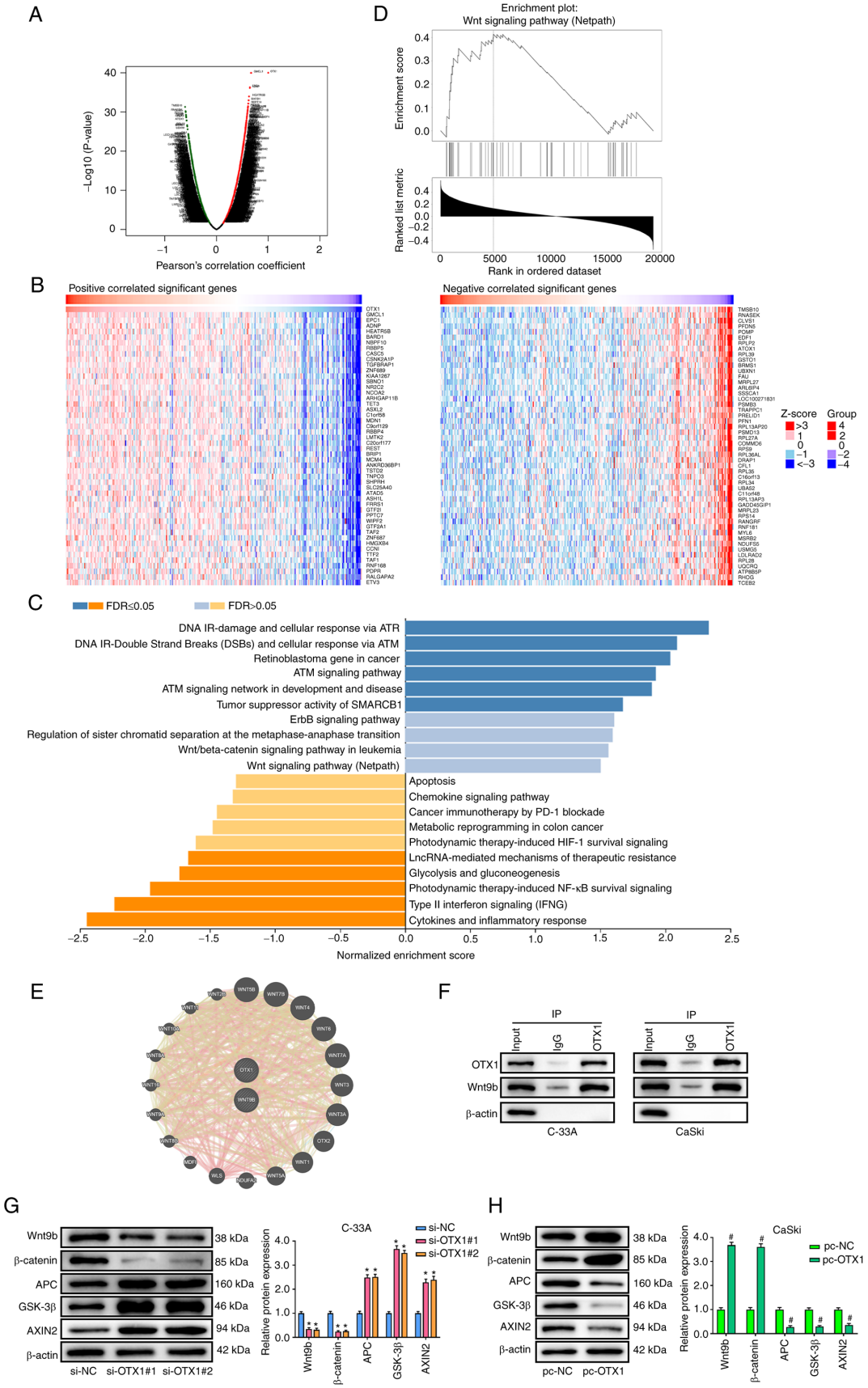

Figure 4. OTX1 activates the Wnt signaling pathway. (A) OTX1 co-expressed genes in cervical cancer were analyzed by Pearons's test (LinkedOmics). (B) Top 50 genes associated with OTX1 expression in cervical cancer (LinkedOmics). (C) Pathway analysis of OTX1 co-expressed genes by LinkedOmics using GSEA. (D) GSEA showed that OTX1 activated the Wnt signaling pathway. (E) Interaction network between OTX1 and Wnt proteins using GeneMANIA. (F) Co-IP assay was used to analyze the interaction between OTX1 and Wnt9b. Western blot for protein expression of Wnt9b, β-catenin, APC, GSK-3β and AXIN2 in (G) C-33A and (H) CaSki cells. All experiments were performed in triplicate. *P<0.05 vs. si-NC; #P<0.05 vs. pc-NC. OTX1, orthodenticle homolog 1; si, small interfering; NC, negative control; GSEA, gene set enrichment analysis; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; AXIN, axis inhibition protein; co-IP, co-immunoprecipitation; FDR, false discovery rate.

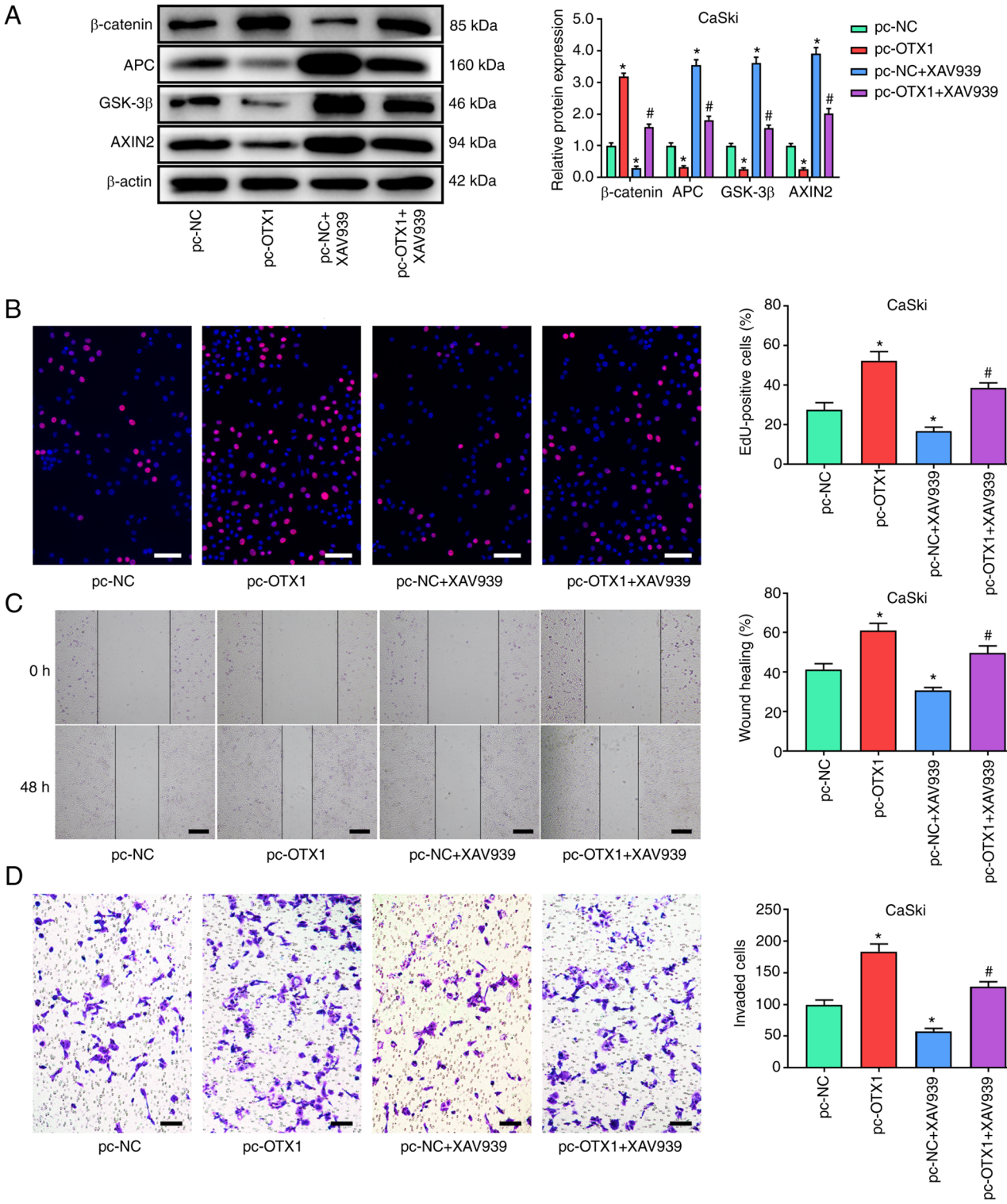

Figure 5. Inhibition of Wnt signaling pathway eliminates the effect of OTX1 on cervical cancer cells. (A) Western blot for protein expression of β-catenin, APC, GSK-3β and AXIN2 in CaSki cells. (B) EdU assay for proliferation, (C) wound healing assay for migration (scale bar, 100 μm) and (D) Transwell assay for invasion of CaSki cells (scale bar, 200 μm). All experiments were performed in triplicate. *P<0.05 vs. pc-NC; $^{\#}$P<0.05 vs. pc-OTX1. OTX1, orthodenticle homolog 1; si, small interfering; NC, negative control; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; AXIN, axis inhibition protein.

overexpressed in cervical cancer tissue and cells. Furthermore, OTX1 promoted proliferation, migration and invasion of cervical cancer cells. Silencing OTX1 significantly inhibited proliferation, migration and invasion of cervical cancer cells. These results demonstrated that OTX1 served a carcinogenic role in cervical cancer.

Wnt signaling pathway promotes tumor progression and metastasis in various types of cancer, such as gastric cancer, pancreatic cancer, lung cancer, and cervical cancer (24-29). Previous studies have shown that the Wnt/β-catenin pathway is activated in numerous types of cancer, including cervical cancer (30-32). Zhang *et al* (29) suggested that activation of

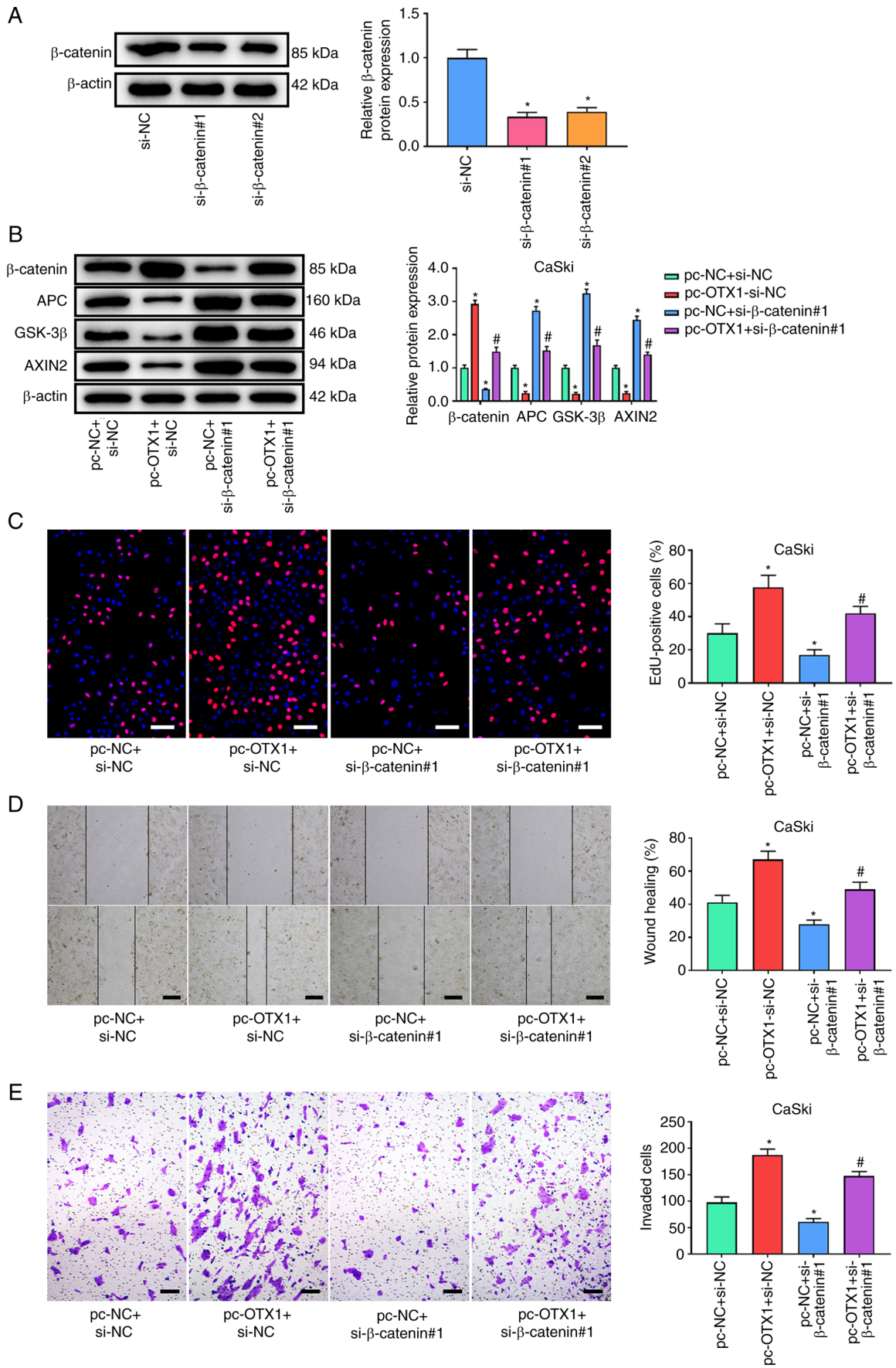

Figure 6. Silencing of Wnt signaling pathway eliminates the effect of OTX1 on cervical cancer cells. (A) Western blot analysis of protein expression of β-catenin in CaSki cells. *P<0.05 vs. si-NC. (B) Western blot for protein expression of β-catenin, APC, GSK-3β and AXIN2 in CaSki cells. (C) EdU assay for proliferation, (D) wound healing assay for migration (scale bar, 100 μm) and (E) Transwell assay for invasion of CaSki cells (scale bar, 200 μm). All experiments were performed in triplicate. *P<0.05 vs. pc-NC + si-NC; #P<0.05 vs. pc-OTX1 + si-NC. OTX1, orthodenticle homolog 1; si, small interfering; NC, negative control; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; AXIN, axis inhibition protein.

Wnt signaling pathway significantly enhances cell viability and migration in cervical cancer. Inhibition of Wnt/β-catenin signaling pathway decreases cell viability and proliferation in ovarian cancer (33). Here, OTX1 activated the Wnt signaling pathway and inhibition of Wnt signaling pathway by XAV939 significantly inhibited proliferation, migration, and invasion of cervical cancer cells promoted by OTX1 overexpression. The results in the present study were consistent with these aforementioned findings. All these data suggested that OTX1 may serve a carcinogenic role in cervical cancer by regulating Wnt signaling pathway.

β-catenin, APC, GSK-3β and AXIN2 are canonical Wnt/β-catenin signaling transducers (34). Studies have confirmed that β-catenin and APC are key factors involved in the regulation of numerous types of cancer, such as lung (35), colorectal (36), breast (37), oral (38) and gastric cancer (39). AXIN2, a direct target of Wnt signaling pathway, restricts the Wnt signaling pathway via a negative feedback loop (40). AXIN2 gene is a negative regulator of the Wnt signaling pathway in colorectal cancer (41). Here, OTX1 silencing significantly decreased levels of Wnt9b and β-catenin and increased levels of APC, GSK-3β and AXIN2. Conversely, OTX1 overexpression enhanced levels of Wnt9b and β-catenin and suppressed levels of APC, GSK-3β and AXIN2. More importantly, XAV939 (Wnt signaling pathway inhibitor) inhibited the increase of proliferation, migration and invasion in OTX1-overexpressing cells. These data indicated that OTX1 promoted the development of cervical cancer by activating the Wnt signaling pathway.

There are some limitations of the present study. This study lacks experimental data about OTX1 silencing in CaSki cells and OTX1 overexpression in C-33A cells. The interactions between OTX1 and other proteins should be investigated in future study. Additionally, *in vivo* study should be investigated in future trials to confirm the findings.

In summary, OTX1 promoted proliferation, migration and invasion of cervical cancer cells by activating the Wnt signaling pathway. These findings indicated that OTX1 may be a treatment target for cervical cancer.

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Availability of data and materials

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

Authors' contributions

LZ and HL conceived and designed the study. LZ, DZ, LC and HD performed experiments. HL, YY and TW analyzed and interpreted data. All authors wrote the manuscript. LZ and HL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Maternal and Child Health Hospital of Hubei Province [(2021) IEC (xm017)], Tongji Medical College, Huazhong University of Science and Technology. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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