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SALL4 promotes the tumorigenicity of cervical cancer cells through activation of the Wnt/β-catenin pathway via *CTNNB1*

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Abstract

SALL4 is overexpressed in many cancers and is found to be involved in tumorigenesis and tumor progression. However, the function of SALL4 in cervical cancer remains unknown. Here, we showed that the expression of SALL4 was gradually increased from normal cervical tissue to high-grade squamous intraepithelial lesions and then to squamous cervical carcinoma. SALL4 was upregulated or downregulated in cervical cancer cells by stably transfecting a SALL4-expressing plasmid or a shRNA plasmid targeting SALL4, respectively. In vitro, cell growth curves and MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assays showed that SALL4 promoted the cell proliferation of cervical cancer cells. In vivo, xenograft experiments verified that SALL4 enhanced the tumor formation of cervical cancer cells in female BALB/c Nude mice. Cell cycle analysis by fluorescence-activated cell sorting found that SALL4 accelerates cell cycle transition from the G_0/G_1 phase to the S phase. TOP/FOP-Flash reporter assay revealed that SALL4 significantly upregulates the activity of Wnt/β-catenin pathway. Western blotting showed that the expression levels of β-catenin and important downstream genes, including c-*Myc* and *cyclin D1*, were increased by SALL4 in cervical cancer cells. Furthermore, dual-luciferase reporter and chromatin immunoprecipitation assays confirmed that SALL4 transcriptionally activated *CTNNB1* by physically interacting with its promoters. Taken together, The results of this study demonstrated that SALL4 may promote cell proliferation and tumor formation of cervical cancer cells by upregulating the activity of the Wnt/β-catenin signaling pathway by directly binding to the *CTNNB1* promoter and *trans*-activating *CTNNB1*.

KEYWORDS
cervical cancer, *CTNNB1*, SALL4, tumorigenicity, Wnt/β-catenin

1 | INTRODUCTION

Cervical cancer is the fourth most common cause of cancer-related deaths in women worldwide.[1] Despite advances in detection, treatment, and prevention, nearly 80% of cervical cancer deaths occur in developing countries.[2] It is known that infection with high-risk human papilloma viruses (HPV) is intimately related to the development of cervical cancer, however not all patients infected with HPV ultimately develop cervical cancer. Infection with HPV is not sufficient for cervical carcinogenesis and tumor

progression.[3-5] To date, the molecular mechanisms involved in the development and progression of cervical cancer remain poorly understood. Researchers have reported that various factors that activate oncogenes (*RAS*, *CLDN1*) and inactivate tumor suppressor genes (*P21*, *P53*) are involved in the proliferative and aggressive nature of human cervical cancer.[6-9] Recently, some studies have found that several stem cell-related transcription factors are associated with tumorigenesis in cervical cancer. For example, KLF4,[10] UTF1,[11] SOX9[12] and Slug[13] have been found to suppress cervical tumor growth. In contrast, NANOG,[14] OCT4,[15] LGR5[16] and EZH2[17] have been reported to promote the tumorigenesis of cervical cancer.

SALL4 (sal-like 4), a member of the mammalian homologs of *Drosophila* homeotic gene *spalt* (sal), is an important zinc finger transcription factor.[18] Human SALL4 has been mapped to chromosome 20.q13.2 and has two isoforms, SALL4A and SALL4B, that have resulted from different internal splicing patterns in exon 2.[18-20] Researchers have reported that SALL4 is an essential factor for maintenance of the pluripotency and self-renewal of embryonic stem cells.[21-24] During early embryogenesis, SALL4A and SALL4B are able to form homodimers or heterodimers with distinct DNA-binding sites and exhibit different roles. SALL4B, but not SALL4A, can maintain the pluripotent state of mouse embryonic stem cells.[25] High expression of SALL4 has been observed in several tumors including liver cancer,[26,27] lung cancer,[28] acute/chronic myeloid leukemia,[29-31] gastric cancer,[32] prostate cancer,[33] colorectal cancer,[34] breast cancer,[35] and endometrial cancer.[36] *SALL4* acts as a novel oncogene that plays an important role in the initiation and progression of tumors.

However, as far as we know, there has been no report exploring the role of SALL4 in cervical carcinogenesis. In this study, SALL4 was found to be involved in the development and progression of cervical cancer. SALL4 promoted cell proliferation and tumor formation of cervical cancer cells by upregulating the activity of the Wnt/β-catenin signaling pathway via *trans*-activation of *CTNNB1*.

2 | MATERIALS AND METHODS

2.1 | Tissue specimens of normal cervical and various cervical lesions

From 2013 to 2015, 34 normal cervical tissues (NC), 30 high-grade squamous intraepithelial lesion (HSIL) and 48 squamous cervical cancer tissues (SCC) were obtained from patients at the First Affiliated Hospital of Xi'an Jiaotong University Medical College, China for immunohistochemical analysis. Histological classifications and clinical staging were based on the International Federation of Gynecology and Obstetrics classification system. None of the subjects had received immunotherapy, chemotherapy, or radiotherapy. Written informed consent was obtained from all subjects before specimen collection. All of the procedures were approved by the Ethics Committee of the Medical College of Xi'an Jiaotong University.

2.2 | Cervical cancer cell lines and cell culture

Human cervical cancer cell lines HeLa, SiHa, C33A, and CaSki were purchased from the American Type Culture Collection (ATCC) and cultured in our laboratory. At 37°C and in an atmosphere of 5% CO_2 in air, HeLa, SiHa, and C33A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) and CaSki cells were cultured in RPMI1640 medium (Sigma-Aldrich, USA). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen).

2.3 | Immunostaining

The immunohistochemical staining procedure was performed as previously described.[9] Primary antibodies included were against SALL4 (1:100 dilution; sc-101147; Santa Cruz), β-catenin (1:200 dilution; sc-7963; Santa Cruz), c-*Myc* (1:100 dilution; sc-40; Santa Cruz), Cycline D1(1:100 dilution; sc-8396; Santa Cruz) and Ki-67 (1:200 dilution, sc-23900; Santa Cruz). Immunohistochemical staining was divided into two categories according to the immunoreactivity score (IRS): negative (0-3) or positive (4-12). Staining intensity was scored as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Staining extent was scored according to the percentage of positively stained cells: 0 (<5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%), 4 (76%-100%). The final IRS = intensity score × quantity score.

For immunocytochemistry, cells were seeded onto autoclaved coverslips; after 48 h, cells were fixed in 4% paraformaldehyde for 30 mins and then permeabilized with 0.2% Triton X-100 for 20 min at room temperature. Cells were then incubated with the SALL4 antibody described above.

2.4 | Western blotting

Western blotting analyses were performed as previously described[9] using 50 μg protein samples from fresh tissues and cells. Primary antibodies included SALL4 (1:500 dilution; sc-101147; Santa Cruz), GSK3β (1:1000 dilution; sc-53931; Santa Cruz), β-catenin (1:1000 dilution; sc-7963; Santa Cruz), c-*Myc* (1:500 dilution; sc-40; Santa Cruz), Cycline D1(1:500 dilution; sc-8396; Santa Cruz) and GAPDH (1:1000 dilution; sc-47724, Santa Cruz). The relative densities of the western blot bands were quantified using the Alpha View system (Cell Biosciences).

2.5 | Vector construction and transfection

The coding sequence (CDS) of the human *SALL4* gene (NM 001318031.1) was amplified by polymerase chain reaction (PCR) using cDNA obtained from SiHa cells, using the Premix PrimeSTAR HS kit (TaKaRa) and the following primers:

F5′-CCGGAATTCGCCACCATGTCGAGGCGCAAGCAGGCGAAAC-3′;
R 5′-CGCGGATCCTTAGCTGACCGCAATCTTGTTTTCTTCC-3′.

To construct the pIRES2-AcGFP-SALL4 recombinant vector, the SALL4 CDS fragment was cloned into the pIRES2-AcGFP expression vector (Clontech) at *Eco*RI and *Bam*HI restriction enzyme sites (TaKaRa). To construct shRNA vectors targeting SALL4, the following two SALL4-Homo sequences were used, which were obtained from GenePharma Co., Ltd.:

SALL4-Homo-636: 5′-GCAAAGTGGCCAACACTAATGT-3′;
SALL4-Homo-1171: 5′-GCTAGACACATCCAAGAAAGGT-3′.

All transfection experiments were performed using Lipofectamine 2000 reagent (Invitrogen). The SALL4 overexpression plasmid was transfected into SiHa and HeLa cells, and the SALL4 shRNA plasmids were transfected into C33A cells. Transfected cells were treated with medium containing G418 (Calbiochem) for approximately 3 wk, then drug-resistant colonies were collected, expanded, and identified.

2.6 | Cell growth and cell viability assays

Cervical cancer cells were seeded in triplicate onto six-well plates at a concentration of 1×10^4 cells/well and cultured for 7 d. Using a hemocytometer under a light microscope, the numbers of cells were counted every 2 d. Then, cell growth curves were plotted to assess cell growth. Cell viability was assessed using MTT (Sigma-Aldrich) dye added to cells; six parallel samples were used for each condition; absorbance at 490 nm was measured (Bio-Rad) every 2 d.

2.7 | Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) Calibur flow cytometry (Becton Dickinson) was used to detect the cell cycle distribution of cells. Approximately 1×10^6 cells in logarithmic phase were collected and fixed overnight in 70% cool ethanol at 4°C. Before FACS analysis, cells were treated with 20 mg/mL propidium iodide (Sigma-Aldrich) and 10 U/mL RNase A for 30 min at room temperature.

2.8 | Tumor xenograft experiment

BALB/c Nude female mice, 4-6 wk old, were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China) and housed in a specific-pathogen-free (SPF) room at constant temperature (22-25°C) and humidity (40%-50%). SALL4-modified cervical cancer cells (1×10^6) were inoculated subcutaneously into each female mouse. To assess the tumor volumes, tumor sizes were measured every 3 d, and tumor volumes were calculated using the standard formula: length × width^2/2. At the termination of the experiment, xenograft tumors were dissociated and weighed. Tissues from the xenograft tumors were paraffin-embedded for histological analysis.

2.9 | TOP-Flash/FOP-Flash reporter assay

In brief, tumor cells (5×10^4) were seeded into a 24-well plate, and TOP-Flash reporter plasmids and pTK-RL plasmids were transiently co-transfected into the cells using Lipofectamine 2000 (Invitrogen). After transfection for 48 h, a Dual-Luciferase Assay Kit (Promega) was used according to the manufacturer's instructions to detect the activities of both firefly and Renilla luciferase reporters. TOP-Flash reporter activity was calculated as the relative ratio of firefly luciferase activity to Renilla luciferase activity.

2.10 | PCR analysis

RNA from cervical cancer cells and tumor tissues was extracted using TRIzol Reagent (Invitrogen), and then the cDNA was obtained through reverse transcription using a PrimeScript™ RT Reagent Kit (TaKaRa). Real-time quantitative PCR was performed in triplicate for each cell sample, using a SYBR Premix Ex *Taq II* Reagent Kit (TaKaRa). The designed primers are listed in Table S1.

2.11 | Dual-luciferase reporter assay

For analysis of the *CTNNB1* promoter, five fragments (from position −1712 bp to 44 bp, −1428 bp to 44 bp, −1144 bp to 44 bp, −844 bp to 44 bp, −440 bp to 44 bp) were respectively cloned into the pGL3-Basic vector (Promega, Madison, WI, USA) to generate *CTNNB1* promoter reporter plasmids. The designed primers are shown in Table S2. Plasmids containing firefly luciferase reporters and pTK-RL plasmids were co-transfected into tumor cells, then the activities of both firefly and Renilla luciferase reporters were determined 48 h after transfection using Dual-Luciferase Assay Kit (Promega). The specific promoter activity in different groups was calculated as the relative ratio of firefly luciferase activity to Renilla luciferase activity.

2.12 | Quantitative chromatin immunoprecipitation

An EZ-ChIP™ Assay Kit (Cat#17–371; Millipore) was used to perform quantitative chromatin immunoprecipitation (qChIP) assays according to the manufacturer's protocol. In brief, cells were treated with 37% formaldehyde to crosslink proteins, and then the reaction was terminated with 0.125 M glycine. After sonication, chromatin–protein complexes were immunoprecipitated with 5 μg of anti-Sall4 antibody (sc-101147; Santa Cruz). Regions of interest were amplified in triplicate from precipitated samples by real-time PCR, and the amount of precipitated DNA was calculated as a percentage of the input sample. The primers used in quantitative ChIP assays are listed in Table S3.

2.13 | Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc.). All data are shown as means ± SD. For comparison among groups, Student's *t* test, one-way analysis of variance (ANOVA) and chi-squared test were performed. The expression variance analysis of CESC was based on the Wilcox test. For correlation analysis, Pearson's correlation test and logistical regression analysis were used. In all tests, $P < .05$ was defined as statistically significant.

3 | RESULTS

3.1 | SALL4 expression in samples of normal cervix and various cervical lesions

To identify whether the endogenous SALL4 is involved in cervical carcinogenesis, immunohistochemistry (IHC) was used to detect the expression of SALL4 in 34 NC, 30 HSIL, and 48 SCC samples. Representative SALL4 staining was observed in various cervical tissues (Figure 1A). The percent of specimens with positive SALL4 expression was 14.71% (5 of 34) in the NC, was gradually increased to 36.67% (11 of 30) in the HSIL, and was 79.17% (38 of 48) in the SCC (Figure 1B and Table S4; NC vs SCC, $P < .01$; HSIL vs SCC, $P < .01$; NC vs HSIL, $P > .05$). Additionally, analysis of the IRS of SALL4 staining also revealed that the score gradually increased from 2.74 ± 2.29 in NC to 3.73 ± 3.29 in the HSIL and 7.69 ± 3.80 in the SCC (Figure 1C, NC vs SCC, $P < .01$; HSIL vs SCC, $P < .01$; NC vs HSIL, $P > .05$). Western blot assays were used to examine the expression of SALL4 protein in eight fresh NC samples and eight fresh SCC lesions from patients undergoing surgery (Figure 1D); the relative expression levels of SALL4A and SALL4B were higher in the SCC than for those in the NC group (Figure 1E, $P < .05$). Expression of SALL4 was analyzed using The Cancer Genome Atlas (TCGA) database, the results showed that SALL4 expression was significantly increased in 306 cervical cancer specimens (Figure 1F, $P < .05$). All these results suggested that SALL4 was involved in the development and progression of cervical cancer.

3.2 | SALL4 promotes the proliferation of cervical cancer cells in vitro

To explore the effect of SALL4 on cervical cancer cells, we identified the expression of SALL4 protein in the cervical cancer cell lines SiHa, HeLa, C33A; and CaSki by immunocytochemistry and western blotting. A high level of SALL4 expression was found in C33A cells, a low level of SALL4 expression was detected in SiHa and CaSki cells, and almost no SALL4 expression was observed in HeLa cells (Figure 2A,B). SALL4 was upregulated in HeLa and SiHa cells by stable transfection with a SALL4-expressing plasmid (Figure 2C,D). SALL4 was downregulated in C33A cells by stable transfection with an shRNA plasmid targeting SALL4 (Figure 2E). Western blot assays were used to confirm the effects of the upregulation and

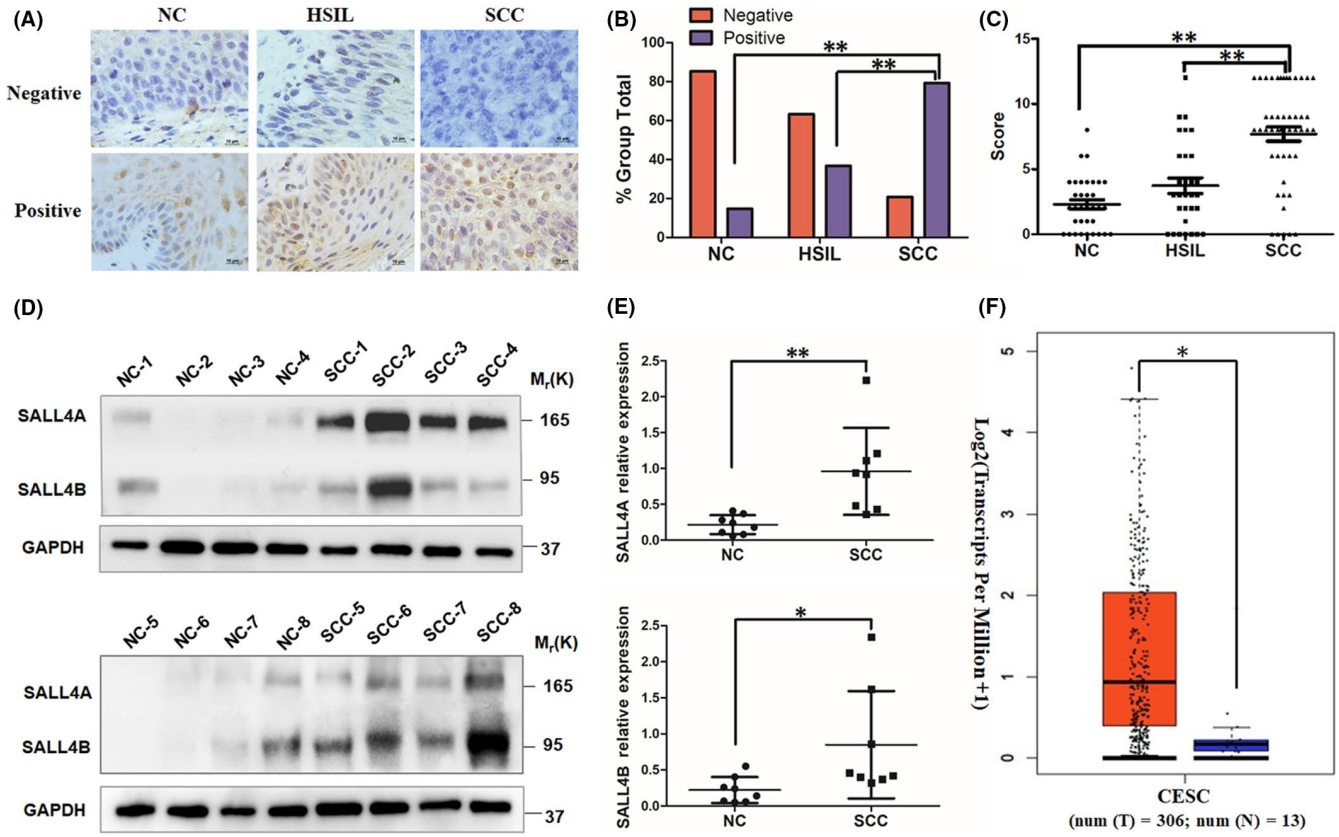

FIGURE 1 SALL4 expression in samples of normal cervix and various cervical lesions. A, Immunohistochemical (IHC) staining for SALL4 expression is shown in normal cervical tissue (NC), high-grade squamous intraepithelial lesion (HSIL) and squamous cervical cancer tissues (SCC); original magnification, ×1000 . B, Bar graph showing the percentage of SALL4-positive and SALL4-negative staining in 34 NC, 30 HSIL and 48 SCC. C, The IHC score of SALL4 staining in NC, HSIL and SCC. D, Representative western blots are shown of the SALL4 expression in eight NC and eight SCC. E, The relative expression of SALL4 in NC and SCC. F, The Cancer Genome Atlas Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (TCGA-CESC) database was used to analyze the expression of SALL4 in cervical cancer. Values are shown as the mean ± SD. *$P < .05$ vs control, **$P < .01$ vs control

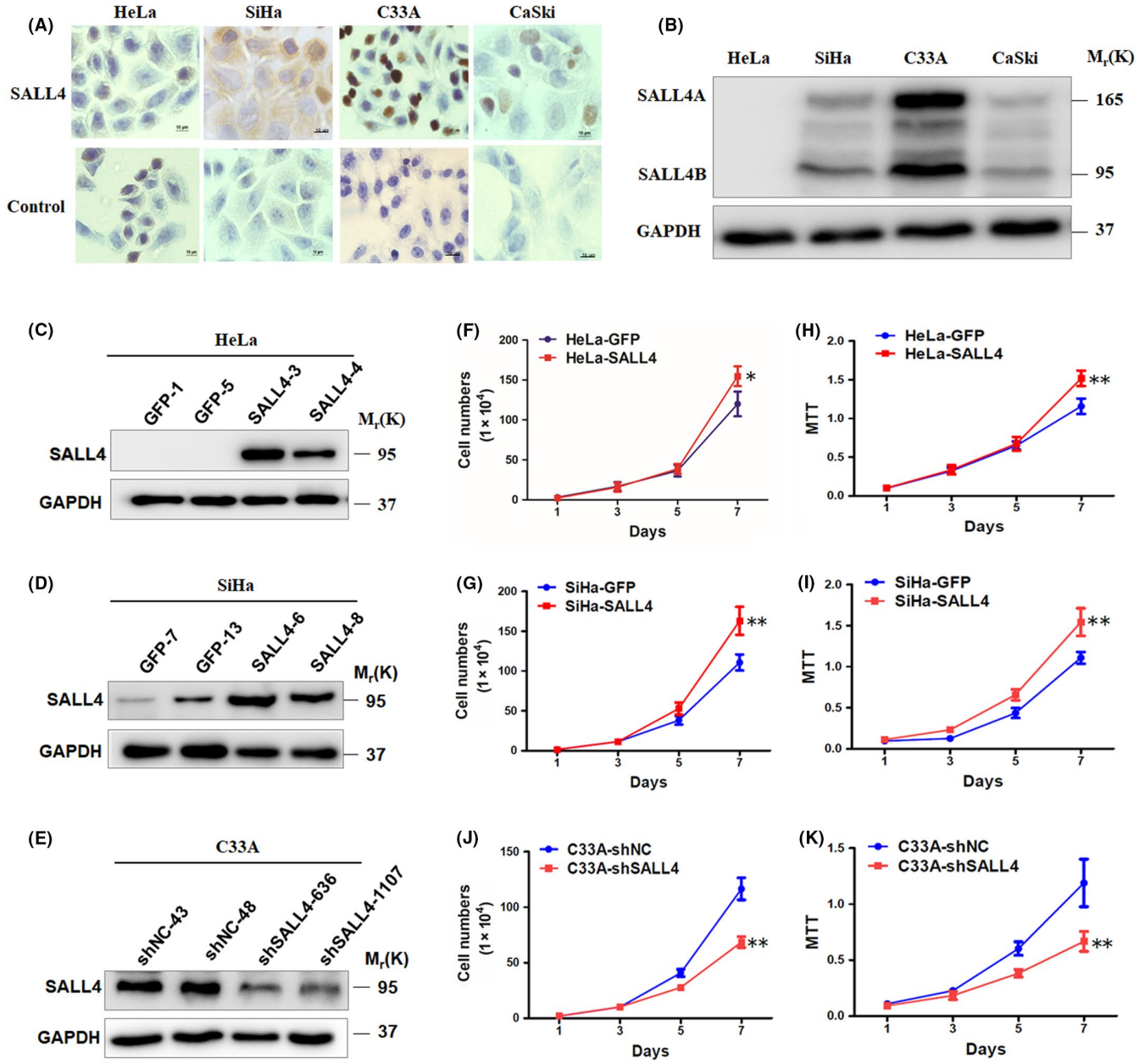

FIGURE 2 SALL4 promotes the proliferation of cervical cancer cells in vitro. A, Immunocytochemical staining showing SALL4 expression in HeLa, SiHa, C33A and CaSki cells; original magnification, ×1000. B, Representative western blots are shown of the SALL4 expression in HeLa, SiHa, C33A and CaSki cells. SALL4-modified cervical cancer cell lines were identified by western blot: C, control (HeLa-GFP) and *SALL4*-overexpressing HeLa (HeLa-SALL4) cells; D, control (SiHa-GFP) and *SALL4*-overexpressing SiHa (SiHa-SALL4) cells; E, control (C33A-shNC) and *SALL4*-silenced C33A (C33A-shSALL4) cells. The growth and viability of cervical cancer cells were detected using growth curves and the 3-(4,5-dimethylthiazole-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in HeLa-GFP and HeLa-SALL4 cells (F, H), SiHa-GFP and SiHa-SALL4 cells (G, I), and C33A-shNC and C33A-shSALL4 cells (J, K). Values are shown as the mean ± SD obtained from three separate experiments. **P* < .05 vs control, ***P* < .01 vs control

downregulation of *SALL4* expression in cervical cancer cells and their controls.

Cell growth curves and MTT assays were used to uncover the proliferative ability among SALL4-modified cervical cancer cells and their controls. The SALL4-overexpressing HeLa (HeLa-SALL4) cells and the SALL4-overexpressing SiHa (SiHa-SALL4) cells grew much faster than their respective control cells (HeLa-GFP and SiHa-GFP; Figure 2F, *P* < .01; Figure 2G, *P* < .01). The cell viabilities of HeLa-SALL4 cells and SiHa-SALL4 cells were significantly higher than their respective control cells (HeLa-GFP and SiHa-GFP; Figure 2H, *P* < .01; Figure 2I, *P* < .01). Furthermore, SALL4-silenced C33A (C33A-shSALL4) cells had significantly weaker cell growth and cell viability than the control cells (C33A-shNC; Figure 2J, *P* < .01; Figure 2K, *P* < .01). All these results demonstrated that SALL4 may promote the proliferation of cervical cancer cells in vitro.

3.3 | SALL4 enhances the cell proliferation and tumor formation of cervical cancer cells in vivo

To identify the effects of SALL4 on tumor formation of cervical cancer cells, 1×10^6 cells were subcutaneously inoculated into each nude mice for the tumor formation assay. The growth of the tumors formed by SALL4-overexpressing cells was much faster than those formed by the control cells (Figure 3A,B, $P < .05$); the tumor-free survival in SALL4-overexpressing cells group was significantly shorter than that in controls (Figure 3C, $P < .05$). Furthermore, the average tumor weight formed by SALL4-overexpressing cells was much heavier than that formed by control cells (Figure 3D, $P < .05$). All these results suggested that SALL4 may enhance tumor formation of cervical cancer cells in vivo. To determine whether cell proliferative ability is related to the tumor formation ability of SALL4-modified cells, a well known cell proliferation marker, Ki67 was stained in tumor xenograft tissues formed by SALL4-overexpressing cells and the control cells. As shown in Figure 3E,F, both Ki67 and SALL4 staining were stronger in the tumor xenograft tissues formed by SALL4-overexpressing cells than those formed by the control cells. All these results indicated that SALL4 promotes tumor formation of cervical cancer cells probably by enhancing the cell's proliferative ability.

3.4 | SALL4 accelerates cell cycle transition from G_0/G_1 phase to S phase of cervical cancer cells

To explore how the SALL4 protein affects the cell proliferation of cervical cancer cells, FACS was used to analyze the cell cycle distribution of the SALL4-modified cervical cancer cells and the control cells. As shown in Figure 4A and B, 34.45% of the HeLa-SALL4 cells were in the G_0/G_1 phase, which was much lower than the number of HeLa-GFP cells in the G_0/G_1 phase (53.94%; $P < .01$); 30.49% of the HeLa-SALL4 cells were in the S phase, which was much higher than that of the HeLa-GFP cells (22.24%; $P < .05$). Consistent with these results, the percentage of SiHa-SALL4 cells in the G_0/G_1 phase was 57.20%, much lower than that of the SiHa-GFP cells (67.53%; Figure 4C,D, $P < .05$); the percentage of SiHa-SALL4 cells in the S phase was 28.47%, much higher than that of the SiHa-GFP cells (20.83%; Figure 4C,D, $P < .05$). Furthermore, 49.09% of the C33A-shSALL4 cells were in G_0/G_1 phase cells, much higher than that of the C33A-shNC cells (38.28%; Figure 4E,F, $P < .01$); and 32.51% of C33A-shSALL4 cells were in the S phase, much lower than that of the C33A-shNC cells (46.30%; Figure 4E,F, $P < .01$). All these results indicated that SALL4 accelerates the transition of the cell cycle from the G_0/G_1 phase to the S phase in cervical cancer cells.

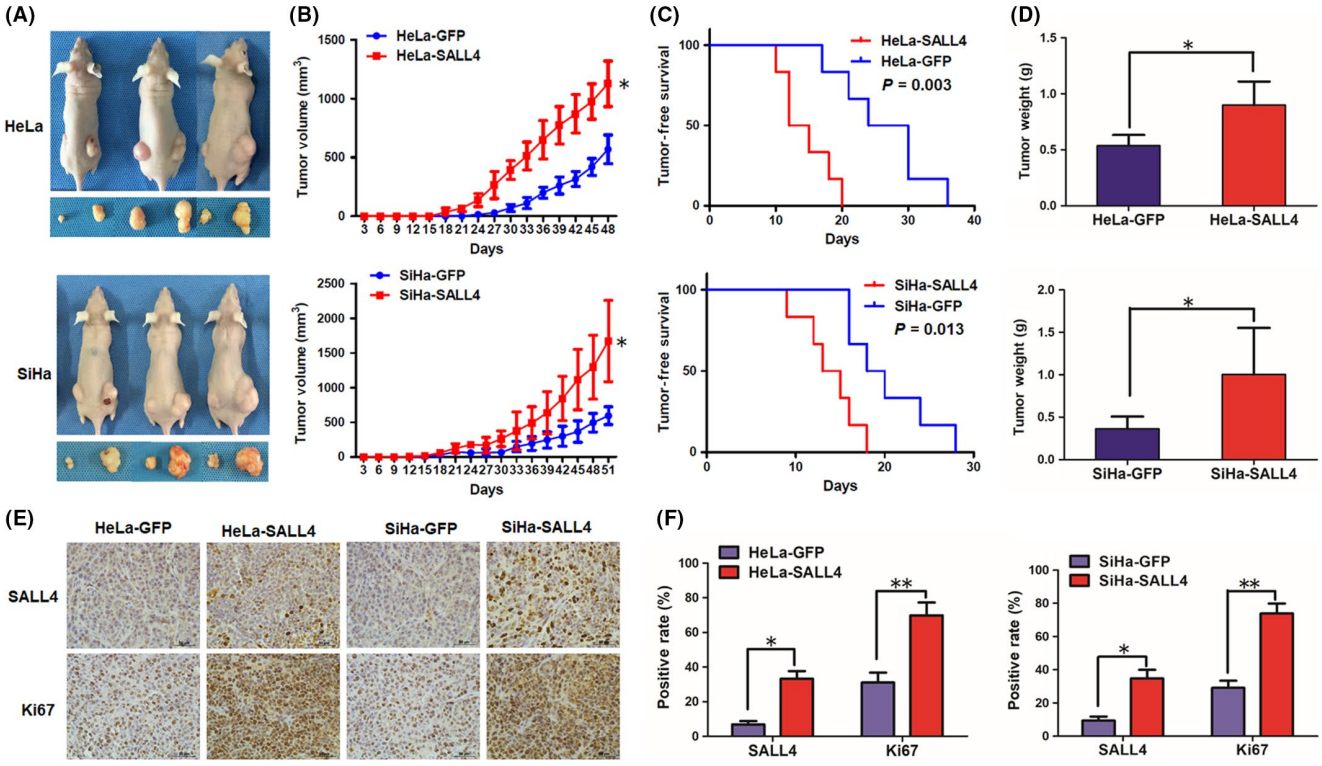

FIGURE 3 SALL4 enhances the cell proliferation and tumor formation of cervical cancer cells in vivo. Tumor formation experiments were performed with eight female nude mice per group. A, The representative tumor xenografts formed by *SALL4*-overexpressing cervical cancer cells and control cells. Tumor growth curve (B), tumor-free survival (C) and (D) tumor weight (D) are shown in *SALL4*-overexpressing cervical cancer cells and control cells, respectively. E, F, IHC staining for SALL4 and Ki-67 is shown for tumor xenografts formed by *SALL4*-overexpressing cervical cancer cells and control cells, respectively; original magnification, ×400 . Values are shown as the mean ± SD. *$P < .05$ vs control, **$P < .01$ vs control

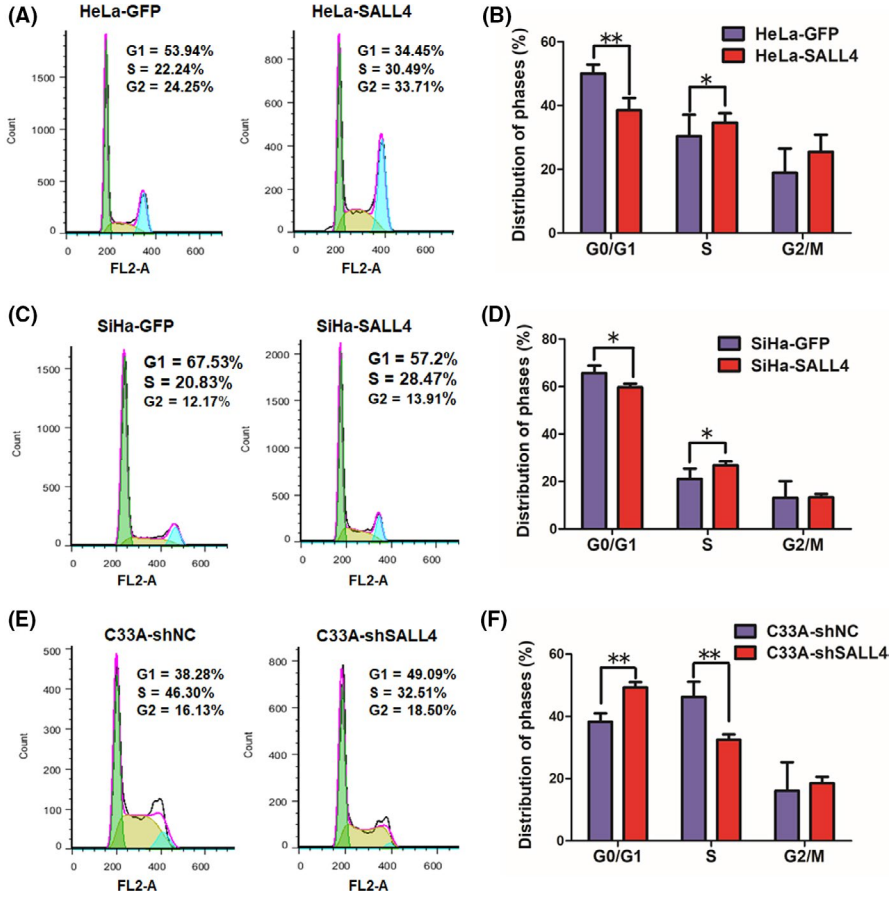

FIGURE 4 SALL4 accelerates cell cycle transition from G_0/G_1 to S phase of cervical cancer cells. FACS was used to analyze the cell cycle distribution of the SALL4-modified cervical cancer cells, and a quantitative analysis of the cell cycle distribution is shown. A, B, The representative diagram and quantitative analysis of HeLa-GFP and HeLa-SALL4 cells. C, D, The representative diagram and quantitative analysis of SiHa-GFP and SiHa-SALL4 cells. E, F, The representative diagram and quantitative analysis of C33A-shNC and C33A-shSALL4 cells. Values were obtained from three separate experiments. *P < .05 vs control, **P < .01 vs control

3.5 | SALL4 activates the Wnt/β-catenin signaling pathway in tumorigenicity of human cervical cancer cells

It had been reported that SALL4 induced myelodysplastic syndrome and acute myeloid leukemia by activating the Wnt/β-catenin signaling pathway.[30,37] In esophageal squamous cell carcinoma, inhibition of SALL4 reduces the tumorigenicity via the Wnt/β-catenin signaling pathway.[38] We investigated whether the function of SALL4 in cervical cancer cells was also associated with the Wnt/β-catenin signaling pathway. As shown in Figure 5A, the TOP/FOP-Flash reporter activities in SALL4-overexpressing cells were significantly increased compared with the control cells (HeLa, P < .01; SiHa, P < .05). Furthermore, the TOP/FOP-Flash reporter activity in SALL4-silenced C33A cells was significantly decreased compared with the control cells (P < .01). All these results indicated that SALL4 may enhance the activity of the Wnt/β-catenin signaling pathway in cervical cancer cells.

GSK3β and β-catenin are crucial molecules in the Wnt/β-catenin signaling pathway; c-*Myc* and Cyclin D1 are important downstream target genes of this pathway. Therefore, we measured the expression of GSK3β, β-catenin, c-*Myc*, and Cyclin D1 using western blotting in SALL4-modified cells and control cells. As shown in Figure 5B,C, the expression levels of β-catenin, c-*Myc* and Cyclin D1 in SALL4-overexpressing cells were significantly increased compared with that in the control cells (P < .05). Furthermore, the expression levels of β-catenin, c-*Myc* and Cyclin D1 in SALL4-silenced C33A cells were all significantly decreased compared with the control cells (Figure 5D, P < .05). However, the expression of GSK3β failed to show a significant difference in SALL4-modified cervical cancer cells and the control cells (Figure 5B, C, and D, P > .05). These results demonstrated that SALL4 accelerates protein expression of key molecules in the Wnt/β-catenin signaling pathway including β-catenin, cyclin D1, and c-*Myc*, but not GSK3β.

Next, an inhibitor of Wnt/β-catenin, XAV939, which accelerates the degradation of β-catenin by stabilizing axin,[39] was used to block the Wnt/β-catenin pathway in SALL4-overexpressing cells. As shown in Figure 5E,F, when the SALL4-overexpressing cells and their respective control cells were treated for 48 h with XAV939, the relative expression of β-catenin, c-*Myc* and Cyclin D1 was lower than that in the cells treated with DMSO (P < .05). Meanwhile, the cell growth and viability were significantly inhibited by XAV939 in SALL4-overexpressing cells and their respective control cells (Figure 5G,H, P < .05). These results suggested that β-catenin might be the key molecule by which SALL4 promotes the proliferation of cervical cancer cells by upregulating the activity of the Wnt/β-catenin signaling pathway.

To validate the correlation between the expression of SALL4 and Wnt/β-catenin pathway-related proteins in cervical cancer

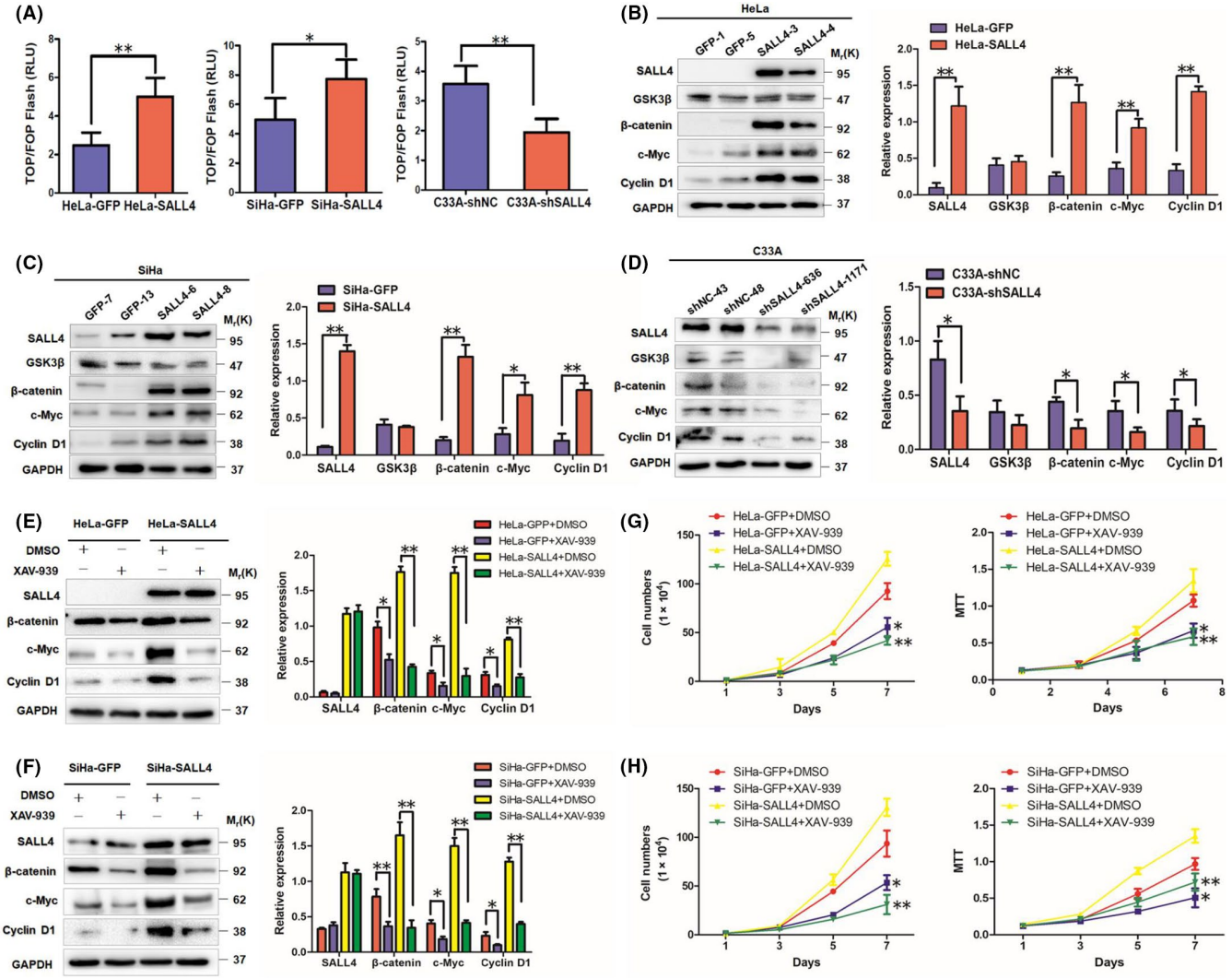

FIGURE 5 SALL4 upregulates the activity the Wnt/β-catenin pathway in cervical cancer cells. A, The TOP/FOP reporter assay was used to examine the activity of the Wnt/β-catenin pathway in *SALL4*-overexpressing HeLa and SiHa cells and *SALL4*-silenced C33A cells. B-D, Western blotting was used to detect the expression of GSK3β, β-catenin, c-*Myc* and Cyclin D1 in SALL4 mediated cervical cancer cells and the control cells, and the quantitative analysis is shown. An inhibitor of β-catenin, XAV-939, was used to treat the *SALL4*-overexpressing cells and the control cells for 48 h. E, F, The expression of β-catenin, c-*Myc*, and Cyclin D1 was measured by western blot in SALL4-overexpressing cells and the control cells. Meanwhile (G, H) the effects of XAV-939 on the growth and viability of SALL4-overexpressing cells and control cells were evaluated by the cell growth curve and 3-(4,5-dimethylthiazole-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Values are shown as the mean ± SD obtained from three separate experiments. **P* < .05 vs control, ***P* < .01 vs control

specimens, the expression levels of SALL4, β-catenin, c-*Myc*, and cyclin D1 were detected by IHC staining in 22 randomly selected cervical cancer samples. The results revealed that, as SALL4 expression increased, expression levels of β-catenin, c-*Myc* and cyclin D1 also increased in the human cervical cancer tissues (Figure S1A). Logistical regression analysis showed that SALL4 expression was significantly positively correlated with β-catenin, c-*Myc*, and cyclin D1 (Figure S1B, *P* < .05). These results supported the idea that SALL4 acts as a positive regulator of Wnt/β-catenin signaling pathway in clinical cervical cancer tissues. All these results demonstrated that the tumorigenicity of SALL4 in cervical cancer cells is mediated, at least in part, by upregulating the activity of the Wnt/β-catenin signaling pathway.

3.6 | SALL4 *trans*-activates the expression of *CTNNB1* by directly binding to the promoter of *CTNNB1* in cervical cancer cells

To elucidate the possible mechanism by which SALL4 upregulates the activity of the Wnt/β-catenin signaling pathway in cervical cancer cells, we examined mRNA expression levels of *GSK3β*, *CTNNB1*, c-*Myc*, and *CCND1* in SALL4-modified cervical cancer cells using real-time PCR. Relative RNA levels of *CTNNB1*, c-*Myc* and *CCND1* were significantly increased in *SALL4*-overexpressing cells (Figure 6A,B, *P* < .05). Furthermore, in *SALL4*-silenced C33A cells, the relative RNA levels of *CTNNB1*, c-*Myc* and *CCND1* were significantly decreased (Figure 6C, *P* < .05). However, mRNA expression of *GSK3β* failed

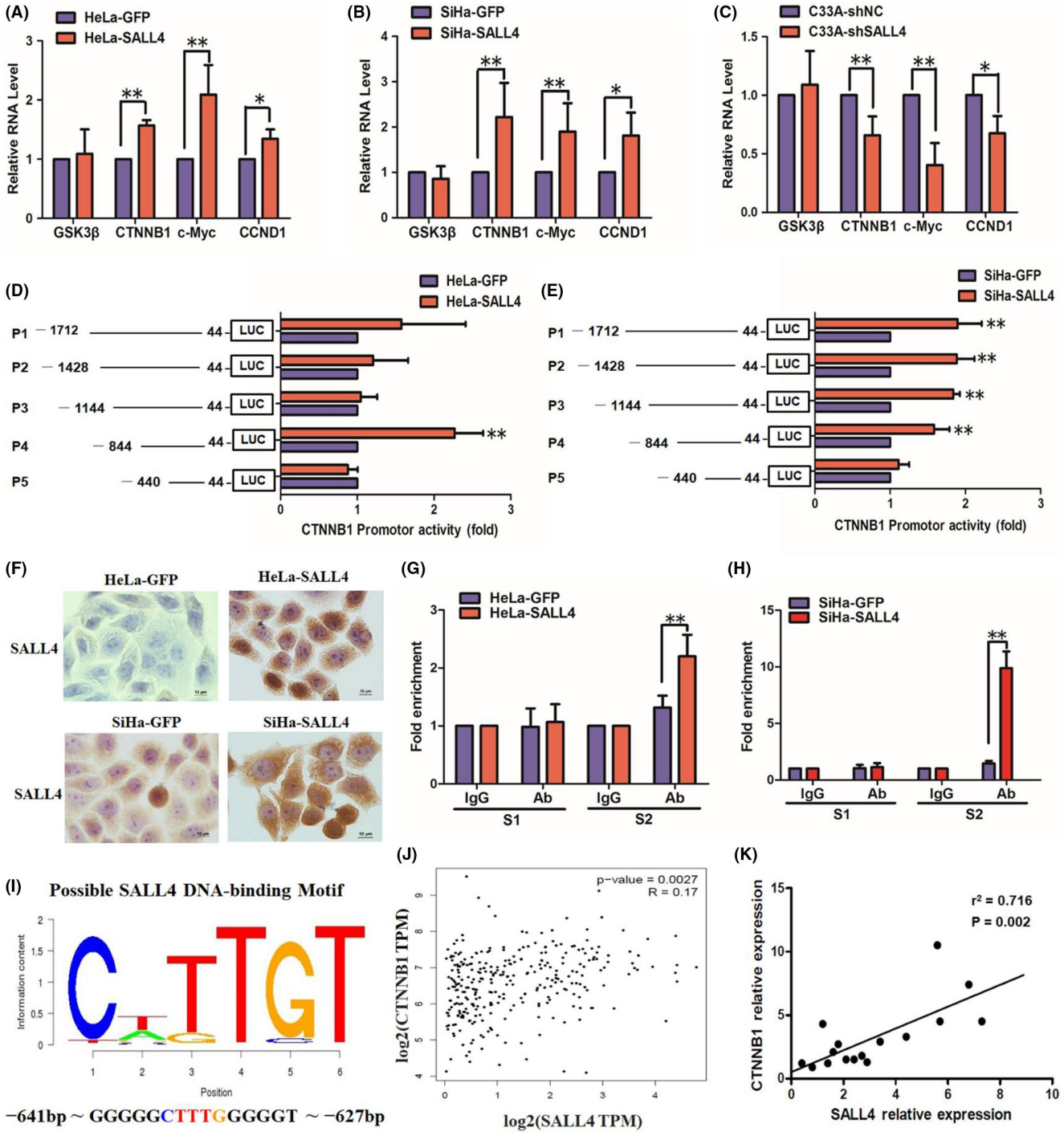

FIGURE 6 SALL4 *trans*-activates the expression of *CTNNB1* in cervical cancer cells. A-C, Quantitative real-time-PCR assay was performed to determine the relative mRNA levels of *GSK3β*, *CTNNB1*, *c-Myc*, and *CCND1* in SALL4-modified cervical cancer cells. *CTNNB1*: the gene name of β-catenin; *CCND1*: the gene name of Cyclin D1. D, E, The activity of the *CTNNB1* promoter was measured by dual-luciferase assay and shown as the fold change in the rate of SALL4-overexpressing cells vs the control cells. F, Immunocytochemistry was used to detect the expression of SALL4 in *SALL4*-overexpressing cells and control cells. G, H, Quantitative ChIP assay was performed in the SALL4-overexpressing cells. Immunoglobulin G (IgG) was used as a negative control. I, A possible transcription factor binding site for SALL4 was found in the Cistrome Data Brower. J, The Cancer Genome Atlas (TCGA) database was used to analyze the correlation between *SALL4* and *CTNNB1* in cervical cancer. K, Real-time PCR was used determined the relative expression of *SALL4* and *CTNNB1* in 15 cervical cancer specimens, also, the correlation of relative expression were analyzed. Values are shown as the mean ± SD obtained from three separate experiments. *$P < .05$ vs control, **$P < .01$ vs control

to show a significant fold change in SALL4-modified cervical cancer cells (Figure 6A-C, $P > .05$). All of these three genes, *CTNNB1*, c-*Myc*, and *CCND1* were upregulated by SALL4 at the transcriptional level in cervical cancer cells, but *CTNNB1* is the upstream gene compared with c-*Myc* and *CCND1* in Wnt/β-catenin signaling pathway. Therefore, these results may suggest that SALL4 upregulates the activity of the Wnt/β-catenin signaling pathway at least partly through directly *trans*-activating *CTNNB1* in cervical cancer cells.

To confirm this hypothesis, a dual-luciferase reporter assay was performed to determine whether SALL4 activates the promoter activities of *CTNNB1*. Five luciferase reporters were constructed to contain *CTNNB1* promoter fragments with different deletions between −1712 and +44 upstream of the *CTNNB1* gene transcriptional start site. The results showed that the luciferase activities of the P4 promoter (−844 to +44) in HeLa-SALL4 cells were more than two-fold higher than those in control cells (Figure 6D, $P < .01$). In the other promoter regions, including P1 (−1712 to +44), P2 (−1428 to +44), P3 (−1144 to +44), and P5 (−440 to +44), the luciferase activities of HeLa-SALL4 cells showed that differences were not significant compared with the control cells (Figure 6D, $P > .05$). Similarly, the luciferase activities of P4 (−844 to +44) promoter in SiHa-SALL4 cells were more than 1.6-fold higher than those in control cells (Figure 6E, $P < .01$). The luciferase activities of P5 (−440 to + 44) promoter failed to show a significant difference between SiHa-SALL4 cells and control cells (Figure 6E, $P > .05$). These results showed that the sequence between the nucleotides −844 and −440 in the *CTNNB1* promoter may contain the SALL4-binding sites. To clarify the nuclear localization of SALL4, immunocytochemistry was used to detect the expression of SALL4 in *SALL4*-overexpressing cells and control cells. The results showed that the expression of SALL4 mainly locates in the nucleus (Figure 6F). All these results identified that SALL4 directly *trans*-activates the expression of *CTNNB1* in cervical cancer cells.

To further identify the specific binding sites of the SALL4 protein in the *CTNNB1* promoter, a quantitative chromatin immunoprecipitation (ChIP) assay was performed. Two pairs of primers were designed to amplify the specific region of the *CTNNB1* promoter, primer 1 was designed to amplify S1 (sequence from −844 bp to −644 bp) and primer 2 was designed to amplify S2 (sequence from −644 bp to −440 bp). The results showed that the use of primer 1 led to an amplification that was not significantly different between *SALL4*-overexpressing cells and the control cells, but the use of primer 2 led to an amplification that was more than 1.7-fold higher in HeLa-SALL4 cells than that in HeLa-GFP cells (Figure 6G, $P < .01$) and eight-fold higher in SiHa-SALL4 cells than that in SiHa-GFP cells (Figure 6H, $P < .01$). All these results revealed that SALL4 directly binds to the sites between the nucleotides −644 and −440 in the *CTNNB1* promoter region. As showed in Figure 6I, an experimentally defined transcription factor binding motif of SALL4, the binding sequence 5′-CTTTG-3′, was found in the Cistrome Data Brower (http://cistrome.org/db/#/). 5′-CTTTG-3′ locates between nucleotides −641 and −627 in the S2 region of the P4 promoter, indicating that SALL4 might recognize and bind to the 5′-CTTTG-3′ site and/or other unknown sites between nucleotides −644 and −440 in the *CTNNB1* promoter region in cervical cancer cells.

In addition, the correlation between SALL4 and *CTNNB1* was analyzed using the TCGA Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (TCGA-CESC) data collection, the

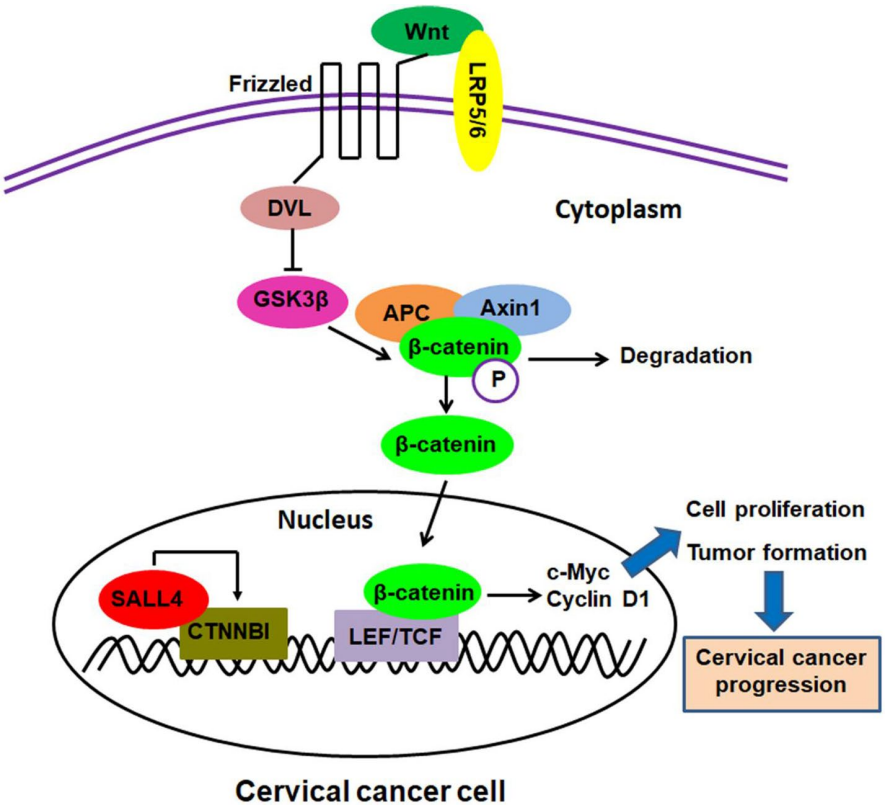

FIGURE 7 Schematic diagram of the functional roles of SALL4 in cervical cancer cells. SALL4 protein could recognize and bind to the *CTNNB1* promoter region as a transcription activator and accelerate the expression of β-catenin, resulting in the upregulation of downstream target genes, such as c-*myc* and *cyclin D1*. Then, the activity of Wnt/β-catenin pathway was enhanced, promoting cell proliferation and tumor formation in cervical cancer cells

results showed that SALL4 expression was positively correlated with *CTNNB1* (Figure 6J, $P < .01$). In clinical specimens, the expression of SALL4 also showed positive correlation with *CTNNB1* at the transcriptional level (Figure 6K; $P < .01$). Therefore, these results further supported the idea that SALL4 *trans*-activates *CTNNB1* in cervical cancer.

4 | DISCUSSION

It is known that SALL4 plays a vital role in stem cell self-renewal and pluripotency through different mechanisms, depletion of SALL4 results in early embryonic development defects.[40-42] Enhanced expression of SALL4 was first found to be associated with carcinogenesis in acute myeloid leukemia.[37] Subsequently, overexpression of SALL4 has been demonstrated to promote tumorigenesis, tumor growth, and tumor progression in various cancers. *SALL4* is an important oncofetal gene in a subset of hepatocellular carcinomas with an aggressive phenotype,[43,44] and blocking the action of this gene with a short peptide could have therapeutic potential.[27] In colorectal cancer (CRC), *SALL4* is a new oncogene and a critical biomarker for efficiently screening patients to detect early stages of CRC.[34] Zhang et al[45] reported that SALL4 has oncogenic roles in gastric cancer through the modulation of epithelial-mesenchymal transition (EMT). SALL4 also promotes gastric cancer progression by directly activating CD44 expression.[32] Elevated expression of SALL4 was found in endometrial cancer samples and is associated with poor survival in patients.[36] Although there have been many studies on SALL4 involvement in solid tumors, to our knowledge no available reports have detailed the expression and function of SALL4 in cervical cancer.

In the present study, our results revealed an important role for SALL4 in the development and progression of cervical cancer. We showed that SALL4 is upregulated in cervical cancer relative to normal cervix tissues, and that SALL4 promotes the cell proliferation of cervical cancer cells in vitro and in vivo. We demonstrated that upregulation of SALL4 potently activates Wnt/β-catenin pathway to promote cervical cancer development and progression. Mechanistically, SALL4 activates the Wnt/β-catenin signaling pathway by directly binding to the *CTNNB1* promoter and *trans*-activating *CTNNB1*, therefore identifying *SALL4* as an oncogenic driver in cervical carcinogenesis.

The Wnt/β-catenin signaling pathway was activated by SALL4 in leukemogenesis and downstream target genes, such as c-*Myc* and *Cyclin D1*, were upregulated in SALL4B transgenic mice.[37] In addition, knockdown of *SALL4* in TE7 cells markedly decreased the expression of Wnt3a and β-catenin at both the mRNA and protein level, suggesting that SALL4 could activate the Wnt/β-catenin signaling pathway in esophageal squamous cell carcinoma.[38] Here, we confirmed that the tumorigenicity of SALL4 in cervical cancer cells is mediated, at least in part, by upregulating the activity of the Wnt/β-catenin signaling pathway, and revealed that SALL4 upregulates the activity of the Wnt/β-catenin signaling pathway by directly binding to the sites between the nucleotides −644 and −440 in the *CTNNB1* promoter and *trans*-activating *CTNNB1*. Although, a possible SALL4 DNA-binding motif 5′-CTTTG-3′ found in the Cistrome Data Brower locates between the nucleotides −641 and −627 in *CTNNB1* promoter, this result need to be further validated in cervical cancer cells. To our knowledge, the SALL4 protein can directly bind to the β-catenin protein in acute myeloid leukemia,[37] but there are no literature reports on the relationship between the SALL4 protein to the *CTNNB1* promoters. Of course, further experiments will be required to confirm the specific motifs of *CTNNB1* that were occupied by the SALL4 protein. In endometrial cancer cells, SALL4 specifically binds to the c-*Myc* promoter region and regulates the expression of c-*Myc*, indicating that c-*Myc* is one of the SALL4 downstream targets in endometrial cancer.[36] In this study, protein expression and mRNA expression of c-*Myc* were upregulated by SALL4 in *SALL4*-overexpressing cervical cancer cells, further experiments will be required to determine whether SALL4 directly *trans*-activates c-*Myc* in cervical cancer cells.

In summary, this is the first study to demonstrate that SALL4 enhances cell proliferation and tumor formation in cervical cancer cells. Based on previous published literature and our results, we propose that SALL4 promotes cell proliferation and tumor formation of cervical cancer cells by upregulating the activity of the Wnt/β-catenin signaling pathway by directly binding to the *CTNNB1* promoter and *trans*-activating *CTNNB1* (Figure 7).

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DISCLOSURE

All authors declared that they had no conflicts of interest.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
3. Sigurdsson K. HPV vaccination and cervical cancer screening. *Laeknabladid*. 2007;93:819, 821.
4. Bosch FX, Castellsague X, de Sanjose S. HPV and cervical cancer: screening or vaccination? *Br J Cancer*. 2008;98:15-21.
5. Grigore M, Matei M, Pristavu A, Iordache F, Teleman S. HPV and cervical cancer awareness among HPV vaccinated women and non-vaccinated women. *Int J Gynecol Cancer*. 2016;26:74-.
6. Mitra S, Misra C, Singh RK, Panda CK, Roychoudhury S. Association of specific genotype and haplotype of p53 gene with cervical cancer in India. *J Clin Pathol*. 2005;58:26-31.

7. Zhang WN, Li W, Wang XL, et al. CLDN1 expression in cervical cancer cells is related to tumor invasion and metastasis. *Oncotarget*. 2016;7:87449-87461.
8. Mora N, Rosales R, Rosales C. R-Ras promotes metastasis of cervical cancer epithelial cells. *Cancer Immunol Immun*. 2007;56:535-544.
9. Liu X, Yang WT, Zheng PS. Msi1 promotes tumor growth and cell proliferation by targeting cell cycle checkpoint proteins p21, p27 and p53 in cervical carcinomas. *Oncotarget*. 2014;5:10870-10885.
10. Yang WT, Zheng PS. Promoter hypermethylation of KLF4 inactivates its tumor suppressor function in cervical carcinogenesis. *PLoS ONE*. 2014;9:e88827.
11. Wu XL, Zheng PS. Undifferentiated embryonic cell transcription factor-1 (UTF1) inhibits the growth of cervical cancer cells by transactivating p27Kip1. *Carcinogenesis*. 2013;34:1660-1668.
12. Wang HY, Lian P, Zheng PS. SOX9, a potential tumor suppressor in cervical cancer, transactivates p21WAF1/CIP1 and suppresses cervical tumor growth. *Oncotarget*. 2015;6:20711-20722.
13. Cui N, Yang WT, Zheng PS. Slug inhibits the proliferation and tumor formation of human cervical cancer cells by up-regulating the p21/p27 proteins and down-regulating the activity of the Wnt/beta-catenin signaling pathway via the trans-suppression Akt1/p-Akt1 expression. *Oncotarget*. 2016;7:26152-26167.
14. Gu TT, Liu SY, Zheng PS. Cytoplasmic NANOG-positive stromal cells promote human cervical cancer progression. *Am J Pathol*. 2012;181:652-661.
15. Wang YD, Cai N, Wu XL, Cao HZ, Xie LL, Zheng PS. OCT4 promotes tumorigenesis and inhibits apoptosis of cervical cancer cells by miR-125b/BAK1 pathway. *Cell Death Dis*. 2013;4:e760.
16. Chen Q, Cao HZ, Zheng PS. LGR5 promotes the proliferation and tumor formation of cervical cancer cells through the Wnt/beta-catenin signaling pathway. *Oncotarget*. 2014;5:9092-9105.
17. Chen Q, Zheng PS, Yang WT. EZH2-mediated repression of GSK-3 beta and TP53 promotes Wnt/beta-catenin signaling-dependent cell expansion in cervical carcinoma. *Oncotarget*. 2016;7:36115-36129.
18. Al-Baradie R, Yamada K, St Hilaire C, et al. Duane radial ray syndrome (Okihiro syndrome) maps to 20q13 and results from mutations in SALL4, a new member of the SAL family. *Am J Hum Genet*. 2002;71:1195-1199.
19. Zhang X, Yuan X, Zhu W, Qian H, Xu WR. SALL4: an emerging cancer biomarker and target. *Cancer Lett*. 2015;357:55-62.
20. Uez N, Lickert H, Kohlhase J, et al. Sall4 isoforms act during proximal-distal and anterior-posterior axis formation in the mouse embryo. *Genesis*. 2008;46:463-477.
21. Zhang JQ, Tam WL, Tong GQ, et al. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. *Nat Cell Biol*. 2006;8:1114-1123.
22. Yang JC, Chai L, Fowles TC, et al. Genome-wide analysis reveals Sall4 to be a major regulator of pluripotency in murine-embryonic stem cells. *Proc Natl Acad Sci U S A*. 2008;105:19756-19761.
23. Oikawa T, Kamiya A, Kakinuma S, et al. Sall4 regulates cell fate decision in fetal hepatoblasts. *Hepatology*. 2008;48:617a-618a.
24. Abboud N, Moore-Morris T, Hiriart E, et al. A cohesin-OCT4 complex mediates Sox enhancers to prime an early embryonic lineage. *Nat Commun*. 2015;6:6749.
25. Rao S, Zhen S, Roumiantsev S, McDonald LT, Yuan GC, Orkin SH. Differential roles of Sall4 isoforms in embryonic stem cell pluripotency. *Mol Cell Biol*. 2010;30:5364-5380.
26. Gilbert JA. SALL4, a new target in aggressive liver cancer. *Lancet Oncology*. 2013;14:e300.
27. Suzuki E, Chiba T, Yokosuka O. Oncofetal gene SALL4 in aggressive hepatocellular carcinoma. *New Engl J Med*. 2013;369:1170-1171.
28. Yong KJ, Li AL, Ou WB, et al. Targeting SALL4 by entinostat in lung cancer. *Oncotarget*. 2016;7:75425-75440.
29. Ballerini P, Boelle PY, Deswarte C, et al. Prognostic significance of SALL4 expression levels in paediatric acute myeloid leukaemia (AML). *Blood*. 2008;112:782.
30. Chai L, Cui W, Chang JC, Di CH, Amin H, Ma YP. SALL4, a novel oncogene induces myelodysplastic syndrome and acute myeloid leukemia via Wnt/beta-catenin pathway. *Blood*. 2005;106:397a-a.
31. Liu L, Leung LH, Cooney AJ, et al. Knockdown of SALL4 protein enhances all-trans retinoic acid-induced cellular differentiation in acute myeloid leukemia cells. *J Biol Chem*. 2015;290:10599-10609.
32. Yuan X, Zhang X, Zhang W, et al. SALL4 promotes gastric cancer progression through activating CD44 expression. *Oncogenesis*. 2016;5:e268.
33. Lai YM, Huang J, Guo ZH, et al. The transcription factor SALL4 is a marker of poor prognosis in prostate cancer promoting invasion and metastasis by regulating tumor angiogenesis. *Int J Urol*. 2016;23:130-131.
34. Khales SA, Abbaszadegan MR, Abdollahi A, Raeisossadati R, Tousi MF, Forghanifard MM. SALL4 as a new biomarker for early colorectal cancers. *J Cancer Res Clin*. 2015;141:229-235.
35. Kobayashi D, Kuribayshi K, Tanaka M, Watanabe N. SALL4 is essential for cancer cell proliferation and is overexpressed at early clinical stages in breast cancer. *Int J Oncol*. 2011;38:933-939.
36. Li A, Jiao Y, Yong KJ, et al. SALL4 is a new target in endometrial cancer. *Oncogene*. 2015;34:63-72.
37. Ma Y, Cui W, Yang J, et al. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice. *Blood*. 2006;108:2726-2735.
38. He J, Zhou MX, Chen XF, et al. Inhibition of SALL4 reduces tumorigenicity involving epithelial-mesenchymal transition via Wnt/beta-catenin pathway in esophageal squamous cell carcinoma. *J Exp Clin Canc Res*. 2016;35:98.
39. Huang SMA, Mishina YM, Liu SM, et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature*. 2009;461:614-620.
40. Jeong HW, Cui W, Guo Y, et al. Sall4, a stem cell factor, promotes chemoresistance by regulates the side population cell phenotype. *Blood*. 2009;114:1203.
41. Oikawa T, Kamiya A, Zeniya M, et al. Sal-like protein 4 (SALL4), a stem cell biomarker in liver cancers. *Hepatology*. 2013;57:1469-1483.
42. Yang FK, Yao YX, Jiang YP, Lu L, Ma YP, Dai W. Sumoylation is important for stability, subcellular localization, and transcriptional activity of SALL4, an essential stem cell transcription factor (vol 287, pg 38600, 2012). *J Biol Chem*. 2016;291:428.
43. Yin F, Han X, Yao SK, Wang XL, Yang HC. Importance of SALL4 in the development and prognosis of hepatocellular carcinoma. *World J Gastroentero*. 2016;22:2837-2843.
44. Leake I. Cancer: importance of oncofetal gene, SALL4, in a subset of hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol*. 2013;10:441.
45. Zhang L, Xu Z, Xu X, et al. SALL4, a novel marker for human gastric carcinogenesis and metastasis. *Oncogene*. 2014;33:5491-5500.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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