



Odd-skipped related 1 inhibits lung cancer proliferation and invasion by reducing Wnt signaling through the suppression of SOX9 and β -catenin

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The odd-skipped related 1 (*OSR1*) gene encodes a zinc-finger transcription factor. The expression and significance of *OSR1* in human tumors remains unclear. We found that *OSR1* was downregulated in lung cancers, and its expression was correlated with poor differentiation. Overexpression of *OSR1* by *OSR1* gene transfection into H1299 cells (H1299-*OSR1*) inhibited the proliferation and invasion of lung cancer cells. Knockdown of *OSR1* with small interfering (si)RNA against *OSR1* in A549 cells (A549-si*OSR1*) enhanced the proliferation and invasion of lung cancer cells. Western blot analysis showed that the expression level of GSK3 β increased, while that of p-GSK3 β , nuclear β -catenin, cyclin D1, c-Myc and matrix metalloproteinase 7 significantly decreased in the H1299-*OSR1* cells, and this pattern was reversed in the A549-si*OSR1* cells compared to that in the control cells. Furthermore, upregulation of sex-determining region Y-box 9 (*SOX9*) by *SOX9* gene transfection increased the expression of β -catenin, which was inhibited by *OSR1*. The mRNA and protein expression levels of *SOX9* and β -catenin were reduced in H1299-*OSR1* cells and increased in A549-si*OSR1* cells. In conclusion, the expression of *OSR1* was more reduced in lung cancer tissues than in normal lung tissues, and was correlated with poor differentiation. *OSR1* downregulated the activity of the Wnt signaling pathway by suppressing the expression of *SOX9* and β -catenin.

KEYWORDS

β -catenin, lung cancer, *OSR1*, *SOX9*, Wnt signaling pathway

1 | INTRODUCTION

Lung cancer is the second most common malignant tumor and the leading cause of cancer deaths worldwide.^{1,2} Many complex factors play important roles in the growth and invasion of tumor cells.^{3,4} The overall 5-year survival of patients with lung cancer remains very low. Effective biomarkers for the early detection,

diagnosis, prognosis and monitoring of lung cancer are urgently needed.^{4,5}

The odd-skipped related 1 (*OSR1*) gene, located on human chromosome 2p24.1, encodes a zinc-finger transcription factor.⁶ *OSR1* plays important roles in the development of the intermediate mesoderm. During this process, multiple factors interact with *OSR1*. Bone morphogenetic protein⁷ and 1,25-dihydroxyvitamin D3 can activate

OSR1,⁸ whereas runt related transcription factor 2 and IKAROS family zinc finger 1 can repress it.⁹ OSR1 is also essential for embryonic heart and urogenital formation, and the development of the tongue and kidneys.^{10–14} However, the biological relationship between OSR1 and cancer biology has not yet been well-characterized, except in gastric cancer and renal cell carcinoma.^{15,16} Otani et al¹⁵ demonstrated that OSR1 mediated tumor suppressive effects through the activation of p53 pathway and the repression of the Wnt/ β -catenin signaling pathway in gastric cancer. The Wnt signaling pathway is essential in both normal embryonic development and a variety of cancers.^{17–19} This signaling pathway is exquisitely regulated by a large and complex array of proteins, among which β -catenin is the key protein (Figure 1).²⁰ When the Wnt signal is weak, β -catenin is incorporated in a destruction complex that contains glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), axin 1 and casein kinase 1, which results in the phosphorylation of β -catenin and its degradation by a ubiquitin-mediated proteasomal pathway.²¹ However, in human cancers, the dissociation of β -catenin from this destruction complex results in the accumulation of β -catenin in the cytoplasm and nucleus, which activates the target genes of the Wnt pathway, such as cyclin D1, matrix metalloproteinase 7 (MMP7) and c-Myc.^{22,23} The detailed mechanism of OSR1-mediated regulation of the Wnt signaling pathway is unclear. The expression and function of OSR1 in human lung cancer and its underlying molecular mechanisms also need to be explored.

In this study, we examined the expression of OSR1 in lung cancer tissues and corresponding normal lung tissues, and analyzed its correlations with clinicopathological factors. We also investigated the underlying mechanisms of OSR1-mediated regulation of the Wnt signaling pathway, and its effects on the proliferative and invasive abilities of lung cancer cells.

2 | MATERIALS AND METHODS

2.1 | Patient data and tissue samples

Tissue specimens from 250 lung cancer patients who underwent complete surgical resection at the First Affiliated Hospital of China Medical University between 2012 and 2015 were selected from the archival files of the Department of Pathology. Some lung cancer samples (126 cases) were accompanied by corresponding normal lung tissue samples. The ages of patients ranged from 20 to 81 years old, with a mean age of 60 years. There were 172 male and 78 female patients. The histological diagnoses and differentiation grades of the tissue samples were classed according to the World Health Organization classification system as squamous cell carcinoma ($n = 119$) and adenocarcinoma ($n = 131$).²⁴ The cancers were classified as well-differentiated (40 cases), moderately differentiated (143 cases) and poorly differentiated (67 cases). According to the seventh edition of the International Union against Cancer TNM Staging System for Lung Cancer,²⁵ patients were categorized as stage I ($n = 41$), II ($n = 147$), III ($n = 51$) or IV ($n = 11$). Lymph node metastases were found in 126 cases. We also collected 30 pairs of fresh

specimens, including both tumor tissues and corresponding normal tissues, which were stored at -80°C immediately after resection. The study was conducted under the regulations of the Institutional Review Board of China Medical University.

2.2 | Immunohistochemistry

All resected specimens were fixed in 10% neutral formalin, embedded in paraffin, and sectioned serially into 4- μm slices. Immunostaining was performed using a streptavidin-peroxidase method. All sections were deparaffinized in xylene, rehydrated in a graded alcohol series, and boiled in 0.01 mol/L citrate buffer for 2.5 minutes in an autoclave. Endogenous peroxidase activity was blocked using hydrogen peroxide, followed by incubation with normal goat serum to reduce non-specific binding. The sections were incubated with anti-OSR1 rabbit polyclonal antibody (1:100; Abcam, Cambridge, MA, USA) at 4°C overnight. Then, the sections were incubated with biotinylated goat anti-rabbit serum IgG secondary antibody and HRP-conjugated streptavidin-biotin (MaiXin, Fuzhou, China). Visualization was performed using DAB chromogen (MaiXin, Fuzhou, China).

Two investigators blinded to the clinical data scored the slides. Five views were examined per slide, and 100 cells were observed per view at $400\times$ magnification. The positive rate for each case was obtained by calculating the percentage of positively stained cells in each slide. The percentage score for each case was categorized as

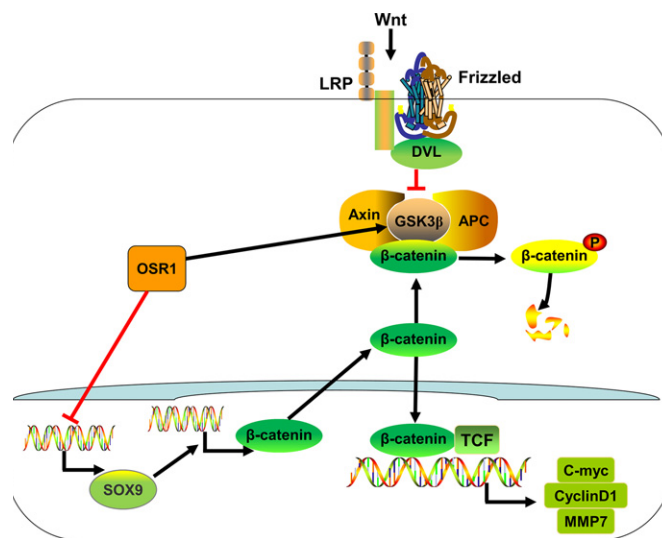


FIGURE 1 Schematic diagram. When the Wnt signal is weak, β -catenin is incorporated in a destruction complex that contains glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC) and axin 1, which results in the phosphorylation of β -catenin and its degradation. OSR1 can enhance the expression level of GSK3 β and downregulates the activity of the Wnt signaling pathway. SOX9 enhances the transcription and expression level of β -catenin. The high level of β -catenin increases the formation of the β -catenin-TCF/LEF complex and the transcriptional activities of Wnt target genes. OSR1 suppresses the transcription of SOX9, indirectly reverses the activating effect of SOX9 on β -catenin and the Wnt signaling pathway

follows: (i) 1%-25%, (ii) 26%-50%, (iii) 51%-75% and (iv) 76%-100%. The intensity of immunostaining was scored as 0, 1, 2 or 3, if negative, weak, moderate, or marked, respectively. The scores from each tumor sample were multiplied to give a final score ranging from 0 to 12, and the tumors were categorized based on their scores, with ≤ 6 and ≥ 8 indicating low and high expression, respectively.²⁶

2.3 | Cell culture and transfection

The normal human bronchial epithelial cell line HBE and the human lung cancer cell lines H292, LK2, H460, H661, A549 and H1299 were purchased from American Type Culture Collection (Manassas, VA, USA). The HBE and LK2 cells were cultured in Minimal Essential Medium (Gibco, Invitrogen, NY, USA), whereas the other cells were cultured in RPMI-1640 (Gibco, Invitrogen, NY, USA), both supplemented with 10% FBS (Gibco, Invitrogen, NY, USA) at 37°C in 5% CO₂. The cells were grown in sterile culture dishes and passaged every 1 or 2 days using 0.25% trypsin (Gibco, Invitrogen, NY, USA).

For transfection, cells were seeded in a 6-well plate 24 hours before the experiment. The pCMV6-OSR1 plasmid, pCMV6-SOX9 plasmid, pCMV6-CTNNB1 plasmid and the control empty vector pCMV6 were purchased from Origene (Rockville, MD, USA). Small interfering (si)RNA against OSR1 (OSR1-siRNA), SOX9-siRNA, CTNNB1-siRNA and control siRNA were synthesized by RIBOBIO (Guangzhou, China). The plasmids or siRNA were transfected into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Semi-stably transfected cell lines were screened for 4 weeks with G418.

2.4 | Real-time PCR

Real-time PCR was performed using the SYBR PrimeScript RT-PCR Kit II (Takara, Dalian, China) in a total volume of 20 μ L on a 7500HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) as follows: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. The primer sequences are listed in Table 1.

2.5 | Western blot

Total protein was extracted from cells in cell lysis buffer (Pierce, Rockford, IL, USA) and quantified using the Bradford method.²⁷

TABLE 1 List of primer sequences

Primer name	Sequence (5'-3')
OSR1-F	CGGTGCCTATCCACCCTTC
OSR1-R	GCAACGCGCTGAAACCATA
SOX9-F	AGCGAACGCACATCAAGAC
SOX9-R	CTGTAGGCGATCTGTTGGGG
β -catenin-F	AAAGCGGCTGTAGTCACTGG
β -catenin-R	CGAGTCATTGCATACTGTCCAT
GAPDH-F	ACAACCTTTGGTATCGTGAAGG
GAPDH-R	GCCATCACGCCACAGTTTC

Nuclear/cytoplasmic proteins were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA), according to the manufacturer's protocol. A total of 60 μ g of protein was separated using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with antibodies against OSR1 (1:150, Santa Cruz Biotechnology), SOX9 (1:150, Santa Cruz Biotechnology), β -catenin (1:100, Santa Cruz Biotechnology), active β -catenin (1:500, Cell Signaling Technology), GSK3 β (1:500, Cell Signaling Technology), p-GSK3 β (1:500, Cell Signaling Technology), cyclin D1 (1:100, Santa Cruz Biotechnology), c-Myc (1:200, BD Biosciences), MMP7 (1:100, Santa Cruz Biotechnology), LDL receptor related protein 6 (LRP6; 1:100, Santa Cruz Biotechnology), axin (1:100, Santa Cruz Biotechnology), dishevelled segment polarity protein 1 (DVL1; 1:100, Santa Cruz Biotechnology), lymphoid enhancer binding factor 1 (LEF1; 1:100, Santa Cruz Biotechnology), snail (1:500, Cell Signaling Technology), E-cadherin (1:500, Cell Signaling Technology), N-cadherin (1:500, Cell Signaling Technology) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2000, Santa Cruz Biotechnology). After washing, the membranes were incubated with peroxidase-conjugated anti-mouse/rabbit IgG (1:2000, Proteintech) at 37°C for 2 hours. Protein bands were visualized with ECL (Pierce) and detected using a bioimaging system (DNR Bio-Imaging System, Jerusalem, Israel). The relative protein levels were calculated using GAPDH protein as a loading control. The bands were quantified with Image J software.

2.6 | Cell proliferation assay

Twenty-four hours after transfection, cells were plated in 96-well plates (3000 cells/well) in medium containing 10% FBS. Cell proliferation was detected using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Tokyo, Japan). CCK-8 reagent was added to each well at 1:10 (v/v) per 100 μ L and incubated for 2 hours at 37°C. The results were quantitated spectrophotometrically using a test wavelength of 450 nm.

2.7 | Colony formation assay

Cells were plated in 6-cm cell culture dishes (800 cells/dish) 24 hours after transfection, then incubated for 14 days. We changed the medium every 4-5 days. Cells were fixed with 4% paraformaldehyde for 20 minutes and stained with hematoxylin for 10 minutes. We counted the colonies with more than 50 cells.

2.8 | Matrigel invasion assay

We used Matrigel (BD Biosciences, San Jose, CA, USA) and Transwell chambers with a pore size of 8 μ m (Costar, Cambridge, MA, USA), according to the manufacturers' instructions, to assess the invasive ability of transfected cells. Briefly, 100 μ L Matrigel (1:7 dilution) was added to each insert. Then, we placed the chambers

in a 37°C incubator for at least 2 hours; after gelation of the Matrigel, we added 8×10^4 cells in 100 μ L medium supplemented with 2% FBS to the upper chamber. Medium supplemented with 20% FBS was added to the lower chamber as the chemoattractant. After 20 hours of incubation, the filters were fixed with 4% paraformaldehyde for 20 minutes and stained with hematoxylin for 10 minutes. The non-invading cells on the upper surface were removed by scrubbing with a cotton swab. The numbers of invasive cells were counted in 10 randomly selected high-power fields under a microscope. The experiments were performed in triplicate.

2.9 | Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (Chicago, IL, USA) and GraphPad Prism 6.0 software (La Jolla, CA, USA). The Chi-square-test was used to examine possible correlations between OSR1 expression and clinicopathological factors. Other results were analyzed using Student's *t*-test or the Pearson correlation coefficient. $P < .05$ was considered to indicate a statistically significant result.

3 | RESULTS

3.1 | OSR1 expression is downregulated and negatively correlated with β -catenin expression in lung cancer tissues

The expression of OSR1 was examined in 250 lung cancer tissues and 126 corresponding normal lung tissue specimens using immunohistochemistry. OSR1 was mainly expressed in the cytoplasm, accompanied by nuclear expression in some cells. In corresponding normal lung tissues, 95 cases (75.4%) showed high expression of OSR1 in normal bronchial epithelial cells or alveolar cells (Figure 2A,B), and 31 cases (24.6%) exhibited low expression. In lung cancer tissues, 111 cases (44.4%) had low expression of OSR1 (Figure 2C,E), whereas 139 cases (55.6%) had high expression (Figure 2D,F). The expression rate of OSR1 was lower in lung cancers than in normal lung tissues ($P < .001$) (Table 2). As listed in Table 2, the low expression of OSR1 correlated significantly with poor differentiation of lung cancers ($P = .037$). The expression of OSR1 did not correlate with patient age ($P = .437$) or gender ($P = .919$), or the histological type ($P = .767$), TNM stage ($P = .130$) or lymphatic metastasis ($P = .123$) of lung cancers.

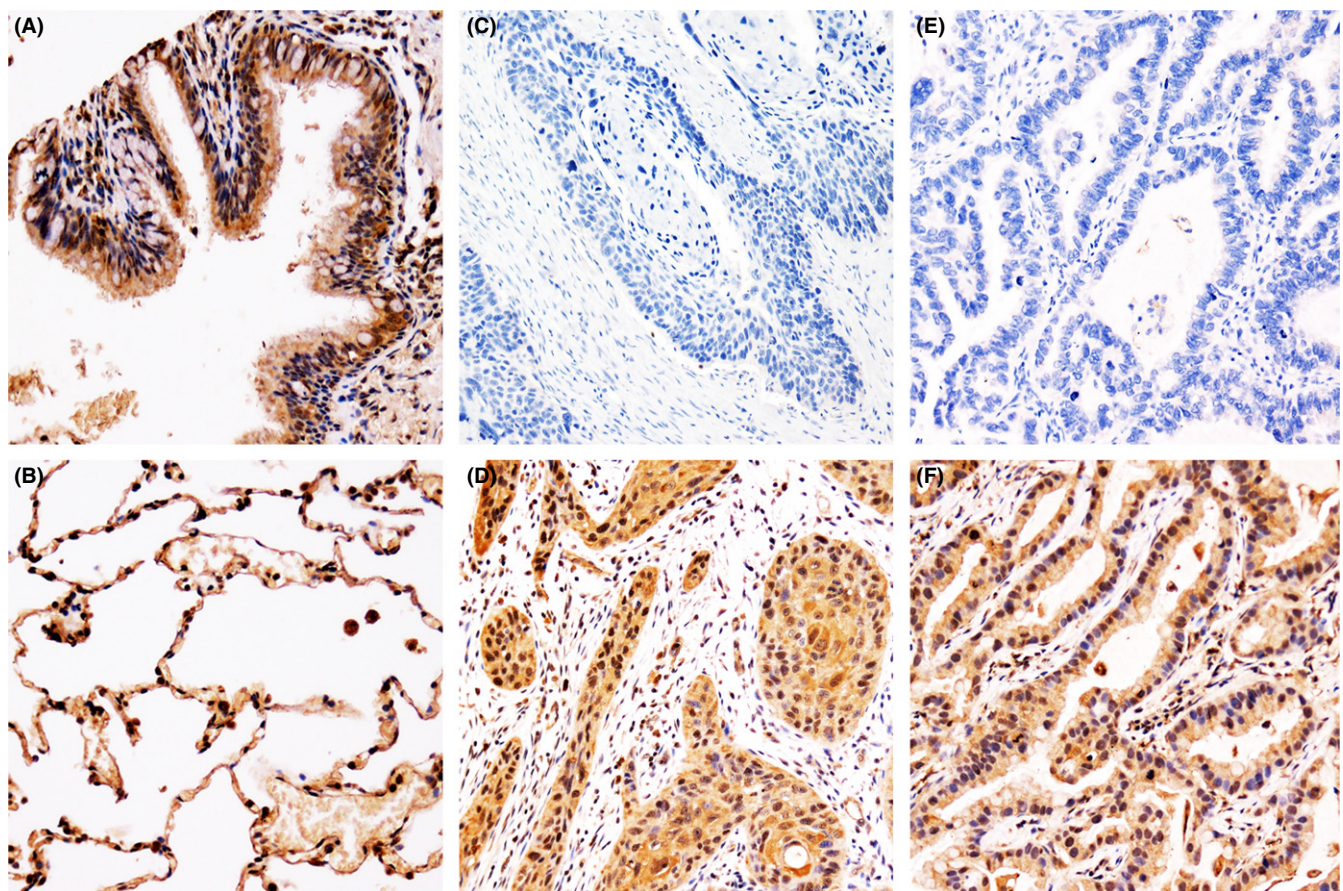


FIGURE 2 Immunohistochemistry staining of OSR1 in lung cancers. A, Positive expression of OSR1 in normal bronchial epithelial cells (200 \times). B, Positive expression of OSR1 in normal alveolar cells (200 \times). C, Absent expression of OSR1 in a lung squamous cell carcinoma case (200 \times). D, Positive expression of OSR1 in a lung squamous cell carcinoma case (200 \times). E, Absent expression of OSR1 in a lung adenocarcinoma case (200 \times). F, Positive expression of OSR1 in a lung adenocarcinoma case (200 \times)

TABLE 2 Correlations between OSR1 expression and clinicopathological factors in lung cancers

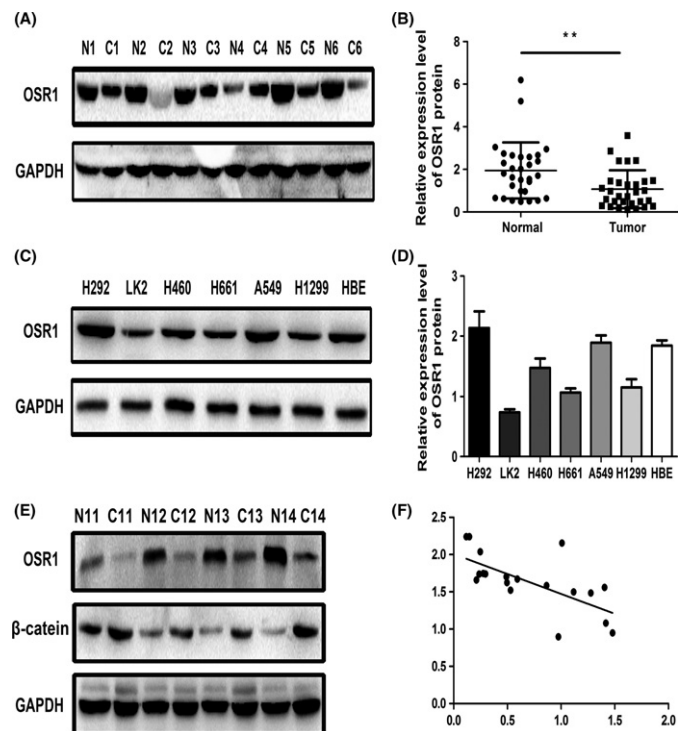
	n	OSR1 low expression	OSR1 high expression	P-value
Tissues				
Normal	126	31 (24.6%)	95 (75.4%)	<.001
Lung cancer	250	111 (44.4%)	139 (55.6%)	
Age				
<60	124	52 (41.9%)	72 (58.1%)	.437
≥60	126	59 (46.8%)	67 (53.2%)	
Gender				
Male	172	76 (44.2%)	96 (55.8%)	.919
Female	78	35 (44.9%)	43 (55.1%)	
Histological type				
Squamous cell carcinoma	119	54 (45.4%)	65 (54.6%)	.767
Adenocarcinoma	131	57 (43.5%)	74 (56.5%)	
Differentiation				
Well-moderate	183	74 (40.4%)	109 (59.6%)	.037
Poor	67	37 (55.2%)	30 (44.8%)	
TNM stages				
I	41	13 (31.7%)	28 (68.3%)	.130
II	147	72 (49.0%)	75 (51.0%)	
III-IV	62	26 (42.0%)	36 (58.0%)	
Lymphatic metastasis				
Yes	126	62 (49.2%)	64 (50.8%)	.123
No	124	49 (39.5%)	75 (60.5%)	

FIGURE 3 The expression of OSR1 in lung cancers and normal lung tissues. A, Western blot analysis of OSR1 in lung cancer tissues and corresponding normal lung tissues. GAPDH served as an internal control. N: corresponding normal lung tissue. C, lung cancer tissue. B, The relative expression level of OSR1 in lung cancer tissues and corresponding normal lung tissues ($n = 30$). $**P < .01$. C, Western blot analysis of OSR1 in cell lines. D, The relative expression levels of OSR1 in cell lines. GAPDH served as an internal control. E, Western blot analysis for OSR1 and β -catenin expression in lung cancer tissues and corresponding normal lung tissues. GAPDH served as an internal control. F, The relative expression levels of OSR1 and β -catenin in lung cancer tissues ($n = 20$). $**P < .01$

Western blot analysis confirmed that the expression levels of OSR1 were significantly higher in the normal lung tissues than in lung cancer tissues (1.95 ± 0.24 vs 1.07 ± 0.16 ; $n = 30$; $P < .01$) (Figure 3A,B). We examined the expression of OSR1 in 6 lung cancer cell lines and a normal bronchial epithelial cell line (HBE). We found that the expression of OSR1 was lower in 4 of 6 lung cancer cell lines, especially in LK2, H661 and H1299, than in HBE cells (Figure 3C,D). The expression levels of OSR1 and β -catenin were also examined in 20 pairs of lung cancer tissues and corresponding normal lung tissues. We found that OSR1 expression was negatively correlated with that of β -catenin in lung cancer tissues ($n = 20$; correlation coefficient = -0.675 ; $P < .01$) (Figure 3E,F).

3.2 | OSR1 inhibited the proliferative and invasive abilities of lung cancer cells

To examine the function of OSR1 in lung cancer cells, we elevated the expression of OSR1 by OSR1 gene transfection in H1299 cells (H1299-OSR1) or downregulated the expression with OSR1 siRNA in A549 cells (A549-siOSR1). Compared with H1299 cells transfected with the empty control vector (NC), the H1299-OSR1 cells had significantly higher mRNA and protein expression levels of OSR1 (Figure 4A, B). Overexpression of OSR1 inhibited the invasive ability ($P < .01$), colony formation ($P < .01$) and proliferation rate of the lung cancer cells (day 5, $P < .05$) (Figure 4E-G). In contrast, compared with A549 cells transfected with control siRNA (siNC), A549-siOSR1 cells had significantly lower mRNA and protein levels of OSR1 (Figure 4C,D). The downregulation of OSR1 enhanced the invasive ability ($P < .01$), colony formation ($P < .01$) and proliferation rate of cancer cells (day 5, $P < .05$) (Figure 4E-G).



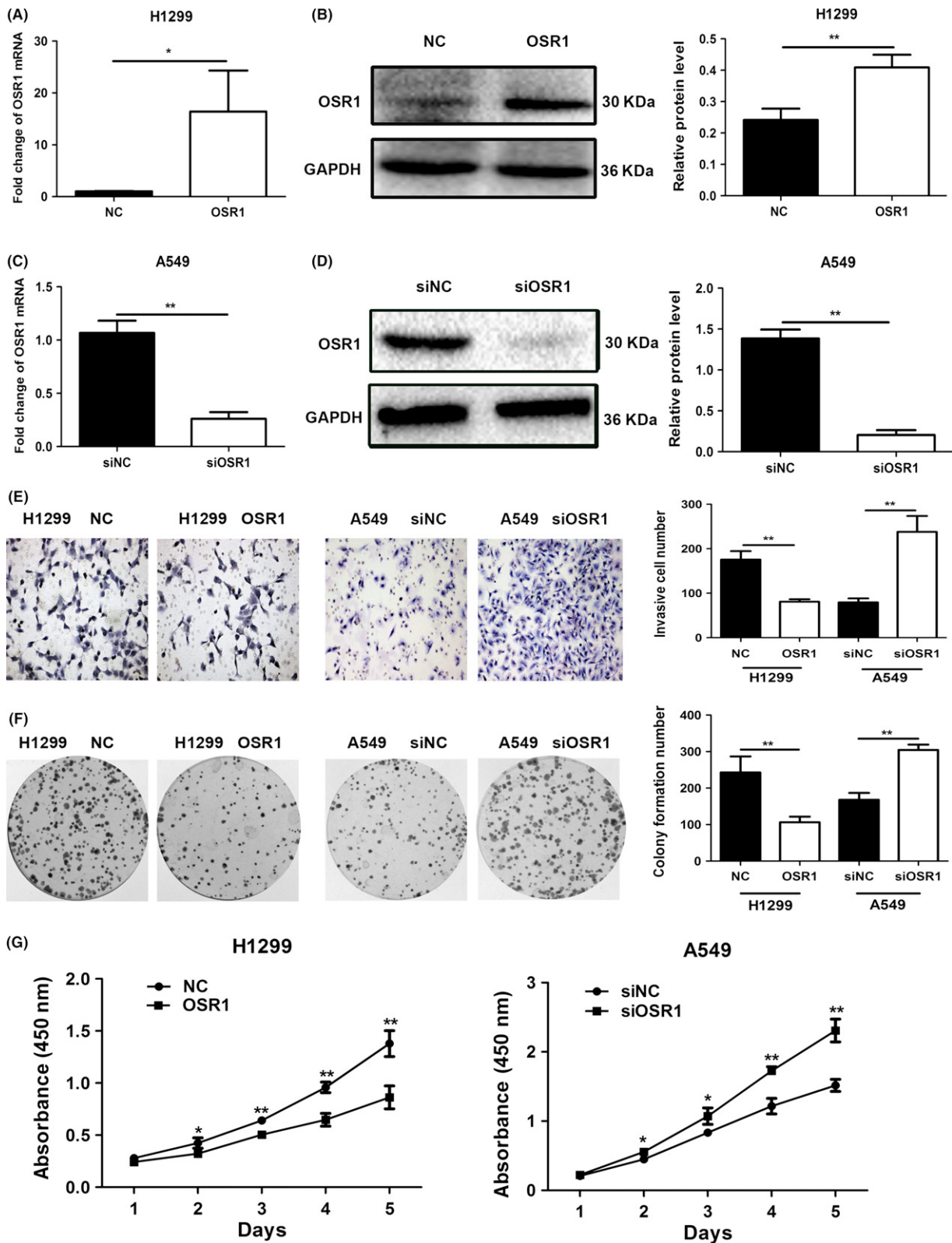


FIGURE 4 OSR1 inhibited the proliferative and invasive abilities of lung cancer cells. A, The mRNA levels of OSR1 in H1299 cells transfected with pCMV6-OSR1 (H1299-OSR1) and cells transfected with control empty vector (NC). B, The western blot analysis and relative expression level of OSR1 in H1299-OSR1 and NC cells. C, The mRNA levels of OSR1 in A549 cells with OSR1-siRNA (A549-siOSR1) and cells transfected with control siRNA (siNC). D, The western blot analysis and relative expression level of OSR1 in A549-siOSR1 cells and siNC cells. E, Representative images of the Matrigel invasion assay for H1299-OSR1 cells, A549-siOSR1 cells, and their control cells. The invasive cell number for each group is shown in the histogram. F, Representative images of the colony formation assay for H1299-OSR1 cells, A549-siOSR1 cells, and their control cells. The number of colonies formed by each group is shown in the histogram. G, The cell growth curve of H1299-OSR1 cells, A549-siOSR1 cells, and their control cells. * $P < .05$; ** $P < .01$

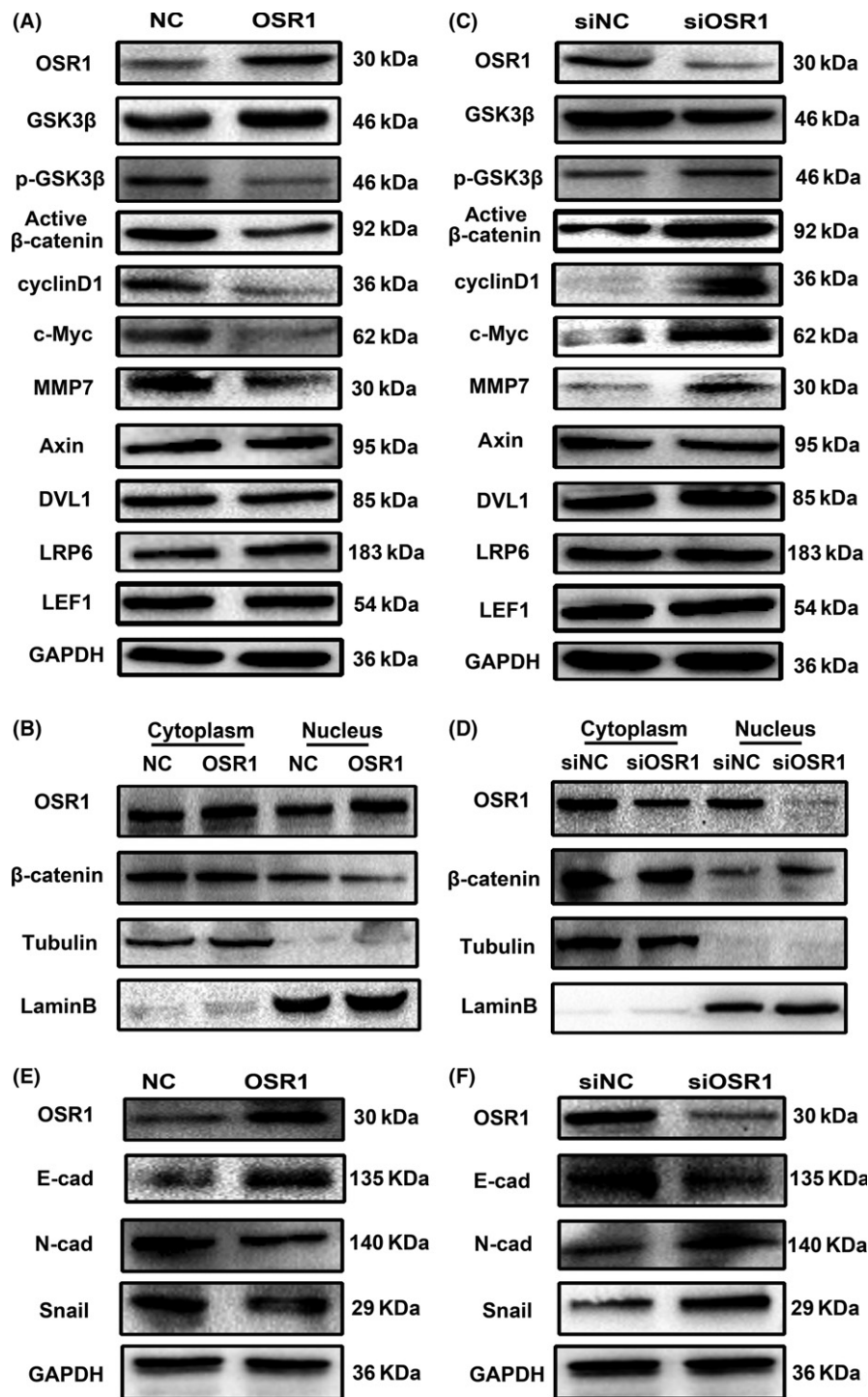


FIGURE 5 The expression of proteins relevant to Wnt signaling pathway in lung cancer cells after OSR1 overexpression or knockdown. A, Western blot analysis for OSR1, GSK3β, p-GSK3β, active β-catenin, cyclin D1, c-Myc, MMP7, axin, DVL1, LRP6 and LEF1 in H1299-OSR1 and NC cells. GAPDH served as an internal control. B, Western blot analysis for evaluating the cytoplasmic levels of OSR1 and β-catenin, and nuclear levels of OSR1 and β-catenin in the H1299-OSR1 and NC cells. Tubulin and Lamin B served as the internal controls. C, Western blot analysis for OSR1, GSK3β, p-GSK3β, active β-catenin, cyclin D1, c-Myc, MMP7, axin, DVL1, LRP6 and LEF1 in A549-siOSR1 and siNC cells. GAPDH served as an internal control. D, Western blot analysis showing the cytoplasmic levels of OSR1 and β-catenin, and nuclear levels of OSR1 and β-catenin in the A549-siOSR1 and siNC cells. Tubulin and Lamin B served as the internal controls. E, F, Western blot analysis for OSR1, E-cadherin, N-cadherin and Snail in H1299-OSR1 and NC cells; A549-siOSR1 and siNC cells. GAPDH served as an internal control

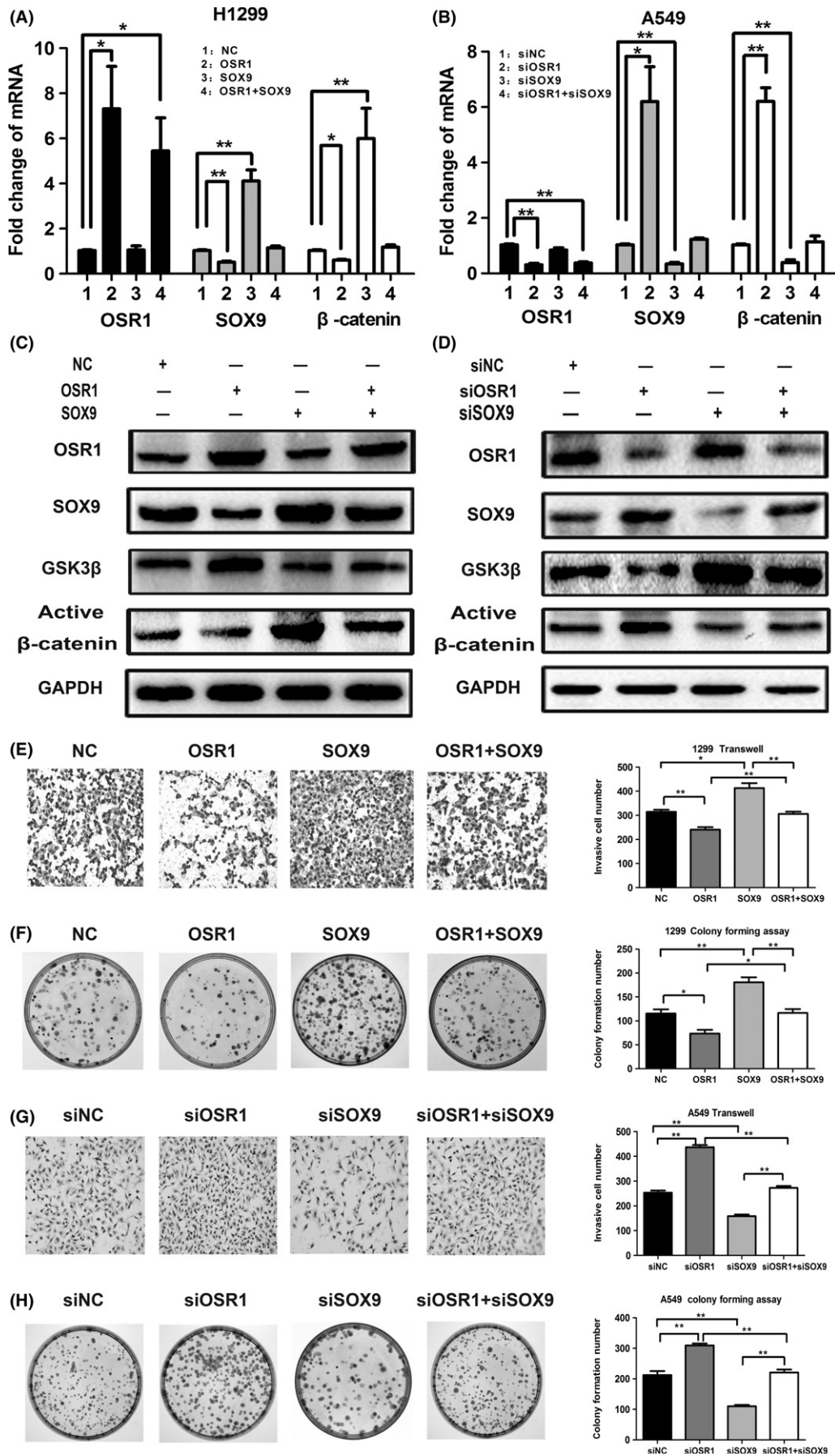


FIGURE 6 Regulation of SOX9 and β -catenin by OSR1. A, The mRNA levels of OSR1, SOX9 and β -catenin (*CTNNB1*) in H1299-OSR1 cells, cells transfected with pCMV6-SOX9 (H1299-SOX9), cells transfected with both pCMV6-OSR1 and pCMV6-SOX9 (H1299-OSR1 + SOX9) and NC cells. B, The mRNA levels of OSR1, SOX9 and β -catenin (*CTNNB1*) in A549-siOSR1 cells, cells transfected with SOX9-siRNA (A549-siSOX9), cells transfected with both OSR1-siRNA and SOX9-siRNA (A549-siOSR1 + siSOX9), and siNC cells. C, The western blot analysis of OSR1, SOX9, GSK3 β and active β -catenin in H1299-OSR1 cells, H1299-SOX9 cells, H1299-OSR1 + SOX9 cells and NC cells. GAPDH served as an internal control. D, The western blot analysis of OSR1, SOX9, GSK3 β and active β -catenin in A549-siOSR1 cells, A549-siSOX9 cells, A549-siOSR1 + siSOX9 cells and siNC cells. GAPDH served as an internal control. E,F, Representative images of the Matrigel invasion assay and colony formation assay for the H1299-NC, H1299-OSR1, H1299-SOX9 and H1299-OSR1 + SOX9 cells. The cell number for each group is shown in the histogram. G,H, Representative images of the Matrigel invasion assay and colony formation assay for the A549-siNC, A549-siOSR1, A549-siSOX9 and A549-siOSR1 + siSOX9 cells. The cell number for each group is shown in the histogram. * $P < .05$; ** $P < .01$

3.3 | OSR1 restrained the expression of β -catenin and the activity of the Wnt signaling pathway, and regulated the expression of epithelial–mesenchymal transition-related proteins

We used western blotting to examine important proteins in the Wnt signaling pathway to explore the underlying mechanisms of the effects of OSR1 on lung cancer cell proliferation and invasion. Compared to the control cells, the expression level of GSK3 β increased, while that of p-GSK3 β , active β -catenin and nuclear β -catenin significantly decreased in the H1299-OSR1 cells ($P < .05$). Concomitantly, the expression levels of target genes of the Wnt signaling pathway, such as cyclin D1, c-Myc and MMP7, were also significantly reduced in H1299-OSR1 cells ($P < .05$) (Figure 5A,B). However, the expression levels of axin, DVL1, LRP6 and LEF1 were not markedly changed ($P > .05$). The expression level of E-cadherin increased, while N-cadherin and Snail significantly decreased in the H1299-OSR1 cells compared to the control cells ($P < .05$) (Figure 5E). In contrast, compared to the control cells, the expression level of GSK3 β decreased, while that of p-GSK3 β , active β -catenin and nuclear β -catenin significantly increased in the A549-siOSR1 cells ($P < .05$). The expression levels of target genes of the Wnt signaling pathway, such as cyclin D1, c-Myc and MMP7, were also significantly increased in A549-siOSR1 cells ($P < .05$) (Figure 5C,D). As in the cells that overexpressed OSR1, the expression levels of axin, DVL1, LRP6 and LEF1 were not markedly changed ($P > .05$). The expression level of E-cadherin decreased, while N-cadherin and Snail significantly increased in the A549-siOSR1 cells compared to the control cells ($P < .05$) (Figure 5F).

3.4 | OSR1 downregulated the expression of β -catenin by inhibiting the transcription of SOX9

A previous study showed that overexpression of OSR1 suppressed the expression of endogenous SOX9 mRNA.¹¹ The expression of β -catenin increased in the SOX9-overexpressing glioma cells, suggesting a regulatory role for SOX9 in β -catenin signaling.²⁸ Thus, we hypothesized that OSR1 may inhibit the expression of β -catenin by suppressing SOX9 expression. We altered the expression levels of OSR1 and SOX9 in H1299 or A549 cells, both individually and simultaneously. As shown in Figure 6A,B, the mRNA level of β -catenin was enhanced in H1299 cells transfected with SOX9 compared to control H1299 cells, and reduced in A549 cells transfected with

SOX9 siRNA compared to control A549 cells. In addition, compared to the control H1299 cells, the protein level of GSK3 β was lower, while that of β -catenin was higher in the H1299 cells transfected with SOX9. In contrast, compared to the control A549 cells, the protein level of GSK3 β was enhanced, while that of β -catenin was reduced in the A549 cells transfected with SOX9 siRNA (Figure 6C, D). Thus, SOX9 enhanced the expression of β -catenin at both mRNA and protein levels in lung cancer cells. However, the mRNA and protein levels of SOX9 were reduced in H1299-OSR1 cells and enhanced in A549-siOSR1 cells compared to control cells. Overexpression of OSR1 reversed the SOX9-induced upregulation of β -catenin and downregulation of GSK3 β . Thus, we found that OSR1 inhibited the transcription and expression of SOX9 in lung cancer cells, thereby indirectly downregulating the expression of β -catenin. We also found that SOX9 could reverse the effects of OSR1 on invasive ability and colony formation of lung cancer cells (Figure 6E-H).

3.5 | Overexpression of β -catenin can reverse the inhibitory effects of OSR1 in lung cancer cells

To confirm whether the inhibitory effects of OSR1 on lung tumor cell proliferation and invasion were mediated via the inhibition of β -catenin activity, we altered the expression level of OSR1, or that of OSR1 and β -catenin (*CTNNB1*) simultaneously in the H1299 or A549 cells. As shown in Figure 7, overexpression of OSR1 inhibited the invasive ability ($P < .01$) (Figure 7A), colony formation ($P < .05$) (Figure 7C), and proliferation rate of lung cancer cells (day 5, $P < .05$) (Figure 7E). However, when cotransfected with the OSR1 and β -catenin (*CTNNB1*) plasmids simultaneously, the inhibitory effects of OSR1 on invasive ability, colony formation and proliferation rate were reversed (Figure 7A,C,E) ($P < .05$). In contrast, the downregulation of OSR1 enhanced the invasive ability ($P < .01$) (Figure 7B), colony formation ($P < .01$) (Figure 7D) and proliferation rate of cancer cells (day 5, $P < .01$) (Figure 7F). These effects were reduced when OSR1 and β -catenin (*CTNNB1*) were simultaneously knocked down (Figure 7B,D,F) ($P < .05$).

4 | DISCUSSION

Previous studies on OSR1 were mostly in the field of embryonic development. Reports of OSR1 in tumors were rare.^{15,16} Therefore,

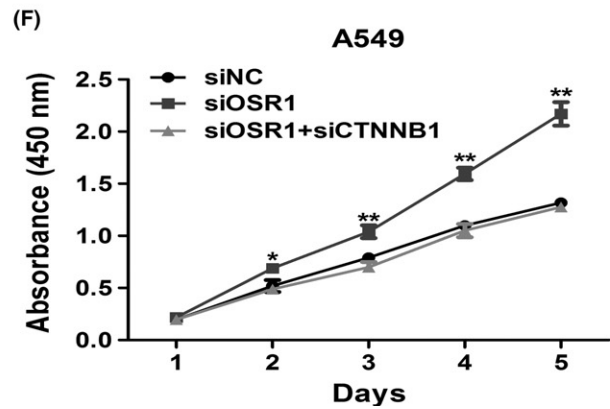
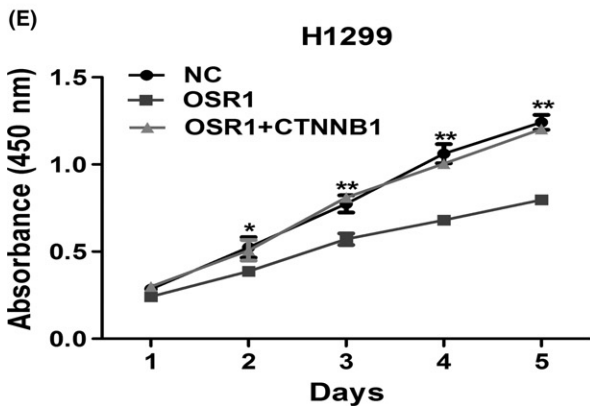
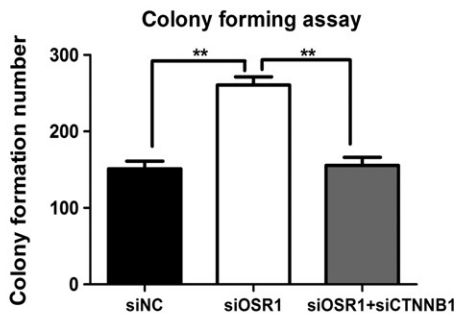
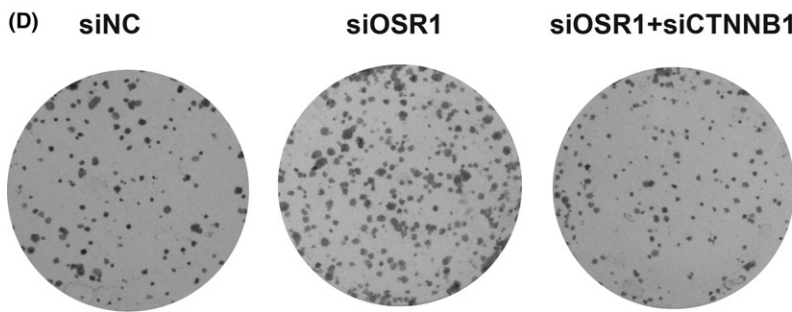
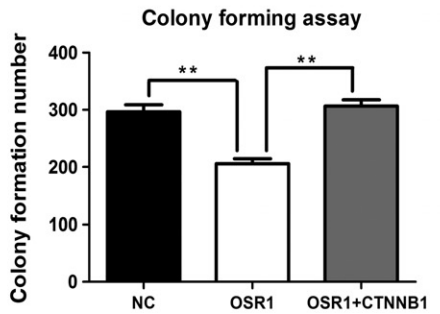
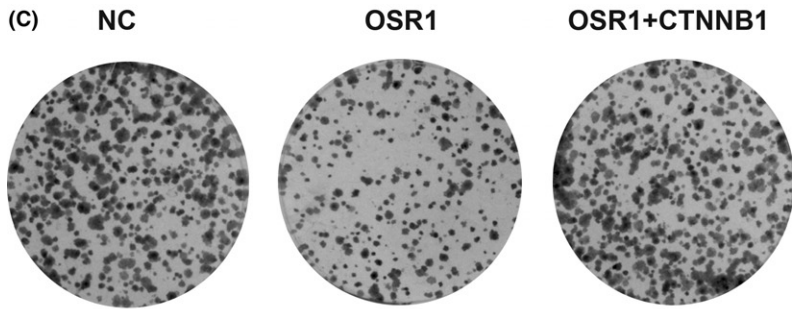
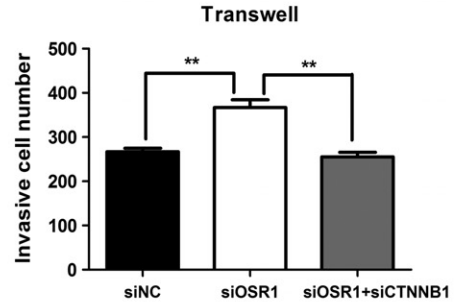
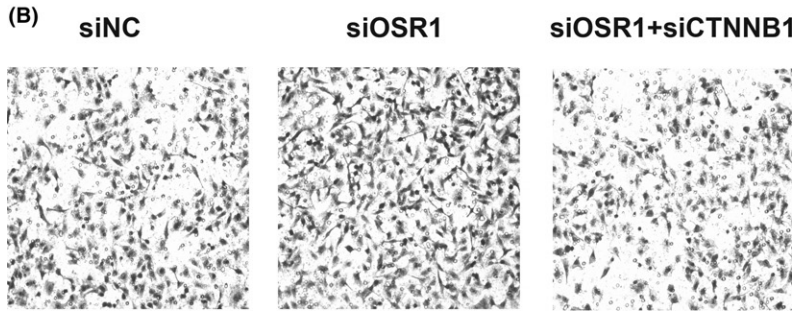
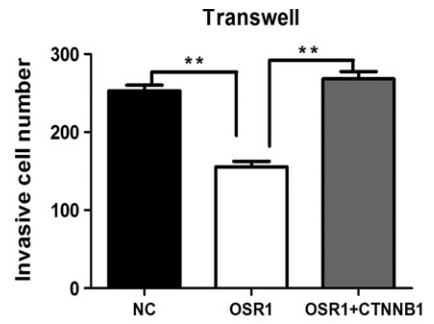
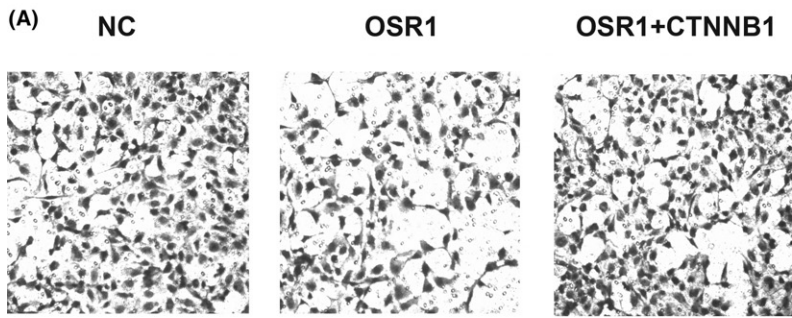


FIGURE 7 Overexpression of β -catenin can reverse the inhibitory effects of OSR1 in lung cancer cells. A,C, Representative images of the Matrigel invasion assay and colony formation assay for the H1299-NC, H1299-OSR1 and H1299-OSR1 + CTNNB1 cells. The cell number for each group is shown in the histogram. B,D, Representative images of the Matrigel invasion assay and colony formation assay for the A549-siNC, A549-siOSR1 and A549-siOSR1 + siCTNNB1 cells. The cell number for each group is shown in the histogram. E, The growth curves of H1299-NC, H1299-OSR1 and H1299-OSR1 + CTNNB1 cells. F, The growth curves of A549-siNC, A549-siOSR1 and A549-siOSR1 + siCTNNB1 cells. * $P < .05$, ** $P < .01$

the biological relationship between OSR1 and cancer development has not been well-documented. In our study, we demonstrated that the expression of OSR1 was significantly reduced in lung cancer tissues compared to normal lung tissues, and it correlated with the poor differentiation of lung cancers. These findings were confirmed by the analysis based on TCGA datasets (Figure S1). To date, this is the first report concerning the expression pattern and clinical significance of OSR1 in human lung cancers.

Further in vitro experiments confirmed that the expression of OSR1 inhibited the proliferative and invasive abilities of lung cancer cells. We showed that the expression of OSR1 enhanced the expression of GSK3 β , inhibited the expression and activation of β -catenin, and the expression of Wnt target genes, such as cyclin D1, c-Myc and MMP7. OSR1 also regulated the expression of epithelial-mesenchymal transition (EMT)-related proteins, such as E-cadherin, N-cadherin and Snail. Thus, OSR1 acts as a tumor suppressor in lung cancers by restraining the activation of the Wnt signaling pathway and EMT. Otani et al¹⁵ also demonstrated that OSR1 activated the p53 pathway and repressed Wnt/ β -catenin signaling in gastric cancers. However, they did not investigate the detailed mechanism of OSR1-mediated regulation of the Wnt signaling pathway.

As a transcription factor, OSR1 can bind to the promoter of some important genes and regulate their transcriptions. Liu et al¹¹ found that OSR1 could bind to the promoter of the SOX9 gene, and that the overexpression of OSR1 suppressed the expression of endogenous SOX9 mRNA. SOX9 is a high mobility group box transcription factor, which plays critical roles in embryogenesis and human cancers.²⁹⁻³² In chondrocyte fate determination and maintenance, SOX9 inhibits Wnt signaling by promoting β -catenin phosphorylation and subsequent degradation.³³ However, Liu et al²⁸ found that the expression of β -catenin was increased in SOX9-overexpressing glioma cells, suggesting a regulatory role for SOX9 in β -catenin signaling. We found, using the Jaspar database (<http://jaspar.genereg.net/>), that the promoter region of β -catenin (CTNNB1) contained several SOX9 binding sites. Based on previous reports and our findings, we hypothesized that OSR1 may inhibit the expression of β -catenin by suppressing SOX9. Our studies confirmed our hypothesis. SOX9 can regulate β -catenin at the transcriptional level, as well as the stability of proteins GSK3 β and β -catenin. OSR1 inhibited the expression of SOX9 mRNA and protein, and reversed the activating effect of SOX9 on β -catenin. Thus, OSR1 inhibits the activity of the Wnt signaling pathway by inhibiting the expression of β -catenin, which is achieved indirectly through the suppression of the transcription of SOX9 (Figure 1).

In conclusion, OSR1 is a novel tumor suppressor gene, which is commonly downregulated in lung cancers, making it a potential tumor malignancy marker and therapeutic target. OSR1

downregulates the expression of β -catenin and the activity of the Wnt signaling pathway by increasing the expression of GSK3 β and suppressing the expression of SOX9.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

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

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