ORIGINAL RESEARCH

Downregulation of OSR1 Promotes Colon Adenocarcinoma Progression via FAK-Mediated Akt and MAPK Signaling

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Introduction: Odd-skipped related transcription factor 1 (OSR1) is a newly identified tumor suppressor in many tumor types. However, the role and mechanism of OSR1 in colon adenocarcinoma (COAD) remain unknown.

Methods: OSR1 expression was detected in COAD tissues and cells. COAD cells with OSR1 overexpression or knockdown were analyzed by in vitro CCK-8, transwell and flow cytometry assays, and by in vivo xenograft model.

Results: OSR1 expression was downregulated in COAD and low expression level of OSR1 was positively correlated with tumor stage and lymph node metastasis. Furthermore, low OSR1 expression was significantly associated with poor overall survival (OS) and distant metastasis-free survival (DMFS). Lentivirus-mediated restoration of OSR1 expression-inhibited proliferation, invasion and migration while induced cell cycle arrest and apoptosis in COAD cells in vitro, and inhibited tumor growth in vivo. In contrast, OSR1 knockdown promoted proliferation, invasion and migration in COAD cells in vitro. Mechanistically, OSR1 exerted anticancer effects by inhibiting FAK-mediated activation of Akt and MAPK pathways.

Conclusion: Our findings suggest that OSR1 functions as a tumor suppressor in COAD by suppressing FAK-mediated activation of Akt and MAPK pathways.

Keywords: OSR1, colon adenocarcinoma, tumor suppressor, FAK, Akt, MAPK

Introduction

Colon adenocarcinoma (COAD) is one of the most common malignancies worldwide. The incidence of COAD ranks the third among malignancies, and the lethality of COAD ranks the second among malignancies.[1] Despite the development of advanced diagnostic and therapeutic techniques, more than half of COAD patients die every year, mainly because they are diagnosed at an advanced stage.[2] Therefore, it is urgent to further understand the mechanism of COAD and identify the key molecules involved in COAD progression.

The odd-skipped related transcription factor 1 (OSR1) gene is located at human 2p24.1.[3,4] OSR1 is a protein of 266 amino acids containing three highly conserved C2H2 zinc finger domains, a tyrosine kinase phosphorylation site (Tyr 203) and several hypothetical proline-XX-proline (PXXP) SH3 binding motifs. OSR1 is expressed in the human colon, small intestine, bladder, testicles, fetal lungs, mesenchymal stem cells and osteoblasts.[5] OSR1 is an important regulator of embryo, heart and genitourinary development.[6,7] In recent years, increasing studies have suggested that OSR1 exerts

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antitumor effect in multiple tumors, including gastric cancer,[4] tongue squamous carcinoma,[8] renal cell carcinoma,[9] and lung adenocarcinoma.[10,11] However, the role of OSR1 in COAD is not fully understood. Therefore, in our study, we focused on the role and mechanism of OSR1 in COAD.

Materials and Methods
Patient Samples and Immunohistochemistry (IHC)

Total 21 fresh COAD and corresponding paracancerous colon tissue samples were collected from patients who underwent surgery at the First Affiliated Hospital of Chongqing Medical University for mRNA detection, and 91 formalin-fixed, paraffin-embedded COAD tissue samples were collected from patients who underwent surgery at the First Affiliated Hospital of Chongqing Medical University between 2012 and 2013 for IHC. The patients were enrolled based on the following inclusion criteria: (1) no radiotherapy or chemotherapy before surgery and (2) no other history of surgery. Our protocol was in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by Ethical Review Committee of the First Affiliated Hospital of Chongqing Medical University. All patients signed written informed consent. IHC was conducted using IHC kit (ZSGB-BIO, China) according to the manufacturer's protocols, and the results were evaluated based on staining intensity (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) and extent (1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%).

Cell Culture and Transfection

SW480, HT29, HCT116, HCT-8, SW620, and LoVo human COAD cells were purchased from the American Type Culture Collection (USA), and cultured in RPMI 1640 medium (HyClone, USA) containing 10% fetal bovine serum at 37°C with 5% CO_2. COAD cells were divided into seven groups: the Vector group (cells transfected with blank lentivirus pEZ-Lv105-vector), the OSR1 group (cells transfected with recombinant lentivirus pEZ-Lv105-OSR1), the siCtrl group (cells transfected with a negative control siRNA), the siOSR1#1 group (cells transfected with the siRNA#1 targeting OSR1), the siOSR1#2 group (cells transfected with the siRNA#2 targeting OSR1), the PF573228 group (cells treated with the PF573228), the PF573228+siOSR1 group (cells transfected with the siRNA#1 or 2 targeting OSR1 and treated with the PF573228), and the PF573228+OSR1 group (cells transfected with recombinant lentivirus vector

pEZ-Lv105-OSR1 and treated with the PF573228). The recombinant lentivirus vector pEZ-Lv105-OSR1 (OSR1 group) and blank pEZ-Lv105-vector (vector group) were purchased from Genecopoeia (USA). The recombinant lentivirus was transfected into COAD cells. Seventy-two hours after transfection, puromycin was used to select the stably transfected cell lines. Two small interfering RNAs (siRNAs) targeting OSR1 (siOSR1#1: 5'-ACCGGTGAGACTGGTC CAATA-3' and siOSR1#2: 5'-TTCTCCGAACGTGTCAC GT-3'), and a negative control (siCtrl) were provided by RiboBio (Guangzhou, China).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted from tissue or cells using RNAiso Plus (Takara Biotechnology, Japan). RNA was reverse transcribed into cDNA using a PrimeScript™ RT Master Mix cDNA Synthesis Kit (Takara Biotechnology, Japan). qRT-PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Shanghai) and a SYBR Premix Ex Taq II Kit (Takara Biotechnology, Japan). The primer sequences for OSR1 and β-actin were as follows: OSR1 F: 5'- CGCG CTCTTCTTTCTCCAAA-3' and R: 5'-GACATGAGGGA ACCAGGGAA-3'; human β-actin F: 5'-CCACGAAACTA CCTTCAACTCC-3' and R: 5'- GTGATCTCCTTCTGC ATCCTGT-3'. The relative expression levels of OSR1 were measured using the $2^{-\Delta\Delta Cq}$ method.[12]

Cell Counting Kit-8 (CCK-8) and Colony Formation Assays

Cells were seeded onto 96-well plates (2×10^3 cells per well). After cell adhesion (6–8 h after cell seeding), the medium was replaced with 100 μL complete medium containing 10 μL CCK-8 detection reagent (Bimake, USA). After incubation for 2 h, the absorbance (450 nm) was detected at 0, 24, 48, and 72 h with a microplate reader (Bio-Rad, Shanghai, China). For colony formation assay, cells (500 cells per well) were seeded onto 6-well plates. After incubation for 2 weeks, the cells were washed with cold PBS, fixed with 4% paraformaldehyde, stained with crystal violet, photographed, and counted.

Transwell Migration and Invasion Assays

Cells in serum-free medium were seeded on the upper layer of the Transwell chamber (1×10^4 cells per well), and 700 μL complete medium was added to the lower layer of the chamber. After incubation for 48 h, the

penetrated cells were washed, fixed with 4% paraformaldehyde, stained with crystal violet, photographed, and counted. For invasion assay, 1640 medium and Matrigel (BD Biosciences, USA) were fully mixed at a proportion of 7:1 and quickly added to the upper layer of the chamber, and then incubated for 3 h at 37°C. A total of 8×10^4 cells in 200 μL serum-free medium were seeded onto the upper layer of the chamber, and 700 μL complete medium was added to the lower layer of the chamber. The next steps were the same as those of Transwell migration assay.

Flow Cytometry Assay

For cell cycle assay, cells at 80–100% confluence were collected and fixed with 75% alcohol overnight, centrifuged, and washed again with cold PBS twice. The cells were resuspended in 400 μL PBS containing 2.5 μL RNase A (20 mg/mL; Sigma-Aldrich) and incubated for 30 min at 37°C in the dark. Next, 5 μL propidium iodide (PI; BD Biosciences, USA) was added and incubated for 30 min at 37°C in the dark. Finally, cell cycle analysis was performed using flow cytometry. For apoptosis assay, cells at 80–100% confluence were stained using an Annexin V-fluorescein isothiocyanate (FITC)/PI kit (BD Biosciences, USA) in the dark. Finally, cell apoptosis was detected by flow cytometry.

In vivo Tumorigenicity

All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, and were performed following the guidelines of the National Institutes of Health guide for the care and use of laboratory animals. HCT-8 cells from the OSR1 and vector groups were injected subcutaneously into BALB/c mice (4–6 weeks old). The tumor volumes were measured every 3 days with calipers according to the following formula: volume= length x width^2/2. The mice were sacrificed 30 days postinjection, and the tumor samples were dissected for further analysis.

Western Blot Analysis

Cell was lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethanesulfonyl fluoride, and protein concentrations were detected using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai) according to the manufacturer's protocol. The proteins were then separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked in 5% milk for 2 h at room temperature and incubated with antibodies for OSR1 (Santa Cruz Biotechnology, sc-376545, 1:1000), FAK (Santa Cruz Biotechnology, sc-271126, 1:1000), p-FAK (Tyr397) (Santa Cruz Biotechnology, sc-81493, 1:1000), AKT (Wanleibio, WL0003b, 1:500), p-AKT (Ser473) (Wanleibio, WLP001a, 1:500), p38 (Wanleibio, WL00764, 1:500), p-p38 (Thr180/Tyr182) (Wanleibio, WLP1576, 1:500), ERK 1/2 (Cell Signaling Technology), p-ERK1/2 (CST), E-cadherin (Wanleibio, WL01482), N-cadherin (Wanleibio, WL01047), Vimentin (Wanleibio, WL01960), p21 (Wanleibio, WL0362), Cyclin D1 (Wanleibio, WL01435a), Snail (Wanleibio, WL01863), c-Myc (Wanleibio, WL01781), Bcl-2 (Wanleibio, WL01556), and GAPDH (BOSTER, BM1623, 1;2000) at 4°C overnight. Then, the blots were washed and incubated with second antibodies (1:5000, Bioss) for 1 h at room temperature. Finally, the blots were washed and visualized with enhanced chemiluminescence (ECL) reagents (Wanleibio, Shenyang).

Statistical Analysis

Statistical analyses were performed with the GraphPad Prism 5.00 (GraphPad Software, USA) and SPSS 19.0 (USA). Differences were compared using Student's *t*-test. Categorical data were analyzed by Fisher's exact tests. Survival curves were determined by the Kaplan-Meier method and Log rank tests. Cox's proportional hazards regression model was used to analyze independent prognostic factors. P<0.05 was considered statistically significant.

Results
OSR1 Is Downregulated in COAD and Is a Predictor of COAD Patient Prognosis

Data from The Cancer Genome Atlas (TCGA) dataset showed that OSR1 expression was lower in COAD tissues than in normal tissues (P<0.001) (Figure 1A). qRT-PCR analysis demonstrated that OSR1 expression was lower in 21 pairs of COAD tissues than in paracancerous tissues (P<0.001) (Figure 1B). Furthermore, we detected OSR1 protein levels in another 89 COAD tissues by IHC (Figure 1C–F). Notably, OSR1 staining intensity decreased gradually in tumor samples from patients with stage I, II to III COAD (Supplemental Figure 1A–C). The results indicated that OSR1 expression was lower in COAD tissues than in normal tissues (P<0.001) (Table 1), and low OSR1 level was associated with tumor stage and lymph node metastasis but not with age, sex, tumor location, tumor size or tumor grade (Table 2). The Kaplan–Meier survival curve showed that patients with low OSR1 expression had worse overall survival (OS) (Figure 1G,
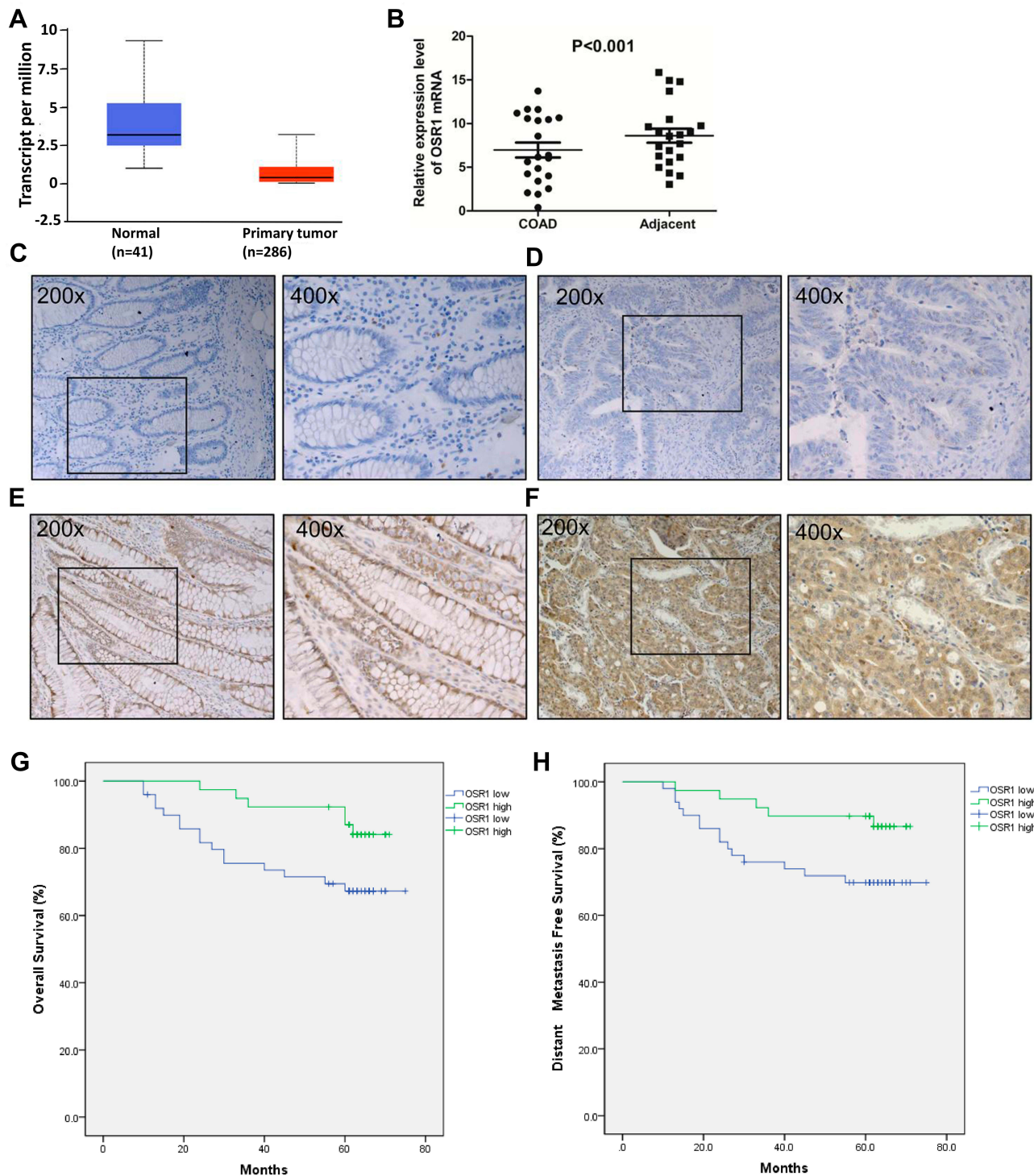
Figure I Expression of OSR1 in COAD tissues. (**A**) Expression of OSR1 in TCGA samples (COAD vs normal, p<0.001) (http://ualcan.path.uab.edu/analysis.html). (**B**) qPCR results showed OSR1 expression levels in 21 COAD patients (COAD vs adjacent, p<0.001). (**C**) Negative staining of OSR1 in adjacent normal colon tissues. (**D**) Negative staining of OSR1 in COAD tissues. (**E**) Positive staining of OSR1OSR1 in adjacent normal colon tissues. (**F**) Positive staining of OSR1 in COAD tissues (200× and 400×). The Kaplan–Meier survival curve showed that patients with low OSR1 expression had worse overall survival (**G**) and distant metastasis-free survival (**H**).

p<0.05) and distant metastasis-free survival (DMFS) (Figure 1H, p<0.05). Moreover, multivariate Cox regression analyses indicated that OSR1 expression, sex, and TNM stage could be independent predictors for OS and DMFS (Table 3).

OSR1 Inhibits the Proliferation, Migration, and Invasion of COAD Cells

OSR1 expression levels showed difference in COAD cell lines (SW480, HT29, HCT116, HCT-8, and LoVo) (Figure 2A). To

Table 1 Immunohistochemistry Analysis of OSR1 Expression in COAD and Paracancerous Normal Tissues

Variables	All Cases	None or Low OSR1 Expression	High OSR1 Expression	P value
Paracancerous	25	8	17	<0.05
Cancer	89	50	39	

Table 2 Correlation of OSR1 Protein Expression with Patient Features

Variables	All Cases	High OSR1 Expression (n=50)	Low OSR1 Expression (n=39)	P value
Age at Surgery				0.395
<60	45	23	22	
≥60	44	27	17	
Gender				0.050
Male	54	35	19	
Female	35	15	20	
Location				0.521
Left	48	25	23	
Right	41	25	16	
Tumor Size (cm^3)				0.828
≤22.45	48	28	20	
>22.45	41	22	19	
pN				0.000***
N0	54	39	15	
N1/N2	35	11	24	
Grade				0.7971
G1+ G2	70	40	30	
G3	19	10	9	
Tumor Stage				0.000***
I+II	53	38	15	
III+IV	36	12	24	

Notes: ***P<0.001. pN, lymph node metastases.

determine the role of OSR1 in COAD, we altered OSR1 expression levels by transfecting cells with recombinant lentiviral vectors or siRNA (Figure 2B, Supplemental Figure 2).
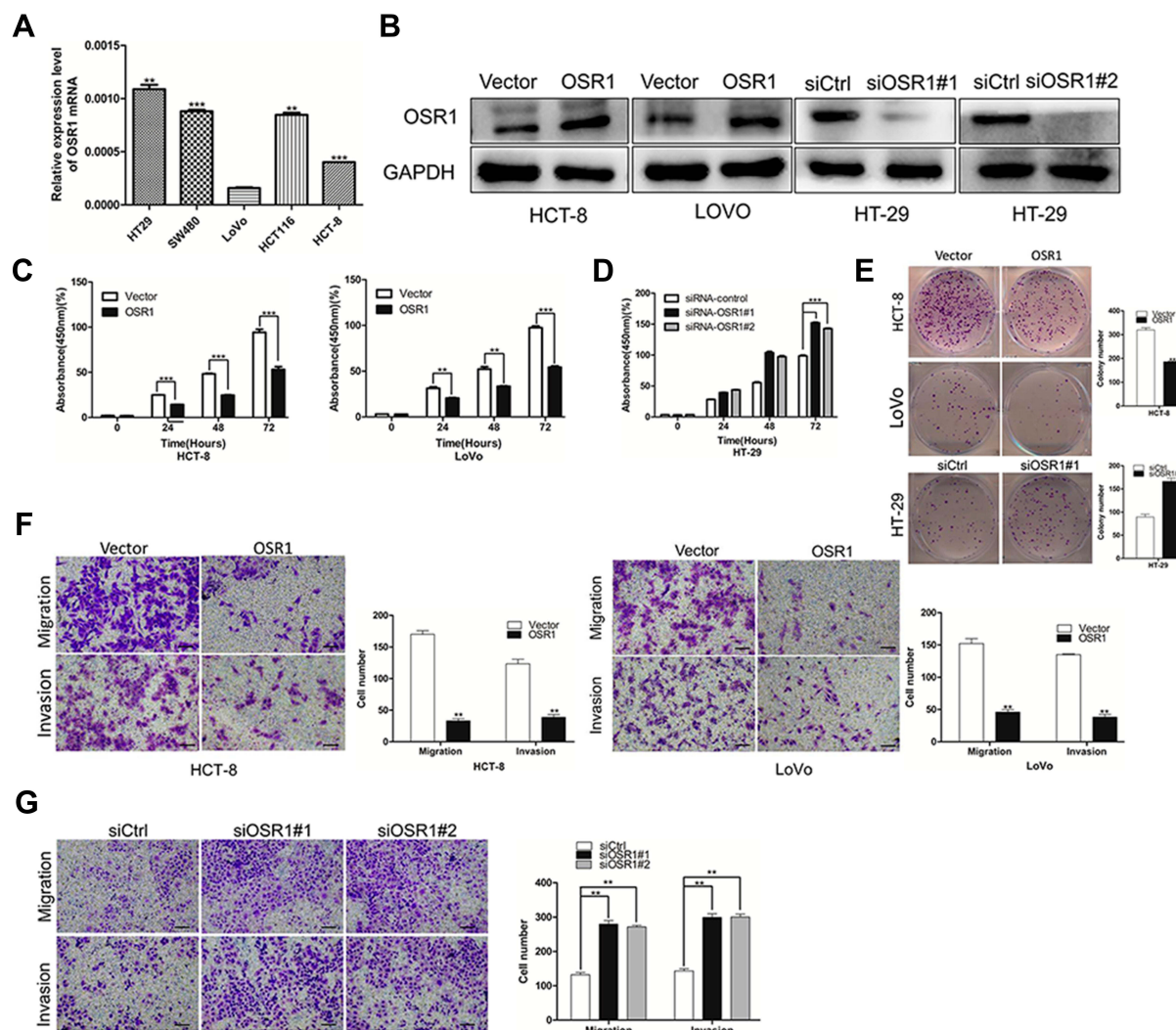
CCK-8 assay demonstrated that the cell viability was lower in the OSR1 group than in the vector group (Figure 2C). On the other hand, the cell viability was higher in the siOSR1 group than in the siCtrl group (Figure 2D). Furthermore, the colony formation assay demonstrated that the number of cell colonies was lower in the OSR1 group than in the vector group, and the number of cell colonies was higher in the siOSR1 group than in the siCtrl group (Figure 2E). Moreover, the number of penetrated COAD cells was significantly lower in the OSR1 group than in the vector group (Figure 2F), and the number of penetrated COAD cells was significantly higher in the siOSR1 group than in the siCtrl group (Figure 2G). Collectively, these results indicate that OSR1 inhibits the proliferative, invasive, and migratory ability of COAD cells.

OSR1 Induces Cell Apoptosis and Cycle Arrest

Cell apoptosis assay showed that OSR1 overexpression significantly increased the number of apoptotic HCT-8 and LoVo cells (Figure 3A). In addition, OSR1 overexpression induced G0-G1 phase arrest in HCT-8 cells, while the number of cells in G2-M and S phase decreased (Figure 3B). OSR1 overexpression induced G0-G1 phase arrest in LoVo cells, while the number of cells in S phase decreased (Figure 3C). On the other hand, OSR1 knockdown induced S phase arrest in HT-29 cells, while the number of cells in G2-M phase decreased (Figure 3D).

OSR1 Inhibits Akt and MAPK Pathways in COAD Cells

A previous study showed that OSR1 suppressed integrin signaling and regulated cell communication and adhesion.[13] Therefore, we detected FAK related signaling pathways in COAD. Western blot analysis demonstrated that OSR1 overexpression significantly decreased the levels of p-FAK, p-p-38, p-ERK1/2, and p-AKT (Ser473), while the levels of total FAK, total p38, total ERK1/2, and total AKT were unaffected. Conversely, the levels of p-FAK,

Table 3 Multivariate Regression Analysis of 5-Year Overall Survival and Distant Metastasis-Free Survival of COAD Patients

Variables	Overall Survival			Distant Metastasis-Free Survival		
	HR	95% CI	P-value	HR	95% CI	P-value
OSR1 (low vs high)	0.181	0.06–0.543	0.002**	0.162	0.051–0.518	0.002**
Sex (male vs female)	0.285	0.093–0.878	0.029*	0.296	0.091–0.962	0.043*
TNM (I/II vs III/ IV)	5.451	2.044–14.537	0.001**	6.921	2.384–20.090	0.000***

Notes: *P<0.05, **P<0.01, ***P<0.001.
Abbreviations: HR, hazard ratio; CI, confidence interval.

Figure 2 OSR1 inhibited COAD cell proliferation, migration, and invasion. (**A**) RT-qPCR analysis of OSR1 expression in five COAD cell lines. (**B**) Western blot analyses confirmed that OSR1 expression was upregulated or downregulated. (**C–E**) CCK-8 assay and colony formation assay showed that OSR1 overexpression or knockdown regulated the proliferation rate of COAD cells. Viability was expressed as a percentage of the control population. (**F**) Transwell migration and invasion assays showed that OSR1 overexpression reduced the migration and invasion abilities of COAD cells. (**G**) Transwell migration and invasion assays showed that OSR1 knockdown promoted the migration and invasion abilities of COAD cells. *p<0.05; **p<0.01; ***p<0.001. Scale bar: 100 μm.

p-p38, p-ERK1/2, and p-AKT were markedly increased in cells treated with OSR1 siRNA (Figure 4A).

In addition, Western blot analyses showed that the expression levels of N-cadherin, vimentin, cyclin D1, snail, c-myc, and Bcl-2 were significantly decreased and the expression levels of E-cadherin, p21, caspase-3, and caspase-9 were significantly increased in OSR1 overexpression group, and opposite results were observed in siOSR1 group (Figure 4B, Supplemental Figure 3).

Furthermore, we treated COAD cells with PF573228, which is an ATP-competitive inhibitor of FAK to inhibit the phosphorylation but not the expression level of FAK. We found that cell proliferation was lower in HT-29 cells treated with both PF573228 and siOSR1 than in HT-29 cells treated with

siOSR1 alone (Figure 5A). Transwell invasion assay showed that the number of penetrated HT-29 cells treated with both PF573228 and siOSR1 was significantly lower that of HT-29 cells treated with siOSR1 alone (Figure 5B). On the other hand, CCK-8 assay showed that the proliferation of HCT-8 cells treated with both PF573228 and OSR1 overexpression vector was inhibited compared to that of the cells treated with PF573228 and empty vector (Figure 5C). Transwell invasion assay showed that the number of penetrated HCT-8 cells treated with both PF573228 and OSR1 overexpression vector was lower than that of the cells treated with PF573228 and empty vector (Figure 5D). These data indicate that OSR1 may inhibit proliferation and invasion of COAD cells by suppressing FAK-mediated Akt and MAPK pathways.
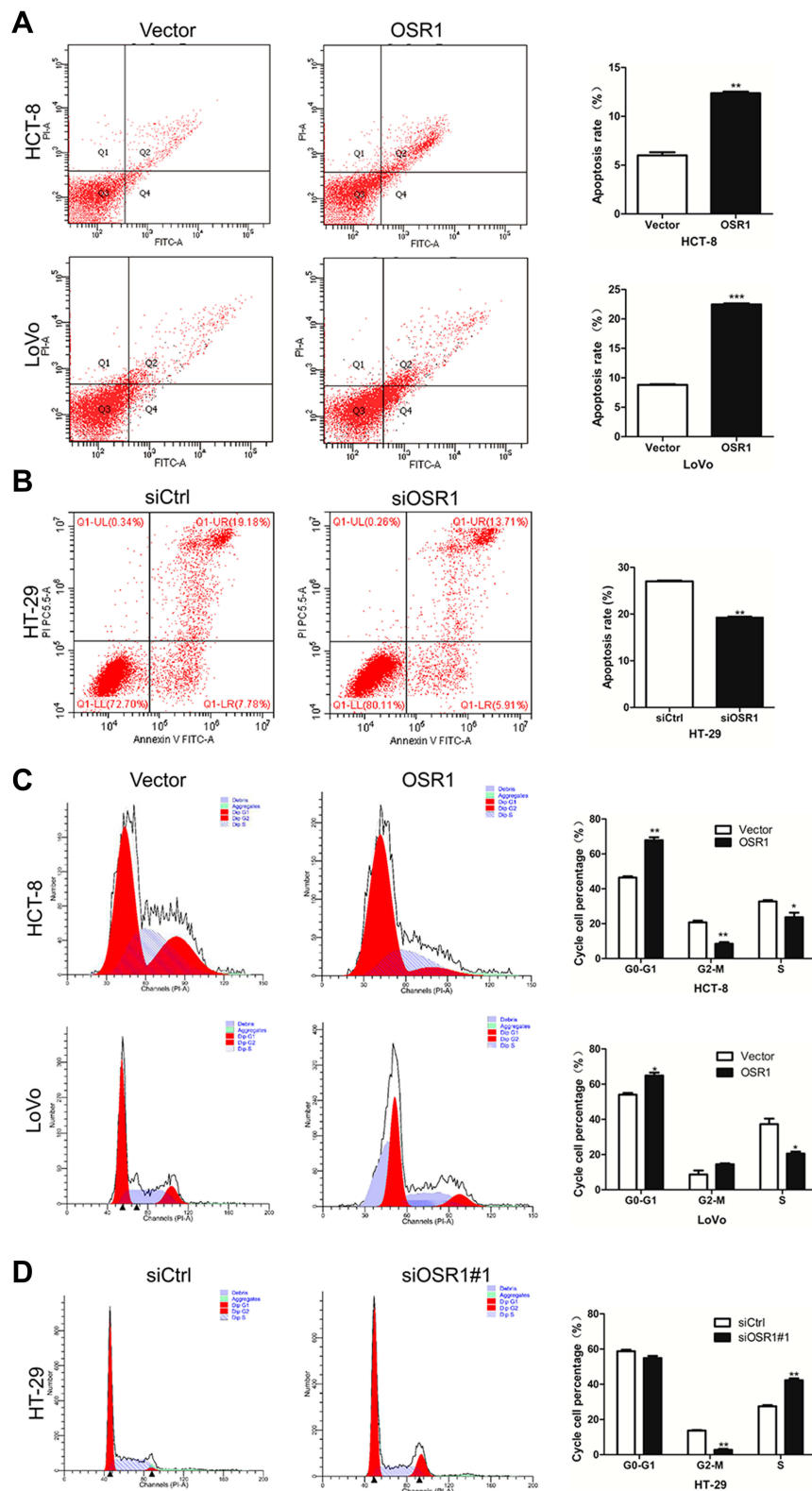
Figure 3 OSR1 induced apoptosis and cell cycle arrest. Flow cytometry showed that OSR1 overexpression induced cell apoptosis (**A**), while OSR1 knockdown inhibited cell apoptosis (**B**). For cell cycle distributions, HCT-8 and LoVo (OSR1 group) cells were significantly arrested in the G0/G1 phase (**C**), while HT-29 (siOSR1#1) cells were significantly arrested in the S phase (**D**). *p<0.05; **p<0.01; ***p<0.001.
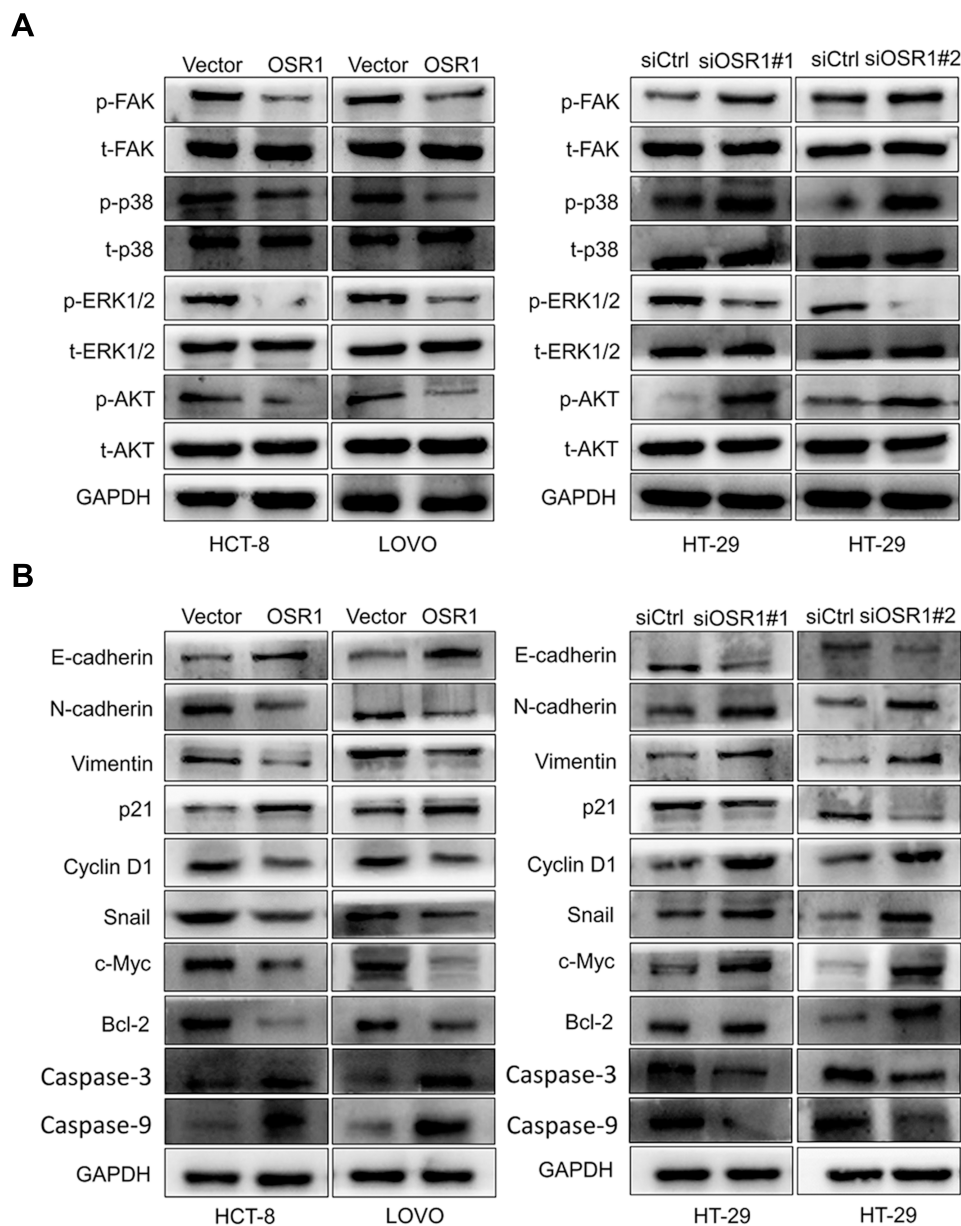
Figure 4 OSR1 inhibited Akt and MAPK signaling pathways. (**A**) Western blot analysis of the levels of total FAK, p-FAK (Tyr397), total p38, p-p-38 (Thr180/Tyr182), total ERK1/2, p-ERK1/2, total AKT, and anti-p-AKT (Ser473) in COAD cells with OSR1 knockdown or overexpression. (**B**) Western blot analysis of expression levels of E-cadherin, N-cadherin, vimentin, p21, cyclin D1, snail, c-myc, caspase-3, caspase-9, and Bcl-2 in COAD cells with OSR1 knockdown or overexpression.

## OSR1 Suppressed the Growth of COAD Cells in vivo

To confirm that OSR1 inhibited COAD in vivo, nude mice were injected subcutaneously with HCT-8 cells treated with either OSR1 overexpression vector or empty vector. OSR1 overexpression inhibited tumor growth in vivo (Figure 6A), and tumor tissues were remarkably smaller in OSR1 overexpression group than in empty vector group (Figure 6B). Furthermore, Western blot analysis of the dissected tumor tissues showed lower levels of p-FAK,

p-p-38, and p-AKT in OSR1 overexpression group than in empty vector group (Figure 6C, Supplemental Figure 4).

Discussion

Recently, low expression of OSR1 has been reported to be associated with multiple malignancies, including gastric cancer,[4] tongue squamous cell carcinoma,[8] renal cell carcinoma,[9] and lung adenocarcinoma.[10,11] In addition, OSR1 downregulation is associated with OSR1 methylation in gastric cancer,[4] renal cell carcinoma,[9] and lung
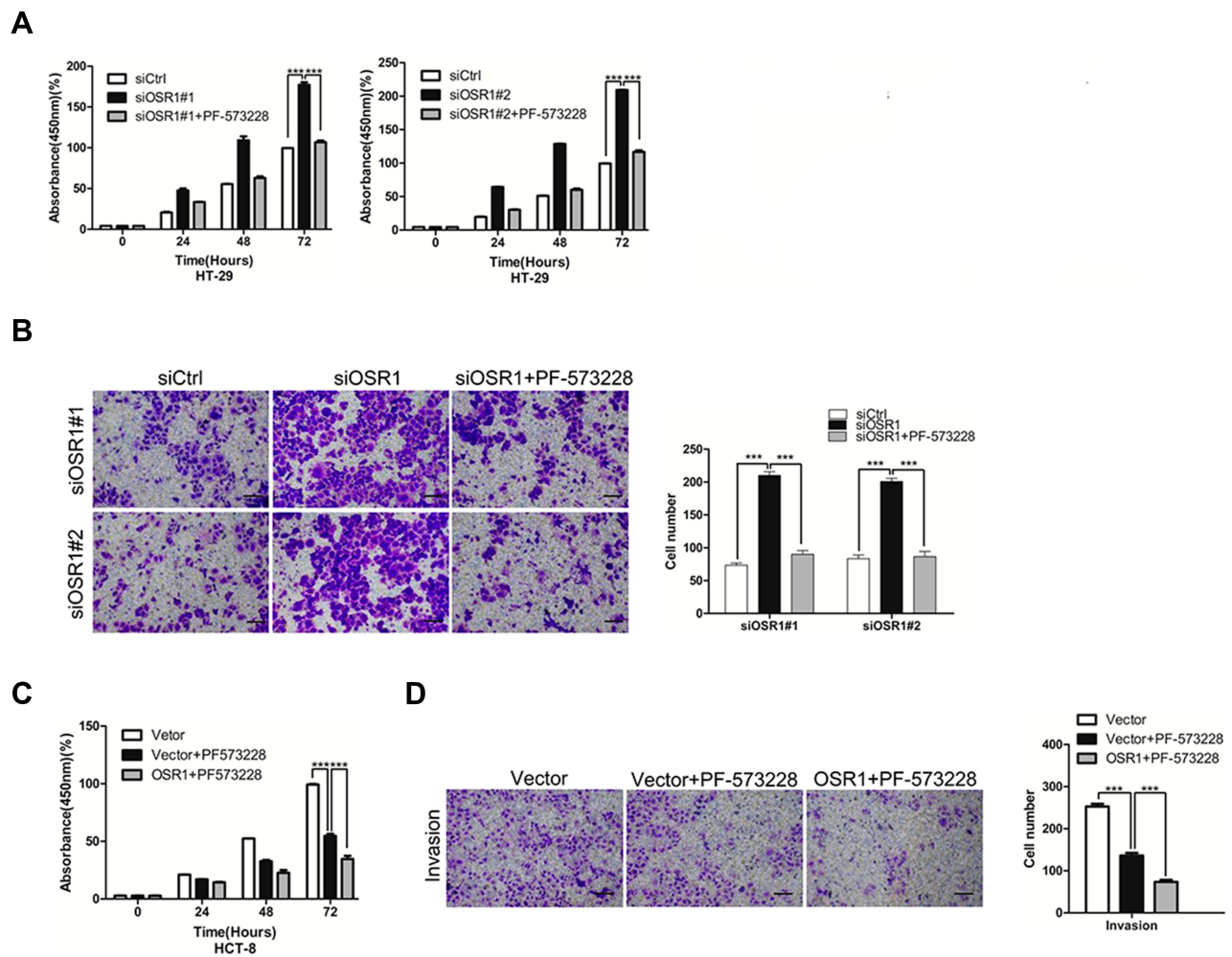
Figure 5 FAK inhibitor inhibits the proliferation and invasion of COAD cells. (**A**) PF573228 reversed the oncogenic effects of siOSR1 on HT-29 cell proliferation. HT-29 cells transfected with OSR1 or negative control siRNA were treated with or without 10 μM PF573228 for 72 h, cell viability was detected at 0, 24, 48, and 72 h by CCK-8 assay. Viability was expressed as a percentage of the control. (**B**) PF573228 reversed the oncogenic effects of siOSR1 on HT-29 cell invasion. HT-29 cells transfected with OSR1 siRNA were treated with or without 10 μM PF573228 for 48 h, cell migration was detected by Transwell assay. (**C**) PF573228 enhanced the inhibitory effects of OSR1 overexpression on HCT-8 cell proliferation. HT-29 cells transfected with OSR1 overexpression vector or blank vector were treated with or without 10 μM PF573228 for 72 h, cell viability was detected at 0, 24, 48, and 72 h by CCK-8 assay. Viability was expressed as a percentage of the control. (**D**) PF573228 enhanced the inhibitory effects of OSR1 overexpression on HCT-8 cell invasion. HT-29 cells transfected with OSR1 overexpression vector or blank vector were treated with or without 10 μM PF573228 for 48 h, cell migration was detected by Transwell assay. Scale bar: 100 μm. ***$p<0.001$.

adenocarcinoma.[10] OSR1 may suppress the migration and invasion of tongue squamous cell carcinoma by inhibiting NF-KB pathway.[8] However, the expression and role of OSR1 in COAD remain unknown.

In this study, qRT-PCR and IHC assays demonstrated that OSR1 is downregulated in COAD tissues and OSR1 downregulation is positively related to tumor stage, lymph node metastasis and poor prognosis. The Kaplan-Meier survival curve showed that patients with low OSR1 expression had worse OS and DMFS. Moreover, multivariate Cox regression analyses indicated that OSR1 expression could be an independent predictor of OS and DMFS. These findings suggest that OSR1 is crucially involved in COAD progression.

Therefore, we further investigated the function of OSR1 as a tumor suppressor in COAD growth and metastasis in vitro and in vivo. CCK-8 and colony formation assay demonstrated that OSR1 overexpression inhibited the proliferation of COAD cells while OSR1 knockdown yielded the opposite effects. Transwell migration and invasion assays showed that OSR1 overexpression impaired the migration and invasion of COAD cells. In addition, flow cytometry analysis showed that OSR1 overexpression induced COAD cell G0-G1 phase arrest and apoptosis, while OSR1 knockdown yielded the opposite effects. Consistent with the in vitro results, OSR1 overexpression inhibited COAD tumor growth in vivo. These findings
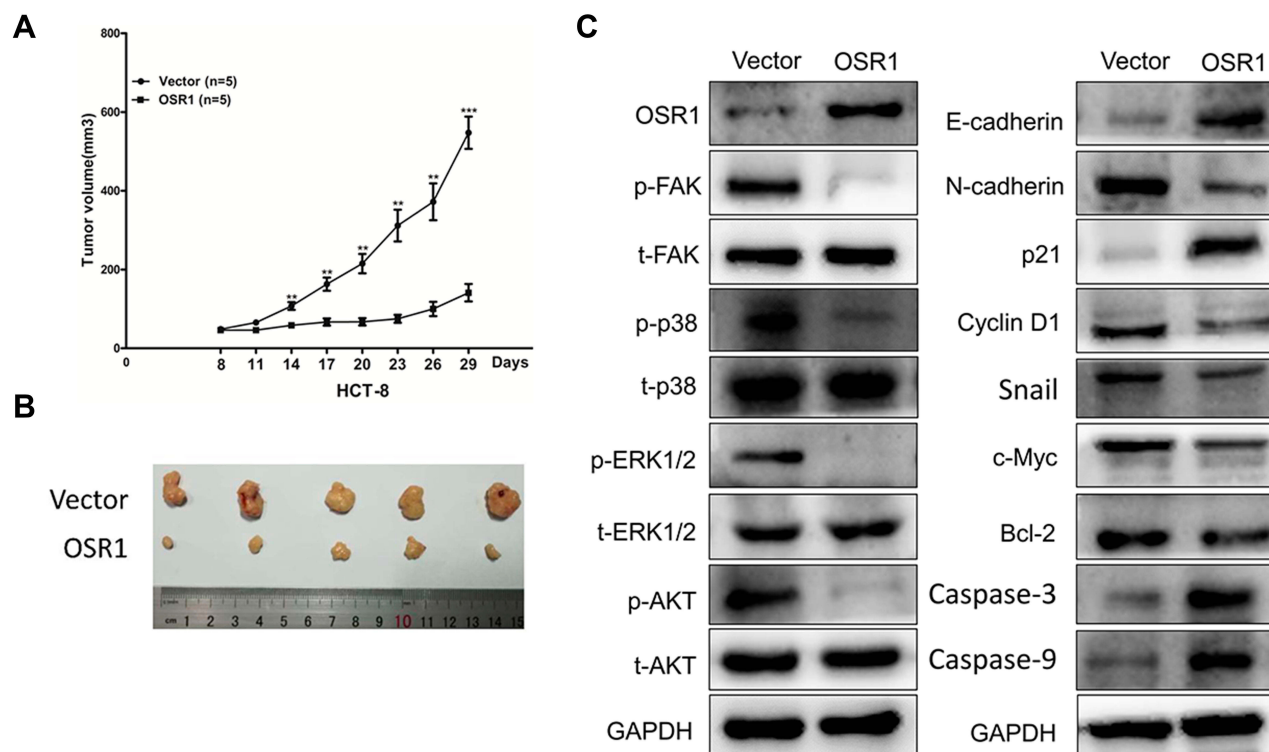
Figure 6 OSR1 suppressed COAD growth in vivo. (**A**) OSR1 overexpression inhibited tumor growth in vivo. ****p<0.01; ***p<0.001 vs Vector. (**B**) After 30 days postinjection, all mice were sacrificed, and subcutaneous tumors were collected. (**C**) Western blot analysis of the levels of total FAK, p-FAK (Tyr397), total p38, p-p-38 (Thr180/Tyr182), total ERK1/2, p-ERK1/2, total AKT, p-AKT (Ser473), E-cadherin, N-cadherin, vimentin, p21, cyclin D1, snail, c-myc, caspase-3, caspase-9, and Bcl-2 in subcutaneous tumors from OSR1 overexpression group and vector group.

suggest that OSR1 serves as a tumor suppressor in the development of COAD.

Furthermore, we investigated the molecular mechanism underlying anti-cancer effects of OSR1 on COAD. OSR1 overexpression suppressed integrin signaling pathway which is involved in cell-cell adhesion and communication.[13] As a critical mediator that connects integrin and the downstream signaling molecules, FAK can be activated by integrin phosphorylation and subsequently activates downstream PI3K/AKT pathway and MAPK pathway.[14–21] To validate whether OSR1 exerts anti-cancer effects via FAK in COAD, we detected the activation of FAK based on p-FAK level. The results indicated that OSR1 overexpression inhibited the activation of FAK.

Activated FAK stimulates the downstream kinase PI3K by binding and phosphorylating the p85α subunit of PI3K (regulatory subunit of PI3K).[22] As a downstream signaling pathway of FAK activation, PI3K/AKT could regulate cancer cell proliferation, migration, apoptosis, cell cycle, and survival.[14,23-25] AKT activation could upregulate cyclin D1[14] and c-Myc,[26] and downregulate cyclin D kinase (CDK) inhibitors p21.[27] Therefore, we investigated the

changes in the activation of PI3K/Akt pathway and MAPK pathway and the expression of critical downstream proteins such as cyclin D1, Bcl-2, c-Myc, and p21. We demonstrated that OSR1 overexpression significantly decreased the levels of p-p38, p-ERK, cyclin D1, Bcl-2, and c-Myc and promoted the expression of p21. In contrast, knockdown of OSR1 significantly increased the levels of p-p38, p-ERK, cyclin D1, Bcl-2, and c-Myc and downregulated the expression of p21. These results suggest that OSR1 could inhibit FAK-mediated activation of Akt and MAPK pathways to induce COAD cell cycle arrest and apoptosis.

Epithelial–mesenchymal transition (EMT) has been recognized as a vital process in the development of multiple tumors, including COAD.[28–30] Therefore, we examined the expression of epithelial marker E-cadherin, mesenchymal markers N-cadherin and vimentin, and EMT-inducing transcription factor snail. We observed that OSR1 overexpression markedly increased E-cadherin expression and reduced N-cadherin, vimentin, and snail expression, while OSR1 knockdown yielded opposite results. These results suggest that OSR1 could regulate COAD cell migration and invasion via EMT.

To further prove the involvement of FAK in tumor suppressor function of OSR1, we treated COAD cells with FAK inhibitor PF573228. The results showed that PF573228 abolished the increase in proliferation and invasion in COAD cells with OSR1 knockdown, while enhanced the decrease in proliferation and invasion in COAD cells with OSR1 overexpression. These results confirm that OSR1 inhibits FAK to suppress COAD cell proliferation and invasion.

In summary, OSR1 suppresses COAD cell proliferation, invasion, and migration and induces apoptosis and cell cycle arrest via inhibiting FAK-mediated Akt and MAPK pathways. In addition, OSR1 might serve as a prognostic marker of COAD.

Disclosure

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J Clin*. 2017;67(1):7–30. doi: 10.3322/caac.20073.
2. Vermeulin T, Lucas M, Lahbib H, Froment L, Czernichow P, Josset V, Jusot F, Di Fiore F, Launoy G, Merle V. Compliance of the Initiation Time to Adjuvant Chemotherapy for Colon Cancer: Development of a Quality Indicator Based on the French Diagnosis-Related Group-Based Information System (PMSI).Oncologie 2018; 20(3-4): 42–48. doi:10.3166/onco-2018-0014
3. Katoh M. Molecular cloning and characterization of OSR1 on human chromosome 2p24. *Int J Mol Med*. 2002;10(2):221–225. doi:10.3892/ijmm.10.2.221
4. Otani K, Dong Y, Li X, et al. Odd-skipped related 1 is a novel tumour suppressor gene and a potential prognostic biomarker in gastric cancer. *J Pathol*. 2014;234(3):302–315. doi:10.1002/path.4391
5. Verlinden L, Kriebitzsch C, Eelen G, et al. The odd-skipped related genes Osr1 and Osr2 are induced by 1,25-dihydroxyvitamin D3. *J Steroid Biochem Mol Biol*. 2013;136:94–97. doi:10.1016/j.jsbmb.2012.12.001
6. Wang Q, Lan Y, Cho ES, Maltby KM, Jiang R. Odd-skipped related 1 (Odd 1) is an essential regulator of heart and urogenital development. *Dev Biol*. 2005;288(2):582–594. doi:10.1016/j.ydbio.2005.09.024
7. James RG, Kamei CN, Wang Q, Jiang R, Schultheiss TM. Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells. *Development*. 2006;133(15):2995–3004. doi:10.1242/dev.02442
8. Chen W, Zou A, Zhang H, Fu X, Yao F, Yang A. Odd-skipped related transcription factor 1 (OSR1) suppresses tongue squamous cell carcinoma migration and invasion through inhibiting NF-kappaB pathway. *Eur J Pharmacol*. 2018;839(15):33–39. doi:10.1016/j.ejphar.2018.09.020
9. Zhang Y, Yuan Y, Liang P, et al. OSR1 is a novel epigenetic silenced tumor suppressor regulating invasion and proliferation in renal cell carcinoma. *Oncotarget*. 2017;8(18):30008–30018. doi:10.18632/oncotarget.15611
10. Daugaard I, Dominguez D, Kjeldsen TE, et al. Identification and validation of candidate epigenetic biomarkers in lung adenocarcinoma. *Sci Rep*. 2016;6:35807. doi:10.1038/srep35807
11. Wang Y, Lei L, Zheng YW, et al. Odd-skipped related 1 inhibits lung cancer proliferation and invasion by reducing Wnt signaling through the suppression of SOX9 and beta-catenin. *Cancer Sci*. 2018;109 (6):1799–1810. doi:10.1111/cas.13614
12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. 2001;25(4):402–408. doi:10.1006/meth.2001.1262
13. Orgeur M, Martens M, Leonte G, et al. Genome-wide strategies identify downstream target genes of chick connective tissue-associated transcription factors. *Development*. 2018;145(7):pii: dev161208. doi:10.1242/dev.161208
14. Zong D, Jiang N, Xu JH, et al. ZNF488 is an independent prognostic indicator in nasopharyngeal carcinoma and promotes cell adhesion and proliferation via collagen IV/FAK/AKT/Cyclin D1 pathway. *Cancer Manag Res*. 2019;11:5871–5882. doi:10.2147/CMAR.S200001
15. Yu R, Li Z, Zhang C, et al. Elevated limb-bud and heart development (LBH) expression indicates poor prognosis and promotes gastric cancer cell proliferation and invasion via upregulating Integrin/FAK/Akt pathway. *Peer J*. 2019;7:e6885. doi:10.7717/peerj.6885
16. Liu SQ, Xu CY, Wu WH, et al. Sphingosine kinase 1 promotes the metastasis of colorectal cancer by inducing the epithelial-mesenchymal transition mediated by the FAK/AKT/MMPs axis. *Int J Oncol*. 2019;54 (1):41–52. doi:10.3892/ijo.2018.4607
17. Song G, Xu S, Zhang H, et al. TIMP1 is a prognostic marker for the progression and metastasis of colon cancer through FAK-PI3K/AKT and MAPK pathway. *J Exp Clin Cancer Res*. 2016;35(1):148. doi:10.1186/s13046-016-0427-7
18. Bartolome RA, Garcia-Palmero I, Torres S, Lopez-Lucendo M, Balyasnikova IV, Casal JI. IL13 receptor alpha2 signaling requires a scaffold protein, FAM120A, to activate the FAK and PI3K pathways in colon cancer metastasis. *Cancer Res*. 2015;75(12):2434–2444. doi:10.1158/0008-5472.CAN-14-3650
19. Shi S, Zhong D, Xiao Y, et al. Syndecan-1 knockdown inhibits glioma cell proliferation and invasion by deregulating a c-src/FAK-associated signaling pathway. *Oncotarget*. 2017;8(25):40922. doi:10.18632/oncotarget.16733
20. Ichihara E, Westover D, Meador CB, et al. SFK/FAK signaling attenuates osimertinib efficacy in both drug-sensitive and drug-resistant models of EGFR-mutant lung cancer. *Cancer Res*. 2017;77(11):2990–3000. doi:10.1158/0008-5472.CAN-16-2300
21. Sugase T, Takahashi T, Serada S, et al. Suppressor of cytokine signaling-1 gene therapy induces potent antitumor effect in patient-derived esophageal squamous cell carcinoma xenograft mice. *Int J Cancer*. 2017;140(11):2608–2621. doi:10.1002/ijc.30666
22. Flamini MI, Uzair ID, Pennacchio GE, et al. Thyroid hormone controls breast cancer cell movement via integrin αV/β3/SRC/FAK/PI3-kinases. *Horm Cancer*. 2017;8(1):16–27. doi:10.1007/s12672-016-0280-3
23. Wang C, Lin C, Tao Q, Zhao S, Liu H, Li L. Evaluation of calcium-binding protein A11 promotes the carcinogenesis of hypopharyngeal squamous cell carcinoma via the PI3K/AKT signaling pathway. *Am J Transl Res*. 2019;11(6):3472–3480.
24. Zheng Q, Wang B, Gao J, et al. CD155 knockdown promotes apoptosis via AKT/Bcl-2/Bax in colon cancer cells. *J Cell Mol Med*. 2018;22(1):131–140. doi:10.1111/jcmm.13301
25. Huang GL, Chen QX, Ma JJ, Sui SY, Wang YN, Shen DY. Retinoic acid receptor alpha facilitates human colorectal cancer progression via Akt and MMP2 signaling. *Onco Targets Ther*. 2019;12:3087–3098. doi:10.2147/OTT.S200261
26. Fu Y, Su L, Cai M, et al. Downregulation of CPA4 inhibits non small-cell lung cancer growth by suppressing the AKT/c-MYC pathway. *Mol Carcinog*. 2019;58(11):2026–2039. doi:10.1002/mc.23095
27. Zhou X, Yang Y, Ma P, et al. TRIM44 is indispensable for glioma cell proliferation and cell cycle progression through AKT/p21/p27 signaling pathway. *J Neurooncol*. 2019;145(2):211–222. doi:10.1007/s11060-019-03301-0

28. Wu T, Bao T, Liang D, Wang L. Epithelial-mesenchymal transition contributes to malignant phenotypes of circulating tumor cells derived from gastric cancer. *Biocell*. 2019;43(4):293–298. doi:10.32604/biocell.2019.07841

29. Wang L, Wu XT, Wang BW, Wang Q, Han LY. miR-145 regulates proliferation and chemotherapy sensitivity of ovarian carcinoma. *Eur J Gynaecol Oncol*. 2018;39(4):634–640. doi:10.3892/ol.2018.9446

30. Ma T, Wang F, Wang X. LncRNA LINC01772 promotes metastasis and EMT process in cervical cancer by sponging miR-3611 to relieve ZEB1. *Biocell*. 2019;43(3):191–198. doi:10.32604/biocell.2019.06989

31. Siegel RL, Miller KD, Jemal A. Cancer Statistics 2017. *CA Cancer J Clin*. 2017;67(1):7–30. doi:10.3322/caac.20073