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RESEARCH ARTICLE

Specificity protein 1 regulates fascin expression in esophageal squamous cell carcinoma as the result of the epidermal growth factor/extracellular signal-regulated kinase signaling pathway activation

Xiao-Feng Lu · En-Min Li · Ze-Peng Du · Jian-Jun Xie · Zhang-Yan Guo · Shu-Ying Gao · Lian-Di Liao · Zhong-Ying Shen · Dong Xie · Li-Yan Xu

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Abstract The overexpression of fascin in human carcinomas is associated with aggressive clinical phenotypes and poor prognosis. However, the molecular mechanism underlying the increased expression of fascin in cancer cells is largely unknown. Here, we identified a Sp1 binding element located at −70 to −60 nts of the *FSCN1* promoter and validated that Sp1 specifically bound to this element in esophageal carcinoma cells. Fascin expression was enhanced by Sp1 overexpression and blocked by Sp1 RNAi knockdown. Specific inhibition of ERK1/2 decreased phosphorylation levels of Sp1, and thus suppressed the transcription of the *FSCN1*, resulting in the down-regulation of fascin. Stimulation with EGF could enhance fascin expression via activating the ERK1/2 pathway and increasing phosphorylation levels of Sp1. These data suggest that *FSCN1* transcription may be subjected to the regulation of the EGF/EGFR signaling pathway and can be used as a viable biomarker to predict the efficacy of EGFR inhibitors in cancer therapies.

Keywords Fascin · Sp1 · EGF · ERK1/2 · Esophageal squamous cell carcinoma · *FSCN1*

Abbreviations

AhR	Aryl hydrocarbon receptor
CDK	Cyclin-dependent kinase
c-erbB-2	v-erb-b2 Erythroblastic leukemia viral oncogene homolog 2
CK II	Casein kinase II
CREB/AP-1	cAMP responsive element binding protein/ active protein-1
CBP	CREB binding protein
CTGF	Connective tissue growth factor
CYR61	Cysteine-rich protein 61
DMNB	4,5-Dimethoxy-2-nitrobenzaldehyde
DIG	Digoxigenin
DNA-PK	DNA-dependent protein kinase
DRB	5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
ESCC	Esophageal squamous cell carcinoma
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
IP	Immunoprecipitation
JNK	C-Jun N-terminal kinase
KLF	Kruppel-like factor

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X.-F. Lu · Z.-P. Du · S.-Y. Gao · L.-D. Liao · Z.-Y. Shen · L.-Y. Xu (✉)
Institute of Oncologic Pathology,
The Key Immunopathology Laboratory of Guangdong Province,
Shantou University Medical College, No. 22, Xinling Road,
Shantou 515041, People's Republic of China
e-mail: liyanxu1130@yahoo.com.cn

X.-F. Lu · E.-M. Li (✉) · J.-J. Xie · Z.-Y. Guo · S.-Y. Gao · D. Xie (✉)
Department of Biochemistry and Molecular Biology,
Shantou University Medical College, Shantou,
People's Republic of China

D. Xie
Laboratory of Molecular Oncology,
Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences,
Shanghai, People's Republic of China

MAPK	Mitogen-activated protein kinases
MEK	MAPK/ERK kinase
MMA	Mithramycin A
PCR	Polymerase chain reaction
p-ERK1/2	Phosphorylated ERK1/2
PKA	Protein kinase A
PKC	Protein kinase C
p-Sp1	Phosphorylated Sp1
rhSp1	Recombinant human Sp1 protein
RIPA	Radioimmunoprecipitation assay
RNAi	RNA interference
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
Sp1	Specificity protein 1
TGF-β1	Transforming growth factor-β 1
TCF	Transcription factor

Introduction

Fascin, an actin-bundling protein, was initially identified in the extracts of unfertilized sea urchin eggs [1–3]. It is highly expressed in normal mesenchymal, endothelial, dendritic, and neuronal cells, but generally absent or at the low levels of expression in normal epithelial cells [4]. Fascin is a cytoplasmic protein functioning to form the parallel actin bundles that support the lamellipodial and filopodial cell protrusions, which are key cellular structures for environmental guidance and cell migration [5, 6]. The overexpression of fascin has been reported in various human carcinomas examined to date [7–15]. High expression levels of fascin in various primary carcinomas were consistently shown to correlate with aggressive tumor phenotypes and poor prognosis [7–15]. In esophageal squamous cell carcinomas (ESCCs), fascin was reported to also be overexpressed and its increased expression was associated with the poor prognosis of ESCC patients [16]. Our previous studies revealed that the overexpression of fascin in ESCCs was an early event in tumor development and fascin depletion via RNAi resulted in attenuated proliferation and invasion of ESCC cells [17, 18]. Recently, we further demonstrated that fascin promoted the proliferation and invasion of ESCC cells by modulating the expression of connective tissue growth factor (CTGF) and cysteine-rich protein 61 (CYR61) via the transforming growth factor, beta 1 (TGF-β1) pathway [19]. These findings suggest that fascin might play an important role in the development and progression of ESCCs. However, the molecular mechanism underlying the up-regulation of fascin in ESCC cells is largely unknown.

Human fascin is encoded by *FSCN1* located at chromosome 7p22. The core promoter region of *FSCN1*, harboring a putative GC box, a composite cAMP responsive element/active protein-1 binding site (CREB/AP-1) and a TATA box, is located at 100 bps upstream from the transcription start site [20, 21]. Moreover, evidence has shown that the key elements of *FSCN1* were located between −74 and −41 nts of the promoter [22]. The expression level of fascin was lower in CREB binding protein (CBP)-depleted NT2 neuronal precursor cells compared to controls, suggesting that CBP might participate in the transcriptional regulation of this gene [23]. Recently, Hashimoto et al. [21] identified novel roles for CREB and the aryl hydrocarbon receptor (AhR) as major specific regulators of *FSCN1* transcription in human breast and colon carcinoma cells. It has also been reported that the β-catenin-TCF (transcription factor) signaling pathway was involved in the regulation of *FSCN1* transcription in human colorectal cancer cells [10]. However, in v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (c-erbB-2) over-expressed breast cancer cells with elevated fascin levels, the result of luciferase reporter analysis did not support the involvement of the β-catenin-TCF signaling pathways in the modulation of fascin expression [24]. These data indicated that distinctive regulatory elements contributed to the promoter activity of *FSCN1* in different cell types. We hypothesized that there may be novel signaling pathways involved in the up-regulation of *FSCN1* transcription in ESCC cells through increased binding of the transcriptional factors to the key elements of *FSCN1* promoter. The purpose of this study was to identify the transcriptional factor(s) that bind to the key element(s) of the *FSCN1* promoter region and to investigate the signaling pathway(s) that control fascin expression through activating the transcriptional factor(s) in ESCC cells.

Materials and methods

Cell lines, plasmids, and other materials

EC109 [25], EC18 (also named EC/CUHK2) [26], KYSE150, and KYSE180 [27] human ESCC cell lines were cultured in 199 medium (Invitrogen, Carlsbad, CA, USA) plus 10% newborn calf serum. HeLa human cervical cancer and BGC823 stomach cancer cell lines obtained from the Type Culture Collection of Chinese Academy of Sciences were grown under standard conditions. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. CMV-Sp1 and CMV-Sp3 expression plasmids were kindly provided by Dr. Guntram Suske (Marburg, Germany). ΔN-TCF4 (a TCF4 deletion mutant lacking 30 N-terminal amino acids and being capable of binding to DNA but not to

β-catenin), the reporter constructs TopFlash and β-catenin constructs, generated through site-directed mutagenesis, were generous gifts of Dr. Arnold J. Levine, which included the following: wt β-catenin (wild-type β-catenin), 4145β-catenin (a β-catenin mutant having a threonine and serine at positions 41 and 45, respectively, mutated to alanine and being a stable mutant with longer half-life), 4145TV (4145β-catenin mutant having an additional double-point mutation that changed Thr-120 and Val-122 to alanines at the α-catenin-binding region), NLS (4145β-catenin mutant containing SV40 large T antigen nuclear localization signal sequence; localized to nucleus [immunofluorescence]) and 4145ΔC (4145β-catenin mutant having a deletion of the C-terminus [putative transcriptional activation domain]). Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor (U0126) and p38 MAPK inhibitor (SB203580) were purchased from Promega (Madison, WI, USA). C-Jun N-terminal kinase (JNK) inhibitor (SP600125), protein kinase A (PKA) inhibitor (H-89), protein kinase C (PKC) inhibitor (Bisindolylmaleimide I), casein kinase II (CK II) inhibitor (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, DRB), DNA-dependent protein kinase (DNA-PK) inhibitor (4,5-dimethoxy-2-nitrobenzaldehyde, DMNB) and cyclin-dependent kinase (CDK) inhibitor (Olomoucine) were purchased from Calbiochem (La Jolla, CA, USA).

Assembly of reporter constructs

Four overlapping fragments of the human *FSCN1* promoter (−2,902/+122, −255/+122, −74/+122, and −40/+122) were cloned by polymerase chain reaction (PCR) and inserted into pGL3-Basic reporter vector (Promega) to create the −2,900, −255, and −74 constructs [22]. Six or eleven nucleotides in the −74 construct were mutated by PCR-based site-directed mutagenesis. The fragments were generated by PCR amplification of the wild-type −74 construct, using the upstream mutant primer with the downstream primer R-Fasn and Pfu DNA polymerase (Promega). The primer sequences are listed in Table 1. The product was digested with *Mlu*I and *Hin*dIII, and ligated into corresponding sites in pGL3-Basic reporter vector. The resulting substitution construct was sequenced to confirm the desired mutation.

Electrophoretic mobility shift assay

Nuclear extracts from ESCC cells were prepared using the nuclear extraction kit (Active Motif, Tokyo, Japan) according to the manufacturer's instructions. Electrophoretic mobility shift assay (EMSA) was performed as described [28]. Equimolar amounts of complementary and single-stranded oligonucleotides were annealed and labeled with digoxigenin (DIG)-ddUTP by terminal transferase using a DIG Gel Shift kit (Roche, Mannheim, Germany). The oligonucleotide probes used in EMSAs are listed in Table 2. These probes corresponded to human *FSCN1* sequence from nt −74 to nt −41. For supershift analysis, 1 μg of antibody to Sp1 (Santa Cruz, California, CA, USA) was added to the extracts or recombinant human Sp1 protein (rhSp1) followed by pre-incubation at 4°C for 1 h

Table 1 Primers used in this study

Primers	Sequences	Names of constructs
Primers for site-directed mutation constructs		
F −74/−69s	5′-ACGCGT*GTCGAC*GGGCGTGGCCTGGTGGCGCTGACGTCAC-3′	−74/−69s
F −70/−60s	5′-ACGCGTACAG*TATCTATGTTA*CTGGTGGCGCTGACGTCAC-3′	−70/−60s
F −68/−63s	5′-ACGCGTACAGGG*GTCGAC*GGCCTGGTGGCGCTGACGTCAC-3′	−68/−63s
F −66/−57s	5′-ACGCGTACAGGGGG*TATGTTAAGT*GTGGCGCTGACGTCAC-3′	−66/−57s
F −62/−57s	5′-ACGCGTACAGGGGGGCGT*GAATTC*GTGGCGCTGACGTCAC-3′	−62/−57s
R-Fasn	5′-AAGCTTGGTGGCAGTAGACGAGAGGCCGCTG-3′	
Primers for quantitative RT-PCR		
F-fascin	5′-GTCTGCCAATCAGGACGAGGA-3′	
R-fascin	5′-TCACGCCACTCGATGTCAAAG-3′	
F-Sp1	5′-CGGATGAGCTACAGAGGCACAA-3′	
R-Sp1	5′-TCCTCATGAAGCGCTTAGGACAC-3′	
F-Sp3	5′-GCTTGCACCTGTCCCAACTGTA-3′	
R-Sp3	5′-CTCCAGAATGCCAACGCAGA-3′	
F-GAPDH	5′-GCACCGTCAAGGCTGAGAAC-3′	
R-GAPDH	5′-TGGTGAAGACGCCAGTGGA-3′	

F forward primer, *R* reverse primer, *GAPDH* glyceraldehydes 3-phosphate dehydrogenase. The cutting sites are *underlined*. The substituted sites and sequences are shown in *italics*

Table 2 Probes and competitors for EMSA

Names	Sequences (from −74 to −41)	Substituted sites
−74/−41w	5′-ACAGGGGGGCGTGGCCTGGTGGCGCTGACGTCAC-3′	–
	5′-GTGACGTCAGCGCCACCAGGCCACGCCCCCCTGT-3′	
−74/−69s	5′-*GTCGAC*GGGCGTGGCCTGGTGGCGCTGACGTCAC-3′	−74 to −69
	5′-GTGACGTCAGCGCCACCAGGCCACGCCC*GTCGAC*-3′	
−70/−60s	5′-ACAG*TATCTATGTTA*CTGGTGGCGCTGACGTCAC-3′	−70 to −60
	5′-GTGACGTCAGCGCCACCAG*TAACATAGATA*CTGT-3′	
−68/−63s	5′-ACAGGG*GTCGAC*GGCCTGGTGGCGCTGACGTCAC-3′	−68 to −63
	5′-GTGACGTCAGCGCCACCAGGCC*GTCGAC*CCCTGT-3′	
−66/−57s	5′-ACAGGGGG*TATGTTAAGT*GTGGCGCTGACGTCAC-3′	−66 to −57
	5′-GTGACGTCAGCGCCAC*ACTTAACATA*CCCCCTGT-3′	
−62/−57s	5′-ACAGGGGGGCGT*GAATTC*GTGGCGCTGACGTCAC-3′	−62 to −57
	5′-GTGACGTCAGCGCCAC*GAATTC*ACGCCCCCCTGT-3′	
−50/−45s	5′-ACAGGGGGGCGTGGCCTGGTGGCG*GAATTC*TCAC-3′	−50 to −45
	5′-GTGA*GAATTC*CGCCACCAGGCCACGCCCCCCTGT-3′	
−50/−41s	5′-ACAGGGGGGCGTGGCCTGGTGGC*TAGTGTTGACA*-3′	−50 to −41
	5′-*TGTCAACACTA*GCCACCAGGCCACGCCCCCCTGT-3′	

The substituted sequences are shown in *italics*

prior to the binding reactions. For blocking Sp1 binding to DNA, DNA probes were pre-incubated for 1 h at 4°C with increasing concentrations of mithramycin A (MMA) (Sigma-Aldrich, St. Louis, MO, USA) before the binding reactions. In specific competition experiments, a 125-fold molar excess of unlabeled oligonucleotides was added to the binding reactions.

Transient transfections and luciferase reporter assay

Cells were seeded in 96-well plates at 1.5×10^5 cells/ml, grown to 50–80% confluency and co-transfected with 0.5 μg of the experimental reporter vectors described above, which contains a modified coding region for *firefly* (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells, and 0.01 μg of *Renilla* luciferase plasmid pRL-TK (Promega) containing a cDNA (*Rluc*) encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis,* as an internal control for transfection efficiency using Superfect Transfection Reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After transfection, cells were incubated for 48 h and harvested in Passive Lysis Buffer (Promega). The luciferase reporter activity of the lysates was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations. For analyzing the effect of kinase inhibitors or recombinant human TGF-β1 or recombinant human epidermal growth factor (EGF) (Invitrogen) on the luciferase reporter activity, after transfection with the reporters for 24 h, the cells were treated with kinase inhibitors or selective growth factors for another 24 h before being harvested. All transfections were performed in triplicate, and the experiment was repeated three times.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the EC109 cells with Trizol reagent (Invitrogen) and reverse transcribed to cDNA using the PrimeScript™ reverse transcription polymerase chain reaction (RT-PCR) kit (TaKaRa, Dalian, China). The quantitative RT-PCR assay was carried out with the Rotor-Gene 6000 system (Corbett Life Science, Sydney, Australia) using SYBR® Premix Ex Taq™ (TaKaRa) according to the manufacturer's instructions. Primers for quantitative RT-PCR are shown in Table 1. The relative value from the vehicle-treated control group was considered equal to one arbitrary unit. All PCR reactions were performed in triplicate, and the experiment was repeated three times.

Western-blot analysis

Total cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium desoxycholate, 0.1% (wt/vol) SDS] containing a complete protease inhibitor cocktail (Santa Cruz). Western-blot analysis was performed as described [28] with the following primary antibodies: mouse anti-fascin (Dako,

Glostrup, Denmark), mouse anti-β-actin (Sigma), mouse anti-Sp1 (Abcam, Cambridge, UK) and mouse anti-nucleoporin p62, mouse anti-β-catenin, mouse anti phosphorylated ERK1/2 (p-ERK1/2) and rabbit anti-ERK1/2 (Santa Cruz). The experiments were repeated three times.

Small interfering RNA

RNAi Human/Mouse Starter Kit and silencer small interfering RNA (siRNAs) against human Sp1 in a FlexiTube format were purchased from QIAGEN. Four species of Sp1 siRNAs (Sp1 siRNA1-4) against different Sp1 target sequences (5′-TTGGGTAAGTGTGTTGTTTAA-3′; 5′-TCCCAGAAAGTATATACTGAA-3′; 5′-CAGCAAGTTCTGACAGGACTA-3′; 5′-CTAGGACGCAATAAATTTATA-3′) were used. EC109 cells at 70–80% confluency were transfected with a negative control siRNA or Sp1 siRNAs at 5 nM using HiPerFect Transfection Reagent according to the manufacturer's recommendations. After 48 h, the cells were harvested to determine the effect of siRNAs on the endogenous mRNA expression of Sp1 and fascin using quantitative RT-PCR. For reporter assays, at 24 h post-transfection of the siRNAs, EC109 cells were transfected with the appropriate promoter–reporter construct and pRL-TK using SuperFect transfection reagent (QIAGEN) and cultured for another 24 h. The luciferase reporter activity was then measured as described above. For Western-blot analysis, at 48 h post-transfection of the siRNAs, the cells were harvested and total proteins were extracted with RIPA buffer.

Immunoprecipitation

Immunoprecipitation (IP) was carried out as described [29] with minimal modifications. Briefly, cells were grown until confluence, washed twice with phosphate-buffered saline, and lysed at 4°C in Phosphosafe Extraction Reagent (Calbiochem). After centrifugation at 20,000 × g for 20 min at 4°C and having been precleared by incubation with protein A agarose bead slurry (Sigma) for 2 h at 4°C, the lysates were incubated overnight at 4°C with mouse monoclonal anti-phosphoserine IgG conjugated to agarose beads (Sigma). The agarose beads were collected by centrifugation, washed twice in ice-cold RIPA buffer, boiled in Laemmli sample buffer, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblot analysis. Three independent experiments were carried out.

Statistical analysis

Comparisons between data sets were performed using the χ^2 test and t test when appropriate. $p < 0.05$ was used to determine statistical significance. All statistical tests were performed with SPSS statistic software (SPSS® 13.0 by SPSS Inc).

Results

FSCN1 transcriptional activation does not depend on the β-catenin-TCF signaling pathway in ESCC cells

Given the presence of multiple conserved TCF binding sites, but no significant similarities within mouse *Fscn1* [10] and human *FSCN1* promoters (GeneBank accession no. EU486847, Supplementary Fig. S1) and the finding that β-catenin-TCF transcription factor complex can regulate fascin expression in human colorectal [10], but not breast cancer cells [24], it is important to assess the ability of human *FSCN1* promoter fragments to respond to various β-catenin and TCF mutants in ESCC cells. The −2,900 construct for *FSCN1* promoter was co-transfected with various β-catenin mutant plasmids and TCF constructs (see "Materials and methods" for further details) into EC109 and KYSE150 cells characterized by low and high endogenous expression levels of β-catenin, respectively, and comparable fascin protein levels (Fig. 1a, b). Cotransfection of the wt β-catenin, 4145β-catenin, 4145TV or NLS construct enhanced the luciferase activity of the −2,900 *FSCN1* promoter construct by less than twofold in both EC109 cells (Fig. 1c, left) and KYSE150 cells (Fig. 1d, left). These β-catenin plasmids readily activated TopFlash, the luciferase reporter used as the positive control, transcriptionally (Fig. 1c, d, right). Cotransfection of the 4145ΔC construct encoding a protein not able to bind to TCF, or ΔN-TCF4 plasmid, encoding a protein able to bind to DNA but not to β-catenin, appeared to slightly enhance the luciferase response of the −2,900 *FSCN1* promoter construct, in contrast to the decreased luciferase response observed for TopFlash (Fig. 1c, d). Similar results were obtained from transient cotransfections using the −74 *FSCN1* promoter reporter in both EC109 cells and KYSE150 cells (Supplementary Fig. S2). These results suggest that the β-catenin-TCF transcription factor complex does not regulate the transcriptional activity of *FSCN1* promoter in ESCC cells.

Interaction between nuclear proteins and the core promoter region of *FSCN1*

Since the segment between −74 and −41 seems to be critically important to the *FSCN1* promoter activity [22], we looked for potential factors interacting with this region by EMSA. The nuclear extracts prepared from EC109 cells were incubated with DIG-labeled oligonucleotides

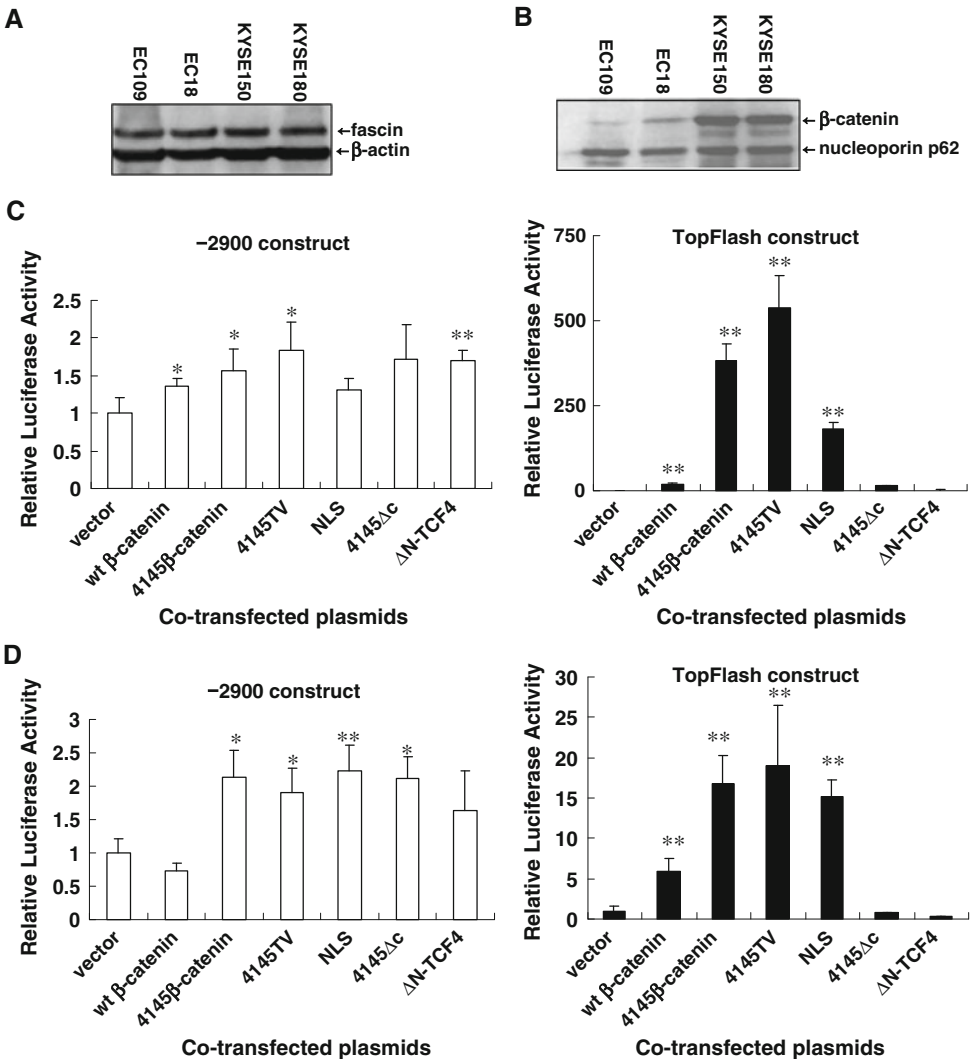


Fig. 1 The *FSCN1* promoter is not responsive to elevated levels of *β*-catenin or TCF protein. **a** The expression of fascin protein in ESCC cells. Whole cell extracts from ESCC cells were prepared with RIPA buffer. The blot (25 μg of protein per lane) was hybridized with an antibody against fascin or *β*-actin as a control for loading. **b** The expression of *β*-catenin protein in ESCC cells. Nuclear extracts from ESCC cells were prepared and analyzed by Western-blot using 60 μg of protein per lane. The blot was hybridized with an antibody against *β*-catenin or nucleoporin p62 as a control for loading. **a**, **b** Representative blot from three independent experiments is shown. **c**, **d** The effect of transient expression of *β*-catenin or TCF on the activity of *FSCN1* promoter in ESCC cells. Transient transfections were performed in EC109 cells (**c**) and KYSE150 cells (**d**), using 1.0 μg of total plasmids (0.5 μg of reporter plasmid and 0.5 μg of *β*-catenin or TCF plasmid or empty vector) and 0.01 μg of *Renilla* luciferase plasmid pRL-TK as an internal control. The reporter constructs used here are the −2,900 construct of the *FSCN1* promoter and TopFlash as a positive control. The reported luciferase values were normalized to the *Renilla* luciferase activity and then presented relative to that of the empty vector, which was set as 1. The results of a representative experiment were presented as mean ± SD of the three independent samples. All experiments were repeated three times. $*p < 0.05$ or $**p < 0.01$, compared with the group transfected with the empty vector

spanning from nt −74 to −41 (−74/− 41w) of the fascin promoter. This binding reaction generated one retarded protein–DNA complex (shift band) (Fig. 2a, lane 2). To determine the specificity of the binding complex, we added 125-fold molar excess of unlabeled −74/− 41w oligonucleotide to the reaction system. As a result, the shift band was completely ablated (Fig. 2a, lane 3). Based on the putative transcription factor binding site information presented in Supplementary Fig. S1, an Sp1 binding site located between nt −70 and −60 and a CREB/AP-1 binding site located between nt −50 and −41 of the promoter were identified. Next, we tried to determine which one of these two sites was responsible for forming these protein–DNA complexes. Interestingly, when 125-fold molar excess of unlabeled oligonucleotides covering the same region but each containing a substituted sequence (Fig. 2a, *lanes 4 through 10*) as listed in Table 1, were added to the binding reaction, only the substituted

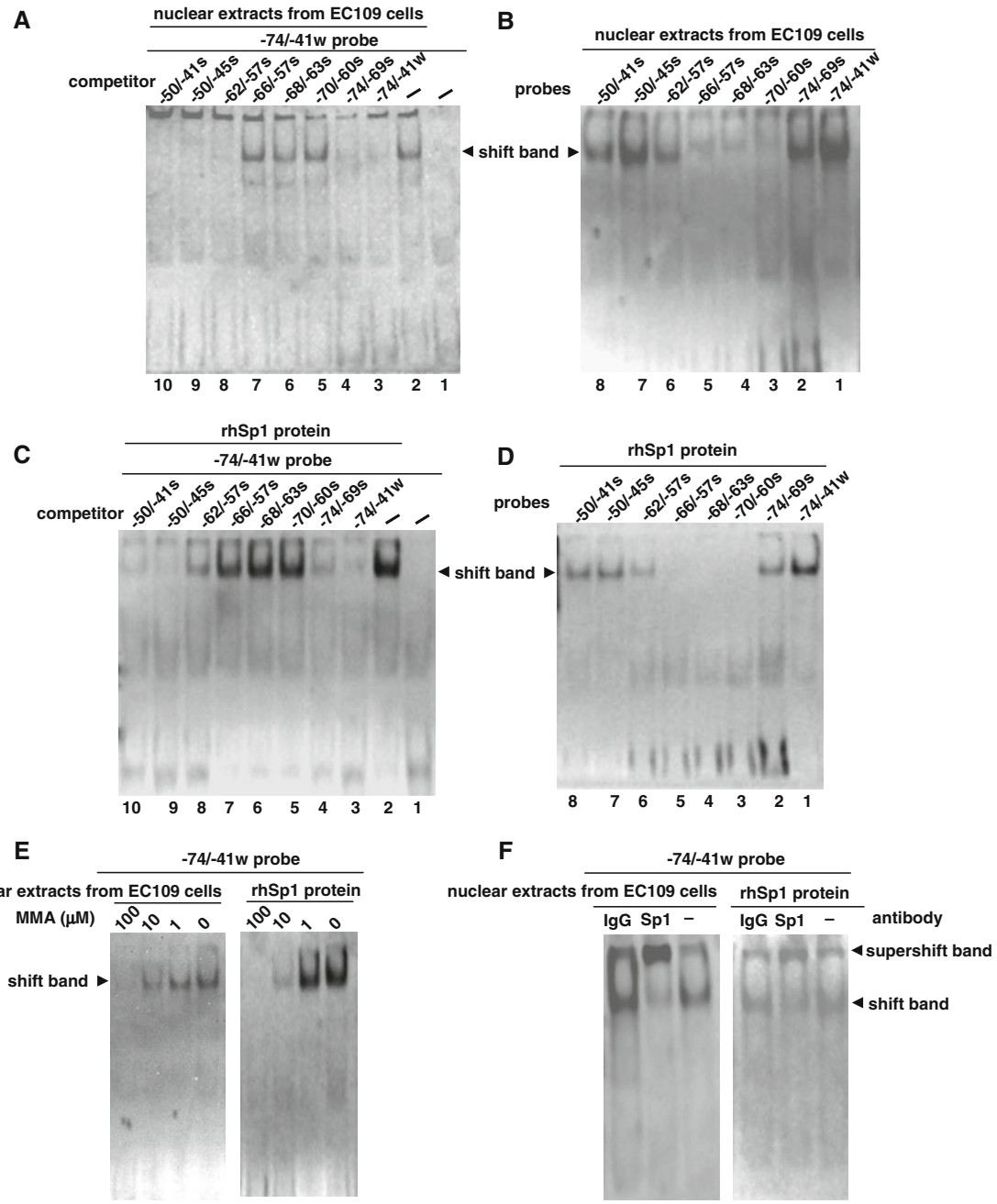


Fig. 2 Specific interaction between nuclear proteins and a Sp1 binding site in the *FSCN1* promoter. In **a**, the nuclear extract was prepared from EC109 cells. Two micrograms of extracts was incubated with a DIG-labeled oligonucleotide spanning the segment from −74 nt to −41 nt (−74/−41w; *lanes 2 through 10*) of the *FSCN1* promoter. The binding specificity was confirmed by chasing labeled −74/−41w with a 125-fold molar excess of unlabeled −74/−41w (*lane 3*) or the oligonucleotides covering the same segment but containing a substituted sequence (*lanes 4 through 10*), which are listed in Table 2. Labeled probe free of the nuclear extract migrated as shown in *lane 1*. In **b**, 2 μg of nuclear extracts prepared from EC109 cells was incubated with labeled oligonucleotides covering the segment from −74 nt to −41 nt but containing the substituted sequence as described in **a**. In **c**, **d**, recombinant protein rhSp1 (0.2 μg) was incubated with labeled probes and then analyzed by EMSA as described in **a, b**. **e** Result of EMSA to show the effect of MMA on protein-DNA complex formation. DIG-labeled −74/−41w probe was pre-incubated with increasing concentrations of MMA (1, 10, or 100 μM) for 1 h at 4°C before being added to the binding reaction. In **f**, the proteins complexed to the labeled −74/−41w probe were identified by pre-incubating 2 μg of nuclear extracts from EC109 cells or 0.2 μg of rhSp1 with 1 μg of anti-Sp1 antibody or homologous IgG, followed by the binding reaction. Representative data from three independent experiments are shown here

oligonucleotides around the consensus binding site for Sp1 abolished the shift band (*lane 4 and lanes 8–10*), whereas mutated Sp1 binding site did not reduce the complex formation (*lanes 5–7*). The critical role of the putative Sp1 binding site for the complex formation was further confirmed by incubating the nuclear extracts with similarly labeled oligonucleotides covering the segment from nt −74 to −41 but containing the substituted sequence. As shown in Fig. 2b, only the wild-type Sp1 binding site led to the shift band (*lanes 2 and lanes 6–8*), whereas the mutated Sp1 binding sites and the wild-type CREB/AP-1 binding site could not (*lane 3–5*). Collectively, these data demonstrated that a specific interaction existed between nuclear proteins and the sequence containing the Sp1 binding site.

We then tried to identify the nuclear protein involved in forming this DNA–protein complex in EC109 cells. Since the element nt −70/−60 encodes the consensus-binding site for Sp1, the recombinant human Sp1 protein (rhSp1) was tested first. As shown in Fig. 2c, d, when rhSp1 was incubated with DIG-labeled nt −74/−41w or oligonucleotides covering the same segment but containing a substituted sequence in the presence or absence of specific competitors, the nature of the shift band was reminiscent of those shown in Fig. 2a, b. This indicated that the Sp1 transcription factor was present in the nuclear extracts from EC109 cells. For further confirmation, the effect of MMA, an antibiotic competitively preventing Sp family proteins from binding to DNA [30], on the complex formation was analyzed. Consistent with the result from rhSp1 protein (Fig. 2e, right), the complex formation of nuclear extracts from EC109 cells was reduced by MMA treatment in a dose-dependent manner (Fig. 2e, left). Importantly, incubation of the nuclear extracts or rhSp1 protein with the Sp1 antibody could generate the supershift complex as compared to the IgG control (Fig. 2f). Taken together, these data demonstrated that transcription factor Sp1 was indeed bound to this nt −70/−60 sequence within the *FSCN1* promoter in ESCC cells.

Isolation of a Sp1 binding site as a key element in the *FSCN1* promoter

To verify that this Sp1 binding site plays a critical role in promoter activity, we substituted the Sp1 binding site or the sequences around it in the −74 construct and transfected the mutated constructs into four fascin-bearing ESCC cell lines, including EC109, EC18, KYSE150, and KYSE180 cells (Fig. 1a). When compared to the −74 construct, the −74/−69s and −62/−57s constructs retained approximately 40–80% luciferase activity, while the −68/−63s and −66/−57s construct only retained 20–40%. Specially, when the intact Sp1 binding site (−70/−60) was mutated, its luciferase activity was dramatically reduced to 10% (Fig. 3). Similar data were obtained from the cervical carcinoma HeLa cells and gastric cancer BGC823 cells (Supplementary Fig, S3). All these data supported that the Sp1 binding site (−70/− 60) functioned as an essential element of the *FSCN1* promoter in human cancer cells.

Sp1 up-regulates *FSCN1* transcription

The evidence above prompted us to explore the biological function of this Sp1 binding site within the *FSCN1* promoter. EC109 cells were transfected with the expression vectors CMV-Sp1 and CMV-Sp3, respectively, and the empty vector pCMV was used as a control. As shown in Fig. 4, the levels of both fascin mRNA (Fig. 4c) and protein (Fig. 4d) were significantly increased after the

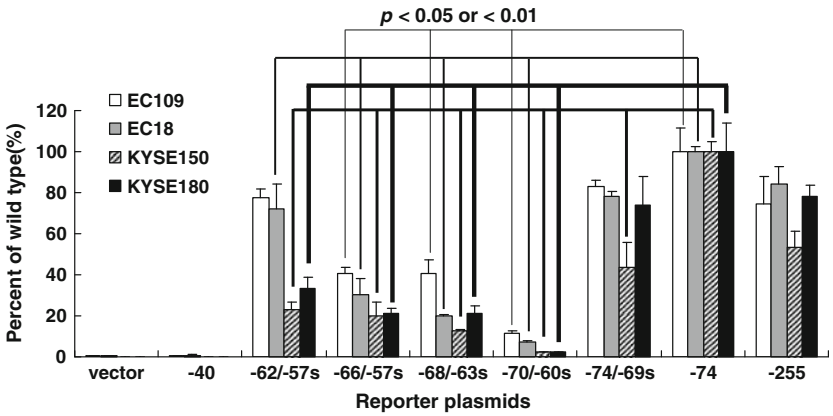


Fig. 3 Identification of an Sp1 binding site as the key element for the *FSCN1* promoter in ESCC cells. The site-directed mutagenesis constructs were prepared as described in "Material and methods" and cotransfected with pRL-TK, an internal control, into ESCC cell lines. The luciferase activities of mutant constructs were shown as a ratio compared to that of the −74 construct, which was set as 100%. The results of a representative experiment were presented as mean ± SD of three independent samples. The experiments were repeated three times

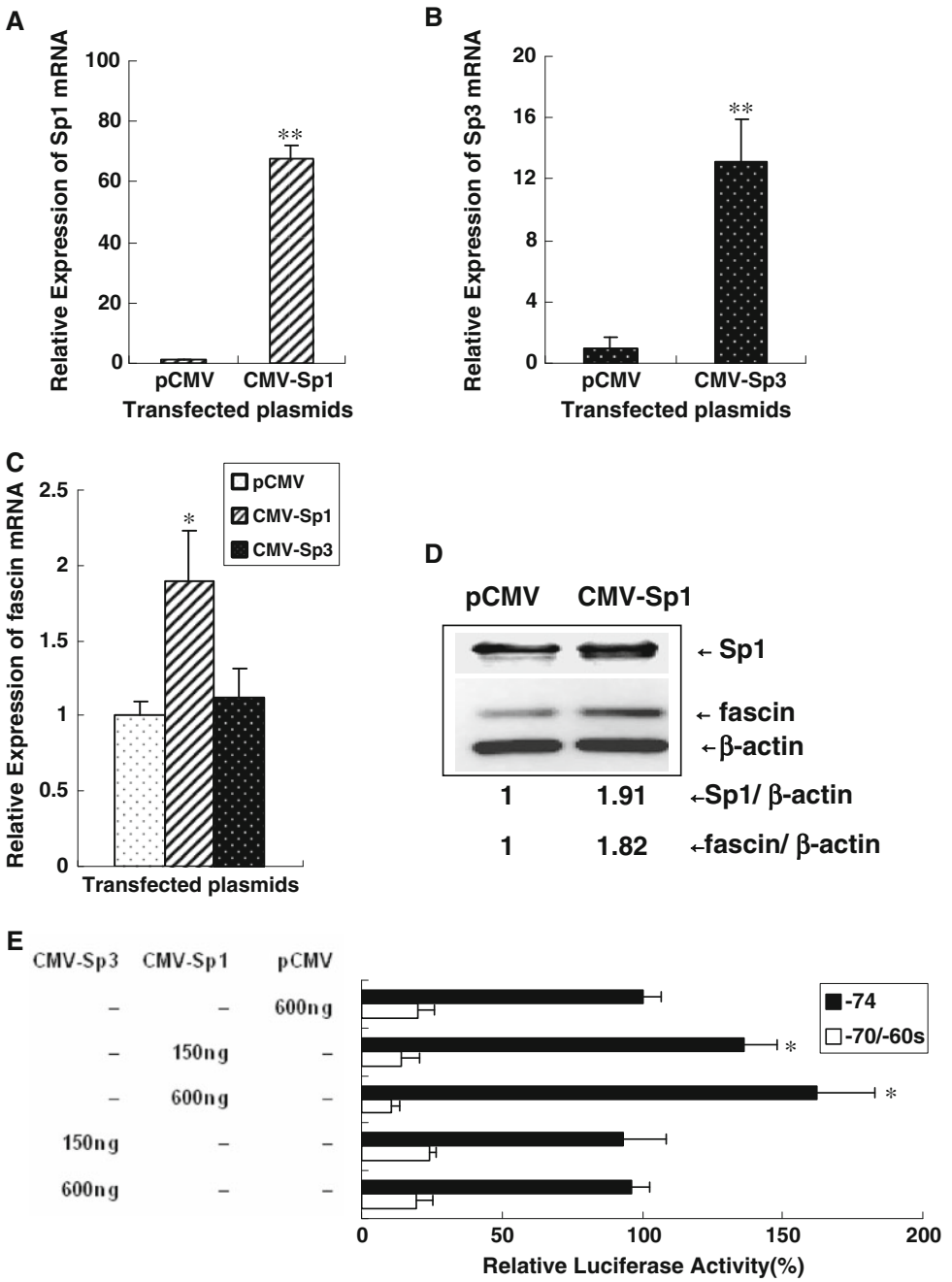


Fig. 4 Up-regulation of fascin expression by elevated levels of Sp1 in EC109 cells. The results of quantitative RT-PCR analysis of Sp1, Sp3, and fascin mRNA expression are shown in **a**–**c**, respectively. EC109 cells were transfected with indicated amounts of the empty vector pCMV, and expression constructs CMV-Sp1 or CMV-Sp3, respectively. The ratio of the expression levels of Sp1 (**a**), Sp3 (**b**) and fascin (**c**) mRNA to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) expression was presented as the relative expression normalized to that of pCMV control. A Western-blot of total proteins (25 μg) prepared from transfected EC109 cells is shown in **d**. The blot was hybridized with the antibody against Sp1, fascin, or β-actin. A representative blot from three independent experiments is shown here. The effect of transient expression of Sp1 or Sp3 on the *FSCN1* promoter activity in the EC109 cells is shown in **e**. The −74 or −70/−60s construct was co-transfected into EC109 cells with pRL-TK and pCMV, CMV-Sp1, or CMV-Sp3 at the indicated amounts. *Firefly* luciferase activity was normalized to *Renilla* luciferase activity and then presented as a percentage to that of the −74 construct in response to the pCMV control. **a–c, e** The results of a representative experiment were presented as mean ± SD of the three independent samples. The experiments were repeated three times. $*p < 0.05$ or $**p < 0.01$, compared to the group transfected with the control vector pCMV

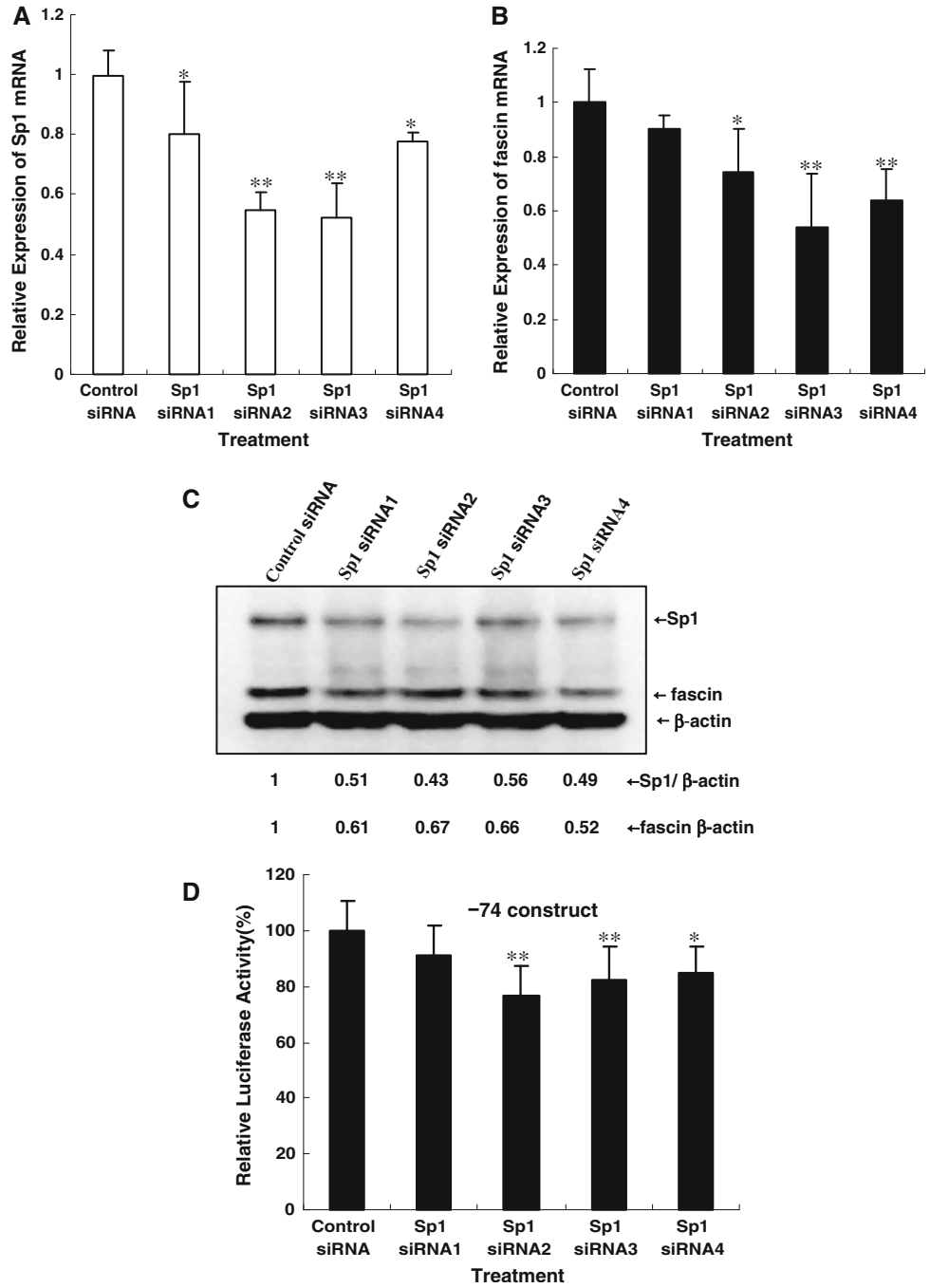


Fig. 5 Inhibition of fascin expression by Sp1 knockdown in EC109 cells. The inhibitory effect of Sp1 siRNAs on the mRNA of Sp1 (**a**) and fascin (**b**) was measured by quantitative RT-PCR. EC109 cells were transfected with 5 nM negative control siRNA or four species of Sp1 siRNAs (Sp1 siRNA1-4) for 24 h. The mRNA level of Sp1 and fascin relative to GAPDH was detected by quantitative RT-PCR and was presented as the relative expression normalized to the control. The result of a Western-blot is shown in **c**. A total protein extract was prepared from EC109 cells after transfection with 5 nM of negative control siRNA or Sp1 siRNAs for 24 h. The blot was hybridized and analyzed as described in Fig. 4d. A representative blot from three independent experiments is shown. The effect of Sp1 siRNAs on the activity of the *FSCN1* promoter was shown in **d**. EC109 cells were transfected with 5 nM of negative control siRNA or Sp1 siRNAs. After 24 h, the cells were co-transfected with the −74 construct and pRL-TK and cultured for another 24 h. The *firefly* luciferase activity was normalized to the *Renilla* luciferase activity and then presented as a percentage compared to that of the cells transfected with control siRNA. **a**, **b**, **d** The results of a representative experiment were presented as mean ± SD of the three independent samples. The experiments were repeated three times. $*p < 0.05$ or $**p < 0.01$, compared with the group transfected with control siRNA

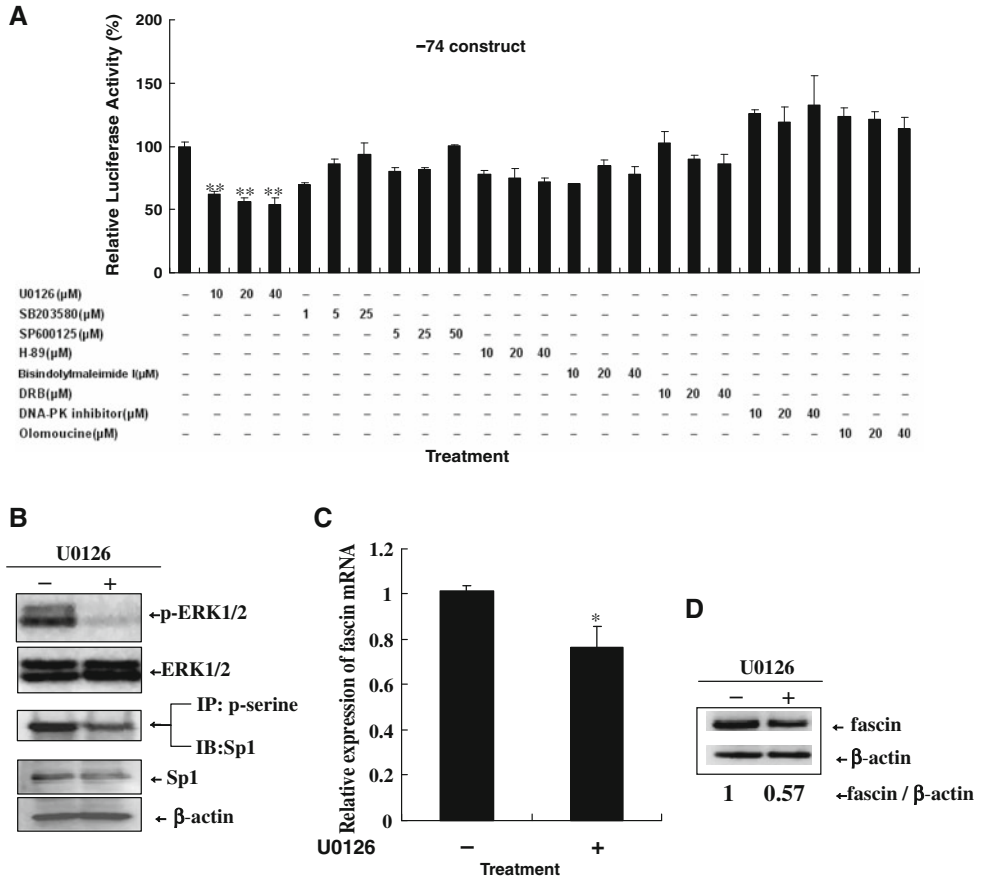
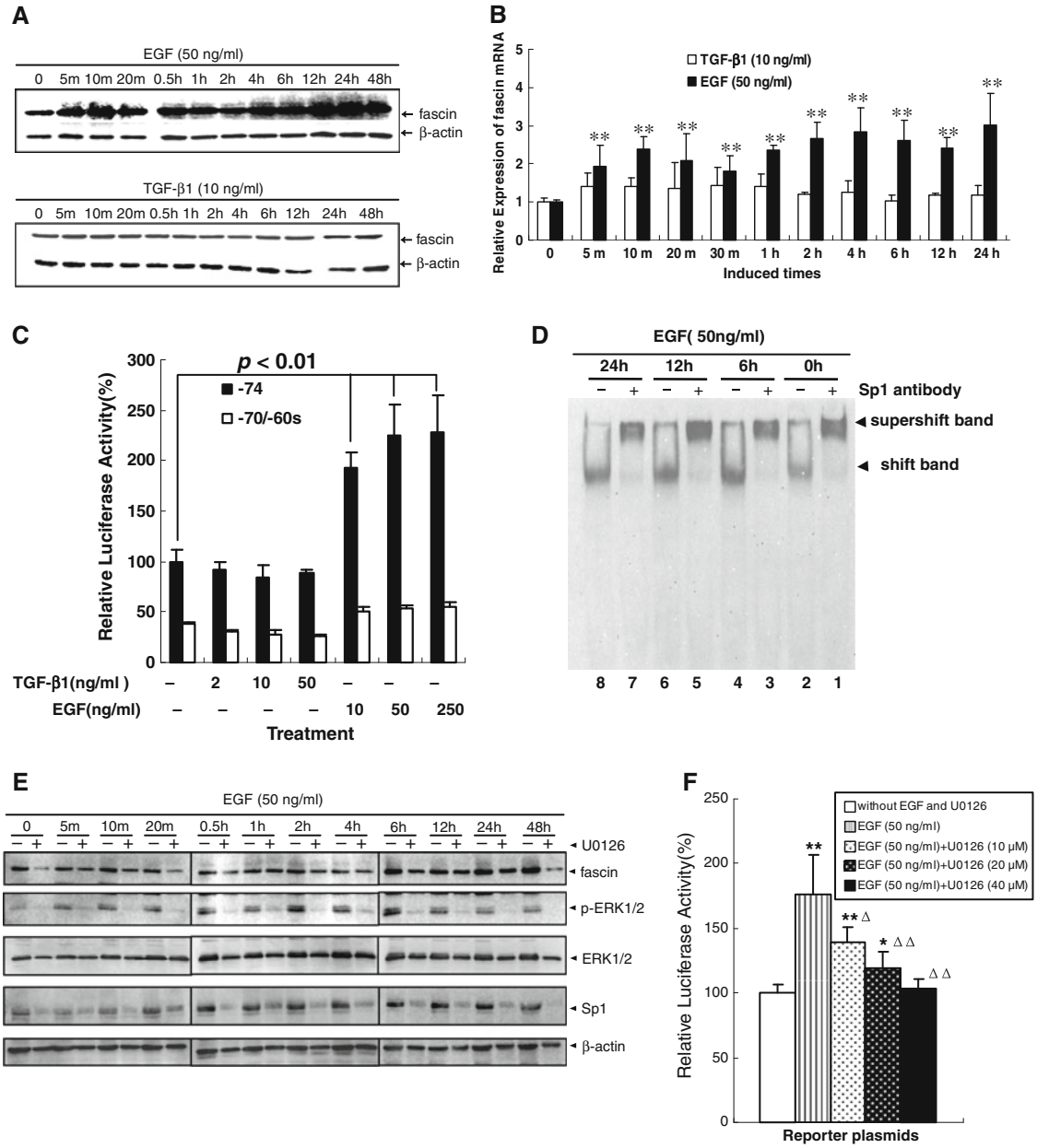


Fig. 6 The ERK1/2 pathway regulated fascin expression via activating Sp1 in EC109 cells. **a** The effect of kinase inhibitors on the activity of *FSCN1* promoter. EC109 cells were co-transfected with the −74 construct and pRL-TK for 24 h before kinase inhibitors were added for another 24 h. The *firefly* luciferase activity of the −74 construct was normalized to the *Renilla* luciferase activity and then presented as a percentage to that of control cells without inhibitor treatment. **b** The inhibitory effect of MEK1/2 inhibitor U0126 on the protein levels of p-ERK1/2 and p-Sp1. EC109 cells were treated with U0126 for 24 h and whole-cell extracts were analyzed by Western-blot analysis. The blot was hybridized with an antibody against p-ERK1/2 or Sp1 and thereafter re-hybridized with an ERK1/2 antibody or *β*-actin. For p-Sp1, the cell lysates (1 mg of total protein) were immunoprecipitated with 50 μl of mouse monoclonal anti-phosphoserine-agarose bead (p-serine) as described in *Materials and methods* and then detected with an antibody against Sp1. **c** The inhibitory effect of the inhibitor U0126 on the mRNA expression of fascin. The mRNA level of fascin was measured by quantitative RT-PCR after EC109 cells were incubated with U0126 for 24 h. **a**, **c** The results of a representative experiment were presented as mean ± SD of the three independent samples. The experiments were repeated three times. $*p < 0.05$ or $**p < 0.01$, compared to the group without kinase inhibitor. **d** The inhibitory effect of the inhibitor U0126 on the protein levels of fascin. The EC109 cells were treated as described in **b**. The blot was hybridized with the antibody against fascin and thereafter re-hybridized with a *β*-actin antibody for the loading control. The relative expression of fascin is indicated via the ratio of band density of fascin to *β*-actin. **b**, **d** A representative blot from three independent experiments is shown

introduction of exogenous Sp1 (Fig. 4a, b), while overexpression of Sp3 (Fig. 4b) showed no effects on the expression of fascin (Fig. 4c). To determine whether Sp1-induced elevation of *FSCN1* mRNA in EC109 cells was through its effects on the activity of the *FSCN1* promoter, the −74 or −70/−60s construct was co-transfected into EC109 cells with pRL-TK, as well as pCMV, CMV-Sp1, or CMV-Sp3. The −70/−60s construct could only generate weak luciferase activity as previously shown in Fig. 3. In contrast, the luciferase activity of the −74 construct was significantly increased if accompanied with Sp1 overexpression, whereas the activity was not affected in the presence of Sp3 overexpression (Fig. 4e). To further confirm the crucial role of Sp1 in the transactivation of *FSCN1*, we down-regulated the expression of Sp1 by RNAi in EC109 cells. As shown in Fig. 5, both quantitative RT-PCR and Western-blot analysis showed that the expression level of endogenous Sp1 was effectively reduced in cells transfected with the specific Sp1 siRNA as compared to the control. Sp1 knock-down inhibited fascin expression as well as the activity of the *FSCN1* promoter concomitantly. These results strongly suggest that Sp1 regulates fascin expression in EC109 cells through transcriptional activation.



The mechanism underlying the regulation of fascin expression by Sp1

Several reports have indicated that phosphorylation of Sp1 via various kinase pathways is important for Sp1-dependent activation of target genes [31]. The ever-growing list of kinases phosphorylating Sp1 includes DNA-PK, CDK, PKA, PKC, CK II, ERK1/2, p38 MAPK, JNK, etc. [32–34]. To investigate the role of Sp1 phosphorylation on fascin expression, EC109 cells were treated with specific inhibitors of several kinases, and the activity of the *FSCN1* promoter was assessed. As shown in Fig. 6a, only MEK1/2 inhibitor U0126 could significantly inhibit the activity of the *FSCN1* promoter, whereas other kinase inhibitors showed no effects. U0126 can block the activation of MEK1/2 and inhibit the subsequent phosphorylation and activation of ERK1/2 [35, 36]. Therefore, we tried to test whether the activation of ERK1/2 plays a critical role in the transactivation of the *FSCN1* transcription through Sp1 phosphorylation. EC109 cells were treated with 40 μM U0126 for 24 h and the total RNA or proteins were extracted for quantitative RT-PCR and Western-blot analysis, respectively. As shown in Fig. 6b, the phosphorylation of both ERK1/2 and Sp1 was inhibited by U0126 and the total protein levels of Sp1 were also reduced, while the total protein levels of ERK1/2 remained unchanged.

◀ **Fig. 7** EGF stimulated fascin expression via activating ERK1/2 and Sp1 in EC109 cells. **a** Changes of fascin protein expression in response to the growth factors. Whole-cell extracts from EC109 cells treated with TGF-β1 or EGF for different time intervals were analyzed by Western-blot. **b** Alteration of fascin mRNA level in response to the growth factor treatment. Facin mRNA expression was analyzed by quantitative RT-PCR after EC109 cells were incubated with TGF-β1 or EGF for different time intervals. $^{**}p < 0.01$, compared to untreated group. **c** Alteration of the *FSCN1* promoter activity in response to the growth factor treatment. EC109 cells were co-transfected with the −74 construct or −70/−60s construct with a mutated Sp1 binding site and pRL-TK for 24 h before various concentrations of EGF or TGF-β1 were added for another 24 h. The *firefly* luciferase activity of constructs was normalized to the *Renilla* luciferase activity and then presented as a percentage to that of cells transfected with the −74 construct without the growth factor treatment. **d** The effect of EGF on protein–DNA complex formation and Sp1 binding to the *FSCN1* promoter region. The nuclear extracts were prepared from EC109 cells after treatment with EGF for various time intervals. The extracts (2 μg) were incubated with a DIG-labeled oligonucleotide covering the fascin promoter region from −74 nt to −41 nt (−74/−41w). The proteins complexed to the labeled probes were further identified by pre-incubating 2 μg of nuclear extracts with 2 μl anti-Sp1 (*lanes 1, 3, 5, 7*) antibody or control IgG (*lanes 2, 4, 6, 8*) before being added to the binding reaction. The representative data from two independent experiments are shown. **e** Alteration of the fascin, p-ERK1/2, and Sp1 protein levels in response to EGF treatment in the presence of specific MEK1/2 inhibitors. EC109 cells were treated with EGF for different time intervals after pretreatment either without or with U0126 (40 μM) for 1 h. The whole-cell extracts were analyzed by Western-blot. The blot was hybridized with an antibody against fascin, p-ERK1/2, ERK1/2, Sp1, or β-actin, which served as a loading control. **f** Alteration of the *FSCN1* promoter activity in response to EGF treatment in the presence of specific MEK1/2 inhibitors. EC109 cells were co-transfected with the −74 construct and pRL-TK. After 24 h, the cells were pretreated with various concentrations of U0126 for 1 h before EGF (50 μM) were added for another 24 h. The *firefly* luciferase activity of constructs was normalized to the *Renilla* luciferase activity and then presented as a percentage to that of cells transfected with the −74 construct without EGF and U0126 treatment. $^{*}p < 0.05$ or $^{**}p < 0.01$, compared to the group without EGF and U0126 treatment. $^{\Delta}p < 0.05$ or $^{\Delta\Delta}p < 0.01$, as compared to the group treated with EGF alone. **a**, **e** Representative blots from three independent experiments are shown. **b**, **c**, **f** The results of a representative experiment were presented as mean ± SD of the three independent samples. The experiments were repeated three times

Moreover, fascin mRNA and protein expression levels were significantly decreased after U0126 treatment (Fig. 6c, d). To confirm the involvement of the ERK1/2 pathway, we transiently transfected EC109 cells with control siRNA, ERK1, or ERK2-specific siRNA. The results of quantitative RT-PCR showed that the mRNA level of fascin was reduced in cells transfected with the specific ERK1 and/or ERK2 siRNA compared to the control (Supplementary Fig. S4). These data support that Sp1 phosphorylation via the activation of the ERK1/2 pathway is important for Sp1-dependent activation of the *FSCN1* gene.

EGF stimulates fascin expression through ERK1/2 and Sp1 activation

Considering that fascin expression is quite low or absent in normal epithelial cells, while being significantly elevated in a variety of cancers [7–15], we believed that fascin expression could be induced by factors which could activate ERK1/2-Sp1 pathway. It was reported that both EGF and TGF-β1 induced target gene expressions through the activation of ERK1/2 and Sp1 [37, 38]. In order to test our hypothesis, we first treated EC109 cells with EGF (50 ng/ml) or TGF-β1 (10 ng/ml) for different time intervals, and then assessed the expression of fascin by Western-blot analysis. We found that fascin protein expression was increased in a time-dependent manner when cells were treated with EGF, but not altered in response to TGF-β1 treatment (Fig. 7a). Consistent with this observation, EGF treatment also up-regulated the mRNA levels of fascin (Fig. 7b). However, the protein levels of fascin did not change when immortalized esophageal epithelium cells [39] was treated with EGF for different time intervals (Supplementary Fig. S5). This indicated that EGF was a specific growth factor that could induce fascin expression in esophageal cancer cells.

To test whether the Sp1 binding site of the promoter mediated EGF-induced fascin expression, EC109 cells co-transfected with the −74 construct or −70/−60s construct containing a mutated Sp1 binding site and pRL-TK were treated with various concentrations of EGF or TGF-β1. In these cells, the −70/−60s construct could only generate weak luciferase activity as previously shown in Fig. 3. However, the luciferase activity of the −74 construct was significantly increased in response to EGF, whereas the activity was not affected by TGF-β1 treatment (Fig. 7c). These results suggest that fascin expression is up-regulated by EGF stimulation through a Sp1 binding site of the *FSCN1* promoter in the ESCC cells.

To determine whether Sp1's binding to this site increases in response to EGF, nuclear extracts were prepared from EC109 cells after EGF treatment for various time periods. The results showed that Sp1 binding was increased in a time-dependent manner (Supplementary Fig. S6, *lanes 2, 4, 6, and 8*; Fig. 7d, *lanes 2, 4, 6, and 8*). For further validation, the nuclear extracts were incubated with Sp1 antibody before the addition of the probe. The results proved that the increase in binding was indeed due to Sp1 (Fig. 7d, *lanes 1, 3, 5, 7*).

We then tried to verify whether EGF up-regulates fascin expression through the ERK1/2-Sp1 pathway activation. For this, we investigated the alteration of fascin expression and its promoter activity in response to EGF for various time periods in the presence of the specific MEK1/2 inhibitor U0126 in EC109 cells. As expected, the levels of

fascin, p-ERK1/2, total Sp1, and phosphorylated Sp1 (p-Sp1) all increased, whereas the total ERK1/2 proteins did not change in response to EGF in the absence of U0126 (Fig. 7e, Supplementary Fig. S7). In contrast, the protein levels of fascin, p-ERK1/2, and total Sp1 and activity of *FSCN1* promoter in response to EGF were significantly reduced in the presence of U0126 (Fig. 7e, F). Moreover, U0126 effectively prevented Sp1 binding (Supplementary Fig. S6, *lanes 3, 5, 7, and 9*). Taken together, these data support that EGF stimulates transcription and expression of *FSCN1* by activating the ERK1/2-Sp1 pathway to increase Sp1 complex.

Discussion

Since the overexpression of fascin has been well established in various human carcinomas examined to date [7–16], fascin has been identified as a new indicator of aggressive phenotypes and poor prognosis in malignant human epithelial carcinomas [7–19]. Besides its significance as an important tumor-promoting factor, convincing evidence also implicates fascin as a novel target for metastasis prevention [10]. However, rare reports exist to discuss the transcriptional regulation of fascin expression in cancer cells. In the present study, we demonstrated that transcription of fascin was activated by native Sp1, which could bind to a typical G-rich element located at nt −70/−60 in the *FSCN1* promoter. The transcriptional activity of Sp1 could be regulated by the ERK1/2 pathway, which was important for Sp1-dependent activation of the *FSCN1* gene. Moreover, EGF up-regulated fascin expression through activating the ERK1/2 -Sp1 signaling pathway and enhancing Sp1 binding to DNA.

The expression of fascin gene is mainly regulated at the transcription level and involves the activation of several well-known transcription factors including β-catenin-TCF and CREB binding protein in human cancer cells. There are conflicting reports on whether the transcriptional activity of *FSCN1* is regulated by β-catenin-TCF [10, 21, 24]. In the present study, we also analyzed the possible link between the β-catenin-TCF signaling pathway and *FSCN1* gene promoter activity in ESCC cells. Although the levels of β-catenin in the nuclear extracts of KYSE150 and KYSE180 cells were higher than that of EC109 and EC18 cells (Fig. 1b), the expression levels of fascin protein in these cells were comparable (Fig. 1a). Furthermore, dominant-negative β-catenin (4145ΔC) or TCF4 (ΔN-TCF4) did not decrease the promoter activity in ESCC cells (Fig. 1c, d). These data support the hypothesis from Hashimoto et al. [21] that the β-catenin-TCF signaling pathway does not play a central role in human breast and colon carcinoma cells. For association of CREB binding protein with the *FSCN1* promoter activity, we previously demonstrated that deletion of the CREB/AP-1 binding site located −50/−41 region of the *FSCN1* promoter did not significantly decrease the activity in ESCC cells [22]. In the current study, the evidence from EMSA further confirmed that there were nuclear proteins binding to −70/−60 region (a Sp1 binding site) but not to −50/−41 region of the *FSCN1* promoter in ESCC cells. Moreover, the transcription factor Sp1 indeed was attached to this site (Fig. 2). Overall, these results suggest that it is the Sp1 binding site but not the CREB/AP-1 site which mainly contributes to the transcriptional regulation of the *FSCN1* in ESCC cells. Importantly, the fascin expression and its promoter activity were enhanced by Sp1 overexpression, while inhibited by Sp1 knockdown via RNAi. These results strongly demonstrated that Sp1 protein was a key transcription factor responsible for the regulation of fascin expression in ESCC cells.

Sp1 and other Sp and Kruppel-like factor (KLF) proteins are members of a family of transcription factors, which bind GC/GT-rich promoter elements through three C_2H_2-type zinc fingers present at their C-terminal domains. Growing evidence has suggested that some Sp proteins play a critical role in the growth and metastasis of various tumors by regulating gene expression [28, 41]. Sp1 and Sp3 both bind to the GC-rich sequences and their interactions with the same DNA sequence can be either cooperative or antagonistic [41, 42]. In the present study, forced expression of Sp3 did not significantly affect fascin expression and its promoter activity in EC109 cells, suggesting that Sp3 was not involved in the transcriptional regulation of *FSCN1*. It was reported that the interaction between Sp1, some Sp/KLF proteins (KLFII), and other transcription factors binding to the GC-rich sites (for example, early growth response protein 1 and nuclear factor-kappa B) was important for regulating gene expressions in different cellular contexts [43, 44]. Thus Sp1 may cooperate with some other GC-rich sites binding factors to activate the transcription of *FSCN1* in ESCC cells.

Sp1 can be phosphorylated by many kinases. Phosphorylation occurs within multiple motifs of the Sp1 molecule, such as the C-terminus, and often results in functional changes, for example, the capability of DNA binding and/or gene-promoter activation. Phosphorylation of Sp1 plays an important role in modulating its transcriptional regulation of multiple genes [45, 46]. In the present study, we demonstrated that ERK1/2-dependent phosphorylation of Sp1 was important for induction of fascin expression in ESCC cells. It has been reported that ERK1/2 directly phosphorylated Sp1 at the residues threonine 453 (Thr453) and threonine 739 (Thr739), resulting in an increase or decrease of the gene-promoter activity [47, 48]. However, we did not observe any signal in the

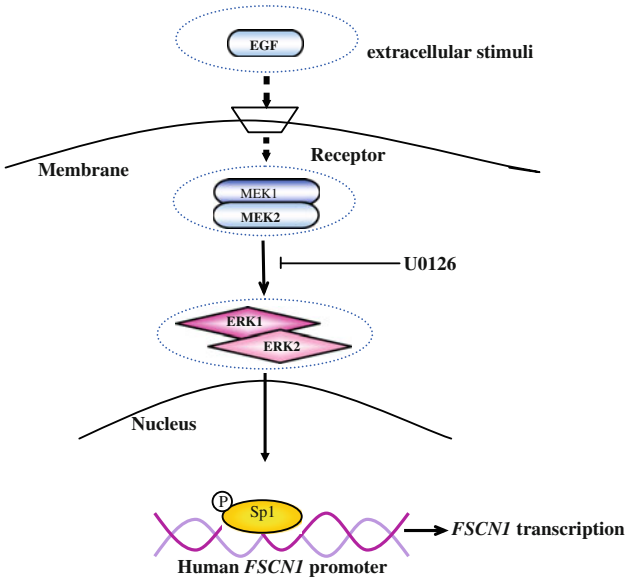


Fig. 8 Hypothetical model elucidating the regulation *FSCN1* expression through the MEK-ERK1/2-Sp1 pathway. Growth factors (such as EGF) induce MEK1/2 to phosphorylate ERK1/2 upon their binding to growth factor receptors, then the activated ERK1/2 phosphorylates Sp1, and enhance its binding to the corresponding sites located in the *FSCN1* promoter, finally resulting in the transcriptional activation and up-regulated expression of fascin

phosphorylation status of Thr453 after MEK1/2 inhibitor U0126 (40 μM) treatment for 24 h (Supplementary Fig. S8). In contrast, the inhibitor U0126 could decrease the level of phospho-serine within Sp1 protein. These data indicated that the phosphorylation of serine residues by ERK1/2 might play an important role in Sp1-dependent regulation of *FSCN1* gene transcription in ESCC cells. However, further studies are required for revealing detailed regulatory mechanisms.

EGF and/or epidermal growth factor receptor (EGFR) are often constitutively activated in many cancers. The functions of EGFRs are frequently dysregulated in malignant human epithelial carcinomas including ESCC and EGFR signaling pathway has been shown to play an important role in cancer progression [49, 50]. In the present study, we observed that the levels of both fascin mRNA and protein were increased in response to EGF. Moreover, this inductive effect exerted by EGF was dependent on ERK1/2 activity, and was linked to increased expression and phosphorylation of Sp1, as well as its enhanced binding to the nt −70/−60 sequence of the *FSCN1* promoter. These results suggested that fascin may be a transcriptional target of the EGF/EGFR signaling pathway (Fig. 8). Inhibition of EGFRs might restrain *FSCN1* expression in some cancer cells, thus inhibiting cancer cell migration, invasion, and metastasis. EGFR inhibitors have provided significant clinical benefit, however not always efficient to all the patients. This clinical observation spurred the search for proper biomarkers to predict the efficacy of EGFR antagonists, as well as to explore the mechanisms underlying different clinical outcomes [51]. Fascin may be employed as a potential biomarker to predict the efficacy of EGFR inhibitors in cancer therapies.

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