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sFRP1 exerts effects on gastric cancer cells through GSK3β/Rac1-mediated restraint of TGFβ/Smad3 signaling

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Abstract. Secreted frizzled-related protein 1 (sFRP1) is an inhibitor of canonical Wnt signaling; however, previous studies have determined a tumor-promoting function of sFRP1 in a number of different cancer types. A previous study demonstrated that sFRP1 overexpression was associated with an aggressive phenotype and the activation of transforming growth factor β (TGFβ) signaling. sFRP1 overexpression and sFRP1 knockdown cell models were established. Immunoblotting was conducted to examine the protein levels of the associated molecules. Immunofluorescence staining followed by confocal microscopy was performed to visualize the cytoskeleton alterations and subcellular localization of key proteins. sFRP1 overexpression restored glycogen synthase kinase 3β (GSK3β) activity, which activated Rac family small GTPase 1 (Rac1). GSK3β and Rac1 mediated the effect of sFRP1 on the positive regulation of cell growth and migration/invasion. Inhibition of GSK3β or Rac1 abolished the regulation of sFRP1 on TGFβ/SMAD family member 3 (Smad3) signaling and the aggressive phenotype; however, GSK3β or Rac1 overexpression increased cell migration/invasion and restrained Smad3 activity by preventing its nuclear translocation and limiting its transcriptional activity. The present study demonstrated a tumor-promoting function of sFRP1-overexpression by selectively activating TGFβ signaling in gastric cancer cells. GSK3β and Rac1 serve an important function in mediating the sFRP1-induced malignant alterations and signaling changes.

Introduction

Aberrant activation of Wnt, an outside-in signaling pathway, is involved in the majority of malignancy types, including gastric cancer. Secreted frizzled-related protein 1 (sFRP1) has been reported to bind to Wnt ligands and modulate their ability to activate signal transduction (1-3). sFRPs are a family of secreted proteins homologous to the Frizzled (Fz) receptors, which bind Wnt ligands (4). They possess only the cysteine-rich domain and lack the seven trans-membrane and intracellular domains of Fz proteins (5). The expression of sFRP1 may vary with disease status or with the stage of development. sFRP1 has been demonstrated to serve a critical role in the development of the lung (6), prostate (7) and gut (8). sFRP1 is expressed in developing tissues but not in mature prostate epithelial cells (7).

Conflicting reports indicate that sFRP1 is able to serve tumor-promoting and tumor-suppressing roles. Transcriptional inactivation of sFRPs has been reported in various cancer types (9-12), supporting the hypothesis that sFRPs function as tumor suppressors; however, contrary results have also been published. Loss of sFRP1 expression has been determined in >80% of invasive breast carcinoma types, excluding the medullary type, and is associated with the presence of hormonal receptors (13). sFRP1 is highly expressed in the basal-like breast cancer (14) and brain relapses, compared with luminal tumor types and bone relapses (15). Similarly, high levels of sFRP1 in carcinomas are associated with the presence of lymphoplasmacytic stroma (13). In addition to its function of inhibiting the Wnt/canonical pathway, sFRP1 is also reported to increase the diffusion of Wnt ligands (16), and interact with Hedgehog (17,18), tumor necrosis factor (19) and integrin signaling (20), which indicates that sFRP1 is a multi-functional protein (20,21).

Gastric carcinoma is the fourth most common malignancy globally, with an estimated 989,000 novel cases and 738,000 mortalities reported in 2008 (22). The depth of invasion and the presence of lymph node metastases are considered to be the most important prognostic factors in gastric cancer (23,24). sFRP1 was overexpressed in aggressive subgroups of human gastric cancer, and was significantly associated with lymph node metastasis and decreased overall survival rate in patients with gastric cancer (25), which is consistent with another previous study that demonstrated that sFRP1 is overexpressed only in metastatic renal carcinoma, but not in primary tumor types (26). sFRP1 overexpression is associated with the activation of the transforming growth

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factor β (TGFβ) signaling pathway and thereby induced cell proliferation, epithelial-mesenchymal transition (EMT) and invasion (25). Expression of sFRP1 decreases the intracellular levels of β-catenin, indicating the inhibition of the Wnt/canonical signaling pathway (5). Crosstalk between the Wnt and TGFβ signaling pathways that are regulated by sFRP1 are substantially associated with one another (25). Despite these data indicating that sFRP1 is able to promote or repress tumorigenesis, the mechanism by which sFRP1 governs cell signaling remains unclear.

In the present study, the molecular mechanism underlying sFRP1-induced signaling alterations was investigated, based on previous data. The critical role of glycogen synthase kinase 3β (GSK3β) and Rac family small GTPase 1 (Rac1) in mediating the sFRP1 signaling, which regulates malignant behaviors and TGFβ signaling in gastric cancer cells, was investigated.

Materials and methods

Cell culture and chemicals. Human gastric cancer cell lines SGC-7901 and BCG823 were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator. GSK3β inhibitor IM-12 (10, 20 and 50 μM) and Rac1 inhibitor NSC23766 (25, 50 and 100 μM) were obtained from Merck KGaA (Darmstadt, Germany). Cells treated with inhibitors were cultured in a 37˚C incubator at 5% CO_2.

Cell proliferation assays. Cell proliferation was assessed using an MTT (Sigma-Aldrich; Merck KGaA) assay. A total of $2x10^3$ cells in 100 μl culture medium were plated in a 96-well plate. MTT reagent (5 mg/ml) was added into each well for 48 h and the plate was returned to a 37˚C incubator for 3 h. The culture medium was aspirated and 150 μl dimethyl sulfoxide (DMSO) was added into each well. The plate was shaken in an orbital shaker for 15 min. The absorbance at an optical density of 590 nm was measured using a microplate reader. GSK3β inhibitor IM-12 (10, 20 and 50 μM) and Rac1 inhibitor NSC23766 (25, 50 and 100 μM) were added 24 h after the cells (SGC-7901/vector and SGC-7901/sFRP1) were plated. Control groups were treated using DMSO.

Plasmids and transfection. sFRP1 vector was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The green fluorescent protein-fused wild-type (WT) Rac1, constitutively active (CA) mutant Rac1 (Q61L), dominant-negative (DN) mutant Rac1 (T17N) (27), Tag5Amyc-GSK3β WT (28), pCS2 Flag Smad3 S204A (29), pCMV5B-Flag-Smad3 (30) and pCMV5 Smad2-HA (31) were purchased from Addgene, Inc. (Cambridge, MA, USA). Top-flash luciferase plasmid (BPS Bioscience, San Diego, CA, USA) is a luciferase reporter plasmid that contains two sets of 3 copies of the wild-type T-cell factor (TCF) binding regions. If the canonical Wnt signaling is activated, β-catenin will translocate to the nucleus to associate with TCF/lymphoid enhancer factor transcription factors to activate transcription of Wnt target genes. pSV-β-Galactosidase control vector was used as an internal control for transfection and was purchased from Promega Corporation (Madison, WI, USA). For plasmid transfection, SGC-7901/vector cells were seeded into 6-well plate and allowed to grow 24 h prior to transfection. Cells in each well were transfected with 4 μg plasmid using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 6 h at 37˚C and the medium was changed subsequent to transfection.

Transient expression reporter gene assay. The transcriptional activity of β-catenin was measured by co-transfection with Top-flash luciferase plasmid and sFRP1 vector (OriGene Technologies, Inc.) or the control vector using Lipofectamine® 2000 for 6 h at 37˚C. TOPFlash encoding the LEF/TCF binding sites (insert gene) linked to firefly luciferase and reflecting Wnt/β-catenin signaling activity was used. After 24 h incubation, the luciferase activity was measured and normalized to β-galactosidase activity (Promega Corporation). The Luciferase Reporter Gene Detection kit (Promega Corporation) and GloMax®-Multi+ Detection system (Promega Corporation) were used according to the manufacturer's protocol. The data presented were the mean value of three independent experiments.

Activity of Rac1 assay. The activation of Rac1 was measured using a Rac1 Activation Assay Biochem kit (Cytoskeleton, Inc., Denver, CO, USA) according to the manufacturer's protocol. Briefly, cell lysates were collected using the lysis buffer at 4˚C for 30 min from the kit. The activated forms of Rac1 were combined by Rac1 activated kinase (PAK)-Rac/Cdc42 (p21) binding domain (PBD) affinity beads. The beads were centrifuged at 5,000 x g at 4˚C for 1 min and activated GTPases were pulled-down into the bead pallets. Bound GTPases were eluted by SDS buffer and analyzed by 12% SDS-PAGE and western blotting. Rac1 levels were analyzed by the specific antibodies.

Western blotting. Whole cell lysates (SGC-7901/vector, SGC-7901/sFRP1, inhibitor treated cells and plasmid-transfected cells) were harvested using radio immunoprecipitation assay cell lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) at 4˚C for 30 min. The nuclear and cytosol extracts were isolated using the nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of the proteins were measured using a DC Protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. A total of 30 μg protein was loaded per lane and loaded into 10-12% SDS-PAGE gels. The proteins were then transferred to a polyvinylidene fluoride membranes followed by blocking with 5% bovine serum albumin (BSA) for 2 h at room temperature on a rocket shaker. Membranes were washed using tris-buffered saline with 0.1% Tween-20 (pH 8.0) three times for 10 min each. Primary antibodies (1:1,000) against sFRP1 (cat no. ab126613), phosphorylated (p-)Smad3L (cat no. ab63402), zinc finger E-box binding homeobox 2 (ZEB2; cat no. ab138222) and lamin A/C (cat no. ab108922) were purchased from Abcam (Cambridge, UK). Antibodies against Smad3 (cat no. 9523), Smad2 (cat no. 5339), Smad4, p-Smad3c (cat no. 9520), p-Smad2c (cat no. 3108), GSK3β (cat no. 12456), p-GSK3β Ser9 (cat no. 9323), p21 (cat no. 2947), β-catenin (cat no. 8480),

p-Rac1/cell division cycle 42 S71 (cat no. 2461) (all obtained from Cell Signaling Technology, Inc., Danvers, MA, USA) and inhibitor of DNA binding 1 (ID1; cat no. 5559-1), Vav guanine nucleotide exchange factor 2 (VAV2; cat no. EP1067Y), plasminogen activator inhibitor 1 (PAI1; cat no. EPR21850-82) (all obtained from Epitomics, Burlingame, CA, USA) were used at 1:1,000 dilutions. Antibodies against GAPDH (cat no. G8795), Lamin A/C (cat no. SAB4200236) and Lamin C (cat no. MAB3540) (Sigma-Aldrich; Merck KGaA) were used at a 1:5,000 dilution. Primary antibodies were diluted in 5% BSA and incubated overnight at 4˚C. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) H&L (cat no. ab6789) and HRP-conjugated goat anti-mouse IgG H&L (cat no. ab6721) secondary antibodies were purchased from Abcam and used at a 1:10,000 dilution in 5% BSA at room temperature for 2 h. Signals were visualized using enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). Membranes were scanned using the ChemiDoc Touch Imaging system (Bio-Rad Laboratories, Inc.) and the images were captured using Image Lab Touch Software (version 1.0.0.15; Bio-Rad Laboratories, Inc.).

Immunofluorescence staining. Cells were fixed with 4% formaldehyde at room temperature for 30 min and then permeabilized with PBS containing 0.2% Triton X-100 for 15 min at room temperature. Slides were blocked using 5% bovine serum albumin at room temperature for 1 h. For F-actin staining, cells were fixed with 4% formaldehyde at room temperature for 30 min and then incubated with Alexa Fluor 555® phalloidin (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 30 min. The nucleus was counterstained using DAPI at room temperature for 5 min. Slides were washed using PBS, mounted and observed under a microscope. Immunofluorescence staining was visualized and captured using a Nikon Digital Sight DS-U2 (Nikon Corporation, Tokyo, Japan) and NIS elements F3.0 software was used (Nikon Corporation). Confocal images were obtained using an inverted ZEISS LSM710 confocal microscope (x40 oil lens; Carl Zeiss AG, Oberkochen, Germany). Zen 2009 Light Edition (Carl Zeiss AG) was used for measurement of the images.

Cell migration assays. Cell migration was analyzed using a Transwell chamber assay. A 24-well plate with 8 μm pore size inserts was used. SGC-7901/vector and SGC-7901/sFRP1 cells treated with vehicle (DMSO), NSC23766 (25 μM) and IM-12 (10 μM) for 24 h were used. A total of $1x10^4$ cells were mixed in 100 μl RPMI-1640 medium and added to the upper chamber of the Transwell insert. A total of 600 μl RPMI 1640-medium with 10% FBS was added to the lower chamber. The Transwell inserts were then placed into the wells of a 24-well plate. After 12 h incubation at 37˚C, the cells were fixed using 4% formaldehyde at room temperature for 30 min and stained by 0.01% (v/v in methanol) crystal violet at room temperature for 10 min. Subsequent to being washed three times by H_2O, the cells on the inner side of the chamber were removed with a cotton swab and the cells on the outer side of the chamber were counted under a light microscope at a magnification of x100. Cells were visualized using an Olympus BX50 microscope, images were captured using Nikon Digital Sight DS-U2 and NIS elements F3.0 software was used for analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cultured SGC7901/vector and SGC-7901/sFRP1 cells using the RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocols, and cDNA was synthesized with oligo (dT) primers by using of a SuperScript first-strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. A total of 1 μg RNA was used to synthesize cDNA. Gene expression was assessed by RT-qPCR using an Applied Biosystems 7500 Fast Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction mixture consisted of QuantiTect SYBR Green PCR master mix (2x QuantiTect SYBR Green kit, containing HotStart Taq® DNA polymerase, QuantiTect SYGB Green PCR buffer, dNTP mix, SYGB I, Rox passive reference dye and 5 mM $MgCl_2$; Qiagen, Inc.), 0.5 μmol/l of each primer and cDNA. The thermocycling conditions were as follows: 95˚C for 30 sec, 40 cycles at 95˚C for 5 sec, 60˚C for 30 sec; and the dissociation stage at 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. The transcript of the housekeeping gene, GAPDH was used as an endogenous control to normalize the expression data. The comparative Cq method was used to calculate the relative changes in gene expression. Expression fold change was calculated using the equation $2^{-(\text{Cq gene - Cq GAPDH})}$ (32). The primers used were as follows: PAI1 forward, 5'-TGGCACGGTGGCCTCCTCAT-3' and reverse, 5'-ACTGTTCCTGTGGGGTTGTGCC-3'; ID1 forward, 5'-CGAGATCAGCGCCCTGACGG-3' and reverse, 5'-GGCCGCCGATCGGTCTTGTT-3'; Smad3 forward, 5'-GGAGAAATGGTGCGAGAAGG-3' and reverse, 5'-GAAGGCGAACTCACACAGC-3'; p21 forward, 5'-GCCGAAGTCAGTTCCTTGTG-3' and reverse, 5'-TTCTGACATGGCGCCTCCT-3'; activating transcription factor 3 (ATF3) forward, 5'-GAGGTGGGGTTAGCTTCAGT-3' and reverse, 5'-TTGATTTTGGGGCAAGGTGC-3'; GAPDH forward, 5'-GGGCTCTCTGCTCCTCCCTGTTCT-3' and reverse, 5'-CAGGCGTCCGATACGGCCAAA-3'.

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Values are presented as the mean ± standard deviation of samples measured in triplicate. $P<0.05$ was considered to indicate a statistically significant difference. Each experiment was repeated three times, unless otherwise indicated. The significance of differences between experimental groups compared to the vehicle control group was analyzed using a paired Student's t-test and two-tailed distribution. Multiple comparisons were analyzed using a one-way analysis of variance (ANOVA) test. Newman-Keuls test was used following ANOVA.

Results

Overexpression of sFRP1 activates Rac1. Firstly, the cell morphological changes induced by sFRP1 overexpression were investigated. SGC-7901/sFRP1 cells exhibited extended and protruded lamellipodia composed of F-actin fibro stained by phalloidin (Fig. 1A, upper); however, SGC-7901/vector cells exhibited a polygon-like shape with less lamellar extensions around the entire cell periphery (Fig. 1A, lower). This phenomenon indicated that Rac1, which is well known to be

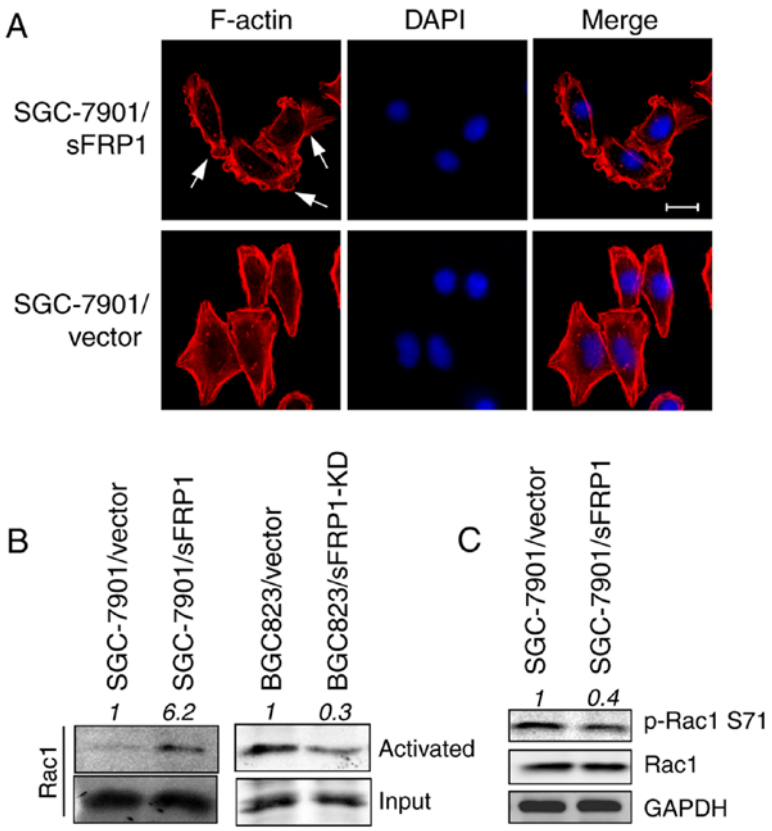

Figure 1. Overexpression of sFRP1 in gastric cancer cells activates Rac1 and GSK3β. (A) Overexpression of sFRP1 in SGC-7901 cells activates Rac1. The morphologies of SGC-7901/vector and SGC-7901/sFRP1 were visualized by confocal microscopy (scale bar, 20 μM) using F-actin with Alexa Fluor 555®-labeled phalloidin. DAPI was used to visualize cell nuclei. White arrows indicate the lamellipodia structures. (B) The activity of Rac1 was investigated by an *in vitro* activity assay. Equal amounts of lysates from SGC-7901/vector and SGC-7901/sFRP1 cells were used (left). Equal amounts of the lysates from BGC823/vector and BGC823/sFRP1-KD cells were used (right). The Rac1 activated kinase-Rac/Cdc42 (p21) binding domain beads were used for precipitation of activated Rac1. Total cell lysates were loaded for input control. (C) Western blotting assays were performed to visualize the inactivated form (p-Rac1 S71) of the Rac1 protein. GAPDH was used as a loading control. Quantification of the intensity of the bands was normalized relative to the SGC-7901/vector, which is depicted on top of the bands. sFRP1, secreted frizzled-related protein 1; Rac1, Rac family small GTPase 1; GSK3β, glycogen synthase kinase 3β; KD, knockdown; p-, phosphorylated.

involved in filopodia and lamellipodia formation and thus control cell movement (33), was activated by sFRP1 overexpression. Therefore, Rac1 activity was measured in control and sFRP1-overexpressing cells by kinase activity assays. In agreement with a previous study (33), sFRP1-overexpressing cells exhibited increased Rac1 activity (Fig. 1B, left); however, the loss of Rac1 activity/activation was observed in sFRP1-knockdown BGC823 cells, compared with vector only control cells (Fig. 1B, right). Immunoblotting also demonstrated a lower level of its inactivated form p-Rac1 Ser71 in sFRP1-overexpressing cells (Fig. 1C). These data indicated that sFRP1 activates Rac1 activity.

sFRP1 overexpression restores GSK3β activity. In addition, it was reported previously that sFRP1 abrogates GSK3β inactivation by preventing its phosphorylation at the Ser9 residue (34). The present study also demonstrated a lower level of p-GSK3β Ser9 in sFRP1-overexpressing cells compared with the control cells (Fig. 2A). In agreement with the notion that sFRP1 is an inhibitor of Wnt signaling, it was determined that TCF-responsive luciferase activity was significantly repressed by sFRP1 overexpression compared with the control cells ($P<0.05$; Fig. 2B) and the nuclear accumulation of β-catenin was attenuated (Fig. 2C). Consistent with other data, the present cell model also demonstrated that sFRP1 overexpression restored GSK3β activity and inhibited the Wnt/canonical pathway.

sFRP1 regulates Rac1 activity through GSK3β. Due to sFRP1 overexpression activating Rac1 and GSK3β, and GSK3β being previously reported to modulate Rac1 activity (35), the present study investigated whether GSK3β regulated Rac1 activity in SGC-7901/sFRP1 cells. Decreased lamellipodia formation, a feature of Rac1 inactivation, was observed in SGC-7901/sFRP1 cells treated with GSK3β inhibitor IM-12 or Rac1 inhibitor NSC23766 compared with vehicle cells (Fig. 3A). As depicted in Fig. 3B, a reduced amount of Rac1 bound to PAK-PBD compared with vehicle cells, which indicated reduced Rac1 activity. Levels of VAV2, a guanine nucleotide exchange factor (GEF) and activator of Rac1 (36), were lower in NSC23766 and IM-12 treated cells that were precipitated by PAK-PBD compared with vehicle cells, indicating that GSK3β or Rac1 inhibition suppressed Rac1 activity. Notably, GSK3β was also one of the components that was precipitated by PAK-PBD beads, and its level was decreased upon Rac1 or GSK3β inhibition compared with the vehicle control cells (Fig. 3B, left). The total levels of Rac1, GSK3β, and VAV2 remained consistent in cells with different treatments (Fig. 3B, right). Due to GSK3β

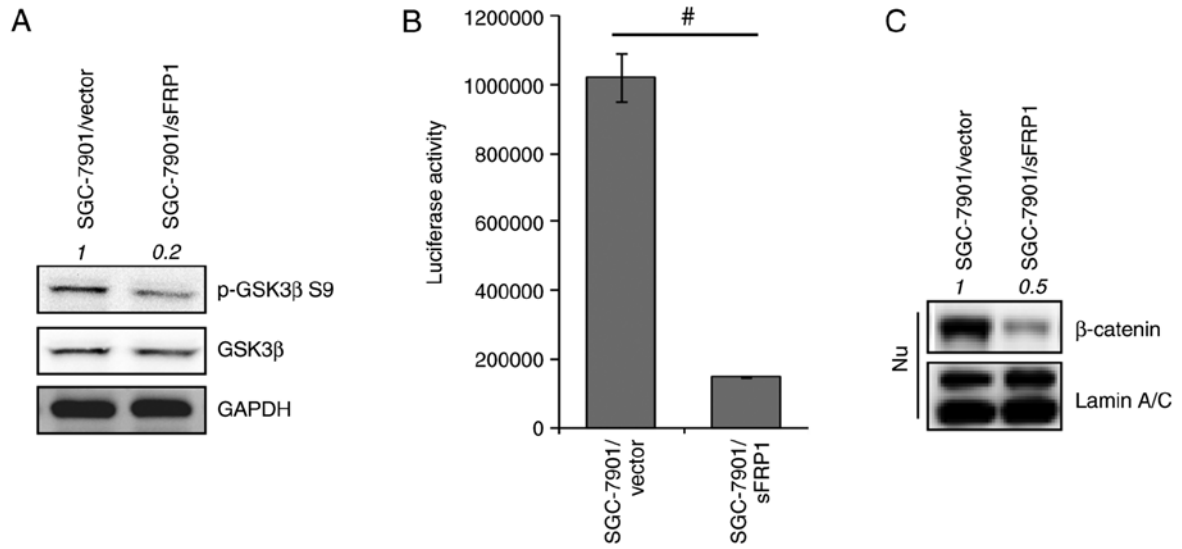

Figure 2. sFRP1 regulates GSK3β activity. (A) Inactive form of GSK3β (p-GSK3β Ser9) and total GSK3β were measured by immunoblotting. GAPDH was used as a loading control. (B) Transcriptional activity of β-catenin was measured by co-transfection with Top-flash luciferase plasmid and sFRP1. The luciferase activity was measured and normalized by β-galactosidase activity. The data are presented as the mean ± standard deviation of three independent experiments ($^{\#}P<0.05$ with comparisons shown by lines). (C) Nuclear accumulation of β-catenin was measured by immunoblotting using nuclear extracts from SGC-7901/vector and SGC-7901/sFRP1 cells. Lamin A/C was used as a loading control. Quantification of the intensity of the bands was normalized relative to the SGC-7901/vector, which is depicted on top of the bands. sFRP1, secreted frizzled-related protein 1; GSK3β, glycogen synthase kinase 3β; p-, phosphorylated.

being precipitated by PAK-PBD, which bound the activated form of Rac1, this indicated that GSK3β may directly or indirectly interact with Rac1; therefore, the levels of precipitated GSK3β were decreased in a similar pattern to the levels of the activated-Rac1, indicating that GSK3β may regulate Rac1 activity. Subsequently, a GSK3β overexpression model was used to investigate whether GSK3β was able to regulate Rac1 activity. As expected, a low level of the inactivated form of Rac1 (p-Rac1 Ser71) was observed in GSK3β-overexpressing cells compared with the vector cells (Fig. 3C). Due to NSC23766 inhibiting Rac1-GEF interaction (37) and IM-12 directly suppressed GSK3β activity (38), GSK3β activity may be necessary for regulating Rac1 activity.

Inhibition of Rac1 or GSK3β activity suppresses growth and metastasis in sFRP1-overexpressing cells. Activated Rac1 signaling has been determined to be important in gastric cancer tumorigenesis (39) and induces the high mobility cell phenotype (40). Although GSK3β is a classic inhibitor of the Wnt/canonical pathway, it is able to activate other signaling pathways and promote tumorigenesis (41,42). Subsequently, whether Rac1 or GSK3β mediated the tumor-promoting effects of sFRP1 was investigated. To address this question, a specific Rac1 inhibitor NSC23766 (37) and a small molecule GSK3β inhibitor IM-12 (38) were used, which were demonstrated to inhibit Rac1-GEF interaction and GSK3β kinase activity, respectively. Cell proliferation and migratory ability were then investigated. The significant inhibition of SGC-7901/sFRP1 cell growth by NSC23766 or IM-12 was depicted in Fig. 4A ($P<0.05$). sFRP1-overexpressing cells exhibited significantly inhibited migration following NSC23766 or IM-12 treatment, compared with control cells ($P<0.05$; Fig. 4B, upper). NSC23766 or IM-12 treatment abolished the formation of lamellipodia and membrane ruffles in SGC-7901/sFRP1 cells (Fig. 4B, lower). These data indicated that Rac1 and GSK3β serve essential functions in regulating the growth and migration of sFRP1-overexpressing cells.

Rac1 or GSK3β inhibition abolishes the regulation of sFRP1 on Smad3 activity. As demonstrated previously (25), sFRP1-overexpressing cells retained nuclear Smad2 levels but exhibited notably reduced Smad3 levels, compared with vector control cells, indicating unbalanced Smad2 and Smad3 activity (Fig. 5A). PAI1, ID1 and ZEB2, downstream targets of the TGFβ signaling pathway (43,44), were also upregulated in SGC-7901/sFRP1 cells (Fig. 5A). Rac1 was determined to selectively antagonize TGFβ/Smad3 mediated growth inhibition via its ability to promote Smad2 activation (45). GSK3β was previously reported to be responsible for the linker region of Smad3 and inhibited its transcriptional activity on molecules that mediated the growth inhibition activity of TGFβ signaling (46). Subsequently, whether Rac1 and GSK3β participated in the regulation of TGFβ signaling through sFRP1 was investigated; therefore, immunoblotting was performed using the nuclear extracts from SGC-7901/sFRP1 cells treated with Rac1 or GSK3β inhibitors. As depicted in Fig. 5B, nuclear Smad2 expression levels were not altered, and Smad3 and Smad4 expression levels were decreased following NSC23766 or IM-12 treatment. Inhibition of Rac1 or GSK3β activity also decreased ID1 and ZEB2 levels, which explained why Rac1 or GSK3β inhibition suppressed cell growth and migration.

Elevated mRNA levels of Smad3-responsive genes (47), including p21, ATF3, PAI1 and Smad3, were significantly elevated by Rac1 or GSK3β inhibition, whereas the ID1 mRNA level was significantly inhibited ($P<0.05$; Fig. 5C). To further observe the different gene responses to Smad2 and Smad3 signaling, HA-Smad2 or Flag-Smad3 constructs were

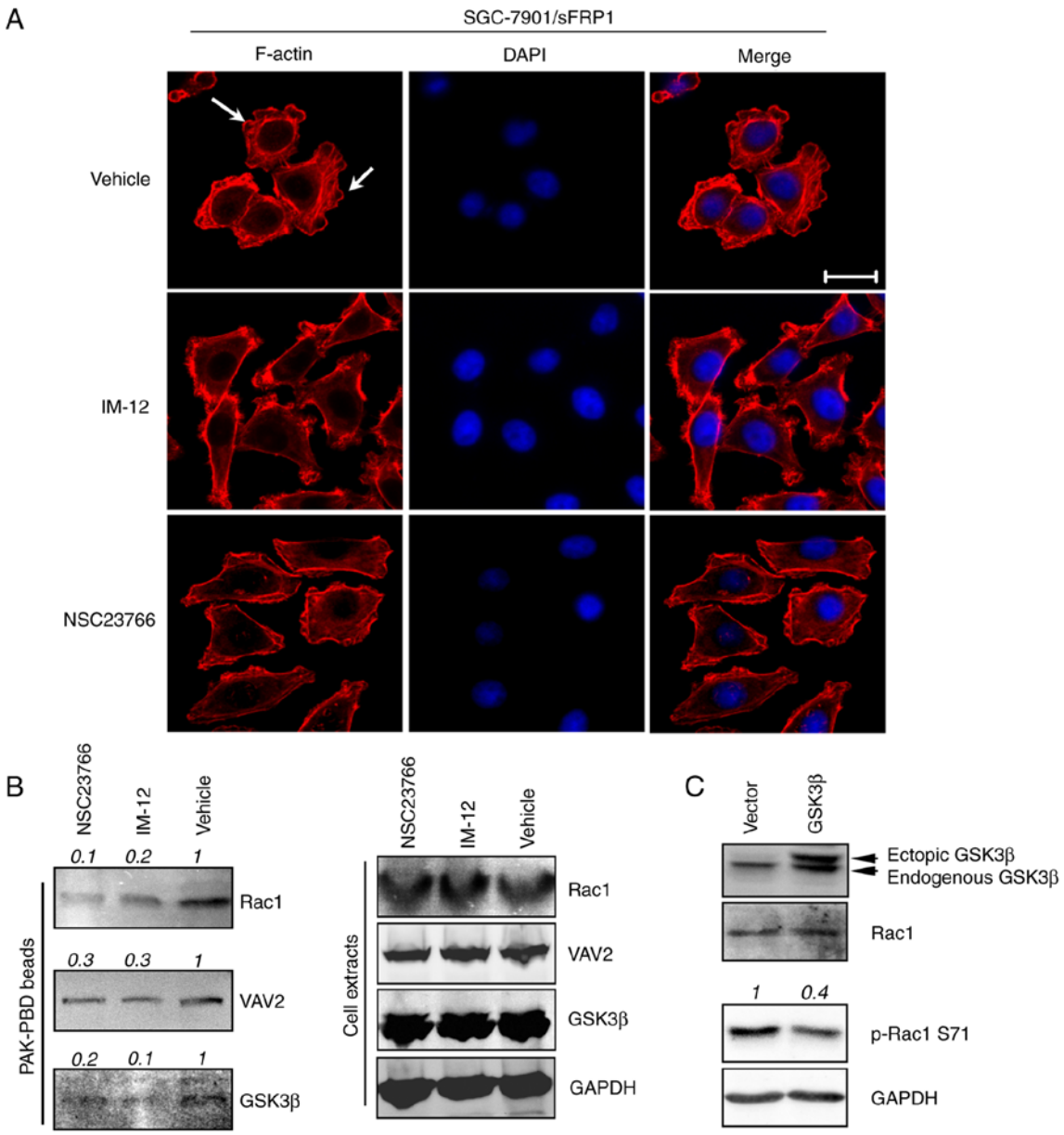

Figure 3. GSK3β regulates Rac1 activity in sFRP1-overexpressing SGC-7901 cells. (A) GSK3β inhibition decreased lamellipodia formation in SGC-7901/sFRP1 cells. SGC-7901/sFRP1 cells were treated with DMSO (vehicle), GSK3β inhibitor (IM-12) or Rac1 inhibitor (NSC23766) under normal culture medium (DMEM with 10% FBS) for 2 h. Cells were then either fixed and F-actin stained using Alexa Fluor 555®-labeled phalloidin to depict the cytoskeleton. Scale bar, 20 μM. (B) SGC-7901/sFRP1 cells were treated with DMSO (vehicle), GSK3β inhibitor (IM-12) or Rac1 inhibitor (NSC23766) under normal culture medium (DMEM with 10% FBS) for 2 h. Cells were collected for the Rac1 activity assays (right). PAK-PBD beads were used for precipitation of activated Rac1. Western blotting assays were performed to visualize the activated form of Rac1 protein, in addition to GSK3β and VAV2 that were also precipitated by PAK-PBD beads. The total levels of Rac1, VAV2, GSK3β and GAPDH were examined using cell extracts from different treatments. (C) Immunoblotting analysis examined the Rac1 activity in the vector only and GSK3β-overexpressing SGC-7901 cells. GAPDH was used as a loading control. Quantification of the intensity of the bands was normalized relative to the vehicle or vector, which are depicted on top of the bands. sFRP1, secreted frizzled-related protein 1; Rac1, Rac family small GTPase 1; GSK3β, glycogen synthase kinase 3β; DMSO, dimethyl sulfoxide; PAK, Rac1 activated kinase; PBD, Rac/Cdc42 (p21) binding domain; VAV2, Vav guanine nucleotide exchange factor 2.

transfected into SGC-7901 cells (Fig. 5D and E). Notably, cells overexpressing Smad3 exhibited high levels of pSmad3C, PAI1, pSmad3L and p21. Transfection of the Smad3-S204 mutant, a mutant form of WT Smad3 with the Ser204 mutant that cannot be phosphorylated by GSK3β, construct into SGC-7901 cells resulted in even higher levels of p21, without exhibiting a pSmad3L band. Additionally, Smad2 overexpression also resulted in elevated PAI1, which was potentially caused by increased pSmad2C levels, as pSmad3C levels were unaltered. These observations supported the observation that sustained Smad2 activity was able to compensate some of the Smad3-responsive functions in sFRP1-overexpressing cells. These data strongly indicated that the Rac1 and GSK3β were able to suppress Smad3 function, whilst retaining the expression of genes that were critical in mediating TGFβ-induced survival and the EMT phenotype.

Ectopic overexpression of Rac1 or GSK3β suppresses the Smad3 activity. To further examine the function of Rac1 or GSK3β in suppressing Smad3 activity, Rac1 or GSK3β were

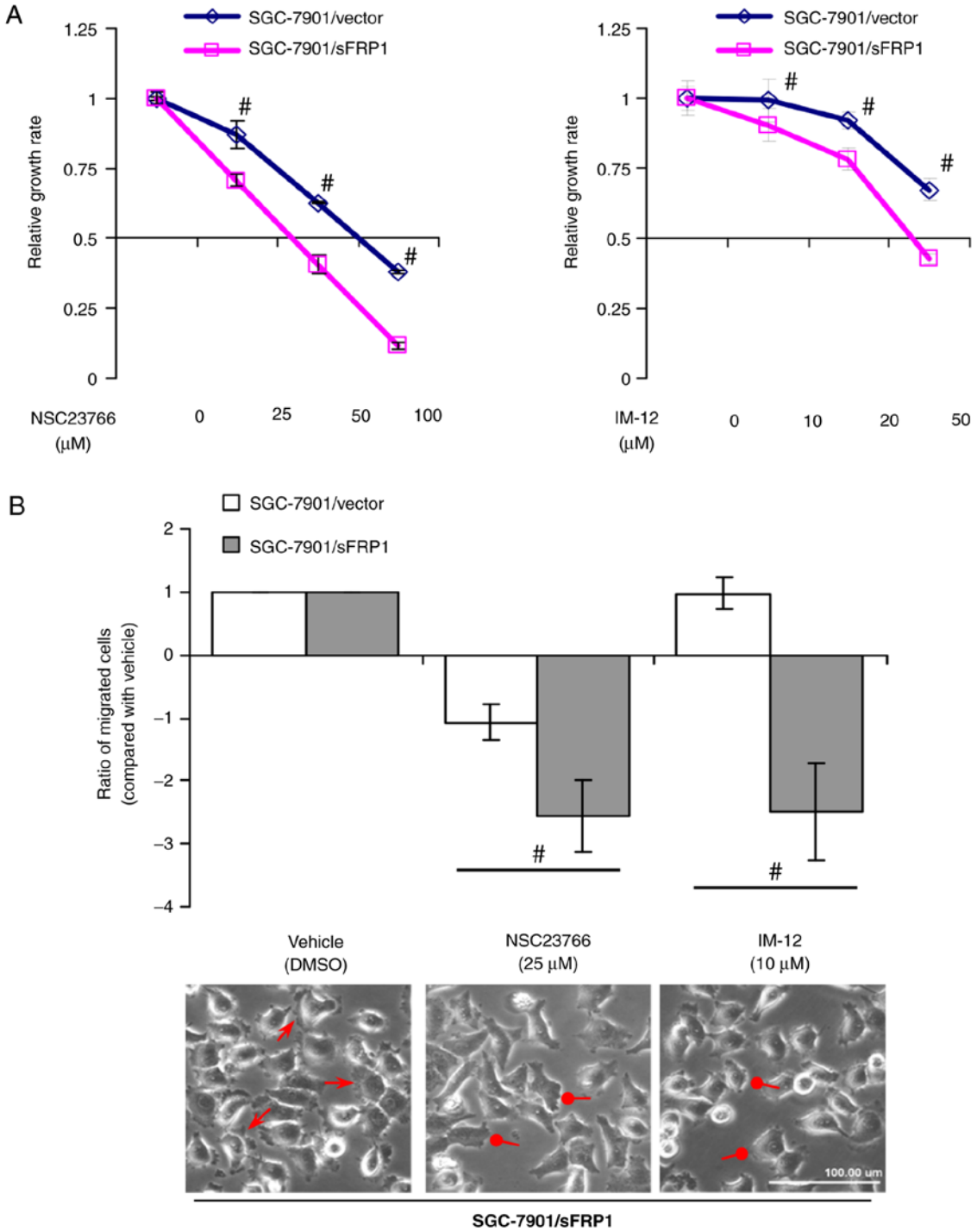

Figure 4. Blocking Rac1 and GSK3β activity attenuates the effects of overexpression of sFRP1 on gastric cancer cells. (A) Rac1 inhibitor NSC23766 and GSK3β inhibitor IM-12 suppressed the growth of sFRP1-overexpressing cells, compared with control cells. Growth curves of SGC-7901/vector and SGC-7901/sFRP1 cells treated with different doses of NSC23766 or IM-12 are plotted. Data represent three independent experiments. (B) Rac1 inhibitor NSC23766 and GSK3β inhibitor IM-12 inhibited the migration of sFRP1-overexpressing cells, compared with control (vehicle-treated) cells. Migration of SGC-7901/sFRP1 cells was measured using Transwell assays ($^{\#}P<0.05$ vs. SGC-7901 vector cells). Negative numbers represent the downregulation fold of migrated cells compared with the vehicle control group. The data are presented as the mean ± standard deviation of three independent experiments. Cell morphologies are depicted (bottom; original magnification, x200). Red arrows indicate spreading edges of SGC-7901/sFRP1 cells. Red round-head arrows indicate the diminishing of spreading edges of SGC-7901/sFRP1 cells treated with either NSC23766 or IM-12. sFRP1, secreted frizzled-related protein 1; Rac1, Rac family small GTPase 1; GSK3β, glycogen synthase kinase 3β; DMSO, dimethyl sulfoxide.

ectopically overexpressed in SGC-7901/vector cells. It is known that GSK3β phosphorylates the linker region (Ser204) of Smad3 and inhibited its transcriptional activity (48). In the present study, higher levels of pSmad3L (Ser204) and unaltered pSmad2 levels (Fig. 6A) were also observed in GSK3β-overexpressing cells compared with the vector cells. Subsequently, the function of Rac1 overexpression on Smad3 activity was investigated by transfecting Rac1-WT and Rac1-CA plasmids into SGC-7901/vector cells. Rac1-WT overexpressing cells exhibited lower pSmad3C

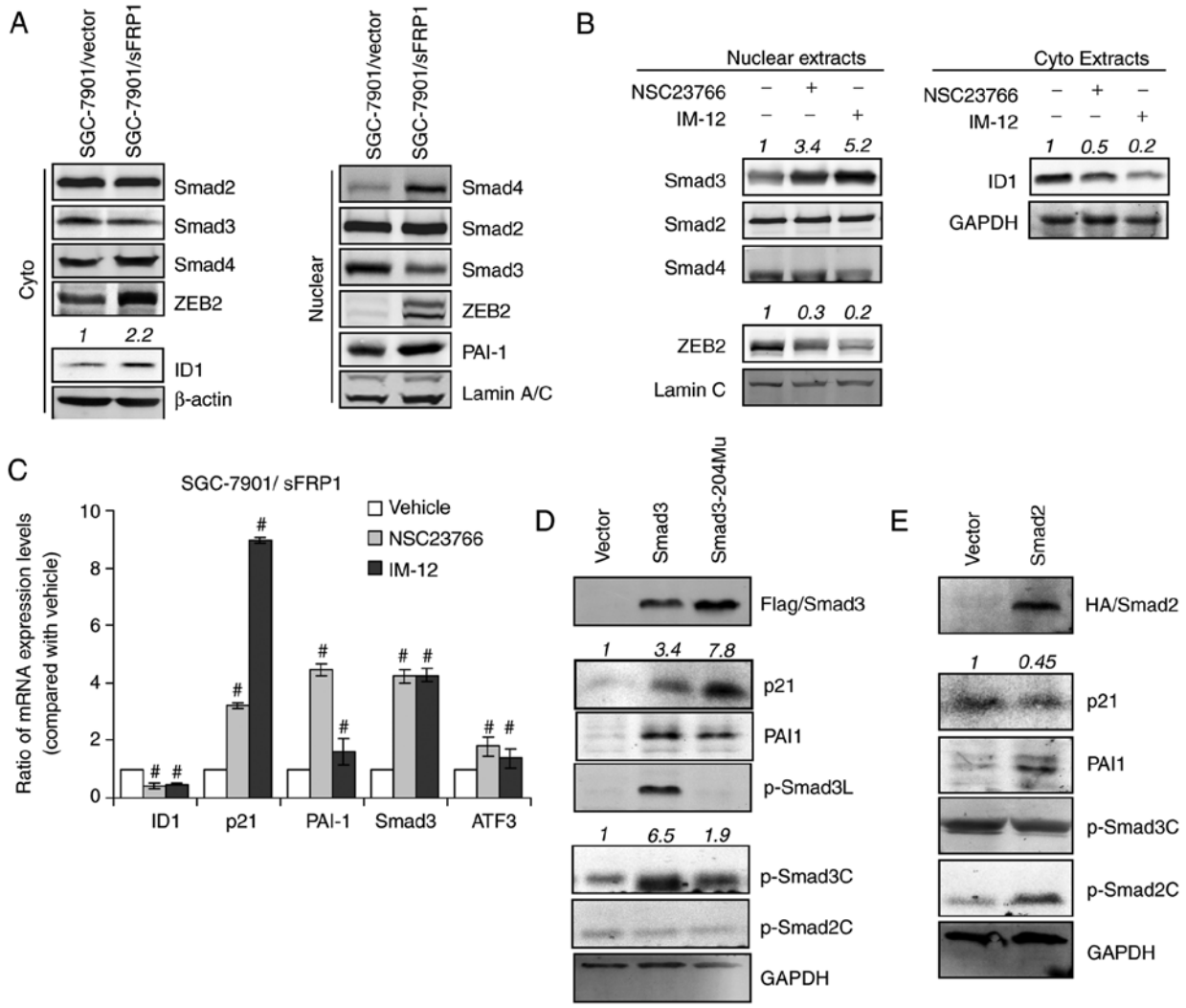

Figure 5. Rac1 and GSK3β participate in the regulation of the TGFβ pathway in sFRP1-overexpressing gastric cancer cells. (A) The expression of TGFβ signaling proteins were examined using immunoblotting. Cytoplasm and nuclear extracts from SGC-7901/vector and SGC-7901/sFRP1 cells were collected using cells cultured for 24 h under normal conditions. Actin and Lamin A/C were used as loading controls for cytoplasm and nuclear proteins, respectively. (B) Nuclear or cytoplasm extracts were collected from SGC-7901/sFRP1 cells treated with IM-12 or NSC23766. TGFβ-signaling downstream targets were examined by immunoblotting. (C) Reverse transcription-quantitative polymerase chain reaction analysis was performed to examine the expression of Smad3-responsive genes in SGC-7901/sFRP1 cells. The cells were treated with dimethyl sulfoxide (vehicle), Rac1 inhibitor (NSC23766) or GSK3β inhibitor (IM-12) in normal culture conditions for 24 h. Relative expression levels, compared with vehicle, are plotted. The data are presented as the mean ± standard deviation of three independent experiments. Statistical analysis was performed for each inhibitor (NSC23766 or IM-12) compared to vehicle individually (ANOVA test). $^{\#}P<0.05$ vs. vehicle. (D) Immunoblotting analysis of protein expression levels in vector-only SGC-7901 cells transfected with vector, WT Flag-Smad3 or Ser204 mutant Flag-Smad3 (the phosphorylation site modulated by GSK3β). (E) Immunoblotting analysis of protein expression levels in vector-only SGC-7901 cells transfected with vector and WT HA-Smad2. GAPDH was used as a loading control. Quantification of the intensity of the bands was normalized in relative to vehicle or vector, which are depicted on top of the bands. sFRP1, secreted frizzled-related protein 1; Rac1, Rac family small GTPase 1; GSK3β, glycogen synthase kinase 3β; TGFβ, transforming growth factor β; WT, wild-type.

expression levels compared with the vector cells (Fig. 6B, left). Rac1-CA overexpressing cells exhibited a more notable decrease in pSmad3C expression, compared with Rac1-WT overexpressing cells (Fig. 6B, left); however, Rac1-DN overexpressing cells exhibited an elevated pSmad3C expression level (Fig. 6B, right). Collectively, these data indicated that GSK3β and Rac1 are responsible for modulating TGFβ/Smad3 signaling in sFRP1-overexpressing cells (Fig. 6C).

Discussion

In the present study, it was demonstrated that there was high GSK3β and Rac1 activity in sFRP1 overexpressing cells. Additionally, it was observed that GSK3β and Rac1 mediated the effect of sFRP1 overexpression on regulating cell proliferation, migration and invasion. sFRP1-overexpression activated TGFβ and suppressed its growth inhibitory effect through activating GSK3β/Rac1.

Rac1 and GSK3β have been reported to be involved in tumorigenesis. Overexpression of Rac1 occurs in a number of tumor types, including breast (49,50), colon (51), bladder (52) and gastric cancer (53,54). Rac1 activation is associated with the progression of gastric cancer (39). *In vitro* studies have implicated Rac1 in cell migration (55,56), cell-cycle progression (57,58) and Ras-induced focus formation (59), indicating a role of Rac1 in tumor development and progression. GSK3β

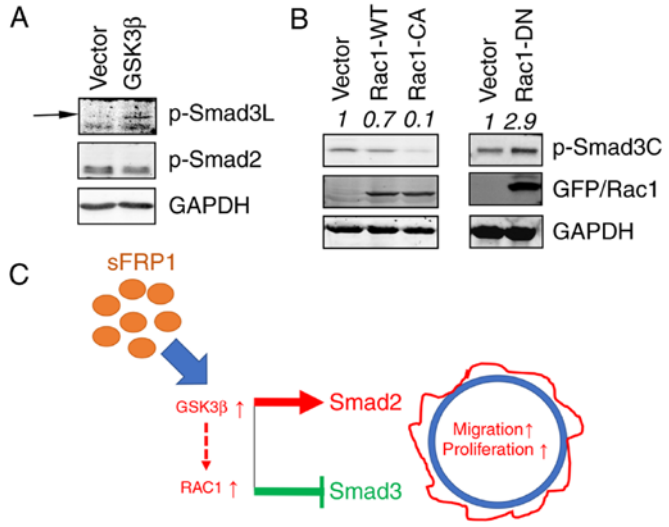

Figure 6. sFRP1 regulates TGFβ signaling. (A) GSK3β overexpression regulated pSmad3C and pSmad3L levels assayed by immunoblotting. (B) Rac1 overexpression (WT, CA and DN) regulated the pSmad3C expression level assayed by immunoblotting. (C) A schematic diagram demonstrating the function of Rac1 and GSK3β in sFRP1 signaling. sFRP1 activates Rac1 and GSK3β and regulates TGFβ signaling by restraining Smad3 activity and retaining Smad2 activity, thus enhancing epithelial-mesenchymal transition and metastasis while suppressing the growth inhibitory effects of TGFβ signaling. The broken line indicates currently unknown mechanisms. Quantification of the intensity of the bands was normalized in relative to the vehicle or vector, which are depicted on top of the bands. sFRP1, secreted frizzled-related protein 1; Rac1, Rac family small GTPase 1; GSK3β, glycogen synthase kinase 3β; TGFβ, transforming growth factor β; WT, wild-type; CA, constitutively active; DN, dominant-negative; p-, phosphorylated; Smad, SMAD family member.

was previously considered as a tumor suppressor, due to its known inhibition of Wnt/β-catenin activity; however, emerging evidence indicated its role in promoting tumor formation and metastasis (41,42). GSK3β activation is observed in gastric cancer and its signaling pathway has been determined to be functional in gastric cancer cells without involving Wnt signaling (60).

The first observation of the morphological changes in sFRP1-overexpressing cells was the formation of lamellipodia, which resulted in Rac1 activation. It was also observed that the level of the inactivated form of GSK3β was reduced in sFRP1-overexpressing cells, which indicated the role of sFRP1 in restoring GSK3β activity. Inhibition of GSK3β or Rac1 suppressed SGC-7901/sFRP1 growth and metastasis, indicating that sFRP1 overexpression may regulate cellular functions through GSK3β and Rac1. GSK3β overexpression and GSK3β inhibition further demonstrated a positive association between GSK3β and Rac1 activity, which is consistent with previous data indicating that Rac1 activity may be regulated by GSK3β (35). Suppression of Wnt signaling may result in the stabilization of Rac1 (61). High TGFβ1 expression levels observed in sFRP1-overexpressing cells (25) may also activate Rac1, thus promoting cell invasiveness (62); therefore, sFRP1-overexpression may activate Rac1 and maintain its sustained activity through multi-pathways. sFRP1 is also known to abrogate GSK3β inactivation, by preventing its phosphorylation at the Ser9 residue (34); thus, the activation of Rac1 may be due to the inhibition of Wnt and/or subsequent GSK3β activation.

A previous study determined that sFRP1 overexpression was associated with the activation of the TGFβ signaling pathway and induced cell proliferation, EMT and invasion (25). Additionally, the EMT-associated gene expression profile and TGFβ-induced growth inhibitory gene expression signature, including the upregulation of p21 and p15 and the downregulation of ID1, were not exhibited in sFRP1-overexpressing cells. This observation indicated that the growth inhibitory effect of TGFβ signaling was suppressed by sFRP1 overexpression. TGFβ1 may serve as a potent inhibitor of proliferation in epithelial cells. This cytostatic activity is dependent on the ability of TGFβ1 to increase the expression of cyclin-dependent kinase inhibitors, including p15Ink4b and p21Cip1. and repress the expression of the growth-promoting factors, including ID family proteins, and is primarily controlled by a Smad3-dependent signal (48); however, Smad2 was not responsible for the growth inhibition and the response of migratory induced by TGFβ1 (45). Loss of the negative regulation is considered to contribute to tumor development (63-65).

Different mechanisms regarding how cells evade TGFβ-meditated growth inhibition have been investigated. Among these, GSK3β was determined to inhibit Smad3 activity as a pro-apoptotic effector of TGFβ signaling in cancer cells (48); however, Rac1 antagonizes TGFβ/Smad3 mediated growth inhibition by promoting Smad2 activation (45). In the present study, it was determined that Rac1 enhanced Smad2 but suppressed Smad3 signal assayed by Rac1 overexpression and inhibition. Smad3 levels and activity were consistently reduced in sFRP1-overexpressing cells. Additionally, GSK3β and Rac1 were demonstrated to have increased activation in SGC-7901/sFRP1 cells, compared with control cells; therefore, GSK3β and Rac1 activity were conversely associated with nuclear Smad3 levels in sFRP1-overexpressing cells.

Recent studies (46,66) indicated that Rac1 and GSK3β can regulate TGFβ signaling. GSK3β phosphorylates the linker region of Smad3 and inhibits its transcriptional activity (46). Consistent with previous data (45,48), it was demonstrated that GSK3β and Rac1 activity were conversely associated with nuclear Smad3 expression levels in sFRP1-overexpressing cells. This regulation by Rac1 may be indirectly through GSK3β, due to Rac1 not combining with Smad3 (data not shown). In the present study, apparent loss or gain of nuclear Smad2 in sFRP1-overexpression cells was not determined. Additionally, nuclear Smad2 expression levels were not notably altered by GSK3β and Rac1 activity. Due to the potential of Smad2/3 activity being influenced by other proteins, including mannosidases α class 1 (67), neural precursor cell expressed developmentally downregulated 4-like E3 ubiquitin protein ligase (29), sterol carrier proteins (68) and protein kinase B (69), the effects from other regulators on Smad2/3 in sFRP1-overexpressing cells cannot be excluded. It was speculated that the regulation towards TGFβ signaling by sFRP1-overexpression is primarily through targeting Smad3.

In conclusion, sFRP1 overexpression promotes gastric cancer cell proliferation and metastasis by activating TGFβ signaling; however, sFRP1 limits the growth inhibitory effect of TGFβ signaling via Rac1 and GSK3β. The present study demonstrated that sFRP1 has a novel role in regulating gastric cancer malignancy and may therefore serve as a therapeutic target for gastric cancer treatment.

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Availability of data and materials

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

Authors' contributions

JXP conceived and designed the study. JXP, SYL and LL performed the experiments. JXP wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Surana R, Sikka S, Cai W, Shin EM, Warrier SR, Tan HJ, Arfuso F, Fox SA, Dharmarajan AM and Kumar AP: Secreted frizzled related proteins: Implications in cancers. Biochim Biophys Acta 1845: 53-65, 2014.
2. Bovolenta P, Esteve P, Ruiz JM, Cisneros E and Lopez-Rios J: Beyond Wnt inhibition: New functions of secreted frizzled-related proteins in development and disease. J Cell Sci 121: 737-746, 2008.
3. Kawano Y and Kypta R: Secreted antagonists of the Wnt signalling pathway. J Cell Sci 116: 2627-2634, 2003.
4. Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA and Nathans J: A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc Natl Acad Sci USA 94: 2859-2863, 1997.
5. Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei LD and Umansky SR: SARPs: A family of secreted apoptosis-related proteins. Proc Natl Acad Sci USA 94: 13636-13641, 1997.
6. Foronjy R, Imai K, Shiomi T, Mercer B, Sklepkiewicz P, Thankachen J, Bodine P and D'Armiento J: The divergent roles of secreted frizzled related protein-1 (SFRP1) in lung morphogenesis and emphysema. Am J Pathol 177: 598-607, 2010.
7. Joesting MS, Cheever TR, Volzing KG, Yamaguchi TP, Wolf V, Naf D, Rubin JS and Marker PC: Secreted frizzled related protein 1 is a paracrine modulator of epithelial branching morphogenesis, proliferation, and secretory gene expression in the prostate. Dev Biol 317: 161-173, 2008.
8. Matsuyama M, Aizawa S and Shimono A: Sfrp controls apicobasal polarity and oriented cell division in developing gut epithelium. PLoS Genet 5: e1000427, 2009.
9. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG and Baylin SB: A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 31: 141-149, 2002.
10. Chung MT, Lai HC, Sytwu HK, Yan MD, Shih YL, Chang CC, Yu MH, Liu HS, Chu DW and Lin YW: *SFRP1* and *SFRP2* suppress the transformation and invasion abilities of cervical cancer cells through Wnt signal pathway. Gynecol Oncol 112: 646-653, 2009.
11. Valencia A, Román-Gómez J, Cervera J, Such E, Barragán E, Bolufer P, Moscardó F, Sanz GF and Sanz MA: Wnt signaling pathway is epigenetically regulated by methylation of Wnt antagonists in acute myeloid leukemia. Leukemia 23: 1658-1666, 2009.
12. Fukui T, Kondo M, Ito G, Maeda O, Sato N, Yoshioka H, Yokoi K, Ueda Y, Shimokata K and Sekido Y: Transcriptional silencing of secreted frizzled related protein 1 (SFRP 1) by promoter hypermethylation in non-small-cell lung cancer. Oncogene 24: 6323-6327, 2005.
13. Ugolini F, Charafe-Jauffret E, Bardou VJ, Geneix J, Adélaïde J, Labat-Moleur F, Penault-Llorca F, Longy M, Jacquemier J, Birnbaum D, *et al*: WNT pathway and mammary carcinogenesis: Loss of expression of candidate tumor suppressor gene *SFRP1* in most invasive carcinomas except of the medullary type. Oncogene 20: 5810-5817, 2001.
14. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y and Pietenpol JA: Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 121: 2750-2767, 2011.
15. Smid M, Wang Y, Zhang Y, Sieuwerts AM, Yu J, Klijn JG, Foekens JA and Martens JW: Subtypes of breast cancer show preferential site of relapse. Cancer Res 68: 3108-3114, 2008.
16. Mii Y and Taira M: Secreted frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range. Development 136: 4083-4088, 2009.
17. Katoh Y and Katoh M: Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (Review). Int J Mol Med 22: 271-275, 2008.
18. He J, Sheng T, Stelter AA, Li C, Zhang X, Sinha M, Luxon BA and Xie J: Suppressing Wnt signaling by the hedgehog pathway through sFRP-1. J Biol Chem 281: 35598-35602, 2006.
19. Häusler KD, Horwood NJ, Chuman Y, Fisher JL, Ellis J, Martin TJ, Rubin JS and Gillespie MT: Secreted frizzled-related protein-1 inhibits RANKL-dependent osteoclast formation. J Bone Miner Res 19: 1873-1881, 2004.
20. Esteve P and Bovolenta P: The advantages and disadvantages of sfrp1 and sfrp2 expression in pathological events. Tohoku J Exp Med 221: 11-17, 2010.
21. De Toni F, Racaud-Sultan C, Chicanne G, Mas VM, Cariven C, Mesange F, Salles JP, Demur C, Allouche M, Payrastre B, *et al*: A crosstalk between the Wnt and the adhesion-dependent signaling pathways governs the chemosensitivity of acute myeloid leukemia. Oncogene 25: 3113-3122, 2006.
22. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
23. Hohenberger P and Gretschel S: Gastric cancer. Lancet 362: 305-315, 2003.
24. Smith DD, Schwarz RR and Schwarz RE: Impact of total lymph node count on staging and survival after gastrectomy for gastric cancer: Data from a large US-population database. J Clin Oncol 23: 7114-7124, 2005.
25. Qu Y, Ray PS, Li J, Cai Q, Bagaria SP, Moran C, Sim MS, Zhang J, Turner RR, Zhu Z, *et al*: High levels of secreted frizzled-related protein 1 correlate with poor prognosis and promote tumourigenesis in gastric cancer. Eur J Cancer 49: 3718-3728, 2013.
26. Saini S, Liu J, Yamamura S, Majid S, Kawakami K, Hirata H and Dahiya R: Functional significance of secreted frizzled-related protein 1 in metastatic renal cell carcinomas. Cancer Res 69: 6815-6822, 2009.
27. Subauste MC, Von Herrath M, Benard V, Chamberlain CE, Chuang TH, Chu K, Bokoch GM and Hahn KM: Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. J Biol Chem 275: 9725-9733, 2000.
28. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M and Hung MC: Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol 6: 931-940, 2004.

29. Gao S, Alarcón C, Sapkota G, Rahman S, Chen PY, Goerner N, Macias MJ, Erdjument-Bromage H, Tempst P and Massagué J: Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signaling. Mol Cell 36: 457-468, 2009.
30. Labbé E, Silvestri C, Hoodless PA, Wrana JL and Attisano L: Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. Mol Cell 2: 109-120, 1998.
31. Hata A, Lo RS, Wotton D, Lagna G and Massague J: Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. Nature 388: 82-87, 1997.
32. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402-408, 2001.
33. Frame MC and Brunton VG: Advances in Rho-dependent actin regulation and oncogenic transformation. Curr Opin Genet Dev 12: 36-43, 2002.
34. Ren J, Wang R, Song H, Huang G and Chen L: Secreted frizzled related protein 1 modulates taxane resistance of human lung adenocarcinoma. Mol Med 20: 164-178, 2014.
35. Koivisto L, Häkkinen L, Matsumoto K, McCulloch CA, Yamada KM and Larjava H: Glycogen synthase kinase-3 regulates cytoskeleton and translocation of Rac1 in long cellular extensions of human keratinocytes. Exp Cell Res 293: 68-80, 2004.
36. Abe K, Rossman KL, Liu B, Ritola KD, Chiang D, Campbell SL, Burridge K and Der CJ: Vav2 is an activator of Cdc42, Rac1, and RhoA. J Biol Chem 275: 10141-10149, 2000.
37. Gao Y, Dickerson JB, Guo F, Zheng J and Zheng Y: Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc Natl Acad Sci USA 101: 7618-7623, 2004.
38. Schmöle AC, Brennführer A, Karapetyan G, Jaster R, Pews-Davtyan A, Hübner R, Ortinau S, Beller M, Rolfs A and Frech MJ: Novel indolylmaleimide acts as GSK-3beta inhibitor in human neural progenitor cells. Bioorg Med Chem 18: 6785-6795, 2010.
39. Pan Y, Bi F, Liu N, Xue Y, Yao X, Zheng Y and Fan D: Expression of seven main Rho family members in gastric carcinoma. Biochem Biophys Res Commun 315: 686-691, 2004.
40. Matsuoka T, Yashiro M, Kato Y, Shinto O, Kashiwagi S and Hirakawa K: RhoA/ROCK signaling mediates plasticity of scirrhous gastric carcinoma motility. Clin Exp Metastasis 28: 627-636, 2011.
41. Tang QL, Xie XB, Wang J, Chen Q, Han AJ, Zou CY, Yin JQ, Liu DW, Liang Y, Zhao ZQ, *et al*: Glycogen synthase kinase-3β, NF-κB signaling, and tumorigenesis of human osteosarcoma. J Natl Cancer Inst 104: 749-763, 2012.
42. Shakoori A, Mai W, Miyashita K, Yasumoto K, Takahashi Y, Ooi A, Kawakami K and Minamoto T: Inhibition of GSK-3 beta activity attenuates proliferation of human colon cancer cells in rodents. Cancer Sci 98: 1388-1393, 2007.
43. Hu H, Wang YL, Wang GW, Wong YC, Wang XF, Wang Y and Xu KX: A novel role of Id-1 in regulation of epithelial-to-mesenchymal transition in bladder cancer. Urol Oncol 31: 1242-1253, 2013.
44. Sánchez-Tilló E, Siles L, de Barrios O, Cuatrecasas M, Vaquero EC, Castells A and Postigo A: Expanding roles of ZEB factors in tumorigenesis and tumor progression. Am J Cancer Res 1: 897-912, 2011.
45. Ungefroren H, Groth S, Sebens S, Lehnert H, Gieseler F and Fändrich F: Differential roles of Smad2 and Smad3 in the regulation of TGF-β1-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: Control by Rac1. Mol Cancer 10: 67, 2011.
46. Guo X, Ramirez A, Waddell DS, Li Z, Liu X and Wang XF: Axin and GSK3-control Smad3 protein stability and modulate TGF-signaling. Genes Dev 22: 106-120, 2008.
47. Wrana JL, Attisano L, Cárcamo J, Zentella A, Doody J, Laiho M, Wang XF and Massagué J: TGF beta signals through a heteromeric protein kinase receptor complex. Cell 71: 1003-1014, 1992.
48. Cohen-Solal KA, Merrigan KT, Chan JL, Goydos JS, Chen W, Foran DJ, Liu F, Lasfar A and Reiss M: Constitutive Smad linker phosphorylation in melanoma: A mechanism of resistance to transforming growth factor-β-mediated growth inhibition. Pigment Cell Melanoma Res 24: 512-524, 2011.
49. Schnelzer A, Prechtel D, Knaus U, Dehne K, Gerhard M, Graeff H, Harbeck N, Schmitt M and Lengyel E: Rac1 in human breast cancer: Overexpression, mutation analysis, and characterization of a new isoform, Rac1b. Oncogene 19: 3013-3020, 2000.
50. Fritz G, Just I and Kaina B: Rho GTPases are over-expressed in human tumors. Int J Cancer 81: 682-687, 1999.
51. Zhu G, Wang Y, Huang B, Liang J, Ding Y, Xu A and Wu W: A Rac1/PAK1 cascade controls β-catenin activation in colon cancer cells. Oncogene 31: 1001-1012, 2012.
52. Kamai T, Shirataki H, Nakanishi K, Furuya N, Kambara T, Abe H, Oyama T and Yoshida K: Increased Rac1 activity and Pak1 overexpression are associated with lymphovascular invasion and lymph node metastasis of upper urinary tract cancer. BMC Cancer 10: 164, 2010.
53. Zhan H, Liang H, Liu X, Deng J, Wang B and Hao X: Expression of Rac1, HIF-1α, and VEGF in gastric carcinoma: Correlation with angiogenesis and prognosis. Onkologie 36: 102-107, 2013.
54. Wu YJ, Tang Y, Li ZF, Li Z, Zhao Y, Wu ZJ and Su Q: Expression and significance of Rac1, Pak1 and Rock1 in gastric carcinoma. Asia Pac J Clin Oncol 10: e33-e39, 2014.
55. Steffen A, Ladwein M, Dimchev GA, Hein A, Schwenkmezger L, Arens S, Ladwein KI, Margit Holleboom J, Schur F, Victor Small J, *et al*: Rac function is critical for cell migration but not required for spreading and focal adhesion formation. J Cell Sci 126: 4572-4588, 2013.
56. Lewis-Saravalli S, Campbell S and Claing A: ARF1 controls Rac1 signaling to regulate migration of MDA-MB-231 invasive breast cancer cells. Cell Signal 25: 1813-1819, 2013.
57. Wang J, Rao Q, Wang M, Wei H, Xing H, Liu H, Wang Y, Tang K, Peng L, Tian Z, *et al*: Overexpression of Rac1 in leukemia patients and its role in leukemia cell migration and growth. Biochem Biophys Res Commun 386: 769-774, 2009.
58. Fritz G and Kaina B: Rac1 GTPase, a multifunctional player in the regulation of genotoxic stress response. Cell Cycle 12: 2521-2522, 2013.
59. Qiu RG, Abo A, McCormick F and Symons M: Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol Cell Biol 17: 3449-3458, 1997.
60. Cho YJ, Yoon J, Ko YS, Kim SY, Cho SJ, Kim WH, Park JW, Youn HD, Kim JH and Lee BL: Glycogen synthase kinase-3β does not correlate with the expression and activity of β-catenin in gastric cancer. APMIS 118: 782-790, 2010.
61. Esufali S, Charames GS and Bapat B: Suppression of nuclear Wnt signaling leads to stabilization of Rac1 isoforms. FEBS Lett 581: 4850-4856, 2007.
62. Binker MG, Binker-Cosen AA, Gaisano HY, de Cosen RH and Cosen-Binker LI: TGF-β1 increases invasiveness of SW1990 cells through Rac1/ROS/NF-kB/IL-6/MMP-2. Biochem Biophys Res Commun 405: 140-145, 2011.
63. Bachman KE and Park BH: Duel nature of TGF-beta signaling: Tumor suppressor vs. tumor promoter. Curr Opin Oncol 17: 49-54, 2005.
64. Akhurst RJ and Derynck R: TGF-beta signaling in cancer-a double-edged sword. Trends Cell Biol 11: S44-S51, 2001.
65. Derynck R, Akhurst RJ and Balmain A: TGF-beta signaling in tumor suppression and cancer progression. Nat Genet 29: 117-129, 2001.
66. Hubchak SC, Sparks EE, Hayashida T and Schnaper HW: Rac1 promotes TGF-beta-stimulated mesangial cell type I collagen expression through a PI3K/Akt-dependent mechanism. Am J Physiol Renal Physiol 297: F1316-F1323, 2009.
67. Kondé E, Bourgeois B, Tellier-Lebegue C, Wu W, Pérez J, Caputo S, Attanda W, Gasparini S, Charbonnier JB, Gilquin B, *et al*: Structural analysis of the Smad2-MAN1 interaction that regulates transforming growth factor-β signaling at the inner nuclear membrane. Biochemistry 49: 8020-8032, 2010.
68. Wrighton KH, Willis D, Long J, Liu F, Lin X and Feng XH: Small C-terminal domain phosphatases dephosphorylate the regulatory linker regions of Smad2 and Smad3 to enhance transforming growth factor-beta signaling. J Biol Chem 281: 38365-38375, 2006.
69. Shepherd RD, Kos SM and Rinker KD: Flow-dependent Smad2 phosphorylation and TGIF nuclear localization in human aortic endothelial cells. Am J Physiol Heart Circ Physiol 301: H98-H107, 2011.