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

JI-XIANG PENG1, SHUN-YU LIANG2 and LI LI1

1Department of Gastrointestinal Surgery, Guangzhou First People’s Hospital, The Second Affiliated Hospital of South China University of Technology; 2Department of Gastrointestinal Surgery, Guangzhou First Municipal People’s Hospital, Affiliated Guangzhou Medical College, Guangzhou, Guangdong 510180, P.R. China

Received March 1, 2018; Accepted October 11, 2018

DOI: 10.3892/or.2018.6838

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...
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.) 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 determined using a Rac1 Activation Assay Biochem kit (Cytoskeleton, Inc., Denver, CO, USA) according to the manufacturer's protocol. The data presented were the mean value of three independent experiments.

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. ab126661), 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. E1067Y), 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 1x10^5 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 phalloidin (Fig. 1A, upper); however, SGC-7901/vector and SGC-7901/sFRP1 cells exhibited extended lamellipodia composed of F-actin fibro stained by phalloidin (Fig. 1A, lower). This phenomenon indicated that Rac1, which is well known to be necessary for cell migration, was activated by sFRP1. The 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 indicates that Rac1, which is well known to be activated by sFRP1, is necessary for cell migration.

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 Quant iTect SYBR Green PCR master mix (2x Quant iTect SYBR Green kit, containing HotStart Taq® DNA polymerase, Quant iTect SYGB Green PCR buffer, dNTP mix, SYGB 1, Rox passive reference dye and 5 mM MgCl2; 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^-ΔΔct (Cq gene - Cq GAPDH) (32). The primers used were as follows: PAI1 forward, 5'-TGGCCAGGTTGCGCTCTCAT-3' and reverse, 5'-ACTGTTCCTGTGGGTTGTTG-3'; ID1 forward, 5'-CGAGATCAGCGCCCTGACCG-3' and reverse, 5'-GGGCCGGCATCGGTCCTTGT-3'; Smad3 forward, 5'-GGAAGATGGTGCAGAAGG-3' and reverse, 5'-GAA GCCGAACTCACAGCGC-3'; p21 forward, 5'-GCCGAAGTCTGTTCTTG-3' and reverse, 5'-TCTGACATGGGCG TCTG-3'; activating transcription factor 3 (ATF3) forward, 5'-GAGGTGTTGTTAGCTTCAGT-3' and reverse, 5'-TTG ATTTTTGCGAACGTGC-3'; GAPDH forward, 5'-GGG CTCCTGCTTCTCCCTCTTCTT-3' and reverse, 5'-CAG GCGTCCGATAAGGCCCC-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
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 beads, and its level was decreased upon Rac1 or GSK3β inhibition compared with the vehicle control 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 beads, and its level was decreased upon Rac1 or GSK3β inhibition compared with the vehicle control cells (Fig. 3A).
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
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 TGFβ1-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
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 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.
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 overexpression (WT, CA and DN) regulated the pSmad3C and pSmad3L levels assayed by immunoblotting. (B) Rac1 led-related protein 1; Rac1, Rac family small GTPase 1; GSK3, glycogen synthase kinase 3; TGFβ, transforming growth factor β; WT, wild-type; CA, constitutively active; DN, dominant-negative; p-, phosphorylated; Smad, SMAD family member.

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-mediated 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 (46,46) 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-overexpressing 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.
Acknowledgements

Not applicable.

Funding

No funding was received.

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. Biochem 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 signaling 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 apoptosis-related proteins. Proc Natl Acad Sci USA 94: 2859-2863, 1997.
5. Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tometi LD and Umansky SR: SARP: 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, Sklepikiewicz P, Thankachen J, Bodine P and D’Armiento J: The divergent roles of secreted frizzled related protein-1 (SFRP1) in lung morphogenesis and embryogenesis. 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. Matsuysama 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, Gabrielsson 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: 658-663, 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 (SFRP1) by promoter hypermethylation in non-small-cell lung cancer. Oncogene 24: 6323-6327, 2005.
13. Ugalini F, Charafe-Jauffret E, Bardou VJ, Geneix J, Adélaïde J, Labat-Moleur F, Renault-Lorca 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, Lehmkuhl M, Arfuso F, Fox SA, Dharmarajan AM and Kumar AP: Secreted frizzled related proteins: Implications in cancers. Biochim Biophys Acta 1845: 53-65, 2014.
15. Smid M, Wang Y, Zhang Y, Siewerts AM, Yu J, Klijn JG, Birnbaum D, et al: Transcriptional silencing of secreted frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range. Development 136: 4083-4088, 2009.
16. Katoh Y and Katoh M: Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (Review). Int J Mol Med 21: 271-275, 2008.
17. 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.
18. 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.
19. 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.
20. 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 crossstalk between the Wnt and the adhesion-dependent signaling pathways governs the chemosensitivity of acute myeloid leukemia. Oncogene 25: 3115-3122, 2006.
21. 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.
22. Hohenberger P and Gretschel S: Gastric cancer. Lancet 362: 805-815, 2003.
23. 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: 714-724, 2005.
24. 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.
25. 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.
26. 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.
27. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M and Hung MC: Dual regulation of SFRP1 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, Eurdjument-Bromage H, Tempst P and Massague J: Ubiquitin ligase Nedd4L targets Smad2/3 to limit TGF-beta signaling. Mol Cell 36: 457-468, 2009.

30. Labbé E, Silvestri C, Hoodless PA, Wranja JL and Attisano L: Smad2 and Smad3 positively and negatively regulate TGF.

31. Hata A, Lo RS, Wotton D, Lagna G and Massague J: Mutations increasing antiinhibition inactive TGF causes 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^−ΔΔCt method. Methods 25: 402-408, 2001.

33. Franne MY and Horton VG: Advances in Rho-dependent actin regulation and oncogenic progression 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, Burrage 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. Schmölle 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-3β 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 genes in mammary gland cancer cells. Biochem Biophys Res Commun 315: 686-691, 2004.

40. Matsubara T, Yashiro M, Kato Y, Shintoh O, Kashiwagi S and Hirakawa K: RhoA/ROCK signaling mediates plasticity of squamous carcinoma cell motility. Clin Exp Metastasis 28: 627-636, 2011.

41. Tang QL, Xie XB, Wang J, Chen Q, Han AJ, Zhou CY, Yin JQ, Liu DW, Liang Y, Zhao ZQ, et al.: Rac function is critical for cell migration but not required for spreading and focal adhesion formation. J Cell Sci 127: 1242-1253, 2014.

42. Sánchez-Tilló E, Siles L, de Barrios O, Cuatrecazas 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.

43. Ungefelden H, Groth S, Sebens S, Lehnhrt H, Gieseler F and Färdrich D: Differential roles of Smad2 and Smad3 in the regulation of TGF-β-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: Control by Rac1. Mol Cancer 10: 67, 2011.

44. Guo X, Ramirez A, Waddell DS, Li Z, Liu X and Wang XF: TGF-β signaling. Mol Cell 36: 457-468, 2011.

45. Whitney 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-β signaling. J Biol Chem 281: 38356-38375, 2006.

46. Brook KD, Kos SM and Rinker KD: Flow-dependent Smad2 phosphorylation and TGF-β nuclear localization in human aortic endothelial cells. Am J Physiol Heart Circ Physiol 301: H98-H107, 2011.