

# Suppressing Wnt Signaling by the Hedgehog Pathway through sFRP-1<sup>\*[5]</sup>

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The hedgehog (Hh) signaling pathway is essential for embryonic development and carcinogenesis. Activation of Hh signaling has been identified in several types of gastrointestinal cancers, including esophageal, gastric, pancreatic, and liver cancers. Several recent studies suggest that Hh signaling activation can inhibit Wnt signaling. However, the molecular basis underlying this inhibition remains unclear. As transcription factors in the Hh signaling pathway, Gli molecules transform cells in culture, and their expression are associated with cancer development. Here we report that expression of a secreted frizzled-related protein-sFRP-1 in mouse embryonic fibroblasts is dependent on Gli1 and Gli2. In human gastric cancer cells, inhibition of Hh signaling reduces the level of sFRP-1 transcript, whereas ectopic expression of Gli1 increases the level of sFRP-1 transcript. Results from chromatin immunoprecipitation indicate that Gli1 is involved in transcriptional regulation of sFRP-1. In 293 cells with Gli1 expression, Wnt-1-mediated  $\beta$ -catenin accumulation in the cytosol and DKK1 expression are all abrogated, which can be reversed by inhibiting sFRP-1 expression. Furthermore, while SIIA cells do not respond to Wnt-1-conditioned medium, inhibition of Hh signaling by smoothened (SMO) antagonist KAAD-cyclopamine (keto-*N*-aminoethylaminocaproyldihydrocinnamoylcyclopamine) leads to Wnt1-mediated  $\beta$ -catenin accumulation in the cytosol. These data indicate that sFRP-1, a target gene of the hedgehog pathway, is involved in cross-talk between the hedgehog pathway and the Wnt pathway.

Hedgehog (Hh)<sup>2</sup> proteins are a group of secreted proteins whose active forms are derived from a unique protein cleavage process and at least two post-translational modifications (1).

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<sup>2</sup> The abbreviations used are: Hh, hedgehog; SMO, smoothened; KAAD-cyclopamine, keto-*N*-aminoethylaminocaproyldihydrocinnamoylcyclopamine; MEF, mouse embryonic fibroblast; siRNA, small interfering RNA; RT, reverse transcription; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinediethanesulfonic acid.

Secreted Hh molecules bind to the receptor patched, thereby alleviating patched-mediated suppression of smoothened (SMO) (2, 3). Expression of sonic hedgehog appears to stabilize SMO protein possibly through post-translational modification of SMO. In *Drosophila*, SMO stabilization triggers complex formation with Costal-2, Fused, and Gli homologue cubitus interruptus, which prevents cubitus interruptus degradation and formation of a transcriptional repressor (4–7). SMO ultimately activates transcription factors of the Gli family. As transcriptional factors, Gli molecules can regulate target gene expression by direct association with a consensus binding site (5'-tgggtg-gtc-3') located in the promoter region of the target genes (8, 9).

Constitutive activation of Hh signaling is detected in a variety of human cancers. The link between the Hh signaling pathway and human cancer has come from genetic analysis of Gorlin syndrome patients, who are predisposed to early onset of multiple basal cell carcinomas (10, 11). In addition to basal cell carcinomas and medulloblastomas, Hh signaling activation occurs frequently in advanced prostate cancer, small cell lung cancer, and several gastrointestinal cancers, including gastric, esophageal, pancreatic, and liver cancers (12–17). Wnt signaling is also known to be involved in several types of gastrointestinal cancers (18). Two recent studies indicate that tumors with activated Hh signaling often do not have nuclear accumulation of  $\beta$ -catenin, a major indicator for the canonical Wnt signaling (16, 19). One study indicates that Indian Hh negatively regulates Wnt signaling (20); however, the molecular basis for such an inhibition remains elusive.

sFRP-1 is a 30-kDa glycoprotein that was first identified as the antagonist of Wnt signaling and regulator of apoptosis (21). The sFRP-1 gene is frequently up-regulated in Hh-activated tumors (22). We have found that expression of sFRP-1 is dependent on Gli1 and Gli2 in MEF cells and regulated by the Hh pathway in gastric cancer cell lines. Gli1 protein appears to be involved in regulation of the endogenous sFRP-1 promoter. As a consequence of Hh signaling activation and consequent sFRP-1 expression, Wnt-1-mediated  $\beta$ -catenin accumulation in the cytosol and DKK1 expression are inhibited. This inhibition can be reversed when sFRP-1 expression is reduced. These data indicate that sFRP-1 serves as the molecular link for Hh signaling-mediated inhibition of Wnt signaling.

## MATERIALS AND METHODS

**Cell Culture and Plasmids**—The SIIA cell line was established in the Department of Surgery in our institution (23). AGS is another cell line from gastric adenocarcinomas used in this study. Both cell lines were kindly provided by Dr. Mark Hellmich and cultured according to the suggested conditions (14). 293 cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Ectopic expression of Gli1 in AGS cells was achieved by the stable retrovirus infection. For retrovirus infection, pLNCX-Gli1 (MYC-tagged) was transfected into Phoenix packaging cells using FuGENE 6 (Roche Applied Science). Supernatant was collected 24–48 h post-transfection (24). Empty pLNCX

vector was used to produce the control retrovirus. Infection for AGS cells was carried out overnight in the presence of 8  $\mu\text{g}/\text{ml}$  polybrene. A day later, 250  $\mu\text{g}/\text{ml}$  G418 was added to the culture medium for selection of a pool of Gli1 stable expressing clones. Western blot was used to detect the Gli1 protein. 293-Wnt1 cells were made from the pLNCX-Wnt1 plasmid (25, 26).

SIIA Cells were treated with 2.5  $\mu\text{M}$  keto-*N*-aminoethylaminocaprolyldihydrocinnamoylcyclopamine (KAAD-cyclopamine, catalog number K171000 from Toronto Research Chemicals Inc., Toronto, Canada) for 10, 12, and 24 h (14).

*siRNA, RNA Isolation, RT-PCR, and Real-time PCR*—Expression of sFRP-1 in 293 cells was knocked down using Ambion's pre-designed siRNA for sFRP-1 (siRNA ID number 121421) following transfection with oligofectAmine according to manufacturer's instruction.

Total RNA of cells was extracted using a RNA extraction kit from Promega according to the manufacturer (Promega, Madison, WI), and quantitative PCR analyses were performed according to a previously published procedure using primers and probes from Applied Biosystems (16). Triplicate  $C_T$  values were analyzed in Microsoft Excel using the comparative  $C_T$  ( $\Delta\Delta C_T$ ) method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amount of target ( $2^{-\Delta\Delta C_T}$ ) was obtained by normalization to an endogenous reference (18 S RNA) and relative to a calibrator.

We performed RT-PCR of Gli1, sFRP-1, and HIP with 32 cycles of 96 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s with the following primers: Gli1, 5'-GGAATTCTGTTTCCCCAGGT-3' (forward primer) and 5'-ACCCCTGGACTCTCTTGAT-3' (reverse primer); sFRP-1, 5'-CCCTCGGGGAAGTGTGTCACA-3' (forward primer) and 5'-GCTCAACAAGAAGTGCACA-3' (reverse primer); HIP, 5'-TGCTAAGCCTCGCATTCCA-3' (forward primer) and 5'-ACAACCCTAAGAATGTGGTCATGA-3' (reverse primer).

*Western Blotting*—Cells were lysed in protein loading buffer, and proteins were separated by 10% SDS-PAGE. After electrophoresis, protein was electrotransferred on nitrocellulose membrane, blocked with 5% nonfat dry milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and blotted against appropriate primary antibodies (sFRP-1 antibodies were from R&D System, Inc., catalog number AF 1384; Myc-tag (9B-11) antibodies were from Cell Signaling Inc., catalog number 2276;  $\beta$ -catenin antibodies were from BD Transduction Laboratory, catalog number 610154;  $\beta$ -actin antibodies were purchased from Sigma) overnight at 4 °C, which was followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h at room temperature. Different dilutions were used for different primary antibodies: anti-sFRP-1, 1:1,000; anti- $\beta$ -catenin, 1:1,000; anti-MYC 9B11, 1:5,000; and anti- $\beta$  actin, 1:5,000. The protein bands were visualized by enhanced chemiluminescence (24).

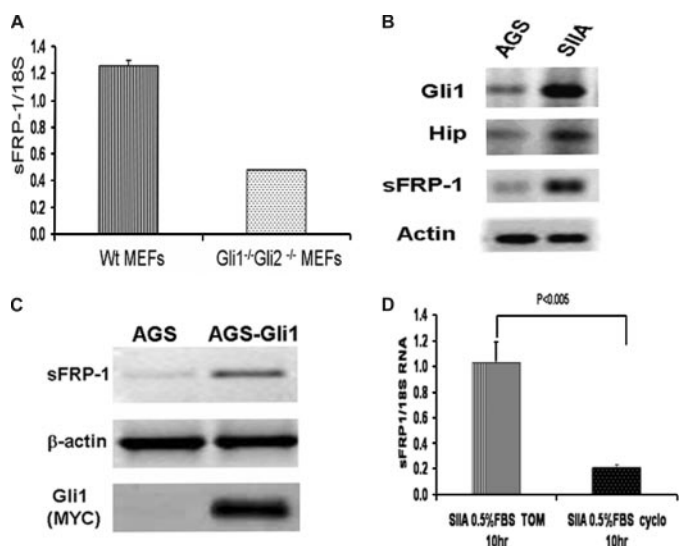
*Chromatin Immunoprecipitation (ChIP) Assay*—Log-phase AGS cells and the AGS cells with ectopic Gli1 expression ( $2 \times 10^7$  cells for each immunoprecipitation experiment) were cultured and fixed by the addition of formaldehyde (1% (w/v) final concentration) for 10 min. After addition of 125 mM glycine, cells were washed three times with cold phosphate-buffered

saline, re-suspended in 900  $\mu\text{l}$  of lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40) containing protease inhibitors and left for 10 min at 4 °C. DNA was sheared by sonication to yield an average length of 500–700 bp and cleared by centrifugation at  $12,000 \times g$  for 5 min at 4 °C. Lysates were diluted with the dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). 100  $\mu\text{l}$  of the sample was used as the "input," and the rest of sample was incubated with 1  $\mu\text{g}$  of MYC or the control HA antibody beads at 4 °C for overnight. Gli1-DNA complexes were isolated by centrifugation at 5,000 rpm in an Eppendorf microcentrifuge for 1 min, and pellets were washed three times with 1 ml of  $1 \times$  dialysis buffer (50 mM Tris-Cl, pH 8.0, 2 mM EDTA) and three times with 1 ml of immunoprecipitation wash buffer (100 mM Tris-Cl, pH 8.0, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid) for 5 min with rotation. After wash, 200  $\mu\text{l}$  of digestion buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% SDS, 100  $\mu\text{g}/\text{ml}$  proteinase K) was added to each sample, incubated at 55 °C for 3 h, followed by 6 h at 65 °C. The samples were extracted with phenol-chloroform following by precipitation with ethanol at  $-80$  °C in the presence of 20  $\mu\text{g}$  of glycogen overnight. The pellets were suspended in 20  $\mu\text{l}$  of TE buffer (100 mM Tris-Cl, 10 mM EDTA, pH 8.0). PCR was performed with 35 cycles of 96 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s using the following primers flanking the putative Gli-binding sites in the human sFRP-1 promoter: sFRP-1F, 5'-GTTGGAGCTGTTTGGCTGTGA-3'; sFRP-1R, 5'-ATGTTTTGGCTTTCCACACC-3'.

*Cell Fractionation*—Following treatment with KAAD-cyclopamine (2.5  $\mu\text{M}$ ) for 24 h (for SIIA cells) or conditioned medium from 293-Wnt1 cells for 3 h (for 293 cells), the cells were rinsed twice with cold phosphate-buffered saline, harvested in 1 ml of cold phosphate-buffered saline for each 10 cm dish, and collected by centrifugation at  $1,500 \times g$  for 5 min. The cell pellets were incubated with 2 ml of hypotonic buffer (1 M Tris-HCl, pH 7.4, 1 M sodium fluoride, 0.5 M,  $1 \times$  proteinase inhibitor). After 20 min on ice, the lysates were centrifuged at  $1,000 \times g$  for 10 min at 4 °C. The supernatant fractions as the cytoplasmic and membrane fraction were ultracentrifuged at  $100,000 \times g$  for 30 min at 4 °C. The supernatant fractions (containing the cytosol) were collected (25, 26). All extracts were normalized for protein amounts determined by Bio-Rad protein assay (Bio-Rad) and separated by 10% SDS-PAGE for further analysis. Antibodies to  $\beta$ -catenin (BD Transduction Laboratory) were used to detect the level of  $\beta$ -catenin in different fractions.

## RESULTS

To identify Hh target genes, we compared gene expression between wild type MEFs and Gli1<sup>-/-</sup>/Gli2<sup>-/-</sup> MEF cells. We found that expression of sFRP-1 was dependent on Gli1 and Gli2. As shown in Fig. 1A, the sFRP-1 mRNA level in wild type MEFs was about 3-fold higher than that in Gli1/Gli2 double knock-out MEF cells. This result was further confirmed by the Northern blot analysis (data not shown). To validate the results in cancer cells, we detected the sFRP-1 mRNA expression in the human cancer cell lines with activated Hh pathway. As shown in Fig. 1B, the Hh activation level was lower in AGS cells than that in SIIA cells. Consistent with Hh signaling activation, a higher level of sFRP-1 mRNA expression was detected in SIIA



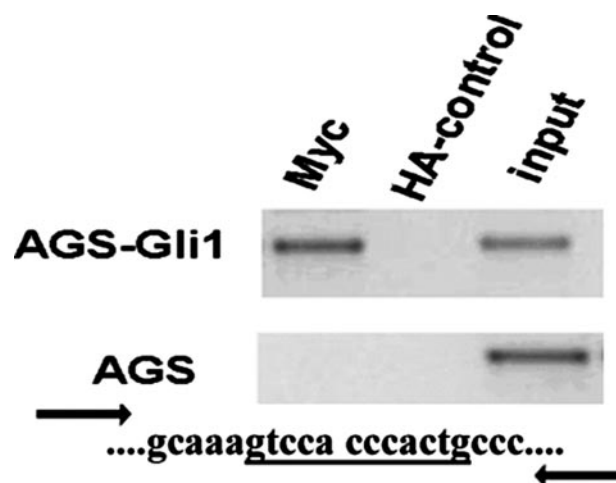
**FIGURE 1. sFRP-1 is regulated by Gli.** *A*, sFRP-1 mRNA of MEF cells and Gli1/Gli2 null MEF cells were detected by real-time PCR using primers and probes from the Applied Biosciences (see "Materials and Methods" for the analysis). *B*, after RNA extraction from AGS and SIIA cells, Gli1, HIP, and sFRP-1 transcripts were detected by RT-PCR. Human  $\beta$ -actin was used as the internal control. *C*, by retrovirus infection, Gli1 was ectopically expressed in AGS cells. MYC-tagged Gli1 was detected by anti-MYC antibodies. Induced expression of sFRP-1 transcript was detected by RT-PCR. *D*, SIIA cells were treated with 2.5  $\mu$ M KAAD-cyclopamine (indicated as *Cyclo* in the figure) for 10 h. The control was tomatidine (indicated as *Tom* in the figure), a compound with a similar structure to cyclopamine but has no specific effects on hedgehog signaling. Real-time PCR was performed to detect the sFRP-1 transcript.

cells (Fig. 1*B*). Immunohistochemistry analysis of primary human cancers further confirmed that sFRP-1 was expressed highly in gastric cancer specimens with activated Hh signaling (data not shown).

Following ectopic expression of Gli1 via retrovirus-mediated gene transfer in AGS cells, we further showed that sFRP-1 expression was induced (Fig. 1*C*). Conversely, treatment of SIIA cells for 12 h with SMO antagonist, KAAD-cyclopamine, decreased the sFRP-1 mRNA level by 80% in SIIA cells (Fig. 1*D*). To confirm the expression of sFRP-1 at the protein level, we performed Western blotting analysis using conditioned medium of SIIA cells since sFRP-1 is a secreted molecule. As shown in supplemental Fig. 1, we found that sFRP-1 protein expression was reduced after KAAD-cyclopamine treatment in SIIA cells. These data indicate that expression of sFRP-1 is regulated by Hh signaling.

To determine whether Gli transcription factors are involved in regulation of the sFRP-1 promoter, we analyzed the promoter sequence of sFRP-1 and found two putative Gli-binding sites. We performed ChIP assays to assess whether Gli1 is involved in binding the endogenous sFRP-1 promoter. As shown in Fig. 2, we found that immunoprecipitation of ectopically expressed Gli1 can pull down the endogenous sFRP-1 promoter DNA sequence containing one Gli-putative binding site, suggesting that Gli1 is involved in regulation of the sFRP-1 promoter.

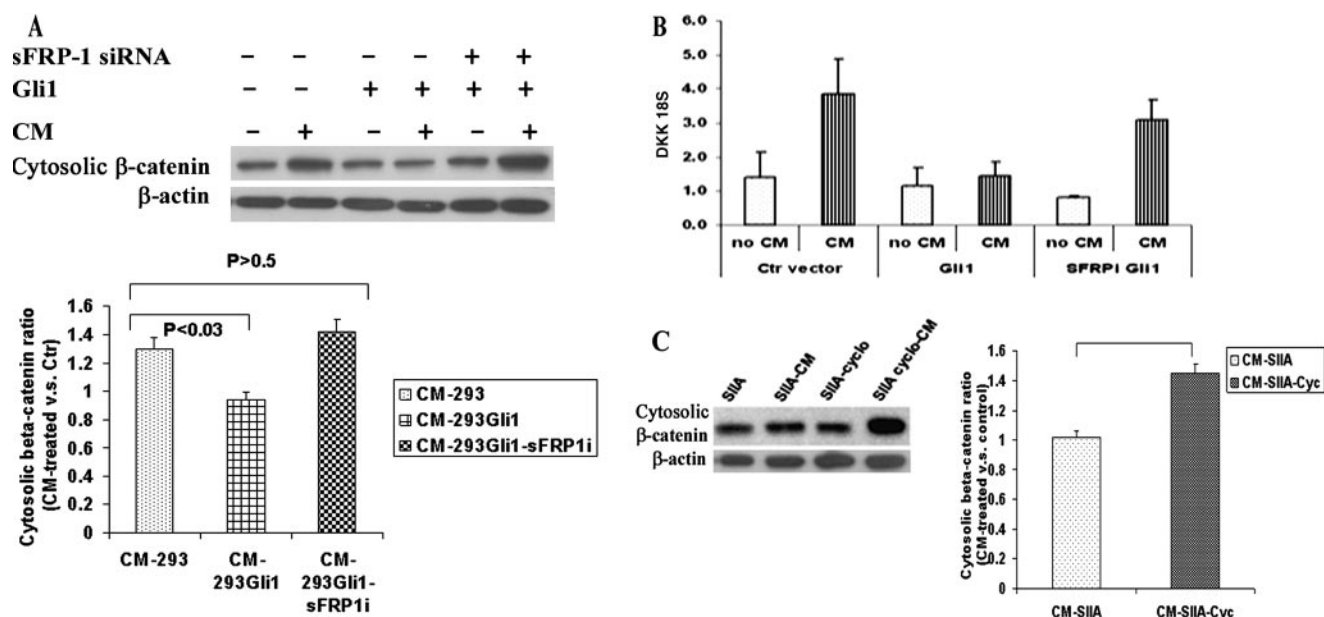
It is known that sFRP-1 is an inhibitor for Wnt signaling (21). To understand if Hh-induced sFRP-1 expression affects Wnt signaling, we assessed  $\beta$ -catenin accumulation in the cytosol of 293 cells in different conditions. Consistent with previous pub-



**FIGURE 2. Direct binding of Gli1 to the sFRP-1 promoter.** Log-phase AGS cells and the AGS cells with ectopic Gli1 expression ( $2 \times 10^7$  cells/immunoprecipitation) were harvested, and ChIP assay was performed to examine the binding of Gli1 to sFRP-1 promoter. Anti-HA antibodies were used as the negative control. Human sFRP-1 primers (sFRP-1F, 5'-GTTGGAGCTGTTGCTGTGA-3' and sFRP-1R, 5'-ATGTTTTGGCTTCCACACC-3') were used to amplify the sFRP-1 promoter region containing the putative Gli-binding site (the underlined sequence).

lished results (25, 26), Wnt-1 conditioned medium significantly induced  $\beta$ -catenin accumulation in the cytosol (Fig. 3*A*). However, following expression of Gli1, Wnt-1-mediated  $\beta$ -catenin accumulation was greatly reduced (Fig. 3*A*). In both cases, no significant changes of membrane  $\beta$ -catenin were observed (supplemental Fig. 2). To confirm this result, we examined the expression of DKK1, a Wnt target gene (27), in these cells. As shown in Fig. 3*B*, while Wnt-1-conditioned medium induced expression of DKK1 by 3-fold, ectopic expression of Gli1 prevented Wnt-1-mediated induction of DKK1. As predicted, Gli1 expression was accompanied by elevated level of sFRP-1 in the conditioned medium (supplemental Fig. 3, *A* and *B*). This induction was independent of new protein synthesis because addition of cycloheximide did not affect the level of sFRP-1 (supplemental Fig. 3*C*). To test whether sFRP-1 is required for Gli1-mediated inhibition of Wnt-1 signaling, we knocked down sFRP-1 in Gli1-expressing 293 cells. We found that following sFRP-1 knockdown (supplemental Fig. 4), Wnt-1-conditioned media were able to induce cytosolic accumulation of  $\beta$ -catenin (Fig. 3*A*) as well as DKK1 expression (Fig. 3*B*). These results demonstrated that Hh signaling attenuates Wnt signaling in 293 cells through regulation of sFRP-1 expression. We hypothesized that if sFRP-1 is induced in cancer cells with activated Hh signaling, these cells should be insensitive to Wnt-1 stimulation. Indeed, we found that Wnt-1-conditioned medium did not cause  $\beta$ -catenin accumulation in SIIA cells, which have active hedgehog signaling. However, following inhibition of Hh signaling by KAAD-cyclopamine (2.5  $\mu$ M), which is accompanied by reduced expression of sFRP-1 (Fig. 1*D*), Wnt-1-conditioned medium was able to stimulate cytosolic accumulation of  $\beta$ -catenin (Fig. 3*C*). These data confirm that Hh signaling affects Wnt signaling through regulation of sFRP-1.

The biological relevance of our findings were reflected by the fact that most gastrointestinal cancer specimens with Hh signaling activation did not have elevated cytoplasmic accumula-



**FIGURE 3. Hh signaling affects Wnt1-induced cytosolic accumulation of  $\beta$ -catenin through sFRP-1.** *A*, 293 cells were transiently transfected with Gli1 (with MYC tag) for 24 h, followed by stimulation with conditioned medium (CM) from 293-Wnt-1 cells for 3 h. Cells were lysed and fractionated. In addition, sFRP-1 was inactivated by siRNA (indicated as *sFRP1i* in the figure) in the Gli1-expressing 293 cells for conditioned medium treatment. Equivalent amounts of protein from each cytosolic fraction were analyzed by Western blotting with monoclonal antibodies against  $\beta$ -catenin (BD Transduction Laboratory). The experiments were repeated three times. The ratio of cytosolic  $\beta$ -catenin between the conditioned medium-treated group and the control group was obtained by comparison of the relative intensity of the  $\beta$ -catenin band and was used to show the Wnt1-mediated signaling. Student's *t* test for two samples were used to assess the significance between the paired samples (*p* value less than 0.05 was considered statistically significant). *B*, expression of the Wnt target gene DKK1 was examined by real-time PCR at the same time point as the  $\beta$ -catenin accumulation studies. The relative level of DKK1 was determined by comparison with the internal control, 18 S RNA. *C*, SIIA cells were treated with 2.5  $\mu$ M KAAD-cyclopamine for 24 h and then stimulated with 293-Wnt1-conditioned medium for 3 h. Cytosolic protein, normalized with  $\beta$ -actin protein expression, was analyzed by Western blotting. An average of three independent experiments was shown and the standard deviations are less than 10%. Student's *t* test was used to show the significance of the difference between the paired samples.

tion of  $\beta$ -catenin (16, 19). As we showed in liver and gastric cancers, only one out of 40 tumors with activated Hh signaling contains nuclear localization of  $\beta$ -catenin (the *p* value < 0.03) (16).

In summary, our data provide direct evidence to support that sFRP-1 is the downstream target gene of Hh signaling that mediates the suppressive effect on Wnt signaling. Our data from ChIP assay suggest that the Hh signaling pathway regulates sFRP-1 expression through the promoter.

## DISCUSSION

The inappropriate activation of Hh and Wnt signaling occurs frequently in cancer. Recent studies indicate that Ihh serves as an inhibitor for Wnt signaling during colon development and in colon cancer (20). Furthermore, it is shown that Gli1 plays an inhibitory role in the development of colorectal cancer involving Wnt signaling (19). We have shown that activation of Hh signaling is rarely associated with nuclear localization of  $\beta$ -catenin in liver cancers (16). Our data indicate that elevated expression of sFRP-1 following activation of the Hh pathway provides the molecular link for the inhibitory effect on Wnt signaling. We have shown that sFRP-1 expression is dependent on Gli1 and Gli2. Tumors with activated Hh signaling have a high level of sFRP-1 expression, and inhibition of Hh signaling also reduces the level of sFRP-1. We further show that Gli1 is involved in binding the endogenous sFRP-1 promoter. SIIA cells in which Hh signaling is activated do not respond to Wnt1-mediated  $\beta$ -catenin accumulation in the cytosol, whereas inhibition of Hh signaling sensitizes Wnt-1 stimulation. We

predict that this inhibitory signaling loop is defective in some cancers in which sFRP-1 is inactivated through promoter methylation (28), which can be tested in future studies. Thus, loss of sFRP-1 not only allows Wnt signaling to occur without proper control but also abrogates the inhibitory effect from the Hh pathway. In addition to elevated expression of sFRP-1, other mechanisms may be also responsible for this inhibitory role, such as  $\beta$ -catenin accumulation (19).

Although the biological activity of sFRPs has been largely attributed to their inhibition of Wnt activity, other mechanisms of action may exist. Recently sFRP-2 was reported to promote cell adhesion and inhibit apoptosis through an association with a fibronectin-integrin complex and stimulation of integrin signaling (29). A screening of peptide library using recombinant sFRP-1 identified several molecules involved in cell-cell signaling, such as RANKL and UNC5H3, a human homologue of *Caenorhabditis elegans* netrin receptor Unc5 (30). It will be interesting to assess if these mechanisms are involved in Hh-mediated signaling in human cancer.

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