Renal cell carcinoma (RCC) occurs in 1 of 30,000 people, with the average age of onset between the fifth and seventh decades of life. Incidence of the disease is twice as common in males as in females, with 50% of patients eventually developing metastatic disease. Incidence and mortality rates for RCC have risen steadily for more than 30 years; this trend is not fully explained by an increase in abdominal imaging (1, 2). The most common histologic subtype is clear cell RCC (cRCC), which accounts for >80% of all RCCs diagnosed in the United States each year. This cancer exhibits resistance to chemother-apy and radiation, and <10% of patients suffering from metastatic disease survive 5 years after diagnosis (3–5). There are currently no clinically proven adjuvant therapies that exist for cRCC patients at high risk of relapse following surgical resection (6). A better understanding of the molecular pathogenesis of cRCC is needed to direct new therapeutic interventions. In the present study, genomic profiling of cRCC and matched normal tissue samples were examined for changes in gene expression between tumor tissue and normal kidney. Analysis of the data revealed that expression levels of several targets of the Wnt signaling pathway are increased in tumor tissue compared with normal and that expression of a secreted Wnt inhibitor was lost in patient tumor samples. A role for Wnt/β-catenin signaling in colon cancer as well as others cancers has been well defined (7). As a consequence of oncogenic activation, β-catenin drives transcription of genes that contribute to the tumor phenotype by regulating processes, such as proliferation, survival, and invasion. Two noncanonical Wnt signaling pathways have been implicated in cancer (8, 9). The planar cell polarity pathway regulates cytoskeletal polarity

---

**Abstract**

**Purpose:** Incidence and mortality rates for renal cell carcinoma (RCC) have been rising for decades. Unfortunately, the molecular events that support RCC carcinogenesis remain poorly understood. In an effort to gain a better understanding of signaling events in clear cell RCC (cRCC), we investigated the antitumor activity of secreted frizzled-related protein 1 (sFRP1), a negative regulator of Wnt signaling.

**Experimental Design:** Genomic profiling of cRCC tumors and patient-matched normal tissues was done and confirmed using quantitative PCR and immunohistochemistry. Methylation-specific PCR was done on patient samples to evaluate the mechanism responsible for sFRP1 loss. sFRP1 expression was restored in cRCC cells and the effects on tumor phenotype were characterized.

**Results:** Genomic profiling, quantitative PCR, and immunohistochemistry indicated that loss of sFRP1 occurred in cRCC and papillary RCC patient tissues. Twelve Wnt-regulated genes were up-regulated in cRCC tissues, including c-myc and cyclin D1, potentiators of cell proliferation and survival. Methylation of the sFRP1 gene was one mechanism identified for attenuation of sFRP1 mRNA. Stable reexpression of sFRP1 in cRCC cells resulted in decreased expression of Wnt target genes, decreased growth in cell culture, inhibition of anchorage-independent growth, and decreased tumor growth in athymic nude mice.

**Conclusions:** To our knowledge, this is the first report to show that stable restoration of sFRP1 expression in cRCC cells attenuates the cRCC tumor phenotype. Our data support a role for sFRP1 as a tumor suppressor in cRCC and that perhaps loss of sFRP1 is an early, aberrant molecular event in renal cell carcinogenesis.
through Rac/Ink– and Rh/ROCK–dependent signaling, whereas the Ca²⁺/protein kinase C pathway regulates cell adhesion. The activities of both of these noncanonical Wnt signaling pathways have implications for invasion and metastasis.

To our knowledge, the noncanonical Wnt pathways have not been investigated in cRCC. Whether or not Wnt/β-catenin signaling is important for cRCC remains a matter of some dispute. Several independent studies on cRCC patient tumors indicated that mutations in adenomatous polyposis coli or β-catenin itself were extremely rare, suggesting that the β-catenin pathway does not play a role in the majority of cRCC cases (10–13). However, a decrease in plasma membrane–associated β-catenin was observed and correlated with advanced stage and a higher grade of cRCC in one study (12). In another study, cytoplasmic accumulation of β-catenin was seen in cRCC but not in chromophobe or papillary carcinomas (11). Examination of 18 cases of cRCC by another group revealed an increase in β-catenin protein in tumor samples compared with normal tissue, but no difference in mRNA levels was observed (14). More recent evidence for the involvement of the Wnt pathway in RCC came from investigation into the expression levels of the frizzled (Fzd) receptors (15). Fzd5 and Fzd8 both exhibited increased mRNA levels in tumor samples compared with normal renal tissue. One mechanism that could be responsible for Wnt pathway activity in cRCC is the loss of expression of a negative inhibitor of the pathway, secreted Fzd-related protein 1 (sFRP1).

sFRP1 is a 35-kDa secreted glycoprotein containing a cysteine-rich domain that is similar to that found in the Wnt family (reviewed in refs. 16, 17). It negatively regulates Wnt signaling at the level of the plasma membrane where it binds and sequesters Wnt molecules from their cognate membrane receptors, the Fzd family. Loss of sFRP1 expression has been observed in many tumors, including cancers of the colon (18), lung (19), ovary (20), breast (21), and, most recently, RCC (22, 23). Methylated-inducing silencing has been confirmed as the most likely mechanism responsible for repression of sFRP1 in several cancer types (reviewed in ref. 17). The effect of sFRP1 loss on Wnt signaling and the cancer phenotype in general is not clear. sFRP1 and other sFRP family members have been shown to have not only tumor suppressor activity but also tumor-promoting activity (17). sFRP1 has shown antiapoptotic activity and increased cell proliferation (24, 25). Conversely, sFRP1 has had an inhibitory effect on anchorage-independent growth in colon cancer (26). Further studies are needed to determine the unique effects of sFRP1 on Wnt signaling in each cancer subtype.

In the current study, our data showed that repression of sFRP1 occurred in stage I localized clear cell and papillary RCC. One might speculate that sFRP1 loss plays a role in RCC carcinogenesis and is likely to contribute to activation of the Wnt pathway in this disease. We observed by gene array and confirmed by quantitative PCR (QPCR) and immunohistochemistry that Wnt-regulated genes were elevated in the tumors of cRCC patients compared with patient-matched normal tissue. We described the loss of sFRP1, a secreted inhibitor of the Wnt pathway, as a common event in cRCC. Using a cell model, we showed that restoration of sFRP1 in cRCC cells attenuated the expression of Wnt target genes identified by genomic profiling of patient RCC tissues. Moreover, we showed that sFRP1 reexpression alters the tumor phenotype of cRCC, such as cell proliferation and tumorigenesis. Collectively, these data link sFRP1 loss with enhanced expression of genes contributing to cRCC tumor phenotype and indicate that Wnt signaling is a novel signaling pathway for targeted therapy in cRCC.

### Materials and Methods

**Patient sample collection.** On ice and under sterile conditions, renal tissue (normal and tumor) was transported to a sterile hood within the pathology department. Tissue was dissected under the direction of a pathologist. A portion of the tissue was frozen in liquid nitrogen for future isolation of RNA, DNA, and protein. Another portion of tissue was processed to establish primary cell cultures, and a third portion of tissue was fixed in formalin for immunohistochemistry. Tissues were collected from The University of Texas M. D. Anderson Cancer Center (C.G.W.) and Mayo Clinic College of Medicine Jacksonville (A.S.P. and J.A.C.) according to Institutional Review Board protocols.

**Microarray data analysis.** Gene expression profiling was done using Affymetrix HU133A and B oligonucleotide gene arrays. Total RNA (Trizol, Invitrogen) was extracted from patient-matched normal renal cortex and tumor tissue from patients diagnosed with stage I or II cRCC. Data were analyzed by a combination of two-dimensional ANOVA, Affymetrix MAS5.0, and hierarchical cluster analysis using Spotfire. Space precludes a detailed description of the procedure that we have used to identify altered expression of large sets of genes, as well as other issues about microarray analyses; a thorough consideration can be found in our review article on this subject (27). All genes have a P value of ≤0.001 with the exception of connexin 43, which has a P value of <0.01. The results of this analysis have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database under the accession number GSE6344.

**Genomic DNA isolation, bisulfite treatment, methylation-specific PCR, and bisulfite genomic sequencing.** Genomic DNA was isolated from human RCCs, their matched normal control tissue, and several RCC cell lines using DNAzol (Molecular Research Center, Inc.) according to the manufacturer's instructions. Briefly, tissue was homogenized and DNA precipitated, washed, and solubilized. The DNA was then treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) and amplified with primers specific for either methylated or unmethylated sFRP1, as described previously (21, 28). Templates for the positive controls for the unmethylation and methylation reactions were, respectively, bisulfite-treated human genomic DNA (Novagen) and CpGenome Universal Methylated DNA (Chemicon). Methylation status of sFRP1 in representative samples was confirmed by bisulfite genomic sequencing using primers that flank the methylation-specific PCR region: 5′-CAACCTCTCCTAAACCTCC-3′ (sense) and 5′-GTAGTTTAGGTGTAATTGTCG-3′ (antisense). One microliter bisulfite-modified DNA was amplified in a total volume of 25 μL containing 1× PCR buffer (Applied Biosystems), 3.0 mmol/L MgCl₂, 200 μmol/L of each deoxynucleotide triphosphate, 400 nmol/L of each primer, and 1.25 units AmpliTaq Gold polymerase (Applied Biosystems). Amplification included 95°C for 10 min, denaturing at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s for 40 cycles, and a final 15-min extension step. PCR products were cut from gels and purified using Qiaquick Gel Extraction kit (Qiagen) and then ligated into pCR 2.1-TOPO cloning vector using a TOPO TA Cloning kit (Invitrogen). For each clone, six colonies were sequenced for detailed methylation status of each CpG site.

**Quantitative real-time PCR.** Two-step quantitative reverse transcriptase-mediated real-time PCR (QPCR) was used to measure changes in mRNA levels of target genes. Total RNA was reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems) per the manufacturer's instructions. Twenty nanograms of cDNA were used as template in subsequent QPCRs. Applied Biosystems’ assays-on-demand 20× assay mix of primers and Taqman MGB probes (FAM dye labeled) for sFRP1

---


A was amplified in a total volume of 25 μL containing 1× PCR buffer (Applied Biosystems), 3.0 mmol/L MgCl₂, 200 μmol/L of each deoxynucleotide triphosphate, 400 nmol/L of each primer, and 1.25 units AmpliTaq Gold polymerase (Applied Biosystems). Amplification included 95°C for 10 min, denaturing at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s for 40 cycles, and a final 15-min extension step. PCR products were cut from gels and purified using Qiaquick Gel Extraction kit (Qiagen) and then ligated into pCR 2.1-TOPO cloning vector using a TOPO TA Cloning kit (Invitrogen). For each clone, six colonies were sequenced for detailed methylation status of each CpG site.
containing 0.05 mg/mL G418. Bethesda MD). Stable clones were selected in the above media pcDNA3.1-sFRP1-myc-his was done using Fugene (Roche) according to A498 cells using empty vector (pcDNA3.1-Neo, Invitrogen) or hybridized with an anti-myc tag antibody (Sigma) overnight at 4 °C, then transferred to Immobilon-P membranes. The membrane was separated on 10% to 20% tris-glycine gels (Invitrogen) and combined with adhered cells in 60-mm dishes and lysed. Protein lysates were transferred on an antibody against human sFRP1 (1:100; R&D Systems), cyclin D1 (1:200; Lab Vision), c-Myc (1:25; DakoCytomation), or fibronectin (1:6,400; Abcam). The Envision Dual Labeled Polymer kit (DakoCytomation) was used according to the manufacturer’s instructions. All slides were placed in antigen retrieval solution (DakoCytomation) using steam heat for 20 min, stained using 3,3′-diaminobenzidine chromogen, and then lightly counterstained with Gill I hematoxylin before dehydration and mounting.

Cell culture. UMRC3 cells were the gift of Dr. Bart Grossman (University of Texas M. D. Anderson Cancer Center, Houston, TX; ref. 30). A498, 786-O, and 769-P cells were purchased from American Type Culture Collection. RCC cell lines were maintained in MEM medium containing 5% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin-ampoterpin B, HEPES, sodium pyruvate, insulin/transferin/selenium, and epidermal growth factor hRE152HTET cells were immortalized using human retroviral telomerase (pBABE-hTERT-hygro, gift of Dr. Robert Weinberg, Massachusetts Institute of Technology, Boston MA) and maintained in the above media containing 100 µg/mL hygromycin. Stable transfection of UMRC3 cells or A498 cells using empty vector (pcDNA3.1-Neo, Invitrogen) or pcDNA3.1-sFRP1-myc-his was done using Fugene (Roche) according to the manufacturer’s instructions. The plasmid pcDNA3.1-sFRP1-myc-his was the gift of Dr. Jeffrey Rubin (National Cancer Institute, Bethesda MD). Stable clones were selected in the above media containing 0.05 mg/mL G418.

Western blot analysis. Conditioned media from UMRC3-Neo or UMRC3-sFRP1 clones 1, 2, and 10 were collected and concentrated from 2 mL to ~600 µL using a Centricon YM-10 filter (Millipore). The concentrate was then purified over a Ni-NTA column (Qiagen). The eluate was used for protein concentration using the bicinchoninic acid assay (Pierce). Protein was loaded on a SDS-PAGE gel (Invitrogen) and then transferred to Immobilon-P membranes. The membrane was hybridized with an anti-myc tag antibody (Sigma) overnight at 4 °C. The secondary antibody used was a horseradish peroxidase–conjugated anti-mouse secondary antibody (The Jackson Laboratory). Detection was done using Supersignal West Pico (Pierce).

Growth in culture. Twenty thousand cells were plated per well in 12-well culture plates in triplicate for UMRC3-Neo and UMRC3-sFRP1 clones 1, 2, and 10. Cells were rinsed in PBS, trypsinized, collected, and counted in a Beckman Coulter Counter. Cell growth was measured after 2, 4, 6, and 8 days of growth. This same experiment was conducted for A498-Neo and A498-sFRP1 clones.

Soft agar assay. Soft agar assays for anchorage-independent growth were done on UMRC3-Neo cells and UMRC3-sFRP1 clones 1, 2, and 10. Each 60-mm plate was first layered with 0.75% agar diluted with 10% fetal bovine serum – supplemented αMEM medium complete with additives. The cell layer was then prepared as previously stated but included 10³ cells per plate. Plates were maintained at 37 °C under 5% CO₂ for 4 weeks. Colonies visible to the naked eye were counted by hand. Assays were done in triplicate.

Ectopic mouse model. Suspensions of 2 × 10⁴/0.1 mL UMRC3-Neo, UMRC3-sFRP1 clone 1, UMRC3-sFRP1 clone 2, or UMRC3-sFRP1 clone 10 cells in αMEM medium were injected s.c. in each flank of 3- to 4-week old athymic female nu/nu mice (Harlan). Tumors were measured every 7 days for 42 days with calipers. Tumor volumes were calculated by the formula: 0.5236 × (a × b × c), where a is the shortest diameter, b is the diameter perpendicular to a, and c is the diameter height. Five mice were used per cell line with two injection sites per mouse.

Proliferation assay. A bromodeoxyuridine (BrdUrd) cell proliferation ELISA (chemiluminescent) assay (Roche) was used to measure cell proliferation, according to the manufacturer’s directions. Seven hundred cells were plated per well in clear-bottomed, black 96-well plates (Corning). Seventy-two hours later, cells were pulse labeled with BrdUrd. Following fixation, cells were incubated with the anti – BrdUrd-POD for 1 h at 37 °C, washed three times for 5 min each on a rocking platform, and then incubated with Substrate Solution for 3 min. Luminescence was measured on a SpectraMax M5 ( Molecular Devices) bottom read plate reader.

Apoptosis. Following 72 h of growth in culture, floating cells were combined with adhered cells in 60-mm dishes and lysed. Protein lysates were separated on 10% to 20% tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membrane for Western blot analysis using anti–poly(ADP-ribose) polymerase (PARP; 1:1,000 dilution; Cell Signaling) and anti–caspase-3 (1:1,000 dilution; Cell Signaling) antibodies. β-Actin was used as a loading control (1:5,000 dilution; Sigma). Horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse (1:4,000 dilution; The Jackson Laboratory) secondary antibodies were used. Chemiluminescence was detected using SuperSignal West Pico.

Statistical analysis. Statistical analyses of the nonmicroarray data were done using the Kruskal-Wallis one-way ANOVA analysis with the Tukey test for error protection (SigmaStat 3.1). Values with P < 0.05 were considered significant.

Results

sFRP1 expression is repressed in cRCC. Affymetrix Hu133A and B oligonucleotide gene arrays were used to identify gene expression in human cRCC. Normal renal cortex and primary tumor tissue were collected from each of five patients diagnosed with local disease (tumor-node-metastasis stage group I and II). Data were analyzed by two-dimensional ANOVA and hierarchical cluster, as described (31). Expression of sFRP1 was down-regulated in 15 of 15 patients tested, with
decreases ranging from 10- to 70-fold. This observation showed that loss of sFRP1 expression may account for activation of Wnt signaling in this disease. Loss of expression was confirmed by analysis of mRNA abundance in an independent set of 33 patient samples representing all stages of cRCC. Using QPCR, decreases of >140-fold were observed in stages I to IV (Fig. 1A). Importantly, down-regulation or loss of sFRP1 mRNA was again observed in every patient sample tested.

Loss of sFRP1 in cRCC was confirmed at the level of protein expression. sFRP1 protein levels were evaluated in an independent set of 39 patient-matched normal and tumor tissue samples (9 or 10 cRCC patients from each of stages I-IV) using immunohistochemistry. The staining intensity of these patient samples was scored by a pathologist on a scale of 0 to 3 (Table 1). More than 70% of the patient samples scored showed a total loss of sFRP1 protein in the tumor compared with the matched normal. sFRP1 expression was observed in proximal tubules of the normal tissue, but there was little to no staining in the tumor samples (Fig. 1B).

Tissue samples from two other RCC subtypes, papillary and chromophobe, and the benign kidney tumor oncocytoma were evaluated as well (Fig. 1C; Table 1). Interestingly, loss of sFRP1 expression in the tumor was observed in papillary tissues. Chromophobe and oncocytoma tumors had equivalent levels of sFRP1 staining compared with the patient-matched normal renal tissue.

The sFRP1 gene is methylated in cRCC. Methylation-induced silencing seems to be the mechanism of sFRP1 loss in several cancer types (18–20, 28). We evaluated the methylation status of the sFRP1 gene in cRCC from 10 patient tissue samples. Bisulfite-treated genomic DNA from cRCC tumors, the respective matched normal tissue, and several cRCC cell lines were analyzed using methylation-specific PCR following sodium bisulfite treatment (representative results in Fig. 2A and B). Overall, the sFRP1 gene was methylated in 8 of 10 cRCC tested. Among the 10 normal samples, sFRP1 methylation was found in one instance. Every tumor tissue sample yielded an unmethylated product, as expected, because these samples are a heterogeneous mix of cells. These results indicated that

| Table 1. sFRP1 protein expression is repressed in cRCC patients |
|---------------------------------|---|---|---|
| Intensity score | Normal* | Tumor* |
| cRCC             | 0  | 1  | 28 |
|                  | 1  | 16 | 10 |
|                  | 2  | 17 | 1  |
|                  | 3  | 5  | 0  |
| Papillary        | 0  | 0  | 4  |
|                  | 1  | 0  | 2  |
|                  | 2  | 0  | 2  |
|                  | 3  | 0  | 0  |
| Chromophobe      | 0  | 0  | 0  |
|                  | 1  | 0  | 4  |
|                  | 2  | 5  | 4  |
|                  | 3  | 3  | 4  |
| Oncocytoma       | 0  | 0  | 0  |
|                  | 1  | 0  | 11 |
|                  | 2  | 16 | 3  |
|                  | 3  | 1  | 7  |

*Number of patients with indicated intensity score.
methylation-induced silencing contributes to the loss of sFRP1 in cRCC. Furthermore, four cRCC cell lines were tested, A498, 786-O, UMRC3, and ACHN (Fig. 2C). Of these, only ACHN cells did not yield a methylated product, providing additional evidence for the role of methylation in the loss of sFRP1 expression. These results were confirmed using bisulfite genomic sequencing (Fig. 2D). A representative normal tissue shows no CpG methylation in six clones (Fig. 2D, open circles), whereas four of six clones in the cRCC patient tissue showed methylation (Fig. 2D, closed circles). Three cRCC cell lines, A498, UMRC3, and ACHN, show CpG methylation (Fig. 2D, 3-5, closed circles). Interestingly, the bisulfite genomic sequencing seems more sensitive versus that of methylation-specific PCR because methylation is shown in ACHN cells using bisulfite genomic sequencing (Fig. 2D, open circles) versus that of methylation-specific PCR (Fig. 2D, closed circles).

**Wnt pathway target genes are activated in cRCC.** At least 12 Wnt pathway targets were up-regulated in tumor tissue compared with normal tissue in early-stage cRCC according to the genomic profiling data, as indicated by the heat map in Fig. 3A. To confirm changes in mRNA levels of these Wnt targets, QPCR was done on a subset of Wnt target genes (Fig. 3B). RNA extracted from patient-matched normal and tumor tissues was used as template. At least 38 independent patient-matched normal and tumor samples representing all four stages of cRCC were analyzed. Greater than 4-fold increases were observed for cyclin D1, VEGF, c-Myc, connexin 43, endothelin-1, fibronectin-1, vimentin, SPARC, and TIMP1. All of these genes are regulated through either noncanonical (activator protein-1 mediated) or canonical Wnt signaling (32, 33). Statistically significant increases in tumor samples compared with normal were found for all targets tested ($P \leq 0.001$). To confirm this up-regulation at the level of protein, a subset of these Wnt targets was chosen for evaluation by immunohistochemistry. Protein levels of cyclin D1, c-Myc, endothelin, and fibronectin, all known to be involved in regulation of cell proliferation, survival, invasion, and metastasis (26, 34–38), were increased in tumor samples compared with the patient-matched normal tissue (Fig. 3C; Table 2). For instance, in Table 2, the percentage (%) of patients with a score of 3 in the tumor compared with normal for each protein is the following: cyclin D1, 30% versus 0%; c-myc, 73% versus 24%; endothelin, 91% versus 12%; and fibronectin, 46% versus 17%. Together, these results suggest that Wnt signaling is active in cRCC.

Reexpression of sFRP1 in cRCC cells inhibits expression of Wnt targets. UMRC3 cells, derived from the primary tumor of a patient diagnosed with metastatic cRCC, were chosen as a model of cRCC. These cells grow on soft agar and in athymic nude mice (30). UMRC3 cells were evaluated for sFRP1 expression. Expression levels of sFRP1 were undetectable in UMRC3 cells by QPCR, recapitulating the clinical results about loss of sFRP1 in patient tumors. To test the hypothesis that loss of sFRP1 in cRCC contributes to the tumor phenotype of cRCC, we stably transfected UMRC3 cells to express sFRP1. Expression of sFRP1 was confirmed in three representative clones by Western blot analysis of conditioned media (Fig. 4A). The presence of sFRP1 in the media indicated that the protein was produced by the cells and properly secreted. We evaluated the effect of sFRP1 on the Wnt targets described in Fig. 3 by measuring endogenous expression levels of these targets in the three UMRC3-sFRP1 clones compared with UMRC3-Neo cells. QPCR revealed decreases in expression for cyclin D1, c-Myc, VEGF, fibronectin-1, and vimentin in at least two of the three clones (Table 3).

sFRP1 inhibits growth of cRCC cells. Characterization of the stably expressing sFRP1 clones revealed inhibition of the growth properties of these cells compared with cells transfected with empty vector (UMRC3-Neo). Growth in culture of UMRC3-Neo, UMRC3-sFRP1 clone 1, UMRC3-sFRP1 clone 2, and UMRC3-sFRP1 clone 10 was measured over the course of 8 days (Fig. 4B). Whereas UMRC3-Neo cell number nearly doubled every 2 days (comparable with wild-type UMRC3 cells), growth of each of the sFRP1-expressing clones was dramatically impaired. Soft agar assays were done to evaluate the effect of sFRP1 expression on anchorage-independent growth of UMRC3 cells (Fig. 4C). Significant decreases of at least 90% were observed in the number of colonies formed for all three sFRP1-expressing clones compared with UMRC3-Neo cells. This same phenomenon was confirmed in another cRCC cell line, A498 cells. A498 cells were stably transfected with either empty vector (A498-Neo) or sFRP1-myc-his. A498 cells stably expressing sFRP1 exhibited a growth-inhibited phenotype similar to that
Fig. 3. Wnt targets are up-regulated in cRCC patients. A, a heat map was generated from microarray data comparing expression of Wnt-regulated genes in five stage I (S1) and five stage 2 (S2) cRCC patient-matched normal and tumor tissue samples. Expression level of the transcript with low (green), intermediate (black), and high (red) expression. B, QPCR was used to confirm increases in mRNA levels of Wnt targets in cRCC. Changes in expression between normal (black columns) and tumor tissue (gray columns) for all stages of cRCC. Columns, mean (n = between 38 and 40 patients for each target tested); bars, SE. *, P < 0.001. C, immunohistochemistry was done to evaluate expression levels of several Wnt pathway targets in patient-matched cRCC tumor and normal samples. Paraffin-embedded tissues were used. Representative photographs.
of UMRC3 cells with 90% inhibition of growth (data not shown).

**sFRP1 inhibits cell proliferation in cRCC.** To determine if the growth-inhibited phenotype of sFRP1-expressing UMRC3 cells was due to a decrease in proliferation or an increase in apoptosis, we measured whether there were differences in either process between the sFRP1 clones compared with UMRC3-Neo (Fig. 5). To evaluate differences in proliferation, BrdUrd incorporation was measured using an ELISA. Statistically significant levels of inhibition, ranging from about 65% to 80%, were observed for all three sFRP1-expressing clones, indicating that the inhibited growth properties of these cells was due to decreased proliferation (Fig. 5A). To evaluate the effect of sFRP1 on apoptosis, the sFRP1 clones and UMRC3-Neo cells were evaluated for caspase-3 activation and PARP cleavage, both markers of programmed cell death. HeLa cells treated with 50 nmol/L Taxol were used as a positive control for apoptosis. Western blot analysis did not indicate significant differences in levels of activated caspase-3 or cleaved PARP in the sFRP1 clones compared with UMRC3-Neo cells (Fig. 5B). UMRC3-sFRP1 clone 10 cells did exhibit a low level of PARP cleavage, but it was not significant compared with the positive control. Together, these results indicated that the growth-inhibitory effects of sFRP1 were due primarily to decreased cell proliferation rather than an increase in apoptosis.

**sFRP1 acts as a tumor suppressor in cRCC.** We next measured the effects of sFRP1 expression on the tumor phenotype in vivo. Two million cells of UMRC3-Neo, UMRC3-sFRP1 clone 1, UMRC3-sFRP1 clone 2, or UMRC3-sFRP1 clone 10 were injected ectopically into the flanks of athymic, nude mice (five mice per cell line, two injection sites per mouse). Tumor volumes were measured every 7 days for 6 weeks following the injections (Fig. 6). By week 4, tumors in UMRC3-Neo mice began to double in size, whereas tumors in the UMRC3-sFRP1 clones 1, 2, and 10 mice continued to decrease in size. By the end of week 7, tumors in the UMRC3-sFRP1 clones 1, 2, and 10 mice were an average of 3% of the size of the tumors in the UMRC3-Neo animals. In fact, of the original 29 sFRP1 tumors, 8 had completely disappeared.

**Discussion**

In our genomic profiling of tissue samples from cRCC patients, we identified sFRP1, a known inhibitor of Wnt signaling, as a candidate marker that was down-regulated in cRCC tumors compared with patient-matched normal samples.
This observation was confirmed in an independent validation set of patient tissue samples at both the level of mRNA and protein expression. In examining RCC histologic subtypes, we identified that sFRP1 was also down-regulated in papillary RCC. The clinical observations presented here revealed that many targets of Wnt signaling were up-regulated in cRCC. Together with loss of sFRP1, these data suggest that the Wnt pathway is activated in cRCC. Moreover, QPCR and immunohistochemistry confirmed changes in the mRNA and proteins levels of sFRP1 and several Wnt pathway targets. These clinical observations led to the hypothesis that loss of sFRP1 contributes to the tumor phenotype of cRCC.

Our cell model of cRCC confirmed that stable reexpression of sFRP1 in UMRC3 cells resulted in decreased mRNA levels of endogenous Wnt targets. The down-regulation of Wnt targets in sFRP1-expressing UMRC3 cells was not as dramatic as expected. However, it is important to note that the gene targets tested are regulated by other pathways as well. Genes, such as cyclin D1 and VEGF, are critical to the tumorigenicity of many cancer types and it is highly likely that inhibition of a single regulatory pathway, such as Wnt, is insufficient to completely abrogate expression. Cyclin D1 and VEGF are regulated by other transcriptional factors, such as hypoxia-inducible factors, activator protein-1, and specificity protein-1 (39). Furthermore, other putative Wnt inhibitors, such as Wnt-inhibitory factor, Dickkopf-3 (40), and other sFRP family members (22), may also play a role in regulating Wnt signaling in cRCC. Indeed, our genomic profiling data showed that Wnt-inhibitory factor expression was down-regulated in several tumor samples (data not shown). The effect of sFRP1 on the tumor phenotype of UMRC3 cells was dramatic. Growth in culture and anchorage-independent growth were inhibited in sFRP1-expressing UMRC3 cells. Most strikingly, sFRP1 had tumor-suppressive effects in a nude mouse model. Whereas sFRP1 has been shown to affect cell proliferation and apoptosis (17), growth inhibition of sFRP1 seemed to be the result of inhibited cell proliferation in our cell culture model. sFRP1 has been shown to inhibit cell proliferation in myostatin knockout mice (41). This effect of sFRP1 on cellular proliferation is also consistent with evidence from prostate cancer, where retroviral-mediated expression of sFRP1 resulted in inhibited cellular proliferation but had no effect on apoptosis (42). Our observation that restoration of sFRP1 expression attenuated the malignant phenotype of cRCC is further supported by reports from colon and lung cancer cell models in which reexpression of sFRP1 resulted in decreased colony formation (19, 26). To our knowledge, our report is the first to show antitumor activity of sFRP1 in an animal model.

Our observation that loss of sFRP1 in cRCC is likely due to methylation of the gene is supported by recent reports on the methylation status of Wnt pathway inhibitors in RCC (22, 23). During the preparation of our manuscript, Urakami et al. (22) published a report in which they described methylation of the sFRP1 gene and restoration of sFRP1 expression in RCC cell lines by a methylation inhibitor. Dahl et al. (23) also showed hypermethylation of the sFRP1 gene in RCC. Thus, our findings as well as those of Urakami et al. and Dahl et al. indicate that the sFRP1 gene is methylated in RCC. A role for sFRP1 as a tumor suppressor has been proposed in many cancers based on its loss in patient tumors (17). Its frequent inactivation by methylation-induced silencing is consistent with it behaving as a tumor suppressor. Indeed, the sFRP1 gene is located at chromosome 8p11, a region that is frequently lost in many cancer types (18). Investigations into the short arm of chromosome 8 led several investigators to the finding that epigenetic silencing of sFRP1 via methylation, rather

### Table 3. Wnt targets are down-regulated in UMRC3-sFRP1 clones

<table>
<thead>
<tr>
<th>Target</th>
<th>Fold change compared with UMRC3-Neo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>-2.2  -2.7  -1.4</td>
</tr>
<tr>
<td>VEGF</td>
<td>-1.7  -2.8  -2</td>
</tr>
<tr>
<td>c-Myc</td>
<td>-3.6  -2.1  -2</td>
</tr>
<tr>
<td>Fibronectin-1</td>
<td>-2.8  -3.2  -3.2</td>
</tr>
<tr>
<td>Vimentin</td>
<td>-1.3  -2.3  -1.6</td>
</tr>
</tbody>
</table>

Fig. 5. sFRP1 inhibits proliferation of UMRC3 cells. A, a BrdUrd ELISA was used to evaluate BrdUrd incorporation as a measure of cell proliferation. Cells were pulse labeled with BrdUrd for 6 h after reaching log-phase growth (t = 3 d). An enzyme-linked antibody against BrdUrd was used to measure BrdUrd incorporation following incubation with a chemiluminescent substrate. Columns, mean (n = 3); bars, SE. *P ≤ 0.02. B, caspase-3 activation and PARP cleavage were evaluated in UMRC3-Neo and UMRC3-sFRP1 clones 1, 2, and 10 as a measure of apoptotic activity. Cell lysates were analyzed using Western blot with an anti-caspase-3 antibody (recognizes procaspase-3 as well as the cleaved form of the protein; Cell Signaling), an anti-PARP antibody (recognizes full-length and cleaved PARP; Cell Signaling), or β-actin (Sigma). HeLa cells treated with 50 mmol/L Taxol were used as a positive control for caspase-3 activation and PARP cleavage.
than loss of heterozygosity, is responsible for its loss in many cancers, including colorectal (18), non–small cell lung cancer (19), breast cancer (21), bladder cancer (43), esophageal adenocarcinoma (28), ovarian cancer (20), and, most recently, cRCC (22). Although these reports were all consistent with a role for sFRP1 as a tumor suppressor, the mechanism of its action has not been well defined. Two reports on colon cancer offer opposing views. Caldwell et al. (18) proposed a role for sFRP1 as a tumor suppressor and speculated that due to the nature of β-catenin activation in colon cancer, it was likely that sFRP1 acted to inhibit noncanonical Wnt signaling. Suzuki et al. (26) reexpressed sFRP1 in a colon cancer cell line and showed that it had inhibitory effects on canonical Wnt/β-catenin transcriptional activity as well as growth in soft agar. The present study does not distinguish between the types of Wnt signaling, although targets of both pathways are represented in our identified Wnt-regulated genes. Whether or not sFRP1 acts to inhibit canonical, noncanonical, or both components of Wnt signaling may well be determined by cancer- and tissue-specific factors.

Which Wnt pathways are affected by sFRP1 also depends on the Wnt and Fzd receptor molecules present in the tumor. According to our microarray and QPCR data (data not shown), Wnt2, Wnt5, Wnt7, and Wnt11 are expressed in cRCC tumors as well as normal tissue. Levels of expression did not vary between normal and tumor samples; however, loss of sFRP1 in cRCC tumors could allow increased signaling activity of these molecules. Another determinant of Wnt signaling is the Fzd receptor. Our genomic data indicated that Fzd1, Fzd4, Fzd7, and Fzd8 are expressed in cRCC. To completely define the molecules involved in the sFRP1-mediated inhibition of the cRCC tumor phenotype, it will be necessary to use a systematic approach to identify the specific Wnts and Fzds involved.

At the present time, there are few effective therapeutic options for metastatic cRCC that result in increased longevity and quality of life (6, 44). sFRP1 represents a possible therapeutic agent (17) given its dramatic effects on tumor growth in our preclinical mouse model. We have also shown in cRCC cell lines (UMRC3, ACHN, A498, and 786-O cells) a dose-responsive inhibition of cell growth using 5-aza-2′-deoxycytidine (data not shown), suggesting that methylation inhibitors may play a role as a chemotherapeutic strategy to antagonize cRCC tumor growth. 5-Aza-2′-deoxycytidine has been shown recently to synergistically augment antiproliferative effects of IFN-α-2 and IFN-β as well as tumor necrosis factor–related apoptosis-inducing ligand (45) in cRCC cells. Indeed, a 5-aza-2′-deoxycytidine analogue, azacitidine, and IFN-α2B are currently in phase I clinical trials for stage IV RCC patients (ongoing clinical trials YALE-HIC-27409, NCT00217542, and NCI-7317).

Inhibitors of Wnt signaling may have therapeutic benefits as well. A recent report suggests that docosahexaenoic acid may exhibit antitumoral effects through induction of β-catenin degradation (46). Curcumin and retinoic acid are both known inhibitors of activator protein-1, a downstream component of both canonical and noncanonical Wnt signaling (7). Curcumin is widely known for its chemopreventive and anticancer effects (47) and had proapoptotic effects in a RCC cell line, Caki-1 (48). Retinoic acid has been in clinical trials for cRCC in the past, in combination with IFN, with mixed results (49, 50). The use of sFRP1 or Wnt inhibitors as therapeutics could benefit patients diagnosed with a variety of cancers, as loss of sFRP1 has been described in several different cancers, including many solid tumors (18, 20, 21) and hematopoietic malignancies (51). For example, blockade of Wnt-1 signaling induced apoptosis in colorectal cancer cells, showing that blockade of membrane signaling has therapeutic potential (52).

To our knowledge, the results presented here are the first evidence of the role of sFRP1 in suppressing the tumor phenotype in cRCC. Furthermore, we have identified a subset of Wnt-regulated genes that are likely antagonized by sFRP1 in cRCC. These observations have wide implications for treatment of cRCC and possibly other cancers. Loss of sFRP1 could also serve as a diagnostic marker given that loss or repression of sFRP1 expression was observed in every patient tested by QPCR and complete loss of sFRP1 protein expression occurred in >70% of patients tested by immunohistochemistry.

References

Fig. 6. sFRP1 acts as a tumor suppressor in cRCC. Athymic nude mice were injected ectopically with approximately two million cells per flank of each of the cell lines indicated. Tumor volumes were measured every 7 d. Points, mean (n = 10 tumours; five mice per cell line, two injection sites per mouse; for clone 2, n = 9); bars, SE.
sFRP1 Loss in cRCC


Secreted Frizzled-Related Protein 1 Loss Contributes to Tumor Phenotype of Clear Cell Renal Cell Carcinoma

Michelle L. Gumz, Hongzhi Zou, Pamela A. Kreinest, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/13/16/4740

Cited articles
This article cites 51 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/13/16/4740.full#ref-list-1

Citing articles
This article has been cited by 25 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/13/16/4740.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.