Functional Significance of Secreted Frizzled-Related Protein 1 in Metastatic Renal Cell Carcinomas

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Abstract

The secreted Frizzled-related protein 1 (SFRP1) is a Wingless-type (Wnt) antagonist that has been associated with various malignancies, including renal cell carcinomas (RCC). However, the functional significance of SFRP1 has never been investigated in metastatic RCC. Here, we investigated the role of this molecule in kidney cancer progression and metastasis. Using Wnt pathway-focused cDNA expression profiling in normal renal, primary RCC, and metastatic RCC cell lines, we identified that *SFRP1* is up-regulated in metastatic RCC. SFRP1 overexpression in metastatic RCC was confirmed by immunostaining in renal tissues. We explored the molecular mechanisms underlying SFRP1 up-regulation by analyzing DNA methylation and histone modification patterns on *SFRP1* promoter. We found that this gene is unmethylated/hypomethylated and enriched in activating histone modifications in metastatic RCC. To understand the functional significance of SFRP1 overexpression in metastatic RCC with regard to tumorigenesis, we used a small interfering RNA–mediated approach to knockdown the gene and monitored cellular proliferation, apoptosis, and metastatic behavior. Proliferation was unaltered and apoptosis increased on attenuation of SFRP1 expression. Also, *SFRP1* depletion decreased the invasive potential of the metastatic RCC cell line, suggesting that the overexpression of this Wnt antagonist may be related to invasiveness and metastatic behavior in RCC. We investigated the molecular basis of the role of SFRP1 in invasion and metastasis and found that matrix metalloproteinase *MMP10* is regulated by *SFRP1*. In conclusion, our data suggest that SFRP1 plays a role in the metastatic potential of RCC. The present findings may be important in the design of treatment modalities for metastatic RCC. [Cancer Res 2009;69(17):6815–22]

Introduction

The incidence and mortality rates of renal cell carcinoma (RCC) have increased in recent years with ~54,390 new cases and 13,010 deaths in 2008 (1). Approximately 50% of RCC cases eventually develop metastases with a very poor survival rate (<5-15%). To date, there is no effective therapy for metastatic RCC. This emphasizes the need for a better understanding of the molecular pathogenesis of metastatic RCC, which could lead to better diagnostic and therapeutic interventions for the disease.

The Wingless-type (Wnt) signaling pathway regulates diverse developmental processes such as cell migration, adhesion, and proliferation. Dysregulation of the Wnt pathway has been implicated in a variety of human malignancies, including RCC (2). "Wnts" constitute a family of secreted glycoproteins that activate several signal transduction pathways following binding to receptors of the Frizzled family (2). A canonical/β-catenin-mediated pathway is activated in most tissues, which leads to cytoplasmic accumulation of β-catenin that translocates to the nucleus and associates with the T-cell transcription factor/lymphocyte enhancer factor proteins and induces transcription of target genes such as *c-myc* and *cyclin D1* (3). In addition, several "noncanonical" Wnt pathways are initiated by Wnt/Frizzled interaction. This includes Wnt-calcium pathway that regulates cell adhesion and cell migration and planar cell polarity pathway that regulates cytoskeletal polarity (4). The noncanonical pathways influence the processes of invasion and metastasis (5). Studies suggest that the β-catenin pathway does not play a major role in RCC (6–12).

Inactivation and attenuation of Wnt signaling is regulated by several secreted Wnt antagonists that includes the secreted Frizzled-related protein (SFRP) class and the Dickkopf class (13). The SFRP class includes SFRP1 to SFRP5 (also known as secreted apoptosis-related factors), Wnt-inhibitory factor-1, and Cerberus. These Wnt antagonists are thought to bind directly to Wnts and presumably block both canonical and noncanonical pathways. It has been postulated that the functional loss of Wnt antagonists can contribute to aberrant activation of the Wnt pathway resulting in carcinogenesis through dysregulation of cellular proliferation and differentiation. Recent publications from our laboratory and others have shown that impaired regulation by hypermethylation of Wnt antagonists is found in many cancers (14–20).

SFRP1 is a 35 kDa secreted glycoprotein that is a prototypical member of the SFRP family and has been reported to bind Wnt ligands and modulate their signaling activity (21, 22). It acts as a biphasic modulator of Wnt signaling, counteracting Wnt-induced effects at high concentrations and promoting them at lower concentrations (22). It is located in a chromosomal region (8p12-p11.1) that is frequently deleted in some cancers and is thought to harbor a tumor suppressor gene (23). Loss of SFRP1 expression has been reported in tumors of various organs, including colon (23), lung (24), breast (25) ovary (26), stomach, pancreas, parathyroid, adrenal gland, gall bladder, endometrium, and testis (ref. 15; reviewed in refs. 27, 28). In kidney, SFRP1 is highly expressed (29, 30) and is required for kidney metanephric development (29). Several recent reports document a loss of SFRP1 expression in primary RCC (14–16). Restoration of *SFRP1* expression led to an attenuation of the tumor phenotype of clear-cell RCC concomitant

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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with a decreased expression of Wnt target genes such as c-myc, cyclin D1, and vascular endothelial growth factor (14). However, the functional role of SFRP1 in metastatic RCC has never been reported.

As molecular interactions underlying the Wnt pathway in various tumor settings are still being explored, the main objective of the present study was to further elucidate the role of Wnt antagonists in the pathogenesis of RCC. Towards this, Wnt pathway-focused microarray profiling was done in RCC cell lines; interestingly, SFRP1 was found to be consistently overexpressed in metastatic RCC. Using a combination of expression profiling, quantitative PCR, and immunohistochemistry, we confirmed the up-regulation of this Wnt antagonist in metastatic RCC. We further investigated the molecular basis of this up-regulation by analyzing promoter methylation and histone modifications at the *SFRP1* locus. To understand the functional significance of *SFRP1* overexpression in metastatic RCC, we depleted the gene by RNA interference and assayed for cellular proliferation, apoptosis, and invasion.

Materials and Methods

Cell lines and cell culture. The nonmalignant SV-40 immortalized renal cell line HK2 and human renal cancer cell lines Caki1, ACHN, Hs891.T, Caki2, and A498 were obtained from the American Type Culture Collection. The HK2 cell line was maintained in keratinocyte serum-free medium (Life Technologies) supplemented with 50 g/mL bovine pituitary extract, 5% L-glutamine, and 5 ng/mL epidermal growth factor. The Caki1 and Caki2 cell lines were incubated in McCoy's 5A medium supplemented with 10% fetal bovine serum. The A498 and ACHN cell lines were maintained in Eagle's MEM and Hs891.T cells were cultured in DMEM each supplemented with 10% fetal bovine serum. All cell lines were cultured in a humidified incubator (5% CO_2) at 37°C.

Reverse transcription and real-time PCR. Total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen) and cDNAs were synthesized with oligo(dT) primers by use of a SuperScript first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocols. Gene expression was assessed by real-time quantitative PCR (RT-QPCR) using an Applied Biosystems 7500 Fast Sequence Detection System and gene-specific TaqMan assay kits for *SFRP1* and *MMP10* (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as endogenous control to normalize expression data. The comparative Ct (threshold cycle) method was used to calculate the relative changes in gene expression. The results are representative of three independent experiments. Data are shown as mean ± SE.

For RT-PCR-based expression profiling of a set of genes of a particular pathway (Wnt or tumor metastasis), pathway-specific PCR arrays (SABiosciences) were used as per manufacturer's instructions. Expression of altered genes was further confirmed by TaqMan gene expression assays (Applied Biosystems).

Western blotting. Whole-cell extracts were prepared in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 0.1% SDS, and 1.0% NP-40] containing protease inhibitor cocktail (Roche). Total protein was electrophoresed by SDS-PAGE and Western blotting was carried out using antibodies for SFRP1 (4690; Cell Signaling) and GAPDH (sc-32233; Santa Cruz Biotechnology) according to standard protocols.

Bisulfite DNA sequencing. Genomic DNA was extracted from RCC cell lines using the genomic DNA extraction kit (Qiagen). Bisulfite modification of genomic DNA was done using the Epi-Tect Bisulfite kit (Qiagen) following the manufacturer's directions. Bisulfite-modified DNA was amplified by a nested PCR approach using primers near the transcription start site of the *SFRP1* promoter. First round of amplification was done with the primers *SFRP1*-F (5′-GTATTTTAGTTTTGTAGTTTT-3′) and *SFRP1*-1R (5′-CCATCTTCTTATAACCCAC-3′) using the PCR conditions: 5 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 48°C, and 1 min at 72°C, and 5 min at 72°C. Aliquots of first PCR were subjected to second round amplifications using primers *SFRP1*-2F (5′-GGAGGTTTTTGGAAGTTTG-3′) and *SFRP1*-2R (5′- CACTAAAATAACTTAATATAAAAAC-3′). The amplification products were sequenced directly by an outside vendor (McLab).

Chromatin immunoprecipitation. Chromatin immunoprecipitation analysis was done using the EZ-ChIP kit (Upstate Biotechnology) according to the manufacturer's directions. Antibodies used in the immunoprecipitation were from Upstate Biotechnology and recognized acetyl-histone H3 (06-599), acetyl-histone H4 (06-866), dimethyl-histone H3 lysine 4 (07-030), trimethyl-histone H3 lysine 4 (07-473), dimethyl-histone H3 lysine 9 (07-441), and trimethyl-histone H3 lysine 9 (07-442). The immunoprecipitated DNA was analyzed by PCR or RT-QPCR using the following primers: *SFRP1*-promoter-F (5′-GTGAGCTTCCAGTCGGACAT-3′) and *SFRP1*-promoter-R (5′-CCATCTTCTTGTAGCCCAC-3′). PCR conditions were 5 min at 94°C, 28 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, and 5 min at 72°C. The amplified DNA was electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide. For quantitative PCR, Power SYBR Green PCR master mix (Applied Biosystems) was used for the quantification of the immunoprecipitation fractions. For each immunoprecipitation, the enrichment value was calculated relative to the corresponding input fraction and the relative enrichment ratios were then plotted.

Immunohistochemistry. Immunostaining was done on formalin-fixed, paraffin-embedded renal carcinoma progression tissue microarray (US Biomax). The slides were deparaffinized and antigen retrieval was carried out by microwaving the slides in 10 mmol/L sodium citrate buffer for 10 min. The remainder of the staining process was done using the ImmunoCruz Staining System (Santa Cruz Biotechnology) as per the manufacturer's instructions. Briefly, after antigen retrieval, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 8 min. Subsequently, blocking antibody (normal goat serum) was applied to the slides for 20 min at room temperature. Next, slides were incubated overnight with anti-SFRP1 antibody (H-90; sc-13939; Santa Cruz Biotechnology) at a 1:250 dilution. Anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used as a secondary antibody. Slides were incubated with the prediluted secondary antibody solution followed by peroxidase-conjugated streptavidin complex for 30 min each at room temperature. 3,3′-Diaminobenzidine Plus Substrate System (Lab Vision) was used as chromogen. The slides were then counterstained with Harris' hematoxylin, dehydrated, and mounted under a coverslip. Immunohistochemical staining was evaluated by assessing staining intensity (0-2) using a Olympus BX60 microscope equipped with Spot Advanced imaging software (Diagnostic Instruments). The criteria of intensity were as follows: 0, negative expression; 1+, weakly positive expression; and 2+, strongly positive expression.

***SFRP1* knockdown using small interfering RNA.** At 30% to 50% confluence, Hs891.T/Caki1 cells were transfected using Oligofectamine (Invitrogen) with small interfering RNA (siRNA) duplexes specific for human *SFRP1* (Qiagen) or control nonsilencing (*NS*) siRNA. Initially, four different sets of siRNA duplexes at different concentrations were tested to evaluate the target specificity and knockdown efficiency. siRNA duplex showing the most efficient *SFRP1* knockdown was used for further experiments at 50 nmol/L concentration. The following are the sequences of *SFRP1* siRNA: sense-r(CGCUUAUGUUAAUAGUAAU)dTdT and antisense-r(AUUACUAUUAACAUAAGCG)dAdT. Also, untransfected controls were included. The siRNA experiment was carried out for 72 h. Total RNA and proteins were analyzed by RT-QPCR and Western blotting, respectively.

Invasion assay. Control cells or *SFRP1* siRNA-treated cells were analyzed for invasion/migration through Matrigel (BD Biosciences) according to the manufacturer's protocol. Briefly, 48 h post-transfection, cells were placed in Matrigel inserts or control inserts at 1×10^5 cells/mL in serum-free medium and were allowed to migrate for 20 h at 37°C. Nonmigrating cells were removed from the top of the filter and cells that migrated were fixed and stained with a Hema 3 kit (Fisher Chemicals). The number of cells that migrated to the bottom side of the insert was counted manually and presented as percentages of invasion.

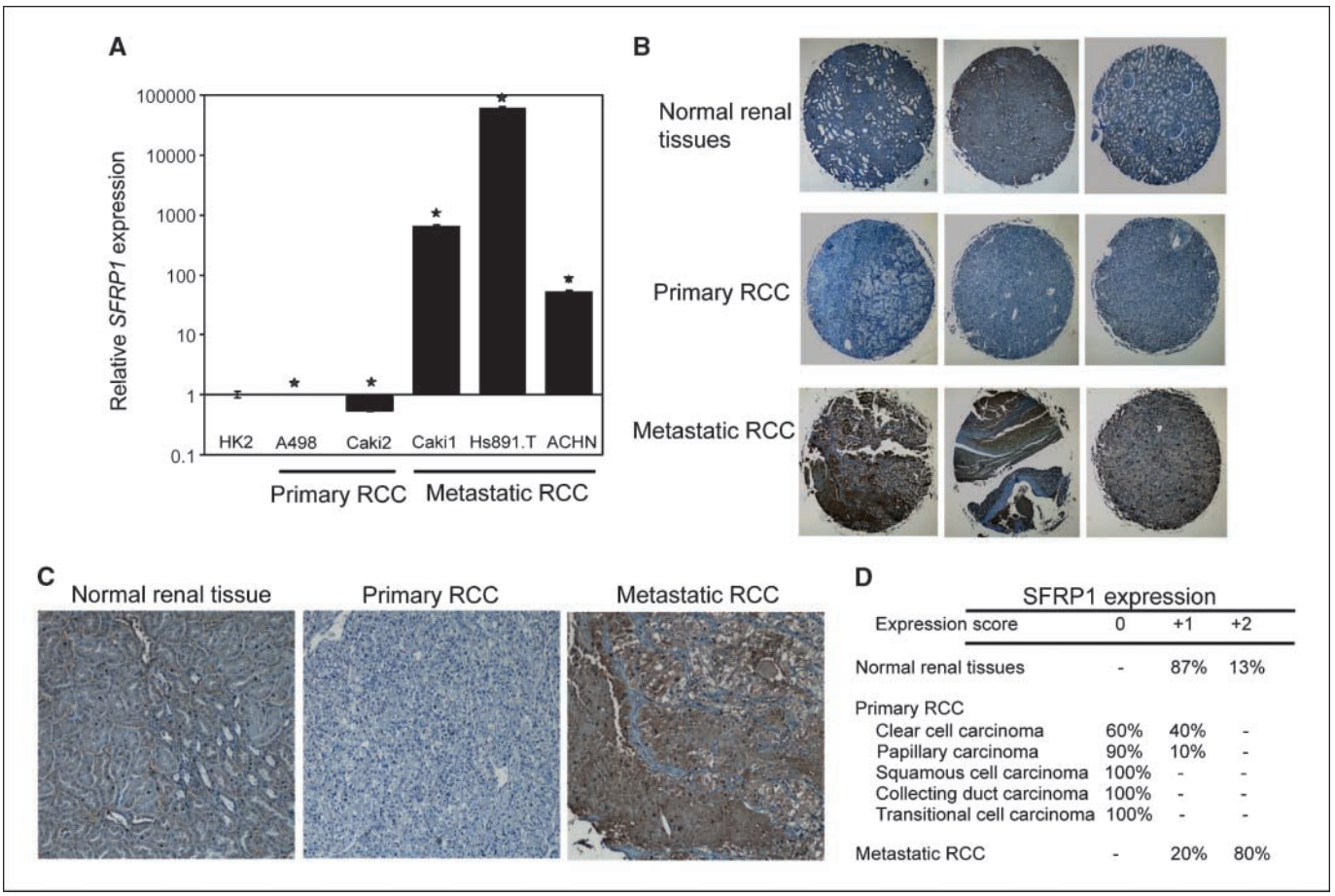

Figure 1. SFRP1 is overexpressed in metastatic RCC. *A,* total RNA from metastatic RCC (Caki1, Hs891.T, and ACHN) as well as primary RCC (A498 and Caki2) and normal kidney (HK2) cell lines was used to examine endogenous *SFRP1* mRNA levels by RT-QPCR analysis. Data were normalized to *GAPDH. B,* renal tissue microarray was immunostained with SFRP1 antibody to examine SFRP1 expression in normal kidney, primary RCC, and metastatic RCC. Representative portion of tissue microarray (magnification, ×100; hematoxylin counterstained) showing high SFRP1 expression in metastatic RCC (*bottom*) compared with primary RCC tissues (*middle*) and normal renal tissues (*top*). *C,* representative examples of SFRP1 immunostaining in renal tissues (magnification, ×200) showing SFRP1 expression in normal renal (*left*), primary RCC (*middle*), and metastatic RCC (*right*) tissues. *D,* summarized results of SFRP1 immunostaining of tissue microarray.

Apoptosis assay. For measuring apoptosis, transfected cells were dual stained with the viability dye 7-amino-actinomycin D and Annexin V-FITC using an Annexin V-FITC/7-amino-actinomycin D kit (Beckman Coulter) according to the manufacturer's protocol. Stained cells were analyzed by flow cytometry (Cell Lab Quanta SC; Beckman Coulter).

Statistical analysis. Statistical analysis was done using StatView version 5.0 for Windows as needed. Data were analyzed using Student's t test and a statistically significant difference was considered to be $P < 0.05$.

Results

SFRP1 is overexpressed in metastatic RCC. To identify dysregulated components of the Wnt pathway in renal carcinogenesis, we performed Wnt pathway-focused cDNA expression profiling using RCC cell lines as a model system. Normal renal (HK2), primary RCC (A498 and Caki2), and metastatic RCC (Caki1, Hs891.T, and ACHN) cell lines were used for this profiling (data not shown). Caki1, Hs891.T, and ACHN cell lines are derived from the skin, lymph node, and pleural effusion metastatic sites, respectively. This identified that *SFRP1* expression is highly up-regulated in metastatic RCC cell lines. To validate this observation, RT-QPCR was done to analyze relative *SFRP1* expression in normal renal and primary RCC versus metastatic RCC cell lines (Fig. 1*A*). It was observed that, in ACHN, Caki1, and Hs891.T cell lines, *SFRP1* levels are up-regulated ~54-, 650-, and ~62,200-fold, respectively, compared with HK2. Primary RCC cell lines Caki2 and A498 showed a down-regulation of *SFRP1* consistent with earlier reports (14, 16).

SFRP1 overexpression in metastatic renal carcinomas was further confirmed by immunohistochemical staining in renal tissues. Tissue microarray consisting of 26 cases of clear-cell carcinoma, 10 transitional cell carcinoma, 8 papillary carcinoma, 7 squamous cell carcinoma, 2 collecting duct carcinoma, 2 undifferentiated carcinoma, 5 each of metastatic carcinoma, inflammation, adjacent tissue, adjacent normal tissue, and normal tissue was immunostained (Fig. 1*B-D*). In keeping with earlier studies (14, 15), ~60% of clear-cell carcinomas showed an almost complete loss of SFRP1 expression ($P < 0.0001$). Also, primary tumors from other subtypes exhibited SFRP1 loss to varying degrees (10 of 10 cases in transitional cell carcinomas, 7 of 8 cases of papillary RCC, 3 of 3 cases of squamous cell carcinoma, and 2 of 2 cases of collecting duct carcinomas). However, 80% (4 of 5 cases) of metastatic cases showed strong SFRP1 positivity, whereas one metastatic case exhibited moderate SFRP1 staining, suggesting increased SFRP1 expression in metastatic cases ($P < 0.0161$). Representative examples of SFRP1 immunohistochemistry are

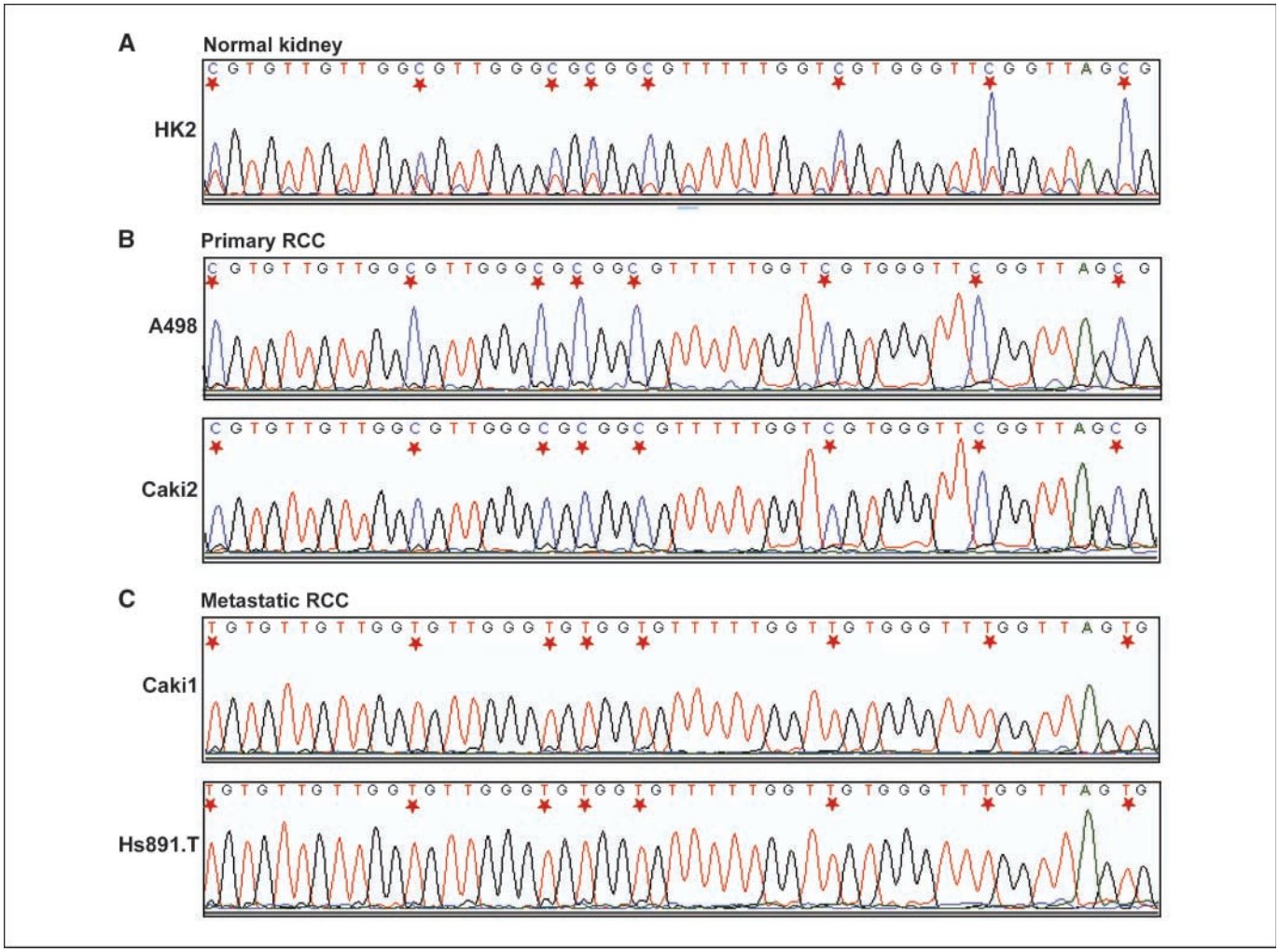

Figure 2. *SFRP1* promoter is unmethylated/hypomethylated in metastatic RCC. *SFRP1* promoter methylation status was assessed by bisulfite DNA sequencing. DNA from the indicated cell lines was subjected to bisulfite conversion, PCR amplified, and sequenced. Representative portion of the *SFRP1* promoter sequence for (*A*) normal renal cells (HK2), (*B*) primary RCC cell lines (A498 and Caki2), and (*C*) metastatic RCC cell lines (Caki1 and Hs891.T). *Red asterisks,* individual CpG sites.

shown in Fig. 1*B* and *C* and a summary of immunostaining data is represented in Fig. 1*D*. Examination of stained normal or tumor tissues showed that the subcellular localization of SFRP1 is cytoplasmic and/or membranous.

***SFRP1* promoter is unmethylated/hypomethylated in metastatic RCC.** Because promoter hypermethylation has been proposed as a predominant mechanism of SFRP1 loss in primary RCC (14–16), we reasoned that the *SFRP1* promoter hypomethylation may play a role in SFRP1 up-regulation seen in metastatic RCC. Therefore, we investigated the promoter methylation status of this gene by bisulfite genomic sequencing in metastatic RCC cell lines (Caki1 and Hs891.T) along with normal renal (HK2) and primary RCC (Caki2 and A498) cell lines. For methylation studies, primers were designed in the CpG-rich region of the *SFRP1* promoter around the transcription start site. Representative DNA sequencing results are shown in Fig. 2. In normal renal cells (HK2; Fig. 2*A*), the *SFRP1* promoter is partially methylated. However, in primary RCC cell lines (Fig. 2*B*), promoter hypermethylation was observed. In earlier reports, SFRP1 promoter hypermethylation has been observed in primary RCC (14–16). Hence, our results in primary RCC were consistent with these studies. Interestingly, in metastatic cell lines Caki1 and Hs891.T, the *SFRP1* promoter is totally unmethylated (Fig. 2*C*).

Chromatin structure of *SFRP1*. In addition to DNA methylation, histone modifications play an important role in epigenetic regulation of gene expression. To examine the pattern of histone modifications at the SFRP1 promoter, chromatin immunoprecipitation analyses was done. We looked for histone modifications associated with active genes (histone H3 dimethylated and trimethylated at lysine 4 and acetylated histones H3 and H4) as well as histone modifications that silence gene expression (histone H3 dimethylated and trimethylated at lysine 9) in normal renal (HK2) versus metastatic RCC (Caki1) and primary RCC (A498) cell lines (Fig. 3). The primers used for amplification of chromatin immunoprecipitation products were designed around the transcriptional start site. The metastatic RCC cell line Caki1 was found to be associated with a high level of activating histone modifications, acetylated histones H3, H4, and H3 dimethylated and trimethylated lysine 4 compared with A498 and HK2 cell lines. However, we did not detect significant changes in levels of trimethylated H3-K9 on the *SFRP1* promoter though dimethylated H3-K9 was reduced 2-fold in Caki1 cells

compared with HK2, whereas A498 cells exhibited ~10-fold increase in this repressive modification. This pattern of histone modifications on the *SFRP1* promoter suggests that the chromatin structure of the *SFRP1* promoter in metastatic cell lines is relatively open/transcriptionally active.

***SFRP1* knockdown modulates the invasive potential of metastatic cancer cell lines.** To explore the functional consequence of SFRP1 overexpression in metastatic RCC with regard to tumorigenesis, we used siRNA to knockdown endogenous *SFRP1* in metastatic RCC cell line Hs891.T (Fig. 4*A* and *B*) followed by functional assays. *SFRP1*-specific siRNA resulted in ~80% depletion of endogenous SFRP1 (Fig. 4*A* and *B*). We then examined the effects of *SFRP1* depletion on the invasive properties of the metastatic cells in an *in vitro* Matrigel invasion assay (Fig. 4*C*). Cells treated with *SFRP1* siRNA showed significantly less (9%) invasion than *NS* control oligonucleotide (40%)–treated cells or untreated cells (32%; Fig. 4*C*). Thus, these findings indicate that *SFRP1* can modulate the invasive properties of metastatic RCC and its up-regulation may be linked to invasion and metastatic behavior in RCC.

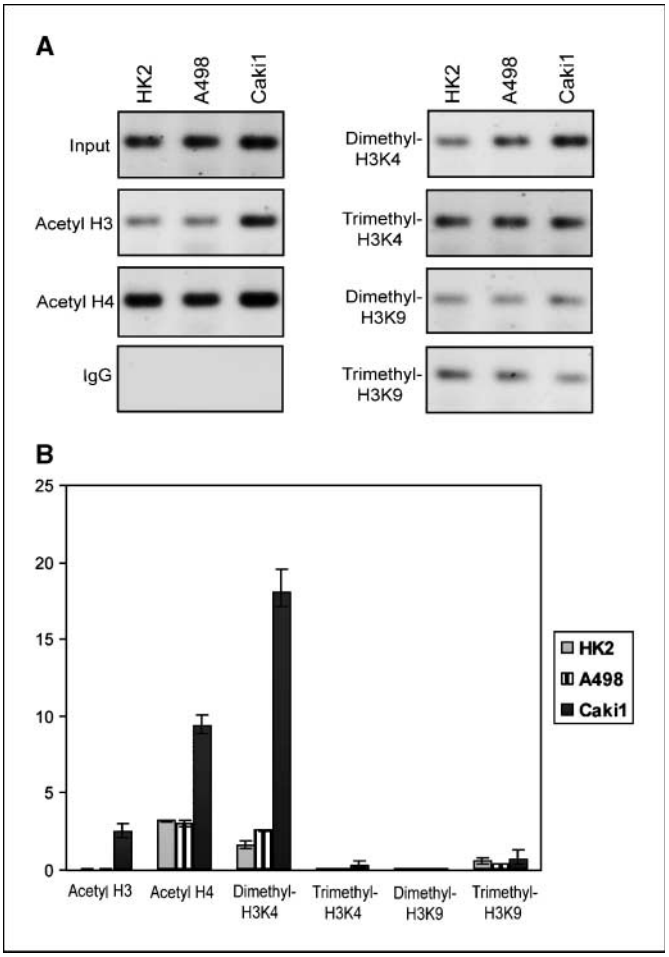

Figure 3. *SFRP1* promoter is enriched in active histone modifications in metastatic RCC. To assess histone modification patterns at the *SFRP1* promoter, chromatin immunoprecipitation assay was done on normal renal (HK2), primary RCC (A498), and metastatic RCC (Caki1) cell lines using the indicated antibodies. *A,* PCR products from the immunoprecipitation fractions were resolved on 2% agarose gels along with the corresponding inputs and visualized by staining with ethidium bromide. *B,* RT-QPCR analysis of chromatin immunoprecipitation samples. For each immunoprecipitation sample, the enrichment value was calculated relative to the corresponding input.

SFRP1 influences apoptosis in metastatic RCC. To further understand the consequences of SFRP1 up-regulation in metastatic RCC, we also evaluated the effect of *SFRP1* knockdown on cellular proliferation and apoptosis in metastatic RCC cell lines. Proliferation assays suggest that SFRP1 depletion does not alter the proliferative capabilities of metastatic RCC (data not shown). Apoptosis was examined with Caki1 cells, which were transiently transfected with *NS* siRNA (Fig. 5*A*) or *SFRP1* siRNA (Fig. 5*B*). The apoptotic cell fractions (early apoptotic + apoptotic) were significantly increased in *SFRP1* depleted cells (17%) compared with control siRNA-treated cells (7%).

Expression of matrix metalloproteinase *MMP10* is regulated by *SFRP1* in metastatic RCC. To understand the molecular basis of SFRP1-mediated altered invasiveness in metastatic RCC, we examined the expression of key genes important for tumor metastasis using RT-PCR–based arrays. Interestingly, we found that *MMP10* expression was down-regulated ~3.5-fold on *SFRP1* depletion (Fig. 6), suggesting that *SFRP1* regulates this matrix metalloproteinase in metastatic RCC.

Discussion

SFRP1 has been thought to bind to Wnt molecules, thereby preventing their binding to the cognate Frizzled receptors and therefore act as a negative modulator or "antagonist" of the Wnt pathway. Loss of SFRP1 has been reported in many human malignancies including RCC (14–16, 27). In the present study, we show that SFRP1 is overexpressed in metastatic RCC. SFRP1 up-regulation was identified by cDNA expression profiling in RCC cell lines and confirmed by RT-QPCR and immunohistochemical staining. In agreement with previous studies (14–16), we found that SFRP1 is down-regulated in primary RCC cell lines (A498 and Caki2) and also in tissue samples representing different subtypes of primary RCC. Interestingly, the observed augmentation of SFRP1 expression in metastatic RCC is a novel finding.

Elevated levels of SFRP1 and other Wnt antagonists including SFRP2, SFRP4, Dickkopf 1, and Wnt-inhibitory factor-1 have been reported in various tumors (27, 28, 31–33). SFRP1 overexpression has been observed in uterine leiomyomas (31) and prostate carcinoma–derived stromal cells (33). These studies suggest that Wnt antagonists, including SFRP1, may augment tumor formation or growth in these particular contexts.

Further, consistent with the expression data, we found that the *SFRP1* locus is epigenetically regulated in metastatic RCC. Two main epigenetic events, DNA methylation and histone modifications, are known to influence gene expression patterns (34–38). In our present study, we found that both DNA methylation status and histone modifications influence the epigenetic state of the *SFRP1* locus. In metastatic RCC cell lines, *SFRP1* promoter is hypomethylated/unmethylated in contrast to the hypermethylation observed in primary RCC. Also, this locus is enriched in active histone modifications, suggesting an open, transcriptionally active chromatin structure of this gene in metastatic RCC. Analysis of the *SFRP1* gene in RCC for possible genetic alterations showed that there were no mutations within the *SFRP1* gene or in the promoter region, suggesting that mutational inactivation does not regulate *SFRP1* expression in RCC (15). Instead, promoter methylation-induced alterations underlie *SFRP1* expression changes in RCC (14–16, 18).

To examine the functional significance of the observed *SFRP1* expression in metastatic RCC, we knocked down the expression of

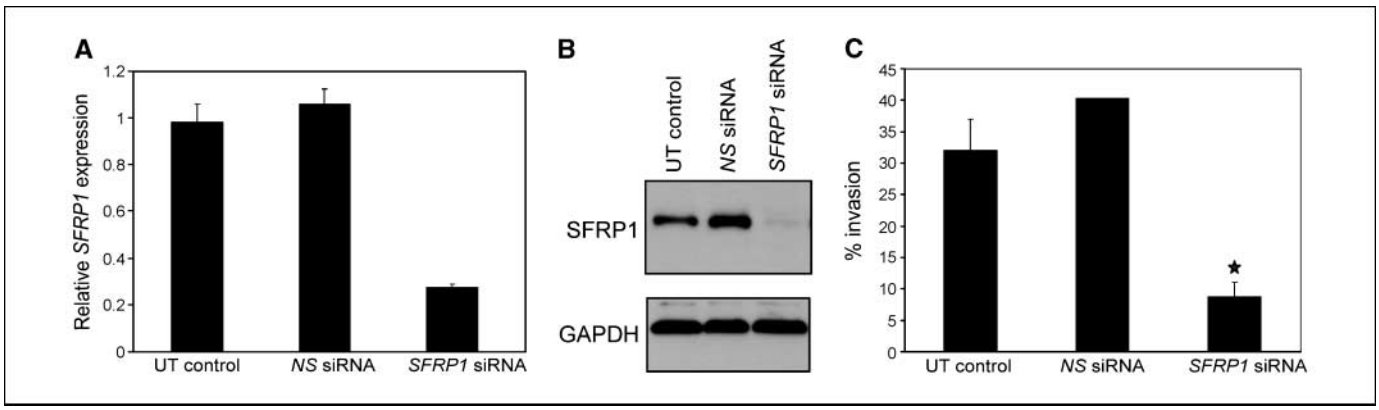

Figure 4. *SFRP1* modulates the invasive potential of metastatic RCC. *A,* siRNA-mediated knockdown of *SFRP1*. Hs891.T cells were treated with *SFRP1* siRNA (50 nmol/L) or control *NS* siRNA (50 nmol/L) for 72 h, following which cells were harvested for RNA and protein analysis. Efficiency of *SFRP1* knockdown was assessed by RT-QPCR of treated cells and untreated (*UT*) controls. Expression data were normalized to GAPDH. *B,* whole-cell lysates were analyzed for SFRP1 protein by Western blotting. GAPDH was used as an internal loading control. *C,* invasion assay. Untreated RCC (Hs891.T) cells (control) or cells treated with either *SFRP1* or *NS* siRNA (50 nmol/L) for 72 h (1×10^5 cells) were plated on Matrigel or control inserts for an invasion assay. Cells were allowed to migrate for 20 h at 37°C. Cells that migrated were counted in 10 random fields. Data are presented as percentages of invasion relative to control inserts.

this gene by RNA interference followed by functional assays. We examined the cell cycle profile and proliferation after *SFRP1* depletion and found no significant differences between *SFRP1* siRNA-treated cells and control cells. Significantly, we found that the *SFRP1* knockdown in metastatic RCC cells leads to attenuation of invasive properties, suggesting that SFRP1 governs/influences the metastatic potential in RCC. In prostate cancer, SFRP1 expression has been shown to be associated with invasiveness of the tumor, where SFRP1 expression was acquired by progressively more invasive carcinoma cells in an experimental carcinoma model (27, 33).

In the present study, *SFRP1* depletion also increased apoptosis in metastatic RCC. In view of this, we suggest that *SFRP1* knockdown may suppress metastasis, at least in part, by promoting apoptosis. Up-regulation of SFRP1 in metastatic RCC *in vivo* may partly regulate metastatic behavior by influencing apoptosis following dissemination of cells to secondary sites. An antiapoptotic role of SFRP1 has been reported in uterine leiomyomas (31) and prostatic epithelial cells (33). This role of SFRP1 has been associated with the regulation of apoptosis-related genes including those encoding p53, caspase-3, caspase-9, and Bcl-2 interacting killer (27, 39).

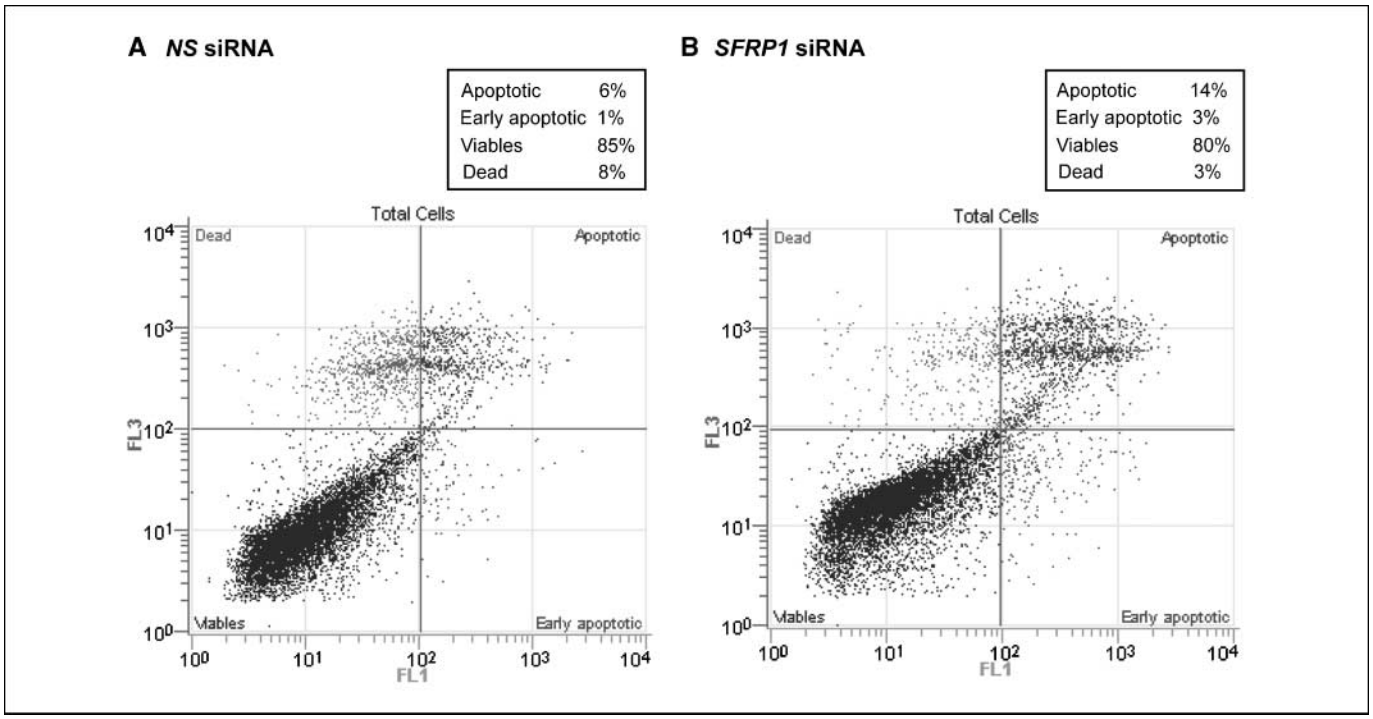

Figure 5. SFRP1 influences apoptosis in metastatic RCC. Apoptosis was measured in Caki1 cells treated with either *NS* siRNA (*A*) or *SFRP1* siRNA (*B*) for 72 h by dual staining with the viability dye 7-amino-actinomycin D and Annexin V-FITC followed by flow cytometric analysis. A typical dual staining is represented in the biparametric histograms (*A* and *B*) and shows cells in early (*bottom right quadrant*) and late apoptotic states (*top right quadrant*). Viable cells are double negative (*bottom left quadrant*).

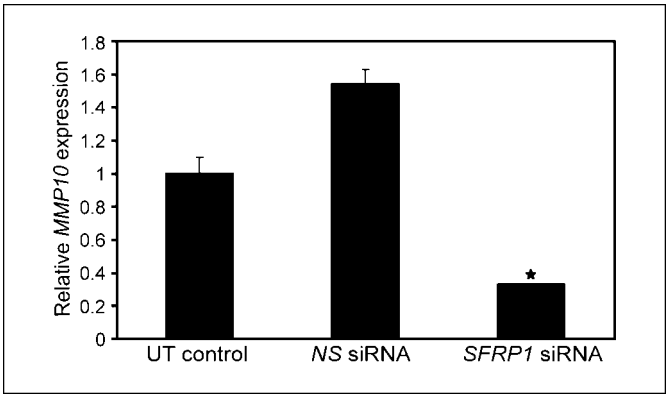

Figure 6. Expression of matrix metalloproteinase *MMP10* is regulated by *SFRP1* in metastatic RCC. *MMP10* transcript levels were assessed in control and *SFRP1* or *NS* siRNA-treated Caki1 and Hs891.T cell lines by RT-QPCR analysis. Representative result from Hs891.T cells. Data were normalized to *GAPDH*.

We hypothesized that augmentation of SFRP1 expression influences metastatic behavior in RCC and SFRP1 may influence other steps of the metastatic cascade as well. Metastasis is a complex process involving a cascade of molecular events that are not completely understood. The factors that enable the cancer cells to invade and metastasize are complex. The matrix metalloproteinases are a family of extracellular matrix–degrading enzymes that have been associated with cancer cell invasion and metastasis based on their up-regulation in virtually all human tumors (40). We examined the mechanistic basis of the SFRP1-mediated invasiveness in metastatic RCC on a molecular level using limited gene expression analysis and identified *MMP10* as a *SFRP1*-regulated gene. MMP10, also known as stromelysin 2, degrades multiple components of the extracellular matrix, such as proteoglycans, fibronectin, laminin, and collagen III and IV (41). *MMP10* overexpression is associated with malignant potential and tumor progression in lung (42), prostate (43), and esophageal cancers (44). In RCC, MMP10 has been shown to play an important role in invasion and is a potentially useful therapeutic target to prevent conventional RCC tumor progression (45).

Several mechanisms have been put forth to account for the potential oncogenic roles of SFRP1 and other Wnt antagonists in various malignancies (27). It has been proposed that SFRPs may act as agonists of Wnt signaling in particular contexts. In fact, SFRP1 has been shown to act as a biphasic modulator of Wnt signaling (22). Another study suggests that SFRP1 binds to Frizzled 2 and functions as an agonist for noncanonical signaling (46). Alternatively, it has been proposed that the growth-promoting effects of SFRPs might be due to inhibition of noncanonical Wnt signaling that negatively regulates β-catenin transcriptional activity (27). In the present study, we analyzed the expression of the key canonical pathway mediator β-catenin by RT-PCR and Western blotting (Supplementary Fig. S1). Active β-catenin expression was unaltered in *SFRP1* siRNA-transfected cells compared with untransfected or *NS* siRNA-treated control cells, suggesting that SFRP1 may not affect the canonical Wnt signaling pathway in this setting.

Wnt-independent interactions of Wnt antagonists have also been explored to understand the expression patterns and activities of these secreted proteins (27). It has been suggested that the secretion of Wnt antagonists might alter the activity of neighboring cells in a manner that would favor the proliferation and metastasis of malignant cells (27). Importantly, recent studies on the process of metastasis suggest that the metastatic dissemination of cells is an early event in tumorigenesis; hence, the genetic and epigenetic alterations in the metastatic tumor cells may diverge from the primary tumors (47). The present study suggests that increased SFRP1 expression is an attribute of metastatic behavior in RCC and the molecular mechanism may be through activation of MMP10-mediated pathways.

Metastatic RCC is associated with a poor survival rate and only limited therapeutic options are available (48, 49). Therefore, it is imperative to understand the molecular basis of the disease. In conclusion, our present study suggests that SFRP1 may act as an oncogene and plays a role in conferring metastatic potential to RCC. To our knowledge, this is the first report showing that metastatic RCC is associated with increased expression of this Wnt antagonist. The present findings can increase our understanding of the role of SFRP1 and may influence the diagnostic and therapeutic interventions for metastatic RCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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