Research Article

Molecular Cancer Therapeutics

Oncogenic Functions of Secreted Frizzled-Related Protein 2 in Human Renal Cancer

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Abstract

The secreted Frizzled-related proteins (sFRP) are modulators of the Wnt signaling pathway, which is involved in embryonic development and tumor progression. The functions of sFRP2 have not been studied in renal cancer. Transient transfection of sFRP2 promoted cell growth in renal carcinoma cells, whereby the largest effect was observed in A498 cells. To further study the functions of sFRP2 gene in renal carcinoma cells, we established A498 renal cancer cell lines, which stably expressed sFRP2. Stably expressed sFRP2 significantly promoted cell proliferation *in vitro* and *in vivo* tumor growth. The stably expressed sFRP2 cells were also found to have reduced UV-induced apoptosis and increased G_2 phase of the cell cycle. The phosphorylation level at $Ser^{33/37}$/Thr^{41} of β-catenin was lower in the stable sFRP2 cell lines compared with the control cell line. sFRP2 significantly activated T-cell factor/lymphoid enhancer factor transcriptional activity. In the stable sFRP2 cell line, expression of c-Fos, Bcl2, Bcl-w, cyclin B2, and cyclin E2 genes was significantly increased and p53 expression was decreased. This is the first report documenting that sFRP2 activates the canonical Wnt pathway and promotes cell growth by evoking diverse signaling cascades in renal cancer cells. This study may provide better strategies for the management of renal cancer through regulation of sFRP2 pathways. *Mol Cancer Ther; 9(6); 1680–7. ©2010 AACR.*

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Introduction

The Wnt proteins are palmitoylated secreted glycoproteins that activate the Wnt signaling pathway. Wnt activity is regulated at the cell surface by different transmembrane proteins (1–3). Frizzled receptors, G protein–coupled receptors, are the major proteins that bind to Wnt proteins on the plasma membrane and trigger Wnt pathway signaling, which leads to activation of T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription. This signaling pathway is involved in embryonic development and tumor progression. Activation of the canonical Wnt pathway and noncanonical Wnt signaling pathways has been reported in various cancers (4, 5).

The Wnt signaling pathway is partly regulated by Wnt antagonists, including members of the Dickkopf and secreted Frizzled-related protein (sFRP) families, and Wnt inhibitory factor 1 (6, 7). It has been suggested that secreted Wnt antagonists act as tumor suppressors, being that their expression is silenced by promoter hypermethylation in many cancers.

The sFRPs are a family of soluble glycoproteins, which contain cysteine-rich domains homologous to the putative Wnt-binding sites of Frizzled proteins. sFRPs are modulators of the Wnt signaling pathway, interact with Wnt proteins, and prevent Frizzled receptors from binding to Wnt proteins, thereby downregulating Wnt signaling (8).

Loss or significant downregulation of sFRP expression has been documented in human tumors and is often caused by epigenetic promoter hypermethylation (9). Epigenetic inactivation of sFRP2 by promoter hypermethylation has been reported for human gastric cancer (10), colorectal cancer (11–13), and breast cancer (14, 15), suggesting that sFRP2 is a tumor suppressor. sFRP2 suppresses the transformation and invasive abilities of cervical cancer cells (16). Other reports indicate that sFRP2 promotes tumor progression in glioma (17), breast cancer cells (18), and cell proliferation in the intestine (19). Currently, the functional significance of sFRP2 in renal cancer has not been reported.

Here, we report that transient transfection of sFRP2 promotes cell growth in various renal carcinoma cells. A further study revealed that overexpression of sFRP2 activates the canonical Wnt pathway and promotes cell growth by evoking diverse signaling cascades in renal cancer cells.

Materials and Methods

Cell culture

Human renal carcinoma cells, A498 [primary renal cell carcinoma (RCC), p53 wild-type], Caki1 (metastatic

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clear cell RCC, p53 wild-type), Caki2 (primary clear cell RCC, p53 wild-type), and ACHN (primary RCC, p53 wild-type) cells were purchased from the American Type Culture Collection. A498, Caki1, and Caki2 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. ACHN cells were cultured in MEM Eagle's medium supplemented with 10% fetal bovine serum.

RNA extraction and reverse transcription-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription reactions were done with 1 μg of total RNA using a Reverse Transcription System kit (Promega). Quantitative real-time PCR analysis was done in triplicate with an Applied Biosystems Prism7500 Fast Sequence Detection System using Taqman universal PCR master mix according to the manufacturer's protocol (Applied Biosystems, Inc.). Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems).

Plasmid construction

A human sFRP2 expression vector, pcDNA-sFRP2, was constructed by subcloning the full-length cDNA of sFRP2 (Invitrogen) with a FLAG epitope-tagged sequence at the COOH terminus into the *Hin*dIII-*Xho*I site of the pcDNA3.1(+) vector (Invitrogen).

Generation of stable sFRP2 cell lines

A498 cells were transfected with pcDNA-sFRP2 or pcDNA3.1(+) (control) using FuGENE HD (Roche Diagnostics) according to the manufacturer's instructions, and the transfected cells were selected with 500 μg/mL G418. Single colonies were picked and stable cell lines were generated.

Cell proliferation assay

Cell viability was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), a colorimetric assay that measures the activity of reductase enzymes. Cells were seeded at a density of 1×10^3 per well in flat-bottomed 96-well plates. At the indicated times, CellTiter 96 Aqueous One reagent was added to each well according to the manufacturer's instructions. Cell viability was determined by measuring the absorbance at 490 nm using a kinetic microplate reader (SpectraMax 190, Molecular Devices Co.). Data are the mean ± SD of three independent experiments.

***In vivo* tumor growth**

Suspensions of the stable sFRP2-expressing cells or the control cells (1×10^7 in 200 μL RPMI 1640) were s.c. injected into female nude mice (strain BALB/c *nu/nu*, 4–5 wk old; Charles River Laboratories, Inc.). Tumor size was measured with calipers once per week for 8 weeks, and tumor volume was calculated on the basis of width (x) and length (y): $x^2y/2$, where $x < y$.

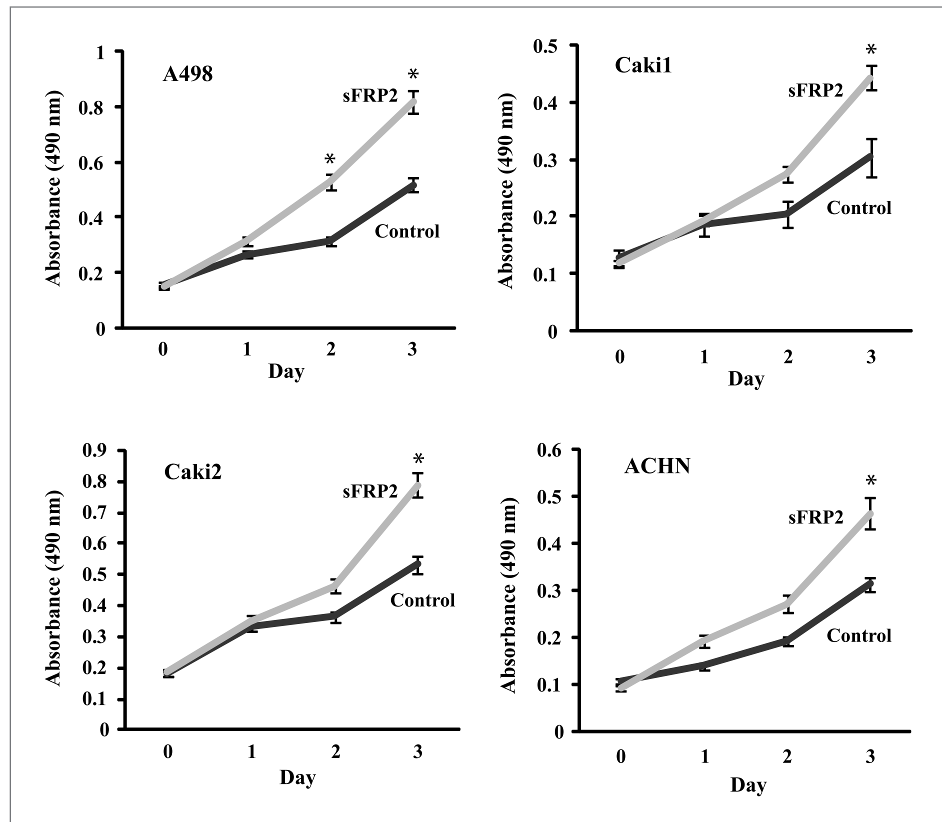

Figure 1. Transient transfection of sFRP2 promotes cell proliferation of renal carcinoma cells. Renal cancer cells were seeded at a density of 1.5×10^3 per well in 96-well plates. The cells were transiently transfected with the vector only [pcDNA3.1(+)] for control or sFRP2-pcDNA3.1(+). Cell viability was assayed at the indicated times. *, $P < 0.05$, compared with control.

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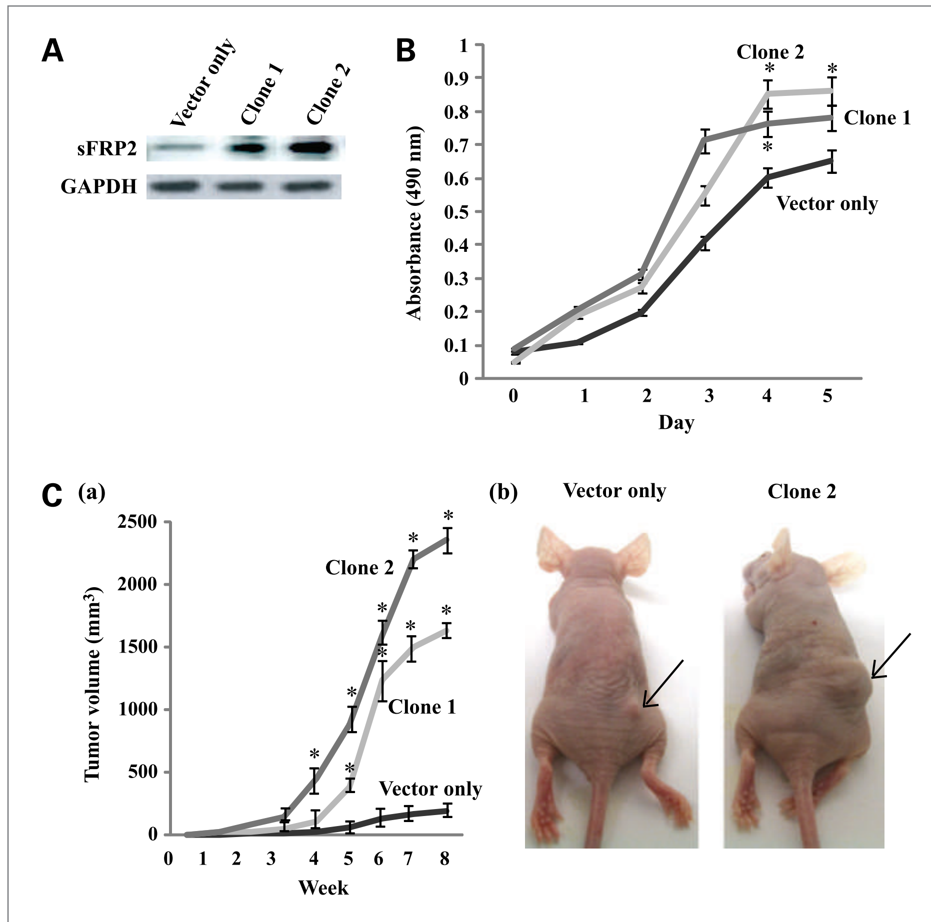

Figure 2. Ectopic expression of sFRP2 promotes proliferation of A498 cells. A, Western blot analysis showing expression levels of sFRP2 in A498 stable cell lines. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, cell viability of sFRP2 stable cell lines or control cell lines (vector only) was assayed at the indicated times. *, $P < 0.05$, compared with control. C, *in vivo* tumor growth of sFRP2 stable cell line. a, time course of tumor growth in nude mice. *, $P < 0.01$, compared with control. b, representative images of tumors at 8 wk after s.c. inoculation of sFRP2 stable cell line or control cell line to nude mice.

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Apoptosis analysis

Apoptosis was measured using flow cytometry (Cell Lab Quanta SC, Beckman Coulter, Inc.) with Annexin V-FITC/7-aminoactinomycin D labeling. Measurements were repeated independently thrice.

Cell cycle analysis

Cell cycle was analyzed using flow cytometry (Cell Lab Quanta SC) with 4′,6-diamidino-2-phenylindole staining. Measurements were repeated independently thrice.

Western blot

Protein extracts were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (Hybond-P, GE Healthcare) membranes, followed by incubation with the indicated primary and secondary antibodies conjugated to horseradish peroxidase (GE Healthcare). Signals were detected using the enhanced chemiluminescence detection system (ECL Plus Western Blotting Detection System, Amersham). Antibodies against sFRP2, c-Fos, and cyclin E were purchased from Abcam. Antibodies against β-catenin, phospho-β-catenin ($Ser^{33/37}$/Thr^{41}), phospho-β-catenin (Ser^{552}), phospho-β-catenin (Ser^{675}), cyclin E2, cyclin D1, c-Jun, Bcl-w, Rac1/2/3, focal adhesion kinase (FAK), and glyceraldehyde-3-phosphate dehydrogenase were purchased from Cell Signaling Technology. Antibodies against cyclin B2, cyclin-dependent kinase 2 (CDK2), and Bcl2 were purchased from Santa Cruz Biotechnology. An antibody against p53 was purchased from GeneTex. Antibodies against phospho-c-Jun (Thr^{239} and Ser^{243}) were purchased from Signalway Antibody.

Luciferase reporter assay

Cells in 24-well plates were transfected with a TCF reporter plasmid, TOPflash or FOPflash (Millipore), using FuGENE HD according to the manufacturer's instructions. TOPflash contains wild-type TCF-binding sites, whereas FOPflash contains mutated TCF-binding sites. The pRL-TK *Renilla* luciferase (Promega) was cotransfected to normalize for transfection efficiency. All transfection experiments were done in triplicate. Luciferase activity was assayed at 48 hours after transfection using a dual-luciferase reporter assay system (Promega).

Statistical analysis

Data are shown as mean values ± SD. The Student's *t* test was used to compare the two different groups. *P* values of <0.05 were regarded as statistically significant.

Results

Transient transfection of sFRP2 promotes cell proliferation of renal carcinoma cells

To study the effect of sFRP2 on the cell growth of renal carcinoma cells, we transiently transfected A498, Caki1, Caki2, and ACHN cells with the vector only [pcDNA3.1(+)] for control or sFRP2-pcDNA3.1(+). The transient transfection of sFRP2 increased sFRP2 mRNA levels (Supplementary Fig. S1) and promoted cell growth in all of the cell lines (Fig. 1). The growth ratios of the cells transfected with sFRP2 versus the cells transfected with the vector only at 48 hours after the transfection were 2.4, 2.0, 1.5, and 2.1 for A498, Caki1, Caki2, and ACHN cells, respectively. The largest effect of sFRP2 on cell growth was observed in A498 cells.

Generation of sFRP2 stable A498 cell lines

To further study the functions of sFRP2 in primary cancer cells, we established A498 cell lines, which stably expressed sFRP2, because the largest effect of sFRP2 on cell growth was seen in A498 cells (Fig. 1). For controls, A498 was transfected with the vector pcDNA3.1(+) only. Western blot shows that the expression levels of sFRP2 were dramatically increased in the stable sFRP2 cell lines compared with that in the control cell line indicated as vector only (Fig. 2A). Increased sFRP2 mRNA level in the stable sFRP2 cell lines is shown in Supplementary Fig. S2.

sFRP2 promotes cell proliferation

We also examined the effects of ectopic expression of sFRP2 on cell growth using the A498 cells that stably expressed sFRP2. The cell proliferation assay showed that the ectopic expression of sFRP2 promoted cell growth (Fig. 2B).

To examine the effects of the ectopic expression of sFRP2 on *in vivo* tumor growth, we s.c. injected the stable sFRP2 or the control cell line into nude mice. Tumor volumes were measured every 7 days for 8 weeks following the injection. Tumor of xenografts from A498 cells overexpressing sFRP2 grew significantly faster than

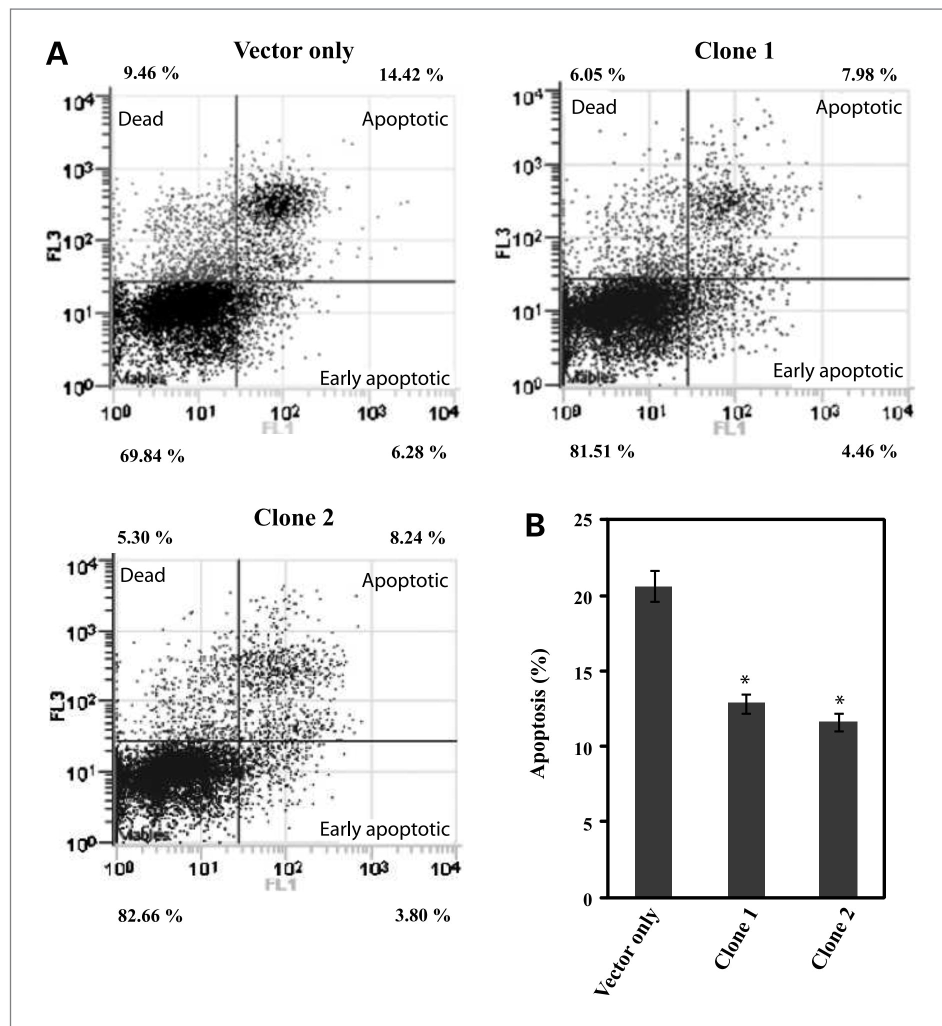

Figure 3. Ectopic expression of sFRP2 reduces UV-induced apoptosis in A498 cells. A, after irradiation with UV, stable sFRP2 or control cell lines were stained with Annexin V-FITC/7-aminoactinomycin D and apoptosis was analyzed by flow cytometry. B, proportion of early apoptotic and apoptotic cells with UV irradiation. The data expressed are the percentage of early apoptotic and apoptotic cells out of the total cell population of A498 cells. *, $P < 0.05$, compared with control.

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xenografts from the control cells. At week 8, tumor sizes of sFRP2 xenografts were six to nine times larger than those of control xenografts, indicating that the ectopic expression of sFRP2 significantly promoted tumor growth *in vivo* (Fig. 2C, a and b).

sFRP2 reduces UV-induced apoptosis

Because ectopic expression of sFRP2 stimulated cell growth, we next studied the effects of sFRP2 on apoptosis. To examine the apoptotic activity of sFRP2 in A498 cells, stably expressed sFRP2 or control cell lines were irradiated with UV and subjected to flow cytometry for apoptosis measurements because sFRP2 overexpression was found to decrease the susceptibility of mammary cancer cells to UV-induced apoptosis (18). We also found that ectopic expression of sFRP2 resulted in about a 50% reduction in UV-induced apoptosis in A498 cells (Fig. 3), showing that sFRP2 has antiapoptotic activity in A498 cells.

sFRP2 promotes cell cycle progression

We also did cell cycle analysis of the stable sFRP2 cell lines using flow cytometry because ectopic sFRP2 promoted cell proliferation. The cell population in the G_0-G_1 phase was decreased, whereas the cell populations in the S and G_2 phases were increased in the sFRP2 stable cell line (Fig. 4), indicating that ectopic expression of sFRP2 promotes cell cycle progression.

sFRP2 activates Wnt signaling pathway

In the Wnt signaling pathway, β-catenin enters the nucleus and interacts with TCF/LEF family transcription factors to activate gene expression. We examined β-catenin expression in the stable sFRP2 cell lines by Western blot. sFRP2 did not change the level of β-catenin; however, the phosphorylation level at $Ser^{33/37}/Thr^{41}$ of β-catenin was lower in the stable sFRP2 cell lines compared with the control cell line. This indicates that sFRP2 activated the canonical Wnt signaling pathway because the phosphorylation at $Ser^{33/37}/Thr^{41}$ by glycogen synthase kinase-3 promotes the degradation of β-catenin (Fig. 5A; ref. 20). sFRP2 overexpression did not significantly change the level of phosphorylation at Ser^{552} and Ser^{675} in β-catenin, which induces β-catenin accumulation and increases its transcriptional activity (21, 22). We did luciferase assays using the TOPflash/FOPflash reporter system to study the effect of sFRP2 on β-catenin–dependent TCF/LEF transcriptional activity. The TCF/LEF transcriptional activity with TOPflash was activated

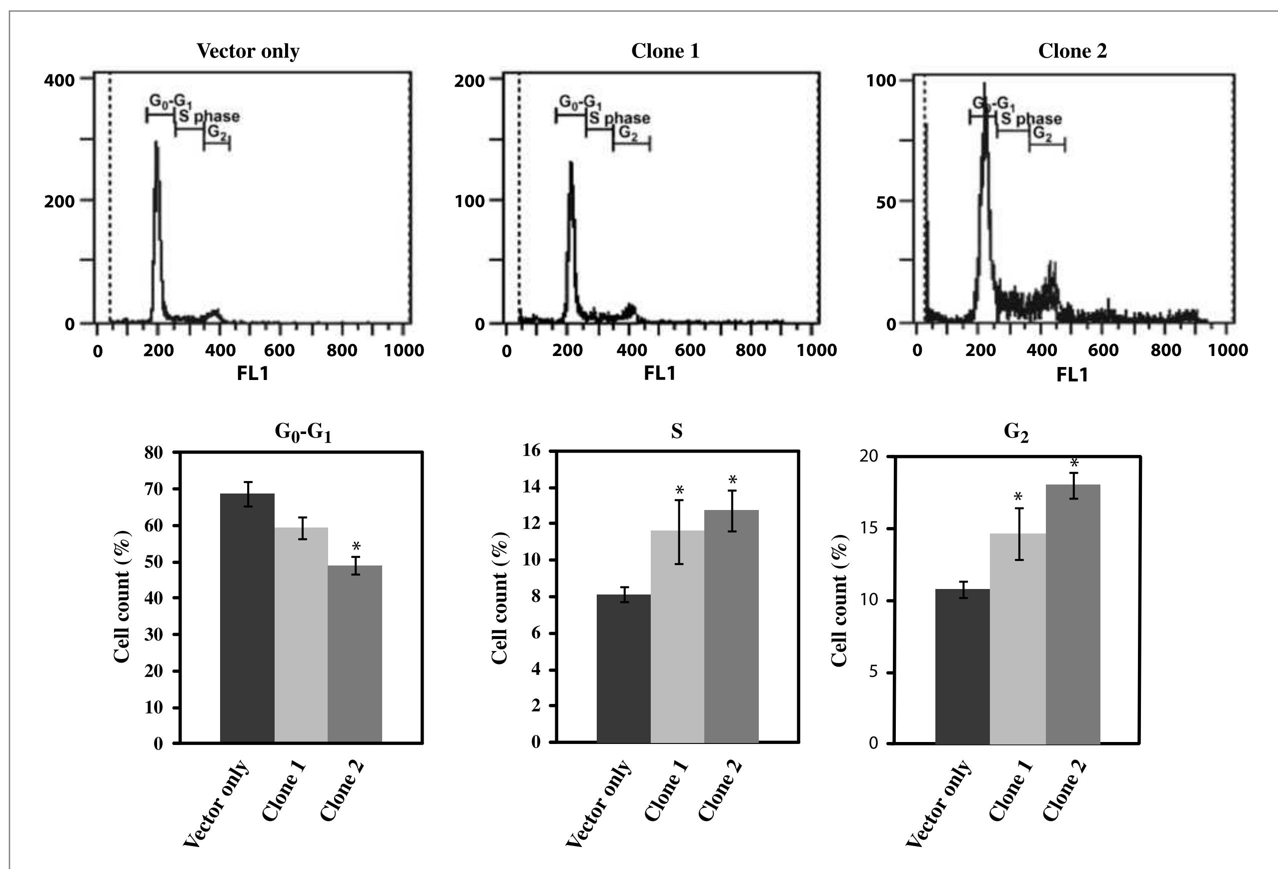

Figure 4. Ectopic expression of sFRP2 promotes cell cycle progression. Stable sFRP2 or control cell lines were stained with 4′,6-diamidino-2-phenylindole, and cell cycle was analyzed by flow cytometry. *, $P < 0.05$, compared with control.

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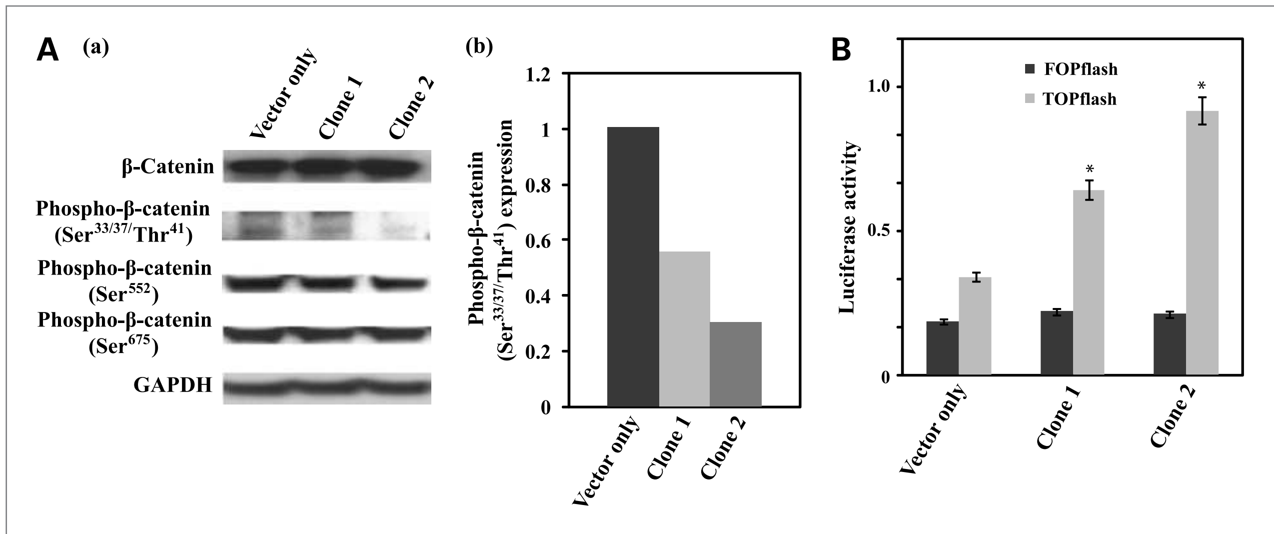

Figure 5. sFRP2 activates the canonical Wnt signaling pathway. A, a, sFRP2 reduced the phosphorylation level of β-catenin, as analyzed by Western blot; b, quantification of the phosphorylation level of β-catenin in a, normalized to β-catenin expression. B, β-catenin/TCF transcriptional activities. The cells were transfected with TOPflash or FOPflash with pRL-TK *Renilla*. Luciferase reporter assay was done at 24 h after the transfection. *, $P < 0.05$, compared with control.

up to 3-fold in the stable sFRP2 cell lines compared with that of the control cell line, whereas luciferase activity with FOPflash, which has mutated TCF-binding sites, was the same in the stable sFRP2 and control cell lines (Fig. 5B). These results also indicate that sFRP2 activated the canonical Wnt signaling pathway in stable sFRP2 cell lines.

sFRP2 alters the expression levels of various genes

Because we observed that sFRP2 promoted *in vitro* and *in vivo* cell growth and cell cycle progression and showed antiapoptotic activity, we examined the expression of genes that regulate proliferation, apoptosis, and the cell cycle by Western blot analysis. sFRP2 has been reported to be in the c-Fos network and (23), and c-Fos was significantly increased in sFRP2 stable cell lines compared with the control cell line (Fig. 6A). Expression of p53, a tumor suppressor gene, was found to be significantly lower in sFRP2 stable cell lines (Fig. 6A and B). Among antiapoptotic genes, ectopic sFRP2 expression significantly increased Bcl2 and Bcl-w with and without UV irradiation (Fig. 6A and B). In genes that regulate the cell cycle, ectopic sFRP2 expression significantly increased cyclin B2 and cyclin E2 (Fig. 6A); however, CDK2, cyclin E, and cyclin D1 were not changed (data not shown). Expression levels of c-Jun and FAK were not changed, and the phosphorylation level of c-Jun was not altered in sFRP2 stable cell lines (data not shown).

Discussion

sFRPs are a family of proteins that contain a cysteine-rich domain homologous to the extracellular region of the Frizzled Wnt receptors. sFRPs bind Wnt molecules through this cysteine-rich domain and inhibit the Wnt-mediated signaling cascade. In humans, five family members, sFRP1 to sFRP5, have been identified and are recognized as modulators in development and disease processes.

The importance of sFRP2 has been shown in cancer biology. Several studies have suggested that sFRP2 is an inhibitor of the Wnt pathway (6), and epigenetic inactivation of sFRP2 has been shown in various cancers (10–15), suggesting that sFRP2 functions as a tumor suppressor. In contrast to these results, others have found that sFRP2 promotes tumor progression. Thus, ectopic expression of sFRP2 reduced apoptosis (17, 18, 24, 25), significantly promoted the growth of glioma xenografts in nude mice, and stimulated cell proliferation (19). However, the functional significance of sFRP2 in renal cancer has not been reported.

In this study, we have shown that transient expression of sFRP2 promotes cell proliferation in renal carcinoma cells, A498, Caki1, Caki2, and ACHN cells. Similarly, stably expressed sFRP2 promoted cell proliferation *in vitro* and *in vivo*, decreased apoptosis, and stimulated cell cycle progression in A498 kidney cancer cells, suggesting that sFRP2 has oncogenic properties in these cells.

During our investigation of the molecular mechanisms by which sFRP2 promotes proliferation and the cell cycle and reduces apoptosis, we examined the expression of various genes that regulate these pathways. Expression of the p53 tumor suppressor gene was found to be significantly suppressed by sFRP2 overexpression, which may result in reduction of apoptosis and promotion of the cell cycle (26).

In genes that regulate proliferation, c-Fos increased significantly in sFRP2 stable cell lines compared with the control cell line. sFRP2 was found to be in the

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c-Fos network (23), and the level of c-Fos gene transcripts has been reported to be 100-fold greater in human term fetal membranes than in other normal human tissues and cells (27). Therefore, c-Fos is thought to contribute to the oncogenic properties induced by sFRP2 in A498 cells.

Activation of the Wnt pathway results in increased amounts of β-catenin, which consecutively stimulates transactivation of the transcription factor TCF/LEF. We observed that ectopic expression of sFRP2 did not alter the expression level of β-catenin but reduced the phosphorylation level at $Ser^{33/37}/Thr^{41}$, which is reported to be promoted by glycogen synthase kinase-3 in β-catenin (20). This was observed in the stable sFRP2 cell lines compared with the control cell line, suggesting that the canonical Wnt pathway was activated because phosphorylation results in the degradation of β-catenin (20). This has also been reported in glioma cells in which sFRP2 was overexpressed (17). However, sFRP2 overexpression did not change the levels of phosphorylation at Ser^{552} and Ser^{675}, which induce β-catenin accumulation and increase its transcriptional activity (21, 22). Luciferase assays using the TOPflash/FOPflash system confirmed that sFRP2 activated TCF/LEF (i.e., the canonical Wnt pathway in our experiments).

In breast cancer cells that ectopically expressed sFRP2, the phosphorylation of c-Jun was decreased and the c-Jun NH_2-terminal kinase pathway was suppressed (24). In contrast to these observations, the phosphorylation level of c-Jun was not altered in the sFRP2 cell lines in our study (data not shown).

sFRP2 has been reported to attenuate the susceptibility of MCF7 breast carcinoma cells to UV-induced apoptosis (25). The antiapoptotic activity of sFRP2 was found to be caused by the phosphorylation of FAK, the activation of NF-κB, and the suppression of activity of Janus kinases in breast cancer cells (24) and *Xenopus* embryos (28). However, the phosphorylation levels of c-Jun and FAK were not altered in our sFRP2 cell lines (data not shown).

In contrast to these results, we found in genes that regulate apoptosis that the ectopic expression of sFRP2 significantly increased the level of expression of Bcl2 and Bcl2-w. Bcl2 inhibits a major apoptotic pathway by preventing the release of cytochrome *c* from the mitochondria, thereby blocking caspase-induced apoptosis (29), and is overexpressed in many human solid tumors (30). The increase of Bcl2 expression may be caused by the suppression of p53 in sFRP2 stable cell lines because Bcl2 is one of the target genes of p53 (26). Bcl2-w is also an antiapoptotic and proapoptotic regulator and is increased in solid and hematologic malignancies (31). Therefore, the increased expression of antiapoptotic genes may contribute to the suppression of apoptosis by sFRP2.

In genes that regulate the cell cycle, the ectopic expression of sFRP2 significantly increased the level of the expression of cyclin B2 and cyclin E2. Cyclin B2 is one of essential components of the cell cycle regulatory machinery and has important roles in the control of the cell cycle at the G_2-M–phase transition (32). The accumulation of cyclin B2 was also found in human malignant tumors (33–35). Cyclin E2 is a regulatory subunit of the CDK2 complex and accelerates the G_1-S–phase transition (36). Deregulation of cyclin E2 may promote oncogenesis via genomic instability (37). These increases in the cyclins may account for the promotion of the cell cycle by sFRP2.

Interestingly, these genes are not direct targets of the Wnt signaling pathway, although the Wnt pathway may be activated by reduction of phosphorylated β-catenin

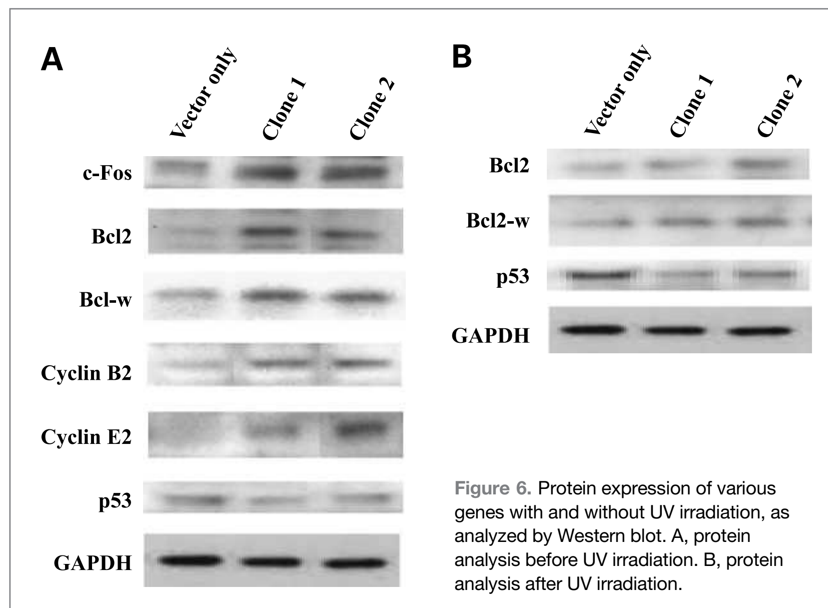

Figure 6. Protein expression of various genes with and without UV irradiation, as analyzed by Western blot. A, protein analysis before UV irradiation. B, protein analysis after UV irradiation.

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as discussed above. This suggests that ectopic sFRP2 expression elicits alternative signaling pathways in A498 cells, including suppression of p53 signaling.

In this study, we have presented the first evidence of the oncogenic function of sFRP2 in renal carcinoma cells. We have identified various sFRP2-regulated genes, suggesting that sFRP2 is able to activate diverse signaling pathways. These findings have important implications for the treatment of renal cancer and possibly other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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