Secreted frizzled-related protein 2 (sFRP2) is a negative modulator of the Wingless-type (Wnt) signaling pathway, and shown previously that methylation analysis of Wnt antagonist genes may have diagnostic and prognostic value in the detection and management of RCC. However, there have been no other previous reports documenting involvement of histone modifications in sFRP2 repression in RCC.

Therefore, in our study, to gain a further understanding of the epigenetic mechanisms involved in sFRP2 expression, such as DNA methylation, and histone modifications, we used a chromatin immunoprecipitation (ChIP) assay to assess histone acetylation and methylation. We found that DNA methylation was the major epigenetic mechanism for a repressed sFRP2 gene in RCC cells, while occasionally it was silenced by histone modification without DNA methylation. Expression of the sFRP2 gene silenced by methylation could be restored by a DNA methyltransferase inhibitor or histone deacetylase inhibitor. However, the histone deacetylase inhibitor could reactivate the silenced sFRP2 gene only in hypomethylated cells. Our results provide evidence for the underlying mechanisms of the epigenetic regulation of the sFRP2 gene in RCC cells.

Material and methods

Tissue samples and cell culture

A total of 20 newly diagnosed RCC tissues from radical nephrectomy were obtained from the Veterans Affairs Medical Center San Francisco, CA. Informed consent was obtained from all patients. HK-2, a SV-40-immortalized human renal tubular cell line and human RCC cell lines Caki-1, Caki-2, A-498 and ACHN were obtained from the American Type Culture Collection (Manassas, VA). The HK-2 cell line was maintained in keratinocyte serum-free medium (GIBCO Laboratories, Grand Island, NY) supplemented with 50 μg/mL bovine pituitary extract, 5% t-glutamine and 5 ng/mL epidermal growth factor (EGF). The Caki-1 and Caki-2 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 minimal essential medium supplemented with 10% fetal bovine serum. All cells were maintained in a humidified incubator (5% CO2) at 37°C. In the drug treatment experiments, cells were split on Day 0 at 8 × 10⁷/100 mm tissue culture dish. For 5-aza-2'-deoxycytidine (DAC; Sigma, St. Louis, MO) treatment, DAC was added on day 1 at a dose of 1.0 μM. Culture medium was changed on Day 4, and fresh DAC was added. Cells were incubated for a total 7 days. For trichostatin A (TSA, Upstate Biotechnology, Lake Placid, NY) treatment, 300 nM TSA was added to the media on Day 2 and cells were incubated with TSA for 24 hr. For combined treatment, cells were split on Day 0 and treated with 1.0 μM DAC starting on Day 1. Culture
medium was changed on Day 4, and fresh DAC was added. Culture medium was changed on Day 7, and 200 nM TSA was added during the last 24 hr. Total RNA and genomic DNA were isolated using the AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA) following the manufacturer’s directions.

### Immunohistochemistry

Immunostaining was done on formalin-fixed, paraffin-embedded sections. Antigen retrieval was carried out by microwaving the slides in 10 mmol/L sodium citrate buffer. Slides were incubated overnight with a 1:200 dilution of anti-sFRP2 antibody (sc-13939, Santa Cruz Biotechnology, Santa Cruz, CA). Negative controls were done using non-specific immunoglobulin. A pathologist not involved in the present study evaluated the immunostaining under blind conditions. The immunohistochemical staining was graded on an arbitrary scale from 0 to 1+; 0 representing negative expression (0–20% positive cells), 1+ representing weakly positive expression (20–50% positive cells) and 2+ representing strongly positive expression (50–100% positive cells). The scale was determined according to the average number of positive cells in 10 random fields of all slides.19

### Reverse transcription and real-time PCR

Reverse transcription reactions were conducted with 1 μg of total RNA using Reverse Transcription System (Promega, Madison, WI). Reverse transcription-PCR (RT-PCR) for the detection of sFRP2 expression in kidney cell lines was carried out using REDTag DNA polymerase (Sigma, St. Louis, MO). Samples were defined as sFRP2 positive if a PCR product was detectable after 32 cycles of amplification. Referring to a previous report,16 primer sequences were designed. The primer sequences and PCR conditions are shown in Table I. One additional primer set was used to amplify a 166-bp fragment of the GAPDH gene as an internal control. Each PCR reaction was initially set up using different amounts of ChIP immunoprecipitated DNA was eluted in a total volume of 50 μL. Aliquots of 2 μL of first PCR reactions were subjected to second round amplifications using a nested primer pairs (BSP-1, BSP-2) in a total volume of 30 μL. The amplification products were confirmed by electrophoresis on a 2% agarose gel and sequenced directly by an outside vendor (McLab, South San Francisco, CA).

### Bisulfite DNA sequencing

Bisulfite modification of genomic DNA was done using the EpiTect Bisulfite kit (Qiagen, Valencia, CA) following the manufacturer’s directions. Primers for bisulfite genomic sequencing PCR was designed manually or by using the online program MethPrimer.20 The primer sequences and PCR conditions are shown in Table I. All reactions were subjected to 2 rounds of amplifications using a nested primer approach. Bisulfite-modified DNA (1 μL) was amplified using Pan-1 and Pan-2 primer pairs in a total volume of 20 μL. Aliquots of 2 μL of first PCR reactions were subjected to second round amplifications using a pair of nested primer pairs (BSP-1, BSP-2) in a total volume of 30 μL. The amplification products were confirmed by electrophoresis on a 2% agarose gel and sequenced directly by an outside vendor (McLab, South San Francisco, CA).

### Chromatin immunoprecipitation assay

ChIP assays were performed on cell line DNA using the EZ-ChIP Kit (Upstate Biotechnology, Lake Placid, NY) and followed the manufacturer’s protocols. Chromatin was prepared from confluent cells and sonicated to DNA lengths between 200 and 1,000 bp, then immunoprecipitated with each antibody. Antibodies for acetylated histone H3 (Abcam, Cambridge, MA), acetylated histone H4 (Abcam, Cambridge, MA) were used in the immunoprecipitations. The immunoprecipitated DNA was eluted in a total volume of 50 μL, and 2 μL of each DNA sample was analyzed by PCR. We designed primers to separately amplify 3 regions in the sFRP2 promoter area (Fig. 2c). The primer pairs used for ChIP assays are shown in Table I. One additional primer set was used to amplify a 166-bp fragment of the GAPDH gene as an internal control. Each PCR reaction was initially set up using different amounts of ChIP sample with varying PCR cycle numbers, and we selected the final PCR conditions accordingly. PCR products were analyzed on 2.0% agarose gels and visualized by staining with ethidium bromide. Densitometric analysis of the observed bands was performed using ImageJ software (http://rsb.info.nih.gov/ij). Fold

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| Primer sequence (5‘-3‘) | Annealing temperature (°C), PCR cycles | Product size (bp) |
|------------------------|----------------------------------------|------------------|
| RT-PCR primers         |                                        |                  |
| sFRP2-S                |                                        |                  |
| sFRP2-AS               |                                        |                  |
| GAPDH-AS               |                                        |                  |
| BSP primers            |                                        |                  |
| PAN-1-S                | 50, 45                                 | 322              |
| PAN-1-AS               | 50, 35                                 | 291              |
| PAN-2-AS               | 50, 45                                 | 499              |
| BSP-2-S                | 50, 35                                 | 285              |
| ChIP primers           |                                        |                  |
| ChIP-1-S               | 55, 33                                 | 170              |
| ChIP-1-AS              | 55, 33                                 | 216              |
| ChIP-2-AS              | 55, 33                                 | 190              |
| ChIP-3-AS              | 55, 33                                 | 166              |
| ChIP-3-S               | 55, 33                                 | 190              |
| GAPDH-S                | 55, 33                                 | 166              |
| ChIP-1-AS              | 55, 33                                 | 216              |
| ChIP-2-AS              | 55, 33                                 | 190              |
| ChIP-3-AS              | 55, 33                                 | 166              |

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enrichment in each immunoprecipitation was determined by the ratio to input DNA. Two independent ChIP experiments were performed for each analysis, and PCR was done twice for each of the ChIP-DNA samples.

Western blotting
Protein extracts (80 μg/lane) were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting. The membranes were immersed in 3% skim milk in 0.02 mol/L Tris-HCl (pH 8.0), 0.4 mol/L NaCl, and 0.05% Tween 20 buffer at room temperature for 2 hr. After blocking, proteins were detected by overnight incubation with 1 μg/mL monoclonal antibody against sFRP2 (MAB1169; R&D Systems, Minneapolis, MN) and monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (MAB374; Millipore, Bedford, MA) at 1:500 dilution. After washing, the membranes were incubated with goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad, Richmond, CA) at room temperature for 2 hr. The specific complexes were visualized using the ECL detection system (GE Healthcare, Buckinghamshire, UK).

Statistical analysis
Statistical analysis was performed using one-way ANOVA followed by the Fisher’s protected least-significant-difference test. p values of less than 0.05 were regarded as statistically significant. All statistical analyses were performed using StatView version 5.0 for Windows.

Results
Expression level of sFRP2 in human kidney samples
To examine the expression levels of sFRP2 in malignant and normal kidney tissues, immunostaining was performed on 20 RCC and 20 normal kidney specimens using antibody to sFRP2. Typical immunostaining of sFRP2 in normal kidney (a) and RCC (b) specimens are shown in Figure 1. Strongly positive expression

FIGURE 1 – sFRP2 expression in normal kidney, RCC samples and RCC cells. (a) Immunostaining of sFRP2 in normal kidney tissue (×200, hematoxylin counterstained). Strong membrane expression of sFRP2 was observed. (b) sFRP2 expression in RCC tissue (×200, hematoxylin counterstained) with no membrane staining. (c) Summary of immunostaining data. (d) Equivalent amounts of RNA were analyzed by quantitative real-time RT-PCR in each cell line. Results are the mean ± standard deviation of 3 independent experiments. * indicates the statistical difference between HK-2 and each RCC cell line (p < 0.05).

FIGURE 2 – (a) Schematic representation of the promoter region of the human sFRP2 gene and primer location. The vertical lines represent the location of CpG dinucleotides, and the gray boxes show CpG islands. The black box indicates the first exon, and arrows show the approximate position of the transcription start site. The doubled horizontal lines indicate the region examined by bisulfite DNA sequencing. The 3 horizontal bars indicate the location of the DNA fragments amplified by ChIP-PCR. Primer sequences and expected PCR product sizes are shown in Table I. (b) Methylation mapping of 46 CpG sites of the sFRP2 promoter obtained from bisulfite sequencing in kidney cell lines.
was observed in 70% of normal kidney specimens, whereas in RCC specimens the majority of samples showed negative immunostaining. In contrast, negative or weakly positive expression in normal kidney specimens was significantly lower than that in RCC specimens (0 or 30% vs. 85 or 15%, respectively) as shown in Figure 1c. To determine relative expression levels of sFRP2 in kidney cell lines, equivalent quantities of RNA were analyzed by quantitative real-time RT-PCR. As shown in Figure 1d, sFRP2 mRNA was expressed at a significantly higher level in normal (HK-2) cells, whereas it was down-regulated in all tumor cell lines \((p < 0.05)\). Two RCC cell lines, A-498 and ACHN, had no sFRP2 expression. Caki-1 and Caki-2, showed a significant decrease of expression that was ~20% and 10% of the normal level, respectively.

Methylation status of the sFRP2 promoter region

To determine whether loss of sFRP2 expression resulted from promoter hypermethylation, the methylation status at 46 CpG sites of the 2 CpG islands, CGI 1 and CGI 2, across 0.6 kb of the sFRP2 locus was characterized in these cell lines by bisulfit genomic sequencing (Fig. 2a). The detailed CpG methylation statuses of kidney cell lines are shown in Figure 2b. These analyses indicated that the 2 CpG islands in each cell line showed essentially the same methylation status. Most of the CpG sites of the sFRP2 promoter were not methylated in HK-2 cells that expressed a relatively high level of sFRP2 gene. In contrast, Caki-2, A-498, and ACHN cell lines that expressed a low level of sFRP2 gene displayed dense methylation in the sFRP2 promoter. All the samples with methylated promoters were sFRP2 negative, indicating that promoter DNA methylation is tightly correlated with and possibly be responsible for sFRP2 repression. Compared to these 3 cell lines, a lower degree of methylation was found in Caki-1 cells. These results indicated that, in addition to promoter methylation, other mechanisms probably exist to regulate sFRP2 expression in this cell line.

Histone modifications associated with the sFRP2 promoter region

To clarify the role of epigenetic suppression of the sFRP2 gene, we examined local histone acetylation and H3 methylation in the chromatin associated with the sFRP2 promoter region using a ChIP assay. The histone-associated DNAs, immunoprecipitated with antibodies against AcH3, AcH4, H3K4 and H3K9, were individually amplified with 3 primer sets covering the sFRP2 promoter region (Fig. 2a). The results in Figure 3 show marked differences in the levels of histone acetylation and H3 methylation between normal cells and RCC cells. In HK-2, which was the sFRP2 positive cell line with an unmethylated promoter, levels of AcH3, AcH4 and H3K4 were high and that of H3K9 was very low. This pattern of histone modifications is known to mark an open chromatin structure associated with active gene expression. All RCC cell lines, which represented the sFRP2 negative cell lines, showed a contrary pattern (low levels of AcH3, H4Ac and H3K4, and a high level of H3K9), known as repressive histone modifications. These results indicated that the histone modifi-
cations at the sFRP2 promoter region were strongly correlated with sFRP2 expression.

Effects of DAC and TSA on sFRP2 gene expression in RCC cell lines

The association of sFRP2 promoter DNA methylation and gene silencing in relation to histone modifications after drug treatment has not been reported previously. To establish this functional link, we examined the changes of sFRP2 expression and DNA methylation and histone modification in the promoter region after treatments of cells with DAC, an inhibitor of DNA methyltransferases (DNMTs) or TSA, a histone deacetylase (HDAC) inhibitor. In all RCC cell lines, treatment with DAC restored mRNA and protein expression of sFRP2 (Fig. 4a). The expression levels of sFRP2 were quantified by real-time RT-PCR (Fig. 4b). On the other hand, Caki-2, A-498 and ACHN cell lines, sFRP2 negative cells with methylated promoters, showed no reactivation of sFRP2 when the cells were treated with TSA. In contrast, TSA treatment reactivated sFRP2 expression in Caki-1 cells, with a partially methylated promoter.

To confirm the demethylation effect of DAC, bisulfite sequencing was done on treated cell lines (Fig. 5). In Caki-1 cells, a hypomethylated promoter was observed after DAC treatment. In the other 3 cell lines, partial demethylation was detected after DAC treatment. We then asked whether histone modifications in the promoter also changed during sFRP2 reexpression induced by treatment with DAC, and/or TSA in Caki-1 and ACHN cells. The ChIP assays revealed that TSA treatment induced drastic histone modification changes in Caki-1 cells (Fig. 6a). There were substantial increases of AcH3, AcH4 and H3K4 and a simultaneous decrease of H3K9, that is, changes to an active pattern of histone modifications. In contrast, treatment with DAC showed little effect on histone modification in these cells. However, histone modifications were induced by DAC treatment in ACHN cells (Fig. 6b). AcH3, AcH4 and H3K4 levels were higher, whereas H3K9 levels were lower in the promoter region. Treatment with TSA alone caused similar but only moderate changes in histone modifications.

Discussion

In this report, we have shown that the expression levels of sFRP2 were higher in normal kidney tissues compared with RCC specimens. sFRP2 mRNA was also expressed at a significantly higher level in normal cultured cells (HK-2), whereas it was down-regulated in all tumor cell lines. Next, we have demonstrated that the 5' CpG island in the sFRP2 gene promoter region is methylated in 3 sFRP2 negative RCC cell lines, while a lower degree of methylation was observed in Caki-1 cells that also expressed low levels of the sFRP2 gene. Several antagonists of Wnt signaling have been identified and can be divided into 2 functional classes: the secreted frizzled-related protein (sFRP) class and the Dickkopf (Dkk) class. The former class, which includes the sFRP gene family (sFRP1, sFRP2, sFRP3, sFRP4 and sFRP5), Wnt inhibitory factor-1 (WIF-1), and Cerberus, inhibits Wnt signaling by directly binding to Wnt proteins. In the sFRP gene family, recent publications from our laboratory and others have shown that impaired regulation by hypermethylation of the sFRP1 gene was found in many cancers. In addition, hypermethylation of the sFRP1 gene in RCC has been reported. On the other hand, hypermethylation of the sFRP2 gene has been also reported in gastric, colorectal and bladder cancers. However, there is no report concerning sFRP2...
**FIGURE 5** – Methylation status of the *sFRP2* promoter in control RCC cells and in RCC cells treated with DAC (1 μM) for 7 days was determined by bisulfite DNA sequencing of 5 individual DNA strands.

|        | No Treat | DAC   |
|--------|----------|-------|
| Caki-1 |          |       |
|        |          |       |
| Caki-2 |          |       |
|        |          |       |
| A-498  |          |       |
|        |          |       |
| ACHN   |          |       |
|        |          |       |

**FIGURE 6** – Quantification of ChIP-PCR results on Caki-1 (hypomethylated) and ACHN (hypermethylated) cells after treatment with DAC, and/or TSA. Two independent ChIP experiments were performed for each analysis, and PCR was done twice for each of the ChIP DNA samples to confirm reproducibility of the results.
Several tumor suppressor genes are known to be repressed by hypomethylated promoters. These effects indicate that aberrant DNA methylation and histone modifications work together to silence many tumor suppressor genes in human cancer. In our present study, ChIP assay revealed that histone acetylation and H3K4 methylation were increased in HK-2 cells, that showed sFRP2 mRNA expression. These histone patterns are known as an open chromatin structure with associated with active gene expression. There was no acetylation or methylation of these same sites in RCC cells that had no mRNA expression. However, H3K9 methylation, a repressive histone modifications indicative of gene silencing, was increased in all RCC cells. We also examined the effect of a DNA methyltransferase inhibitor (DAC), and/or a histone deacetylase inhibitor (TSA) on histone acetylation (AcH3, AcH4), H3K4 and H3K9 methylation associated with sFRP2 promoter region. In hypomethylated Caki-1 cells, the effects of DAC treatment on histone modifications in the promoter were less than those observed following TSA treatment. Conversely, histone acetylation and methylation were altered by DAC compared to TSA treatment in ACHN cells with a hypermethylated promoter. In these methylated cell lines, treatment with DAC changed not only the DNA methylation status, but also the histone modifications to an active pattern in the promoter region. These results are in agreement with previous reports of decreased H3K9 methylation levels in other epigenetically silenced genes following DAC treatment. Wozniak et al. reported that DAC treatment reduced dimethyl-H3-K4 to the levels seen in normal epithelial cells, even in the absence of significant DNA demethylation in breast cancer cells. These results indicate that while aberrant DNA methylation and repressive histone modifications work in combination to silence many important tumor suppressor genes in human cancers, DAC can act at both levels, either singly or concomitantly, to reactivate expression of these genes. Meanwhile, McGarvey et al. described that when transcription of DNA hypermethylated genes was activated in cancer cells after drug treatment, several repressive histone modifications remained in colorectal cancer cells. These differences are attributed to cell type- and specific loci. In addition, we observed synergistic effects of DAC and TSA on mRNA reexpression leading to reactivation of sFRP2 gene in all RCC cell lines. Several reports have demonstrated that synergistic effects of DNA hypermethylation and local histone deacetylation are crucial factors for chromatin structure alteration leading to transcriptional suppression. DAC is a potent inhibitor of DNA methyltransferase (DNMT) activity, through irreversible binding of DNMTs to 5-aza-2′-deoxycytidine-substituted DNA. The methylation of genomic DNA is catalyzed by DNA methyltransferases (DNMTs), which include DNMT1, DNMT3a and DNMT3B. In particular, DNMT1 is considered to take part in tumorigenesis. DNMT1 overexpression has been shown in colon, lung, liver and prostate cancers, and it causes genomic hypermethylation. Recently, it has been reported that TSA not only alters histone acetylation, but also may affect DNA methylation. Januchowski et al. stated that TSA decreased DNMT1 mRNA stability, and therefore may exhibit a double effect on epigenetic reactivation of silenced genes by increasing hyperacetylation of histones and hypomethylation of DNA. TSA may also lower the threshold for DNMT inhibitors to induce DNA hypomethylation, and may be one of the mechanisms for the synergistic effects of DAC and TSA on DNA hypermethylation and histone modifications. On the other hand, TSA did not induce acetylation of histone H3 and H4 as much in methylated promoters as it did in the unmethylated promoters. TSA may induce unmethylated promoter DNA to fully induce histone acetylation. In addition, TSA has been shown to suppress growth of ACHN cells via cell cycle arrest in association with p27 or apoptosis. The anti-growth effects of TSA may be more influential than the effects on histone modifications in RCC cells with methylated promoters.

In conclusion, this is the first report showing that aberrant DNA methylation and histone modifications work together to silence the sFRP2 gene in human kidney cancer cells. The majority of sFRP2 negative cells had a methylated promoter. Meanwhile, though sFRP2 expression was repressed in Caki-1 cells that have hypomethylated promoter, these cells also had repressive histone modifications. Therefore in Caki-1 cells, sFRP2 was reactivated by TSA, accompanied by an active pattern of histone modification. Repressive histone modifications were also observed in RCC cells with hypermethylated promoters, but sFRP2 was reactivated only by DAC and not by TSA. However, the activation of the silenced sFRP2 gene could be achieved in all cells using a combination of DAC and TSA. These results suggest that it is important to look at alterations in DNA methylation and histone modifications if epigenetic modulating drugs are used for kidney cancer chemotherapy. In our study, we focused on DNA methylation and histone modifications in the sFRP2 promoter using cultured cell lines. A larger series of human kidney cancer samples will need to be studied to confirm whether or not DNA methylation and histone modifications associated with sFRP2 repression are relevant mechanisms in renal cell carcinoma.

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