

Wnt Pathway Inhibitors Are Strongly Down-Regulated in Pituitary Tumors

Marianne S. Elston, Anthony J. Gill, John V. Conaglen, Adele Clarkson, Janet M. Shaw, Andrew J. J. Law, Raymond J. Cook, Nicholas S. Little, Roderick J. Clifton-Bligh, Bruce G. Robinson, and Kerrie L. McDonald

Cancer Genetics Unit (M.S.E., J.M.S., R.J.C.-B., K.L.M.), Kolling Institute of Medical Research, Department of Anatomical Pathology (A.J.G., A.C.), Royal North Shore Hospital, and Department of Neurosurgery (R.J.C., N.S.L.), Royal North Shore and North Shore Private Hospitals, Sydney, New South Wales 2065, Australia; University of Sydney (A.J.C., A.C.), and Faculty of Medicine (B.G.R.), Sydney, New South Wales 2006, Australia; Department of Endocrinology (J.V.C.), Waikato Hospital, Waikato 3204, New Zealand; and Department of Neurosurgery (A.J.J.L.), Auckland City Hospital, Auckland 1023, New Zealand

The etiology of sporadic pituitary tumors is currently unknown. The Wnt pathways have been implicated in the pathogenesis of a variety of human tumors, but the role of these pathways in pituitary tumors is unclear. Microarray analysis using the Affymetrix HG U133 plus 2.0 GeneChips identified four secreted frizzled-related protein (sFRP) family members of Wnt pathway inhibitors that were differentially expressed in both nonfunctioning and clinically functioning pituitary tumors (n = 20) compared with normal pituitary controls (n = 3). Reduced tumor expression of Wnt inhibitory factor-1 (*WIF1*), *sFRP2*, and *sFRP4* mRNA was confirmed by real-time quantitative RT-PCR ($P < 0.001$ and $P = 0.002$ and 0.013 , respectively) in all pituitary subtypes. Hypermethylation of the *WIF1* promoter was present in 88% of the pituitary tumors (n = 41). Seventy-six percent of pituitary tumors demonstrated absent or weak cytoplasmic *WIF1* staining by immunohisto-

chemistry (n = 41), although preserved staining was seen in some functioning tumors, with strong staining in 92% of normal pituitary controls (n = 13). The Wnt pathway target gene cyclin D1 was found to be up-regulated specifically in the nonfunctioning pituitary tumors compared with controls at both mRNA and protein level, supportive of activation of the Wnt- β -catenin pathway. Nuclear accumulation of β -catenin, however, was not observed in any pituitary tumors (n = 70). By transfecting GH3 cells with *WIF1*, decreased cell proliferation and colony formation was observed compared with empty vector controls. In conclusion, our data suggest that *WIF1* may be a tumor suppressor, specifically in nonfunctioning pituitary tumors, and that the Wnt pathways are important in pituitary tumorigenesis. (*Endocrinology* 149: 1235–1242, 2008)

PITUITARY TUMORS COMPRISE approximately 10–25% of all intracranial tumors (1). Most pituitary tumors are benign, although they may cause significant morbidity and premature mortality from mass effect and hormonal dysfunction. Pituitary tumors usually occur sporadically and are subgrouped according to hormonal status into functioning and nonfunctioning adenomas (NFAs) and further subdivided based on size into microadenomas (<1 cm) and macroadenomas (≥ 1 cm). The etiology of pituitary tumors is not fully understood but is thought to be a multistep process involving both intrinsic molecular defects and hormonal/growth factor stimulation (1).

Aberrant Wnt signaling has been described in many tumor types. There are at least three Wnt pathways, and the Wnt- β -catenin, or canonical, pathway is the best characterized of these. The canonical pathway has been studied most extensively in colon cancer (2) but has also been implicated in the

pathogenesis of other human malignancies (see review, Ref. 3). In the canonical pathway, binding of the Wnt ligand to the Frizzled-lipoprotein-related protein receptor complex results in stabilization of β -catenin such that it accumulates in the cytoplasm and translocates into the nucleus to regulate target gene transcription. Secreted Wnt antagonists are involved in regulating the Wnt pathways. These Wnt inhibitors are divided into two main families containing either secreted frizzled-related proteins (sFRPs) or the Dickkopf (DKK) proteins. The sFRP family comprises five sFRPs [sFRP 1–5; sFRP3 is also known as Frizzled B (FRZB)] and Wnt inhibitory factor 1 (*WIF1*). The sFRPs bind directly to Wnt ligands, thereby preventing their interaction with the Frizzled-lipoprotein-related protein receptor complex and downstream Wnt signaling. Down-regulation of *WIF1* and *sFRP1–5* gene expression has been reported in a number of tumor types (4–8), and epigenetic silencing for *WIF1* and the *sFRP* family by CpG island methylation has been demonstrated in several cancers (5, 6, 8–15). Recent work has shown that reduced expression of Wnt pathway inhibitors may occur in colorectal tumors even if they also contain other downstream Wnt pathway mutations, suggesting that loss of these inhibitors may have an additional role in tumor growth (12).

Data on the Wnt pathways in pituitary tumors is limited. Immunohistochemical staining of pituitary tumors for

First Published Online December 13, 2007

Abbreviations: ACTH-S, ACTH-secreting; DKK, Dickkopf; EV, empty vector; GH-S, GH-secreting; NFA, nonfunctioning adenoma; qPCR, quantitative PCR; sFRP, secreted frizzled-related protein; *WIF1*, Wnt inhibitory factor-1.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

β -catenin has been reported, but results have been conflicting with respect to the frequency of cytoplasmic and nuclear protein accumulation (16–20). Nuclear accumulation of β -catenin has been reported to be present in 57% of pituitary tumors (17); however, an independent study demonstrated nuclear staining in only 1% of tumors (18). Mutations in the genes encoding β -catenin, AXIN1, adenomatous polyposis coli, and glycogen synthase kinase 3 β have been assessed but appear to be infrequent even in those pituitary tumors reported to show nuclear β -catenin accumulation (18, 21, 22). The only report of a Wnt inhibitor in pituitary tumors comes from a microarray study where elevated *sFRP1* mRNA expression in NFAs was identified (23).

The aim of the current study was to find genes potentially involved in pituitary tumor pathogenesis using microarray analysis. We report the reduced expression of *WIF1* and three other sFRPs common to both nonfunctioning and functioning pituitary tumors. Further confirmation by real-time quantitative PCR (qPCR) and immunohistochemistry suggests that aberrant Wnt pathway activity may be associated with pituitary tumorigenesis. The downstream target of activated Wnt pathway signaling, cyclin D1, was also found to be up-regulated in pituitary tumors but not normal pituitary. Putative *WIF1* tumor suppressor function was investigated, and restoration of *WIF1* in rat pituitary GH3 cells resulted in reduced cell proliferation.

Materials and Methods

Tumor collection and characterization

Pituitary tumors were collected from patients undergoing surgery at Royal North Shore and North Shore Private Hospitals, Sydney, Australia, and Auckland City Hospital, New Zealand. Informed consent was obtained from all patients, and the study was performed with permission and in accordance with the local ethics committees' guidelines. The tumors were snap-frozen in liquid nitrogen at the time of surgery and stored at -80°C until used.

Tumors were classified according to functional subtype based on clinical and hormonal evaluation. Additional information was provided by histological assessment and immunohistochemical staining for all anterior pituitary hormones. All tumors were sporadic, and there were no carcinomas.

Microarray studies were performed on a core group of 20 pituitary tumors and three normal controls. The qPCR was performed on an expanded group of 42 patients that included all 20 tumors used for the microarray with an additional 22 tumors and a total of five normal controls. All samples used for qPCR also had methylation assessment of the *WIF1* promoter except one tumor in which there was insufficient DNA. Forty-one tumors were used for immunohistochemistry for *WIF1* with an additional 29 tumors included for β -catenin immunohistochem-

istry to obtain representative numbers for each tumor subtype. Twenty-seven of the samples from the qPCR were included in the immunohistochemistry and so had matched expression, methylation, and immunohistochemical data. Thirteen normal pituitary controls (comprising normal pituitary gland away from resected adenomas) were included in these immunohistochemical studies. The patient clinical characteristics are shown in Table 1.

RNA extraction and preparation for microarray and qPCR

Total RNA was extracted from fresh-frozen pituitary tumors (15–50 mg) using TRIzol reagent according to the manufacturer's protocol (Invitrogen, San Diego, CA). RNA was further purified by precipitation with 2.5 mol/liter lithium chloride according to the manufacturer's protocol (Ambion, Inc., Austin, TX) and stored at -80°C until used. RNA concentration and purity was measured by UV absorbance at 260/280nm (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, DE) and quality assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA). Four normal pituitary RNA samples were purchased commercially (Ambion), and an additional normal pituitary RNA sample was obtained from a consented patient from our institution within 2 h of death. Three of the commercial RNA controls were included in the microarray study. All four commercial RNA controls plus the postmortem RNA sample were included in the qPCR analysis.

Tumor subtype confirmation and exclusion of normal pituitary contamination

RT-PCR for pituitary transcription factor-1 (*Pit-1*) and proopiomelanocortin (*POMC*) was performed on all tumors and controls included in microarray and qPCR analyses, both to confirm subtype and to exclude significant normal anterior pituitary contamination as previously described (24). The primer sequences used for *POMC* were 5'-AGGACCT-CACCACGGAAAG-3' (forward) and 5'-CATGGAGTAGGAGCGCTTG-3' (reverse). The primers for *Pit-1* were 5'-GGAAACCAGCCATCAACCTA-3' (forward) and 5'-ACTTTTCCGCCTGAGTTCCT-3' (reverse).

Microarray

RNA labeling, hybridization to the Affymetrix GeneChip Human Genome U133 plus 2.0 Arrays and scanning was performed by the Clive and Vera Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia).

Preprocessing of the CEL file and normalization were performed using Robust Multichip Average (RMA) using R statistical software version 2.3.1 libraries contained in Bioconductor (<http://www.bioconductor.org/>). The AffyGUI analysis suite (Bioconductor), which uses a moderated *t* statistic (25), was applied to the normalized data set using the Benjamini-Hochberg correction, and differentially expressed genes were identified using an *M*-value of less than or equal to -1 or greater than or equal to 1 (log 2-fold change) and a *B*-statistic (log-odds expression) of greater than or equal to 2. Four separate analyses were performed, all tumors *vs.* normal pituitary, NFA *vs.* normal, GH-secreting (GH-S) *vs.* normal and ACTH-secreting (ACTH-S) *vs.* normal.

TABLE 1. Patient clinical characteristics

	Microarray, n = 20	qPCR/methylation, n = 42	WIF1 IPx, n = 41	Normal RNA controls, n = 5	Normal DNA controls, n = 6
Median age, yr (range)	56 (32–87)	58 (32–87)	57 (20–87)	78 (19–88)	63 (19–86)
Gender (n)					
Male	14	27	24	2	4
Female	6	15	17	3	2
% Macroadenomas	85	90	88 ^a	NA	NA
NFA	13	31	28		
GH-S	5	7	6		
ACTH-S	2	3	7		
TSH-S		1			

IPx, Immunohistochemistry, NA, not applicable.

^a Tumor size information unavailable for two patients.

qPCR

Total RNA from each tumor and normal tissue was reverse-transcribed into cDNA using random hexamers and the Superscript III first-strand synthesis system (Invitrogen). qPCR was performed on a Corbett Rotor-Gene 3000 (Corbett Research, Mortlake, New South Wales, Australia) using gene-specific TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Each PCR was performed as a duplex reaction with the gene-specific FAM-TAMRA-labeled TaqMan probe and a VIC-labeled TaqMan probe and ribosomal 18S RNA as the internal control for normalization (Applied Biosystems). All samples were run in triplicate and repeated a minimum of two times. Analysis of the qPCR results was performed using the relative expression software tool (REST 2005) (26) with relative expression ratios calculated based on the PCR efficiency and crossing points.

DNA extraction

DNA was extracted from fresh-frozen pituitary tumor tissue (15–50 mg) and one normal pituitary gland using TRIzol reagent according to the manufacturer's protocol (Invitrogen). DNA concentration and purity was measured by UV absorbance at 260/280nm (Nanodrop ND-1000; Biolab). Five additional normal pituitaries were extracted to use as comparison with the tumors in methylation studies.

Bisulfite treatment and genomic methylation sequencing

Bisulfite treatment of DNA was performed using the Methyl Easy DNA Bisulfite Modification Kit according to the manufacturer's protocol (Human Genetic Signatures Pty. Ltd., Sydney, Australia). Briefly, 1 µg DNA was treated with 3 M NaOH, followed by overnight bisulfite treatment (15 h). Two overlapping PCR were performed to amplify a 661-bp area (–543 to +118, relative to the translation start site) of the *WIF1* promoter containing 64 CpG dinucleotides. The primers used were as previously reported: forward 5'-TAGGGGTTTTGAGTGTTC-3' and reverse 5'-ACCTAAATACCAAAAAACCTAC-3' (5); forward 5'-GTAGGTTTTTGGTATTTAGG-3' and reverse 5'-TCCATAAATA-CAAATCTCCTC-3' (14). CpGenome universal methylated and unmethylated controls were included with each reaction (Chemicon International Inc., Temecula, CA). Sequencing was performed by Sydney University Prince Alfred Macromolecular Analysis Centre (SUPAMAC) using the ABI PRISM 3700 platform (Applied Biosystems).

Immunohistochemistry

Immunohistochemistry for β -catenin (n = 70), *WIF1* (n = 41), and cyclin D1 (n = 26) was performed on formalin-fixed paraffin-embedded tissue using a mouse monoclonal antibody for β -catenin (17C2; Novocastra, Newcastle-upon-Tyne, UK) and *WIF1* (133015; R&D Systems, Inc., Minneapolis, MN) and a rabbit monoclonal antibody for cyclin D1 (Clone SP4, catalog item RM-9104-S; Neomarkers Inc., Fremont CA). Thirteen normal pituitary controls were included in each analysis. Representative blocks from each tumor were sectioned at 4 µm onto positively charged slides (SuperFrost Plus; Menzel-Glaser, Braunschweig, Germany). Slides were then stained using the Vision Biosystems Bond-Max Autostainer (Vision Biosystems, Mount Waverley, Victoria, Australia) according to the manufacturer's protocol. Briefly, slides were dewaxed in Bond Dewax solution (AR9222; Vision Biosystems) and hydrated in Bond Wash solution (AR9590; Vision Biosystems). Antigen retrieval was performed at an alkaline pH using Epitope Retrieval 2 solution (AR9640; Vision Biosystems) for 20 min at 100 C. Slides were then incubated with the primary antibody at a concentration of 1:100 (β -catenin), 1:50 (*WIF1*), or 1:25 (cyclin D1) for 30 min at room temperature. Antibody detection was performed using the biotin-free Bond Polymer Defined Detection System (DS9713; Vision Biosystems). Slides were counterstained with hematoxylin. External positive and negative controls were examined with each batch of stains.

Staining for β -catenin was evaluated as previously outlined (18). Briefly, immunostaining results were scored as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong) for each of the locations: cytoplasmic membrane (CM), cytoplasm (C), and nuclear (N). For *WIF1*, cytoplasmic staining was evaluated based on the scoring system above. For cyclin D1,

a score of 0 was assigned to tumors that were completely negative, 1+ indicated occasional positive cells (less than 5% of total tumor cells), 2+ indicated moderate staining of tumor (5–30% of tumor cells positive), and a score of 3+ indicated diffuse strong positive staining (defined as more than 30% of tumor cells positive, but usually all tumor cells showing diffuse positive staining). For the purposes of binary analysis, scores of 0 and 1+ can be considered negative and scores of 2+ and 3+ positive. The pituitaries were evaluated by a single observer (A.J.G.) in conjunction with a hematoxylin- and eosin-stained slide. The observer was blinded as to other information.

In vitro studies

The rat pituitary GH3 cell line was a generous gift from C. Chen (Prince Henry's Institute of Medical Research, Melbourne, Australia). Cells were grown in 45% DMEM, 45% Ham's F12, with 10% fetal bovine serum and cultured at 37 C with 5% CO₂.

RNA was extracted from cells to assess endogenous *WIF1* levels using the Trizol method as above.

Whole-cell lysates were obtained by harvesting the cell cultures and lysing in SDS protein lysis buffer and denatured (95 C, 10 min) before electrophoresis on 8% SDS-polyacrylamide gels. For Western blot analysis, separated proteins were transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ) and probed with *WIF1* (133015; R&D Systems) at 1:1600 and α -tubulin (DM1A; Sigma Chemical Co., St. Louis, MO) at 1:10,000. The Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) were used for protein detection.

Full-length human *WIF1* cDNA was cloned into pcDNA3 and pcDNA4 T/O. Cells were transfected with either *WIF1*-containing pcDNA3 or pcDNA3 empty vector (EV) using Effectene Transfection Reagent according to the manufacturer's instructions (QIAGEN, Valencia, CA).

Cell proliferation studies were performed by plating 1 × 10⁵ cells per well in a 96-well plate 24 h before transfection, and proliferation was assessed using CellTiter 96 Aqueous MTS Reagent (Promega, Madison, WI). Experiments were performed in at least triplicate and repeated three times.

Colony formation assay was performed by plating EV- or *WIF1*-transfected cells in soft agar at 5,000 and 10,000 cells per well in six-well plates, using G418 400 µg/ml to select transfected cells. Plates were stained with 0.5% crystal violet 3 wk after transfection and colonies counted.

Statistics

Generalized linear modeling using the binomial distribution with probit link was used to assess the relationship between methylation status and tumor type, and methylation status *vs.* mRNA expression. ANOVA followed by post-estimation matrix testing was used to assess the significance of mRNA expression for the tumors compared with normal pituitary. Logistic regression was used to assess immunohistochemistry results (absent/weak *vs.* moderate/strong staining). A *P* value below 0.05 was considered significant.

Results

Subtype confirmation and exclusion of normal pituitary contamination

All pituitary tumors and normal controls used for the microarray and qPCR were screened using RT-PCR for *Pit-1* and *POMC*. The normal pituitary controls showed positive bands on RT-PCR for *Pit-1* and *POMC*. In all of the functioning tumors, *Pit-1* and *POMC* correctly identified the tumor subtype, *i.e.* ACTH-S tumors (n = 3) tested positive for *POMC* and negative for *Pit-1*, whereas the GH-S (n = 7) and TSH-S (n = 1) tumors were positive for *Pit-1* and negative for *POMC*. Three NFAs showed a positive band for *Pit-1* consistent with possible normal pituitary tissue contamination and were excluded from the study.

Microarray

A total of 1253 genes were differentially expressed in all pituitary tumors when compared with the normal controls (supplemental Tables 1–4, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). After separating the tumors into the clinical subtypes (NFAs, GH-S, and ACTH-S), the NFAs showed more differentially expressed genes compared with the functional tumors (876 *vs.* 377, respectively) consistent with a previous study (27).

One of the most differentially expressed genes (both in terms of fold change and significance) in the pituitary tumors compared with normal pituitary was *WIF1* (Table 2). This gene was consistently down-regulated across all three tumor subtypes: NFAs, GH-S, and ACTH-S (97-, 91-, and 91-fold, respectively). Because *WIF1* is a member of the sFRP family of Wnt inhibitors, we reviewed the list of differentially expressed genes to see whether the other five sFRPs were differentially expressed. Three other members of the sFRP family, *sFRP2*, *FRZB*, and *sFRP4*, were also significantly down-regulated when comparing the total group of pituitary tumors to the normal controls, and *sFRP1* was also differentially expressed (increased 2.5-fold), although this was not significant (B-statistic, -6) (Table 2).

qPCR

Validation of the microarray results was performed using qPCR. Consistent with the microarray results, *WIF1* mRNA expression was significantly down-regulated in pituitary tumors (n = 42) compared with normal controls (n = 5) ($P < 0.001$) (Fig. 1). After separating the tumors into their clinical subtypes, *WIF1* mRNA expression was significantly under-expressed in all groups when compared with the normal pituitary controls (down 3468-fold in the NFAs, $P = 0.001$; down 867-fold in the GH-S, $P = 0.011$; down 1734-fold in the ACTH-S, $P = 0.034$). Both *sFRP2* and *sFRP4* were also significantly reduced in pituitary tumors when compared with normal controls ($P = 0.002$ and 0.013 , respectively) (Fig. 1). The mRNA expression of *sFRP2* was significantly under-expressed in the NFAs (down 339-fold, $P = 0.001$) and ACTH-S (down 47-fold, $P = 0.034$) but did not reach statistical significance in the GH-S tumors (down 268-fold, $P = 0.059$). *sFRP4* mRNA expression was significantly down-regulated in the NFAs (down 13-fold, $P = 0.004$), GH-S (down 9-fold, $P = 0.008$), and ACTH-S (down 59-fold, $P = 0.034$). *FRZB* mRNA expression was down 4-fold compared with the con-

TABLE 2. Microarray fold change of *WIF1* and *sFRP1-5* in pituitary tumors compared with normal pituitary controls

Gene	M-value ^a	B-statistic ^b
<i>WIF1</i>	-6.5	35
<i>sFRP1</i>	1.3	-6
<i>sFRP2</i>	-4.3	7
<i>FRZB</i>	-2.9	2
<i>sFRP4</i>	-3	6
<i>sFRP5</i>	0.2	-5

The expression values shown in *bold* meet our predefined cutoff ($M \leq -1$ or > 1 and $B \geq 2$).

^a Gene expression result in a log₂ scale.

^b Log-odds expression that the gene is differentially expressed.

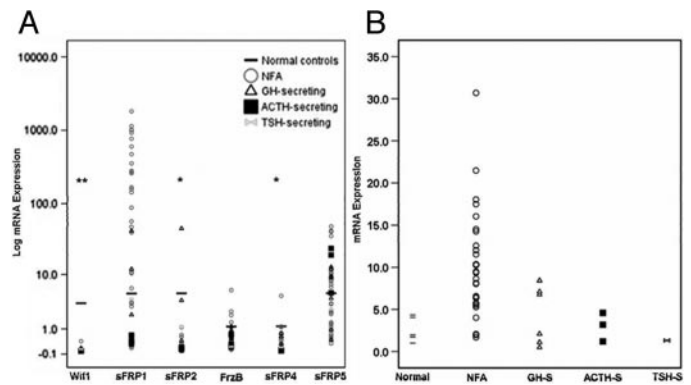


FIG. 1. qPCR mRNA expression values. A, Results for *WIF1* and *sFRPs1-5*. Log mRNA expression is shown for each gene normalized to the endogenous control ribosomal 18S and relative to a common reference sample. The median of the normal controls for each gene set is represented as a horizontal line. *, $P < 0.05$; **, $P < 0.001$. B, Results for cyclin D1. mRNA expression is normalized to the endogenous control ribosomal 18S and relative to a common reference sample.

trois; however, this decrease did not reach statistical significance ($P = 0.073$). Because *sFRP1* has previously been reported to be elevated in NFAs (23), qPCR for *sFRP1* was also performed. *sFRP1* mRNA expression showed marked variation in the NFAs (Fig. 1A) with a subset demonstrating elevated mRNA expression compared with the controls. However, when analyzed as a group, *sFRP1* mRNA expression in the NFAs did not significantly differ from the controls ($P = 0.230$) but was significantly higher when compared with the other tumors ($P = 0.006$). The mRNA expression levels of the remaining sFRP family member, *sFRP5*, was also assessed; however, this gene was not significantly different in the tumors compared with the controls ($P = 0.707$) (Fig. 1A). The downstream Wnt target gene cyclin D1 was also assessed by qPCR. The mRNA expression levels were significantly elevated in all pituitary tumors (n = 42) compared with normal pituitary controls (n = 5) ($P = 0.0112$). However, when the tumors are categorized into functioning and non-functioning subtypes, only the NFAs significantly differed from normal pituitary controls ($P = 0.0022$). No significant differences were measured for the clinically functioning subtypes when compared with the normal pituitary controls ($P = 0.29$ and $P = 0.65$ for the GH-S and ACTH-S adenomas, respectively) (Fig. 1B).

Methylation

The significantly reduced expression of *WIF1* in all pituitary tumors was the most striking result from both the microarray and qPCR. *WIF1* has a CpG island in the promoter and has previously been reported to be methylated in a number of human malignancies. Therefore, this gene was selected for promoter methylation sequencing. *WIF1* bisulfite sequencing was performed on 47 samples (41 tumors and six normal pituitary controls) analyzing a 661-bp area of the promoter. With the exception of one control (two of 61 CpG dinucleotides methylated), the normal pituitary controls did not display methylation (Fig. 2). Methylation was present in 36 of 41 tumors (88%), significantly higher than the controls ($P = 0.001$). The 31 NFAs showed a higher percentage of

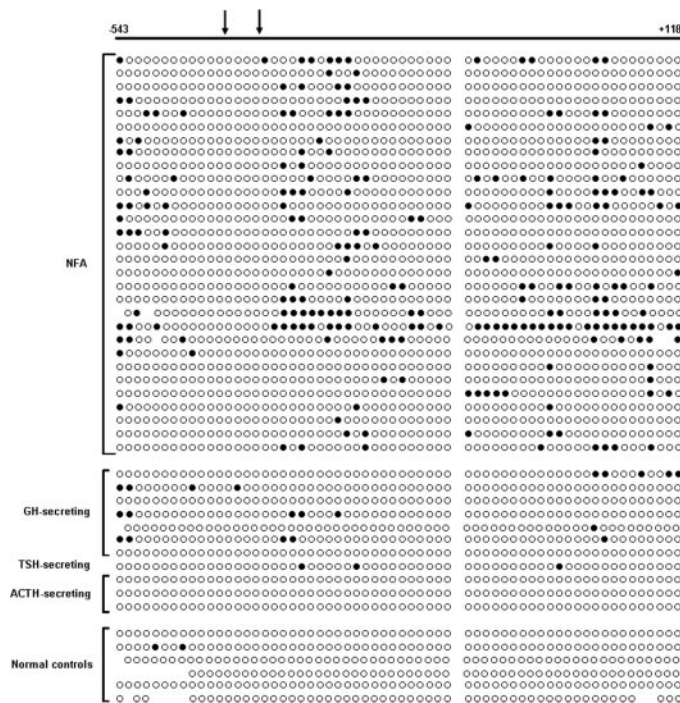


FIG. 2. Methylation status of the 661-bp region of the *WIF1* promoter sequenced. Each line represents a sample, and each circle represents a CpG dinucleotide. Open circles are unmethylated and filled circles methylated CpG dinucleotides. The tumors are grouped according to clinical subtype. The two putative Sp1 binding sites within the hypomethylated region between -438 and -380 are marked by arrows.

methylation compared with the 10 functioning tumors ($P = 0.002$). Five tumors had no methylation (all three ACTH-S and two of the seven GH-S). Although heterogeneous methylation was typically observed in the tumors, an extensive area between -438 and -380 bp, relative to the translational start site, was consistently unmethylated (Fig. 2).

Immunohistochemistry

WIF1 (Table 3). Normal pituitary tissue ($n = 13$) demonstrated moderate (2+) to strong (3+) cytoplasmic staining for *WIF1* in 92% of samples. In many of the positively staining cells, relatively prominent nuclear membrane staining for *WIF1* was also noted (Fig. 3A). When compared with the normal pituitary *WIF1*, cytoplasmic staining was significantly reduced in 31 of 41 (76%) pituitary tumors ($P < 0.001$) (Fig. 3B). Of the 10 pituitary tumors that did not show loss of *WIF1* staining, seven were functional [five GH-S (Fig. 3C) and two ACTH-S].

TABLE 3. Cytoplasmic *WIF1* immunohistochemical staining in pituitary tumors

WIF1 score	Normal pituitary, n = 13	NFA, n = 28	GH-S, n = 6	ACTH-S, n = 7
0	0	18	0	29
1	8	71	17	42
2	31	11	17	29
3	61	0	66	0

Results shown as percentages.

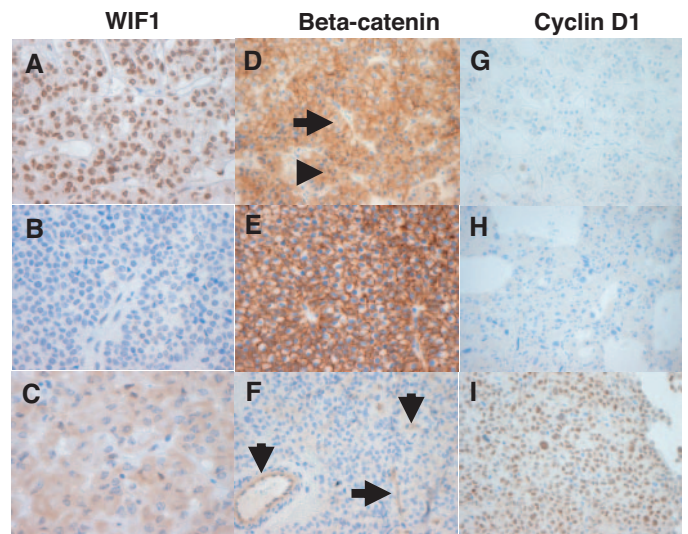


FIG. 3. Photomicrographs showing representative immunohistochemical staining of *WIF1* (A–C), β -catenin (D–F), and cyclin D1 (G–I). For all images, original magnification is $\times 400$; hematoxylin counterstained. A, Representative immunostaining of *WIF1* in normal pituitary. There is diffuse cytoplasmic staining. In this area, nuclear membrane accentuation is particularly prominent. B, Typical immunostaining of *WIF1* in NFAs. *WIF1* staining is markedly decreased compared with normal pituitary. C, Typical immunostaining of *WIF1* in GH-S adenomas. Cytoplasmic staining is preserved. D, Typical immunostaining of β -catenin in normal pituitary. Note the heterogeneity of staining patterns. Some cells display intense membrane staining and little cytoplasmic staining (arrow), whereas others show predominantly cytoplasmic staining with little membrane staining (arrowhead). Many granular eosinophilic cells displayed negative membrane staining with 1+ to 2+ cytoplasmic staining. Nongranular eosinophilic cells and most basophilic cells showed 2+ to 3+ membrane staining with less cytoplasmic staining. E, A NFA positive for β -catenin. Strong cytoplasmic membrane staining is present with no significant cytoplasmic staining. F, A NFA completely negative for β -catenin. Positive staining of the endothelial cells (arrows) provides an internal positive control. G, Representative immunostaining of cyclin D1 in normal pituitary. Most nuclei are negative. H, A GH-S adenoma with absent cyclin D1 staining. I, A typical NFA positive for cyclin D1. Most nuclei stain positive.

β -Catenin. In the normal pituitary gland, β -catenin displayed a predominantly cytoplasmic membrane pattern of staining with less but definite cytoplasmic accumulation. There was some heterogeneity of staining in the normal pituitary gland (Fig. 3D). No nuclear accumulation of β -catenin was present in any tumor or normal tissue. Lower values of *WIF1* and *sFRP5* mRNA expression were associated with higher levels of cytoplasmic β -catenin staining, but these did not reach statistical significance ($P = 0.058$ and $P = 0.053$, respectively). Cytoplasmic staining did not vary according to tumor type ($P = 0.704$) (Fig. 3, D–F). Cytoplasmic membrane staining did not correlate with the mRNA expression of *WIF1* and *sFRPs* in the tumors. The NFAs demonstrated higher cell membrane β -catenin staining than the other tumor types ($P = 0.036$) (Fig. 3E).

Cyclin D1. Normal pituitary tissue demonstrated weak or absent nuclear staining for cyclin D1. In contrast, in 73% ($n = 26$) of pituitary tumors, moderate (2+) to strong (3+) staining was observed. Similar to the findings for the mRNA expression, the NFAs showed increased staining compared with the

functioning tumors ($P = 0.01$) (Fig. 3, H–I). Immunohistochemistry strongly correlated with qPCR results ($P = 0.002$).

In vitro studies

GH3 WIF1 expression. The rat pituitary GH3 cell line was previously untested for endogenous levels of *WIF1*. To assess its suitability as a functional model, *WIF1* mRNA levels were measured using qPCR. *WIF1* expression was significantly reduced (22-fold) when compared with normal human pituitary ($P < 0.001$).

Cell proliferation studies. Because we found that *WIF1* was underexpressed in the GH3 pituitary cell line, we transfected *WIF1* into these cells to determine whether loss of expression of *WIF1* contributes to pituitary tumor cell proliferation. Three days after transfection, we found significantly reduced cell proliferation in the cells transfected with *WIF1* compared with the cells transfected with the EV using MTS assay ($P = 0.015$) (Fig. 4A). Reduced colony formation was also seen in *WIF1*-transfected cells compared with EV ($P = 0.039$) at 3 wk after transfection (Fig. 4B).

Discussion

The Wnt pathways have been implicated in the pathogenesis of a number of human tumors but to date have received limited attention in pituitary tumors. Using microarray analysis to compare pituitary tumors with normal pituitary gland, we have demonstrated that *WIF1* mRNA expression is markedly underexpressed in all pituitary tumor subtypes, and we confirmed that *WIF1* protein expression was reduced in 76% of tumors using immunohistochemistry. In addition, we also found significantly reduced mRNA expression of two other Wnt pathway inhibitors, *sFRP2* and *sFRP4*.

Reduced mRNA expression of *WIF1* is common to all four subtypes examined (NFA, GH-S, ACTH-S, and TSH-S). The otherwise diverse biological behavior of these clinical subtypes of pituitary tumors suggests that reduced *WIF1* expression may be a common and early pathogenetic hallmark. Decreased expression of *WIF1* has been identified in 73% of colorectal adenomas (10) and therefore also appears to occur early in colorectal tumor development. Interestingly, the protein expression of *WIF1* was more variably affected with loss of expression predominantly seen in the NFAs despite similar levels of mRNA expression. The reason for this differential expression between subtypes is currently unclear.

By restoring *WIF1* levels in the rat pituitary GH3 cell line, we demonstrated reduced cell growth and inhibited colony formation. Our data support previous work that identifies *WIF1* as a potential tumor suppressor gene (7, 28). In other studies, by restoring *WIF1* expression to esophageal and colorectal cancer cell lines, a reduction in growth rate and colony numbers was also observed (10) and apoptosis was increased (13). Overexpression of the sFRPs has been demonstrated to result in decreased colony formation and increased rates of apoptosis in colorectal cell lines (12), and there is increasing evidence for the role of these genes as tumor suppressors in other tissues (29–32). However, although most studies have supported the role of the sFRP family as having tumor suppressor effects, a few studies

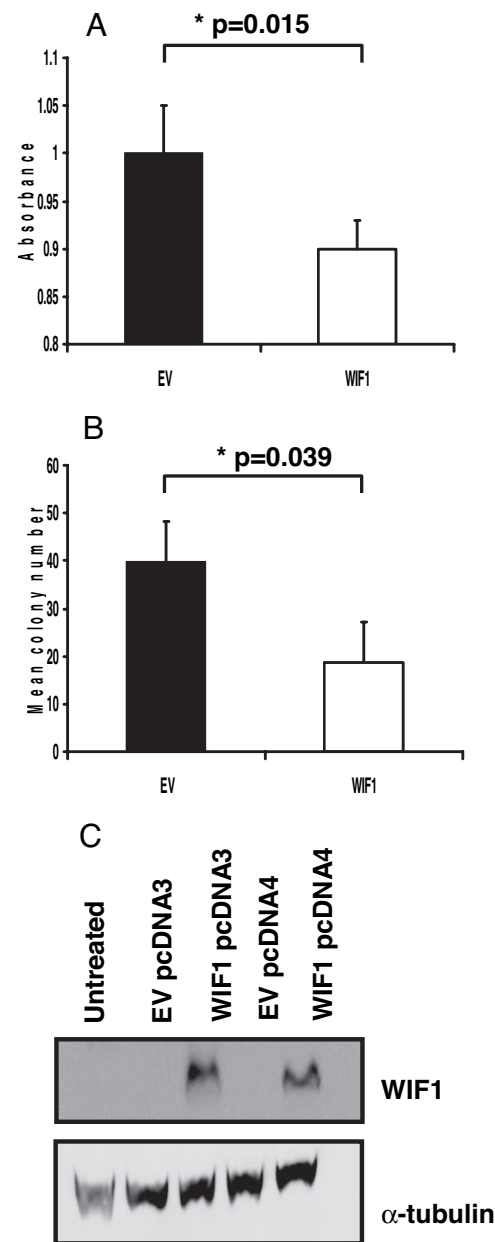


FIG. 4. Cell proliferation studies. A, Day 3 of MTS assay for EV and *WIF1*, 50 ng per well. Data are shown as the mean of the three experiments plus SE. Absorbance (y-axis) was measured at 492 nm ($OD_{492-650}$). B, Three-week colony formation assay in soft agar. Data are representative of triplicate experiments. C, Western blot analysis showing *WIF1* protein expression in untreated and EV controls compared with *WIF1*-transfected GH3 cells.

suggest that in certain tissues and contexts they may have the opposite effect, *i.e.* growth promotion (reviewed in Ref. 33), which necessitates further investigation into these factors in more tissue types and pathological conditions.

Approximately half of all human genes contain a CpG island in their promoters (34), and methylation of these CpG dinucleotides can lead to gene silencing (35). *WIF1* contains a CpG island within its promoter (14), and low expression due to promoter methylation has been demonstrated in a number of human tumors (5, 6, 9, 10, 14, 15). In addition,

treatment of tumor cell lines with demethylating agents has been shown to restore expression of *WIF1*, suggesting that promoter methylation is the likely mechanism of the reduced gene expression (6, 10, 11, 14). Our data show heterogeneous methylation of the *WIF1* promoter in 88% of tumors examined. We identified a hypomethylated region in our study, –438 to –380, which contains two putative Sp1 binding sites (GGGCGG) on sequence analysis. Sp1 binding sites have previously been demonstrated to be more resistant to methylation (36), and this hypomethylated region is consistent with the findings of Ai *et al.* (6). The clinically functioning tumors had significantly lower rates of methylation than the NFAs, and five clinically functioning tumors (including the three ACTH-S) showed no *WIF1* promoter methylation. Increased methylation was not associated with reduced *WIF1* mRNA expression levels. Our data suggest that methylation may contribute to the reduced *WIF1* expression in NFAs but that there may be other mechanisms involved in the functioning pituitary tumors.

β -Catenin is the central mediator of the canonical Wnt signaling pathway, and reports on the presence of abnormal nuclear β -catenin accumulation in pituitary tumors have been conflicting (16–20). In this study, we observed no abnormal nuclear β -catenin accumulation in 70 tumors, which is in agreement with the reports suggesting that nuclear accumulation of β -catenin is uncommon in pituitary tumors (16, 18–20). Furthermore, we have shown heterogeneous β -catenin staining within normal pituitary, which has not been previously reported. In the 27 tumors with combined mRNA expression data for *WIF1* and *sFRP1–5* and immunohistochemistry for β -catenin available, association between cytoplasmic β -catenin staining and *WIF1* and *sFRP5* mRNA expression levels was of borderline significance. One possible explanation for the lack of nuclear β -catenin and only weak cytoplasmic staining in the NFAs is the elevation in *sFRP1* seen in a subset of these tumors because *sFRP1* has been reported to be a target of the hedgehog pathway, which can inhibit Wnt signaling (37, 38). However, even in tumors with reduced expression of all six inhibitors compared with normal pituitary, there was no nuclear β -catenin staining. We have not assessed the expression of the DKK family of Wnt inhibitors in pituitary tumors using qPCR. From our microarray data, only *DKK2* was differentially expressed, and this was down-regulated 2.6-fold, suggesting that these family members are unlikely to be contributing to the lack of nuclear β -catenin accumulation. However, we have demonstrated increased expression of the Wnt target gene, cyclin D1, at both the mRNA and protein levels in the NFAs, which is supportive of activation of the canonical Wnt pathway in this pituitary tumor subtype. Larger numbers of functioning tumors are needed to elucidate whether a different pathway is operational in the functioning subtypes. Further work into this pathway in pituitary tumorigenesis is required.

In conclusion, pituitary tumors demonstrate down-regulation of *WIF1* mRNA, which is common to all pituitary tumor subtypes with a reduction in WIF1 protein levels predominantly limited to the NFAs. In addition, the other sFRP family members are affected in a more variable manner, suggesting that the Wnt pathways are important in pituitary tumorigenesis. Our *in vitro* studies show that WIF1 acts to

slow pituitary cell growth, and these data are consistent with our clinical data in that loss of WIF1 is associated with pituitary tumorigenesis. Our data suggest that *WIF1* mRNA down-regulation in pituitary tumors is an early event because it is reduced in all tumor subtypes and that later genetic events may explain the differences in tumor behavior seen in the different pituitary tumor subtypes.

Acknowledgments

We are grateful to Wendy Shaw for her time and effort with the collection of the New Zealand pituitary tumors.

Received May 2, 2007. Accepted December 4, 2007.

Address all correspondence and requests for reprints to: Marianne S. Elston, M.B. Ch.B., Cancer Genetics Unit, Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards, New South Wales 2065, Australia. E-mail: marianne@med.usyd.edu.au.

This work was supported by a project grant-in-aid from the Waikato Medical Research Foundation. M.S.E. was supported by a National Health and Medical Research Council medical postgraduate research scholarship and New South Wales Cancer Institute Research Scholars Award.

Disclosure Statement: All authors have nothing to declare.

References

- Asa SL, Ezzat S 1998 The cytogenesis and pathogenesis of pituitary adenomas. *Endocr Rev* 19:798–827
- Segditsas S, Tomlinson I 2006 Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 25:7531–7537
- Barker N, Clevers H 2006 Mining the Wnt pathway for cancer therapeutics. *Nat Rev Drug Discov* 5:997–1014
- Byun T, Karimi M, Marsh JL, Milovanovic T, Lin F, Holcombe RF 2005 Expression of secreted Wnt antagonists in gastrointestinal tissues: potential role in stem cell homeostasis. *J Clin Pathol* 58:515–519
- Urakami S, Shiina H, Enokida H, Kawakami T, Tokizane T, Ogishima T, Tanaka Y, Li LC, Ribeiro-Filho LA, Terashima M, Kikuno N, Adachi H, Yoneda T, Kishi H, Shigeno K, Konety BR, Igawa M, Dahiya R 2006 Epigenetic inactivation of Wnt inhibitory factor-1 plays an important role in bladder cancer through aberrant canonical Wnt/ β -catenin signaling pathway. *Clin Cancer Res* 12:383–391
- Ai L, Tao Q, Zhong S, Fields CR, Kim WJ, Lee MW, Cui Y, Brown KD, Robertson KD 2006 Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis* 27:1341–1348
- Wissmann C, Wild PJ, Kaiser S, Roepcke S, Stoehr R, Woenckhaus M, Kristiansen G, Hsieh JC, Hofstaedter F, Hartmann A, Knuechel R, Rosenthal A, Pilarsky C 2003 WIF1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer. *J Pathol* 201:204–212
- Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weisenberg MP, Herman JG, Baylin SB 2002 A genomic screen for genes up-regulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 31:141–149
- Chim CS, Chan WW, Pang A, Kwong YL 2006 Preferential methylation of Wnt inhibitory factor-1 in acute promyelocytic leukemia: an independent poor prognostic factor. *Leukemia* 20:907–909
- Taniguchi H, Yamamoto H, Hirata T, Miyamoto N, Oki M, Noshio K, Adachi Y, Endo T, Imai K, Shinomura Y 2005 Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* 24:7946–7952
- Lin YC, You L, Xu Z, He B, Mikami I, Thung E, Chou J, Kuchenbecker K, Kim J, Raz D, Yang CT, Chen JK, Jablons DM 2006 Wnt signaling activation and WIF-1 silencing in nasopharyngeal cancer cell lines. *Biochem Biophys Res Commun* 341:635–640
- Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB 2004 Epigenetic inactivation of sFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 36:417–422
- He B, Reguart N, You L, Mazieres J, Xu Z, Lee AY, Mikami I, McCormick F, Jablons DM 2005 Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations. *Oncogene* 24:3054–3058
- Mazieres J, He B, You L, Xu Z, Lee AY, Mikami I, Reguart N, Rosell R, McCormick F, Jablons DM 2004 Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. *Cancer Res* 64:4717–4720
- Batra S, Shi Y, Kuchenbecker KM, He B, Reguart N, Mikami I, You L, Xu Z,

- Lin YC, Clement G, Jablons DM 2006 Wnt inhibitory factor-1, a Wnt antagonist, is silenced by promoter hypermethylation in malignant pleural mesothelioma. *Biochem Biophys Res Commun* 342:1228–1232
16. Buslei R, Nolde M, Hofmann B, Meissner S, Eyupoglu IY, Siebzehnrubl F, Hahnen E, Kreutzer J, Fahlbusch R 2005 Common mutations of β -catenin in adamantinomatous craniopharyngiomas but not in other tumours originating from the sellar region. *Acta Neuropathol (Berl)* 109:589–597
 17. Semba S, Han SY, Ikeda H, Horii A 2001 Frequent nuclear accumulation of β -catenin in pituitary adenoma. *Cancer* 91:42–48
 18. Tziortzioti V, Ruebel KH, Kuroki T, Jin L, Scheithauer BW, Lloyd RV 2001 Analysis of β -catenin mutations and α -, β -, and γ -catenin expression in normal and neoplastic human pituitary tissues. *Endocr Pathol* 12:125–136
 19. Qian ZR, Li CC, Yamasaki H, Mizusawa N, Yoshimoto K, Yamada S, Tashiro T, Horiguchi H, Wakatsuki S, Hirokawa M, Sano T 2002 Role of E-cadherin, α -, β -, and γ -catenins, and p120 (cell adhesion molecules) in prolactinoma behavior. *Mod Pathol* 15:1357–1365
 20. Xu B, Sano T, Yoshimoto K, Yamada S 2002 Downregulation of E-cadherin and its undercoat proteins in pituitary growth hormone cell adenomas with prominent fibrous bodies. *Endocr Pathol* 13:341–351
 21. Oikonomou E, Barreto DC, Soares B, De Marco L, Buchfelder M, Adams EF 2005 β -Catenin mutations in craniopharyngiomas and pituitary adenomas. *J Neurooncol* 73:205–209
 22. Sun C, Yamato T, Kondo E, Furukawa T, Ikeda H, Horii A 2005 Infrequent mutation of APC, AXIN1, and GSK3B in human pituitary adenomas with abnormal accumulation of CTNBN1. *J Neurooncol* 73:131–134
 23. Moreno CS, Evans CO, Zhan X, Okor M, Desiderio DM, Oyesiku NM 2005 Novel molecular signaling and classification of human clinically nonfunctional pituitary adenomas identified by gene expression profiling and proteomic analyses. *Cancer Res* 65:10214–10222
 24. Morris DG, Musat M, Czirjak S, Hanzely Z, Lillington DM, Korbonits M, Grossman AB 2005 Differential gene expression in pituitary adenomas by oligonucleotide array analysis. *Eur J Endocrinol* 153:143–151
 25. Smyth GK 2004 Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3:Article3
 26. Pfaffl MW, Horgan GW, Dempfle L 2002 Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30:e36
 27. Altenberger T, Bilban M, Auer M, Knosp E, Wolfsberger S, Gartner W, Mineva I, Zielinski C, Wagner L, Luger A 2006 Identification of DLK1 variants in pituitary- and neuroendocrine tumors. *Biochem Biophys Res Commun* 340:995–1005
 28. Rubin JS, Barshishat-Kupper M, Feroze-Merzoug F, Xi ZF 2006 Secreted WNT antagonists as tumor suppressors: pro and con. *Front Biosci* 11:2093–2105
 29. Gumz ML, Zou H, Kreinest PA, Childs AC, Belmonte LS, LeGrand SN, Wu KJ, Luxon BA, Sinha M, Parker AS, Sun LZ, Ahlquist DA, Wood CG, Copland JA 2007 Secreted frizzled-related protein 1 loss contributes to tumor phenotype of clear cell renal cell carcinoma. *Clin Cancer Res* 13:4740–4749
 30. Horvath LG, Lelliott JE, Kench JG, Lee CS, Williams ED, Saunders DN, Grygiel JJ, Sutherland RL, Henshall SM 2007 Secreted frizzled-related protein 4 inhibits proliferation and metastatic potential in prostate cancer. *Prostate* 67:1081–1090
 31. Zi X, Guo Y, Simoneau AR, Hope C, Xie J, Holcombe RF, Hoang BH 2005 Expression of Frzb/secreted Frizzled-related protein 3, a secreted Wnt antagonist, in human androgen-independent prostate cancer PC-3 cells suppresses tumor growth and cellular invasiveness. *Cancer Res* 65:9762–9770
 32. Lee AY, He B, You L, Dadfarmay S, Xu Z, Mazieres J, Mikami I, McCormick F, Jablons DM 2004 Expression of the secreted frizzled-related protein gene family is downregulated in human mesothelioma. *Oncogene* 23:6672–6676
 33. Shi Y, He B, You L, Jablons DM 2007 Roles of secreted frizzled-related proteins in cancer. *Acta Pharmacol Sinica* 28:1499–1504
 34. Bird AP 1986 CpG-rich islands and the function of DNA methylation. *Nature* 321:209–213
 35. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarr JR, Linehan WM, Baylin SB 1994 Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci USA* 91:9700–9704
 36. Macleod D, Charlton J, Mullins J, Bird AP 1994 Sp1 sites in the mouse *aprt* gene promoter are required to prevent methylation of the CpG island. *Genes Dev* 8:2282–2292
 37. He J, Sheng T, Stelzer AA, Li C, Zhang X, Sinha M, Luxon BA, Xie J 2006 Suppressing Wnt signaling by the hedgehog pathway through sFRP-1. *J Biol Chem* 281:35598–35602
 38. Katoh Y, Katoh M 2006 WNT antagonist, SFRP1, is Hedgehog signaling target. *Int J Mol Med* 17:171–175

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.