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Secreted WNT antagonists as tumor suppressors: pro and con

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1. ABSTRACT

Dysregulation of Wnt signaling is common in a variety of human malignancies. Activation of the canonical Wnt or beta-catenin pathway has been especially well documented in cancer, although other non-canonical Wnt signaling pathways also have been implicated in neoplasia. In most instances, constitutive signaling through the beta-catenin pathway involves activation of effector molecules or loss of tumor suppressor function downstream of Wnt binding to its cell surface receptors. Nonetheless, in recent years increasing evidence suggests that secreted Wnt antagonists act as tumor suppressors, with their expression often silenced by promoter hypermethylation. This implies that maximal constitutive signaling in cancer requires unimpaired Wnt stimulation at the cell surface as well as enhanced signal propagation within the cell. However, an understanding of the role secreted Wnt antagonists may play in cancer is complicated by the multiplicity of these proteins, their potential Wnt-independent activities and observations indicating that sometimes they may promote tumor growth. Just as the particular function of Wnt signaling in development and homeostasis varies with the setting, the impact of secreted Wnt antagonists on neoplasia depends on the molecular, cellular and tissue context.

2. WNT SIGNALING AND CANCER

Before considering the role of secreted Wnt antagonists as tumor suppressors, it is helpful to review the connection of Wnt signaling to cancer. In this section, we briefly describe the canonical Wnt or beta-catenin pathway, and review the evidence linking its aberrant activity to neoplasia. We also summarize a couple of non-canonical Wnt pathways that have been implicated in tumorigenesis.

In the absence of Wnt stimulation, soluble beta-catenin turns over rapidly by a mechanism that involves its binding to a scaffolding protein, Axin (or its homolog, Axin2), in a degradation complex that includes the adenomatosis polyposis coli (APC) protein, glycogen synthase kinase 3-beta (GSK3-beta) and casein kinase I-alpha (CKI-alpha). CKI-alpha phosphorylates beta-catenin at serine-45, priming the molecule for additional phosphorylation by GSK3-beta at threonine-41, serine-37 and serine-33 [reviewed in (1, 2)]. Phosphorylated beta-catenin binds to beta-transducin repeat containing protein (beta-TrCP), which facilitates its ubiquitylation and subsequent proteosomal degradation. Activation of the Wnt/beta-catenin pathway normally requires the binding of Wnt ligand to both a member of the Frizzled (FZD), seven-

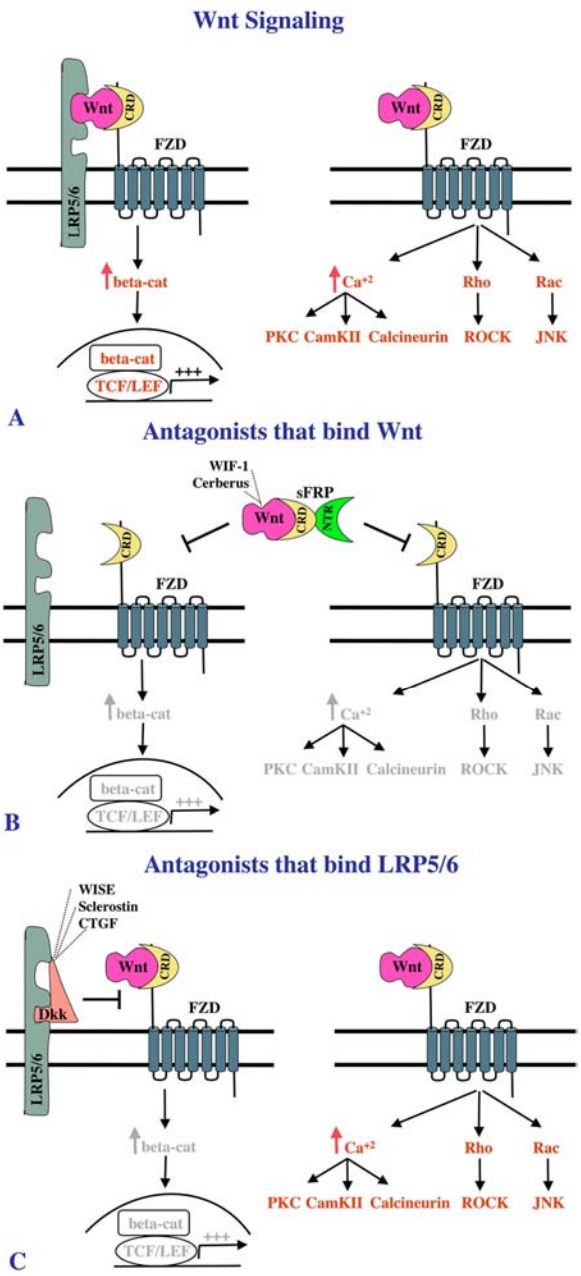

Figure 1. Wnt signaling and its regulation by secreted antagonists. A. Simplified version of canonical Wnt/beta-catenin (beta-cat) pathway (left panel), and non-canonical Wnt/Ca^{+2} and PCP pathways (right panel). Activated effectors are shown in red. B. sFRPs, WIF-1 and Cerberus bind directly to Wnts, blocking both beta-catenin and non-canonical signaling (gray indicates inhibition). C. Dkk, Wise, sclerostin and CTGF bind to Wnt co-receptors LRP5 or LRP6, inhibiting the beta-catenin pathway but leaving non-canonical signaling intact.

pass transmembrane receptor family and either low-density lipoprotein receptor related protein (LRP) 5 or LRP6. This results in disruption of the Axin/APC/beta-catenin/GSK3-beta/CKI-alpha complex by a process that involves Disheveled, Axin recruitment to the plasma membrane and subsequent Axin degradation. Consequently, non-phosphorylated beta-catenin accumulates in the cytosol and then the nucleus, where, in combination with members of the DNA-binding, T cell factor/lymphoid enhancer factor (TCF/LEF) family, it up-regulates the expression of specific genes that participate in cell proliferation, differentiation and other processes (Figure 1A).

Dysregulation of this pathway occurs in several malignancies and by multiple genetic mechanisms (2, 3). Mutations in beta-catenin that interfere with its phosphorylation and degradation have been documented in twenty different kinds of tumors. Loss of function mutations in APC almost invariably perturb its Axin binding site, thereby destabilizing the Axin complex and enabling the accumulation of nuclear beta-catenin. Such *APC* mutations occur in ~80% of human colorectal cancer, and have been reported in melanoma and medulloblastoma (4-6). Similarly, loss of function defects in the *AXIN* gene have been documented in hepatocellular carcinoma (7, 8) and medulloblastoma (9, 10). Many genes are up-regulated as a consequence of constitutive beta-catenin transcriptional activity, and they promote tumorigenesis in a variety of ways (2). For instance, c-Myc and cyclin D1 stimulate cell proliferation or sustain a stem cell phenotype, cyclooxygenase-2 and survivin inhibit apoptosis. and CD44 enhances tissue invasion and metastasis (2, 3). While changes in the beta-catenin gene (*CTNNB1*), *APC* and *AXIN* are well-established mechanisms of constitutive beta-catenin activation, over-expression of various Wnts, FZDs, Dishevelleds and LRP5 has been noted in an assortment of malignancies where they also may increase signaling through this pathway [see (2, 11) for references].

In contrast to Wnt/beta-catenin signaling, a number of "non-canonical" Wnt pathways are initiated by Wnt/FZD interaction, without binding to LRP5/6 (Figure 1A). The Wnt/calcium pathway relies on the release of Ca+2 from intracellular stores to activate enzymes such as protein kinase C (PKC) isoforms, calmodulin kinase II (CaMKII) and calcineurin that stimulate cell differentiation, motility, gene expression and morphogenesis (12, 13). The planar cell polarity (PCP) pathway controls epithelial cell polarity and cell movement in various model systems via the small GTPases RhoA and Rac, which increase the activity of Rho-associated kinase (ROCK) and c-Jun N-terminal kinase (JNK), respectively (12).

Although less well documented than the beta-catenin pathway, non-canonical Wnt signaling has been described in the setting of cancer. A recent report suggested that beta-catenin and JNK signaling act synergistically in the development of intestinal carcinogenesis (14). Wnt-5a was identified as a pro-metastatic factor in melanoma that stimulated cell motility through a PKC-dependent process (15). Both RhoA and PKC were implicated in the Wnt-dependent, invasive properties of multiple myeloma cells (16). Just as these pathways stimulate epithelial-mesenchymal transition during development, they might have a similar effect

during tumorigenesis (11). However, other studies suggest that certain Wnts, particularly Wnt-5a, have tumor suppressor activity (17-19), potentially by blocking beta-catenin transcriptional activity through non-canonical mechanisms (20-22).

3. SECRETED WNT ANTAGONISTS

Several secreted proteins have been identified as Wnt antagonists. They can be divided into two broad groups based on their mechanisms of action: (1) factors that bind directly to Wnts, and presumably block all Wnt signaling pathways (Figure 1B); (2) factors that bind to LRP5 or LRP6, and consequently only inhibit the beta-catenin pathway (Figure 1C). Prominent among the first group are the secreted Frizzled-related proteins (sFRPs), which contain a FZD-type cysteine-rich domain (CRD) that has Wnt-binding properties (23-25). There are five sFRPs in mammals with amino acid sequences that are 25-55% identical to each other, and CRDs that are typically 30-50% identical to the corresponding regions of FZDs. In addition to the CRD, sFRPs possess a netrin (NTR) domain that facilitates binding to heparan proteoglycan and may be involved in other interactions (26-28). SFRPs typically have distinct though overlapping patterns of expression, consistent with the idea that they have both unique and redundant functions (29).

Wnt inhibitory factor-1 (WIF-1) was first identified in fish, amphibia and mammals, shown to bind Wnt proteins *in vitro* and regulate somitogenesis, consistent with its expression in paraxial mesoderm (30). Interaction with Wnts was localized to an amino-terminal ~150 amino acid residue WIF-1 domain that is not related to the FZD CRD. Interestingly, Derailed, a cell surface protein in *Drosophila* (RYK in mammals, LIN-18 in *C. elegans*) with a WIF-1 domain, functions as a receptor for Wnt-5, reinforcing the Wnt-binding capability of this domain (31). Recently, the WIF-1 ortholog in *Drosophila*, Shifted, was reported to control the diffusion of Hedgehog, but not interact with Wnt proteins (32, 33). Cerberus is a protein that lacks a FZD CRD and a WIF-1 domain, but binds and antagonizes Wnt in *Xenopus* (34), although mammalian Cerberus orthologs have not been shown to inhibit Wnts (35). Besides Wnts, Cerberus also associates with Nodal and bone morphogenetic proteins (BMPs) (34). Coco is structurally related to Cerberus and has Wnt antagonist activity in *Xenopus*, but this activity has not been confirmed in mammals (36).

The Dickkopfs (Dkks) were the first proteins reported to block the beta-catenin pathway by binding to LRP5/6 and disrupting the interaction of Wnt with its co-receptor (37-39). Dkk-1 was originally identified as a Wnt antagonist expressed in Spemann's organizer with head-inducing activity (40). While there are conflicting reports, it appears that the four Dkk proteins in human all can inhibit the Wnt/beta-catenin pathway, at least in some experimental models (40-45). The mechanism, at least for Dkk-1 and Dkk-2, involves concomitant binding to LRP5 or LRP6 and Kremen proteins; the latter mediate the internalization of LRP5/6, accounting for their down-regulation from the cell surface (43, 46). However, there are circumstances in which Dkk-2 stimulates, rather than inhibits the beta-catenin pathway (42, 47). Recently, WISE (48) and the related protein sclerostin (49, 50) have been shown to bind LRP5 or LRP6 and inhibit beta-catenin signaling, although WISE has bifunctional activities (48) and sclerostin also has been reported to interact with BMPs (51). Connective tissue growth factor (CTGF), a CCN family member, is another molecule that associates with LRP6 and inhibits beta-catenin signaling (52), although it also has Wnt-independent mechanisms of action involving integrins (53).

4. SECRETED WNT ANTAGONISTS AS TUMOR SUPPRESSORS

Given the important role of Wnt signaling in cancer, the idea that secreted Wnt antagonists function as tumor suppressors is logical. However, the potential impact of agents that block Wnt/receptor interactions at the cell surface might be limited, because of the tendency for constitutive Wnt signaling in malignancies to result from defects in downstream regulation of the beta-catenin pathway. Three lines of evidence suggest this is not the case. Firstly, there are numerous examples of tumor cell lines with autocrine Wnt loops, where inhibition of Wnt/receptor signaling attenuated the tumor phenotype (54, 55). Secondly, sFRPs decrease beta-catenin stabilization and promote cell death even in cells that have downstream mutations in the beta-catenin pathway (56), suggesting that receptor activation at the cell surface augments signal propagation associated with these mutations. Thirdly, as mentioned above, beta-catenin and JNK signaling act synergistically in the intestines to promote tumorigenesis (14). Therefore, at least in some situations, factors that block non-canonical Wnt signaling would be expected to undermine the tumorigenicity of activating mutations in the beta-catenin pathway. Table 1 contains a list of secreted Wnt antagonists, and related family members, that have been reported to possess tumor suppressor activity.

4.1. sFRPs

In addition to its activity as a Wnt antagonist, the chromosomal location of *SFRP1* at 8p11-12 provided an early indication that it might be a tumor suppressor (57). This is a site commonly associated with deletions and loss of heterozygosity (LOH) in a variety of cancers (58). Similarly, the chromosomal location of *FRZB/SFRP3* at 2q31-33 is an area associated with LOH in lung and colorectal carcinomas as well as neuroblastomas (59). When expressed ectopically in the human mammary tumor cell line MCF7, sFRP-1 had pro-apoptotic activity, implying that its loss might enhance tumor cell survival (60). *SFRP1* transcript was elevated in cells exposed to pro-apoptotic conditions, consistent with the idea that it could mediate apoptosis in these situations, while expression was reduced in breast cancer cell lines and in breast, ovary and kidney tumor specimens (61). A more extensive survey of breast cancers indicated that *SFRP1* expression was undetectable in 78% of 90 malignant samples, but only 16% of benign tumors (62). Subsequently, *in situ* hybridization analysis demonstrated that *SFRP1* was expressed by normal mammary epithelial

Table 1. Wnt antagonists and tumor suppressor activity

Secreted Wnt antagonists	Chromosomal location (human)	Tumor suppressor activity (references)
sFRP-1 (SARP2, FzA)	8 p12-p11.1	56, 60-75
sFRP-2 (SARP1)	4 q31.3	56, 68
sFRP-3 (Frzb-1, Fritz)	2 q31-33	-
sFRP-4 (DDC-4, Frzb-2, frpHE)	7 p14.1	56, 68, 74, 75
sFRP-5 (SARP3)	10 q24.1	56, 68, 74, 75
WIF-1	12 q14.3	76, 77
Dkk-1	10 q11.2	78-83
Dkk-2	4 q25	-
Dkk-3 (REIC)	11 p15.2	45, 84-88
Dkk-4	8 p11.2-p11	-

Note: Crescent and Sizzled are secreted Frizzled-related proteins with Wnt antagonist activity that are expressed in *Xenopus* and chicken, but have not yet been detected in mammals.

cells and the *in situ* component of ductal carcinomas, but was absent from over 80% of invasive carcinomas, except the medullary type (63). A recent large tissue microarray analysis confirmed that sFRP-1 protein expression was frequently reduced or absent in cases of invasive breast carcinoma (64). Others also have reported that loss of *SFRP1* expression is common in breast cancer, particularly in pre-menopausal patients and in high-grade lesions regardless of menopausal status. Interestingly, they noted that a decline in expression often occurred in the absence of a decrease in gene copy number (65). *SFRP1* expression also was reduced in gastric cancer (66) and cervical cancer (67); in the latter setting, restoration of expression increased apoptosis when cells were maintained in serum-free medium.

The idea that *SFRP*s are tumor suppressor genes was reinforced by the discovery that many of them contain dense CpG islands that are frequently hypermethylated in cancer (68). Epigenetic silencing by hypermethylation is a mechanism that often accounts for the loss of expression of tumor suppressor genes (69). In their seminal study, Suzuki and colleagues demonstrated that expression of the *SFRP1* gene was repressed by hypermethylation in all colorectal and gastric carcinoma lines tested, and in a few mammary and prostate cancer lines as well. They noted that *SFRP2*, *SFRP4* and *SFRP5*, but not *FRZB/SFRP3*, also had dense CpG islands extending from the 5'-flanking region into the first exon, and that these sequences were often modified by hypermethylation. Specifically, in an analysis of 124 primary colorectal tumor samples, silencing due to hypermethylation occurred in 95.1% of the cases for *SFRP1*, 89.5% for *SFRP2*, 29.0% for *SFRP4* and 58.9% for *SFRP5*. Silencing of at least one *SFRP* gene occurred in all but one of the 124 specimens, and expression of all four genes was absent in 24.1% of the tumors (68). A high rate of epigenetic silencing of *SFRP1* in colorectal cancer was documented by another laboratory, although in this report chain-terminating mutations in exon 1 also were observed in a minority of samples (70). Importantly, a subsequent study by Baylin's group demonstrated that restoration of sFRP expression, especially of sFRP-1 or sFRP-2 and to a lesser extent sFRP-5, attenuated Wnt signaling as measured by a decline in cytosolic and nuclear beta-catenin as well as a decrease in beta-catenin transcriptional activity (56). There was a corresponding increase in apoptosis and inhibition of colony formation; the latter was seen with sFRP-4 expression as well, even though restoration of sFRP-4 expression had little effect on beta-catenin signaling or apoptosis in the colorectal carcinoma cell lines (56). Of particular note, sFRP-dependent inhibition of Wnt signaling and cell survival was observed in cells that had mutations in APC or beta-catenin. Downstream mutations in the beta-catenin pathway were not sufficient for maximal activity; upstream events sensitive to sFRP regulation had a major impact on Wnt signaling, cell survival and presumably tumorigenesis. A high frequency of *SFRP* silencing in pre-malignant colorectal lesions emphasized their potential importance in the etiology of colorectal cancer (56).

SFRP epigenetic silencing by hypermethylation has been observed in cancers from several other organs. In bladder, *SFRP1* expression was decreased or undetectable in 38% of carcinoma lines and primary tumors as determined by RT-PCR analysis, and in 66% of cases in a tissue microarray evaluated by immunohistochemistry (71). Promoter hypermethylation, rather than homozygous deletions or mutations, was documented in this setting. Hypermethylation of the *SFRP1* gene also was demonstrated in a small percentage of ovarian carcinoma cell lines and primary tumors, but was not seen in ovarian endometrial cysts (72). In non-small cell lung cancer, promoter methylation was detected in approximately 50% of tumor cell lines (15/29) and primary lung tumors (44/80). But LOH at the *SFRP1* locus occurred in 38% of surgical specimens, indicating that both epigenetic and genetic mechanisms contributed to the decline in *SFRP1* expression. Transfection of tumor lines with an *SFRP1* cDNA inhibited beta-catenin transcriptional activity and colony formation, implying that the absence of *SFRP1* expression had enhanced beta-catenin signaling and tumor growth (73). Suppression of *SFRP1*, *SFRP4* and *SFRP5* expression by methylation also was common in esophageal carcinoma and its precursor, Barrett's esophagus (74), analogous to findings in colorectal cancer and the same genes were frequently down-regulated in malignant pleural mesothelioma (75).

4.2. WIF-1

Currently there is less evidence of a role for WIF-1 as a tumor suppressor compared to the sFRPs. However, one study demonstrated a down-regulation of *WIF1* expression in prostate, breast, lung and bladder cancer based on DNA microarray and immunohistochemical

analysis (76). Hypermethylation of CpG islands in the promoter region of the *WIF1* gene has been observed in lung cancer cell lines and in a high proportion of freshly resected lung cancers (15/18) (77). Thus, there are indications that loss of *WIF1* expression also may contribute to carcinogenesis.

4.3. Dkks

Dkk-1 is the prototype of Wnt antagonists that specifically block the beta-catenin pathway by binding to Wnt co-receptors LRP5/6 and mediating their down-regulation by concomitant binding to kremen proteins (46). As such, it is a candidate for tumor suppressor activity. Indirect support for this function came from a report that *DKK1* expression was up-regulated by the tumor suppressor p53, but not by a mutated form of p53 (78). Subsequent work showed that *DKK1* expression was enhanced by genotoxic stimuli regardless of the status of the p53 gene, and stable ectopic expression of Dkk-1 altered Bcl-2/Bax expression, shortened telomere length and increased sensitivity to apoptotic stimuli (79). Pro-apoptotic activity of Dkk-1 also was detected during embryonic development where it correlated with inhibition of Wnt/beta-catenin signaling (80). Another study indicated that in some instances Dkk-1 promoted apoptosis by a beta-catenin-independent mechanism that may involve JNK activation (81). A connection between Dkk-1 and pro-apoptotic activity independent of beta-catenin regulation also was suggested by an analysis of putative tumor suppressor genes in HeLa cervical carcinoma cell revertants (82). In this experimental model, *DKK1* was identified as a gene markedly up-regulated in HeLa cells that had lost their tumorigenic properties. Dkk-1 expression in HeLa cells did not affect beta-catenin transcriptional activity, but it did sensitize the cells to UV-induced apoptosis. When HeLa cells expressed Dkk-1, growth in soft agar was markedly reduced and there was a delay in tumor formation when these cells were injected into mice. Moreover, tumors that formed after injection of Dkk-1-expressing HeLa cells almost uniformly lacked Dkk-1, strongly suggesting that loss of Dkk-1 was important for tumorigenesis in this model. Consistent with this view, recently *DKK1* was shown to be a target gene for TCF/beta-catenin signaling. However, its expression was absent from colon tumors that have a constitutively active beta-catenin pathway, again implying that loss of Dkk-1 was important for neoplasia (83).

DKK3 exhibits the characteristics of a tumor suppressor gene, although Dkk-3 function as a Wnt antagonist is controversial. The gene was identified in a screen to detect transcripts that were often lacking in immortalized cells and tumor-derived cell lines; hence it was provisionally termed Reduced Expression in Immortalized Cells, *REIC* (84). Correspondingly, expression was highest in organs with a preponderance of post-mitotic cells (brain and heart). Transfection of Dkk-3 cDNA into SaOS-2 osteosarcoma cells inhibited DNA synthesis but did not increase TUNEL staining, implying that it primarily had an anti-proliferative rather than pro-apoptotic activity (85). There was no change in the nuclear localization of beta-catenin in cells transfected with Dkk-3, implying that its mechanism of action did not involve inhibition of the beta-catenin pathway. While this view is in agreement with some reports (41, 43), it differs from others (44, 45). In particular, Hoang and colleagues claimed to see a shift of beta-catenin from the nucleus to the cell membrane in SaOS-2 cells expressing Dkk-3, and this was associated with an increase in cell-cell adhesion and a decrease in cell penetration through a Matrigel-coated membrane (45). Although the chromosomal location of *DKK3*, 11p15, is frequently associated with LOH, deletions or mutations in the gene appear to be rare. Rather, hypermethylation of the promoter occurred frequently in a variety of tumor lines (11/21) and in fresh non-small cell lung cancer specimens (14/24) (86). Dkk-3 RNA and protein expression were reduced in >90% of human renal clear cell carcinoma specimens (87). Another study revealed down-regulation of *DKK3* expression in 29/48 human cancer specimens from kidney, bladder, prostate, pancreas and lung (88). Ectopic expression of Dkk-3 in tumor cell lines inhibited cell growth *in vitro* and tumor formation *in vivo*, effects largely attributed to increased apoptosis (88).

5. SECRETED WNT ANTAGONISTS AS PROMOTERS OF TUMOR GROWTH

Contrasting with the literature summarized above, several reports offer another view of the expression and activity of secreted Wnt antagonists in the tumor setting. These discrepancies may reflect subtle distinctions in the function of structurally related molecules, or alternative activities of molecules when expressed in different contexts. In this section, we describe a number of observations that contradict the view that secreted Wnt antagonists are tumor suppressors.

SFRP-2 (SARP-1, secreted apoptosis-regulated protein-1) was identified as a factor that increased both the nuclear staining of beta-catenin and the resistance of a human mammary carcinoma line, MCF7, to apoptotic stimuli (60). When expressed ectopically in malignant glioma cells, sFRP-2 enhanced their clonogenicity and resistance to serum starvation, although it did not reduce their susceptibility to a variety of other apoptotic stimuli. However, it promoted the growth of intracranial xenografts in nude mice, while inhibiting cell motility *in vitro* (89). Subsequent work with spontaneously occurring canine mammary tumors revealed a marked up-regulation of *sFRP2* expression in benign and malignant tumors relative to normal mammary tissue. Again, this correlated with an increase in beta-catenin located in the nucleus (90). Over-expression of sFRP-2 in mammary carcinoma cells inhibited apoptosis associated with UV exposure, while increasing cell-substrate adhesion. The stimulation of fibronectin/integrin interaction, which appeared to involve a physical association of this complex with sFRP-2, was instrumental in the anti-apoptotic effect (91). Taken together, these results suggested that either anti-apoptotic or growth-promoting activity of sFRP-2 might contribute to tumorigenesis.

SFRP1 expression also has been seen in tumors, specifically in uterine leiomyomas where it appeared to act

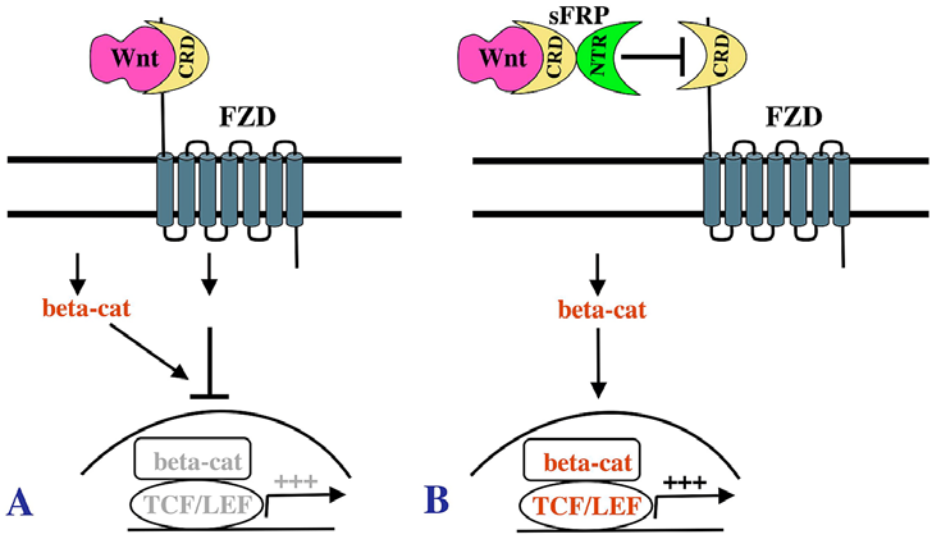

Figure 2. Hypothetical activation of beta-catenin pathway by sFRP. A. Wnt-5a inhibits beta-catenin transcriptional activity (indicated by gray) elicited by any of multiple ligand/receptor signaling mechanisms that increase soluble beta-catenin concentration (red). B. sFRP binds to Wnt-5a, repressing its inhibition of beta-catenin transcriptional activity.

primarily as an anti-apoptotic agent (92). Inhibition of apoptosis by sFRP-1 was reported in a non-tumor setting as well: suppression of endogenous *sFRP1* expression by RNA interference increased apoptosis in cultured periodontal ligament fibroblasts, while ectopic expression in gingival fibroblasts reduced apoptosis. These changes were associated with the regulation of apoptosis-related genes including those encoding p53, caspase-3, caspase-9 and BCL-2 interacting killer (BIK) (93). In a separate study, recombinant sFRP-1 stimulated growth of embryonic mouse prostate in organ culture, while the ectopically expressed protein increased prostate epithelial cell proliferation and inhibited apoptosis. Notably, the *SFRP1* gene was up-regulated in prostate carcinoma-derived stromal cells compared with stroma cells from normal tissue, and in a prostate carcinoma experimental model, progressively more advanced carcinoma cells acquired the expression of *SFRP1* (94). Thus, sFRP-1 exhibited the characteristics of a survival factor that might augment tumor formation or growth in particular circumstances.

Over-expression of other Wnt antagonists has been described in various tumors. *SFRP4* was up-regulated in the stroma of endometrial carcinomas and invasive breast carcinomas (95). It also was increased in breast carcinoma cells in a minority of tumor specimens; this was correlated with a reduced incidence of axillary metastases (96). *SFRP4* expression was elevated in ~80% of primary prostate carcinomas (76), and membranous immunostaining of sFRP-4 over-expressed in prostate cancer was reported to be a favorable prognostic indicator (97). Marked over-expression of *SFRP4* in tumors associated with osteomalacia led to the discovery that systemic sFRP-4 released from the tumor cells was a potent phosphaturic agent (98). The potential impact of this activity on tumor growth has not been determined. While *SFRP* and *DKK1* expression often is suppressed in colorectal carcinoma, WIF-1 transcript level was elevated in colonic adenomas in a mouse model of colorectal cancer, and was detected in two human colon adenocarcinoma cell lines (99). Dkk-1 is produced by multiple myeloma cells and thought to contribute to the osteolytic lesions common in this disease by impairing the differentiation of osteoblast precursors (100). *DKK1* expression also has been seen with high frequency in hepatoblastomas (101). The functional consequences of these patterns of over-expression with regard to tumorigenesis are largely unknown. While one report suggested that a membranous distribution of sFRP-4 signified a less virulent phenotype, overall the fact that these proteins are expressed at elevated levels in particular cancers argues against their function as tumor suppressors in these contexts.

6. POSSIBLE MECHANISMS TO ACCOUNT FOR DISPARATE ACTIVITIES OF WNT ANTAGONISTS

The range of effects described above or inferred from expression patterns cannot be easily rationalized based on the simple models of Wnt inhibition portrayed in Figure 1. At present, detailed mechanistic explanation of these diverse activities is lacking. In this section, we propose alternative models and speculate about possible mechanisms to account for the paradoxical behavior of the secreted Wnt antagonists.

The growth-promoting effects of sFRPs might be due to inhibition of non-canonical Wnt signaling that negatively regulates beta-catenin transcriptional activity (Figure 2). Wnt-5a has been reported to block beta-catenin activity by at least three different pathways: (1) calcium-dependent cascade involving CaMKII, TAK1 and Nemo-like kinase, resulting in TCF/LEF phosphoryation that reduces its affinity for beta-catenin (21); (2) PKC phosphorylation of Dishevelled (20); (3) GSK3-

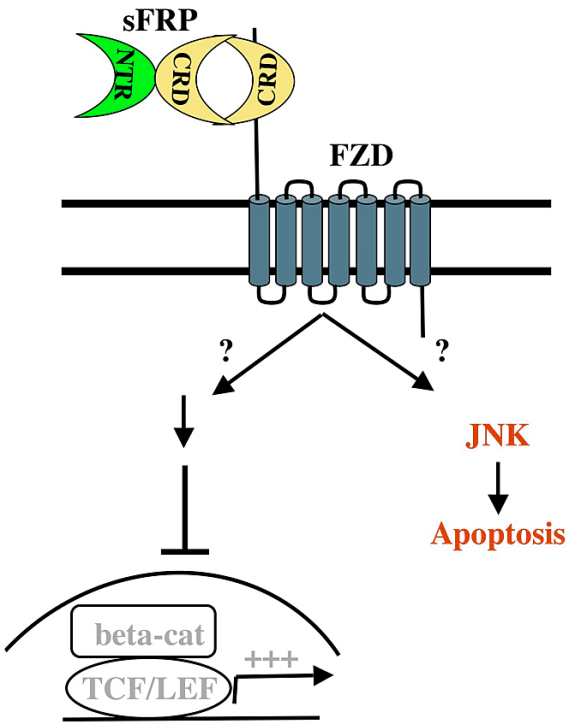

Figure 3. Hypothetical signaling by sFRP as a FZD ligand. Alternate mechanisms of sFRP tumor suppressor activity: mimicking non-canonical inhibition of beta-catenin transcriptional activity; stimulation of a proposed FZD/JNK pro-apoptotic pathway (111).

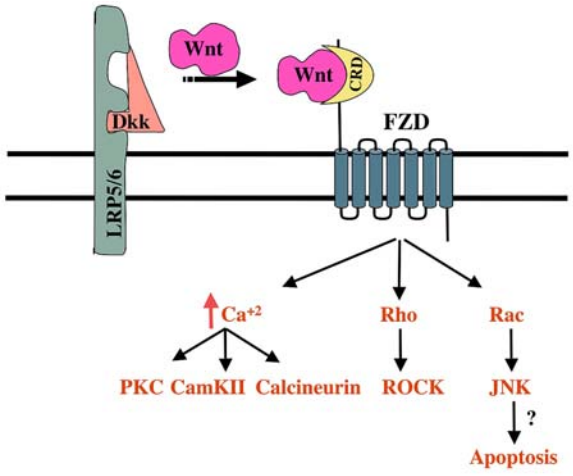

Figure 4. Proposed increase in non-canonical Wnt signaling by Dkk. Inhibition of Wnt binding to LRP5/6 should increase concentration of Wnt/FZD complexes, thereby stimulating non-canonical signaling. JNK activation might contribute to pro-apoptotic activity of Dkk (81).

independent, beta-catenin degradation (22). By preventing Wnt-5a binding the to FZD, sFRP could overcome the inhibition of beta-catenin signaling. This effect would prevail whether the impetus for beta-catenin activity came from a canonical Wnt mechanism, integrin/integrin-linked kinase activity (102) or one of the many growth factor/tyrosine kinase receptor combinations that have been shown to increase soluble beta-catenin levels and stimulate beta-catenin transcriptional activity (103-107).

Other mechanisms of sFRP activity also may be operative in specific settings. As suggested in one study (27), rather than sequestering Wnt protein, at low concentrations sFRPs may stimulate Wnt signaling, perhaps by presenting the ligand to its receptors. Crystallographic analysis revealed that FZD and sFRP CRDs formed dimers at high concentrations (108), while co-immunoprecipitation analysis demonstrated that FZD/sFRP association could occur under more physiologic conditions (109). Until recently, the functional significance of such interactions was unknown. However, a recent article suggests that sFRP-1 binds to FZD-2 and functions as an agonist for non-canonical signaling (Figure 3) (110). Such signaling could have various consequences, including inhibition of the beta-catenin pathway (mimicking rather than blocking the activity of Wnt-5a) or stimulating a proposed FZD/JNK pro-apoptotic pathway (111).

Dkks typically have been viewed strictly as inhibitors of the Wnt/beta-catenin pathway. Perhaps, though, by eliminating Wnt-LRP5/6 binding they enhance signaling that emanates solely from Wnt-FZD complexes (Figure 4). This could account for Dkk-1 stimulation of JNK, which sometimes has been linked to its pro-apoptotic activity (81).

Wnt-independent interactions should not be discounted when trying to understand the expression pattern and activities of secreted Wnt antagonists. For instance, the expression of WIF-1 in colonic adenomas and colorectal carcinoma lines is not consistent with the loss of expression of other Wnt/FZD inhibitors such as sFRPs and Dkk-1 in this setting (99). The recent finding that the *Drosophila* ortholog of WIF-1 facilitates Hedgehog diffusion raises the possibility that mammalian WIF-1 may enhance Hedgehog activity (32, 33), which has been reported to antagonize Wnt signaling in colonic epithelial cell differentiation (112). Similarly, the anti-apoptotic activity of sFRP-2 in mammary carcinoma cells has been linked to a novel association with fibronectin/integrin complexes (91). Considering the conflicting data pertaining to Dkk-3 inhibition of Wnt/beta-catenin signaling, its putative tumor suppressor activity may be attributable to other mechanisms involving novel binding partners.

The secretion of Wnt antagonists might alter the activity of neighboring cells in a manner that would favor the proliferation and metastasis of malignant cells. For instance, inhibition of osteoblast differentiation by Dkk-1 released from multiple myeloma cells results in osteolytic lesions that may enable expansion of the tumor mass (100).

7. PERSPECTIVE

Several observations over the past few years suggest that a number of secreted Wnt antagonists are

tumor suppressor candidates. Their expression is frequently reduced in a variety of cancers, and restored expression has an anti-tumorigenic effect. Mechanistically, the putative tumor suppressor activity is consistent with inhibition of Wnt signaling, particularly beta-catenin transcriptional activity, which is elevated in many neoplasias. Even though there is not yet evidence of germ-line inactivating mutations predisposing to inherited increased cancer risk, and only limited evidence that LOH and somatic mutations occur in sporadic tumors, the high incidence of epigenetic silencing by hypermethylation seen in some of these genes is consistent with the hypothesis that they are *bona fide* tumor suppressors. The most suggestive data pertain to members of the sFRP family, with additional support for WIF-1, Dkk-1 and Dkk-3.

In contrast to the hypothesis that these agents are tumor suppressors, other reports have shown that some also have growth-promoting and/or anti-apoptotic effects in various contexts. We can speculate why this happens and cite the diversity of Wnt functions in different settings as precedent for a wide range of biological activities. Tumor suppressor and tumor promoter activities may make sense in different contexts. To resolve this paradox, we need a better understanding of their molecular interactions, both Wnt-related and potentially Wnt-independent, in the distinct settings. That would enable us to draw more definitive conclusions about the roles of these proteins in tumor biology.

The proposed function of secreted Wnt antagonists as tumor suppressors may have clinical utility. Because of the multiplicity of Wnts expressed in many normal tissues, it is likely that systemic delivery of Wnt antagonists would have unacceptable side effects. This view was borne out by a study in which adenoviral delivery of Dkk-1 was lethal in mice due to destruction of the small intestines (113). Clearly, a more specific route of administration would be required to avoid such consequences. The use of DNA methyl transferase inhibitors to reverse epigenetic silencing is an attractive option, although it is not certain that levels of gene expression attainable with these reagents would be sufficient to control Wnt signaling. In cell culture model systems, restoration of antagonist activity typically was achieved with cDNA expression vectors rather than with reagents that prevented or reversed hypermethylation (56). Perhaps the most likely application of the present information would involve the development of cancer diagnostic screening tools, as epigenetic silencing of *SFRP*s appears to be an early event in tumorigenesis observed with a high frequency in pre-malignant lesions (56, 74).

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