Acta Pharmacol Sin 2007 Sep; 28 (9): 1499–1504

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Invited review

# Roles of secreted frizzled-related proteins in cancer[1]

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## Key words

Wnt signaling pathway; secreted frizzled-related proteins; cancer

[1] This work was partially supported by a grant from the National Institutes of Health (RO1 CA 093708-01A3), the Larry Hall and Zygielbaum Memorial Trust, and the Kazan, McClain, Edises, Abrams, Fernandez, Lyons & Farrise Foundation.
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Received 2007-04-27
Accepted 2007-07-23

doi: 10.1111/j.1745-7254.2007.00692.x

## Abstract

The Wnt signaling pathway is implicated in a variety of biological processes ranging from developmental cell fate to human disease. The components involved in Wnt signaling have been under intense investigation over the last 2 decades. Aberrant canonical Wnt activation has been linked to tumor formation and involves activation of effector molecules or loss of tumor suppressor function. Secreted frizzled-related proteins (sFRPs) are Wnt antagonists. In recent years, accumulating evidence has suggested that sFRPs act as tumor suppressors because their expression is frequently silenced in cancer by promoter hypermethylation. However, sFRPs may also promote cell growth in some contexts. Here, we focus on the known knowledge of sFRPs in tumorigenesis.

## Introduction

The Wnt signaling pathway plays an important role in embryonic development, carcinogenesis, and neurodegenerative disease. Human cancer is the most extensively studied disease related to dysregulation of Wnt signaling. In recent years, aberrant activation of the Wnt signaling pathway has been well documented in various human cancers including colorectal cancer [1], melanoma [2], non-small cell lung cancer (NSCLC) [3], leukemia [4], and mesothelioma [5]. Wnt signaling consists of canonical and non-canonical pathways. In the canonical Wnt signaling pathway, β-catenin is a key mediator. In the resting state (Figure 1), no Wnt ligand binds to the frizzled/low density lipoprotein receptor-related protein (LRP) receptor complex. Thus, cytosolic β-catenin is recruited to a multi-protein "destruction complex" consisting of adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase-3β (GSK-3β) [6–9]. β-catenin is phosphorylated by GSK-3β and subsequently targeted for degradation via the ubiquitin proteosome pathway [10–12]. In the activated state (Figure 2), Wnt protein binds to the Frizzled/LRP receptor complex and transduces a signal to Dishevelled (Dvl) [13–15]. Increased activity of Dvl alters the composition of the "destruction complex," and the degradation of β-catenin is inhibited. Consequently, β-catenin accumulates in the cytoplasm and subsequently translocates to the nucleus. In the nucleus, β-catenin interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and turns on the TCF target genes[16,17]. In the non-canonical Wnt pathway, however, β-catenin is dispensable. This pathway is not as well defined as the canonical Wnt pathway but is interesting to many researchers as well. The non-canonical pathway may proceed through calcium flux, G proteins, and JNK [18].

For a long time, constitutive activation of the Wnt pathway was thought to result from gain-of-function mutations in CTNNB1/β-catenin or loss-of-function mutations in tumor suppressor genes, for example APC and AXIN1. However, recent studies have shown that expression of secreted Wnt antagonist genes is silenced due to promoter

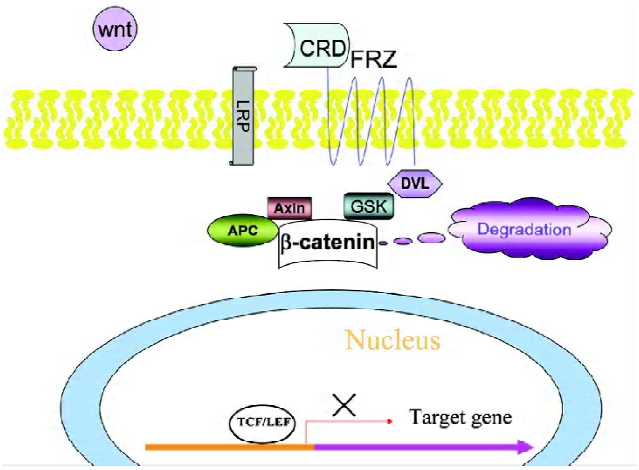


**Figure 1.** Resting state of the canonical Wnt signaling pathway.

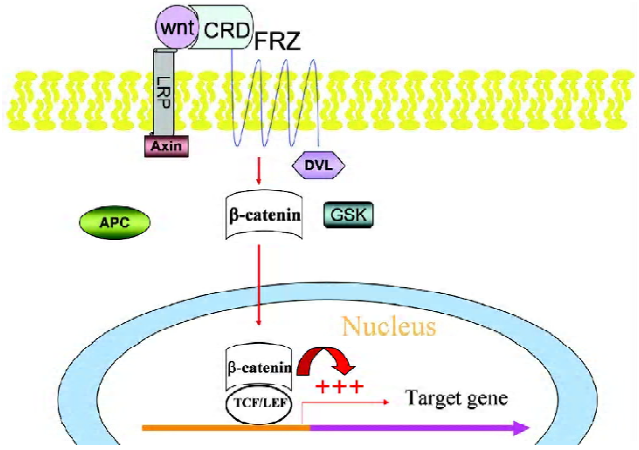


**Figure 2.** Activated state of the canonical Wnt signaling pathway.

hypermethylation in carcinogenesis[19–22]. These studies imply that silencing of Wnt antagonists stimulates Wnt signaling at the cell surface level and acts as a tumor suppressor. Wnt antagonists can be divided into 2 classes based on their mechanisms of action. The first class includes the secreted frizzled related proteins (sFRPs) family, Wnt inhibitory factor (WIF)-1, and Cerberus[23]. This class of Wnt antagonists binds to Wnt proteins directly and possibly blocks all Wnt signaling pathways. The second class consists of members of the Dickkopfs (Dkks) family that bind to Wnt co-receptors and are thought to inhibit only the canonical β-catenin pathway [23]. In this review, we focus on the sFRP family, and discuss the evidence linking its dysfunction to cancer.

## sFRPs family

sFRPs contain a frizzled (FRZ) -type cysteine-rich domain (CRD) that has Wnt-binding properties[24,25]. There are 5 sFRPs existing in mammals. They were simultaneously discovered and named by different groups but are now uniformly designated as sFRP1-5. In 1996, sFRP3 was the first identified member of the sFRP family and was isolated as a chondrogenic factor in bovine cartilage extracts. It was originally named Frzb because its N-terminal domain contains a characteristic CRD that shares homology with the Drosophila Fz CRD[26]. The CRD in Frzb led to the prediction that the protein regulates the Wnt signaling pathway. Indeed, further studies showed that Frzb interacts with Xwnt-8 and antagonizes Xwnt-8 signaling in Xenopus embryos[27,28]. Moreover, Frzb was reported to interact with Wnt-1 and inhibit Wnt-1 induced accumulation of β-catenin in human embryonic kidney cells[29,30], thereby blocking the Wnt signaling pathway. Following the discovery of Frzb, 4 members of the sFRP family including Frzb were identified in a 1997 study that searched expressed sequence tag (EST) databases for sequences homologous to Fz receptors[24]. The newly discovered proteins were then named sFRP1-4. sFRP1 and sFRP4 were first identified in this manner. sFRP3 was found to be identical to Frzb, and sFRP2 happened to be identical to a previously cloned but incompletely characterized protein, SDF5[31]. At the same time, another lab isolated a novel protein during purification of hepatocyte growth factor/scatter factor in a conditioned medium from a human embryonic lung fibroblast line [32]. This protein was called frizzled-related protein (FRP) and later proved to be identical to sFRP1. Soon thereafter, 3 members of the sFRP family were isolated in another study aimed at identifying human genes associated with apoptosis[33]. These sFRPs were initially named secreted apoptosis-related proteins (SARP) 1–3, but turned out to be identical to sFRP2, 1, and 5, respectively. Moreover, sFRP5 was further characterized by Chang *et al* in 1999 and was found to act by modulating Wnt signal transduction[34].

## Molecular structure of sFRPs

The CRD of sFRPs is one of the most important characteristics of the sFRP molecular structure. The CRD includes 10 conserved cysteine residues and some additional conserved residues. It is located in the N-terminal of the protein and shares 30%–50% sequence similarity with the CRD domains of Fz proteins[33]. CRD is not restricted to Fz proteins and sFRPs. An Ensemble search revealed 46 genes in the human genome incorporating the CRD domain (InterPro domain IPR000024) including receptor tyrosine kinase, recep-

tor tyrosine kinase-like orphan receptor 1 and 2, type XVIII collagen, serine peptidase, and others. Through their CRD domain, sFRPs may interact with Wnt ligands, thus antagonizing Wnt signaling[30]. It is also possible that the CRD of sFRPs interacts with Fz to form nonfunctional complexes, thereby interfering with the Wnt signaling pathway[35]. In addition to CRD, the C-terminal of sFRPs contains a netrin (NTR) domain, which is defined by 6 cysteine residues and several conserved segments of hydrophobic residues. The NTR domain has also been found in tissue inhibitors of metalloproteases, type I procollagen C-proteinase enhancer proteins, and complement proteins C3, C4, and C5[36]. However, the function of the NTR domain in sFRPs is not yet well defined.

## sFRPs suppress tumor growth

**Chromosomal deletion and loss of heterozygosity in cancers** Frequent allele loss at chromosomal regions is strong evidence for the possibility that tumor suppressor genes are present in these regions. The chromosomal location of sFRP1 at 8p11-12 provided the early prediction that it might be a tumor suppressor because in various cancers, deletion and loss of heterozygosity (LOH) are frequently observed in this region[32,37,38]. Indeed, a study in surgically removed lung cancer specimens found that 15 of 40 cases (38%) had LOH in the sFRP1 gene locus[39]. Likewise, the chromosomal location of SFRP3 at 2q31-33 is also an area often associated with LOH in lung cancers, colorectal carcinomas, prostate carcinomas, and neuroblastomas[28,40,41] . One study in prostate cancer reported LOH at 2q32-36 in 6 of 14 cases (42%)[41]. However, 2q32-36 is the chromosomal region that partially harbors the sFRP3 gene and partially harbors the inhibin alpha-subunit gene. To our knowledge, there is no evidence demonstrating that deletion and/or LOH have occurred precisely within the sFRP3 gene locus.

**Transcriptional inactivation of sFRPs in cancer** Transcriptional inactivation of sFRPs has been reported in various cancers[19,39,42–50]. Epigenetic silencing due to hypermethylation of the promoter region of genes is a common mechanism for transcriptional inactivation of tumor suppressor genes in cancer[51,52]. The observation that sFRPs are transcriptionally inactivated in tumors by hypermethylation supports the hypothesis that sFRPs are tumor suppressors. In a genomic screen for genes hypermethylated in colorectal cancer, 4 genes in the sFRP family (sFRP1, 2, 4 and 5) were found to be frequently hypermethylated in colorectal cancer cell lines and in primary colorectal tumor samples[19]. This hypermethylation is associated with a lack of basal expression that is restored by a demethylation agent, 5-aza-2'-deoxycytidine. In an analysis of 124 primary colorectal cancer samples, gene silencing due to hypermethylation was found in 118 cases (95.1%) for SFRP1, 111 cases (89.5%) for SFRP2, 73 cases (58.9%) for SFRP5, and to a lesser extent, 36 cases (29.0%) for SFRP4. Subsequent studies from the same lab further demonstrated that exogenous overexpression of sFRP1, sFRP2, and sFRP5 attenuated Wnt signaling in colorectal cancer cells with downstream mutations, as did overexpression of sFRP4, but to a lesser degree[20]. Originally, downstream APC or β-catenin mutations in colorectal cancer were thought to lead to accumulation of free β-catenin and activation of downstream target genes independent of upstream signals. However, the high frequency of sFRP silencing suggests that upstream Wnt signals at the cell surface level may play important roles in colorectal tumorigenesis.

Besides colorectal cancer, sFRP epigenetic silencing by hypermethylation has been reported in several other cancers. Our lab has demonstrated that sFRP1, 4 and 5 gene promoters are frequently methylated in more than 80% of malignant mesothelioma primary tissues, and we have shown that the restoration of sFRP4 results in growth suppression and apoptosis in cell lines[44]. In lung cancer, hypermethylation of sFRP1 was found in 15 of 29 (52%) non-small cell lung cancer (NSCLC) cell lines, 2 of 25 (8%) small cell lung cancer (SCLC) cell lines, and 44 of 80 (55%) primary lung tumors[39]. In other labs, promoter methylation of sFRP1 was detected in 29% of bladder cancer cases[46]. In ovarian cancer, SFRP1 was found inactivated by promoter methylation in 4 of 13 (13%) ovarian cancer cell lines, and 2 of 17 (12%) primary ovarian cancers[45]. In esophageal carcinoma and its precursor, Barrett's esophagus, hypermethylation of sFRP1, 4, and 5 was also observed[47,48]. In summary, epigenetic silencing due to hypermethylation of the promoter regions of sFRP1, 2, 4, and 5 has been found in different cancers, suggesting tumor suppressor function of these sFRPs.

Downregulation of sFRP3 has been found in malignant mesothelioma and osteogenic sarcoma[44,50]. Unlike other sFRPs, sFRP3 does not have CpG-islands in its promoter region[19]. Therefore, the mechanism underlying downregulation of sFRP3 remains unclear. sFRP3 was hypothesized to be a tumor suppressor gene because sFRP3 binds both Wnt-8 and Wnt-1 and inhibits Wnt signaling in Xenopus embryos[27,28,53]. Furthermore, the sFRP3 gene is located at 2q where frequent deletion and LOH are observed in many cancers, which further evinces sFRP3's role as a tumor suppressor[28,40,41]. Recently, it was reported that expression of sFRP3 in human prostate cancer PC-3 cells suppresses

tumor growth and cellular invasiveness[54]. This result, together with the loss of sFRP3 expression in mesothelioma and osteogenic sarcoma, provides strong evidence that sFRP3 is a tumor suppressor gene.

## sFRPs stimulate cell growth

**Overexpression of the sFRP genes in cancers** sFRP4 overexpression was first reported in the stroma of endometrial carcinomas and in invasive breast carcinomas by differential display techniques[55]. Later on, sFRP4 overexpression was found in primary prostate carcinomas[56], endometrial stromal sarcomas[49] and colorectal carcinomas[57]. The molecular mechanisms responsible for the overexpression of sFRP4 and the effect of this overexpression on tumors are not well studied. However, increased levels of sFRP4 in tumor samples are evidence against the hypothesis that sFRP4 functions as a tumor suppressor in these tumor models. Like sFRP4, sFRP1 overexpression has also been reported in uterine leiomyomas, where it appears to have stimulated cell growth[58].

**sFRPs promote Wnt signaling** In addition to the observed sFRP overexpression in cancer, there are examples in both tumor and non-tumor settings where sFRP1 does not antagonize Wnt signaling, but instead potentiates it. It was reported that sFRP1 has a biphasic effect on Wnt signaling [59] in which high concentrations of recombinant sFRP-1 block Wnt signaling, whereas lower concentrations of sFRP-1 enhance Wnt signaling. sFRP1 was also found highly upregulated in cultured human periodontal ligament fibroblasts during ceramide-induced apoptosis[60]. Moreover, silencing of sFRP1 by RNA interference in cultured periodontal ligament fibroblasts increased apoptosis, while ectopic overexpression of sFRP1 in gingival fibroblasts decreased cell death[60]. Overall, these studies suggest that sFRP1 may contribute to stimulating cell growth under specific circumstances.

Like sFRP1, sFRP2 was also reported to promote cell growth by enabling MCF-7 breast cells to resist TNF-induced apoptosis[33]. Expression of sFRP2 appears to increase the intracellular levels of β-catenin, suggesting an activation of the Wnt signaling pathway. In this work, the function of sFRP1 was also studied. Unlike the behavior observed with sFRP2, expression of sFRP1 decreases the intracellular levels of β-catenin, suggesting an inhibition of the Wnt signaling pathway[33]. The inconsistent function of sFRP1 and sFRP2 in different settings complicates our understanding of their function. Additionally, there is other evidence indicating that sFRP-2 stimulates cell growth. For example, in malignant glioma cells, ectopic expression of sFRP-2 strongly promotes the growth of intracranial glioma xenografts in nude mice[61]. Also, in canine mammary tumor samples, sFRP2 was upregulated in tumor tissue compared to normal tissue. Considered together, these results suggested that sFRP2 might stimulate cell growth by activating Wnt signaling.

## Conclusions and Perspective

Recent studies have revealed that sFRPs are tumor suppressor candidates. The expression of sFRPs is frequently silenced by promoter hypermethylation in a variety of cancers. Restored expression of sFRPs has been shown to inhibit cell growth *in vitro* and tumor growth *in vivo*. These lines of evidence suggest anti cancer potential in those compounds that could reverse promoter hypermethylation. Also, recombinant sFRP proteins developed to inhibit the activated Wnt signaling pathway could be potential cancer therapeutics as well. However, to our knowledge, the development of therapeutics specifically targeting the aberrant Wnt pathway is still in the preclinic stage. So far, there are no drug candidates in clinical trials yet. Moreover, there is increasing evidence showing that some sFRPs have oncogenic functions. Under certain circumstances, some sFRPs are overexpressed in cancer or have cell growth-promoting or anti-apoptotic effects. The apparently contradictory roles of sFRPs in these studies might be due to the different Wnt ligands present in different cells, tissue-specific responses to different stimuli, biphasic responses to different concentrations of sFRPs, and the binding affinities and specificities of different sFRPs for Wnts[23]. Better understanding of the specific relationship between sFRPs and Wnt signaling will result from further work – particularly loss-of-function studies and the availability of purified recombinant Wnt and sFRP proteins[23]. We anticipate that within the next few years, further studies of the roles of sFRPs in cancer will eventually lead to successful therapeutic development targeting the Wnt signaling pathway.

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