

## **Wnt signaling in lung cancer**

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### **Abstract**

Wnt signaling has recently emerged as a critical pathway in lung carcinogenesis as already demonstrated in many cancers and particularly in colorectal cancer. We critically discuss in this review the individual components of the Wnt pathway and their role in lung cancer development. We propose that activation of the Wnt-mediated signal occurs in a different manner in lung cancer than in colorectal cancer. In lung cancer, mutations of APC or  $\beta$ -catenin are rare and the Wnt pathway appears to be activated upstream of  $\beta$ -catenin. We identified at least three mechanisms of activation: overexpression of Wnt effectors such as Dvl, activation of a noncanonical pathway involving JNK and repression of Wnt antagonists such as WIF-1. The respective relevance of each event and their likely relationship remain unclear. Nevertheless, we propose that many of the studied components of the Wnt pathway may serve as potential targets in the search for therapeutic agents and we can reasonably argue that blockade of Wnt pathway may lead to new treatment strategies in lung cancer.

## 1. Introduction

Lung cancer is a highly aggressive and challenging cancer that now represents the leading cause of cancer deaths within the United States and throughout the world. Although advances in chemotherapy have provided some improvement in overall survival for patients with advanced non-small-cell lung cancer (NSCLC), outcomes remain poor. More recently there has been modest success using novel agents, typically small molecules and monoclonal antibodies, to target signaling transduction pathways, growth factor receptors, oncogenes and tumor-suppressor genes known to be aberrant in lung cancer. Some of these agents have entered clinical trials with promising results [1]. Further elucidation of the molecular mechanisms underlying lung cancer is essential for the development of new effective therapeutic agents.

Secreted Wingless type (Wnt) ligands have been shown to activate signal transduction pathways and trigger changes in gene expression, cell behavior, adhesion and polarity. The role of Wnt signaling in cancer was first suggested 20 years ago with the discovery of Wnt-1 as an integration site for mouse mammary tumor virus (MMTV) in mouse mammary carcinoma [2]. Over time, a wealth of evidence have implicated Wnt signaling in tumor development and progression [3, 4]. Numerous reports have demonstrated aberrant activation of the Wnt signaling pathway in disparate human cancers including colorectal cancer [5, 6], head and neck carcinoma [7], melanoma [8] and leukemia [9]. Less is known about the Wnt pathway activation in lung cancer. However, we and others have recently described the pivotal role of the Wnt pathway in thoracic malignancies such as non-small cell lung cancer and mesothelioma.

The role Wnt signaling plays in tumorigenesis has been covered in recent exhaustive and informative reviews [4, 10-12]. The aim of this review is to critically appraise the role of the components of the Wnt pathway in lung cancer development.

## 2. The Wnt pathway: a brief overview

Wnt proteins comprise a family of highly conserved secreted signaling molecule. Wnt proteins signal through at least three known pathways. The best understood of these, commonly called the canonical pathway, involves binding of Wnt ligands to two distinct families of cell-surface receptors: the Frizzled (Fz) receptor family and the LDL-receptor-related protein (LRP) family [12]. Intracellularly, Wnt signaling activates Dishevelled (Dvl) proteins, which inhibit glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). The offset of GSK-3 $\beta$  is mediated through casein kinase 1 $\epsilon$ , which binds to the protein interaction domain PDZ and phosphorylates Dvl. This, in turn prevents GSK-3 $\beta$  from phosphorylating  $\beta$ -catenin. Thus, free  $\beta$ -catenin is stabilized and accumulates in the cytosol allowing its translocation into the cell nucleus where it can bind to transcription factors such as those of the LEF/TCF family. The  $\beta$ -catenin-Tcf/Lef complex then induces transcription of important downstream target genes, many of which have been implicated in cancer [13] (see also <http://www.stanford.edu/~rnusse/pathways/targets.html>). The other two pathways that Wnt proteins can signal through either activate calmodulin kinase II and

protein kinase C (the Wnt/Ca<sup>++</sup> pathway) or Jun N-terminal kinase (the planar cell polarity pathway) [14].

### **3. Wnt signaling in lung development and diseases**

The numerous functions of Wnt signaling in animal development include crucial roles in the morphogenesis of many organs including the gastrointestinal tract [15], mammary glands [16], cardiovascular system [17] and bone marrow [18]. The first indication that Wnt proteins had a dual role in embryogenesis and carcinogenesis came when it was learned that the drosophila homolog of the mouse mammary oncogene *int-1* was identical to the polarity gene wingless [19]. Recent data highlights the role played by the Wnt pathway in both embryonic and adult lung development. Wnt signaling regulates important aspects of both epithelial and mesenchymal development during gestation [20]. More precisely, knockout mice studies demonstrated the importance of *Wnt-2*, *Wnt-5a* and *Wnt-7b* in lung maturation [21-24]. Using oligonucleotide arrays, Bonner *et al.* demonstrated the contribution of the Wnt pathway to various stages of murine lung development [25]. Lastly, the Wnt/ $\beta$ -catenin pathway has been shown to be activated in lung inflammatory processes including idiopathic pulmonary fibrosis [26]. The Wnt pathway thus appears to play a multifaceted role; its activation is critical for lung development, while its deregulation can lead to inflammatory diseases and cancer transformation.

### **4. Wnt signaling and cancer stem cells**

Recent evidence suggests that stem cells may be the source of the mutant cells that give rise to cancerous tumors and maintain their growth [27]. Wnt signaling has been shown to promote self-renewal of stem cells, a phenomenon clearly established in the hematopoietic system [18, 28]. Very recently, it has been demonstrated that activation of  $\beta$ -catenin in CML granulocyte–macrophage progenitors is able to enhance the self-renewal activity leading to leukemic potential [29]. Wnt signaling has also been shown to be required for the maintenance and self-renewal of gut epithelial cells [15]. Studies of transgenic mice suggest that activation of the Wnt pathway in epidermal stem cells may lead to epithelial cancers [30]. Consistent with these findings, Clevers *et al.* have found that gene expression patterns in colon cancer cells resembled those in colon stem cells [31].

Although the extent of cancer stem cells involvement in solid tumor development is unknown, the potential of the Wnt pathway to control the fate of cancer stem cells and their self-renewal brings another argument for its critical role in carcinogenesis and raises the therapeutic interest of developing Wnt inhibitors.

### **5. Wnt signaling and lung cancer**

As discussed above, Wnt signaling is intricately involved in lung development, in cancer stem cell self-renewal and in carcinogenesis. We thus hypothesized that the Wnt pathway plays a critical role in lung carcinogenesis.

We and others recently reported the activation of the Wnt pathway in thoracic malignancies through alterations of many of its components.

## **5.1. Wnt ligands**

Nineteen Wnt proteins have thus far been identified in mammals. Wnt proteins signal via interaction with their corresponding Frizzled receptors which have 10 identified family members.

### **5.1.1 Wnt-1**

Wnt-1 was first identified from retroviral integration that caused mammary tumors in mice [2] and was found upregulated in various human cancers [32, 33]. Moreover, cancer cells expressing Wnt-1 are resistant to therapies that mediate apoptosis [34]. We recently demonstrated that Wnt-1 is overexpressed in NSCLC cell lines and primary tumor tissues [35]. Inhibition of Wnt1 by either siRNA or a specific monoclonal antibody induces apoptosis. Additionally, the monoclonal anti-Wnt-1 antibody suppresses tumor growth *in vivo* [35]. Rhee *et al.* found similar results in head and neck squamous cell carcinoma [7] suggesting that Wnt-1 mediated signaling is of key importance in controlling of apoptosis in cancers of epithelial origin.

### **5.1.2. Wnt-2**

The human *Wnt-2* gene, located on chromosome 7q31.3, is highly expressed in fetal lung and weakly expressed in placenta [36]. The link between *Wnt-2* and tumorigenesis was first proposed after data indicated that *Wnt-2* (known as *int-2* at the time) was amplified in human cancers [37]. Similarly *Wnt-2* has been implicated in mouse mammary tumorigenesis through gene amplification [38]. Wnt-2 was later shown to be upregulated in gastric cancers [39, 40], colorectal cancers [41-43] and melanoma [44]. We recently demonstrated the overexpression of Wnt-2 in non-small cell lung cancer [45]. Following the same study design as for Wnt-1, we demonstrated that inhibition of Wnt-2-mediated signaling by siRNA or a monoclonal antibody induces programmed cell death in NSCLC cells [45]. Moreover, the anti-Wnt-2 antibody has the potency to inhibit cell growth of primary cultures obtained from patients suffering from NSCLC (unpublished data)

### **5.1.3. Other Wnts**

A role for Wnt-7a has recently been proposed in lung cancer. First, it has been reported that expression of Wnt-7a is downregulated in most lung cancer cell lines and tumor samples [46]. It has been further hypothesized that Wnt-7a positively regulates E-cadherin expression in lung cancer cells [47]. Interestingly, Wnt-7a functions through a  $\beta$ -catenin-independent pathway during limb development [48] and appears to function through the canonical pathway in lung cancer even if TCF-LEF transcriptional activity is not directly targeted [47].

Similarly, Wnt-5a is a Wnt protein initially known for activating the Wnt/ $Ca^{++}$  noncanonical pathway during development [49], whose role in carcinogenesis remains controversial. Wnt-5a is upregulated in some cancers

[50] and can increase invasion of metastatic melanoma in a  $\beta$ -catenin independent manner [8]. Yet, Wnt-5a also behaves as a tumor suppressor gene in hematopoietic malignancies [51]. Since Wnt-5a has also been shown to be involved in the occurrence of lung metastasis in human sarcoma [52], its role in lung cancer is of high interest.

## 5.2. Dishevelled (Dvl)

Dvl proteins are positive mediators of Wnt signaling located downstream of the frizzled receptors and upstream of  $\beta$ -catenin. Three dishevelled genes have thus far been characterized, Dvl-1 to Dvl-3. Dishevelled proteins possess three conserved domains, an N-terminal DIX domain that binds to Axin [53], a central PDZ domain involved in protein-protein interactions [54], and a C-terminal DEP domain found in proteins that regulate RhoGTPases [55]. We showed that Dvl-3 was overexpressed in 75% of fresh NSCLC microdissected samples compared to autologous matched normal tissues [56]. Moreover targeted inhibition of Dvl-1, -2 and -3 decreased  $\beta$ -catenin expression, Tcf-dependent transcription and inhibited cell growth in human NSCLC cell lines [56]. We also demonstrated that a PDZ domain deletion mutant of Dvl could suppress tumorigenesis in pleural malignant mesothelioma [57]. Collectively, this data support the novel hypothesis that Wnt signaling is activated through Dvl overexpression in thoracic malignancies. A number of proteins have been shown to interact with dishevelled including Daam-1, Casein kinase 1 and 2, Notch and  $\beta$ -arrestin [58]. While most contribute to carcinogenesis, their respective interplay in the activation of Dvl proteins in lung cancer remains unknown.

## 5.3. $\beta$ -catenin

$\beta$ -catenin is a key player in the Wnt pathway, transmitting Wnt signals to the nucleus and playing a crucial in tumorigenesis either through the regulation of oncogenes (including cyclin D1 and c-myc) or through its own sporadic mutations.

Overexpression of  $\beta$ -catenin has been reported in lung cancer. It has been proposed that increased expression of  $\beta$ -catenin is associated with a high proliferative index and surprisingly with a better prognosis [59]. Two other independent studies reported that reduced expression of  $\beta$ -catenin might be associated with a poor prognosis in adenocarcinoma [60, 61]. The real significance of  $\beta$ -catenin expression in lung cancer is still controversial and this might be linked to the complexity of its effects since  $\beta$ -catenin also function as a cadherin-mediated cell adhesion component [62].

Moreover, mutations in the  $\beta$ -catenin gene appear uncommon in lung cancer. Sunaga *et al.* screened 46 lung cancer cell lines and 47 primary tissues and found mutations in the  $\beta$ -catenin gene, CTNNB1, in only one cell line and two surgical specimens, all adenocarcinomas, suggesting that genetic alteration of CTNNB1 is rare in lung cancer and restricted to the adenocarcinoma subtype [63]. Likewise, only 1 adenocarcinoma out of 90 primary lung cancer and 3 out of 76 lung cancer cell lines studied has been reported to have a mutation [64]. In another study, none of the 93 lung cancer cells analyzed displayed the mutations

[65]. Only fetal-type adenocarcinomas, a very unusual subtype of lung cancer, appear to more consistently possess the activating  $\beta$ -catenin mutations [66].

Based upon the previous studies, it appears that  $\beta$ -catenin, at least in itself, plays a less important role in lung carcinogenesis than in other malignancies, particularly colorectal cancer.

#### **5.4. APC**

APC has been identified as a tumor suppressor gene and is mutated in both sporadic and hereditary colorectal tumorigenesis. A frequent genetic alteration occurring in 40% of NSCLC is an allelic loss on chromosome 5q21 which contains the APC gene [67]. Nevertheless, among the 32 tumors studied in the former study, no mutation was found in the APC gene by single-strand conformational polymorphism analysis [67]. Other studies similarly failed to find APC mutations in 55 lung cancers analyzed by an RNA protection assay [68] and in 29 NSCLC analyzed by a yeast-based assay [69]. One recent study revealed 2 cases of APC mutation in 44 squamous cell carcinoma and 1 case in 32 small cell lung carcinoma suggesting that APC mutations may be involved in the pathogenesis of a small subset of lung carcinoma [70].

#### **5.5. Non-canonical pathway**

The vast majority of the data published about the Wnt pathway in cancer concerns the canonical Wnt pathway, which signals through the stabilization of  $\beta$ -catenin. However, more is becoming known about the so-called non-canonical,  $\beta$ -catenin independent Wnt pathway and its role in cancer [14]. Mechanisms of the non-canonical pathways are varied, including signaling through calcium flux, JNK and heterotrimeric G proteins.

The planar cell polarity (PCP) pathway is known to act through small GTPases. For example, Rho, Rac and Cdc 42 have been implicated in vertebrate noncanonical Wnt signaling [71-73]. The link between Rho proteins and Wnt signaling has been recently reinforced by showing the interactions of Tiam1 (a Wnt-responsive gene) and Rac [74], WISP-1 and Rac [75] and of Wrch-1 and Cdc42 [76]. Additionally, Wnt/Fz signaling has been shown to activate Rac and Rho through dvl proteins [77]. Interestingly, many of these small GTPases including RhoA [78], RhoB [79], RhoC [80], Rac [81] and Cdc 42 [82] are known to be involved in lung carcinogenesis but how they contribute to cancer development remains unclear. We can thus hypothesize that crosstalks between Rho and Wnt signaling may contribute in some way to lung tumorigenesis, particularly to the processes of cell adhesion and migration [83].

Another putative mediator of non-canonical Wnt signaling is the JNK pathway [14]. The role of JNK in Wnt-mediated signaling is unclear. It has been shown that Dvl induces JNK activity through its DEP domain in planar cell polarity [84] and that this activation may involve some Rho GTPases [77, 85]. We recently provided new insights into the field by showing that Wnt-1 blockade by either siRNA or a monoclonal antibody induces apoptosis in  $\beta$ -catenin deficient mesothelioma cell lines underlining the role of the Wnt noncanonical pathway(s) in this process. Interestingly, JNK was found to be up-regulated in these cell lines

after blocking Wnt1 signaling. Moreover, treatment of these cell lines with a selective JNK-1, JNK-2, JNK-3 inhibitor, SP600125, significantly inhibits the apoptotic cell killing induced by Wnt-1 blockade. This data suggests that both canonical and noncanonical pathways may be involved in the Wnt-1-mediated apoptosis [86].

## **6. Wnt antagonists**

Wnt-mediated signals are modulated extracellularly by secreted proteins. Wnt antagonists can be divided into two groups: the first group includes the sFRP family, WIF-1 and Cerberus and inhibits Wnt signaling by direct binding to Wnt molecules. The second group including the Dickkopf (DKK) family inhibits Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex [87]. These inhibitors have been studied extensively in developmental studies. Recently, their involvement in oncogenesis has been demonstrated in cervical carcinomas [88], breast cancers [89], gastric cancers [90] and colorectal tumorigenesis [91-93]. Their contribution to lung carcinogenesis has recently been highlighted.

### **6.1. Wnt inhibitory factor-1 (WIF-1)**

*WIF-1* is a highly conserved gene first identified from the human retina. Overexpression of WIF-1 in *Xenopus* embryos blocks the Wnt-8 pathway and induces abnormal somitogenesis [94]. Recently, Wissman *et al.* reported the downregulation of WIF-1 in several cancer types including lung cancer using a chip hybridization assay and immunohistochemistry [95]. By using MSP (methylation specific PCR) and sequence analysis after bisulfite treatment, we recently demonstrated frequent hypermethylation of CpG islands in the functional *WIF-1* promoter region that correlated with its transcriptional silencing in human lung cancer cell lines [96].

We also studied WIF-1 expression in freshly resected lung cancers and showed a downregulation in 83% of cases. This silencing also correlates with *WIF-1* promoter methylation [96]. We thus propose that methylation-silencing of *WIF-1* is a common and likely important mechanism of aberrant activation of the Wnt signaling pathway in lung cancer pathogenesis.

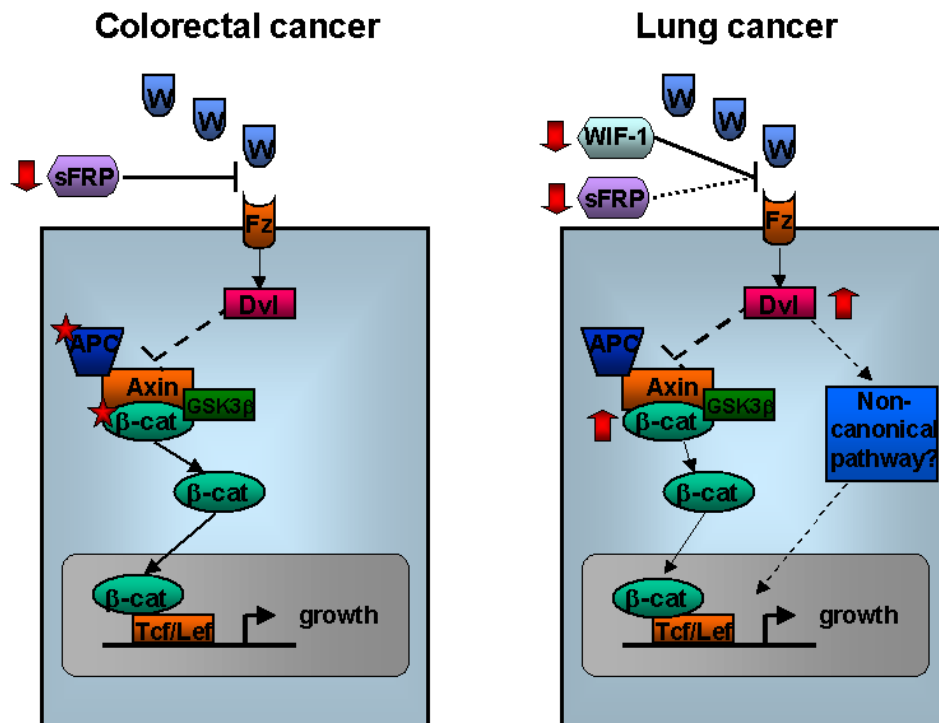
### **6.2. Secreted frizzled-related proteins (sFRP)**

sFRP are endogenous modulators of Wnt signaling that compete with the Wnt ligands for the binding to the frizzled receptors. Previous studies have shown sFRP downregulation in colorectal cancer, gastric cancer [91] and invasive breast tumors [33]. Interestingly, Suzuki *et al.* demonstrated that restoration of sFRP function in colorectal cancer cells attenuates Wnt signaling even in the presence of  $\beta$ -catenin mutation and may complement downstream mutations in the development of colorectal cancer [92]. We recently showed that sFRP was frequently downregulated in NSCLC and mesothelioma cell lines [97]. Moreover, sFRP gene promoter was hypermethylated in more than 80% of mesothelioma primary tissues [97]. Methylation of sFRP appears to occur less frequently in NSCLC (unpublished data). Our results suggest that methylation

silencing of sFRP may be one of the important mechanisms of aberrant signaling activation in mesothelioma and to a lesser extent in NSCLC.

### 6.3. DKK

The DKKs (DKK-1; -2 ; -3) are secreted glycoproteins that have the ability to antagonize Wnt-mediated signal [98]. The REIC/DKK-3 gene has been found downregulated in many cancer cell lines including a NSCLC cell line [99] and in 63% of freshly resected NSCLC tissues [100]. It has also been shown that forced expression of Dkk-3 is able to inhibit cell growth [101]. Moreover, no mutation has been found within the REIC/DKK-3 gene. The gene is instead silenced by promoter hypermethylation in a high proportion of lung cancers [102].



**Figure 1.** Overview of Wnt pathway activation in colorectal and lung cancer. Binding of Wnts (W) to Frizzled receptors (Fz) activates Dishevelled proteins (Dvl) which in turn blocks the function of a multi-protein complex including Axin, APC and GSK-3 $\beta$ . In the presence of Wnt, phosphorylation and degradation of  $\beta$ -catenin is blocked which allows the association of  $\beta$ -catenin with TCF transcription factors. The TCF/ $\beta$ -catenin complexes bind to DNA and activate Wnt target genes, leading to cell growth. In colorectal cancer, the Wnt pathway is mostly activated through mutation of APC and  $\beta$ -catenin. Downregulation of sFRP by methylation has also been shown to be a mechanism of Wnt activation even in presence of the mutations. In lung cancer, Dishevelled (Dvl) proteins are overexpressed, leading to activation and cytosolic release of  $\beta$ -catenin. WIF-1 and to a lesser extent sFRP are downregulated by epigenetic methylation. Moreover,  $\beta$ -catenin-independent pathways involving probably JNK and RhoGTPases (here shown as “?” due to the lack of data in this field) are also triggered by the Wnt proteins. Lines ending with arrows or bars indicate activating or inhibitory effects, respectively. Red stars indicate mutations whereas red arrows indicate under- ( $\downarrow$ ) or over- ( $\uparrow$ ) expression of the adjacent protein. Dashed lines indicate that the process is still unclear whereas solid lines describe proved connections.

## **7. Conclusions**

Wnt signaling has clearly emerged as a critical pathway in lung carcinogenesis, similar to its role in other cancers, particularly colorectal cancer. Here we propose that in lung cancer, a different mechanism exists than in colorectal cancer for activation of the Wnt-mediated signal (Fig. 1). Specifically in lung cancer, mutations of APC or  $\beta$ -catenin are rare and the Wnt pathway appears to be activated upstream of  $\beta$ -catenin. We identified at least three mechanisms of activation: overexpression of Wnt effectors such as Dvl, activation of a noncanonical pathway involving JNK and repression of Wnt antagonists such as WIF-1 (Fig. 1). The role and scope of each mechanism and their relationship to one another remain unclear.

Nonetheless, we suggest that many of the studied components of the Wnt pathway have been sufficiently studied here to present them as attractive targets for potential therapeutic agents. We propose that the design of compounds to block the Wnt pathway represents a novel and plausible treatment strategy for lung cancer.

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