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# WNT signaling in malignant mesothelioma

**Simon Fox [1] and Arun Dharmarajan [2]**

*[1] Pharmacogenetics Laboratory, School of Pharmacy, Curtin University of Technology and West Australian Biomedical Research Institute,; Bentley, WA, Australia, [2] School of Anatomy and Human Biology, The University of Western Australia; Crawley, WA, Australia*

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## 1. ABSTRACT

Neoplastic transformation of mesothelium is commonly associated with exposure to asbestos and gives rise to malignant mesothelioma, an aggressive disease that has proved particularly refractory to conventional anti-cancer therapies. The Wnt signaling pathways play key roles in fundamental processes, which include both development and homeostasis. The importance of these pathways in tumorigenesis is emphasized by the many cancers which show aberrations in Wnt signaling. In this review we examine the current evidence for activation of Wnt signaling and the abnormal expression of specific molecules in malignant mesothelioma.

## 2. INTRODUCTION

Malignant mesothelioma (MM) is a particularly aggressive cancer that is characterized by rapid progression, late metastases and poor prognosis [reviewed in (1)]. Although this tumor is relatively uncommon, the incidence is expected to continue to rise at least over the next decade as a consequence of high past asbestos use and the long latency period between exposure to asbestos and tumor development. MM is highly resistant to conventional forms of anti-cancer therapy and both radiotherapy and chemotherapy have limited effect. Despite the exploration of a variety of new therapeutic approaches with some success, treatment remains palliative at best (2). The

typically advanced stage of the disease at presentation and the inherent properties of mesothelioma cells are contributory factors to this resistance to therapy. Future improvements in the therapy of this disease will rely upon the application of knowledge regarding mesothelioma biology derived from ongoing basic research.

Tumorigenesis in the mesothelium gives rise to a neoplasm, which is characterized by phenotypic variation in histopathological appearance. Mesotheliomas manifest epithelial or sarcomatous morphology and often these may be present simultaneously (1). Tumors with sarcomatous morphology are associated with a poorer prognosis and rapid progression. In culture, mesothelioma cell lines derived from tumors can exhibit either an epithelial morphology with a classical cobblestone appearance or a spindle shaped fibroblastic morphology. In a number of these mesothelioma cell lines experimental induction by varying the serum constituents of the culture media results in differentiation between epithelial and fibroblast-like phenotypes (3).

The Wnt proteins activate a number of signaling pathways that play a key role in fundamental processes of development and the maintenance of homeostasis in adult tissue. The pathways activated by Wnts include those that signal via beta-catenin (canonical) and at least 3 others that utilize other mechanisms (non-canonical). It is well recognized that the deregulation of homeostatic mechanisms controlling cell proliferation and death is a critical step in tumorigenesis. Loss of control of Wnt signaling pathways has been widely described in cancer and is thought to be an important factor in tumor development (4). Investigations of Wnt signaling in cancer have focused upon the genesis of cancer, probably for historical reasons. However, numerous studies have demonstrated the contribution of Wnts to anti-apoptotic signaling, both directly and by crosstalk with other pathways, in normal tissue and neoplasia (5). The means by which Wnt signaling influences apoptosis have yet to be fully elucidated, however the evidence to date and the complexities of this pathway indicate that there are multiple mechanisms. Activation of apoptotic death pathways is the primary mechanism by which both cytotoxic drugs and immune cells kill cancer cells. Defects in apoptotic signaling and regulation are thought to be significant contributors to resistance to anti-tumor therapy (6) and from a practical perspective therapies that target Wnt signaling may be an avenue to address this resistance.

Investigation of the role that Wnt signaling plays in the pathogenesis, progression and resistance to apoptosis of MM has received limited attention until recently in comparison with other neoplasms. Both mesothelial cells and the tumors that arise from them display notable features, particularly in their inherent plasticity, which makes them of interest with regard to investigation of the role of Wnt signaling in both pathological and normal processes.

## 3. THE MESOTHELIUM

The mesothelium consists of a specialized monolayer of cells that covers the surface of the body's three serosal cavities (pleural, pericardial and peritoneal) and covers the internal organs within them. This layer sits upon a thin basement membrane supported by a layer of vascularized connective tissue containing lymphatics and subserosal mesenchymal cells. Morphologically, mesothelial cells most commonly have a flattened, epithelial-like appearance with a diameter of about 25 micrometers. From a functional perspective the mesothelium has a number of important regulatory and physical properties. The classical roles ascribed to the mesothelium were to provide a protective barrier both to microbial infection and physical damage as well as enabling free movement of the organs by generating a low friction interface between serosal surfaces. More recently, it has been recognized that this tissue performs a number of other functions including transport of solutes and particulates, regulation of inflammation and leukocyte trafficking and control of tissue repair, coagulation and fibrinolysis [reviewed in (7) and (8)].

Embryologically, the mesothelium is derived from the mesoderm; however, mesothelial cells are unique in that they show patterns of expression that are characteristic of both mesenchymal and epithelial cells. Mesothelial cells express mesenchymal markers such as vimentin and desmin but also express characteristic epithelial cytokeratins. Emphasizing the duality of mesothelial cells is their expression of factors that are considered characteristic of mesenchymal-epithelial interactions such as keratinocyte growth factor and hepatocyte growth factor as well as their cognate receptors (8). Another feature that distinguishes mesothelium is the markedly different mechanism of regeneration from injury. In contrast to epithelia where wounds heal from the edges, in the mesothelium healing occurs diffusely over the area of the injury apparently driven by cells that settle on the wound via the serosal fluid (9). Current evidence has indicated the existence of a population of mesothelial progenitor cells that are capable of switching phenotype in response to local signaling (10). Wnt proteins are thought to regulate stem/progenitor cell biology (4) and this is likely to also be the case in mesothelial cells although to date neither Wnts nor the role of such cells in the genesis of mesothelioma have been explored.

## 4. MESOTHELIAL AND MESOTHELIOMA CELL PLASTICITY

As described above mesothelioma cells may manifest epithelial or fibroblastic morphology both *in vivo* and *in vitro* with similar phenotypic differentiation inducible in culture. Mesothelial cells also exhibit this capacity to change phenotype in a process of transdifferentiation that resembles the changes found in epithelial to mesenchymal transition (EMT). In culture, following several passages, mesothelial cells lose expression of cytokeratins and manifest a fibroblastic phenotype (figure 1) but revert to an epithelial-like phenotype when reintroduced to an intact mesothelium (9). Various growth factors can also induce similar phenotypic changes in mesothelial cells, for example transforming growth factor beta 1 (TGF-beta1) induces EMT in

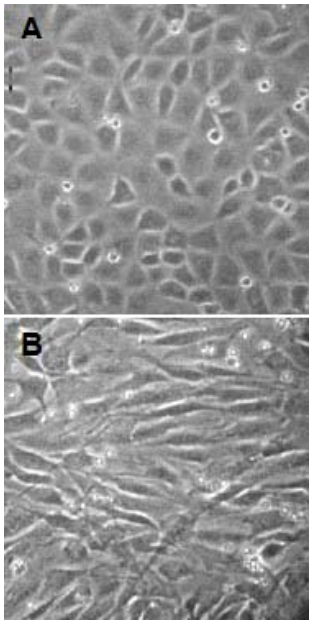


**Figure 1.** Morphology of mesothelial cells in culture. Human pericardial mesothelial cells display (A) epithelial-like or (B) fibroblastic phenotypes in the same cell population at different passage numbers. Adapted from (10).

mesothelial cells (11) and similar observations have been made for epidermal growth factor (EGF) (10). In cultures of MM cells both EGF and insulin-like growth factor 1 (IGF-1) have been reported to induce fibroblastic morphological changes (12). EMT in mesothelioma cells has also been associated with changes in the expression of cell surface proteoglycans: syndecans and glypicans (3). Interaction with these proteoglycans is known to regulate the stability and local distribution of growth factors and Wnts (13,14). They are also thought to modulate cell signaling by facilitating the interaction between these ligands and their receptors (13,14). Thus far investigation of this aspect of mesothelial and mesothelioma cell biology has been directed to the involvement of various growth factors. However, the contribution of Wnt/FZD signaling to EMT in development and tumor progression is well recognized [reviewed in (15)]. Wnt/FZD signaling is likely to be of similar relevance to the regulation of morphological behaviour in MM. Given the association of cellular phenotype with aggression in MM (1) this aspect of the Wnt pathway warrants further investigation in this tumor.

## 5. WNT/FRIZZLED SIGNALING IN MESOTHELIOMA

### 5.1. Beta-catenin function and signaling

Beta-catenin plays a central role in the canonical Wnt/Frizzled (FZD) pathway, transmitting Wnt signals and in cancer, promoting tumorigenesis via transcriptional regulation of oncogenes such as cyclin D1 and c-myc (figure 2). Assessment of differences in beta-catenin protein levels between neoplastic and normal tissues has been frequently used as an indicator of upregulated canonical signaling. Two studies have provided preliminary evidence that canonical Wnt signaling is active in mesothelioma (16,17). Immunohistochemical evidence for increased beta-catenin levels has been found in mesothelioma tumors, primary tumor cultures and cell lines (16,17). Given the complexities of the regulation of beta-catenin levels including interactions with the cadherin pathway [reviewed in (18)] the specificity of these findings requires further confirmation.

Mutational events that result in constitutive Wnt signaling have been described in a variety of cancers focusing predominantly on the genes encoding beta-catenin itself or direct regulators such as adenomatous polyposis coli (APC) and axin (4). Activating mutations of the beta-catenin gene (*CTNNB1*) have not been found to date in MM. Abutaily *et al* (16) screened 63 primary MM tissues and did not find mutations in the *CTNNB1* gene. Similarly, a smaller study (19) of 8 mesothelioma cell lines and 2 tumors did not find activating mutations in *CTNNB1*, although in one MM cell line (NCI-H28) the majority of the gene was deleted. This latter finding may be of little significance to mesothelioma where tumors are invariably positive for beta-catenin (16,17,20) but this beta-catenin null cell line has proved useful for investigations of non-canonical Wnt signaling as described below. Although loss of function mutations in axin and, notably, APC have been implicated in tumorigenesis for many cancer types, thus far these genes have not been investigated in mesothelioma. Abutaily *et al* (16) described possible C-terminal truncations of APC in 23% of a cohort of 63 mesothelioma tumors investigated using immunohistochemistry, however, confirmatory genetic studies have not been reported.

In addition to mutational events, perturbed expression of many other molecular components of Wnt signaling pathways have been demonstrated in a variety of cancers (4,21). As yet there are only sporadic reports in the literature regarding the expression of individual molecules in MM although our own laboratory has undertaken a systematic survey of MM cell lines (manuscript in preparation). He *et al* (22) used immunoblotting to demonstrate expression of Wnt1 in a range of tumor cell types including 5 mesothelioma cell lines and went on to show that blockade of Wnt1 increased apoptosis, although these blocking experiments were not performed in the mesothelioma cells. More recently, Mazieres *et al* (23) have employed a Wnt specific gene array to analyze differences between MM and matched normal pleura in 8 patients and demonstrated Wnt2 upregulation as the most frequent event. This result was confirmed at the protein level and also demonstrated in MM cell lines. Treatment of these cell lines with Wnt2 antibody or downregulation of Wnt2 by RNA interference resulted in reduced cytosolic beta-catenin and dishevelled-3 (Dvl-3) as well as decreasing cell proliferation and increasing apoptosis. While the mechanism behind Wnt2 upregulation is unclear this does mirror evidence regarding Wnt2 in other cancers [reviewed in (24)] and provide impetus for further examination of this molecule in MM. Increased levels of Dvl-3 have also been reported in MM tissue relative to

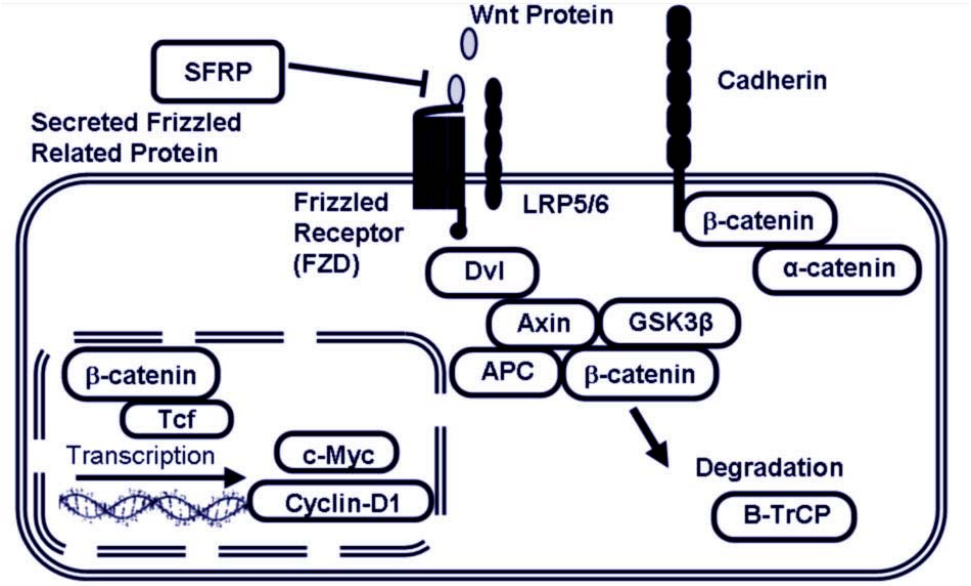


**Figure 2.** Role of specific molecules in canonical Wnt signaling. In the absence of Wnt signal free beta-catenin is degraded following phosphorylation through interactions with the protein kinase GSK3beta, axin and APC. Wnt ligands bind to a Frizzled/LRP receptor complex activating dishevelled (Dvl) which then inhibits the phosphorylation and subsequent degradation of beta-catenin. As beta-catenin accumulates it translocates to the nucleus and activates Tcf-Lef dependent transcription of a number of target genes, which may vary between cell types and often include c-Myc and cyclin D1. Beta-catenin is also found bound to cadherin at the cell membrane where it participates in cell adhesion. The role of this cadherin bound pool in Wnt signaling remains unclear.

normal pleura (17). However, since Wnt2 regulated Dvl-3 in these cell lines (23) it is not clear whether this increased Dvl-3 represents genetically determined overexpression of this gene in isolation or indicates general upregulation of Wnt signaling.

**5.2. Secreted regulators of Wnt/Frizzled signaling in mesothelioma**

Given the significance of Wnt signaling in determination of cell fate it is not surprising that this pathway is regulated via a diverse array of strategies. These include secretory factors among which are the secreted frizzled related proteins (sFRPs), which are structurally related to the FZD receptors [reviewed in (25,26)]. The sFRPs are believed to act principally as antagonists of Wnt signaling by blocking Wnt/FZD interactions. This is consistent with their frequent downregulation in carcinomas and postulated role as tumor suppressors. There is evidence that sFRP molecules may interact with both Wnts and FZDs. However, recent evidence does suggest that the role of sFRPs is somewhat more complex and depends upon context and cell type since they may also potentiate Wnt signaling under some circumstances (25). sFRP4 is a member of this family whose role in cancer is somewhat conflicting with reports of both down and up regulation in tumor cells and tissues (27-31). sFRP4 has been implicated in apoptosis (32-34), regulation of proliferation (27) and tumor progression (27,28,31). Recently Lee *et al* (35) have reported a study that used RT-PCR to show that expression of sFRPs 1, 3, 4 and 5 were detectable in normal pleura but apparently downregulated in mesothelioma tissue samples and two cell lines. There is also evidence from both overexpression (35) and RNAi experiments (36) that in MM sFRP4 may suppress growth and induce apoptosis. In our own laboratory we have found that sFRP4 was expressed in 4/4 primary human mesothelial cell cultures but only 1/4 human MM cell lines (unpublished data). Similarly, we have examined mouse models of mesothelioma and found downregulated expression of sFRPs relative to normal mesothelial cells (Table 1). These results indicate that murine models of MM are likely to be useful for in vivo investigations of the role of sFRPs in tumor biology.

**5.3. Non-canonical signaling in malignant mesothelioma**

By far the majority of studies of Wnt signaling in cancer have concerned the canonical pathway, which is the best characterized to date. However, there are a number of alternate pathways through which Wnts may signal, independent of beta-catenin, with diverse downstream consequences predominantly involved in morphogenesis. Two non-canonical pathways have typically been described: the planar cell polarity (PCP) and the Wnt/Calcium pathways. However, these pathways are ill-defined and current evidence suggests that it may be more appropriate to divide them into at least three distinct pathways [reviewed in (37)]. The diverse mechanisms in these pathways that transduce Wnt/FZD signals include heteromeric GTP-binding proteins, calcium dependent kinases (CamKII and PKC) and JNK. These alternate pathways also play a role in regulating canonical signaling and several mechanisms by which calcium dependant crosstalk antagonizes beta-catenin signaling have been described (37).

**Table 1.** Expression of sFRP mRNA in mouse mesothelioma and mesothelial cells

| | **Mesothelioma cells** | | | | | **Mesothelial cells** | |
|---|---|---|---|---|---|---|---|
| Gene | AC16 | AC29 | AB1 | AB12 | AE17 | CBA | Balb/c |
| SFRP1 | - | - | - | - | - | + | + |
| SFRP2 | + | - | - | - | + | + | + |
| SFRP3 | - | - | - | - | - | + | + |
| SFRP4 | + | - | - | - | - | + | + |
| SFRP5 | - | - | - | - | - | - | - |

Expression was assayed by RT-PCR using gene specific primers. Mouse MM cell lines were derived from asbestos inoculated CBA/CaH (AC16, AC29), Balb/c (AB1, AB12) or C57BL/J mice (AE17) (41,42)**.** Mouse mesothelial cells were primary peritoneal mesothelial cultures from CBA or Balb/c mice.

In MM the identification of a beta-catenin null cell line (19) has prompted investigation of non-canonical pathways in these cells. Dickkopf-1 (Dkk-1) is an extracellular regulator of Wnt/FZD signaling which is thought to interact with LRP5/6 and FZDs to inhibit canonical Wnt signaling (26). Studies in MM cells have shown that Dkk-1 also inhibits proliferation and induces apoptosis by a beta-catenin independent mechanism that is mediated by JNK (38). This indicates that Dkk-1 may also have a role in regulation of non-canonical signaling. Similar studies of sFRP4 showed that this molecule could inhibit proliferation and induce apoptosis in both beta-catenin expressing and non-expressing cell lines (36). Blockade of Wnt1 signaling in beta-catenin null cells also indicated that downstream signaling induced by this Wnt1 had a non-canonical component, which was mediated by JNK and contributed to proliferation and apoptotic resistance (39). These beta-catenin deficient cells have also proved useful in demonstrating that another member of the catenin family, gamma-catenin, can activate Wnt signaling in the absence of beta-catenin (40). While the physiological relevance of these findings to typically beta-catenin positive MM cells is unknown, these studies have provided useful insights into non-canonical signaling pathways.

## 6. PERSPECTIVE

Investigations of Wnt/FZD signaling have indicated that this pathway plays a role in MM cell proliferation and resistance to apoptosis. Research to date using whole MM tumor tissue has faced the conundrum presented by all such studies that the tissue, unless microdissected, includes a large proportion of inflammatory and other cells which may confound the specificity of gene expression findings. Conversely *in vitro* studies of cell lines may not always be physiologically relevant. Even so, these findings have prompted the view that therapies targeting this pathway may have application to MM as has been suggested for other pathways. Investigation of patterns of expression in clinical tumor samples and *in vitro* experiments can provide essential data regarding molecular mechanisms and interactions in mesothelioma. However, it is also necessary to investigate the role of specific molecules and Wnt signaling within the more complex physiological milieu. For example, growth factor signaling, cell adhesion and other factors produced by stromal cells within the tumor microenvironment result in a different molecular and cellular context which may influence Wnt signaling and its effects upon both tumor and non-tumor cells. Unraveling the role of this complex pathway in MM and in mesothelial cell biology may provide us with a better understanding of the pathogenesis of this disease and is likely to lead to improved therapies.

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**Abbreviations:** APC: adenomatous polyposis coli; CamKII: calcium/calmodulin-dependent kinase II; Dkk: dickkopf; Dvl: dishevelled; EGF: epidermal growth factor; EMT: epithelial to mesenchymal transition; FZD: frizzled; GSK3beta: glycogen synthetase kinase 3 beta; IGF-1: insulin-like growth factor 1; JNK: Jun-N-terminal kinase; LRP: low density lipoprotein receptor related protein; MM: malignant mesothelioma; PCP: planar cell polarity; PKC: protein kinase C; RNAi: RNA interference; RT-PCR: reverse transcription polymerase chain reaction; sFRP/SFRP: secreted frizzled related protein; TcF/LeF: T-cell factor/lymphoid enhancer factor; TGF-beta1: transforming growth factor beta 1.

**Key Words:** Mesothelioma, Wnt, Frizzled, sFRP, Apoptosis, Review

**Send Correspondence to**: Prof. Arun Dharmarajan, School of Anatomy and Human Biology, The University of Western Australia, Crawley, WA 6009, Australia. Tel: 618-6488-2981, Fax: 618-6488-1051, E-mail: dharma@anhb.uwa.edu.au

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