Review

Cancer Research

Wnt Signaling in Cancer: Not a Binary ON:OFF Switch

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Abstract

In the March 1 issue of *Cancer Research*, we identified the Wnt receptor Fzd7 as an attractive therapeutic target for the treatment of gastric cancer. In summary, we showed that pharmacological inhibition of Wnt receptors, or genetic deletion of *Fzd7*, blocks the initiation and growth of gastric tumors. Inhibiting Fzd receptors, specifically Fzd7, inhibits the growth of gastric cancer cells even in the presence of *adenomatous polyposis coli* (*Apc*) mutation. Apc is located in the cytoplasm downstream of Fzd7 in the Wnt signaling cascade and *APC* mutations activate Wnt/β-catenin signaling, therefore, this result seems counterintuitive. Here, we analyze this result in greater detail in the context of current knowledge of Wnt signaling and discuss the wider implications of this aspect of Wnt signaling in other cancers.

Regulation of Wnt Signaling Intracellularly and at the Plasma Membrane

The Wnt signaling pathway regulates many cell functions, including proliferation, migration, apoptosis, and differentiation (1). It is essential during embryonic development and also in homeostasis of several adult tissues, including the GI tract (2, 3), liver, breast, and skin (1), and is deregulated in many cancers, including colon, gastric, breast, and liver (4). Wnt ligands are secreted glycoproteins that bind to a "U" shaped pocket in the dimer of Fzd receptors via a lipid modification of palmitoleic acid to form a receptor complex with co-receptors, including Lrp5/6 and Lgr4/5 (1). This complex interacts with components of a cytoplasmic "destruction complex" that contains Gsk3, Axin, Ck1, and Apc, to form a large signalosome (1). In the absence of a Wnt ligand, newly translated β-catenin proteins are bound and phosphorylated by the destruction complex. However, in the presence of Wnt/Fzd binding, signalosome assembly disrupts this process and β-catenin is free to accumulate, translocate into the nucleus and associate with transcription factors Tcf/Lef to regulate target gene transcription (Fig. 1A; ref. 1).

Nuclear β-catenin, a hallmark of active Wnt signaling (1), is observed in approximately 30% of human gastric tumors (5). Next-generation sequencing has revealed that Wnt signaling is deregulated in gastric tumors at several points in the pathway, including the ligand, receptors, and intracellular transduction components (6, 7). In addition to mutations, epigenetic changes are also observed in gastric tumors to Wnt inhibitors such as *sFRP* (binds directly to Wnt ligands) and *Dkk1* (binds to and inhibits Lrp5/6 receptors), resulting in activation of Wnt signaling at the level of the receptor/ligand (1). Indeed, RNF43, an E3 ubiquitin ligase that turns over Fzd on the cell surface (8), is mutated in approximately 18% of human gastric tumors, whereas many Wnt ligands are overexpressed (7). This is around the same frequency (∼18%) observed for *APC* mutations in gastric cancer, approximately 37% of which also contain an *RNF43* mutation, indicating compound activation of Wnt signaling in the same tumor (7). This observation that Wnt signaling is regulated intracellularly and at the plasma membrane is consistent with our experimental data in which we could inhibit the growth of *Apc*-mutant gastric tumors by deleting *Fzd7* and prompts a frequently asked question when presenting this work, "how can a cell with mutant *Apc* respond to inhibition of a Fzd receptor upstream?"

Heterogenous Wnt signaling regulates distinct cell functions in a tumor

To answer this question, we first need to highlight that Wnt signaling is not a binary system whereby the pathway is on or off, but rather is highly regulated in a compound fashion to provide spatial and temporal variation depending on multiple environmental factors (9). This was first demonstrated by Thomas Brabletz in 1998, who showed heterogenous expression of nuclear β-catenin (a surrogate marker of active Wnt signaling) within human colorectal cancers, despite all tumor cells harboring *APC* mutation (Fig. 1B; ref. 10). Furthermore, Wnt signaling is promoted by macrophage-derived TNF-α (11) and *H. pylori* infection can activate the Wnt pathway via Fzd7 (12), highlighting a role for the microenvironment for variable Wnt activity in tumors. This indicates that additional factors can regulate Wnt signal strength even in *APC*-mutant cells. This can occur because the *APC* gene is not deleted but truncated and mutant *APC* is still transcribed and translated into a protein that is able to partially function in the destruction complex and respond to WNT ligands, albeit at a reduced level (13, 14). Indeed, Wnt3 secretion maintains high Wnt signaling in *APC*-mutant colon cancer cells (14), whereas *APC* is methylated, and subsequently inhibited further during

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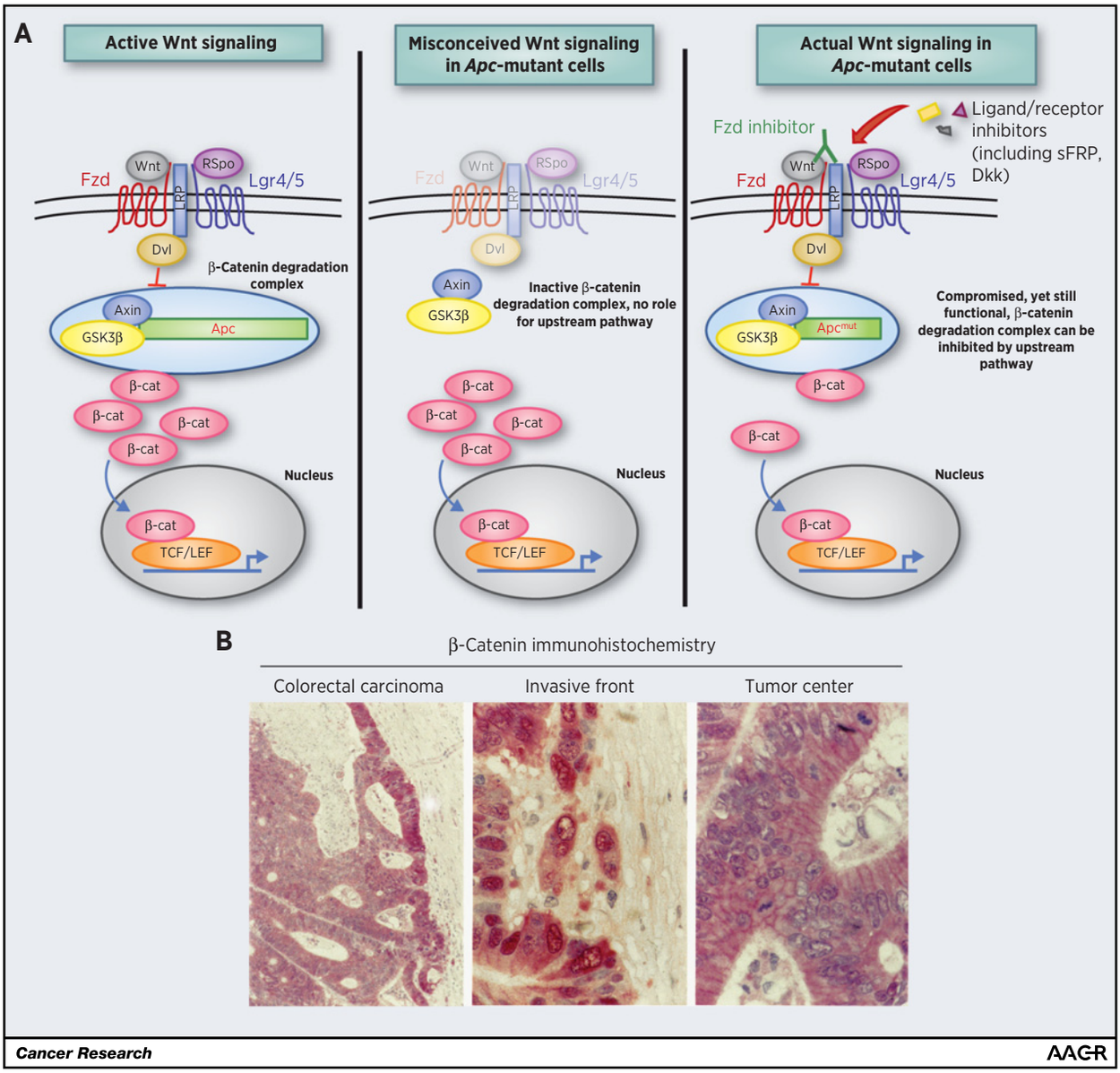

Figure 1.

Wnt signaling with mutant *Apc*. **A,** The levels of cytoplasmic β-catenin are regulated by the degradation complex that is inhibited when Wnt signaling is active (left). In *Apc*-mutant cells, it is often misconceived that Apc is completely deleted and therefore the degradation complex is nonfunctional and Wnt signaling cannot be regulated upstream of the degradation complex at the level of the receptor/ligand (illustration faded out; middle). However, mutant *Apc* is transcribed and translated, resulting in a compromised, yet functional, β-catenin degradation complex that explains how upstream factors, including sFRP, Dkk, and Fzd inhibitors, can still modulate Wnt signal activity (right). **B,** Immunohistochemistry for β-catenin in a human colorectal carcinoma showing increased nuclear localization, as a surrogate marker of active Wnt, in the invasive front compared with the tumor center. **B** is reprinted with permission from Brabletz et al. Pathol Res Pract. 1998;194:701–4 (10).

tumor progression in colon tumors, highlighting variable Wnt activity as a feature of colon tumors (15). This explains why *APC*-mutant cells are sensitive to Fzd inhibition in our work, but what is the function of this variable Wnt signal?

Approximately 37% of *APC*-mutant gastric tumors also contain *RNF43* mutations, indicating compound activation of Wnt signaling in the same tumor (3). However, only 5.5% of colon tumors have *APC* and *RNF43* mutations, suggesting that gastric cancer and colorectal cancer preferentially select different mechanisms of optimal, "just right," levels of Wnt signaling required for tumor growth and progression. The "just right" model of Wnt signaling proposes *APC* mutations are selected for sub-maximal levels of Wnt signaling to provide a Wnt signal that is sufficient to transform cells, but not excessive and cytotoxic (16). Experimental evidence from *in vivo* data helped confirm these models. For instance, the $Apc^{1322T/+}$ mouse model retains a

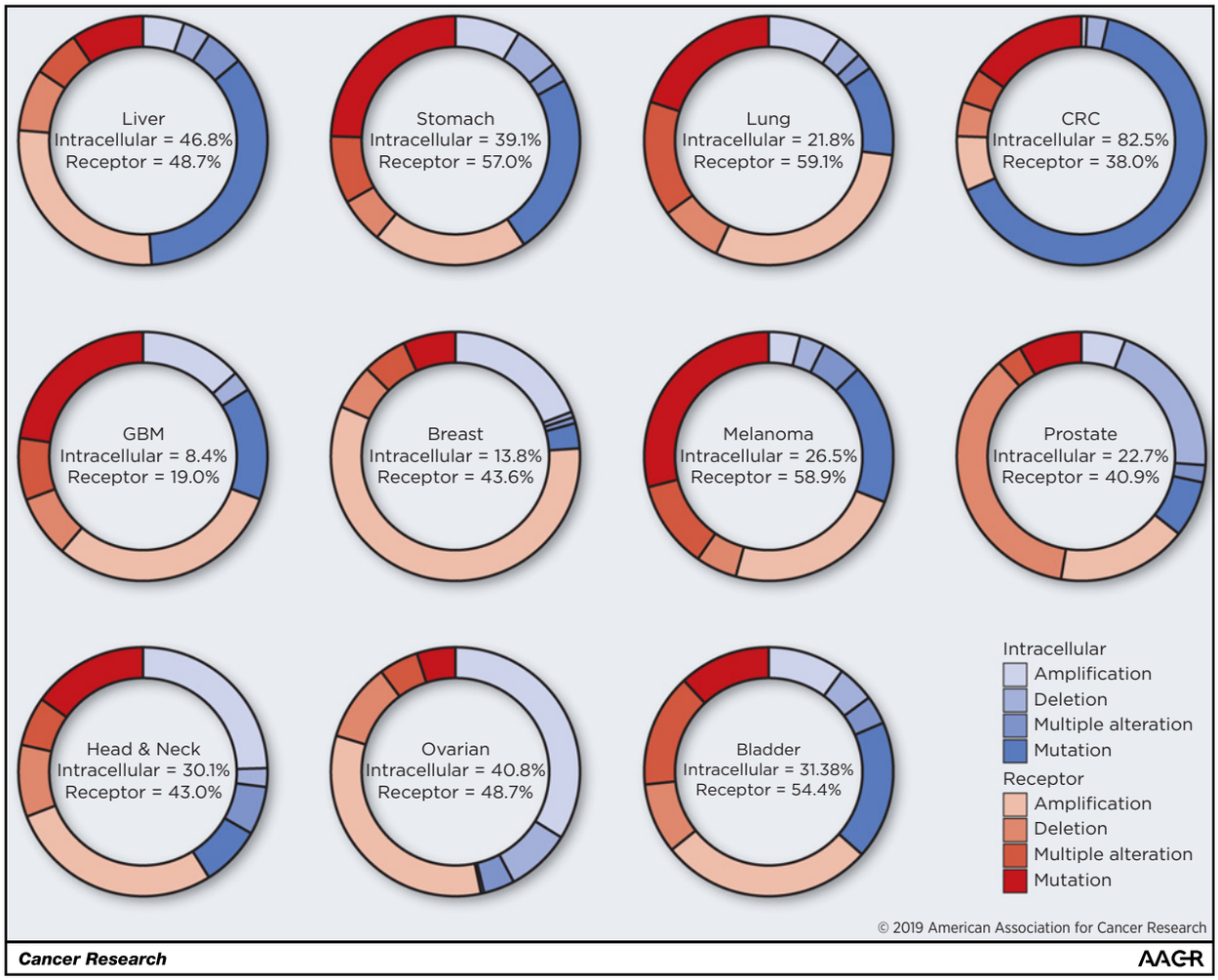

Figure 2.

Alteration of Wnt pathway components in epithelial cancers. Analysis of selected datasets from The Cancer Genome Atlas for mutations, amplifications, and deletions of either intracellular (APC, β-catenin, Axin2, and Dvl) or receptor (e.g., WNTs, FZDs, RNF43, and sFRP) pathway components. Relative frequency of genetic alterations is shown as percentages. CRC, colorectal cancer; GBM, glioblastoma. This figure was generated using data available at www.cbioportal.org.

single β-catenin binding domain in the mutant Apc^{1322T} allele, which develops more severe intestinal tumors, but with lower levels of Wnt signaling compared with other *Apc*-mutant models that lack β-catenin–binding capacity (17). In addition, we have previously shown that intestinal tumorigenesis is reduced in $Cited1^{+/-}$; $Apc^{Min/+}$ mice due to very high, cytotoxic Wnt signaling (18). These data correlate with observations of improved clinical outcomes after relapse for molecular subtypes of colon cancer that display high Wnt signaling (19).

Notably, a common theme is emerging for epithelial tumors that indicates that cancer cells harbor mutations of intracellular components of the Wnt pathway with additional mutations or epigenetic modifications at the level of the receptor/ligand to permit further regulation of Wnt signaling (ligand and receptor overexpression; Fig. 2). The net result, at least in colon cancer, appears to be to constrain Wnt signaling within an optimal "just right" spectrum in the cancer cells, with bursts of intense signaling in a context-dependent manner, for example, at the invasive front of colon tumors (Fig. 1). Given the context-dependent manner of Wnt signaling during development (9) this is perhaps not surprising. Importantly, despite multiple mutations within a cancer cell, correcting or inhibiting one gene in the Wnt pathway that is altered in cancer can potently inhibit tumor growth. This has important implications for cancer therapy. For example, restoring the function of APC in *APC*-mutant colon cancer cells inhibits tumor growth (20, 21), implying that mutation of *APC* is not just an initiating event in the genesis of colon cancer but provides the genetic platform that sustains the tumor. Restoring wild-type *APC* removes that platform. Furthermore, restoring *sFRP* for example (22), or blocking Fzd7 signaling in *APC*-mutant colon cancer cells (23) or gastric cancer cells (7), inhibits Wnt signaling and tumor growth, again, despite the tumor cells harboring many other mutations, implicating the importance of "just right" Wnt signaling. Notably, Wnt signaling can be further stimulated by inhibiting GSK3 or addition of WNT3A in *APC*-mutant colon cancer cells (14, 24), which suggests potential regulation also at

the destruction complex even if *APC* is mutated (24). Furthermore, other members of the Fzd family of Wnt receptors have been implicated in many cancer types, including gastric cancer in which we observed that Fzd2 and Fzd6 were also upregulated in human gastric cancer cells (7), and therefore future research will help determine their functional roles.

Another important function of variable levels of Wnt signaling in tumors is to regulate cancer stem cell (CSC) activity. Stromal myofibroblast-derived hepatocyte growth factor (HGF) can increase Wnt signaling in *APC*-mutant colon cancer cells and endow them with CSC features of self-renewal (25). CSCs also display invasive and metastatic features, with $CD44^+$ CSCs frequently observed at the invasive front of gastric tumors (26). There are considerable similarities between migrating cells in the developing embryo and metastasizing tumor cells. During gastrulation populations of cells undergo an epithelial to mesenchymal transition (EMT) and become motile to set up the complex tissue patterning of the developing embryo (27). Nuclear β-catenin is observed in migrating cells during early gastrulation, and β-catenin deficient mouse embryos fail to develop mesoderm (28). Areas of increased Wnt signaling activity are often observed at the leading edge of tumors as can be seen in Fig. 1 that shows increased expression of β-catenin at the invasive front of a human colon tumor. These data suggest an environmental regulation of Wnt signaling in *APC*-mutant colon cells is high-jacking a developmental process to promote tumor invasion/metastasis.

Non-canonical PCP Wnt signaling is also critical for correct polarization of tissues and cell migration during gastrulation and is implicated in metastasis of several cancer types (29). During neural crest migration, the small GTPase, and PCP signaling component, RhoA, acts to promote the retraction of cell protrusions (30). In colon tumors, RhoA levels are reduced in metastatic sites compared with the primary tumor, and inactivation of RhoA increases canonical Wnt signaling to promote metastasis (31). Furthermore, RhoA mutations are recurrent in genomically stable gastric tumors, suggesting that they contribute to the invasive phenotype of diffuse gastric tumors (32).

The processes of EMT and MET are also regulated by Wnt signaling, with several Wnts and Fzds now implicated in both EMT and MET, including FZD7 (24, 33, 34). Wnt signaling can promote invasion of cancer cells by regulating genes involved in cell adhesion, including Eph/Ephrins and E-cadherin (35). Several matrix metalloproteinase (MMP) genes are also regulated by Wnt signaling in numerous cancers (36, 37), including gastric cancer (38), which degrade proteins in the surrounding stroma, including collagen, gelatin, fibronectin, and laminin, to provide a permissive microenvironment to allow invading cells to migrate. These data illustrate that the heterogenous activation of Wnt signaling in tumors is regulated by deregulation of the pathway, at the level of the ligand/receptor, which is supported by RNASeq data (Fig. 2), and that different levels of Wnt activity regulate different cellular functions, including stemness, EMT/MET and invasion during cancer progression.

It is worth mentioning that many right-sided colorectal cancers, which are associated with poor prognosis, are not mutant for *APC* and instead have mutations to *RNF43* (cbioportal website) or methylation of *Dkk1* (39) or *sFRP* (22), which facilitate elevated Wnt signaling (Fig. 2). In the absence of an *APC* mutation, colorectal cancers can acquire gain-of-function *CTNNB1* mutations that render β-catenin resistant to proteasomal degradation (40). However, the selection bias towards *APC* in preference to *CTNNB1* mutations has been shown to reflect the abundance of colonic E-cadherin that can sequester mutant β-catenin, acting as a sink to prevent transformation (41). Hepatocellular carcinoma (HCC) is the most common form of liver cancer, around 50% of which harbor similar mutations in the *CTNNB1* gene (42). Similar to colorectal cancer and gastric cancer, HCC also exhibit deregulation of Wnt components at the receptor/ligand level, including overexpression of Wnt ligands and FZD receptors, and downregulation of sFRPs (4). Here, too, *CTNNB1* mutation alone does not cause liver cancer in mouse models but instead provides a platform in which tumor progression is accelerated in association with other factors, including Kras (43) and Lkb1 (44).

Context specificity of *Apc*-mutant cells to modulation of Wnt signaling

Despite compelling evidence outlined above that demonstrates Wnt signaling can be further regulated at the level of the receptor in *Apc*-mutant cells, recent work indicates this phenomenon is context dependent. Intestinal stem cells (ISC) are continuously engaged in a stochastic competitive process termed neutral-drift (45). This process is neutral as all ISCs have equal probability of replacing their neighbor or being replaced. However, oncogenic mutations, in genes such as *Apc*, bias this competition in favor of *Apc*-mutant cells such that they displace all wild-type ISCs cells from the niche and the crypt will become clonal with *Apc*-mutant progeny (46). Indeed, lowering the overall level of Wnt ligands in an *Apc*-mutant background further exacerbates the decreased fitness of non-mutant ISCs, allowing for the rapid clonality of *Apc*-mutant ISCs, which accelerates intestinal tumor formation (47). Similar size changes to the ISC pool and rate of clonality were observed following selective inhibition of a single binding site for R-Spondin, which is required to potentiate Wnt signaling (48), indicating that fluctuations in Wnt signaling can impact the dynamics of ISCs and in turn influence tumor initiation.

Some important insight has been gained recently into how *APC*-mutant cells activate Wnt signaling. Wnt pathway activation was observed in *APC*-mutant colorectal cancer cells, which were unable to secrete Wnt ligands due to a mutation in *PORCN* (or treatment with PORCN inhibitor), and this activation was partially inhibited (~50%) in *LRP6*-mutant cells (49). This response was not observed in *CTNNB1*-mutant colorectal cancer cells, suggesting a specific role for mutant *APC*. Further analysis revealed that *APC* knockdown, but not *APC2* knockdown, triggered the assembly of the Wnt signalasome and clathrin-mediated endocytosis to activate Wnt signaling in colorectal cancer cells. However, as we observed that *APC*-mutant gastric cancer cells can respond to PORCN inhibitors (7), further research will be needed to determine whether similar mechanisms exist in other cancer types.

The recent molecular subtyping of multiple cancers has greatly enhanced our understanding of cancer genetics and disease stratification. Using a network-based approach, four consensus molecular subtypes (CMS) of colorectal cancer were recently identified, of which, CMS2 and CMS3 display the highest levels of Wnt signaling and superior patient outcome. Interestingly, approximately 66% of CMS4 colorectal cancers have mutations to *APC* and also display high stromal infiltrate, TGFβ signaling and have the poorest survival (19). However, poor clinical prognosis in CMS4 colorectal cancer subtypes is associated with receptor-mediated Wnt activation, whereas oncogenic Wnt activation (mutant *APC*) is associated with improved patient outcome and CMS2 (50). Collectively, this indicates disease/tumor progression

needs to be considered when selecting the type (agonist vs. antagonist) of WNT therapeutic in colorectal cancer, as increasing Wnt activity could be beneficial to patients with CMS4 tumors.

Summary

There is now substantial evidence that targeting the Wnt pathway at the level of the receptor/ligand is an attractive therapeutic strategy for inhibiting tumor growth, and potentially metastasis. Indeed, several drugs targeting the Wnt pathway at the level of the ligand/receptor are currently in clinical trials, some of which focus on metastatic disease. The monoclonal antibody Vantictumab (OMP-18R5 Oncomed/Bayer) binds to Fzd1, 2, 5, 7, and 8, is in Phase Ib clinical trials for pancreatic cancer, metastatic breast cancer and non–small cell lung cancer (51). Porcupine inhibitors, which prevent the secretion of all Wnt ligands, are also in phase I clinical trials for advanced solid tumors (NCT01351103). We demonstrated Vantictumab was able to inhibit the growth of the growth of gastric tumors, with and without *Apc* mutations (7), suggesting this approach could also benefit patients with gastric cancer. Further research in pre-clinical models is required to determine the therapeutic potential of targeting the Wnt pathway at the level of the ligand/receptor for metastatic disease, but the growing molecular evidence suggests that this is an attractive strategy, and current clinical trials will help resolve this in the future, along with continued drug discovery programs to target Wnt signaling, and combination therapies to optimize treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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