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Special Section on New Opportunities in Targeting WNT Signaling – Minireview

Wnt Signaling and Drug Resistance in Cancer

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ABSTRACT

Wnts are secreted proteins that bind to cell surface receptors to activate downstream signaling cascades. Normal Wnt signaling plays key roles in embryonic development and adult tissue homeostasis. The secretion of Wnt ligands, the turnover of Wnt receptors, and the signaling transduction are tightly regulated and fine-tuned to keep the signaling output "just right." Hyperactivated Wnt signaling due to recurrent genetic alterations drives several human cancers. Elevated Wnt signaling also confers resistance to multiple conventional and targeted cancer therapies through diverse mechanisms including maintaining the cancer stem cell population, enhancing DNA damage repair, facilitating transcriptional plasticity, and promoting immune evasion. Different classes of Wnt signaling inhibitors targeting key nodes of the pathway have been developed and show efficacy in treating Wnt-driven cancers and subverting Wnt-mediated therapy resistance in preclinical studies. Several of these inhibitors have advanced to clinical trials, both singly and in combination with other existing US Food and Drug Administration–approved anti-cancer modalities. In the near future, pharmacological inhibition of Wnt signaling may be a real choice for patients with cancer.

SIGNIFICANCE STATEMENT

The latest insights in Wnt signaling, ranging from basic biology to therapeutic implications in cancer, are reviewed. Recent studies extend understanding of this ancient signaling pathway and describe the development and improvement of anti-Wnt therapeutic modalities for cancer.

Introduction

Wnt signaling is an evolutionarily conserved signaling transduction pathway that plays important roles in embryonic development and adult tissue homeostasis. Dysregulated Wnt signaling causes human cancers, and an increasing number of studies reveal that elevated Wnt signaling contributes to drug resistance in cancer therapy. Given the core role of Wnt signaling in multiple cancers, the Wnt pathway has been one of the hottest targets of drug development. Several Wnt pathway inhibitors have been developed and show promising efficacy in treating Wnt-driven cancers and subverting Wnt-mediated therapy resistance in preclinical studies. In this review, we will present an update on recent findings in the Wnt signaling transduction pathway and advances in pharmacological targeting of Wnt signaling. We will focus on the roles of Wnt signaling in cancer drug resistance as well as anti-Wnt signaling-based combination therapies. As multiple Wnt inhibitors are being widely used in preclinical studies and some have advanced to clinical trials, resistance to Wnt blockade is also observed in certain Wnt-dependent normal tissue and cancers. Here we will also summarize the potential mechanisms that confer resistance to Wnt inhibitors.

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Wnt Signaling Pathway

Wnt Genes and Proteins. Wnts are secreted proteins encoded by Wnt genes that are present in all clades of metazoans (Holstein, 2012). The first mammalian Wnt gene, mouse *Int-1*,

ABBREVIATIONS: AA/P, abiraterone acetate/prednisone; ABC, ATP-binding cassette; AKT, Ak strain transforming; APC, adenomatous polyposis coli; AXIN, axis inhibition protein; BCC, basal cell carcinoma; BET, bromodomain and extra terminal protein; BRAF, V-Raf murine sarcoma viral oncogene homolog B; CK1α, casein kinase 1 alpha; CRD, cysteine-rich domain; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; EGFR, epidermal growth factor receptor; FOXO, O subclass of the Forkhead family; Fzd, Frizzled; GSK3, glycogen synthase kinase 3; iCRT, inhibitor of β-catenin responsive transcription; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; MMTV, mouse mammary tumor virus; MYC, V-Myc Avian Myelocytomatosis viral oncogene homolog; PARP, poly (ADP-ribose) polymerase; PDGFR, platelet-derived growth factor receptor; PORCN, porcupine; RNF43, ring finger protein 43; RSPO, R-Spondin; TCF/LEF, T-cell factor/lymphoid enhancer-binding factor; USP6, ubiquitin-specific protease 6; Wnt, Wingless and Int; ZNRF3, zinc and ring finger 3.

was identified in 1982 as a proto-oncogene in the mouse genome whose expression was activated by mouse mammary tumor virus (MMTV) proviral DNA integration leading to mouse mammary cancers (Nusse and Varmus, 1982). The *Int-1* gene was subsequently found to be a homolog of the *Drosophila Wingless* gene that controls segment polarity in fly larval development (Rijsewijk et al., 1987). Therefore, genes of this family are called *Wnt* as a blend, or portmanteau, of *Wingless* and *Int* (Nusse et al., 1991). There are 19 Wnt genes in most mammalian genomes, including the human genome, which can be categorized into 12 subfamilies.

Wnt proteins are 350–400 amino acids in length and ~40 kDa in size. They contain 22 or 24 conserved cysteine residues that form 11 or 12 intramolecular disulfide bonds that are important for the proper folding of the peptides into functionally active proteins (Macdonald et al., 2014). Wnt proteins undergo post-translational modification after translation. Besides glycosylation, all Wnt proteins require a post-translational modification, the addition of a palmitoleate moiety on a conserved serine residue, catalyzed by a membrane-bound O-acyltransferase called PORCN in the endoplasmic reticulum (ER) (Takada et al., 2006; Najdi et al., 2012; Proffitt and Virshup, 2012). This serine residue is conserved in all Wnts across different species except in a distantly related Wnt gene named *WntD* in Drosophila that regulates NF-κb rather than β-catenin signaling (Fig. 1, A and B) (Ching et al., 2008). The palmitoleation is necessary for the interaction of Wnts with a cargo receptor called WLS that transports Wnts from ER to the plasma membrane (Coombs et al., 2010; Yu et al., 2014). The palmitoleic moiety is also required for direct binding sites for Wnt ligand and its receptor Frizzled on the cell membrane (Fig. 1, C and D) (Janda et al., 2012; Hirai et al., 2019). Previous studies from our group have shown that all assessable human Wnts lost signaling activity when *PORCN* was knocked out, and this could be rescued by *PORCN* re-expression (Najdi et al., 2012). Because of the important role of *PORCN* in Wnt signaling, mutations of the X-linked *PORCN* gene lead to embryonic lethality in males, and focal defects due to random X inactivation in surviving females (Wang et al., 2007). The O-linked palmitoleate makes Wnt proteins hydrophobic and therefore limits the biologic activity of secreted Wnt proteins to a short range, unlike circulating protein hormones such as insulin that act at a distance.

Wnt Receptors. As secreted but hydrophobic morphogens, Wnts travel locally to bind to cell surface receptors and coreceptors to activate downstream signaling transduction cascades. Wnts have several types of receptors. The best known are the 10 Frizzleds (Fzds) and their coreceptors LRP5/6. However, an increasing number of coreceptors such as GPR124, Reck, and TMEM59 (Zhou and Nathans, 2014; Posokhova et al., 2015; Vanhollebeke et al., 2015; Cho et al., 2017; Gerlach et al., 2018), and alternative receptors including Ror and Ryk have been identified (Niehrs, 2012). The Frizzled receptors are seven-transmembrane proteins and have N-terminal extracellular cysteine-rich domains (CRDs). The crystal structure of *Xenopus* WNT8 in complex with mouse Frizzled-8 CRD reveals the multiple interacting surfaces of Wnt-Fzd binding, including a hydrophobic groove in the CRD that binds to the hydrophobic palmitoleate on Wnt (Janda et al., 2012). Additional structures have extended these results, suggesting palmitoleate binding serves to dimerize Frizzled CRDs (Hirai et al., 2019; Nile and Hannoush, 2019).

The abundance of cell surface Frizzled proteins is regulated by post-translational modification. Recent studies identified two highly homologous Wnt target genes called *RNF43* and *ZNRF3*, encoding two transmembrane RING domain-containing E3 ubiquitin ligases. RNF43 and ZNRF3 can ubiquitinate the cytosolic domain of Frizzleds causing the internalization and degradation of Frizzleds. As Wnt target genes, active Wnt signaling upregulates the expression of *RNF43* and *ZNRF3*, which in turn downregulate the surface Wnt receptors level and the downstream Wnt signaling in a negative feedback loop (Fig. 2, A and B) (Hao et al., 2012; Koo et al., 2012). This RNF43/ZNRF3 mediated membrane clearance of Wnt receptors is tightly regulated by at least two processes. First, the ubiquitin-specific protease 6 (USP6) reverses the effects of RNF43/ZNRF3 by deubiquitinating Frizzleds (Fig. 2C) (Madan et al., 2016b). Second, R-Spondins (RSPO1-4) are secreted protein ligands that bind to the extracellular domains of both RNF43/ZNRF3 and LGR4/5 and lead to their clearance from the membrane (Fig. 2D) (Niehrs, 2012; de Lau et al., 2014).

The Wnt/β-Catenin Signaling Cascade. Binding of Wnt ligands to their various receptors can activate different downstream signaling pathways. The Wnt/β-catenin signal transduction (canonical Wnt pathway) is the most well-studied Wnt pathway. β-catenin, encoded by the *CTNNB1* gene, plays a central role in this pathway. β-catenin is a dual function protein, involved in both cell-cell adhesion and regulation of gene transcription. At the cell membrane, β-catenin is part of a protein complex that forms the adherens junction, which is fundamental for the maintenance of the epithelial cell layers. Nuclear β-catenin acts as transcriptional regulator. The abundance of β-catenin is tightly regulated by a destruction complex that contains the scaffold protein AXIN, APC (encoded by the well-known tumor suppressor gene adenomatous polyposis coli [*APC*]), casein kinase 1 alpha (CK1α), and glycogen synthase kinase 3 alpha and beta (GSK3α/β) (Asuni et al., 2006; Doble et al., 2007; Macdonald et al., 2009).

In the absence of Wnt ligands (Fig. 3, left panel), β-catenin is first phosphorylated by CK1α at Ser45, and subsequently phosphorylated by GSK3α/β at Thr41, Ser37, and Ser33 (Amit et al., 2002; Liu et al., 2002). The F-box protein, β-transducin repeats containing protein recognizes and binds to this phosphorylated β-catenin, mediating its ubiquitination by the Skp1, Cullin, F-box E3 ubiquitin ligase complex and subsequent proteasomal degradation (Jiang and Struhl, 1998; Hart et al., 1999; Liu et al., 1999; Winston et al., 1999).

In the presence of Wnt ligands (Fig. 3, right panel), Frizzleds and the coreceptors LRP5/6 multimerize at the cell surface. This leads to recruitment of the cytoplasmic protein Dishevelled to the cell membrane by interacting with cytoplasmic domains of Frizzleds (Wong et al., 2003; Ma et al., 2019). The Frizzled-bound Dishevelled recruits the AXIN complex through the DIX domains on Dishevelled and AXIN (Schwarz-Romond et al., 2007). GSK3α/β in the AXIN complex initiates phosphorylation of the PPPSP motifs of the LRP5/6 cytoplasmic tail and primes subsequent phosphorylation by CK1s (Zeng et al., 2005). As docking sites, these phosphorylated LRP5/6 PPPSP motifs recruit more AXIN complexes to the cell membrane that further phosphorylate the LRP5/6 PPPSP motifs as a positive feedback loop (Zeng et al., 2008). Collectively, stimulation of Wnt ligands relocalizes the β-catenin destruction complex to the cell membrane. However, the exact mechanism by which this causes β-catenin stabilization remains controversial.

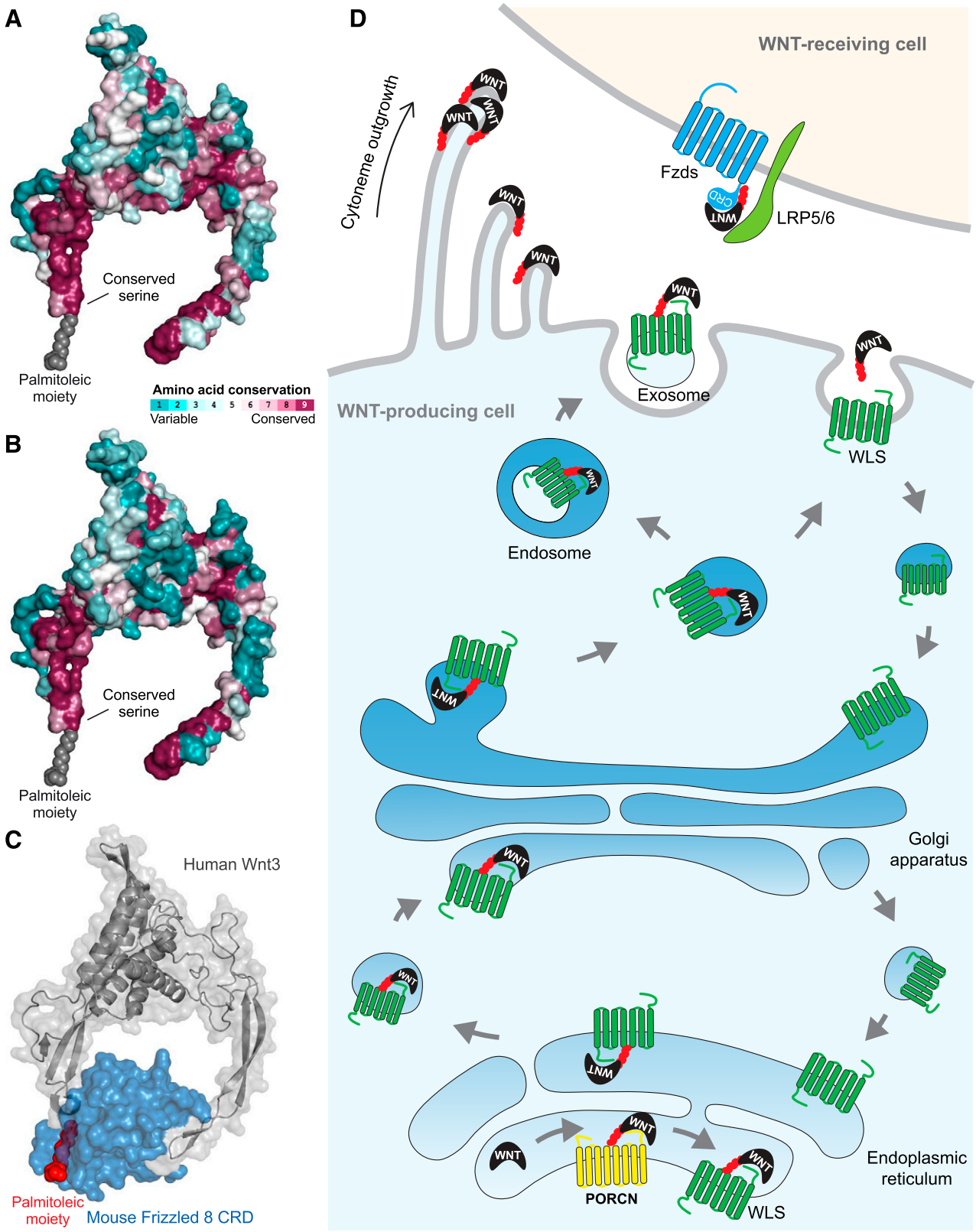

Fig. 1. Wnts are secreted proteins with conserved domains and residues. (A) The consensus modeling of 19 human Wnts. The amino acid sequences of 19 human Wnts were aligned, and the amino acid conservation scores were calculated using The ConSurf Server website (http://consurf.tau.ac.il). The conservation scores were then mapped on the human Wnt3 crystal structure (PDB 6AHY chain B). (B) The consensus modeling of Wnt homologs. The palmitoleation site and Frizzled interaction sites are conserved in all Wnts. 2635 amino acid sequences that are homologs of human Wnt3 were collected from UNIREF90 using the HMMER algorithm. The conservation scores were calculated from 150 amino acid sequences representative of the 2635 sequences using The ConSurf Server website and mapped on the human Wnt3 crystal structure. (C) The crystal structure of human Wnt3 in complex with mouse Frizzled 8 CRD (PDB 6AHY). (D) The Wnt secretion pathway. Wnts are palmitoleated by PORCN in the ER. The palmitoleic moiety (red line) facilitates the interaction of Wnts with the cargo receptor WLS that transports Wnts to the plasma membrane. Multiple routes of Wnt release and extracellular transport including diffusion, exovesicles, and cytoneme-mediated transport have been proposed.

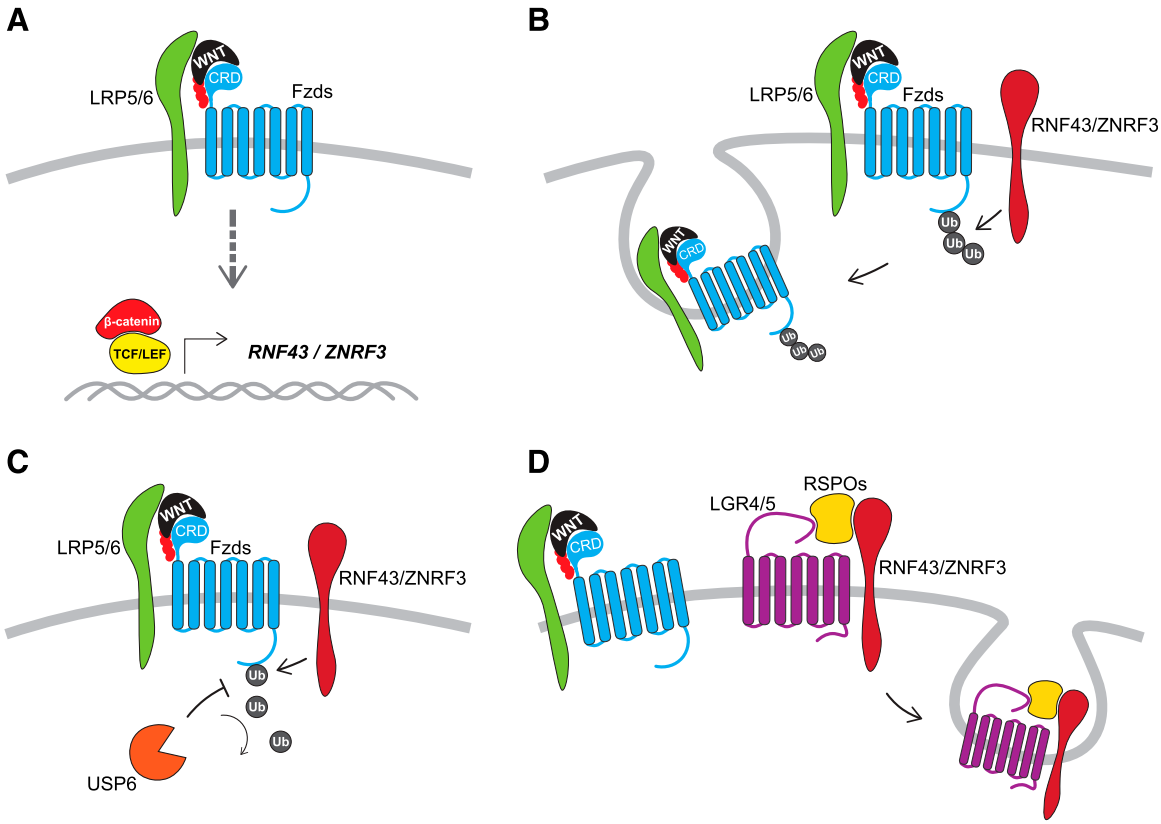

Fig. 2. The abundance of cell surface Wnt receptors is tightly regulated by RNF43/ZNRF3, USP6, and R-Spondins. (A) *RNF43* and *ZNRF3* are Wnt/β-catenin target genes. (B) RNF43 and ZNRF3 ubiquitinate the cytosolic domain of Frizzleds causing the internalization and degradation of Frizzleds. (C) USP6 reverses the effects of RNF43/ZNRF3 by deubiquitinating Frizzleds. (D) R-Spondins bind to the extracellular domains of both RNF43/ZNRF3 and LGR4/5 leading to their membrane clearance. Ub, ubiquitin.

Diverse models have been proposed with supporting evidence, including 1) inhibition of GSK3 activity by the phosphorylated PPPSP motif that binds to the GSK3 catalytic pocket as a "pseudo substrate" (Mi et al., 2006; Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009), 2) sequestration of GSK3 from the cytoplasm into multivesicular bodies (MVBs) that restricts GSK3′s access to substrates (Taelman et al., 2010), 3) dissociation of the ubiquitination machinery from the β-catenin destruction complex (Li et al., 2012), and 4) degradation of AXIN and disassembly of the destruction complex (Tolwinski et al., 2003). Subsequently, the newly synthesized β-catenin protein can accumulate in the cytoplasm and enter the nucleus to regulate gene transcription. Notably, inhibition of AXIN-bound GSK3 in Wnt signaling is independent of the well-studied AKT-mediated phosphorylation on the Ser21 of GSK3α and Ser9 of GSK3β that inhibits their kinase activity (Ding et al., 2000; McManus et al., 2005; Ng et al., 2009), and inhibition of GSK3 by Wnts also blocks degradation of other GSK3 targets including MYC (Taelman et al., 2010; Madan et al., 2018).

β-Catenin regulates gene expression in large part in a TCF-dependent manner (Schuijers et al., 2014). The TCF/LEF family is a group of DNA-bound transcription factors with cognate binding motif 5′-AGATCAAAGG-3′. They recruit Groucho family transcriptional repressors to inhibit gene transcription in the absence of β-catenin. When Wnt is present, β-catenin is stabilized and enters the nucleus, binds to TCF, and converts TCF into a transcriptional activator. However, there is a subset of β-catenin transcriptional targets that do not require TCF/LEF factors for their regulation. In these loci, β-catenin interacts with other factors such as MyoD and FOXO to regulate gene transcription (Fig. 3, right panel) (Doumpas et al., 2019).

The direct Wnt target genes are defined as genes with the conserved TCF binding sites that functionally influence gene transcription. Most Wnt target genes are cell type and developmental stage specific, indicating their promoters are subject to additional tissue-specific regulation (Nakamura et al., 2016). Many of these Wnt/β-catenin targets such as MYC and cyclins regulate core biologic processes such as ribosome biogenesis and cell cycle progression (Madan et al., 2018). It is also interesting that a number of the more robust Wnt target genes are themselves negative regulators of Wnt signaling. These include *AXIN2*, *RNF43*, *ZNRF3*, and *NOTUM*. As described previously, RNF43/ZNRF3 negatively regulate the Frizzleds level on the cell surface (Fig. 2, A and B) (Hao et al., 2012; Koo et al., 2012). NOTUM is a palmitoleoyl-protein carboxylesterase that can remove the palmitoleic group on Wnt ligands (Kakugawa et al., 2015). As this palmitoleic group is necessary for the interaction of Wnt ligand and receptor, Notum inactivates the function of Wnt ligands. These multiple negative feedback mechanisms guarantee that the Wnt signaling is precisely regulated and kept at the just right level.

β-Catenin Independent Wnt Signaling Pathways. β-Catenin–independent Wnt signaling is defined as Wnt- or Frizzled-initiated signaling that is independent of TCF/β-catenin–mediated transcription. These "noncanonical" Wnt signaling pathways are diverse and their role in mammals is less well understood. Some Wnts such as Wnt5a and Frizzleds

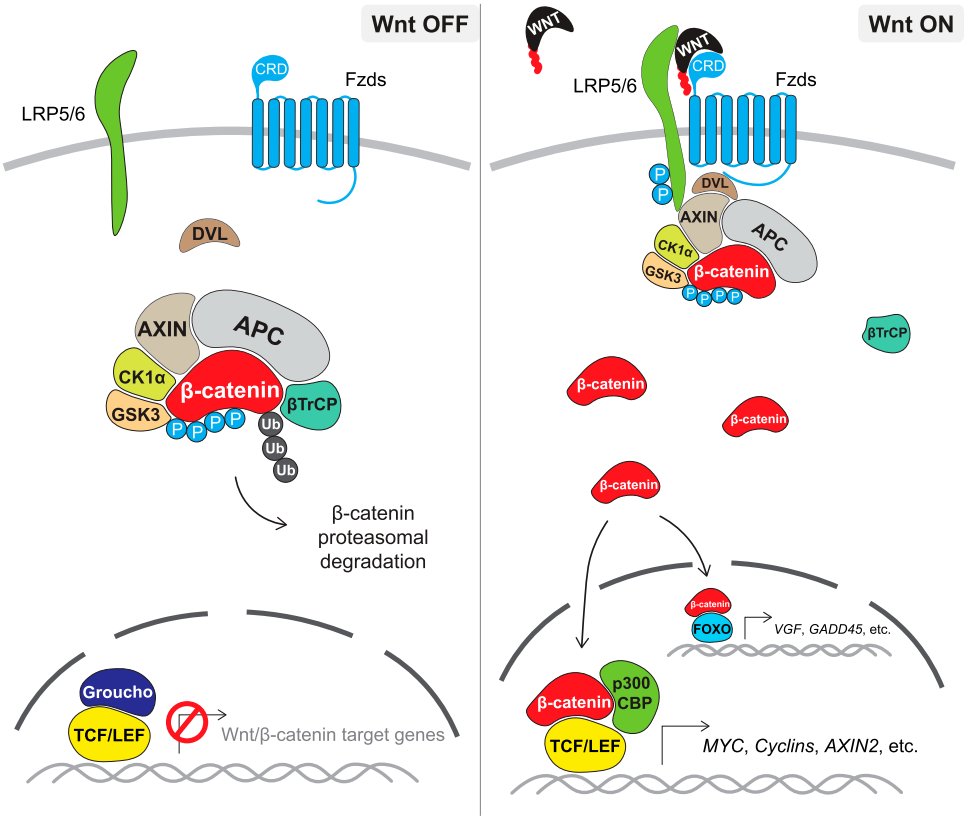

Fig. 3. The canonical Wnt/β-catenin signaling cascade. (Left panel) In the absence of Wnt ligands, β-catenin is phosphorylated, ubiquitinated, and degraded by the β-catenin destruction complex. (Right panel) Binding of Wnt ligands to the receptors relocalizes the destruction complex to the membrane and interferes with its activity. Subsequently, newly synthesized β-catenin accumulates in the cytoplasm and enters the nucleus to regulate gene transcription. Refer to the text for detailed description. DVL, dishevelled.

are involved in the regulation of intracellular calcium levels and planar cell polarity (Kikuchi et al., 2012). Some Wnts can bind to receptor tyrosine kinases such as ROR2 and RYK to activate downstream signaling leading to, e.g., cytoneme extension (Mattes et al., 2018). Notably, one established effect of Wnt5a is antagonism of the canonical Wnt/β-catenin signaling (Ishitani et al., 2003; Topol et al., 2003; Mikels and Nusse, 2006). The specific signaling pathway regulated by individual Wnt ligand is also based on the specific receptors existing on the cell surface and is cell type and developmental stage dependent (Semenov et al., 2007; Najdi et al., 2012).

Roles of Wnt Signaling in Development and Adult Tissue. The Wnt signaling pathway is evolutionarily conserved across various species from sponges to fruit flies to human beings (Loh et al., 2016). As shown by both naturally occurring and experimentally induced loss-of-function and gain-of-function mutations in many species, Wnt signaling plays important roles in both embryonic development and in maintaining adult tissue homeostasis.

The first Wnt gene, *wingless*, was identified in *Drosophila*. As the name suggests, the first mutant of *wingless* gene displayed a phenotype of transformation of wing structures into thoracic notal structures. It was also first found in *Drosophila* that Wnt is critical for the establishment of anterior-posterior polarity during embryonic segmentation (Swarup and Verheyen, 2012). In vertebrates, Wnt signaling also plays a key role in controlling body axis formation. Wnt signaling can induce secondary dorsal structures when misactivated in early *Xenopus* embryos. This axis duplication assay has been widely applied to functionally test a gene candidate's effect on the β-catenin–dependent Wnt signaling pathway. In mice, genetic studies have shown that Wnt signaling is required for gastrulation, and Wnt3, Lrp5/6, and β-catenin knockouts generated similar phenotypes (Wang et al., 2012). In summary, Wnt signaling controls axis patterning, cell fate specification, cell proliferation, and cell migration in embryonic development.

In healthy adult tissue, one of the most important roles of Wnt signaling is to maintain the adult stem cells. Using lineage tracing strategies, the Wnt target gene *Lgr5* has been demonstrated to mark stem cells, first in the intestine (Barker et al., 2007) and then in several other tissues including stomach, pancreas, liver, kidney, ovarian epithelium, mammary gland, hair follicle, inner ear, and taste buds (Leung et al., 2018).

The intestine is one of the best-studied examples of Wnt signaling and stem cell maintenance. The small intestinal epithelial layer is one of the most continuously proliferative tissues in the body and undergoes self-renewal every 3–5 days. It has a crypt-villus structure (Clevers, 2013). Villi are protrusions of the simple columnar epithelium that extend into the gut lumen. Crypts are invaginations of the epithelium that surround the base of each villus. One set of intestinal stem cells (also called crypt base columnar cells) are located at the base of the crypt, surrounded by the Paneth cells. These stem cells then give rise to the transit amplifying cells, which are rapidly dividing committed progenitor cells that differentiate into all the mature cell types of the epithelium. The mature differentiated cells move upward from the crypt region to the villus and are finally shed as part of the self-renewal process of the intestinal epithelium.

As mentioned previously, the Wnt/β-catenin target gene *Lgr5* is exclusively expressed in the crypt base columnar cells, indicating active Wnt signaling in the intestinal stem cells. As a coreceptor for the RSPOs, LGR5 helps to sequester

RNF43/ZNRF3 and upregulates the cell surface Frizzled/LRP levels (Fig. 2D). This may work as a positive feedback loop to further enhance the Wnt signaling in these stem cells. There are additional diverse Wnt ligands expressed in the crypt-villus axis that may help regulate differentiation into other cell types (Gregorieff et al., 2005). Paneth cells reside adjacent to the intestinal stem cells at the base of the crypt and are known for expressing several Wnts, including *Wnt3*. Wnts secreted by Paneth cells can support the ex vivo organoid growth from purified intestinal epithelial cells (Sato et al., 2011). However, one study from our group showed that mice lacking epithelial Wnt activity by genetic ablation of *Porcn* in the intestinal epithelium had normal intestinal homeostasis as well as normal intestinal regeneration capability after radiation injury, indicating that epithelial Wnts (including Wnts secreted by Paneth cells) are dispensable for these processes (Kabiri et al., 2014). Recently, our group identified the PDGFRα^+ pericryptal stromal cells as the critical source of Wnts and RSPO3 for the intestinal stem cells in vivo (Greicius et al., 2018). And three other studies identified stromal cells marked by FOXL1$^+$ (Aoki et al., 2016; Shoshkes-Carmel et al., 2018) and GLI1$^+$ (Degirmenci et al., 2018), respectively, that form the essential Wnt-secreting niche for the stem cells in the gut. Notably, there is significant but incomplete overlap among the PDGFRα^+, FOXL1$^+$, and GLI1$^+$ stromal cells (Greicius and Virshup, 2019).

Importantly, Wnt/β-catenin signaling represses proliferation of the intestinal stem cells. It is known that high Wnt/β-catenin signaling, marked by nuclear β-catenin accumulation and β-catenin target gene expression, is largely restricted to the intestinal stem cells in the crypt base, whereas the highly proliferative transit amplifying cells lack Wnt/β-catenin signaling. Instead, it is well established that the EGFR/RAS/MAPK signaling drives active proliferation in the intestine (Biteau and Jasper, 2011; Jin et al., 2015). One recent study found that Wnt inhibition by PORCN inhibitor induced an initial burst of proliferation of the intestinal stem cells due to conversion of the stem cells into the transit-amplifying cells with a loss of stem cell self-renewal (Kabiri et al., 2018). This is driven by activation of the Wnt-suppressed MAPK signaling. And this will eventually lead to intestinal crypt ablation as the transit-amplifying cells differentiate and stem cells get depleted. Collectively, these findings indicate that the role of Wnt signaling in the intestinal stem cell niche is to maintain the stemness and inhibit differentiation rather than to promote the stem cell proliferation (Greicius and Virshup, 2019).

Aberrant Wnt Signaling in Cancer and Pharmacological Targeting of Wnt Signaling

Common Alterations of Wnt Pathway Components in Cancer

The first mammalian Wnt gene, *Int-1*, was identified because its overexpression caused mouse mammary cancer (Nusse and Varmus, 1982). The first evidence that aberrant Wnt signaling caused human cancer came from studies of hereditary colorectal cancer. In 1991, three groups independently found that the *APC* gene is mutated in the hereditary colon cancer syndrome, familiar adenomatous polyposis, as well as in many cases of the sporadic colorectal cancer (Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). Subsequent studies showed that loss-of-function mutations in the *APC* gene lead to inappropriate stabilization of β-catenin and constitutive transcriptional activation by the β-catenin/TCF complex (Rubinfeld et al., 1996; Korinek et al., 1997; Morin et al., 1997). In agreement with the β-catenin phosphorylation/degradation model (Liu et al., 2002), point mutations in the N-terminal Ser/Thr phosphorylation sites of β-catenin that prevent its degradation were found in a subset of colorectal cancer with wild-type *APC* gene (Morin et al., 1997).

Aberrant activation of Wnt/β-catenin signaling is not restricted to colorectal cancer. Similar alterations in β-catenin, APC, and AXIN1 are found in liver cancer cases (de La Coste et al., 1998; Anastas and Moon, 2013). A growing series of activating mutations in the downstream components of the Wnt signaling pathway have also been reported in various other cancer types, including cancers originating in stomach, pancreas, ovary, endometrium, kidney, adrenal gland, biliary tract, pituitary, and soft tissues (Table 1). Researchers have also developed mouse models that confirm the tumorigenic effect of hyperactive Wnt signaling (Colnot et al., 2004; Taketo and Edelmann, 2009; Koo et al., 2012).

Mutations that activate Wnt/β-catenin signaling fall into two broad categories: Wnt dependent and Wnt independent. Mutations in *APC*, *AXIN1*, and *CTNNB1* that stabilize β-catenin uncouple the downstream Wnt signaling from the upstream Wnt ligands, which means that β-catenin–dependent transcription is constitutively activated regardless of whether the Wnt ligands and receptors are present or not. More recently, studies from several groups identified activating mutations that are Wnt dependent (Table 1). As described in the previous section, the Wnt receptors (Frizzleds and LRPs) are tightly regulated by two homolog E3 ubiquitin ligases (RNF43/ZNRF3) and Wnt agonists (RSPOs). As a negative feedback mechanism, high Wnt signaling upregulates the expression of RNF43/ZNRF3, which leads to the membrane clearance of Wnt receptors and quenches the upstream Wnt signaling (Fig. 2, A and B). Inactivating mutations of *RNF43* and *ZNRF3* were first reported in pancreatic and adrenocortical carcinomas, respectively, and subsequently seen in cancers originating in other tissues including esophagus, stomach, biliary tract, large intestine, endometrium, and ovary (Wu et al., 2011; Assié et al., 2014; Giannakis et al., 2014; Madan and Virshup, 2015). Conversely, RSPOs inhibit the activity of RNF43/ZNRF3 (Fig. 2D), and gain-of-function translocations of *RSPO2* and *RSPO3* were identified first in a subset of patients with *APC* wild-type colorectal cancer (Seshagiri et al., 2012) and subsequently also identified in several other cancer types (Cardona et al., 2014; Li et al., 2018; Picco et al., 2019). Either loss of function of RNF43/ZNRF3 or gain of function of RSPO2/3 increases the surface levels of Frizzleds and LRPs and makes cells harboring these alterations highly sensitive to low levels of Wnt ligands.

In addition, DNA methylation–mediated epigenetic silencing of genes encoding secreted frizzled-related proteins and Dickkopf-related proteins that are negative regulators of Wnt signaling are common in colorectal, breast, gastric, and ovarian cancers (Caldwell et al., 2004; Suzuki et al., 2004; Veeck et al., 2006, 2008; Yu et al., 2009; Zhu et al., 2012).

Targeting the Wnt Pathway in Cancer

Because of the core role Wnt signaling plays in several human cancers, it has been one of the hottest targets in cancer therapeutics. Multiple inhibitors targeting the Wnt pathway

TABLE 1
Common genetic alterations of Wnt pathway components in human cancers

Gene	Type of Mutation	Primary Tissues	% Mutated	Reference
APC	Mainly frameshift and nonsense mutations that lead to truncated APC proteins with compromised ability to degrade β-catenin; LOH	Large intestine	70	[a]
		Stomach	13	[a]
		Endometrium	7	[a]
		Liver	2.7	[a]
AXIN1	Mainly missense mutations, truncating mutations, and deep deletions	Liver	7	[a]
		Stomach	3	[a]
		Large intestine	2.5	[a]
CTNNB1	Mainly missense mutations in the N-terminal Ser/Thr phosphorylation sites of β-catenin that prevent its degradation	Liver	29	[a]
		Endometrium	18	[a]
		Adrenal cortex	16	[a]
		Large intestine	6	[a]
		Stomach	6	[a]
		Pancreas	2.7	[a]
RNF43	Mainly missense mutations and truncating mutations due to frameshift or nonsense mutations, LOH, and homozygous deletion	Ovary (mucinous carcinoma/mucinous borderline tumor)	21/9	Ryland et al., 2013
		Stomach	13	[a]
		Biliary tract (liver fluke-associated cholangiocarcinoma)	9.3	Ong et al., 2012
		Large intestine	9	[a]
		Pancreas	7	[a]
		Endometrium	4	[a]
ZNRF3	Mainly missense mutations and truncating mutations due to frameshift or nonsense mutations, LOH, and homozygous deletion	Adrenal cortex	20	[a]
		Large intestine	4	[a]
		Stomach	2.1	[a]
RSPO2	Chromosome rearrangement leading to the recurrent *EIF3E-RSPO2* gene fusions	Large intestine	2.9	Seshagiri et al., 2012
		Others (lung, head and neck, esophagus, stomach, ovary, and breast)	1–2	Cardona et al., 2014; Li et al., 2018
RSPO3	Chromosome rearrangement leading to the recurrent *PTPRK-RSPO3* gene fusions	Large intestine	7.4	Seshagiri et al., 2012
		Others (lung, head and neck, esophagus, ovary, and breast)	1–11	Cardona et al., 2014

LOH, loss of heterozygosity.
[a]Curated from the cBioPortal database (https://www.cbioportal.org) in July 2019.

have been developed, and a number have advanced to clinical trials (Table 2).

Loss-of-function mutations of APC/AXIN1 or activating mutations of β-catenin represent the most common genetic alterations that constitutively activate Wnt/β-catenin signaling in human cancers (Table 1). As mentioned previously, these downstream mutations uncouple β-catenin signaling from the upstream Wnt ligand stimulation. Therefore, blocking Wnt/β-catenin signaling in these cancers requires inhibitors targeting downstream in the pathway. In many cases of colorectal cancer, the truncated APC still can functionally mediate β-catenin degradation in the AXIN-containing destruction complex, albeit with a weakened capability (Voloshanenko et al., 2013). In this setting, an increase in AXIN protein can restore β-catenin degradation. This can be achieved by inhibition of tankyrases TNKS1 and TNKS2, ADP ribosyltransferases that normally drive degradation of AXIN and other proteins (Huang et al., 2009; Riffell et al., 2012). Several tankyrase inhibitors such as XAV939 have been developed and show significant Wnt/β-catenin signaling suppression and anticancer effect in Wnt-driven cancer with APC mutations (Chen et al., 2009; Huang et al., 2009; Waaler et al., 2011, 2012; James et al., 2012; Lau et al., 2013; Okada-Iwasaki et al., 2016; Thomson et al., 2017). Activation of CK1α in the destruction complex similarly causes β-catenin degradation. This was first demonstrated with pyrvinium (Thorne et al., 2010) and more recently with more selective molecules (Li et al., 2017).

Another approach to inhibiting downstream β-catenin signaling is to block the interaction of β-catenin with TCF/LEF or associated transcriptional coactivators such as CBP/p300. A series of small molecule inhibitors called "inhibitor of β-catenin responsive transcription" (iCRT) inhibits the interactions between β-catenin and TCF4 and thereby suppresses the transcriptional activity of β-catenin (Gonsalves et al., 2011). The small molecule inhibitor ICG-001 binds specifically to CBP but not the related transcriptional coactivator p300, thereby disrupting the interaction of CBP with β-catenin. In preclinical studies, ICG-001 can significantly suppress β-catenin/CBP–mediated transcriptional activation or repression and shows anti-tumorigenesis effects (Emami et al., 2004; Arensman et al., 2014; Gang et al., 2014). Its active enantiomer PRI-724 has entered early phase clinical trials for treating patients with advanced tumors (Ko et al., 2016; El-Khoueiry et al., 2017). Similarly, another small molecule inhibitor called Windorphen selectively targets the association of β-catenin with the transcriptional coactivator p300 but not CBP (Hao et al., 2013).

Upstream Inhibitors. Hyperactive Wnt signaling in cancer can also result from elevated surface Wnt receptor abundance due to alterations in RNF43/ZNRF3 or RSPOs. To suppress Wnt signaling in such cases, studies have focused on the upstream components of the Wnt pathway that include Wnt ligand secretion, Wnt receptor turnover, and Wnt ligand-receptor interactions. As described previously, mammalian Wnts require palmitoleation by PORCN to be secreted. Therefore, small molecule inhibitors targeting PORCN can block secretion of all Wnts and thereby suppress the downstream signaling. Notably, PORCN inhibitors can shut down both

TABLE 2
Inhibitors of the Wnt signaling and their effects in cancer

Targets and Functions	Agent Name	Functional Effects in Cancer	Development Stage	Reference
Small molecule tankyrase (TNKS1/2) inhibitors, stabilizing AXIN1/2	IWRs		Discovery	Chen et al., 2009
	XAV939	Inhibited colony formation of colorectal cancer cell line DLD-1.		Huang et al., 2009
	WIKI4			James et al., 2012
	NVP-TNKS656			Shultz et al., 2013
	JW67, JW74	Both compounds suppressed in vitro proliferation of colorectal cancer cell line SW480; JW74 reduced SW480 in vivo tumor growth and adenoma formation in Apc^{Min} mice.	Preclinical	Waaler et al., 2011
	JW55	Suppressed in vitro proliferation of SW480; decreased adenoma formation in conditional *Apc* knockout mice.		Waaler et al., 2012
	G007-LK	Suppressed in vitro colony formation and in vivo tumor growth of colorectal cancer cell lines COLO-320DM and SW403 and spheroid formation of Apc^{Min} mouse intestinal adenoma.		Lau et al., 2013
	K-756	Suppressed in vitro proliferation of COLO-320DM and SW403; showed dose-dependent inhibition of Wnt signaling in DLD-1 xenografts.		Okada-Iwasaki et al., 2016
Small molecule CK1α activators, promoting β-catenin degradation	Pyrvinium	Suppressed in vitro proliferation of colorectal cancer cell lines SW480, DLD-1, SW620, HCT116, and HT29; reduced adenoma formation in Apc^{Min} mice.	Preclinical	Thorne et al., 2010; Li et al., 2014;
	SSTC3	Suppressed intestinal organoid formation from Apc^{Min} mice and *Apc* knockout mice; reduced adenoma formation in Apc^{Min} mice; suppressed in vitro colony formation of HT29, SW403, and HCT116 and in vivo tumor growth of HCT116; suppressed tumor organoid formation from three patient colorectal tumors and in vivo growth of xenografts.		Li et al., 2017
Small molecule inhibitors blocking β-catenin/TCF interaction	iCRT3, iCRT5, and iCRT14	iCRTs led to cell cycle arrest in HCT116 and HT29; iCRT3 suppressed in vitro growth of primary human colon cancer specimens; iCRT14 reduced HCT116 and HT29 xenograft growth.	Preclinical	Gonsalves et al., 2011
Small molecule inhibitors blocking β-catenin/CBP interaction	ICG-001	Suppressed in vitro growth and led to apoptosis in SW480 and HCT116; suppressed in vivo growth of SW620 xenografts; reduced adenoma formation in Apc^{Min} mice; suppressed in vitro growth of pancreatic cancer cell lines AsPC-1, L3.6pl, MIA PaCa-2, and PANC-1; improved survival of AsPC-1 orthotopic xenograft-bearing mice.	Preclinical	Emami et al., 2004; Arensman et al., 2014
	PRI-724	The active enantiomer of ICG-001; entered Phase 1 and 2 clinical trials for treating advanced cancers.	Phase 1 and 2 clinical trials	ClinicalTrials.gov NCT01764477, NCT01302405, NCT01606579, NCT02413853
Small molecule inhibitor blocking β-catenin/p300 interaction	Windorphen	Suppressed in vitro proliferation of colorectal cancer cell lines SW480 and RKO and prostate cancer cell lines DU145 and PC3.	Discovery	Hao et al., 2013
Small molecule PORCN inhibitors, blocking Wnt secretion	IWPs		Discovery	Chen et al., 2009
	C59	Inhibited MMTV-Wnt1 tumor growth in vivo.	Preclinical	Proffitt et al., 2013
	LGK974	Induced regression of MMTV-WNT1 tumors; inhibited in vitro colony formation of head and neck cancer cell line HN30 and *RNF43*-mutant pancreatic cancer cell lines Patu8988S and HPAF-II; inhibited in vivo tumor growth in xenografts of HN30 and *RNF43*-mutant pancreatic cancer cell lines Capan2 and HPAF-II; entered	Phase 1 and 2 clinical trials	Jiang et al., 2013; Liu et al., 2013; ClinicalTrials.gov NCT01351103, NCT02278133, NCT02649530

(*continued*)

TABLE 2—*Continued*

Targets and Functions	Agent Name	Functional Effects in Cancer	Development Stage	Reference
		Phase 1 and 2 clinical trials for treating cancers.		
	ETC-159	Inhibited in vitro colony formation of teratocarcinoma cell lines PA-1 and *RNF43*-mutant pancreatic and ovarian cancer cell lines HPAF-II, AsPC-1, and MCAS; suppressed in vivo tumor growth in xenografts of HPAF-II, AsPC-1, teratocarcinoma cell lines PA-1 and NCCIT, and patient-derived colorectal tumor xenografts with *RSPO3* fusions; entered Phase 1 clinical trial for treating advanced cancers.	Phase 1 clinical trial	Madan et al., 2016a; ClinicalTrials.gov NCT02521844
	RXC004	Suppressed in vivo tumor growth of Capan2 xenografts; entered Phase 1 clinical trial for treating advanced cancers.	Phase 1 clinical trial	Bhamra et al., 2017; ClinicalTrials.gov NCT03447470
	CGX1321	Suppressed in vivo tumor growth of patient-derived gastric and colorectal tumor xenografts with *RSPO2* fusions; entered Phase 1 clinical trial for treating advanced cancers.	Phase 1 clinical trial	Li et al., 2018; ClinicalTrials.gov NCT03507998, NCT02675946
Decoy Wnt receptor	Ipafricept (OMP-54F28)	Suppressed in vivo tumor growth of patient-derived hepatocellular carcinoma, pancreatic and ovarian cancer xenografts, and decreased the cancer stem cell frequency; entered Phase 1 clinical trials for treating advanced cancers; in a Phase 1 clinical trial, two desmoid tumor, and a patient with germ cell cancer treated with Ipafricept experienced stable disease for >6 mo.	Phase 1 clinical trials	Jimeno et al., 2017; ClinicalTrials.gov NCT02069145, NCT02092363, NCT02050178, NCT01608867
Anti-Frizzled1,2,5,7,8 antibody	Vantictumab (OMP-18R5)	Suppressed in vivo tumor growth of patient-derived colorectal, breast, lung, and pancreatic tumor xenografts and PA-1 xenografts; decreased the cancer stem cell frequency; entered Phase 1 clinical trials for treating cancers.	Phase 1 clinical trials	Gurney et al., 2012; ClinicalTrials.gov NCT01345201, NCT02005315, NCT01957007, NCT01973309
Anti-Frizzled5,8 antibodies	IgG-2919, IgG-2921	Both antibodies suppressed in vitro proliferation of *RNF43*-mutant pancreatic cancer cell lines HPAF-II, Patu8988S, and AsPC-1, and led to cell cycle arrest; IgG-2912 suppressed in vivo tumor growth of HPAF-II and AsPC-1 xenografts and organoid formation of *RNF43*-mutant colorectal cancers.	Preclinical	Steinhart et al., 2017
Anti-LRP6 antibodies	A7-IgG, B2-IgG	A7-IgG specifically inhibited tumor growth of MMTV-Wnt1 xenografts; B2-IgG specifically inhibited tumor growth of MMTV-Wnt3 xenografts.	Preclinical	Ettenberg et al., 2010
Anti-RSPO antibodies	Anti-RSPO1 antibody, anti-RSPO2 antibody, anti-RSPO3 antibodies	Suppressed in vivo tumor growth of patient-derived colorectal, lung, and ovarian tumor xenografts with corresponding RSPO overexpression; Anti-RSPO3 antibody (OMP-131R10) has entered Phase 1 clinical trial for treating advanced solid tumors and metastatic colorectal cancers.	Preclinical, phase 1 clinical trial (OMP-131R10)	Chartier et al., 2016; Storm et al., 2016; ClinicalTrials.gov NCT02482441

canonical and noncanonical Wnt signaling. Several PORCN inhibitors have been developed. They showed potent suppressive effect in preclinical models of Wnt-addicted cancers (Jiang et al., 2013; Liu et al., 2013; Proffitt et al., 2013; Madan et al., 2016; Bhamra et al., 2017; Li et al., 2018) and have entered clinical trials. In addition, neutralizing antibodies targeting Wnt receptors (Frizzleds and LRPs) and agonists (RSPOs) and soluble Fzd-based decoy receptor of Wnt ligands have been developed by different groups. Some of these have also entered clinical trials (Ettenberg et al., 2010; Gurney et al., 2012; Madan and Virshup, 2015; Chartier et al., 2016; Jimeno et al., 2017; Steinhart et al., 2017).

Wnt Signaling Mediated Resistance in Cancer Therapy

Wnt Signaling in Cancer Stem Cell. Stem cells are defined as multipotent cells that can perpetuate themselves through self-renewal and can also differentiate into mature cells of a particular tissue type. They are present in many

adult tissues and organs and help to maintain the normal tissue architecture (Reya et al., 2001). Over the past two decades, more and more studies have revealed the heterogeneity within a tumor population in various cancer types. The cancer stem cell model is one way to explain the intratumor heterogeneity (Visvader and Lindeman, 2008). Similar to normal stem cells, cancer stem cells refer to a subset of tumor cells that undergo self-renewal while generating differentiated cells that comprise the bulk of the tumor. However, compared with the normal tissue stem cells, cancer stem cells proliferate and differentiate aberrantly. Many studies have shown that cancer stem cells are more resistant to traditional chemotherapy and radiotherapy, which could be because of the higher expression of antiapoptotic proteins such as MYC, multidrug resistance genes such as the ATP-binding cassette (ABC) transporters including multidrug resistance (MDR) 1 and MRP1, and more efficient DNA damage repair. Therefore, in many cancers, although chemotherapy or radiotherapy can kill a majority of the proliferating cells leading to tumor shrinkage, the cancer stem cells can survive and lead to cancer relapse. Cancer stem cells were also reported to contribute to tumor metastasis.

Wnt signaling is well-known for regulating stemness in many normal tissues. Several studies have shown that Wnt signaling also plays important roles in maintaining cancer stem cells in various cancer types.

$CD34^+$ bulge stem cells maintain follicular homeostasis in mouse skin. In a mouse cutaneous tumor model induced by chemical carcinogens (DMBA/TPA), $CD34^+$ cells are significantly enriched by 9-fold in the tumor tissues compared with the normal skin. And the $CD34^+$ tumor cells are 100-fold more potent in initiating tumors than unsorted tumor cells in the limiting-dilution transplantation assay, which means this $CD34^+$ population contains the cancer stem cells of this mouse tumor model. It was further shown that Wnt/β-catenin signaling is essential in sustaining the $CD34^+$ cancer stem cell phenotype and that β-catenin deletion leads to the loss of $CD34^+$ cancer stem cells as well as complete tumor regression (Malanchi et al., 2008).

In pancreatic cancer, a small population of cancer cells with high intrinsic Wnt/β-catenin signaling showed properties indicative of cancer stem cells, including higher tumor-initiating capacity and drug resistance, whereas cancer cells with low Wnt activity expressed markers of differentiation. In these tumors, RSPO2 was shown to enhance Wnt/β-catenin signaling to confer stemness traits to susceptible pancreatic cancer cells (Ilmer et al., 2015).

In mouse and human lung adenocarcinomas, two distinct cell subpopulations were identified, one with high Wnt/β-catenin signaling and the other forming the niche that provides the Wnt ligands. The Wnt responding cells expressed the stem cell marker LGR5 and had increased tumor propagation capacity. They comprised a small minority of the tumor but could give rise to the other cell populations of the tumor bulk. The evidence suggests that these cancer cells with active Wnt signaling have features of normal tissue stem cells. Genetic and pharmacological perturbation of Wnt signaling in this model significantly suppressed tumor progression (Tammela et al., 2017).

In colorectal cancer, even though most of the cancer cases have elevated Wnt/β-catenin signaling due to mutations/alterations in the Wnt pathway components, the Wnt signaling level reflected by β-catenin localization and target genes expression is still very heterogeneous within the tumor. It is reported that high Wnt signaling functionally designates the cancer stem cells in colorectal cancer. These Wnt high tumor cells are located close to stromal myofibroblasts and respond to the myofibroblast-secreted factors to activate β-catenin–dependent transcriptions. Notably, myofibroblast-secreted factors can also restore the cancer stem cell phenotype in more differentiated tumor cells, suggesting that the dynamic stemness in colorectal cancer is not just defined by high Wnt signaling, instead is also orchestrated by the tumor microenvironment (Vermeulen et al., 2010).

Recent studies also revealed the involvement of noncoding RNAs in regulating stemness of cancer cells. Many of the reported noncoding RNAs confer stemness traits to cancer cells through activating Wnt/β-catenin signaling (Zhan et al., 2017). microRNA-146a represses Numb and stabilizes β-catenin to maintain the symmetric division of colorectal cancer stem cells (Hwang et al., 2014). In liver cancer stem cells, the long noncoding RNA lncTCF7 is highly expressed and triggers TCF7 expression by recruiting the SWI/SNF complex to *TCF7* promoter, thereby activating Wnt signaling. lncTCF7 is required for the self-renewal and tumorigenic capacity of liver cancer stem cells (Wang et al., 2015). miR-142 promotes mRNA degradation of APC to activate Wnt signaling in breast cancer stem cells (Isobe et al., 2014), whereas miR-582-3p targets mRNAs of Wnt signaling negative regulators *AXIN2*, *DKK3*, and *SRP1* for degradation to maintain stemness features in non–small cell lung cancer (Fang et al., 2015).

Wnt Signaling Mediated Resistance to Conventional Chemotherapy and Radiotherapy. Conventional chemotherapy exposes patients with cancer to cytotoxic agents to block proliferation and lead to cell death of rapidly proliferating cells including but not limited to cancer cells. Radiotherapy applies ionizing radiation to tumor tissues, which damages the DNA of cancer cells and leads to cell death. Cancer stem cells are known to be more resistant to traditional chemotherapeutic agents and radiation compared with the non–stem cell populations of the tumor bulk, and thereby may be able to cause cancer relapse after chemotherapy or radiotherapy. It has been discussed in the previous section that Wnt signaling is important in maintaining the cancer stem cells, therefore contributing to therapeutic resistance in several cancer types.

However, the protective effect conferred by Wnt signaling against chemotherapy and radiotherapy is not restricted to cancer stem cells but also applies to the non–stem cancer cell populations. It has been reported in many cancers that the Wnt/β-catenin activity level positively correlates with the resistance to several common chemotherapeutic drugs and radiation. Chemotherapy or radiation can upregulate Wnt signaling, and upregulation of Wnt signaling protects cancer cells from cell cycle arrest or apoptosis. Although this protective effect has been attributed to the downstream effectors of Wnt/β-catenin signaling such as survivin in one study, more generally the underlining mechanism still remains unclear (Watson et al., 2010; Gao et al., 2013; Nagano et al., 2013; Emons et al., 2017). Two recent studies found that elevated Wnt signaling enhanced the DNA damage repair and thereby conferred resistance to the PARP inhibitor olaparib in ovarian cancers (Fukumoto et al., 2019; Yamamoto et al., 2019). This mechanism could also protect cancer cells from DNA damage caused by other conventional chemo agents or radiation. Another study reported that canonical Wnt signaling is activated in therapy-induced senescence of

cancer cells and can enhance the tumor initiation capacity of cancer cells released or escaping from senescence (Milanovic et al., 2018). In addition, blockade of Wnt signaling by small molecular inhibitors or antibodies targeting Frizzleds or RSPOs can significantly enhance the toxicity of chemotherapy or radiotherapy or resensitize the resistant tumors to the treatment in many studies. Notably, except for those Wnt-driven tumors, anti–Wnt signaling treatment itself normally does not show potent suppressive effect on tumor growth but can significantly enhance the therapeutic effects of conventional chemotherapeutic agents in combinational treatment, further suggesting that Wnt signaling has a specific role in mediating resistance to chemotherapy (Nagaraj et al., 2015; Chartier et al., 2016; Fischer et al., 2017a,b; Trillsch et al., 2017).

Wnt Signaling Mediated Resistance to Targeted Therapy. Wnt signaling also confers resistance against more targeted therapies. Prostate cancer is hormone sensitive; therefore, most patients with prostate cancer who cannot be cured by surgery or radiation undergo androgen deprivation therapy. However, the majority of those patients eventually develop lethal metastatic castration–resistant prostate cancer. Some of the resistance is due to intracrine biosynthesis of androgens, which can be blocked by abiraterone acetate/prednisone (AA/P). But there are still many patients who fail to respond to AA/P. A recent clinical study showed that the AA/P nonresponders have more alterations of Wnt pathway components in their tumors, resulting in elevated Wnt/β-catenin signaling. Organoids derived from tumors of the nonresponders are also resistant to AA/P but can be resensitized to AA/P by Wnt pathway inhibition (Wang et al., 2018). This finding is consistent with preclinical study results that show that Wnt/β-catenin signaling can be activated in prostate cancer cells after androgen deprivation to promote androgen-independent growth, and combining antiandrogen agent with a Wnt pathway inhibitor can achieve enhanced growth suppression effect in both androgen-dependent and -independent prostate cancer cells (Wang et al., 2008; Lee et al., 2015). In addition to the canonical Wnt/β-catenin signaling, it is also reported that the noncanonical Wnt signaling is activated by WNT5A and WNT7B in the circulating tumor cells, which mediates resistance to the androgen receptor inhibitor (Miyamoto et al., 2015). Finally, both canonical and noncanonical Wnt signaling are involved in the bone metastases that are common in prostate cancer (Dai et al., 2008; Nandana et al., 2017).

Active Wnt signaling also contributes to resistance to growth factor signaling pathway inhibitors. A high nuclear β-catenin level is associated with resistance to PI3K/AKT/mTOR inhibitors in patients with colorectal cancer (Arqués et al., 2016). Mechanistically, inhibition of PI3K/AKT signaling promotes nuclear accumulation of the proapoptotic tumor suppressor FOXO3a. However, high nuclear β-catenin confers resistance to the FOXO3a-mediated apoptosis and subverts FOXO3a to promote metastasis in colon cancer, which can be reversed by tankyrase inhibition (Tenbaum et al., 2012; Arqués et al., 2016). In preclinical studies, elevated Wnt signaling has been observed in PI3K inhibitor–treated breast cancer cells and BRAF inhibitor–treated colorectal cancer cells. PI3K inhibition promotes the expression of Wnt ligands in breast cancers, whereas BRAF inhibition upregulates β-catenin signaling through activating the focal adhesion kinase in colorectal cancer. Blocking Wnt/β-catenin signaling by tankyrase inhibitor or PORCN inhibitor can significantly enhance the tumor suppression effect of these small molecular inhibitors targeting the growth factor signaling pathways (Tzeng et al., 2015; Solzak et al., 2017; Chen et al., 2018; Solberg et al., 2018). In EGFR-mutated lung cancer, even though EGFR inhibitors are not observed to significantly upregulate Wnt signaling in preclinical studies, Wnt/β-catenin signaling has been shown to drive resistance to EGFR inhibitors in lung cancer cells because tankyrase inhibitor and EGFR inhibitor in combination can synergistically suppress lung cancer cell growth in vitro and in vivo (Casás-Selves et al., 2012; Scarborough et al., 2017).

Bromodomain and extra terminal protein (BET) inhibitors are first-in-class targeted therapies that entered clinical trials for treating acute myeloid leukemia. Two independent studies found that Wnt/β-catenin signaling promotes primary and acquired resistance to BET inhibition in leukemia (Fong et al., 2015; Rathert et al., 2015). Mechanistically, BET inhibitors disrupt the BRD4-chromatin interaction and repress BRD4-dependent transcription of genes such as *MYC* to suppress cancer progression. However, in the presence of BET inhibitor, β-catenin binds to the promoter regions that are originally occupied by BRD4, and maintains the expression levels of key target genes including *MYC*. This evidence establishes Wnt/β-catenin signaling as a major driver and candidate biomarker of resistance to BET inhibitors in a subpopulation of leukemia cells that show high transcriptional plasticity.

Recently, two independent studies revealed that Wnt signaling mediates resistance to complete response to Hedgehog signaling inhibition in basal cell carcinoma (BCC) (Biehs et al., 2018; Sánchez-Danés et al., 2018). BCC is the most common malignancy in humans and typically arises due to constitutively activation of Hedgehog signaling. Treatment with Hedgehog signaling inhibitor vismodegib mediates differentiation of BCC cells from a hair follicle stem cell like phenotype to an interfollicular epidermis or isthmus cell fate, and thereby leads to tumor regression (Sánchez-Danés et al., 2018). However, BCC tumors always relapse after vismodegib withdrawal (Tang et al., 2016). It was found that a small population of LGR5+ BCC cells with active Wnt signaling escape from vismodegib-induced differentiation and mediate tumor relapse after treatment discontinuation. Importantly, combining vismodegib with PORCN inhibitor led to the eradication of persistent BCC cells and prevented tumor relapse in mouse models.

Wnt Signaling Mediated Resistance to Immunotherapy. The roles of Wnt signaling in regulating normal hematopoiesis remains a controversial topic (Staal et al., 2008; Kabiri et al., 2015). However, increasing evidence from recent studies suggests that Wnt/β-catenin signaling drives the primary, adaptive, and acquired resistance to cancer immunotherapy, first in the anti–CTLA-4/PD-1/PD-L1 treatment of melanoma but in other settings as well (Luke et al., 2019). In human metastatic melanoma samples, activation of Wnt/β-catenin signaling correlates with the absence of a T-cell gene expression signature, which reflects a lack of T-cell infiltration. Activation of tumor intrinsic β-catenin signaling suppresses the expression of tumor-cell–intrinsic chemokines such as CCL4, thereby suppressing the recruitment of $CD103^+$ dendritic cells and subsequent activation of $CD8^+$ T-cells (Spranger et al., 2015). The melanoma derived WNT5A can also activate β-catenin signaling in the dendritic cells in a paracrine mode, which upregulates the expression and activity of the indoleamine 2,3-dioxygenase-1 enzyme in local dendritic cells.

This promotes the development of tolerogenic dendritic cells and drives the differentiation of regulatory T-cells (Holtzhausen et al., 2015; Zhao et al., 2018). Concurrent inhibition of Wnt/β-catenin signaling enhanced the effect of immune checkpoint inhibitors in select preclinical models.

Anti-Wnt Signaling–Based Combination Cancer Therapies. As discussed previously, Wnt signaling mediates resistance to several therapeutic modalities in clinical practice as well as multiple anti-cancer agents developed in preclinical studies. Therefore, several drug combination studies combining Wnt-signaling inhibitors with other conventional cytotoxic agents or targeted inhibitors have been tested in preclinical studies and shown improved efficacy. Some of them have advanced into clinical trials. Moreover, recent technical advances in genome sequencing and genetic screening identified a group of genetically defined cancers that are addicted to Wnt signaling and other oncogenic pathways, such as a subset of colon cancers that harbor concurrent mutations in *BRAF* and *RNF43* or *RSPOs* (Yan et al., 2017) and *RNF43*-mutant pancreatic cancers that show high dependency on PI3K/mTOR signaling (Zhong et al., 2019). These findings inspired the development of PORCN inhibitor and growth factor pathway inhibitor combinational treatment in preclinical studies and clinical trials. Here, we summarize the drug combinations with Wnt pathway inhibitors for cancer therapy in both preclinical studies and clinical trials (Table 3).

Resistance to Wnt Pathway Blockade in Normal Tissue and Wnt-Driven Cancer

As discussed previously, dysregulated Wnt signaling is the driver of a series of genetically defined cancers, and elevated Wnt signaling can mediate resistance to various therapeutic strategies. Therefore, targeting Wnt-signaling pathways has been attracting attention in the field of cancer research for a long time. Researchers have developed different strategies to block Wnt signaling, including antibodies targeting receptors or agonists, PORCN inhibitors, tankyrase inhibitors, etc. These strategies may apply to different cases based on the specific alterations in the Wnt pathway. However, because Wnt signaling also maintains the homeostasis of normal tissue including bone, taste buds, and hair and gut stem cells, one common concern in using Wnt pathway inhibitors is the potential adverse effect on normal tissues with high dependency on Wnt signaling, such as the gastrointestinal tract. Indeed, tankyrase inhibitors have been shown to cause severe toxicity to the gut as well as loss of body weight in mice when used for cancer treatment, which limits the use of tankyrase inhibitors in human patients (Lau et al., 2013). However, surprisingly, PORCN inhibitors can be well tolerated by mice at therapeutic doses for cancer but showing no significant side effect on gut (Liu et al., 2013; Proffitt et al., 2013; Madan et al., 2016). In cancers, even those driven by *RNF43* mutations can develop resistance to Wnt blockade because two pancreatic cancer cell lines with the same *RNF43* loss-of-function mutation show different sensitivity to PORCN inhibitor. Interestingly, they were isolated from the same metastasis of the same patient, but one cell line is sensitive, whereas the other is resistant to PORCN inhibitor (Jiang et al., 2013). In colorectal cancers with *APC* mutations, some cell lines are sensitive to tankyrase inhibitors, whereas others are resistant, even though the expression of Wnt target genes can also be repressed by tankyrase inhibitors in the resistant cell lines (Lau et al., 2013). This observation reveals that there are potential mechanisms that mediates resistance to Wnt pathway inhibitors in normal tissues and cancers. Three mechanisms are described here to explain the resistance.

ABC Transporters. ABC transporters are members of a transport system superfamily. The ABC transporters consist of two ATP-binding domains and two transmembrane domains. These four domains can present in one peptide as a protein or two peptides that form a dimer. The two transmembrane domains typically contain twelve α-helices that form a pore-like structure across the membrane. The ATP-binding domains that have ATPase activity use the energy of ATP binding and hydrolysis to drive the conformational change of the transmembrane domains, thereby energizing the translocation of substrates across the membrane. There are many members in the ABC transporter family that are responsible for uptake or export of various substrates for the cell (Glavinas et al., 2004).

ABC transporters are known to cause the MDR phenotype in cancer cells by pumping diverse anticancer drugs out of the cell. ABCB1 is the most extensively studied ABC transporter related to drug efflux and is also called MDR1 (Robey et al., 2018). Many studies have shown that the expression of ABC transporters is upregulated when tumor cells get resistant to certain drugs, and this always results in multidrug resistance. So far, there is no direct evidence to show that resistance to Wnt pathway inhibitors is because of ABC transporters. However, one recent study from our group shows that in the mouse intestine, the stroma cells that supply Wnts and RSPOs and form the niche for the intestinal stem cells express a subset of ABC transporters, and are therefore resistant to various xenobiotics, including PORCN inhibitors (Chee et al., 2018). This property of the intestinal stroma cells could be due to the unpredictable environment rich in various xenobiotics in the gastrointestinal tract. But it can well explain the differential toxicities that tankyrase inhibitors and PORCN inhibitors have on the gut, as tankyrase inhibitors target the Wnt responding cells (the stem cells) directly, whereas PORCN inhibitors target the Wnt suppling cells, the resistant stroma cells here. This finding provides the rationale for the safety of the gut when using PORCN inhibitors in cancer therapy.

Further Mutations of Wnt Pathway Components. It is generally accepted that upstream Wnt pathway inhibitors such as PORCN inhibitors are less effective in cancer cells with mutations of downstream Wnt pathway components such as *APC* mutations, even though it is also reported that Wnt ligands are required to maintain the high levels of Wnt signaling in colorectal cancer cells with *APC* mutations (Voloshanenko et al., 2013). Besides, it has been observed in many cancer types that inactivating mutations of *RNF43*/*ZNRF3*, *RSPOs* translocation, activating mutations of β-catenin, and loss of *APC* are almost mutually exclusive, which reflects a rule in tumorigenesis that Wnt/β-catenin signaling only need to be activated in one way or another. However, co-occurrence of mutations in both upstream and downstream Wnt pathway does exist in some rare cases, in which the PORCN inhibitors may not work even though alterations of *RNF43*/*ZNRF3* or *RSPOs* can be detected. In addition, mutations in downstream components of the Wnt pathway can happen during the long-term treatment with upstream Wnt pathway inhibitors. These resistant clones may be selected by the treatment and become the dominant population of the tumor. This process has been reported in

TABLE 3
Anti-Wnt signaling-based drug combinations in cancer therapy

Drug A	Drug B (Wnt Pathway Inhibitor)	Drug Combination Rationales/Preclinical Results	Clinical Trials
Paclitaxel and carboplatin (chemo agents)	Ipafricept/OMP-54F28 (decoy Wnt recptor)	The drug combinations showed synergistic and potent tumor growth inhibition in patient-derived ovarian, pancreatic, hepatocellular, breast, lung, and/or colorectal cancer xenografts (Chartier et al., 2016; Fischer et al., 2017a,b; Jimeno et al., 2017).	Phase 1 clinical trial (NCT02092363) in patients with recurrent platinum-sensitive ovarian cancer
Nab-paclitaxel and gemcitabine (chemo agents)			Phase 1 clinical trial (NCT02050178) in patients with previously untreated stage IV pancreatic cancer
Sorafenib (RAF, VEGFR, and PDGFR inhibitor)			Phase 1 clinical trial (NCT02069145) in patients with hepatocellular cancer
Paclitaxel (chemo agent)	Vantictumab/OMP-18R5 (anti-Frizzleds antibody)		Phase 1 clinical trial (NCT01973309) in patients with locally recurrent or metastatic breast cancer
Docetaxel (chemo agents)			Phase 1 clinical trial (NCT01957007) in patients with previously treated non-small-cell lung carcinoma
Nab-paclitaxel and gemcitabine (chemo agents)			Phase 1 clinical trial (NCT02005315) in patients with previously untreated stage IV pancreatic cancer
Taxol, gemcitabine, or irinotecan (chemo agents)	Anti-RSPO1, anti-RSPO2, and anti-RSPO3 antibodies		
FOLFIRI (chemo agents)	OMP-131R10 (anti-RSPO3 antibody)		Phase 1 clinical trial (NCT02482441) in patients with metastatic colorectal cancer
API2 (AKT inhibitor)	NVP-TNKS656 (tankyrase inhibitor)	Tankyrase inhibition reverted resistance to AKT inhibition in patient-derived colorectal cancer xenografts (Arques et al., 2016).	
Gefitinib or AZD9291 (EGFR inhibitors)	AZ1366 (tankyrase inhibitor)	The drug combination significantly slowed down growth of non–small cell lung cancer orthotopic xenografts and improved survival of tumor-bearing mice (Scarborough et al., 2017).	
Vemurafenib (BRAF inhibitor)	ICG-001 (blocking β-catenin/TCF interaction)	BRAF inhibition upregulated β-catenin signaling in colorectal cancer cell lines in vitro. The drug combination led to synergistic tumor growth inhibition in HT29 xenografts (Chen et al., 2018).	
LGX818 (BRAF inhibitor) and Cetuximab (EGFR inhibitor)	LGK974 (PORCN inhibitor)	A subset of colorectal cancers harbor concurrent mutations in *BRAF* and *RNF43* or *RSPOs* (Yan et al., 2017).	Phase 1/2 clinical trial (NCT02278133) in patients with BRAF-mutant metastatic colorectal cancer with Wnt pathway mutations
Buparlisib/BKM120 (pan-PI3K inhibitor)	LGK974 (PORCN inhibitor)	Burparlisib treatment upregulated Wnt signaling in triple negative breast cancer (TNBC) cell lines in vitro. The drug combination led to synergistic tumor growth inhibition in xenografts of TNBC cell line TMD231 (Solzak et al., 2017).	
Multiple PI3K/mTOR inhibitors including GDC-0941, ZSTK474, LY-294002, AS252424, and Ku0063794	ETC-159 (PORCN inhibitor)	ETC-159 in combination with one of the five PI3K/mTOR inhibitors synergistically suppressed 3D colony formation of several Wnt-addicted pancreatic and cholangiocarcinoma cell lines. ETC-159 in combination with GDC-0941 led to synergistic tumor growth inhibition in HPAF-II and AsPC-1 xenografts (Zhong et al., 2019).	
Olaparib (PARP inhibitor)	Pyrvinium (CK1α activator) or XAV939 (tankyrase inhibitor)	Wnt/β-catenin signaling mediated resistance to PARP inhibition in ovarian cancer due in part to upregulation of DNA damage repair. Inhibition of β-catenin signaling reverted resistance to olaparib in ovarian cancer xenografts (Fukumoto et al., 2019; Yamamoto et al., 2019).	
Vismodegib (Hedgehog signaling inhibitor)	LGK974 (PORCN inhibitor) or anti-LRP6 antibody	A $LGR5^+$ tumor cell population maintained by active Wnt signaling persists vismodegib treatment in human and mouse basal cell carcinoma and mediates relapse after treatment discontinuation. The drug combination reduced tumor burden	

(*continued*)

TABLE 3—*Continued*

Drug A	Drug B (Wnt Pathway Inhibitor)	Drug Combination Rationales/Preclinical Results	Clinical Trials
		(vismodegib + anti-LRP6 antibody) or eliminated resistant tumor cells (vismodegib + LGK974) in mouse models (Biehs et al., 2018; SanchezDanes et al., 2018).	
PDR001 (anti-PD1 antibody)	LGK974 (PORCN inhibitor)	Preclinical studies showed that Wnt signaling mediated resistance to immunotherapies, and concurrent inhibition of Wnt/β-catenin signaling enhanced the efficacy of immune checkpoint inhibitors in melanoma mouse models (Holtzhausen et al., 2015; Spranger et al., 2015; Zhao et al., 2018).	Phase 1 clinical trial (NCT01351103) in patients with malignancies dependent on Wnt ligands
Pembrolizumab (anti-PD1 antibody)	ETC-159 (PORCN inhibitor)		Phase 1 clinical trial (NCT02521844) in patients with advanced solid tumor
	CGX1321 (PORCN inhibitor)		Phase 1 clinical trial (NCT02675946) in patients with advanced gastrointestinal tumor

a colorectal cancer cell line VACO6. VACO6 harbors an RSPO3 translocation and is sensitive to the PORCN inhibitor LGK974. However, these cells developed resistance to LGK974 within 3 months when cultured with a sublethal concentration of LGK974. Exome sequencing of the resistant VACO6 clone found truncating mutations in AXIN1 that are absent in the parental cell line (Picco et al., 2017). Interestingly, these truncating mutations have been reported in the Catalogue of Somatic Mutations in Cancer database as cancer-related somatic variants. This study demonstrates that further mutations in downstream components of Wnt signaling can confer secondary resistance to genetically defined cancers that are sensitive to upstream Wnt pathway blockade.

Activation of Other Compensatory Signaling Pathways. Wnt signaling can mediate resistance to inhibitors of other signaling pathways, especially the growth factor signaling pathways, and so it is not surprising that activation of these growth factor signaling pathways can compensate for Wnt pathway blockade. For example, mTOR signaling is reported to mediate resistance to tankyrase inhibitors in colorectal cancer (Mashima et al., 2017). Mechanistically, most of the pro-oncogenic and anti-oncogenic targets are regulated by more than one pathway. For example, c-Myc, as a Wnt-signaling effector, is regulated at the transcriptional level and post-translational level by Wnt signaling, as well as PI3K/AKT/mTOR signaling, MAPK signaling, etc. Therefore, inhibiting one signaling input can be compensated by other regulators and may have a minimal effect on the output. Notably, in many Wnt-driven cancers, the growth factor signaling pathways are also hyperactivated due to mutations in *EGFR*, *KRAS*, *BRAF*, *PI3K*, *PTEN*, etc. Therefore, developing combination drug therapy is a promising solution to maximize the therapeutic effects.

Concluding Remarks

Since the discovery of the first mammalian *Wnt* gene and its function in the mouse mammary cancer nearly four decades ago, the Wnt-signaling pathway has been extensively investigated worldwide. Although these studies comprehensively revealed the molecular mechanisms and functional effects of the Wnt signaling in normal status and diseases, numerous questions still remain to be answered. Recent findings in the Wnt field continue to extend our understanding of this evolutionarily conserved ancient signaling pathway. As a highlight of this review, an increasing number of studies reveal and confirm the key roles of Wnt signaling in cancer initiation, progression, and drug resistance, inspiring pharmacological targeting of Wnt signaling in patients.

Importantly, increasing knowledge in the Wnt field have facilitated the development of multiple Wnt pathway inhibitors along with rational drug combination strategies. These inhibitors and drug combinations showed promising efficacy in carefully selected preclinical cancer models, and some have advanced to clinical trials. However, considering the important roles of Wnt signaling in maintaining normal tissue homeostasis, the on-target adverse effects of Wnt pathway inhibitors cannot be neglected. Notably, although those upstream Wnt pathway inhibitors including PORCN inhibitors and the anti-Frizzleds antibody advanced to clinical trials and showed manageable side effects, several of the downstream inhibitors such as the tankyrase inhibitors have remained at the preclinical stage, possibly due to the toxicity to the gastrointestinal tract. Therefore, it will be important to both develop more selective inhibitors that have minimal toxicity on normal tissue and also develop synergistic drug combinations so that lower dosages can be used to improve both the therapeutic index and the anticancer efficacy.

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Authorship Contributions

Performed data analysis: Zhong, Virshup.

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Wnt signaling and drug resistance in cancer

Zheng Zhong, David M. Virshup

Supplemental files

6ahy_19seq.pdb: the PDB file that contains the structure of human Wnt3 in complex with mouse Frizzled 8 CRD for visualizing the consensus modeling of 19 human Wnts

6ahy_19seq.py: the Python script used for visualizing the consensus modeling of 19 human Wnts

6ahy Global.pdb: the PDB file that contains the structure of human Wnt3 in complex with mouse Frizzled 8 CRD for visualizing the consensus modeling of 2635 Wnt homologues

6ahy Global.py: the Python script used for visualizing the consensus modeling of 2635 Wnt homologues

To visualize the consensus modeling of 19 human Wnts or 2635 Wnt homologues, load the 6ahy_19seq.pdb (the 19 human Wnts) or the 6ahy Global.pdb (2635 Wnt homologues) file in PyMOL and run the corresponding .py file to map the conservation scores on the Wnt3 crystal structure. The PDB files are derived from PDB 6ahy.