Wnt/β-Catenin Signaling Pathway as Novel Cancer Drug Targets

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Abstract: Wnt proteins are a large family of secreted glycoproteins. Wnt proteins bind to the Frizzled receptors and LRP5/6 co-receptors, and through stabilizing the critical mediator β-catenin, initiate a complex signaling cascade that plays an important role in regulating cell proliferation and differentiation. Deregulation of the canonical Wnt/β-catenin signaling pathway, mostly by inactivating mutations of the APC tumor suppressor, or oncogenic mutations of β-catenin, has been implicated in colorectal tumorigenesis. Although oncogenic mutations of β-catenin have only been discovered in a small fraction of non-colon cancers, elevated levels of β-catenin protein, a hallmark of activated canonical Wnt pathway, have been observed in most common forms of human malignancies, indicating that activation of this pathway may play an important role in tumor development. Over the past 15 years, our understanding of this signaling pathway has significantly improved with the identification of key regulatory proteins and the important downstream targets of β-catenin/Tcf transactivation complex. Given the fact that Wnt/β-catenin signaling is tightly regulated at multiple cellular levels, the pathway itself offers ample targeting nodal points for cancer drug development. In this review, we discuss some of the strategies that are being used or can be explored to target key components of the Wnt/β-catenin signaling pathway in rational cancer drug discovery.

Keywords: Wnt, β-catenin, signal transduction, tumorigenesis, cancer targets, high throughput screening.

INTRODUCTION

A general paradigm for the development of human cancers has been developed [1]. In this model, cancer is viewed as a multi-stage disease caused by the accumulation of genetic alterations in tumor suppressor genes and/or oncogenes. Numerous studies suggest that activation of the Wnt/β-catenin signaling pathway plays an important role in human tumorigenesis [2-4]. Therefore, many components of this signaling pathway may serve as rational targets of cancer drug development. Given the complexity of Wnt/β-catenin signaling pathway, it is conceivable that potential cancer drugs can be developed by targeting the different nodal points of this signaling pathway.

The term “Wnt” was coined from a combination of the Drosophila segment polarity gene Wingless [5] and the mouse proto-oncogene Int-1 [6,7]. Wnt signaling is involved in many developmental processes, and many components of this pathway are evolutionarily conserved among Drosophila, Dictyostelium, C. elegans, Xenopus, and mammals [8,9]. Wnt proteins are a large family of secreted glycoproteins with at least 19 known human members. The Wnt signal determines the dorsoventral axis in Xenopus and segment polarity in Drosophila, whereas its function in mammals is less well defined. During development, Wnts play important roles in cell fate specification, tissue patterning, and control of asymmetric cell division. The expression of Wnt genes is developmentally regulated in a coordinated temporal and spatial manner. The Wnt ligands initiate the signaling pathway by binding to the Frizzled (Fz) receptors [10-12] and co-receptors LRP5 and LRP6 [13,14]. Canonical Wnt/β-catenin signaling is only activated when both Fz and LRP5/6 are complexed with Wnt ligands. Soluble forms of Fz (a.k.a., secreted Fz related proteins, sFRPs, or FzBs) act as antagonists by squelching Wnt ligands. Similarly, Dickkopf (Dkk) proteins potently inhibit Wnt signaling by binding LRP co-receptors and sterically hindering Wnt interaction with LRPs. Upon binding of a Wnt ligand, the scaffold protein Axin translocates to the cell membrane where it interacts with the intracellular domains of the LRPs, resulting in destabilization of Axin and activation of β-catenin activity. In parallel, Wnt binding to Fz results in hyperphosphorylation of the Disheveled (Dvl) protein [15], which, through its association with Axin [16-
21] and the APC tumor suppressor [22,23], prevents glycolgen synthase kinase 3β (GSK3β) [24,25] from phosphorylating β-catenin [26,27]. Unphosphorylated β-catenin is stabilized by escaping the recognition of by β-TrCP [28-30], a component of an E3 ubiquitin ligase, and eventually translocates to the nucleus where it engages transcription factors LEF/Tcf-4 (T cell factor-4) [31,32] to activate expression of downstream genes (Fig. 1).

β-Catenin performs dual functions, which include a crucial role in cell-cell adhesion and the Wnt/Wingless signaling pathway. Originally identified as a cytoplasmic protein that interacts with cell adhesion molecules, such as E-cadherin, β-catenin was found to be the mammalian homologue of armadillo, a segment polarity gene involved in the Wingless pathway in Drosophila [9]. Its localization in two subcellular compartments corresponds well to these two functions. Several cellular proteins have been identified as directly or indirectly interacting with β-catenin [33]. In normal and unstimulated cells, the majority of β-catenin protein is present in cell-cell junctions with very little in cytoplasmic or nuclear fractions, due to the rapid turnover of β-catenin promoted by the APC/GSK3β/Axin complex. However, in the presence of Wnt signal, GSK3β activity is inactivated, leading to the accumulation of cytoplasmic and, subsequently, nuclear β-catenin, and the activation of β-catenin/Tcf-4 downstream target genes, such as c-Myc, cyclin D1, c-jun/fra-1, uPAR, PPARδ, MMP-7 and WISP [34-42].

β-Catenin signaling is tightly regulated through Wnt-dependent and Wnt-independent mechanisms. β-Catenin signaling is negatively regulated by several cellular factors, including Tcf-1 [43], Groucho [44,45], ICAT [46], Idax [47], Duplin [48], Axam [49], presenilin 1 [50,51], Brg-1 [52], HBP1 [53], and Suppressor of fused [54]. In addition, the serine/threonine phosphatase PP2A has been shown to associate with and dephosphorylate Axin [55-57], suggesting that PP2A may regulate β-catenin stability in response to the Wnt signal. Casein kinases Iε and II have been shown to phosphorylate the disheveled protein [58-61], suggesting that the casein kinases may be involved in the Disheveled-mediated inhibition of β-catenin degradation. A recent study has demonstrated that an Axin-associated kinase, casein kinase 1α (CK1α), functions as a “priming” kinase and preferentially phosphorylates serine-45 of β-catenin, an event that is presumably required for subsequent GSK3β phosphorylation of the β-catenin protein [62]. As an alternative mechanism of β-catenin degradation, Siah-1, the human homolog of Drosophila seven in absentia, has been shown to interact with APC and promote β-catenin degradation in a p53-regulated fashion [63,64]. An increasing number of reports have indicated that β-catenin signaling may also be activated by Wnt-independent mechanisms in mammalian cells [65], including G-protein [66], integrin-linked kinase (ILK) [67], hepatocyte growth factor (HGF) [68,69], growth factor Gas6 [70], insulin-like growth factor-1 (IGF-1) [71,72], IGF-II [73], FGF-1 [74], and vascular endothelial growth factor (VEGF) [75]. Furthermore, cross talks occur between the Wnt/β-catenin signaling pathway and other pathways, including MAP kinase [76-78], Ca2+/calmodulin kinase II [79,80], TGFβ [81], PKC [82-84] and retinoid signaling [85]. Although the regulatory mechanisms of these factors remain to be defined, these findings clearly indicate that β-catenin signaling is tightly regulated in normal cells.

It is important to note that Dvl is probably a main switchboard of Wnt signaling. While this review focuses on targeting Wnt signaling through the β-catenin/Tcf canonical pathway, two other pathways branch off at the level of Dvl. These non-canonical Wnt pathways transduce Wnt signal to either JNK/planar cell polarity (PCP) pathway or the Ca2+-releasing pathway [86]. In general, activators of the non-canonical Wnt pathways, such as Naked cuticle (Nkd), Strabismus (Stbm), and Dapper (Dpr), inhibit the canonical Wnt/β-catenin signaling activity.

### Aberrant Activation of Wnt/β-Catenin Signaling in Human Tumorigenesis

Although the involvement of β-catenin in tumorigenesis was first established in colorectal cancers, where β-catenin was found to form a complex with the APC tumor suppressor gene product [87,88], the importance of β-catenin in regulating cell proliferation has been highlighted by the discovery of oncogenic mutations of the β-catenin gene in colon cancers containing the wild-type APC gene [89-91]. Mutant β-catenin protein becomes more stable because it is capable of bypassing APC-targeted degradation. Moreover, although at a much lower frequency, β-catenin mutations have been uncovered in a variety of human tumors, including melanoma, hepatocellular carcinoma, hepatoblastoma, Wilms’ tumor, prostate cancer, ovarian cancer, uterine endometrial carcinoma, medulloblastoma, thyroid carcinoma, gastric cancer, renal cancer, osteosarcoma, malignant fibrous histiocytoma (MFH) and pilomatrixoma [91-124]. Thus, these collective genetic data highly indicate that deregulation of β-catenin signaling may be involved in the development of a broad range of human malignancies. This notion is further supported by a long-standing observation that overexpression of β-catenin downstream targets, such as c-Myc and cyclin D1, has been extensively documented in most human tumors [125-127].

Stabilization of the β-catenin protein is the key to its activation. Identification of oncogenic mutations in the GSK3β phosphorylation sites of the β-catenin degradation domain has clearly demonstrated that down-regulation of GSK3β activity and concomitant stabilization of β-catenin are critical to the activation of β-catenin signaling. In contrast to colorectal cancer, where mutations in either APC or β-catenin account for more than 90% of tumors, most non-colon cancers harbor no mutations in APC or a very low percentage of mutations in β-catenin [2,128]. However, a significant fraction of these tumor samples show apparent cytoplasmic and/or nuclear accumulation of β-catenin protein [129-142]. Thus, causes for the elevation of β-catenin activity in most human tumors remain undefined.

### ONCOGENES AND TUMOR SUPPRESSOR GENES IN WNT SIGNALING

#### β-Catenin, APC, Axin, Tcf-1

Several proteins in the Wnt signal transduction pathway are oncogenes or tumor suppressors. To date, documentation of mutations in Wnt itself in human cancers is rare, yet
mutations in its downstream targets are frequent [33,143]. The oncogenic role of β-catenin was highlighted by the discovery in which activating β-catenin mutations were detected in approximately 50% of the colorectal cancers that contained wild type APC [90]. The mutually exclusive feature of APC or β-catenin mutations in colon cancers underlines the critical role of β-catenin in colon cancer development. As discussed previously, oncogenic mutations of β-catenin have been found in many human tumors. In fact, the critical role of β-catenin in tumorigenesis has recently been demonstrated in a variety of animal models. For instance, oncogenic forms of β-catenin have been shown to induce tumor formation in transgenic animals [144,145], whereas mutations in β-catenin gene have been frequently uncovered in tumors induced by either carcinogens or activated oncogenes [93,146].

Although Wnt was first identified as an oncogene in mouse mammary tumors, it was the identification of the APC tumor suppressor gene that drew the multidisciplinary attentions to the ever-growing Wnt signaling pathway. The APC gene was originally discovered as the genetic cause for familial adenomatous polyposis (FAP). FAP patients develop large numbers of colorectal polyps in early adulthood. Without any interventions, many of the polyps can further develop into carcinomas and metastatic colorectal cancers. FAP patients, who have inherit one defective allele of the APC gene, usually develop polyps which are clonal outgrowths of colonic epithelial cells with the second APC allele mutated. Immediately after the discovery of genetic connection between APC and FAP, it became apparent that both alleles of APC were inactivated in nearly 80% of sporadic colorectal cancers [147,148]. Further studies have firmly established that APC tumor suppressor functions as a gatekeeper in colorectal tumorigenesis.

The tumor suppressor Axin is an intracellular protein that binds to the APC/GSK3β/CKIα complex and is pivotal in regulating β-catenin degradation [149-151]. Axin was first identified in mice as a product of the Fused gene which regulated an early step in embryonic axis formation [16]. Humans have two Axin genes that encode for 900 amino acid proteins containing an N-terminal APC binding domain [150]. The central region of Axin contains GSK3β, β-catenin, and CKIα binding domains, while the C-terminal region contains the Dvl binding domain [149,150]. When Axin is bound to the β-catenin/APC/GSK3β/CKIα complex, GSK3β phosphorylates β-catenin which then enters the ubiquitin degradation pathway [151]. Upon Wnt stimulation, Axin translocates to the cell membrane to interact with LRP5 and Dvl [149]. Dvl becomes phosphorylated and subsequently inhibits GSK3β phosphorylation of β-catenin and thereby stabilizing β-catenin. Hence, the loss of function of Axin results in elevated nuclear β-catenin and consequently increases expression of the Wnt downstream targets such as Cyclin D1 and c-Myc. To that end, mutations of the Axin gene and/or its loss of expression have been linked to numerous human neoplasms such as colorectal cancer, esophageal squamous cell carcinoma, and medulloblastoma [152-156]. Mutations in Axin have also been reported in hepatocellular carcinomas and a variety of pediatric neoplasms [155,157]. The common finding among these neoplasms is an increase in nuclear β-catenin because it is able to escape the ubiquitin-dependent degradation pathway.

Upon entering the nucleus, β-catenin binds to the transcription factors Tcf/LEF, which promotes the expression of Tcf-regulated genes. In addition, β-catenin gets acetylated by CBP (CREB-Binding Protein) to enhance transactivation at the c-Myc promoter region [158]. The human Tcf/LEF family of transcription factors consists of Tcf-1, LEF-1, Tcf-3, and Tcf-4, which all contain an 80 amino acid high-mobility group box (HMG) that binds DNA [159]. Although activating mutations in Tcf/LEF genes are rare in human cancers, Duvel et al. reported a frequent frameshift mutation in Tcf-4 in both human colorectal cell lines and primary tumors [160]. Recently, Kramps et al. have identified two new components, BCL9 and pygopus (Pygo), in the β-catenin-Tcf/LEF nuclear complex [161]. Pygo, linked to β-catenin by BCL9, permits the β-catenin-Tcf/LEF complex to transcriptionally activate Wnt target genes [161].

**TARGETING WNT/β-CATENIN SIGNALING AT THE EXTRACELLULAR LEVEL**

Targeting growth factors and/or their receptors have recently generated a great deal of interest especially with the recent success of trastuzumab (Herceptin). Trastuzumab is a monoclonal antibody that selectively binds to the extracellular domain of the human epidermal growth factor receptor 2 (HER2) [162]. In breast cancer, HER2 positivity is correlated with decreased survival while the addition of trastuzumab to the treatment regimen has significantly improved patient survival [162-164]. In addition, VEGF (vascular endothelial growth factor) and its receptor have been the target of many clinical studies in human cancers [165]. It is conceivable that the Wnt ligands themselves can be targeted for anti-cancer therapy.

In an earlier study, antisense and sense Wnt-1 expression vectors containing a synthetic promoter composing of glucocorticoid response elements were introduced into a mouse mammary tumor cell line that expressed high levels of endogenous Wnt-1 and formed rapidly growing tumors when transplanted into syngeneic hosts. It was found that the levels of the expression of endogenous Wnt-1 mRNA and protein were reduced significantly (>80%) in the cells that expressed Wnt-1 antisense RNA following exposure to dexamethasone, and transplantation of the Wnt1-antisense expressing cells produced smaller tumors (approximately 0.2 cm in 16 weeks) compared to the tumors (approximately 2.0 cm in 8 weeks) that were produced by the R/Sa-MT/sense and R/Sa-MT cells [166]. More recently, a monoclonal antibody against Wnt-1 has been developed [167]. It was found that incubation of a monoclonal anti-Wnt-1 antibody induced apoptosis and caused downstream protein changes in a variety of human cancer cell lines, including non-small cell lung cancer, breast cancer, mesothelioma, and sarcomas overexpressing Wnt-1. In contrast, apoptosis was not detected in cancer cells lacking or having minimal endogenous Wnt-1 expression after the antibody incubation. This monoclonal antibody was also shown to suppress tumor growth in vivo [167]. Similarly, You et al. have shown that an anti-Wnt-2 monoclonal antibody induced...
Fig. (1). Schematic representation of Wnt/β-catenin signaling. Wnt binds to its Frizzled receptor and LRP5/6 co-receptor and activates Disheveled, leading to the inhibition of APC/Axin/GSK3β-mediated β-catenin degradation. Stabilized β-catenin forms a transcriptional complex with LEF/Tcf and activates downstream targets such as c-Myc. Negative regulators, such as Dkk3, WIF and FRPs, inhibit the interactions between Wnt ligands and their receptors. There are crosstalks between Wnts and other signaling pathways, such as growth factors that activate receptor tyrosine kinases. See text for details.

apoptosis and inhibited tumor growth in malignant melanomas [168]. These findings hold promise as a novel therapeutic strategy for cancers that exhibit an elevated Wnt expression.

As illustrated in Fig. (1), Wnt signaling is tightly regulated by numerous negative factors that interfere with the interactions between the Wnt ligands and their receptors [169,170]. It is conceivable that these antagonists could be used as natural inhibitors of Wnt signaling in cancer therapy. For example, secreted frizzled-related proteins (sFRPs) comprise of a family of five secreted glycoproteins that antagonize Wnt signaling. Significant transcriptional downregulation of the sFRP gene family by promoter methylation has been found in malignant pleural mesothelioma primary tissues and cell lines, as well as several other cancer cell lines (breast, lung, cervical, and glioma) [171]. Furthermore, transfection of the sFRP gene constructs into mesothelioma cell lines lacking sFRP expression resulted in apoptosis and growth suppression [171]. Interestingly, epigenetic inactivation by methylation of sFRP genes has recently been reported in colorectal cancers [172], even though majority of colon cancers already exhibited constitutive Wnt signaling. Restoration of sFRP function in colorectal cancer cells has shown to attenuate Wnt signaling even in the presence of downstream mutations [172]. Taken together, these findings suggest that exogenous expression of the sFRPs may effectively inhibit the proliferation of cancer cells.

Several recent studies have explored the anti-cancer potential of Dkk proteins, a group of antagonists of the Wnt co-receptors LRP5/6. Four Dkk proteins have been identified in humans, although their exact biological functions remain to be elucidated. For example, the expression of Dkk-3 was down-regulated in many human immortalized and tumor-
derived cell lines [173]. The expression of Dkk-3 was significantly down-regulated in primary non-small cell lung carcinomas, and expression of exogenous Dkk-3 gene in non-small cell lung carcinoma cells inhibited cell growth [174]. More recently, Dkk-3 has been shown to inhibit invasion and motility of Saos-2 human osteosarcoma cells by modulating the Wnt/β-catenin pathway [175]. Expression of Dkk-3 and dominant-negative LRP5 mutant in Saos-2 cells significantly reduces invasion capacity and cell motility, which is associated with changes in cell morphology consistent with a less invasive phenotype [175]. Furthermore, Dkk-3 and dominant-negative LRP5 also induce changes in β-catenin localization consistent with an increase in cell-cell adhesion [175].

A different approach has been taken to explore Dkk-1 as a sensitizing agent for tumor cells that are exposed to genotoxic stresses because Dkk-1 is a transcriptional target of the p53 tumor suppressor [176]. Various chemotherapeutic and other agents, which induce DNA adducts and compromise genome integrity, were shown to significantly increase the expression of Dkk-1. Interestingly, genotoxic damage-induced expression of Dkk-1 occurred in many human tumor cell lines, irrespective of their p53 gene status [176]. When human glioblastoma cell line U87MG, which had undetectable hDkk-1 expression, was engineered to express a moderate level of Dkk-1, marked apoptosis was observed after ceramide treatment [176]. Further, the DNA cross-linking drugs BCNU and cisplatin induced significant cell death in U87MG/Dkk-1 cells [176], suggesting that Dkk-1 may be used to sensitize cancer cells as an adjuvant for conventional chemotherapy. The contribution of Dkk-1 to gastrointestinal epithelial proliferation in adult animals was examined using an adenoviral vector expressing Dkk-1 [177]. It was shown that adenoviral Dkk1 treatment of adult mice repressed the expression of the Wnt target genes within 2 days and markedly inhibited proliferation in both the small intestine and colon. This was accompanied by progressive architectural degeneration with the loss of crypts, villi, and glandular structures [177], indicating that the efficacy of systemic expression of Dkk-1 can be used as a general strategy for conditional inactivation of Wnt signaling in adult organisms.

TARGETING WNT/β-CATENIN SIGNALING AT THE REGULATORY PROTEIN LEVEL

As discussed above, Wnt/β-catenin signaling is subjected to tight regulation exerted by a network of intracellular protein-protein interactions. Many of the proteins are negative regulators, which function as tumor suppressors in this signaling pathway. Consistent with the central role the APC tumor suppressor in the development of human colorectal cancers, the β-catenin binding domain of APC is sufficient for tumor suppression [178]. Specifically, a recombinant adenovirus (Ad-CBR) was constructed to constitutively express the central third of APC, which includes all of the known β-catenin binding repeats. When expressed in colon cancer cells, Ad-CBR blocked the nuclear translocation of β-catenin and inhibited β-catenin/Tcf4-mediated transactivation [178]. Accordingly, Ad-CBR infection of colorectal cancer cell lines with mutant APC but wild type β-catenin resulted in substantial growth arrest followed by apoptosis [178], suggesting that the β-catenin-binding domain of APC is sufficient for its tumor suppressor activity, and this mini-APC fragment may be used as anti-cancer agent. Similarly, Axin, another negative regulator of this signaling pathway, is considered as a tumor suppressor because Axin is mutated in several types of human cancers. Adenovirus mediated gene transfer of wild-type Axin-1 induced apoptosis in hepatocellular and colorectal cancer cells that had accumulated β-catenin as a consequence of either APC, CTNNB1 or Axin-1 mutation [155], suggesting that Axin may be an effective therapeutic molecule for suppressing growth of hepatocellular and colorectal cancers.

Several other negative regulators of Wnt/β-catenin signaling can also be conceptually developed as anti-cancer drugs. These factors include Idax [47], Axam [49], and ICAT [46]. A recent study using a recombinant adenovirus encoding ICAT, reported ICAT inhibited proliferation of colorectal tumor cells mutated in APC or β-catenin and hepatocellular carcinoma cells mutated in AXIN [179]. By contrast, ICAT did not inhibit growth of normal or tumor cells containing the wild-type APC, β-catenin, and Axin genes. ICAT was also shown to inhibit the anchorage-independent growth of colorectal tumor cells and tumorigenic growth of colorectal tumor xenografts [179]. These results suggest that expression of ICAT or drugs, which mimic its effects, may be useful in the treatment of human tumors.

TARGETING THE EXPRESSION OF β-CATENIN BY ANTISENSE AND RNA INTERFERENCE

Direct targeting β-catenin has attracted a broad range of attentions since the commonality of many human cancers with aberrant Wnt signaling results in increased β-catenin levels. Several approaches have been pursued, including antisense, RNA interference, and protein knockdown strategies. An anti-sense approach has gained popularity in the last decade. Currently, there are over 20 antisense drugs in clinical trials [180] while one antisense oligonucleotide, Vitravene, has been in use for CMV-induced retinitis for more than five years. The track record of safety and efficacy of antisense oligonucleotides makes them an attractive molecule for developing into an anti-β-catenin therapy. Several clinical trials are underway using antisense oligonucleotides against bcl-2, c-Myc, H-ras, and PKCα [181-186]. With increasing interest in antisense directed therapy, several investigators have begun to examine β-catenin as a potential target. To date, there have been more than 20 reports published using various strategies to knock down β-catenin. The two major strategies, antisense oligonucleotides and RNA interference, yield the same or similar results: decreasing the amount of mRNA and the amount of protein produced.

Antisense oligonucleotides are single-stranded DNA or RNA or chimeric DNA/RNA that are designed to specifically hybridize to a targeted mRNA and subsequently prevent protein synthesis. Since its inception more than 30 years ago, antisense techniques have been developed and widely applied for investigating gene function, gene expression regulation, and validation of novel drug targets.
The use of antisense oligonucleotides as therapeutic agents has been proposed for the treatment of a variety of human diseases including cancer and infection [180,187,188]. Although the exact underlying mechanisms are not fully understood, it is generally accepted that the inhibition of gene expression by antisense oligonucleotides results from hybrid-arrest and/or activation of RNase H after the oligonucleotides bind to and interact with their complimentary target mRNA in a sequence-specific manner. This blockade of gene translation or transcription often can result in therapeutic effects. Table 1 summarizes the reported sequences used in antisense investigations targeting β-catenin.

As early as 1994, antisense strategies were used to decrease expression of β-catenin. These early antisense studies were done to ascertain the roles of β-catenin in embryonic development and organogenesis [189-195]. These studies helped determine where and during which phases of development β-catenin is necessary. Utilizing antisense oligonucleotides is an excellent approach to temporarily decreasing or completely blocking the expression of a gene of interest. Still another antisense sequence was used to study Wnt signaling in mouse mammary epithelial cells [196]. Haertel-Wiesmann et al. reduced β-catenin mRNA levels by 50-80%, resulting in a 90% reduction in Lef transcription activity [196]. The most interesting finding of this group might be that Wnt3 has β-catenin-independent effects.

Two published papers evaluating the use of antisense oligonucleotides in colon cancer showed that the oligonucleotides decreased β-catenin mRNA in a dose-dependent manner, as well as decreased protein levels, Tcf transcription, and Cyclin D1 expression [197,198]. There was also a reduction in cell proliferation, invasiveness and anchorage-independent growth. These effects were only seen in APC-mutant cell lines (SW480, Colo201 and DLD-1), and no effects were seen in APC wildtype cells [197]. However, these APC wildtype cells were from a different tissue (breast), which may make it difficult to relate the APC status to the differences seen. The same oligonucleotide was used in an in vivo xenograft model (SW480 cells). The oligonucleotide decreased β-catenin protein expression in the tumors and tumor growth, and was able to bring about complete tumor regression in 3 out of 5 animals in one study [198]. This suggests that the antisense oligonucleotides directed against β-catenin are effective in reducing the growth of colon cancer, and may be of therapeutic value.

The same oligonucleotides were also used in five esophageal carcinoma cell lines and gave more than 50% reduction in β-catenin mRNA, as well as decreasing the protein expression and Tcf transcription [199]. The level of β-catenin protein expression in whole cell lysate was not significantly reduced after 48 hours. However, nuclear expression of β-catenin was greatly reduced. More importantly, apoptosis and caspase 3 activity were increased, and cell viability and proliferation were decreased up to 80% [199]. Chung et al. evaluated the effects of β-catenin expression in leukemia and lymphoma cell lines [200]. They found that β-catenin was not usually expressed in normal leukocytes, but was in tumor cells. They did not notice any decrease in cell viability, but their oligonucleotide did result in decreased aggregation of tumor cells [200].

Similar to antisense oligonucleotides, RNAi has been used for basic research. For example, Thompson et al. used RNAi to study the involvement of β-catenin in the Wnt signaling pathway [201]. Their study uncovered a new gene product involved in signal transduction, which likely mediates Tcf or Arm interactions with β-catenin [201]. Another Wnt signaling study using a siRNA clarified the need for nuclear accumulation of β-catenin in order to have Tcf transcription [202]. In addition, Kaplan et al. used RNAi to demonstrate that β-catenin is necessary for cells to form bipolar mitotic spindles [203]. Use of their dsRNA against β-catenin led to incomplete separation of centrosomes [203].

A few studies have evaluated the therapeutic potential of RNAi targeting β-catenin. Van de Wetering et al. created an inducible vector system to express siRNA in LS174T colon cancer cells and demonstrated a decrease in Tcf transcription, increase in G1 cell cycle arrest, and promotion of cell differentiation [204]. In contrast to a decrease in VEGF expression after using antisense oligonucleotides [196], an anti-β-catenin siRNA led to an increase in VEGF mRNA [205]. However, the investigators examined different VEGF

Table 1. Targeting β-Catenin by Antisense Oligos and RNAi

<table>
<thead>
<tr>
<th>Type of Molecule</th>
<th>Sequence(s)</th>
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<td>[190-192]</td>
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<tr>
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<td>5′-caggtcagttgagtcgc</td>
<td>[193,194]</td>
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<td>[196,290]</td>
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<td>Antisense</td>
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<td>[197,199]</td>
</tr>
<tr>
<td>RNAi</td>
<td>5′-ageuguauugagccag and 5′-caguuguguaaucucu-3′</td>
<td>[206]</td>
</tr>
<tr>
<td>RNAi</td>
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<td>[205]</td>
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<td>RNAi</td>
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<td>RNAi</td>
<td>5′-accagcagcataaaatcaggt</td>
<td>[201,203]</td>
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receptor ligands, VEGF-D in the siRNA study and VEGF-A in the antisense study, so the results may not have been in conflict. There also exists the possibility that the effects of β-catenin are dependent upon the cellular environment, and that the differences in fibroblasts (RNAi study) and colon carcinoma cells (antisense study) are large enough to affect the functions of β-catenin.

One siRNA approach was used in both cell cultures and in a xenograft model [206]. In both SW480 and HCT116 cell lines, the siRNA reduced β-catenin protein expression after 48 hours. This decrease in protein lasted for up to ten days after transfection. Similar results were seen in 293 and HeLa cells. In addition to the decreased protein, there was also a decrease in Tcf transcription and colony formation, and after 72 hours both colon cell lines showed a decrease in cell growth. The same siRNA was used in SW480 xenograft model. Cells pre-treated (before implantation) with the siRNA displayed decreased growth. Animals treated with 250 pmol of the siRNA also showed decreased tumor growth as well as increased survival [206].

Although there have been only a few examples of their use in vivo, RNAi also seems to be an effective method for knocking down expression of β-catenin, and has potential for use as a therapeutic agent. In comparison to the antisense technology, RNAi is still in its infancy. However, both our understanding and the growth of this technology are increasing exponentially. Therefore, both techniques may be used to create new therapeutic modalities in the near future. The sequences of the antisense and RNAi oligonucleotides appear in Table 1.

**TARGETING THE EXPRESSION OF β-CATENIN BY PROTEIN KNOCK DOWN**

Stabilization of the β-catenin protein is the key to activation of Wnt/β-catenin signaling. The stability of β-catenin is regulated by the cellular Skp1/Cull 1/F box (SCF) ubiquitination machinery [30,207-209]. Several recent studies have explored the feasibility of using this degradation process to accelerate the turnover rate of oncogenic β-catenin activity in human tumor cells. As a proof of principle of such protein knockdown strategy, Cong et al. engineered a chimeric protein with the β-catenin binding domain of E-cadherin fused to β-TrCP ubiquitin-protein ligase [210]. It was shown that the stable β-catenin mutant was recruited to the cellular SCF ubiquitination machinery for ubiquitination and degradation. Conditional expression of β-TrCP-E-cadherin in DLD1 colon cancer cells selectively knocked down the cytosolic, but not membrane-associated subpopulation of β-catenin. As a result, DLD1 cells were impaired in their growth and clonogenic ability in vitro, and lost their tumorigenic potential in nude mice [210].

In order to selectively eliminate pathogenic β-catenin in colon cancers, Su et al. first analyzed a variety of protein motifs known to interact with β-catenin, including the 15- and 20-amino acid repeats of the APC protein, the carboxy-terminal of E-cadherin, and the amino-terminal domain of Tcf-4 [211]. Although all these protein motifs were shown to interact with β-catenin, the APC 15-amino acid repeat unit (designated as APCbc), when engineered to contain multiple duplicates, exhibited the highest affinity to free β-catenin with minimal competition with E-cadherin-associated β-catenin [211]. By engineering a chimeric F box protein (CFP) with multiple copies of the APC 15-amino acid repeat unit, Su et al. demonstrated that introduction of CFP to colon cancer cells induced targeted ubiquitination and proteolytic degradation of nuclear and cytoplasmic free β-catenin while preserving its cellular adhesion counterpart [211]. It was subsequently shown that elimination of pathogenic β-catenin suppressed constitutive Wingless/Wnt signaling and inhibited in vitro and in vivo tumor cell growth [211]. Using a similar strategy, Liu et al. created chimeric F-box fusion proteins by replacing the WD40-repeat of β-TrCP with the β-catenin-binding domains of Tcf-4 and E-cadherin [212]. Expression of chimeric F-box fusion proteins successfully promotes degradation of β-catenin independent of GSK-3β-mediated phosphorylation. More importantly, this degradation does not require an intact APC protein [212]. Taken together, the above findings support the notion that enforced proximity between selected targets and the ubiquitin-conjugation machinery may be the most important factor in facilitating specific proteolysis. These results also demonstrate a practical utility of an SCF-based knockdown system as a tool in targeting β-catenin protein in human tumors.

**TARGETING β-CATENIN/TCF MEDIATED TRANSACTIVATION**

Directly targeting β-catenin and its functional partners (LEF/Tcf proteins) has attracted a broad range of attentions. Recent structural elucidation of the β-catenin/Tcf complex further highlights the possibility of developing cancer drugs via rational design and high throughput screening [213]. In order to target β-catenin/Tcf activity, Wakita et al. developed a novel mammalian two-hybrid system that selectively killed APC-mutated colon cancer cells [214]. This system consists of GAL4/β-catenin, VP16/Tcf-4, and a gene that is transcribed when GAL4 and VP16 associate. It was reported that GAL4/β-catenin accumulated in human colon cancer cells, and in the presence of VP16/Tcf-4, induced high levels of expression of a suicide gene resulted in selective killing of SW480 cells that harbor APC mutations [214]. This suggests that such a system may be applicable for a broader use of gene therapy by targeting tumors that involve β-catenin degradation. In a recent report, Emami et al. demonstrated that a small molecule that inhibited cyclic AMP response element-binding protein (CBP), a p300-related co-activator of β-catenin/Tcf complex, selectively targeted colon cancer cells and reduced colon cancer growth both in vitro and in vivo [215]. A more direct approach has been developed to selectively target the hyperactive β-catenin/Tcf pathway in colon cancer cells [216]. In this case, a recombinant adenovirus, in which an apoptosis gene FADD was under the control of the promoter containing Tcf-responsive elements, selectively and efficiently killed colon cancer cells in which the β-catenin/Tcf pathway was hyperactivated [216], providing a conceptual proof that aberrantly activated β-catenin/Tcf pathways may be used to selectively target colon cancers. In order to maximize the tumor killing effect in colon cancer cells in which β-catenin/Tcf4 pathway is activated, Kwong et al. constructed...
a recombinant adenovirus AdTOP-CMV-TK, which carries a herpes simplex virus thymidine kinase gene (HSV TK) under the control of a β-catenin/Tcf-response promoter [217]. AdTOP-CMV-TK and ganciclovir (GCV) treatment significantly suppressed the growth of human DLD-1 colon cancer cells, but not in a control liver cancer line, in nude mice [217].

The use of oncolytic viral vectors has generated great interest in cancer gene therapy, and there are clinical trials underway [218-221]. Selectively replicating viruses have a major advantage over non-replicating viruses to target known causal oncogenic defects because the therapeutic effect of the injected viruses is augmented by viruses produced within the tumor. This is a very appealing strategy to target the tumor cells that exhibit aberrantly high β-catenin/Tcf activity. To prove this concept, Bruno et al. developed replicating adenoviruses that express the viral E1B and E2 genes from promoters controlled by the Tcf4 transcription factor [222]. The Tcf-E2 and Tcf-E1B promoters were active in many, but not all, cell lines with activation of the Wnt pathway [222]. Viruses with Tcf regulation of E2 expression replicated effectively in SW480 colon cancer cells (i.e., with highly activated β-catenin/Tcf activity) but exhibited a 50- to 100-fold decrease in replication in H1299 lung cancer cells and WI38 normal fibroblasts [222]. Furthermore, activation of Wnt signaling by transduction of an oncogenic form of β-catenin into normal fibroblasts renders the cells permissive for viral replication [222]. Following a similar strategy, Fuerer and Iggo constructed adenoviruses with Tcf-binding sites in the E1A promoter region in order to target viral replication to colon cancers. They demonstrated that Tcf regulation of the E1A promoter conferred a 100-fold selectivity for cells with activated Wnt signaling in viral burst and cytopathic effect assays [223]. When Tcf-binding sites were inserted in the E1A, E1B, E2 and E4 promoters, the viruses exhibited up to 100 000-fold selectivity for cells with activated Wnt signaling [223]. Interestingly, it was subsequently shown that 5-fluorocytosine increased the cytotoxicity of Wnt signaling-targeting replicating adenoviruses that expressed cytosine deaminase as a late gene [224].

Similarly, Toth et al. constructed an oncolytic adenoviral vector (VRX-009) that targets tumor cells with upregulated Wnt signaling [225]. In this viral vector, the E4 promoter region was substituted with five Tcf-binding sites. The authors demonstrated that the viruses replicated on the orders of 2 magnitudes greater in SW480 colon cancer cells than in A549 lung cancer cells. In addition, viral replication in primary bronchial cells and umbilical endothelial cells was significantly less than SW480 cells. Furthermore, when tested in a xenograft model, VRX-009 was able to suppress growth in SW480 cells but not in A549 cells. This and other adenoviral vectors targeting the up-regulated Wnt pathway provide specific anti-tumor efficacy [216].

In addition to the adenoviral system, Malerba et al. modified the minute parvovirus of mice (MVM) P4 promoter to make it responsive to Wnt signaling by inserting binding sites for the heterodimeric β-catenin/Tcf transcription factor [226]. The hybrid MVM/H-I viruses containing Tcf mutant promoters were responsive to Wnt signaling in the context of the virus [226]. Compared to the parental virus, the burst size of the Tcf mutant viruses was reduced at least 1,000-fold in H1299, 293T, NB324K, and HeLa cells, which had relatively lower Wnt signaling activity, while the burst size and cytopathic effect of the Tcf viruses were comparable to the wild-type virus levels in SW480 and Isreco1 colon cancer cell lines, which had high β-catenin/Tcf activity [226]. These findings suggest that the hybrid MVM viruses exhibit high specificity in targeting cancer cells that contain elevated Wnt signaling activity.

**TARGETING DOWNSTREAM MEDIATORS OF β-CATENIN SIGNALING**

Numerous studies have focused on elucidating downstream signaling mediators of the Wnt/β-catenin pathway. Although there are many genes regulated by β-catenin/Tcf, some of the genes that have been associated with human cancers include c-Myc, cyclin D1, PPARδ, COX-2, CD44, MMP7, and c-Myb [35-37,39,227-229]. As many of these downstream targets may play an important role in tumorigenesis, it is possible that these genes can also be targeted for efficacious cancer therapy.

**c-Myc**

c-Myc is an oncogene overexpressed in a variety of human cancers including melanoma, leukemia, and prostate, breast, and colon carcinomas [230]. Upregulated by Wnt, c-Myc seems to be a viable target for anti-cancer therapy. Iversen et al. created an antisense drug, AVI-4126, targeted against c-Myc and showed promising results in vitro and in Phase I clinical trials [185]. The authors reported significant growth inhibition and induction of apoptosis in PC-3 prostate cancer cells. When tested in a xenograft model, AVI-4126 caused a 75-80% reduction in tumor burden when compared to scrambled oligomer and saline controls. Furthermore, Phase I intravenous administration of AVI-4126 in healthy volunteers demonstrated the drug to be safe. In addition, antisense oligonucleotides against c-Myc have been shown to have anti-proliferative effects in leukemia, lymphoma, melanoma, prostate, breast, and liver cancer cells [185]. Combination therapies with antisense c-Myc and chemotherapeutic agents are under investigation in human cancers [231,232]. Interestingly, Arango et al. demonstrated that elevated levels of both c-Myc and wild type p53 improved the sensitivity of colon carcinoma cells to the chemotherapeutic agent 5-fluorouracil [233]. Therefore, antisense oligonucleotides against c-Myc and the use of 5-FU need to be approached with caution. Nevertheless, these results are encouraging since c-Myc expression is commonly transactivated by β-catenin/Tcf.

**Cyclin D1**

As mentioned previously, cyclin D1 expression is transactivated by the β-catenin/Tcf complex. Cyclin D1 complexes with CDK4 (cyclin dependent kinase 4) and CDK6 to phosphorylate retinoblastoma (Rb), which promote histone acetylation and gene transcription [234,235]. Cell cycle progression is tightly regulated by the cyclin-dependent kinases and the cyclins [236]. To date, several drugs targeting cyclin D1 and the cyclin dependent kinases are under investigation. R-Roscovitine (CYC202) is a cyclin-dependent kinase inhibitor, which causes a decrease in cyclin D1 and Rb protein phosphorylation in HT29 and
KM12 colon cancer cell lines [237]. Similarly, rapamycin and CCI-779 (an ester analog of rapamycin) prevent cyclin dependent kinase activation, inhibit Rb phosphorylation, and decrease cyclin D1 levels [238]. Cyclin D1 is also the target of lung cancer chemopreventative agents such as retinoids [239].

**PPARs and COX-2**

Several studies demonstrated that PPARs and/or COX-2 may be regulated by Wnt/β-catenin signaling pathway [35,240-245]. While the mechanisms are complex and not well defined, COX-2 may also be regulated directly or indirectly by PPARs [246,247]. Over the past decade, significant interest has developed on the role of COX-2 in human cancers due to the association of COX-2 and FAP patients. COX-2 has been implicated in many human cancers such as esophageal squamous cell carcinoma, gastric cancer, breast carcinoma, lung cancer, chondrosarcoma, osteosarcoma, rhabdomyosarcoma, and genitor-urinary cancers [248-254]. The emergence of several COX-2 inhibitors (Celecoxib, Rofecoxib, Valdecoxib) on the market has sparked interest in combinational COX-2 inhibitor/chemotherapy treatment and cancer chemoprevention [255-258].

Initial interest in COX-2 and human cancers began in colorectal cancers [259]. Since then, abundant animal studies and epidemiological studies have demonstrated that COX inhibitors, such as NSAIDs, can effectively inhibit the development of colon cancers [260-263]. It has been shown that reduction of intestinal neoplasia with APC gene replacement and COX-2 inhibition was additive [264]. Lew et al. investigated the effects of liposome-mediated APC gene therapy and a selective COX-2 inhibitor, Rofecoxib (Vioxx), on intestinal neoplasia in vivo [264]. Five-week-old Min mice weaned on a 30% high-fat diet were randomized to receive no treatment (control), APC only, Vioxx only, or APC/Vioxx. PC-treated mice received an expression vector containing the human APC cDNA mixed with a liposome preparation that was administered biweekly. The control mice were treated similarly with a plasmid construct lacking the APC gene. After 2 months, there was a 54% and 70% reduction in the total number of intestinal polyps after APC and Vioxx treatment, respectively, whereas the combined APC/Vioxx therapy reduced polyp formation by 87% [264]. The reduction of intestinal neoplasia by APC gene replacement and COX-2 inhibition suggests that each modality, both individually and together, may prove therapeutic and therefore contribute to new strategies in the prevention and treatment of colorectal cancers. The results of both animal and epidemiological studies have led the United States Food and Drug Administration to approve Celecoxib for the treatment of patients with familial adenomatous polyposis. Interestingly, Vioxx has recently been taken off the market for safety concerns.

As mentioned previously, PPAR δ is a downstream target of Wnt signaling [35,240-245]. The PPARs are nuclear receptors that bind to lipophilic ligands and the receptor for 9-cis-retinoid (RXR) to transactivate genes [265,266]. There are three PPAR genes (PPARα, PPARβ/δ, and PPARγ) with two PPARγ isoforms [267,268]. PPARγ has been implicated in many human cancers, and several studies have shown anti-neoplastic effects of PPARγ agonists in neuroblastoma, astrocytoma, glioma, and prostate, thyroid, colon, pancreatic, hepatocellular, breast, and lung carcinomas [269]. Several clinical trials have been conducted to evaluate PPARγ agonists (troglitazone and rosiglitazone) in liposarcoma and prostate, breast, and colon cancers and have revealed mixed results [269]. For example, one clinical study on nine patients with liposarcoma treated with rosiglitazone demonstrated no clinical response while another study reports adipocyte differentiation of liposarcoma patients treated with troglitazone [270,271]. While the in vitro and animal studies on PPARγ agonists are promising, further investigations in the clinical arena needs to be conducted.

**TARGETING THE SIGNALING SYNERGY BETWEEN Wnt/β-CATENIN AND OTHER PATHWAYS**

An increasing number of reports have indicated that β-catenin signaling may be further activated by mediators of Wnt-independent mechanisms in mammalian cells [65], including G-protein [66], integrin-linked kinase (ILK) [67], hepatocyte growth factor (HGF) [68,69], growth factor Gas6 [70], insulin-like growth factor-1 (IGF-1) [71,72], IGF-II [73], FGF-1 [74], and vascular endothelial growth factor (VEGF) [75]. Furthermore, cross talks occur between the Wnt/β-catenin signaling pathway and other pathways, such as MAP kinase [76-78], Ca++/calmodulin kinase II [79,80], TGFβ [81], PKC [82-84] and retinoid signaling [85]. Thus, simultaneously targeting Wnt and these synergistic signaling pathways may maximize anti-cancer therapeutic potential.

**Receptor Tyrosine Kinase Signaling Pathways**

Increasing numbers of studies suggest that growth factors that activate receptor tyrosine kinases may also regulate β-catenin signaling activity [68-73,75]. In tumor cells expressing the EGF (Epidermal Growth Factor) receptor, there is a GSK3β independent activation of β-catenin [272]. Upon EGF stimulation, β-catenin is tyrosine-phosphorylated and results in increased β-catenin/Tcf transactivation [273,274]. Receptor tyrosine kinase signaling activation of the β-catenin GSK3β-independent pathway provides another target for drug therapies. Currently, there are two FDA approved tyrosine kinase inhibitors (gefitinib/Iressa and imatinib/Gleevec/STI-571) on the market. Gefitinib is a selective EGFR inhibitor that has anti-neoplastic effects in non-small cell lung, breast, and head & neck cancers [275]. Gleevec is a tyrosine kinase inhibitor that targets BCR-ABL, KIT, and PDGFR and has shown efficacy in chronic myeloid leukemia, acute lymphoblastic leukemia, gastrointestinal stromal tumors, and a number of solid tumors [276]. A third tyrosine kinase inhibitor erlotinib (Tarceva, R 1415, CP358774, OSI 774, NSC 718781) which targets the EGFR is now in phase III clinical trials with promising results, particularly in non-small cell lung cancer [277]. There are also numerous tyrosine kinase inhibitors that are currently in phase I clinical trials [276].

We have recently demonstrated that tyrosine kinase inhibitor STI-571/Gleevec down-regulates the β-catenin signaling activity [278]. Our results revealed that STI-571 effectively inhibited the constitutive activity of β-catenin signaling in human colon cancer cells as well as the Wnt1-
induced activation of β-catenin signaling in HOS, HTB-94, and HEK 293 cells [278]. Furthermore, STI-571 was shown to effectively suppress the proliferation of human colon cancer cells. Finally, we demonstrated that the Wnt1-mediated activation of a GAL4-β-catenin heterologous transcription system was effectively inhibited by STI-571 [278]. Thus, our findings suggest that tyrosine phosphorylation may play an important role in regulating β-catenin signaling activity, and inhibition of this signaling pathway by STI-571 may be further explored as an important target for alternative/adjuvant treatments for a broader range of human cancers. Our findings are consistent with an in vivo study on combinatorial chemoprevention of intestinal neoplasia by targeting both Wnt and EGF signaling pathways [279]. A combination of sulindac, a prototypical non-steroidal anti-inflammatory drug with established chemopreventative activity, and EKI-569, an irreversible inhibitor of the EGF receptor kinase, afforded remarkable protection from intestinal neoplasia in APC (Min+/+) mice, a murine model of human familial adenomatous polyposis (FAP) [279]. Although 100% of the untreated APC(Min+/+) mice developed approximately 20 polyps, nearly half the mice treated with these two agents developed no polyps [279]. These results suggest a powerful strategy for the chemoprevention of human colonic neoplasia.

Retinoic Acid and Differentiation Therapy

Retinoids have been shown to be a potent stimulus for differentiation, growth arrest, and apoptosis in normal, premalignant, and malignant cells [280,281]. In addition, retinoids have also been shown to decrease β-catenin/Tcf transactivation of genes [85,282,283]. Shah et al. demonstrated that retinoic acid and its nuclear receptor were able to trans-repress β-catenin signaling via interactions with a co-activator, P300 [283]. To this end, Suzui et al. have shown that hepatoma cells treated with retinoids resulted in decreased mRNA levels of the Wnt downstream target, cyclin D1 [282]. In the SKBR3 breast cancer cell line, retinoic acid stabilized the adherens junctions and stimulated the cells to undergo differentiation [284]. Similarly, retinoic acid was able to cause highly metastatic pancreatic cancer cells (KP-1NL, KP-4, and SUIT-2) to acquire a more epitheliod morphology with increased E-cadherin expression [285]. These findings are encouraging and open the door for drug therapies targeting both Wnt and retinoic acid signaling.

TARGETING WNT/β-CATENIN SIGNALING BY RATIONAL HIGH THROUGHPUT DRUG SCREENING APPROACHES

With the improvement of our understanding of molecular pathways of tumorigenesis, rational cancer drug discoveries can now be conducted by using targeted and high throughput screening approaches. Some of the best examples are illustrated well by the screening and use of Bcr-Abl kinase or epidermal growth factor receptor antagonists in treating chronic myeloid leukemia and breast cancers, respectively [286]. As the pathogenic role of constitutive β-catenin activity [89,287] in colon cancers and many other cancers has been well established [1], small antagonist molecules from various combinatorial libraries can hence be assayed for their ability to disrupt or interfere with the binding equilibrium between β-catenin and LEF/Tcf, resulting in the inhibition of β-catenin/Tcf-mediated transcriptional regulation (Fig (2)).
this chimeric transactivator, indicating that this transactivation system is regulated by β-catenin in a Wnt-responsive fashion [288]. Thus, this transactivation system could be used as a functional reporter to identify potential upstream factors that deregulate β-catenin signaling during tumorigenesis, as well as to screen for potential anti-cancer agents that specifically inhibit β-catenin signaling in human tumors. As mentioned above, we demonstrated that ST571/Gleevec showed an inhibitory activity in this functional assay system [278].

In a more recent report, Lepourcelet et al. have put the high throughput screening strategy into action [289]. In order to identify compounds that inhibit association between Tcf4 and β-catenin, Lepourcelet et al. developed a binding assay for high throughput screening (HTS). Purified β-catenin (amino acids 134–668) was coated on microtiter plates and incubated sequentially with a Tcf4 fragment (residues 8–54) fused to glutathione-S-transferase (GST), anti-GST antibody, and alkaline phosphatase (AP)-conjugated secondary antibody [289]. Compounds that disrupt the Tcf/β-catenin complex thus register reduced AP values relative to the background. Using this high-throughput assay of immunoenzymatic detection of the protein-protein interaction, Lepourcelet et al. screened approximately 7000 purified natural compounds from proprietary and public collections, initially at 10 µM concentrations [289]. Eight compounds displayed reproducible, dose-dependent inhibition of the Tcf4/β-catenin interaction with IC50 < 10 µM, and their structures and purity were confirmed by high-performance liquid chromatography and high-resolution mass spectrometry [289]. This group of compounds shows diverse structural properties, although several of them share polyhydroxylated

Table 2. Summary of Current Approaches to Targeting Wnt/b-Catenin Signaling Pathway for Cancer Therapy

<table>
<thead>
<tr>
<th>Level of Targeting</th>
<th>Target</th>
<th>Approaches</th>
<th>Tumors Targetted</th>
<th>Approved/Investigational Drugs</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular and Membrane</td>
<td>Wnts</td>
<td>Antisense Monoclonal antibodies</td>
<td>Breast, NSCLC, Melanoma, Mesothelioma, SS Sarcoma</td>
<td>[166,167,168]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fzds</td>
<td>sFRPs overexpression</td>
<td>Colon, Mesothelioma</td>
<td>[171,172]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dkks</td>
<td>wt-Dkk overexpression Chemosensitization</td>
<td>Colon, Mesothelioma</td>
<td>[175,177,176]</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>APC</td>
<td>wt-APC overexpression</td>
<td>Colon</td>
<td>[178,291]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Axin</td>
<td>wt-Axin overexpression</td>
<td>Esophageal SCC</td>
<td>[155]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-Catenin</td>
<td>Antisense oligos RNA interference</td>
<td>Breast, Colon, Esophageal SCC, Colon</td>
<td>See Table 1 [210-212]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>COX-2</td>
<td>inhibition of COX-2</td>
<td>Breast, Colon, NSCLC</td>
<td>Celecoxib, Rofecoxib, and Valdecoxib [255-258,260-264]</td>
<td></td>
</tr>
<tr>
<td>Nuclear</td>
<td>LEF/Tcf5</td>
<td>Apoptosis/Suicide HSV-TK/ganciclovir Oncolytic virus</td>
<td>Colon, NSCLC</td>
<td>[214,216,217] [222,223,225,226]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBP</td>
<td>Inhibition of CBP</td>
<td>Colon</td>
<td>ICG-001 [215]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c-Myc</td>
<td>Antisense oligos Chemosensitization</td>
<td>Prostate, Melanoma, Colon</td>
<td>AV-4126 [185,231-233]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cyclin D1</td>
<td>Cdk inhibitor</td>
<td>Colon, RCC, NSCLC, Prostate, SS Sarcoma, Breast, SCC, Uterine, Lymphoma, Melanoma, Glioblastoma, Leukemia</td>
<td>R-Roscovitine, Rapamycin, and CCI-779 [237,238]</td>
<td></td>
</tr>
<tr>
<td>Crosstalk pathways</td>
<td>PPARγ</td>
<td>Agonists/ligands</td>
<td>Hepatocellular, Breast, NSCLC, Neuroblastoma, Astrocytoma, Glioma, Prostate, Thyroid, Gastric, Pancreatic</td>
<td>Troglitazone and Rosiglitazone [269]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tyrosine kinases</td>
<td>Tyrosine kinase inhibitor</td>
<td>DFSP, Glioblastoma, Prostate, NSCLC, Osteosarcoma, Colon, Pancreatic, CML, GIST, ALL, SS Sarcoma</td>
<td>Iressa, Gleevec, TArceva [275-279]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RXRs, RARs</td>
<td>Differentiation therapy</td>
<td>Pancreatic, Colon, Hepatocellular</td>
<td>Retinoids [85,282,283,285]</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: NSCLS: non-small cell lung cancer; SS: soft tissue; SCC: squamous cell carcinoma; RCC: renal cell carcinoma; DFSP: dermatofibrosarcoma protuberans; CML: chronic myeloid leukemia; GIST: gastrointestinal stromal tumor; ALL: acute lymphoblastic leukemia
planar features. The selected compounds were shown to disrupt Tcf/β-catenin complexes in several independent in vitro assays and to potently antagonize cellular effects of β-catenin-dependent activities, including reporter gene activation, c-Myc or cyclin D1 expression, cell proliferation, and duplication of the Xenopus embryonic dorsal axis [289]. Thus, these identified compounds seemingly meet predicted criteria for disrupting Tcf/β-catenin complexes and define a general strategy to establish mechanism-based activity of small molecule inhibitors of the pathogenic protein-protein interaction of the β-catenin/Tcf signaling complex.

CONCLUSIONS AND FUTURE DIRECTIONS

While the Wnt signaling pathway is complex, significant progress has been made over the past decade in identifying key players in the pathway. As discussed in this review, gain or loss-of-function mutations of several members in this pathway, such as β-catenin, Axin or APC, have been found in many types of human tumors. The identification of these important regulatory proteins offers an opportunity to develop new therapies targeting this pathway at the extracellular/membrane, cytoplasmic, and nuclear levels as summarized in Table 2. In addition, a synergistic approach of targeting Wnt-dependent and Wnt-independent β-catenin activating pathways is under investigation. It is also conceivable that more effective anti-cancer modalities can be developed by targeting Wnt signaling pathway at multiple levels.

As shown in Table 2, drugs targeting this signaling pathway at the extracellular/membrane level would include anti-Wnt monoclonal antibodies and agents mimicking the effects of Dkk or sFRPs. Anti-Wnt monoclonal antibodies and proteins that mimic Dkk or sFRPs have the advantage of a simple and specific delivery route. Although monoclonal antibodies can be inexpensive to produce, recombinant protein or polypeptide production can be costly. However, drugs targeting at the extracellular/membrane level would not be effective to treat those tumors containing mutations downstream in the pathway. At the cytoplasmic level, potential drugs would include axin and APC-based viral and DNA vectors, β-catenin antisense oligonucleotides, tyrosine kinase inhibitors, or COX-2 inhibitors. Drugs targeting β-catenin signaling at the nuclear level would include PPAR agonists, retinoids, or cyclin kinase inhibitors. In addition, viral or DNA/RNA based therapies targeting Tcf/Lef, CBP, and c-Myc would inhibit the signaling pathway in the nucleus. One of the challenges of DNA and RNA based therapeutic agents is to develop an efficient delivery system that is specific to cancer cells. Once delivered into the cancer cells, the DNA and RNA based drugs can specifically target this signaling pathway. Viral vectors (such as adenoviral vectors), on the other hand, can efficiently deliver the therapeutic genes, but may be non-specific to the cancer cells, and have a potential to elicit host immune responses. COX-2 inhibitors have been on the market for over five years and have a simple delivery route, although they may not be tumor-specific and cause adverse effects. Tyrosine kinase inhibitors are small molecules that have a simple delivery route, and may emerge as promising anti-cancer agents. Likewise, PPAR agonists, retinoids, and cyclin dependent kinase inhibitors are small molecules with a simple delivery route but may suffer from adverse effects associated with their non-specificity.

While many of these drugs targeting the Wnt/β-catenin signaling pathway are in pre-clinical or early phase clinical trials, a few have been approved by the United States Food and Drug Administration or are in Phase III clinical trials, including Gleevec, Iressa, Erlotinib, and COX-2 inhibitors. Although this review only highlights some of the methods in targeting the Wnt signaling pathway, the stage is set for the refinement of our current strategies and/or the development of rational high throughput screening of novel cancer drugs by targeting the Wnt/β-catenin signaling pathway.

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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
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<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphocyte enhancer factor</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferators-activated receptor</td>
</tr>
<tr>
<td>sFRP</td>
<td>Secreted Frizzled-related protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA; Tcf, T cell factor</td>
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