**Molecular Pathways**

**Targeting Wnt Signaling in Colon Cancer Stem Cells**

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**Abstract**

The identification of cancer stem cell (CSC) populations in virtually all tumor types has widespread clinical consequences. CSCs are suggested to be the only cells within malignancies endowed with tumorigenic capacity and are, therefore, directly implicated in therapy resistance and minimal residual disease. The genetic and molecular mechanisms sustaining CSCs are only currently emerging. For instance, aberrant activation of the Wnt signaling pathway is crucial for many cancer types and especially those of the gastrointestinal tract. Indeed, Wnt signaling activity was shown to designate colon CSCs and is, therefore, an attractive target for new therapeutics. Here, we review some of the latest developments that have been achieved to inhibit the Wnt pathway in the context of colon CSCs. Moreover, we discuss some of the pitfalls that can be anticipated and present new opportunities for therapeutic intervention.

**Background**

Colorectal cancer (CRC) is the second cause of cancer-related death in the Western world with an incidence of more than 1 million new cases each year (1). CRC is one of the best-studied malignancies, and despite recent advances in chemotherapies that have improved survival, patients with late-stage disease still have poor prognosis, and the overall mortality of the disease is around 40% (2). Much of our understanding of the histopathologic and molecular processes underlying the transition from a normal epithelium to an invasive adenocarcinoma relies on seminal work from Fearon and Vogelstein (3). They have described the so-called “adenoma-carcinoma sequence” as the stepwise accumulation of genetic and epigenetic changes in oncogenes and tumor suppressor genes.

More recent insights from the cancer stem cell (CSC) field have reshaped our view of malignancies. The CRC stem cell model poses an interesting framework in which tumors are hierarchically organized tissues with CSCs at the top of the hierarchy driving tumor growth and progression (4). CSCs are defined as cells that are endowed with both self-renewal and multilineage differentiation potential (4, 5) and, as such, are believed to clonally expand and repopulate the various types of differentiation lineages present within the tumor. The more differentiated progeny have lost their self-renewing capacity and are thought to be dispensable for tumor maintenance. The CSC theory, therefore, has dramatic consequences for the way in which we perceive cancer initiation and progression and currently serves as a basis for targeted therapies. However, the development and clinical use of effective therapies will depend on an accurate understanding of the molecular processes regulating CSCs. Here, we review the latest insights on molecular pathways regulating colon CSCs, with specific emphasis on the Wnt signaling cascade. Then, we discuss the rationale behind targeting Wnt signaling and the potential caveats to this approach.

**The Wnt canonical signaling pathway**

The Wnt signaling cascade is conserved throughout the animal kingdom and, depending on the context, plays various roles that encompass stem cell maintenance, cell proliferation, differentiation, and apoptosis (reviewed in ref. 6). The canonical pathway is mainly regulated at the level of β-catenin, a protein kept under low cytoplasmic concentration by the destruction complex. The latter contains the tumor suppressor protein adenomatous polyposis coli (APC); 2 kinases, casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3-β); and Axin2, which scaffolds the complex together. In the absence of Wnt ligands, the membrane receptor complex formed by frizzled (Fzd) and low-density lipoprotein receptor–related protein 5/6 (LRP5/6) is not engaged, and CK1 and GSK3-β phosphorylate β-catenin at specific serine and threonine residues, priming its recognition by the U3 ubiquitin ligase β-transducin repeat-containing protein (β-TRCP). As a consequence, β-catenin is ubiquitinated and targeted for proteosomal degradation (Fig. 1A; ref. 7).

Upon binding of Wnt ligands to the receptors, the destruction complex is dissolved by an ill-defined mechanism (8), and β-catenin is no longer degraded, which leads to its accumulation in the cytosol and, subsequently, translocation into the nucleus. There, it associates with...
the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors, converting them from repressors to activators of transcription. These nuclear events require, in a first step, displacement of the corepressor Groucho (9) and, subsequently, recruitment of the histone acetylase CREB-binding protein (CBP)/p300 and coactivators, like pygopus (PYG) and BCL9 (10). This step triggers a complex transcriptional program that directs cell fate, cell proliferation, and stem cell maintenance (Fig. 1B). Important Wnt target genes include \textit{c-MYC} (11), \textit{Axin2} (12), and \textit{ASCL2} (13), which serve important functions in various stages during embryogenesis, but also during organ homeostasis and CRC development.

**Wnt signaling in homeostasis of the gut**

The role of Wnt signaling in adult tissue homeostasis is best illustrated in the gut, where a gradient of Wnt signaling activity is required for the organization and patterning of the intestinal tract (reviewed in ref. 14). Wnt signaling components are present throughout the crypt-villus axis (15); active canonical signals are critical to maintain the stem cell compartment, located at the bottom of the crypt. Blockade of Wnt signaling, either by artificial deletion of TCF4 or overexpression of the Wnt antagonist Dickkopf-1 (DKK1), results in loss of epithelial cell proliferation and intestinal tissue structure (16, 17). Furthermore, positioning of stem and differentiated cells throughout the crypt-
villus axis is orchestrated by the EphB2 and B3 receptors, which are also TCF4 targets (18). Using the TCF4-induced transcriptional program combined with specific localization of identified Wnt target genes to the bottom of the crypt, Barker and colleagues identified LGR5 as a stem cell marker for both intestine and colon (19). Other Wnt targets exemplify the functional role of Wnt signaling in stem cell maintenance. For instance, ectopic expression or, reciprocally, conditional deletion of ASCL2, a transcription factor that is also restricted to the crypt, results in intestinal hyperplasia and loss of the stem cell compartment, respectively (20). Although beyond the scope of this review, it is important to note that other morphogenetic pathways, such as BMP and Notch signaling, are, in conjunction with Wnt signaling, important regulators of gut homeostasis.

Deregulation of the Wnt pathway and intestinal tumors

Given its fundamental role in homeostasis in adult tissue, it is not surprising that deregulation of the Wnt pathway is associated with various pathologic states, including various types of cancer (21, 22). Indeed, loss of function of Wnt components is critically involved in the pathogenesis of CRC (23). Inactivation of the APC gene or activating mutations of β-catenin is reported in virtually all patients presenting with CRC (24) and is believed to be the critical initiating step in malignant transformation (25). Although of various nature, those mutations ultimately result in stabilization of β-catenin and perpetual activation of the Wnt transcriptional program, even in the absence of any extracellular signals (Fig. 1C).

Interestingly, although most patients contain constitutively activating mutations of the Wnt pathway, such tumors often still reveal a certain degree of regulation of the pathway. Several lines of evidence support this finding. The first example is the histopathologic observation that not all tumor cells deficient for APC display homogeneous nuclear β-catenin staining, a surrogate for Wnt signaling activity (26, 27). This observation has been dubbed the “β-catenin paradox.” Second, the two-hit hypothesis that normally results in inactivation of a tumor suppressor gene is thought to be independent of events. However, for APC this does not seem to be the case because it has been shown that the type of germline APC mutation that is present in familial adenomatous polyposis (FAP) patients influences the nature of the second, “somatic” hit in the APC gene (28). Importantly, this never results in a complete loss of function of the protein and suggests a fine-tuned balance of Wnt activity that is required for optimal cell transformation (29). This principle is often quoted as the “just-right” signaling model. Finally, recent observations from our laboratory have shown that Wnt signaling activity in colon cancer is also characterized by a gradient in which colon CSCs are functionally marked by a highly active Wnt signaling pathway, whereas the differentiated progeny of these cells show markedly lower levels of activity. This gradient is, at least in part, orchestrated by the microenvironment. These observations highlight the role of Wnt signaling pathway regulation in CRC and its role in colon-CSC features. As mentioned above, the CSC theory has widespread consequences on the rationale of cancer treatment. The relevance of Wnt activity levels in defining these cells in CRC provides a potential new interesting target. Accordingly, it has become increasingly clear that various types of malignancies, aside from CRC, are dependent on sustained Wnt activity. Therefore, the therapeutic benefit of drugs successfully targeting Wnt signaling is also evident for various cancer types. Next, we review current emerging drugs, especially for the treatment of CRC.

Clinical-Translational Advances

Targeting Wnt pathway components

An incredible collection of natural and synthetic compounds form the basis of intense efforts in high-throughput drug-screening programs. The past decades have seen major advances in understanding the molecular framework of Wnt signaling, which provides an optimal platform for testing these libraries of compounds (30). In 2009, Chen and colleagues screened diverse chemical libraries and identified 2 classes of molecules with Wnt inhibitory features (31). The first class acts primarily at the level of Wnt ligand production by specifically targeting porcupine (PORCN), an acyltransferase that adds a palmitoyl group to Wnt proteins, an essential step for their secretion. The second class regulates Axin2 stability and, importantly, also targets β-catenin degradation in the presence of APC mutations (31). Additionally, another recent study has highlighted the role of the poly-ADP-ribosylating enzymes tankyrase 1 and 2 (TNKS) in promoting Axin2 degradation. Enzymatic inhibition of TNKS by XAV-939 is able to stabilize Axin2 and promotes degradation of β-catenin (32). Although of potential interest for various Wnt signaling–dependent malignancies, the benefit for CRC is questionable as the first class of inhibitors will, in theory, be inefficient when APC mutations render the tumor Wnt-ligand independent (33). However, as mentioned, APC mutations rarely represent complete null mutations. In agreement, Wnt ligands are expressed in various CRC cell lines, and blockade of Wnt1 with monoclonal antibodies can trigger apoptosis in cell lines bearing APC as well as β-catenin mutations (34, 35). Conversely, Wnt natural inhibitors such as secreted Fzd-related proteins (SFRP) are often methylated and silenced in primary tumors (36). These proteins share similarities with Wnt cell-surface Fzd receptors and can prevent their binding with Wnt ligands and subsequent activation of the pathway (37). Similar to inhibition of Wnt1, repression of SFRP in CRC cell lines or their epigenetic reactivation results in decreased Wnt activity as well as cell death (36). These insights clearly support a rationale for targeting the extracellular machinery upstream of the destruction complex. Therefore, an antibody-targeting approach against Wnt ligands and/or blockade of the Fzr receptor signaling might provide an interesting therapeutic avenue to explore (38). From a more fundamental biological perspective, it also supports the notion that full activation of Wnt signaling...
cannot be explained by APC mutations alone, or alternatively, that Wnt ligands activate crucial noncanonical Wnt signaling routes.

The transcriptional program that initiates malignant transformation requires nuclear localization of TCF/β-catenin, in which abrogation of this complex can block the target gene expression and cell growth in vitro (17). Therefore, targeting the TCF/β-catenin nuclear complex also holds great promise for successful therapy. The recruitment of transcriptional coactivators such as PYG, BCL9, and CBP/p300 is well documented, and their induced absence is expected to prevent proper Wnt activation. As a proof of concept, Emami and colleagues screened for TCF/β-catenin inhibitors and found the leading compound ICG-001, which specifically targets and inhibits the coactivator CBP (39). Treatment of CRC cell lines bearing APC or β-catenin mutations with this compound induces dose-dependent cell death, whereas normal colonic epithelial cells are resistant. The effect is also seen in the ApcMin mouse model and in tumor xenographs. As a result, ICG-001 is expected to shortly enter in clinical phase I trials.

**Indirect targeting of the Wnt signaling cascade**

Although of great potential, most Wnt inhibitors are still in preclinical testing or in the developmental stage. Additionally, the fact that Wnt signaling is such an important pathway involved in regulation of tissue homeostasis, interference with crucial components of this cascade is predicted to be associated with serious adverse events. For example, imbalance of intestinal and hematopoietic homeostasis is a predictable bystander effect of nonspecific Wnt inhibition (40). It is anticipated that drug design will require agents providing great specificity and a certain therapeutic window between normal stem cells and CSCs. For example, drugs that have been studied in other clinical settings also have substantial therapeutic impact partially dependent on their Wnt inhibitory properties. The use of nonsteroidal antiinflammatory drugs (NSAID), like sulindac and aspirin, has been suggested in a number of epidemiologic studies to have a chemoprotective role in CRC (41). Preclinical studies have shown a correlation between efficacy of chemoprevention and the Wnt modulatory effects of these compounds (42). NSAIDs have complex modes of action, and only part of them converge to an inhibition of the cyclooxygenase (COX) enzymes (43). COX-2 expression is seen increasingly in early stages of CRC (44). This enhanced expression drives the production of the prostaglandin E2 (PGE2), which mediates tumor progression, angiogenesis, and metastasis (45). Mechanistically, COX-2–induced PGE2 can prevent β-catenin degradation by inhibiting both GSK-3β and Axin2 and, as a result, activate Wnt signaling (Fig. 2B; refs. 46–47). The inhibition of COX-2 can only partially account for the beneficial effect of NSAIDs and COX-2 specific inhibitors (coxibs), such as celecoxib and rofecoxib, on CRC. NSAIDs and celecoxib can also induce CRC cell death independently of COX-2 expression (48). Furthermore, NSAIDs deprived of COX-2 inhibitory capacities also have an effect on CRC (49). For example, growth inhibition via upregulation of the cell cycle inhibitor p21Waf1 is one of COX-2’s independent modes of celecoxib action (50). Another interesting mechanism involves the tyrosine kinase receptor C-MET (51). C-MET, also known as hepatocyte growth factor (HGF) receptor, is known to influence Wnt signaling. Binding of HGF to its receptor induces dissociation of membrane-bound β-catenin from the E-cadherin complexes (52). Additionally, C-MET activation can activate PI3 kinase signaling and subsequent phosphorylation and inactivation of GSK-3β (27, 53). As a result, β-catenin that is part of the destruction complex is no longer degraded but stabilized. Moreover, β-catenin phosphorylation on ser552 by pAKT/ PKB is a nuclear translocation mark (Fig. 2A; ref. 54) also triggered by PI3 kinase activation. These concomitant events initiated by HGF ultimately boost β-catenin levels in the cytosol and nucleus and, therefore, regulate Wnt activity. On the other hand, celecoxib can block C-MET–dependent phosphorylation of various substrates that are accompanied by an increase in GSK-3β activity, thus resulting in β-catenin degradation and in Wnt signaling inhibition (51). Despite other modes of coxib action that require further clarification, celecoxib is approved by the U.S. Food and Drug Administration (FDA) for the treatment of FAP (55). It is, however, important to note that the potential benefit of coxibs in CRC prevention in the general population is hampered by cardiovascular side effects (55, 56).

As described, small molecule inhibitors and natural compounds have been identified to have potential therapeutic value against cancers associated with aberrant Wnt signaling either by direct or indirect mechanisms. However, their lack of specificity and our lack of knowledge about their precise targets, working mechanisms, or their adverse side effects have precluded the start of clinical trials. Identification of these target molecules and determination of the precise mechanism of action of these agents may provide novel targets.

**Future perspectives and concluding remarks**

The discovery and generation of new cancer drug regimens requires a thorough understanding of the basic biological events that drive cancer initiation, progression, and maintenance. As the CSC theory explains part of these processes, an important effort has been made to scrutinize and define their regulation, which will yield an invaluable new source of therapeutic strategies. It is, however, important to integrate the regulation of CSCs in a more general context. As in normal adult tissue in which stem cells reside in a specific protective microenvironment or niche, tumors are also influenced by microenvironmental cues and are increasingly perceived as aberrant but highly organized tissues (57, 58).

Indeed, we and others have shown that such niche requirements are also found in malignancies where they contribute to the CSC phenotype (27, 59). More importantly, when microenvironmental stimuli, such as HGF that is predominantly secreted by the tumor stroma, are applied on the more differentiated tumor cells, these cells...
undergo a dedifferentiation program and revert back to CSCs (27). This plasticity of differentiated cancer cells suggests a more dynamic interpretation of the CSC model, which has crucial implications, especially for therapies that are aimed at specifically targeting the CSC fraction, which would be counteracted by repopulation of the CSC pool. On a more positive note, this interaction would provide a complete novel therapeutic possibility. In this light, small molecule inhibitors and/or monoclonal antibodies developed to target the C-MET receptor, such as PF-02341066, might prove efficacy for cancer treatment and are in clinical trials (60, 61). In the near future, these new areas of drug development will tackle the various CSC regulatory axes and will hopefully yield efficient therapy regimens resulting in improved clinical outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors would like to thank Tijana Borovski for critical comments on the manuscript.

Grant Support

This work was supported by a NWO-VICI grant from the Netherlands Organisation for Scientific Research and a Dutch Cancer Society (KWF Kankerbestrijding) grant (2009–4416; J.P. Medema) and an Academisch Medisch Centrum (AMC) fellowship (L. Vermeulen and F. de Sousa E Melo).

Received October 8, 2010; accepted October 21, 2010; published OnlineFirst December 15, 2010.
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Clinical Cancer Research

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Clin Cancer Res  Published OnlineFirst December 15, 2010.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-1204

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