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 tumour types has widespread clinical consequences. CSCs are suggested to be the only cells within malignancies endowed with tumourigenic 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 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). Colorectal cancer 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 on the histo-pathological 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 tumour suppressor genes.

More recent insights from the cancer stem cell (CSC) field have re-shaped our view of malignancies. The colorectal cancer stem cell model poses an interesting framework in which tumours are hierarchically organized tissues with CSCs at the top of the hierarchy driving tumour growth and progression (4). CSC are defined as the cells that are endowed with both self-renewal and multi-lineage differentiation potential (4-5) and as such are believed to clonally expand and repopulate the various types of differentiation lineages present within the tumour. The more differentiated progeny have lost their self-renewing capacity and are thought to be dispensable for tumour maintenance. The CSC theory therefore has dramatic consequences for the way 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 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 will 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 tumour...
suppressor protein Adenomatous Polyposis Coli (APC), two kinases: Casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3-β), and Axin2 that scaffolds the complex together. In the absence of Wnt ligands, the membrane receptor complex formed by Frizzled (Fzd) and LRP5/6, are not engaged and CK1 and GSK3β phosphorylate β-catenin at specific serine and threonine residues, priming its recognition by the U3 ubiquitin ligase β-TrCP. As a consequence, β-catenin is ubiquitinated and targeted for proteosomal degradation (Fig. 1.A and 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 LEF/TCF (lymphoid enhancer factor/T cell factor) family of transcription factors converting them from repressors to activators of transcription. These nuclear events require in a first step displacement of the co-repressor Groucho (9) and subsequently recruitment of the histone acetylase CBP/p300 and co-activators, like Pygopus (PYG) and BCL9 (10). This triggers a complex transcriptional program that directs cell fate, cell proliferation and stem cell maintenance (Fig. 1.B). Important Wnt target genes include c-MYC (11), Axin2 (12) and ASCL2 (13) that serve important functions in various stages during embryogenesis, but also during organ homeostasis and colorectal cancer 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 over-expression 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 et al. identified LGR5 as 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 tumours

Given its fundamental role in homeostasis in adult tissue, it is not surprising that deregulation of the Wnt pathway is associated with various pathological states including various types of cancer (21-22). Indeed, loss of function of Wnt components is critically involved in the pathogenesis of colorectal cancer (23). Inactivation of the APC gene or activating mutations of β-catenin is reported in virtually all patients presenting with colorectal cancer (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. 1.C).

Interestingly, although most patients contain constitutively activating mutations of the Wnt pathway, such tumours often still reveal a certain degree of regulation of the pathway. There are several lines of evidence to support this. First, the histopathological observation that not all tumour 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 tumour suppressor gene is thought to be independent events. However, for APC this does not seem to be the case since 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 demonstrated that Wnt signaling activity in colon cancer is also characterized by a gradient where 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 colorectal cancer and its role in colon-CSC features. As mentioned above, the CSCs theory has widespread consequences on the rationale treatment of cancer. The relevance of Wnt activity levels in defining these cells in colorectal cancer provides a potential new interesting target. In this respect it has become increasingly clear that various types of malignancies, aside from colorectal cancer, are dependent on sustained Wnt activity. Therefore the therapeutic benefit of drugs successfully targeting Wnt signaling is evident also for various cancer types. In this second part we review the current drugs that are emerging especially for the treatment of colorectal cancer.

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 that provide an optimal platform for testing these libraries of compounds (30). In 2009, Lum et al. screened diverse chemical libraries and identified two classes of molecules with Wnt inhibitory features. The first class acts primarily at the level of Wnt ligands 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-rybosylating 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 colorectal cancer is questionable as the first class of inhibitors will in theory be inefficient when APC mutations render the tumour 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 Frizzled related proteins (SFRPs) 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, re-expression of SFRP in CRC cell lines or their epigenetic re-activation results in decreased Wnt activity as well as cell death (36). These insights clearly support a rational for targeting the extracellular machinery upstream of the destruction complex. Therefore, an antibody targeting approach against Wnt ligands and/or blockade of the Frz 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 non-canonical Wnt signaling routes.

The transcriptional program that initiates malignant transformation requires nuclear localization of TCF/β-catenin where abrogation of this complex can block the target gene expression and cell growth in vitro (17). Therefore targeting the TCF/β-catenin nuclear complex holds as well great promises for successful therapy. The recruitment of transcriptional co-activators such as PYG, BCL9 and CBP/p300 are well documented and their induced absence is expected to impinge a proper Wnt activation. As a proof of principle, Kahn et al. screened for TCF/β-catenin inhibitors and found the leading compound ICG-001 that specifically targets and inhibits the co-activator CBP (39). Treatment of CRC cell lines bearing APC or β-catenin mutations with this compound induces dose dependent cell death, while normal colonic epithelial cells are resistant. The effect is also seen in the APCmin mouse model and in tumour xenografts. As a result of this 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 developmental stage. Additionally, given 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 non-specific Wnt inhibition (40). This requires
anticipation in designing drugs that will offer 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 anti-inflammatory drugs (NSAIDs), like sulindac and aspirin, has been suggested in a number of epidemiological studies to have a chemoprotective role in colorectal cancer (41). Preclinical studies have shown a correlation between efficacy of chemoprevention and the Wnt modulatory effects of these compounds (42). NSAIDs have complex mode of actions and only part of them converged to an inhibition of the cyclooxygenase (COX) enzymes (43). COX-2 expression is seen increasingly in early stages of colorectal cancer (44). This enhanced expression drives the production of the prostaglandene E2 (PGE2), which mediate tumour 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. 2.B and 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 celocoxib and rofecoxib on colorectal cancer. NSAIDs and celocoxib can also induces colorectal cancer cell death independently of COX-2 expression (48). In agreement, NSAIDs deprived of COX-2 inhibitory capacities also have an effect on colorectal cancer (49). For example, growth inhibition via up-regulation of the cell cycle inhibitors p21Waf1 is one of COX-2 independent mode of actions of celocoxib (50). Another interesting mechanism involves the tyrosine kinase receptor C-MET (54). 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 (51). Additionally, C-MET activation can activate PI3kinase signaling and subsequent phosphorylation and inactivation of GSK-3β (27, 52). 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. 2.A and ref. 53) also triggered by PI3kinase activation. These concomitants events initiated by HGF ultimately boost β-catenin levels in the cytosol and nucleus and therefore regulate Wnt activity. On the other hand, celocoxib 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 (54). Despite other modes of action of coxibs that requires further clarification, celecoxib is approved by the food and drug administration (FDA) for the treatment of familial adenomatous polyposis (FAP) (55). It is however important to note that the potential benefit of coxibs in colorectal cancer 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, our lack of knowledge of their precise targets and working mechanism or their adverse side effects has precluded the start of clinical trials. Identification of these targets 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 thorough understanding of the basic biological events that drive cancer initiation, progression and maintenance. As the CSCs theory explains part of these processes, an important effort has been made to scrutinize and define their regulation that will yield an invaluable new source of therapeutic strategies. It is however important to integrate the regulation of the CSCs in a more general context. As in normal adult tissue where stem cell resides in specific protective microenvironment or niche, tumours are also influenced by micro-environmental 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 micro-environmental stimuli, such as HGF that is predominantly secreted by the tumour stroma, are applied on the more differentiated tumour cells, these cells undergo a de-differentiation program and revert back to CSCs (27). This plasticity of differentiated cancer cells suggests a more dynamic interpretation of the CSC model that has crucial implications, especially for therapies that are aimed at specifically targeting the CSC fraction, which would be counteracted by re-population of the CSC pool. On a more positive note, this interaction would provide a complete novel
therapeutic possibility. In light with this, small molecule inhibitors and/or monoclonal antibody 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 playgrounds of drug development will aim to tackle the various CSCs regulatory axes and will hopefully yield efficient therapy regimens resulting in improved clinical outcome.

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Figure. 1. The Wnt canonical pathway

A. In the absence of Wnt ligands, β-catenin is kept under low cytosolic level by the destruction complex. This latter contains Axin2 and APC that present β-catenin to the two kinases CK1 and GSK3-β, facilitating its phosphorylation at specific serine and threonine residues. This primes β-catenin recognition by β-TRCP, which targets it for proteosomal degradation. In the nucleus, TCF transcription factors are bound to co-repressor (Groucho) and gene transcription is actively repressed (7). B. Wnt ligands bound to Fzd and LRP5/6 co-receptors triggers formation of Dvl-Fzd complex and phosphorylation of LRP by GSK3-β. This recruits the scaffolding protein Axin2 to the co-receptors and as a result the destruction complex is dissolved (8). β-catenin is therefore stabilized, can accumulate in the cytosol and subsequently translocate in the nucleus where it converts TCF into a transcriptional activator. This step is mediated by the displacement of the Groucho protein and recruitment of co-activators that includes CBP, BCL9 and Pygopus (PYG) (10). This ensures efficient transcription of genes that are important regulators of stem cell fate (LGR5, ASCL2), cell proliferation (C-MYC), and as well negative regulators of the pathway (Axin2). C. Truncating mutations in APC are frequently observed in colorectal cancer. As a result, the destruction complex cannot properly form, which results in inefficient targeting of β-catenin for degradation degradation. Therefore, β-catenin can accumulate and form active transcription factor complexes with TCF proteins in the nucleus, even in the absence of external signal. (Fzd, Frizzled; LRP, low-density lipoprotein receptor-related protein; Dvl, Dishevelled; APC, Adenomatous polyposis coli; GSK3-β, Glycogen synthase kinase 3 β; CK1, Casein kinase 1; TCF, T cell factor; CBP, CREB-binding protein, β-TRCP, β-transducin repeat-containing protein).
**Figure. 2. Targeting Wnt signaling**

**A.** HGF is mainly produced by stromal myofibroblasts. Binding to its receptor C-MET triggers activation of PI3 kinase signaling and in turn AKT/PKB phosphorylation. Activated AKT/PKB phosphorylates GSK3-β at a specific serine residue which renders it inactive and unable to prime β-catenin for degradation (27-52). Additionally, AKT/PKB phosphorylates β-catenin at a specific serine residue which enhances its nuclear translocation (53). Altogether, this contributes to an increase in nuclear TCF-β-catenin complexes. **B.** Elevated levels of COX-2 are observed in cancer cells (44). This results in increased prostaglandin PGE2 production (45). Via its receptor, PGE2 can efficiently prevent β-catenin degradation by interfering with both GSK3-β and Axin2 function (46-47). A panel of, direct or indirect, Wnt inhibitors (orange) and their molecular targets are also depicted. For instance, IWR (31) stabilizes Axin2. Celocoxib inhibits COX-2 downstream signaling (43) but also target the receptor C-MET (54). Tankyrase (TNKS) is a poly-ADP-rybosylating enzyme that promotes Axin2 degradation and is targeted by XAV-939. Monoclonal antibodies (R13 and R28) can block HGF/C-MET interaction (60).
References


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