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Striking the Target in Wnt-y Conditions: Intervening in Wnt Signaling During Cancer Progression

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Abstract

Wnt signaling can be divided into three pathways, namely the canonical Wnt/ β -catenin pathway, and the non-canonical (or heretical) Wnt/ Ca^{2+} and planar cell polarity (PCP) pathways, the Wnt/ Ca^{2+} pathway was shown to play a major role in cancer. Moreover, increasing data points to the importance of the non-canonical and PCP pathways in several aspects of tumor progression. The recent advances in understanding the players and mechanisms by which the Wnt pathways contribute to cancer progression have led to the identification of numerous targets that are already or could be considered for therapeutical purposes.

1. Introduction

Wnt signaling is a complex process and has been implicated in a large number of diseases, most notably cancer. The variety of receptors and ligands involved in Wnt signaling lead to a multitude of diverse signal transduction cascades. The Wnt family of proteins consists of 19 known human members. These secreted proteins share 20–85 % amino acid identity and have a conserved pattern of 23–24 cysteine residues. Following their synthesis, these secreted Wnt proteins are modified by glycosylation and can bind to the Frizzled (Fzd) family of receptors. To date, ten members of this family of receptors have been identified, all of which are seven-pass transmembrane proteins characterized by an extracellular N-terminal conserved cysteine-rich domain (CRD) that interacts with both Wnts and other Wnt co-receptors [1,2]. The Fzd co-receptors, low-density lipoprotein receptor-related proteins, LRP5 and -6 are single pass transmembrane proteins and in the presence of the Fzd receptor form a co-receptor complex to which Wnts bind resulting in activation of downstream signaling [3]. The CRD domain that binds Wnt ligands was also shown to be present on the single-pass tyrosine kinase ROR2, which has also been involved in Wnt signaling [4], and other receptors for Wnts are discussed in the last section of this review. Fzd receptors are G-protein coupled receptors and downstream signaling upon Wnt binding requires heterotrimeric G proteins [5]. The activation of specific G protein subunits are dependent on the Wnt ligand subtype binding to different Fzd family members [6,7]

Interaction of Wnts with their receptors and co-receptors are associated with three signaling pathways, namely the canonical Wnt/ β -catenin pathway, and the non-canonical (or heretical) Wnt/ Ca^{2+} and planar cell polarity (PCP) pathways. The Fzd receptors have the ability to discriminate between different Wnt ligands, and as such, activation of one of these three pathways is dictated by the nature of the ligand/receptor interaction. The proteins encoded by the *WNT* genes play a role in normal development but also in tumorigenesis [1], [8] and the

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inappropriate activation of the Wnt pathway results in the onset of several types of cancer [9]. In this review, we will go over the main Wnt pathways, how these pathways are modified in different types of cancers and discuss potential targets in these pathways.

2.1. The Canonical Wnt Pathway

The canonical Wnt signaling pathway involves a key mediator, β -catenin. In the absence of Wnt, β -catenin is phosphorylated and targeted for degradation. In a first step, β -catenin is phosphorylated by CKI α and/or CKI ϵ at residue ser45 [10]. This then allows GSK3 β to phosphorylate β -catenin on residues 41, 37, and 33 [11]. The phosphorylation of β -catenin by CKI and GSK3 β , occurs within a complex of several proteins including the scaffolding protein Axin and the tumor suppressor gene product APC (Adenomatous Polyposis coli) [12]. This complex, often referred to as the destruction complex, also contains a protein closely related to Axin, Conductin (Axin2) [13]. Within this complex, Axin binds directly to GSK3 β and its substrate β -catenin [14]. Phosphorylation by GSK3 β leads to the ubiquitinylation of β -catenin by β TrCP, a component of the E3 ubiquitin ligase, and ultimately to its degradation via the proteasome [15–17]. In the absence of Wnt ligand, this Axin-containing destruction complex is constitutively active, resulting in the phosphorylation and degradation of β -catenin [18]. Under these conditions, and in the absence of β -catenin to stimulate transcription, Tcf (T-cell specific transcription factor) represses transcription of Wnt target genes by interacting with co-repressors HDAC and Groucho [19–21].

Activation of the canonical Wnt pathway requires the binding of Wnt to one of the Fzd receptors in the presence of the co-receptor, LRP 5/6 [3,22]. Formation of this complex leads to the phosphorylation of LRP by CKI, allowing the recruitment of Axin to the complex at the membrane, thereby preventing the activation of the destruction complex responsible for β -catenin phosphorylation by GSK3 β [23–27]. GSK3 β is prevented from binding to Axin by the phosphoprotein Dishevelled (Dvl/Dsh) and its recruitment of the GSK3 β binding protein (GBP), which prevents binding of GSK3 β to Axin (Figure 1). Dishevelled therefore inhibits the β -catenin degradation machinery. Axin has distinct binding sites for GSK3 and β -catenin [12,23,28,29].

Once stabilized, β -catenin can enter the cell nucleus and associate with the transcription factors Lef (Lymphoid enhanced transcription factor) and Tcf (T-cell factor), leading to the transcription of Wnt target genes (Figure 1). The conversion of the Tcf repressor complex mentioned above into a transcriptional activator complex is thought to involve the displacement of Groucho from Tcf/Lef and recruitment of the histone acetylase CBP/p300 that acts as a co-activator by binding to the β -catenin-Tcf complex [30,31]. Other factors that contribute to the activation of transcription include Brg1, a component of the SWI/SNF chromatin remodeling complex [32], as well as Bcl9 bound to Pygopus which mediates the interaction between the complex and chromatin [33–37]. It is important to note that several members of the pathway can be regulated independently of Wnt signaling. In particular, GSK3 β can be inhibited by ILK, (Integrin Linked Kinase) [38], and is at the intersection of numerous pathways that might regulate its expression [39]. Regulation of β -catenin, and in particular its degradation can also be induced by p53 activation [40].

2.2. The Canonical Wnt pathway in Cancer

The stabilization of β -catenin, lack of degradation and ultimately nuclear accumulation is used as evidence of an activated Wnt/ β -catenin pathway. Indeed, such accumulation has been detected by immunohistochemical staining in a number of human tumors including colorectal, lung, breast, cervical, skin, and liver. In hepatocellular carcinoma, β -catenin accumulation has been linked to poorly differentiated morphology [41], high proliferative activity [42], and poor prognosis [41–43]. The fate of β -catenin, namely, its accumulation or degradation, is regulated

by numerous proteins, which, if not regulated or expressed appropriately would account for increased β -catenin expression in cancer. This dysregulation may occur due to mutations in the various members of the signaling pathway, or to epigenetic events. Mutations in Wnt themselves are rare and although *Wnt-1* was identified as a mammary oncogene in mouse transgenic studies [44–47], no mutations in this gene have been linked to cancers in humans. Mutations affecting downstream targets however, are quite frequent in cancer [48] [49].

Amongst these are mutations in β -catenin itself. Indeed, activating β -catenin mutations at one of the sites that are phosphorylated by GSK3 β have been identified in 50% of colon cancers that have wild type APC. These mutations prevent the β -catenin degradation via the proteasome [50–52]. These activating point mutations in β -catenin were found in the rare cases of colorectal cancers with wild type APC, thereby preventing β -catenin degradation and leading to the inappropriate formation of the Tcf/ β -catenin transcription complex [50]. Mutations in β -catenin have also been identified in a number of other tumors such as brain tumors, ovarian cancers, prostate cancer, and hepatocellular carcinomas [53–55]. In addition to these mutations in β -catenin, mutations affecting other components of the canonical Wnt pathway have been detected in different types of tumors. In particular, mutations affecting the interaction of β -catenin with the destruction complex, resulting in an increase in cytoplasmic β -catenin, play a major role in the development of a large number of tumors [9,48].

The Adenomatous Polyposis Coli gene (APC), one of the components of the destruction complex, was identified as a tumor suppressor protein. Germline mutations in the gene coding for this protein is linked to an inherited form of colorectal cancer known as Familial Adenomatous Polyposis (FAP), which is characterized by a large number of colorectal polyps in early adulthood [56,57]. In addition, somatic mutations of the APC gene are found in more than 80% of sporadic colorectal cancers [58–61]. Mutations in the APC protein results in the inability to downregulate β -catenin, which ultimately results in the activation of Wnt target gene transcription mediated by Lef/Tcf. Loss of APC occurs at the very initial steps of colorectal cancer and has been shown to accelerate the process of tumor initiation [62]. Whether it is the loss of APC or mutations in the Phosphorylation sites of β -catenin, the consequence remains a defect in the downregulation of β -catenin and transcription of genes that include cyclin D1 and c-myc [63,64], both of which have been implicated in cell cycle progression and its deregulation. This defect in β -catenin degradation is found in numerous types of tumors, and makes the destruction complex an attractive target in terms of finding mechanism to increase its activation in these types of tumors.

One of the major constituents of the destruction complex is Axin, whose concentration was shown to be the rate-limiting factor in regulating the efficiency of the destruction complex [25,65]. Interestingly, the overexpression of Axin induces β -catenin degradation in cell lines with mutated APC [12,66,67]. Therefore stabilization of Axin would provide a mechanism by which to stimulate β -catenin degradation. Recently, stabilization of Axin was reported in the presence of a small inhibitor of the poly(ADP-ribose) polymerase tankyrase [68]. Furthermore, in this same study, this inhibitor could inhibit the growth of β -catenin-dependent (loss of APC) colorectal cells. Other small molecules acting on Axin protein stability have since been described[69].

At the center of the Wnt/ β -catenin pathway and a member of this destruction complex is, as mentioned above, the protein GSK3 β . To date, no inactivating mutations have been detected in human cancers [70]. Although, from its role in phosphorylating β -catenin, GSK3 β would be predicted to act as a tumor suppressor, its activity was deregulated in colorectal cancer and this was suggested to play a major role in colon cancer cell proliferation and survival [71]. Several studies have described an important role for GSK3 β in nuclear factor- κ B (NF- κ B)-mediated cell survival [72], [73], and GSK3 β has been shown to destabilize p53 [74,75] and

PTEN [76], altogether favoring a role for GSK3 β in promoting cancer. GSK3 β protein expression was also detected in human ovarian carcinoma [77]. However, studies done on breast, lung and non-melanoma skin cancers have shown inactivation of GSK3 β in these cancer tissues and have further demonstrated that its activation induced apoptosis of cancer cells [78–81]. Despite this, studies done recently in prostate, pancreatic, and colorectal cancer cell lines reveal that the use of GSK3 β inhibitors lead to significant decreases in cell growth and proliferation [82,83]. It was also shown that in APC mutant mice, treatment with the GSK3 β inhibitor lithium did not result in a significant increase in the number of tumors in these mice [84]. Therefore, although GSK3 β appears to play a controversial role in cancer, the use of GSK3 β inhibitors seem to correlate with an overall positive outcome, although the mechanism by which the inhibitory effect of inhibitors such as lithium affect GSK3 β in the context of the Wnt pathway has not been well studied.

Unlike colorectal cancer, breast cancers do not display genetic alterations in any of the genes coding for β -catenin (*CTNNB1*), or components of the destruction complex such as APC or AXIN1 [85,86], [87–89]. However, primary breast tumors overexpress the Wnt target gene cyclin D1 [90]. Accumulation of β -catenin and induction of cyclin D1 has been correlated with a poor prognosis in breast cancer [90]. This induction of cyclin D1 was shown to be due to the overexpression of Wnt ligands resulting in autocrine activation of Wnt signaling in breast cancer cells [91]. The use of monoclonal antibodies directed against these Wnts and Fzd receptors have shown promising results *in vitro* [92], [93] [94–98], but targeting the secreted ligands might be challenging due to the ubiquitous expression of the Fzd receptors and the possible activation of both canonical and non-canonical pathways.

2.3. Targeting the Canonical Wnt Pathway In Cancer

Ultimately, the Wnt/ β -catenin pathway is linked to cancer via the activation of Tcf/ β -catenin activated genes. Targeting the Tcf/ β -catenin complex has so far proven to be a promising approach. Indeed, compounds acting on specifically disrupting the Tcf/ β -catenin interaction (small molecule antagonists) have been identified and were shown to inhibit proliferation of colorectal cancer cells (HCC) [99]. These same molecules were also shown to exhibit anti-tumor activity against multiple myeloma [100]. In a recent study by Wei *et al* [101], these antagonists were reported to inhibit human hepatocellular carcinoma cell growth with limited cytotoxicity to normal hepatocytes. This effect was shown to involve the disruption of the interaction between Tcf4 and β -catenin and result in the downregulation of the Tcf4/ β -catenin downstream oncogene c-myc, the expression of which alone was previously shown to induce HCC [102]. This same study showed that these antagonists could slow the growth of xenografts in nude mice in preliminary studies. This approach of targeting the pathway at the level of transcription shows promise and would also have the advantage of acting downstream of mutations contributing to the stabilization and increased presence of β -catenin in the transcription complex.

Amongst the Wnt target genes are *PPAR- γ* and *COX-2*, both of which have been implicated in the development of colorectal carcinomas. Moreover, both of these are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) which have been shown to inhibit colon tumorigenesis. COX-2 produces eicosanoids from arachidonic acid. These eicosanoids are themselves PPAR- γ ligands which, in collaboration with retinoic acid receptors, stimulate transcription. PPAR- γ is upregulated in early carcinogenesis by the Tcf- β -catenin complex. Treatment of mutant mice with a selective COX-2 inhibitor was shown to reduce polyp number [103] and the treatment of FAP patients with the COX-2 inhibitor Celecoxib showed significant decrease in the number of colorectal polyps [104,105]. Also, levels of nuclear β -catenin in FAP patients was reduced substantially in polyps of FAP patients treated with the NSAID sulindac sulphide for 6 months [106]. The use of NSAIDs has been associated with intestinal bleeding

and kidney damage and development of more selective, less toxic NSAIDs are still under development.

3. Endogenous Wnt Antagonists

Activation of the Wnt pathway is regulated by secreted Wnt inhibitors (Reviewed in [49]). These inhibitors affect the binding of the Wnt ligands to the receptors or co-receptors. These Wnt antagonists include the members of the secreted frizzled related proteins (sFRPs) that bind to Wnt proteins directly, and the members of the Dkkopf (Dkk) family that bind to the Wnt co-receptors LRP5/6. Whereas the sFRPs block all three Wnt pathways, the Dkks only inhibit the canonical Wnt/ β -catenin pathway [107]. These secreted Wnt inhibitors function to keep Wnt signaling below a certain level of activation. Because these secreted proteins maintain levels of Wnt signaling below a certain threshold, any mechanism leading to their reduced function would lead to increased β -catenin-mediated transcription, such as that observed in numerous types of cancers. A number of studies have indeed described the silencing via promoter hypermethylation of the genes coding for some of the secreted Wnt antagonists in colorectal cancer [108–111]. The transcriptional inactivation of sFRPs has been detected in a number of cancers including colorectal cancer [108], non-small-cell lung cancer [112], breast cancer [113], ovarian cancer [113,114]. It should be noted that overexpression of sFRPs has also been reported in some types of cancers [115–117], although the mechanisms for this overexpression or its consequence is not fully understood. The ability of sFRPs to inhibit Wnt signaling makes them an attractive target for therapeutic use. It was shown that restoring sFRP expression in colon cancer results in a reduction in Wnt signaling, even in the case of otherwise constitutive pathway activation due to mutations in proteins downstream of receptor activation [109]. Manipulating the expression of these proteins therefore appears to be a promising approach to downregulating Wnt signaling, especially in tumors that are characterized by mutations in the pathway, such as colorectal cancer.

Members of the Dkk family have also been shown to have an inhibitory effect on Wnt signaling, although activation of Wnt signaling, in particular by Dkk2 has been reported [118]. The Dkks inhibit Wnt signaling by binding and modulating the Wnt co-receptors LRP5/6. Dkks bind to their receptor Kremen and to LRP5/6, inducing LRP endocytosis and preventing downstream signaling [119–121]. As is the case for sFRPs, epigenetic inactivation has also been reported for DKKs [111,122]. It is clear that either potentiating the activity of endogenous Wnt antagonists, or designing efficacious drugs against targets of the canonical Wnt pathway could have a beneficial effect on many cancers. However, some cancers, such as melanoma, may not fall into this category. It has been demonstrated that β -catenin, while critical for the initial transformation of melanocytes [123], may actually have protective effects in the later stages. *Chien et al* have shown that activation of β -catenin in melanoma led to increased differentiation, and decreased tumor growth. On the other hand, non-canonical Wnt signaling can increase the metastatic potential of melanoma cells [124]. Thus, therapies targeted towards the β -catenin pathway may have no effect, or detrimental effects in melanoma patients, and instead therapies targeted toward the non-canonical Wnt pathway may be more beneficial.

4. Heretical Wnt Pathways

The non-canonical, or heretical, Wnt pathways are β -catenin-independent Wnt activated pathways. Unlike the canonical Wnts, heretical Wnts are unable to transform mammary epithelial cells [125] and are thought to be involved primarily in cell movement and polarity [126,127]. There are two major heretical Wnt pathways, the planar cell polarity (PCP) pathway, and the Wnt/ Ca^{2+} pathway.

4.1. The Wnt/PCP Pathway

The Wnt/PCP pathway has been best described in development, where it coordinates the polarization of cells along embryonic axes. This involves the activation of STAT3, and JAK/STAT signaling [128]. Wnts that play a role in Wnt/PCP signaling include Wnt5A, Wnt11, and Wnt7a [129]. During Wnt/PCP signaling, Wnt/Fz/ROR2 interactions recruit disheveled to the membrane, which triggers the recruitment of vang and prickle to the membrane of adjacent cells, and the balance between these regulates polarity [130–132]. Disheveled-dependent Wnt/PCP signaling then transduces signals via Jun, Daam, RhoA, Rac, Cdc42 and Profilin, and these have cytoskeletal effects that ultimately control both polarity and motility (Figure 2). Since these features are critical for tumor progression, the role of Wnt/PCP has been implicated in cancer.

4.2. Wnt/PCP pathway in cancer and potential targets

Discerning the Wnt/PCP and Wnt/Ca²⁺ pathways in human cancer is quite a difficult task, since both involve key molecules such as Wnt5A and ROR2. Here we will attempt to make a somewhat shaky distinction between the two by assigning the downstream effectors of Jnk and Rac and Rho to the Wnt/PCP pathway, and those of calcium and PKC to the Wnt/Ca²⁺ pathway. It is important to point out however that in elements of both pathways can be found in each, depending on the context of the tumor. Not much is known about Wnt/PCP signaling in cancer, but two excellent reviews have pulled together the information available to give us a comprehensive insight into this, and we direct the interested reader to these reviews [129, 133]. In Wnt/PCP signaling during development, Wnt5A activates Dsh and Jnk and Rho [134]. LRP6 [135] and Fzd7 [136] seem to be critical receptors in mediating this process. Fzd 7 has been shown to promote hepatocellular cancer and colon carcinoma, and targeting Fzd 7 can inhibit the invasion of these cells [137,138]. Dsh has been shown to be critical for β -catenin induced tumorigenesis as mentioned above, but may also play a role in β -catenin independent metastasis. Rac and Rho are also well known promoters of the metastatic phenotype in many cancer types, resulting in effects on the cytoskeleton [139]. Inhibitors of Rac and Rho therefore, would be useful in cancer therapy, and indeed this is an active field of study. Many of the inhibitors designed to target Rac and Rho actually target Ras, since this is one of the major pathways that activate these molecules. However, small molecule inhibitors of Rac and Rho are under also development. NSC23766 is a small molecule inhibitor of Rac that can suppress the growth and invasion of prostate cancer cells [140]. It has been shown that in a chimeric *in vivo* leukemia model that is clinically similar to human disease, this inhibitor can also suppress the growth and proliferation of leukemia [141]. This inhibitor does not affect either Cdc42 or Rho, and since both of these are also implicated in Wnt/PCP signaling, finding inhibitors to these molecules is also critical. C3 exotransferase has been used *in vitro* to inhibit both Cdc42 and Rho, but its use as a clinical agent is unknown [142]. Cethrin, a Rho inhibitor that has been used in the clinic to treat spinal cord injuries [143], may be of use in cancers that have Rho as an important intermediate.

As with the targeting of many intermediate proteins that are necessary for many cellular processes and in many cell types, the identification and use of a specific mediator expressed in cancer, but not normal cells, could significantly decrease toxicity. For example, the activation of Rho and Rac during Wnt/PCP signaling is mediated by the collagen triple helix repeat containing protein 1 (Cthrc1). Cthrc1 mediates the binding of Wnt5A to both Fzd and Ror2, and is thought to specifically activate Wnt/PCP signaling [144]. Knockout of CTHRC1 synergizes with mutations in Vangl2 to affect PCP signaling [144], and Vangl itself is implicated in the metastasis of tumors [145]. CTHRC1 is upregulated in the invasive stages of many cancers including melanoma, lung, breast, gastric, pancreatic, cervical, ovarian and thyroid cancers [146] making it an attractive target. It is possible that the *in vivo* use of CTHRC1 siRNA may have therapeutic effects. Failing the development of such specific inhibitors,

adjuvant therapy combining a few of the above mentioned inhibitors may have the desired effects on tumor ablation.

4.3. The Wnt Calcium Pathway

The other well-described heretical Wnt pathway involves the release of intracellular calcium downstream of Wnt signaling (Figure 3). Members of the Wnt family involved in the Wnt/ Ca^{2+} signaling pathway include Wnt5a, Wnt11, and Wnt4, and activation of the Fzd receptors by these Wnts was shown to result in the activation of heterotrimeric G proteins [7]. This results in phospho-inositol turnover in the membrane and the release of calcium from its intracellular stores. The increase in intracellular calcium results in the activation of calcium-dependent signaling molecules, such as calmodulin-dependent protein kinase II (CAMKII) and protein kinase C (PKC) [147,148]. These molecules can have a cornucopia of effects on downstream signaling, that is often dependent on the cellular context.

Calcium initiated effects include the activation of CAMKII. Wnt5A was shown to inhibit the activation of the canonical Wnt pathway via a number of different mechanisms including activation of CamKII [149], and through induction of Siah, a member of the E3 ubiquitin ligase complex [150]. Wnt5A, via CAMKII, has also been involved in the recruitment of macrophages during the inflammatory response to lipopolysaccharide [151], and its effect on macrophage activation may also play a role in breast cancer metastasis as discussed below [152]. Wnt5A activation of CAMKII is also critical for the proliferation of HUVEC cells, which may also have implications for angiogenesis and cancer [153]. We have also shown that Wnt5A regulates CAMKII in melanoma cells [154].

In addition to CAMKII, other calcium related molecules such as the calcium activate protease calpain (which will be discussed further in the next section) and calcineurin are activated by Wnt/ Ca^{2+} signaling and these have downstream effects. For example, calcineurin is known to de-phosphorylate NFAT, causing its nuclear translocation [155]. This process is tightly regulated by GSK3 β which phosphorylates NFAT to keep it in the cytoplasm. Wnt5A causes NFAT nuclear translocation in endothelial cells, even in the presence of cyclosporin A and FK506, calcineurin inhibitors, and it is thought that this may be due to the inhibition of GSK3 β [156]. Data from our laboratory also show that Wnt5A inhibits the metastasis suppressor Kiss-1 [154]. Kiss-1 is known to inhibit calcineurin, thus Wnt5A may also increase calcineurin expression via the inhibition of Kiss-1, resulting in NFAT translocation. Once in the nucleus, NFAT is capable of activating transcription programs that can contribute to cancer metastasis. It has been shown in mammary epithelial cells that Wnt5A can activate calcineurin and NFAT via a complex with CKI α [157]. This is a strong and durable interaction, but is regulated by Wnt5a signaling via Yes and Cdc42 [157]. This is a prime example of how complex Wnt signaling can be, even when discussing the same Wnt within the same cell type!

PKC is the other major intermediate activated by Wnt/ Ca^{2+} signaling. PKC is important in many cellular processes and is a critical element for Wnt5A signaling. We and others have shown repeatedly that many of the effects of Wnt5A can be mimicked by phorbol esters, and cannot occur in the presence of PKC inhibitors [154,158,159]. PKC is even important in the expression of Wnt5A, as studies have shown that PKC activation can cause the stabilization of Wnt5A mRNA, thus leading to an increase in Wnt5A levels [160]. In neuronal cells, nerve growth factor requires Wnt5A for axonal branching and growth of neuronal cells, in a PKC-dependent manner [161]. In T-cells, Wnt5A mediates the interaction between the chemokine CXCL12 and its receptor CXCR4, and PKC is critical in the transduction of this signal cascade, that leads to the migration of T-cells in response to CXCL12 [162]. In melanoma cells a similar activation of CXCR4 by Wnt5A involves the activation of PKC, and the formation of a Rac-Actin Myosin polarity complex [163], again blurring the distinction between Wnt/ Ca^{2+} and Wnt/PCP signaling in human cancer.

4.4. Wnt/Ca²⁺ pathway in cancer and potential targets

Wnt5A, in accordance with its different effects in the presence of different receptors, has been shown to have either a tumor suppressive or an oncogenic function, depending on the type of cancer. Its expression is downregulated in colorectal cancer [164,165], neuroblastoma [166], ductal breast cancer [167,168], and leukemias [169–171], and this downregulation was shown to be associated with higher tumor grade [172]. Conversely, Wnt5A was shown to be overexpressed in gastric cancer [173], pancreatic cancer [174], non-small cell lung cancer [175], and prostate cancer [176]. Wnt5A gene expression was found to be increased in more metastatic melanoma cells [177] and increased expression led to increased motility [178].

In melanoma, in which Wnt5A signaling has been well studied, expression of Wnt5A is correlated with a poor prognosis [179]. We have recently shown that the Wnt5A-mediated activation of the calcium-activated protease, calpain, results in the cleavage of the cytoskeletal protein filamin [180]. It has been shown that Wnt5A, via ROR2, can mediate the motility of various cell types [181,182] by regulating the formation of lamellopodia and that ROR2 binding to filamin is essential for this process [183]. Our data indicate that cleavage of filamin by Wnt5A could be inhibited by chelation of calcium by BAPTA-AM, underscoring the importance of calcium in this process.

Over the last decade or so we have slowly begun to unravel the intricacy of Wnt5A signaling in melanoma, and recently summarized these findings in a review [184]. Briefly, our data indicate that Wnt5A binds to its receptor ROR2, and this binding is supported by heparan sulphate proteoglycans such as syndecan 1 and syndecan 4 [185]. Upon ROR2 binding, Wnt5A and ROR are internalized via clathrin, and this process both activates and is mediated by PKC [186]. The result of this is the activation of CD44, suppression of Kiss-1, activation of Snail and Vimentin, and an epithelial to mesenchymal transition [154]. In addition, in melanoma cells, PKC activation leads to the activation of STAT3, which in turn inhibits the expression of melanoma differentiation antigens such as MART-1 and GP100. The effect of this is the decreased immunogenicity of melanoma cells, as these antigens often act as “red flags” to the immune cells [187]. In fact, because of this, MART1 and GP100 are often used as targets of immunotherapy [188], and one can speculate that first down-regulating Wnt5A signaling might increase the efficacy of such drugs.

Increases in PKC activation have been shown to increase the migration of melanoma cells, while its inhibition was able to decrease melanoma metastasis [189–192] and melanoma cell motility [154], making PKC an attractive target, at least for melanoma metastasis. PKC inhibitors have been investigated in the context of cancer therapy (reviewed in [193]). Although several compounds have shown initial activity in melanoma (bryostatin and UCN-01), non-Hodgkin's lymphoma (ISIS 3521, bryostatin, and UCN-01), and ovarian carcinoma (ISIS 3521 and bryostatin) in phase I studies, the activity of these drugs used as single agent in the phase II studies that were reported have been limited [194]. Again, because PKC is such an ubiquitous enzyme, finding specific, potent drugs with limited toxicity pose a problem, and targeting the source of aberrant signaling may hold the answer for future therapy.

Since Wnt5A is the best-described heretical Wnt member in human cancer, it is an obvious and highly specific target. Functional antibodies against Wnt5A have not been made available, however a study a few years ago laid the groundwork for a Wnt5A antagonist that could be used in the clinic. Foxy-5, a hexapeptide that acts as an agonist of Wnt5A was developed for use in breast cancer [195]. In breast, cancer, the expression of Wnt5A is a marker of better prognosis, since it acts as a tumor suppressor. Foxy-5 indeed was very effective in eradicating breast cancer in *in vivo* models, by mimicking Wnt5A expression [196]. Very recently the same group demonstrated that the N-butyloxycarbonyl hexapeptide, Box-5, could inhibit the growth

and invasion of melanoma cells, and Box-5 may prove to be an exciting new molecule for cancer therapy [197].

5. Tyrosine Kinase Receptors: A Wnt-Wnt Situation for Cancer Therapy?

When possible, targeting a receptor rather than a secreted ligand is often more desirable. Recently several tyrosine kinase receptors, which are often good targets for cancer therapy, have been associated with the Wnt pathway. Wnt5A was shown to signal via ROR2, a Wnt co-receptor [4]. Wnt5A is the only known ligand for ROR2, which makes this receptor tyrosine kinase a target of particular interest. It was shown that while Wnt5A knockdown reduced ROR2 levels, the inhibition of ROR2 did not affect Wnt5A expression levels, but did inhibit Wnt5A downstream signaling and resulting increase in metastasis [186]. Therefore, inhibiting specifically ROR2 would result in the specific inhibition of Wnt5A-mediated effects. ROR2 bears homology to neurotrophic tyrosine kinases, most closely, NTRK2. Neurotrophic tyrosine kinase receptors demonstrate increased expression in many cancers. In melanoma cells, for example, the neurotrophic Trks p75NTR [198] and TrkC [199] are overexpressed during metastatic progression. Neurotrophic Trks are of great interest as targets for cancer therapy, because, outside of the brain, they are found quite specifically on malignant cells [200], and inhibitors of nTrks have been shown to have efficacy *in vivo* [201]. NTRK2, the neurotrophic Trk closest in homology to ROR2, can be inhibited by molecules such as K252a and it is entirely possible that these may have some efficacy in the treatment of melanoma. It has been shown that K252a prevents the proliferation of melanoma cells [202], and a derivative of K252a, KT6124, has been shown to inhibit B16 melanomas *in vivo* [203]. It is possible that a similar derivative designed to target ROR2 may also have beneficial effects for melanoma patients.

Another tyrosine kinase receptor of importance in neural development that is involved in Wnt signaling is the tyrosine kinase Ryk, or Derailed (in *Drosophila*). Ryk is essential for transducing Wnt signals during synaptogenesis and axon guidance [204]. Unlike ROR2, Ryk can bind both canonical and heretical Wnts. When complexed with fz8, Ryk can bind Wnt1 and activate TCF/LEF dependent transcription [205]. However, when complexed with Fz7, Ryk binds Wnt11 and activates a beta-arrestin2 mediated pathway [206]. Finally, when complexed with Wnt5A, Ryk can signal via the release of intracellular calcium [204]. Because of its ability to transduce both canonical and heretical Wnt signals effectively, Ryk is a less attractive target than ROR2.

Although Ryk itself may not be a good target for cancer therapy, the partners to which it binds may be. Ryk is known to complex with Ephrin receptors, which can also activate and bind Disheveled [207]. It is unknown whether this association requires Wnt signaling, but certainly Ephrin receptors are associated with the increased malignancy of tumors, specifically colorectal and gastro-intestinal tumors, and have been implicated in angiogenesis [208–210]. Ryk complexes with EphB, and inhibitors to EphB do not seem to be available, although inhibitors such as Dasitinib will target EphA [211]. Dasitinib also targets src, which is activated by Wnt5A/Ryk signaling [212]. Several inhibitors for src exist, including PP2, SU6656 and Dasitinib, which has been used in clinical trials for chronic myeloid leukemia (CML) [213]. It was recently reported that in a 2 year follow-up of 2000 CML patients who received Dasitinib after failing Imatinib, 94% were still surviving, and 80% showed no signs of progressive disease [214].

Finally, Bruton's tyrosine kinase (BTK) is a novel receptor that has been identified as a regulator of Wnt signaling [215]. This tyrosine kinase regulates canonical Wnt signaling, and was identified by a combinatorial small molecule screen, as well as an siRNA screen. Loss of BTK by either pharmacological or genetic intervention resulted in an elevation of canonical Wnt signaling. BTK appears to inhibit β -catenin signaling via a direct interaction with a

molecule known as CDC73, which is a member of the transcriptional elongation complex [36]. Increases in CDC73 repress β -catenin activity, and CDC73 has been reported to be a tumor suppressor gene [216]. Enhancing the expression of BTK or CDC73 in cancers where canonical Wnt signaling is important for progression may be of benefit to the patient.

6. Conclusion

The Wnt signaling pathway is ripe with molecular targets for cancer therapy. In addition to key intermediates downstream of Wnt signaling, the discovery of new tyrosine kinase receptors provides a variety of potential targets. In this review we have not touched upon additional molecules such as norrin, r-spondin, CCN2, etc, but these may also prove to be valuable targets, and we refer the interested reader to two excellent reviews that discuss these proteins [205, 217]. The identification and efficacy of small molecule inhibitors of Wnt, such as Box-5 are extremely promising for cancer therapy. The immense amount of work in the Wnt signaling field is opening up a whole new avenue for molecular therapy, and one can only hope that among the targets identified, we will find one to which we will be able to develop the “next Gleevec”.

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Figure 1. The Wnt/β-Catenin Signaling Pathway and Points of Intervention

Receptor activation upon binding of Wnt 1 or Wnt3A leads to stabilization of β-catenin which localizes to the nucleus where it associates with transcription factors and activates transcription of genes involved in proliferation and tumor progression. In the absence of Wnt, transcription of these genes is prevented by phosphorylation of β-catenin by GSK3β within a destruction complex and its subsequent degradation via the proteasome.

Figure 2. The Wnt/PCP Signaling Pathway and Points of Intervention

Receptor activation by Wnt leads to the recruitment of Dsh, resulting in the activation of Rac1 and RhoA, both of which are involved in cytoskeleton remodeling and establishment of an EMT phenotype.

Figure 3. The Wnt/Ca²⁺ Signaling Pathway and Points of Intervention

Binding of Wnt5A to ROR2 leads to the activation of Ca²⁺ and PKC, which in turn stabilizes Wnt5A mRNA and increases its secretion. Secreted Wnt5A is presented to the receptor by HSPGs, resulting in a positive feedback loop. The Wnt5A-mediated increase in Ca²⁺ results in an increase in calpain-dependent filamin cleavage as well as activation of calcineurin and NFAT. Increases in PKC are associated with changes in the expression of proteins involved in the promotion of a metastatic phenotype including the upregulation of CD44 and SNAIL, loss of KISS1 and ECAD and establishment of an EMT. Increases in PKC are also associated with an increase in phospho-STAT3, which in melanoma leads to a decrease in melanosomal antigens.