The Wnt/β-catenin Signaling Pathway in Epithelial Mesenchymal Transition

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Abstract

Epithelial mesenchymal transition (EMT) is a well conserved process by which polarized, immotile epithelial cells transition into motile mesenchymal cells. EMT plays an important role during normal biological processes such as embryogenesis and wound healing. More recently, EMT has been studied for its role in cancer progression and metastasis. Understanding the molecular mechanisms that regulate EMT are key to developing novel therapeutic interventions for cancer. Dysregulated or uncontrolled activation of the Wnt/β-catenin signaling pathway promotes tumor progression and metastasis. The Wnt/β-catenin signaling pathway is one of the signaling pathways that has been implicated in EMT. In this review, major Wnt target genes that promote EMT as well as the various antagonists and microRNAs that regulate the Wnt/β-catenin pathway to influence EMT during cancer progression will be discussed.

Keywords: β-catenin signaling, cancer, epithelial mesenchymal transition (EMT), Wnt signaling

Introduction

Epithelial mesenchymal transition (EMT) can be described as a process by which polarized epithelial cells lose their apical-basal polarity, reorganize their cytoskeleton and undergo biochemical changes that cause cells to gain a mesenchymal cell phenotype [1]. The discovery that cells could transform from epithelial to mesenchymal phenotypes was identified by Elizabeth D. Hay using a chick primitive streak formation model [2]. However since the identified epithelial mesenchymal transformation was later determined to be a transient and reversible process, it came to be known as epithelial mesenchymal transition. EMT occurs in three distinct biological settings: (a) during implantation, embryogenesis, and organ development; (b) during tissue regeneration and organ fibrosis; and (c) during cancer progression, invasion and metastasis [3]. The relevance of EMT in cancer progression, invasion and metastasis has recently gained importance over the past several years [4-5]. Since treatment options are limited and overall patient prognosis is poor once metastasis has occurred, it is important to understand the molecular mechanisms regulating EMT in order to develop novel therapeutic targets and treatment options for cancer.

One of the molecular pathways that plays a critical role in promoting EMT is the Wnt/β-catenin signaling pathway. This signaling pathway has been extensively studied and plays an essential role in development [6-8]. In the absence of the Wnt ligand, the destruction complex of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α) can assemble and cause phosphorylation (at Ser33, Ser37, Thr41 and Ser45) and ubiquitin-mediated proteosomal degradation of cytoplasmic β-catenin (Figure 1). In the presence of the Wnt ligand, the Wnt signaling pathway is activated. The Wnt ligand initiates signaling extracellularly by binding to the seven-transmembrane domain Frizzled receptor and LRP5/6 (low density lipoprotein receptor-related proteins 5 or 6) co-receptor to initiate signaling (Figure 2). Binding of the Wnt ligand to its receptors results in Dishevelled (Dvl) mediated disruption of the Axin/APC/ GSK3β complex leading to cytoplasmic accumulation of β-

catenin. β-catenin then translocates to the nucleus, where it acts as a transcriptional co-activator and causes transcription of downstream TCF (T-cell factor)/LEF (lymphoid enhancer factor) target genes [9-10]. Under normal cell conditions, Wnt/β-catenin signaling is precisely controlled by a delicate balance of agonists and antagonists. Dysregulated activation of the Wnt signaling pathway can occur because of loss of function mutations in

antagonists of the pathway, or gain of function or constitutive activation of agonists of the pathway [11]. Dysregulated activation of the Wnt signaling pathway promotes tumor development, EMT and metastasis in a variety of cancers [8, 12-14]. In particular, Wnt induced-EMT plays a crucial role in the progression and metastasis of several cancers including prostate [15], breast [16-17], colon [18], and pancreatic cancers [19].
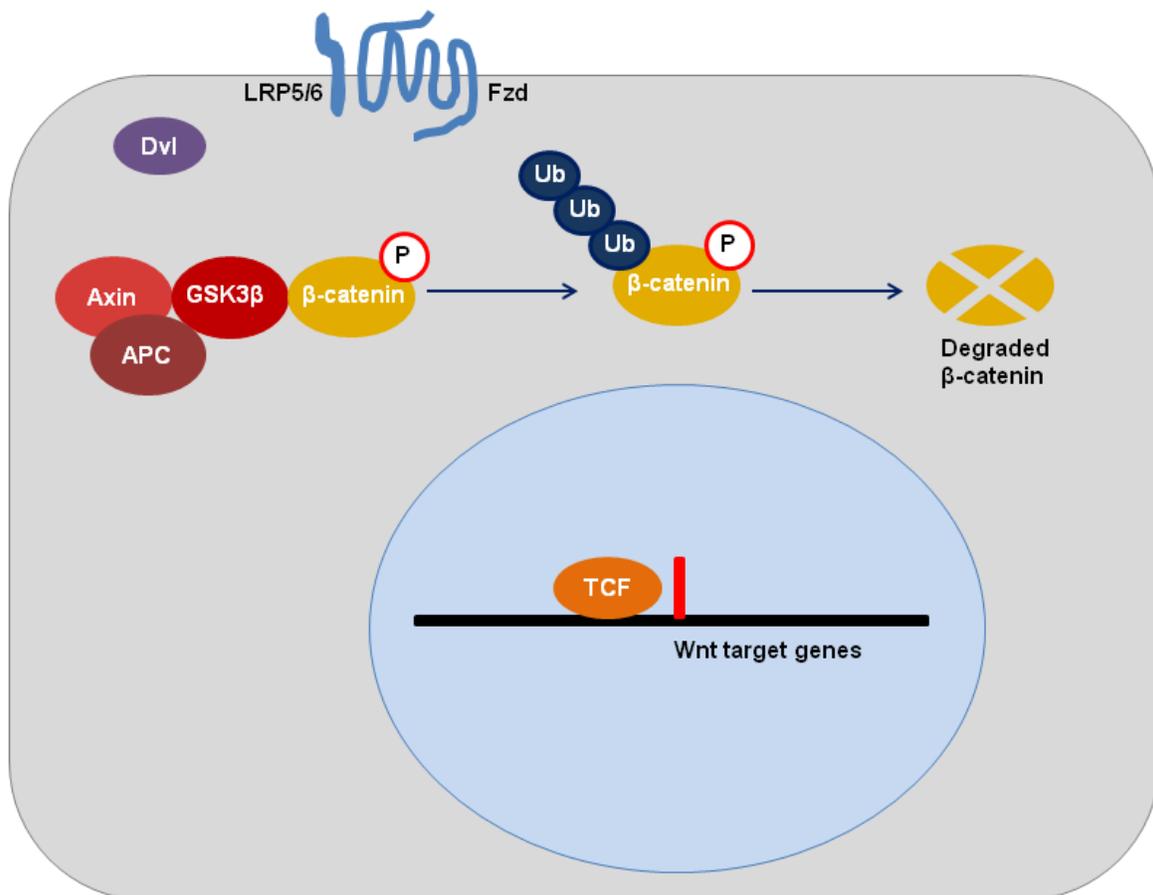

Figure 1. Schematic representation of the Wnt signaling pathway in the absence of Wnt ligands. In the absence of Wnt ligands, the destruction complex of Axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) can assemble. GSK3β then phosphorylates β-catenin at Serine 33, Serine 37, Threonine 41 and Serine 45. Phosphorylated β-catenin gets polyubiquitinated (Ub) by an E3 ubiquitin ligase containing the F-box protein β-TrCP. Polyubiquitinated β-catenin is then degraded by the 26S proteosome and the transcription of Wnt downstream target genes is repressed.
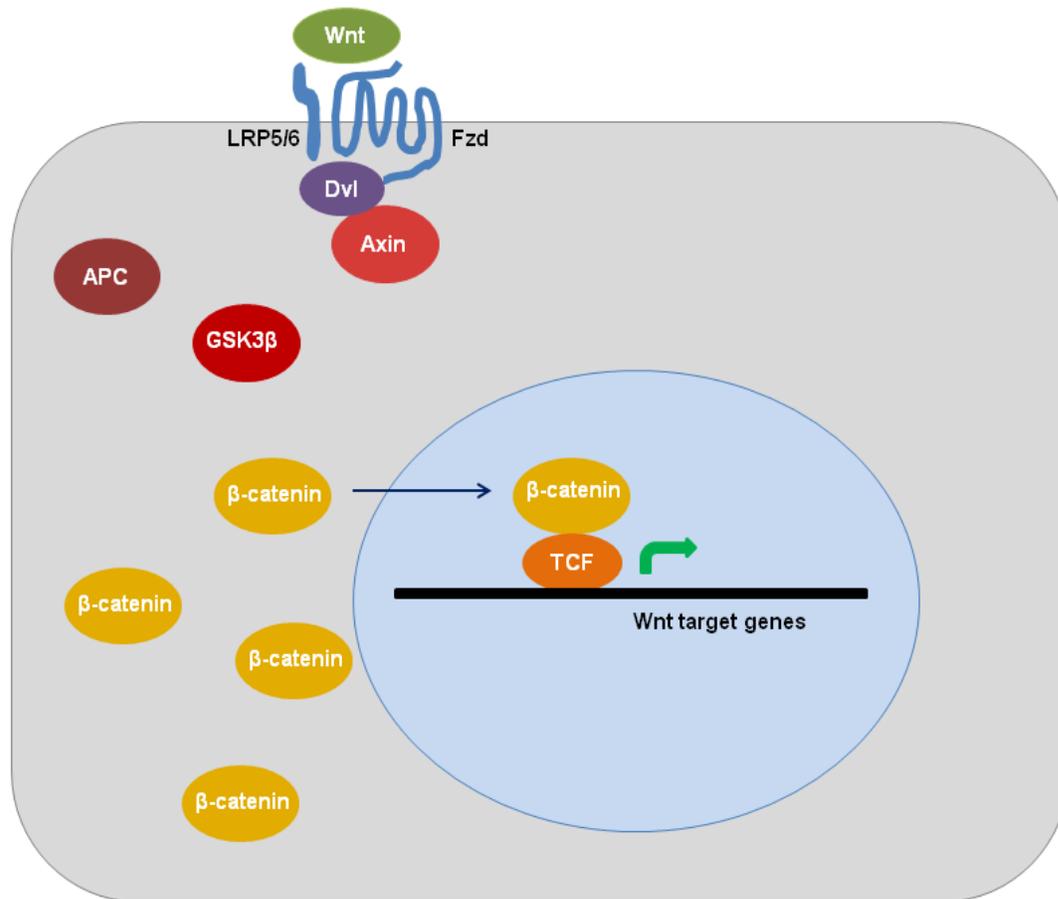
Figure 2. Schematic representation of the Wnt signaling pathway in the presence of Wnt ligands. Wnt ligands present exptracellularly bind to the seven-transmembrane domain Frizzed (Fzd) receptor and LRP5/6 (low density lipoprotein receptor-related proteins 5 or 6) co-receptor to initiate downstream signaling. Binding of Wnt ligands to its receptors results in activation of Dishevelled (Dvl), which inhibits the formation of the destruction complex of Axin, adenomatosis polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β). This results in cytoplasmic accumulation of β-catenin. β-catenin can then translocate to the nucleus where it binds to TCF (T-cell factor)/LEF (lymphoid enhancer factor) and enables transcription of Wnt downstream target genes.

In this review, I will discuss how activation of Wnt signaling enhances EMT, how antagonists of the Wnt signaling pathway inhibit EMT, and the role of microRNAs that regulate Wnt signaling to modulate EMT.

WNT signaling and EMT

Activation of the Wnt signaling pathway increases β-catenin stability and nuclear translocation, ultimately, resulting in transcription of various downstream target genes. Some of the crucial players of EMT, including Slug, Twist, ZEB1 and Vimentin, are Wnt target genes. Slug (Snai2) belongs to the Snail family of zinc-finger transcriptional repressors (Snail and Smuc are the other two members of this family) and can transcriptionally repress the epithelial marker E-cadherin [20]. E-cadherin is essential for epithelial cell-cell

adhesion [21]. Slug also causes a loss and redistribution of other epithelial markers such as Plakoglobin, and an increase in mesenchymal markers such as Fibronectin and Vimentin [20]. Conacci-Sorrell et al. showed that Slug was transcriptionally upregulated in response to Wnt signaling [22]. Wnt signaling was activated using a degradation resistant mutant of β-catenin S33Y (by replacing serine at position 33 with tyrosine, β-catenin could not be phosphorylated and hence was resistant to degradation) and the slug promoter activity was assessed. The Slug promoter was activated in the presence of S33Y β-catenin. Next, Slug promoter activity was assessed in the presence of a dominant negative TCF-4. Dominant negative TCF-4 lacks 31 amino acids at the N terminus and does not associate with β-catenin and hence inhibits transcription of downstream genes. Slug promoter activity was inhibited in the presence of dominant negative TCF-4, indicating Slug was a direct target gene of Wnt signaling [22]. Wu et al. showed that Slug was also controlled by the Wnt/GSK3β/β-TrCP axis [16]. In the absence of Wnt ligands, Slug gets phosphorylated by GSK3β and undergoes ubiquitin-mediated proteosomal degradation. On the other hand, in the presence of Wnt ligands, GSK3β kinase activity is inhibited, allowing accumulation and nuclear translocation of Slug, and downstream target gene transcription and EMT initiation [16]. A recent study further showed that phosphorylation of GSK3β at serine 9 inactivates its kinase activity and causes an increase in slug protein levels and upregulation of EMT [23]. Additionally the ubiquitin ligase (the carboxyl terminus of Hsc70-interacting protein (CHIP), a U-box-type ubiquitin ligase) that degrades phosphorylated Slug was also identified [23].

Twist is a transcription factor that belongs to the basic helix-loop-helix family and is an important regulator of EMT [24-25] . Twist can transcriptionally repress the epithelial marker E-cadherin, by binding to E-box elements of the E-cadherin promoter [26]. Twist also downregulates the expression of other epithelial markers, such as Claudins, Occludin,

Desmoplakin and Plakoglobin [27]. On the other hand, Twist upregulates mesenchymal markers such as N-cadherin, Fibronectin and MMPs [28-31]. Twist expression was assessed following addition of the Wnt ligand, Wnt1, which activates transcription *via* β-catenin/TCF complexes. Twist was upregulated in response to Wnt1 and the Twist promoter was responsive to β-catenin, indicating Twist too was a Wnt target gene [32]. Furthermore, Twist can activate Wnt/β-catenin signaling [24] presumably by release of β-catenin from the membrane as well as inhibition of phosphorylation and degradation of β-catenin. Twist also mediates stabilization of Snail protein [24] thus further enhancing EMT.

The transcriptional repressor zinc-finger E-box binding homeobox 1 (ZEB1) is a crucial inducer of EMT that transcriptionally represses E-cadherin [33] and is another Wnt target gene. β-catenin/TCF4 binds to the ZEB1 promoter to activate ZEB1 transcription and forced translocation of β-catenin to the nucleus resulted in ZEB1 expression [34]. Binding of β-catenin/TCF to the ZEB1 promoter was further confirmed using chromatin immunoprecipitation assays [35]. There was also a strong correlation between nuclear ZEB1 and nuclear β-catenin in tumor samples [35].

Vimentin is a type III intermediate filament expressed in mesenchymal cells. Vimentin was shown to be a Wnt target by Gilles et al. [36]. Vimentin expression correlated with cellular β-catenin distribution and cells with elevated β-catenin/TCF transcriptional activity were Vimentin-positive. Further, cotransfecting β-catenin and TCF4 with the Vimentin promoter resulted in promoter activation, indicating Vimentin was a direct target of Wnt signaling [36].

Matrix metalloproteins (MMPs) degrade the extracellular matrix (ECM), and cell-ECM and cell-cell contacts, allowing detachment and migration of cells. The substrate adhesion molecule Fibronectin [37] and matrix metalloproteinase (such as MMP-7/Matrilysin)

[38] play important roles in induction of EMT. Both Fibronectin [39] and MMP-7 [40] are direct targets of Wnt signaling.

Snail is another key regulator of EMT. Several researchers showed that Snail could trancriptionally repress the epithelial marker E-cadherin [41-44]. In addition to repressing E-cadherin, Snail also represses other epithelial proteins such as Desmoplakin and Claudins [45] and activates expression of mesenchymal markers such as Vimentin, Fibronectin and MMPs [46-47]. Snail (Snai1) is primarily regulated by protein turnover and changes in subcellular localization. Studies examining the role of S33Y β-catenin showed that E-cadherin was significantly decreased [48]. Interestingly, S33Y β-catenin also caused an increase in nuclear Snail protein in a temporal fashion. Snail itself represses E-cadherin transcription and S33Y β-catenin mediates its E-cadherin suppression *via* Snail. Further studies showed that S33Y β-catenin caused an increase in Axin2 levels, which chaperones GSK3β and stabilizes Snail protein to enhance EMT [48]. GSK3β phosphorylates Snail at two phosphorylation motifs. Phosphorylation at one motif results in ubiquitin-mediated proteosomal degradation of Snail. Phosphorylation at the second motif promotes nuclear export of Snail [49]. Thus like β-catenin, in the absence of Wnt signaling, Slug is phosphorylated by GSK3β and undergoes degradation. In the presence of Wnt ligands, GSK3β kinase activity is inhibited and nuclear slug levels increase allowing EMT and increased invasive abilities [16]. Thus GSK3β controls Snail protein turnover and activity.

E-cadherin is a central regulator of epithelial phenotype. Wnt downstream target genes such as Slug, Twist and ZEB1 [20, 26, 33] and Wnt signaling regulated genes such as Snail [41] are transcriptional repressors of E-cadherin. Decrease in E-cadherin level results in a loss of E-cadherin-dependent cell-cell junctions, which are key to an epithelial phenotype. Additionally, decrease in E-cadherin levels result in an increase in free β-catenin cytoplasmic levels,

that can translocate into the nucleus and further activate Wnt signaling [50].

Thus activation of Wnt signaling enhances EMT by several mechanisms, including transcriptional upregulation of key EMT players, and regulation of protein stability and subcellular localization of EMT players and β-catenin.

WNT pathway agonists and EMT

Under normal cell conditions, the Wnt signaling pathway plays a critical role in the control of cell proliferation, cell fate specification and differentiation, and is tightly regulated by several antagonists. There are at least seven known antagonists of the Wnt signaling pathway. These include the secreted Frizzled-related proteins (sFRPs), Cerberus, Crescent, Wnt inhibitory factor-1 (WIF-1), Wise, Naked cuticle homolog 1 (NKD1) and Dickkopfs (DKKs) [11]. Since activation of the Wnt signaling pathway enhances EMT, it can be expected that antagonists of the Wnt pathway would inhibit EMT.

Secreted Frizzled-related proteins and WIF-1 inhibits Wnt signaling by binding to and sequestering Wnt ligands. As would be expected, secreted Frizzled-related protein 1 (sFRP1) caused an inhibition of EMT [51]. Similarly overexpressing sFRP4 caused an increase in E-cadherin expression and a decrease in the expression of Vimentin and Twist, to bring about a reversal of EMT [52]. Overexpressing WIF-1 caused an increase in epithelial markers, E-cadherin, Keratin-8 and Keratin-18 and a decrease in mesenchymal markers, N-cadherin, Fibronectin and Vimentin. Both Slug and Twist expression were also reduced, ultimately resulting in a reversal of EMT [53].

The Dickkopf (DKK) family of proteins are secreted Wnt antagonists that can bind to and sequester the Wnt co-receptor LRP5/6. Five evolutionary conserved members of this family include DKK1, DKK2, DKK3, DKK4 and a unique DKK3-related member, DKKL1 (Dickkopf-like

protein 1, Soggy) [54]. DKK1 itself is a downstream target of Wnt/β-catenin signaling and establishes a feedback loop to control Wnt/β-catenin signaling. As would be expected, DKK1 causes an inhibition of EMT [11]. Overexpressing DKK1 in mesenchymal cancer cells caused an increase in epithelial markers and the expression of both Slug and Twist, direct target genes of Wnt signaling, were reduced [17]. DKK3 also caused a reversal of EMT with an increase in epithelial markers, E-cadherin, Keratin 8 and Keratin 18 and a downregulation of mesenchymal markers, N-cadherin and Fibronectin [55].

Besides the conventional antagonists of the Wnt signaling pathway, several tumor suppressors also inhibit the Wnt pathway to modulate EMT. For example, the tumor suppressive protein DNAJB6 upregulates DKK1 and induces degradation of β-catenin, which results in an inhibition of EMT [56]. Overexpression of DNAJB6 caused a gain of expression of Keratin 18 and a loss of mesenchymal markers, Vimentin, N-cadherin, Twist and Slug. Similarly, oncogenes that upregulate Wnt signaling or inhibit Wnt antagonists can also be expected to enhance EMT.

Thus EMT can be directly inhibited and/or reversed by Wnt antagonists; and tumor suppressors and oncogenes that modulate Wnt signaling can indirectly regulate EMT.

microRNAs regulated by WNT signaling and EMT

microRNAs (miRNAs) are short, 19-23 nucleotide, single-stranded non-coding RNAs that regulate various cellular processes including EMT. miRNAs act at the post-transcriptional level and repress translation or induce cleavage of target mRNA by binding to the 3' or 5' untranslated region (UTR) of the gene. Hundreds of miRNAs have been identified that act as tumor suppressors or oncogenes (also called oncomirs) [57]. We will examine the miRNAs that regulate Wnt signaling as they relate to EMT.

Several miRNA have been identified that regulate EMT *via* the Wnt signaling pathway. Some miRNAs inhibit activation of Wnt signaling and thus block the process of EMT. miR-200a regulates Wnt signaling mediated-EMT by two mechanisms. miR-200a binds to the 3' UTR of β-catenin and directly suppresses Wnt signaling [58]. Secondly, miR-200a inhibits EMT by targeting ZEB1 and ZEB2 to suppress Wnt signaling [59]. ZEB1 and ZEB2 are known repressors of E-cadherin [60-61]. By suppressing ZEB1 and ZEB2, miR-200a causes an increase in total E-cadherin which binds β-catenin and induces formation of cell-cell adhesion complexes. Overexpression of miR-200a also caused a decrease in the expression of mesenchymal markers, N-cadherin, β-catenin, Twist and Slug, and an increase in E-cadherin levels [59]. On the other hand, reducing miR-200a upregulated cytoplasmic and nuclear β-catenin levels and induced EMT [59]. Similarly miR-200c suppressed ZEB1, Snail and N-cadherin and consequently caused an increase in E-cadherin [62]. Interestingly the Wnt antagonist WIF1 causes an increase in the expression and activity of miR-200c [63]. Thus an antagonist of the Wnt signaling pathway upregulates a miRNA that suppresses EMT, further modulating EMT.

miR-203 expression is downregulated in cancer cells and low levels of miR-203 expression is associated with EMT. Studies showed that miR-203 inhibits EMT by indirectly enhancing DKK1 expression and inhibiting Wnt signaling [64]. miR-29b is another miRNA that inhibits activation of Wnt target genes by downregulating coactivators of β-catenin and overexpression of miR-29b caused a reversal of EMT [65]. Similarly loss of miR-101 promotes Wnt signaling-mediated EMT. Using both the degradation resistant β-catenin and a dominant negative TCF-4, there was a strong association between activated Wnt signaling and miR-101 repression [66].

Other microRNAs work in concert with the Wnt signaling pathway to enhance EMT. miR-374a is

upregulated in primary tumors from patients with distant metastases. miR-374a can directly target several antagonists of the Wnt pathway including WIF1, PTEN and Wnt5A thereby activating Wnt signaling. miR-374a thus promotes EMT by activating Wnt signaling [67]. miR-181 is also directly induced when Wnt signaling is activated. The promoters of miR-181a and miR-181b both have β-catenin/TCF-4 binding sites, which upregulate miRNA expression following activation of Wnt signaling [68]. miR-181a has further been identified to induce EMT [69]. Additionally, miR-181a was also shown to target WIF1, an antagonist of Wnt signaling [70]. Thus by targeting antagonists of the Wnt signaling pathway, miR-181a further enhances Wnt signaling and EMT.

Thus miRNA that modulate Wnt signaling also regulate EMT by targeting the activation of Wnt signaling, by upregulating Wnt signaling directly and/or by targeting Wnt antagonists.

Conclusions

The Wnt signaling pathway is an important regulator of tumor progression and metastasis. The finding that Wnt signaling also modulates EMT, an essential step in tumor progression, invasion and metastasis, makes this pathway an attractive target for developing novel therapeutic interventions. US Food and Drug Administration (FDA)-approved drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and the COX2 inhibitor celecoxib were shown to inhibit Wnt signaling in several cancers [71-74].

Small molecules such as pyrvinium, blocking antibodies such as Wnt3A-neutralizing antibodies, and peptides such as Fzd7 extracellular domain peptides have all been assessed to target Wnt signaling in cancer [75]. Thus while several therapies have already been developed to target Wnt signaling [75-77], with increasing information about novel regulation mechanisms of Wnt signaling, we will be able to fine tune currently available therapies for developing better, next-generation therapeutics.

As shown schematically in Figure 3, we can appreciate that the Wnt signaling pathway regulates EMT by multiple intricately connected mechanisms. Several of the downstream target genes of the Wnt signaling pathway are critical mediators of EMT. Further, signaling activated in the presence of Wnt ligands also regulates the protein stability and subcellular localization of EMT mediators. More recent studies have revealed that miRNAs can also regulate EMT *via* the Wnt signaling pathway. By directly targeting β-catenin, miRNAs can inhibit Wnt-mediated EMT. Additionally, by targeting antagonist of the Wnt signaling pathway, miRNAs can promote EMT. As research in the field continues, we might find other novel molecules/miRNAs that also regulate Wnt signaling. As new molecules and signaling mechanisms that regulate Wnt signaling are uncovered, we will have novel therapeutic targets/therapies for the treatment of cancer.
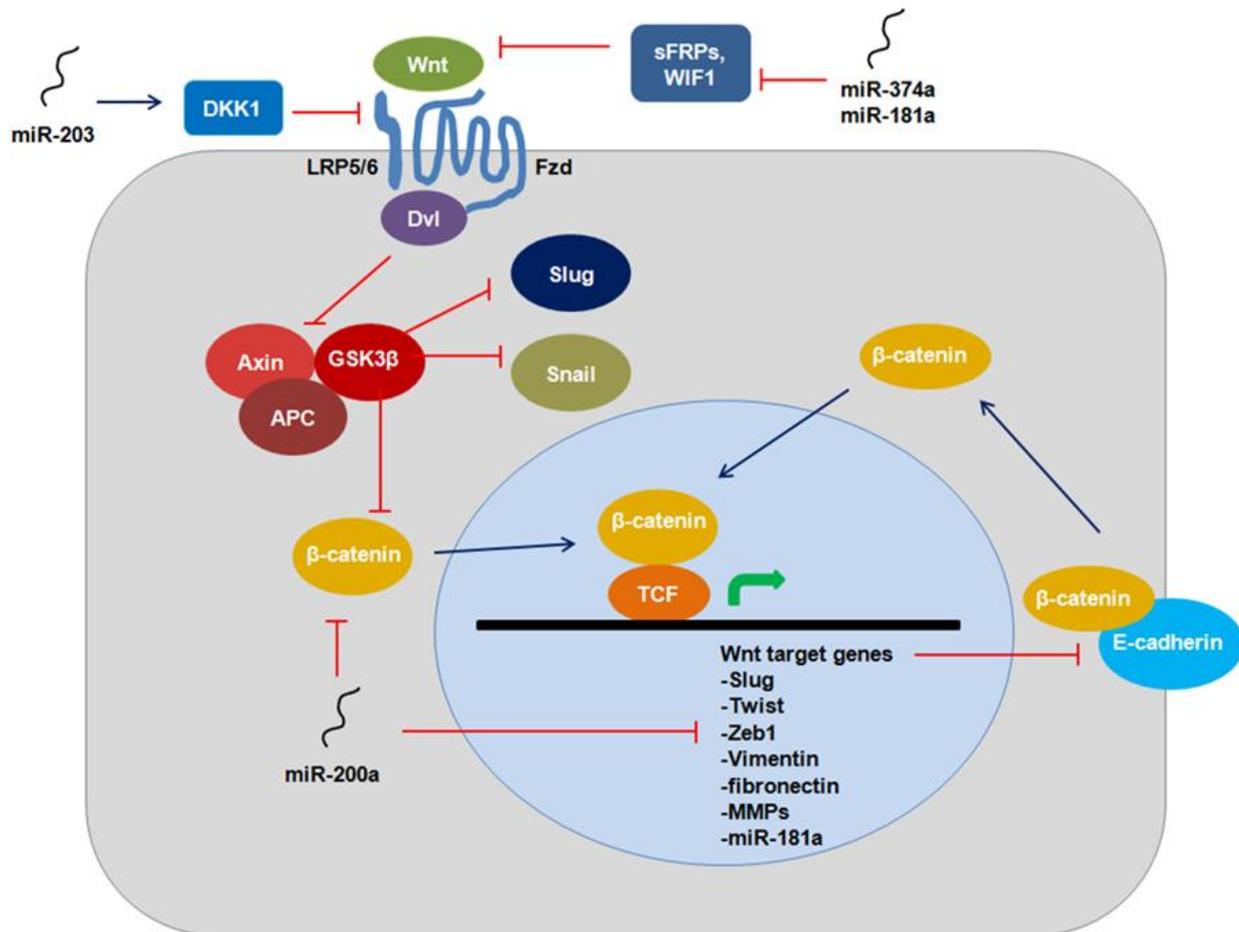
Figure 3. Multiple regulatory mechanisms of Wnt signaling-mediated epithelial mesenchymal transition. Wnt ligands bind to seven transmembrane Frizzled (Fzd) receptor and LRP5/6 coreceptor to initiate signaling. This activates Dishevelled (Dvl) which causes disruption of the destruction complex of Axin, APC and GSK3β (which in the absence of Wnt ligands, causes degradation of β-catenin). β-catenin accumulates in the cytoplasm, translocates to the nucleus and along with TCF (T-cell factor) causes transcription of downstream target genes. Several of these downstream target genes (Slug, Twist, Zeb1, Vimentin, Fibronectin and MMPs) are key regulators of EMT. Some of the Wnt downstream target genes, including Slug, Twist and Zeb1, are transcriptional repressors of E-cadherin. E-cadherin binds to and sequesters β-catenin at cell-cell junctions. However when E-cadherin is repressed, β-catenin is released into the cytoplasm and can translocate to the nucleus and can, in turn, activate Wnt signaling. Activation of Wnt signaling also modulates both protein stability and subcellular localization of Snail and Slug, key regulators of EMT. Activation of Wnt signaling and EMT can be inhibited by the Wnt pathway antagonists - DKK1, sFRPs and WIF1. Another level of regulation is mediated by miRNAs. By directly targeting β-catenin, miRNAs can inhibit Wnt signaling and EMT. On the other hand, by targeting Wnt antagonists, miRNAs can enhance Wnt signaling and EMT.

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