

*Critical Review***The Wnt/ β -Catenin Signaling Pathway as a Target in Drug Discovery**Fumi Takahashi-Yanaga^{1,*} and Toshiyuki Sasaguri¹¹Department of Clinical Pharmacology, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

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Abstract. The cell signaling cascades provoked by Wnt proteins (the Wnt signaling pathways), which are well conserved through evolution, play crucial roles to maintain homeostasis of a variety of tissues such as skin, blood, intestine, and brain, as well as to regulate proliferation, morphology, motility, and fate of cells during embryonic development. Among these pathways, the signal transduction through β -catenin (the Wnt/ β -catenin signaling pathway) has been most intensively studied because this signal regulates the expression of a number of genes essential for cell proliferation and differentiation and also this pathway is perturbed in a number of diseases such as cancers, bone diseases, and cardiovascular diseases. However, there is no therapeutic agents that can selectively modulate the Wnt/ β -catenin signaling pathway, although some existing drugs (e.g., non-steroidal anti-inflammatory drugs, vitamins, and imatinib mesylate) have been suggested to inhibit this pathway. Here we provide an overview of the Wnt/ β -catenin signaling pathway: its roles in physiology and pathology and the possibility as a target in development of new drugs.

Keywords: Wnt/ β -catenin signaling, cancer, calcification, drug development, differentiation-inducing factor

1. Introduction

Cell signaling cascades provoked by Wnt proteins (the Wnt signaling pathways) have been well conserved through evolutionary processes among a variety of species. Regulating cellular processes such as proliferation, differentiation, motility, and survival/apoptosis, the Wnt signaling pathways play a number of key roles in embryonic development and maintenance of homeostasis in matured tissues. Wnt proteins are secreted, cysteine-rich 39 – 46-kDa glycoproteins that act on target cells by binding to Frizzleds (Fz), seven-span transmembrane receptor proteins, and low-density lipoprotein receptor-related protein 5/6 (LRP 5/6), single-span transmembrane co-receptor proteins. Currently, 19 Wnt proteins and 10 different members of Fz have been identified in the human genome (1 – 3).

The Wnt-dependent signaling pathways comprise several branches whose activation depends on the speci-

ficity of the Wnt ligands and Fz receptors, as well as the cellular components. A large variety of responses could be initiated from Wnt/Fz interactions. Until very recently, there were three signaling pathways thought to be regulated by Wnt proteins: 1) the Wnt/ β -catenin (canonical) pathway that regulates the expression of Wnt target genes through β -catenin/T-cell factor (TCF) (2, 3); 2) the planar cell polarity (PCP) pathway that establishes asymmetric cell polarities and coordinates cell shape changes and cellular movement (4); and 3) the Wnt/ Ca^{2+} pathway that regulates cell adhesion and motility (5). Recently a fourth pathway has been identified that involves protein kinase A and plays a role in myogenesis (6). More than 50 component proteins have been identified to transduce the Wnt signals to mediate various cellular responses.

Despite considerable progress in investigating the mechanisms of Wnt signaling pathways, it is only recently that studies have emerged that implicate the Wnt signaling pathways in diseases (7 – 10). In this review, we will focus on the Wnt/ β -catenin signal pathway and discuss the involvement of this pathway in diseases and the possibility to develop new drugs.

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2. Wnt/ β -catenin signaling pathway (canonical pathway)

Among the four Wnt signaling pathways, the Wnt/ β -catenin signaling pathway is best understood (Fig. 1). At least 6 of 19 Wnt proteins, including Wnt1, Wnt2, Wnt3, Wnt3a, Wnt8, and Wnt8b, have been reported to activate this signaling pathway. The activity of this signaling pathway is determined by the amount of β -catenin in cytoplasm. Normally, cytoplasmic β -catenin level is kept low through continuous ubiquitin-proteasome-mediated degradation of β -catenin, which is regulated by a multiprotein complex containing axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3 β (GSK-3 β), and casein kinase 1 α (CK1 α). In general, degradation of proteins by ubiquitin-proteasome involves ubiquitin-activation enzyme (E1), ubiquitin-conjugated enzyme (E2), and ubiquitin ligase (E3). CK1 α and GSK-3 β mediate the degradation of β -catenin molecules

by phosphorylating specific amino terminal residues (Ser³³, Ser³⁷, and Thr⁴¹ by GSK-3 β and Ser⁴⁵ by CK1 α), which marks the protein to be recognized by β -transducin repeat containing protein (β -TrCP), a component of the E3 ubiquitin ligase complex, followed by the degradation by the 26S proteasome complex (11, 12).

Wnt proteins released from or presented on the cell surface initiate intracellular accumulation of β -catenin by binding to a cell surface receptor complex, Fz/LRP. It is poorly understood how binding of the Wnt protein to Fz/LRP receptor elicits signal transduction into the cell. After the binding of Wnt proteins to the receptor complex, cytoplasmic disheveled (Dvl), a downstream protein of the receptor complex, is phosphorylated and inhibits GSK-3 β and CK1 α activities through their retention at the scaffolding protein axin, resulting in the accumulation of non-phosphorylated β -catenin in cytoplasm. Non-phosphorylated β -catenin escapes from recognition by β -TrCP, thereby avoiding degradation

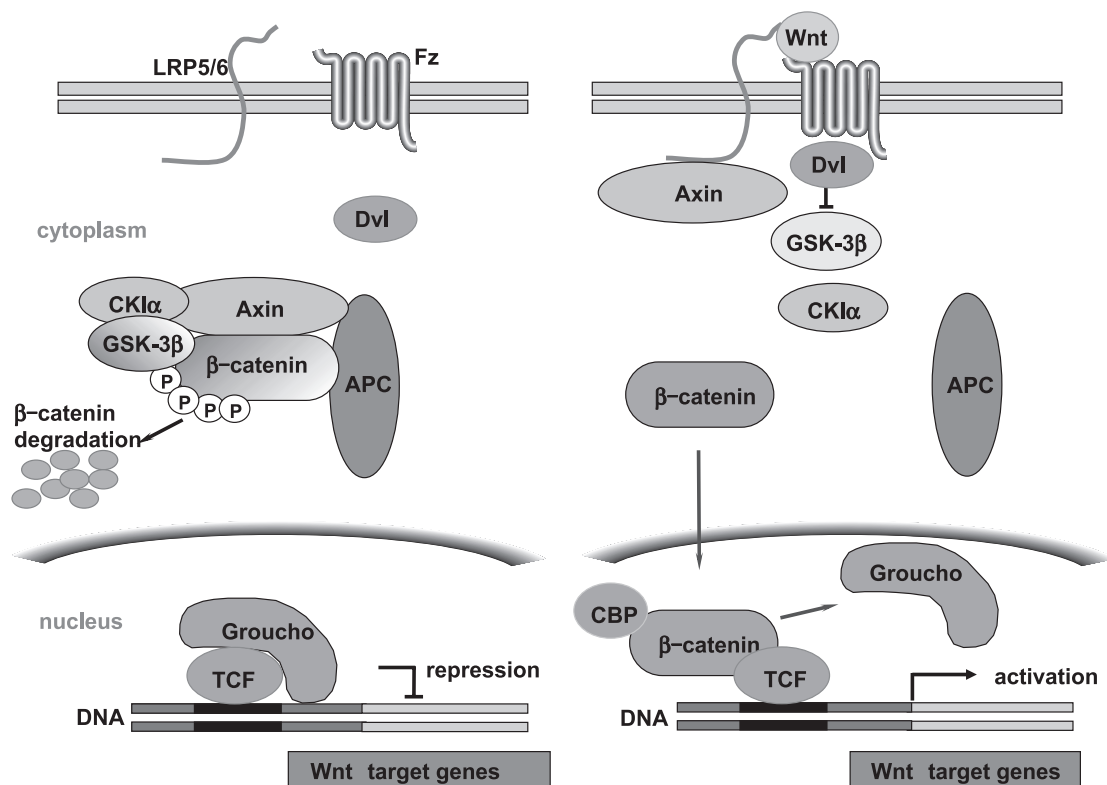


Fig. 1. The Wnt/ β -catenin signaling pathway. In the absence of Wnt, β -catenin binds to the protein complex formed by axin, APC, GSK-3 β , and CK1 α and is phosphorylated by GSK-3 β and CK1 α , resulting in the degradation by the 26S proteasome system (left). Wnt binds to the receptor Fz and co-receptor LRP5/6, and these receptors mediate signals into cells. GSK-3 β is inhibited by activated Dvl, thereby β -catenin escapes from phosphorylation. Unphosphorylated- β -catenin accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, β -catenin releases the inhibitory factor Groucho and activates the transcription of target genes together with TCF and other transcriptional co-factors, such as CBP (right). Fz, Frizzled; LRP, low-density lipoprotein receptor-related protein; Dvl, disheveled; APC, adenomatous polyposis coli; GSK-3 β , glycogen synthase kinase-3 β ; CK1 α , casein kinase 1 α ; TCF, T-cell factor; CBP, CREB binding protein. (Quoted from ref. 100 with minor change).

and being translocated into the nucleus. In the nucleus, β -catenin forms a complex with transcriptional factor, TCF. TCF forms a complex with Groucho in the absence of β -catenin and their interaction has been thought to repress transcriptional activity. β -Catenin interferes with the interaction between TCF and Groucho and induces the expression of down stream target genes including c-myc and cyclin D1, together with TCF and other transcriptional co-factors, such as CREB binding protein (CBP) (13). An up to date list of genes affected by Wnt/ β -catenin signaling can be found on the Wnt Gene Homepage (7).

3. The Wnt signaling pathway and disease

3-1. Cancer

3-1-1. APC

Wnt was initially discovered as a proto-oncogene in mammary tumors activated by integration of the mouse mammary tumor virus (14). Since then, many studies have linked the Wnt signaling pathway to oncogenesis and cancer development. Mutations resulting in constitutive activation of the Wnt signaling pathway may lead to cancer (15). Familial adenomatous polyposis (FAP) is the best-known example of a disease that is caused by a mutation in the Wnt signaling pathway. FAP is an autosomal, dominantly inherited disease and affected individuals usually develop hundreds to thousands of adenomatous polyps in the colon and rectum, which can progress to malignant forms. APC mutations linked to FAP were first identified by two groups in 1991 (16, 17). Most of the mutations found in the APC gene result in truncated gene products that lack axin-binding motifs. Since the axin-binding site is a critical region in APC to regulate β -catenin protein level, APC mutations cause accumulation of β -catenin in cytoplasm, which aberrantly activates cell proliferation due to stimulation of the Wnt signaling pathway, resulting in the formation of adenomatous lesions. Mutations in the APC gene are frequently identified in FAP and colorectal cancers, but are rare in other cancers (18, 19).

3-1-2. β -Catenin

Mutations in the β -catenin gene are found in a wide variety of human cancers including colon cancer, hepatocellular carcinoma, desmoid, pancreatic cancer, gastric cancer, melanoma, ovarian cancer, and prostate cancer (18). β -Catenin protein contains a serine/threonine rich region between Ser²⁹ and Lys⁴⁹ and phosphorylation of key residues within this region triggers degradation of this protein. As described above, phosphorylation of specific amino terminal residues (Ser³³, Ser³⁷, and Thr⁴¹ by GSK-3 β and Ser⁴⁵ by CK1 α) marks the protein to be recognized by the ubiquitin-proteasome

system. Therefore, mutations that affect these critical serine or threonine residues stabilize β -catenin and induce constitutive activation of the Wnt/ β -catenin signaling pathway, which could contribute to the pathogenesis of cancer.

3-1-3. Axin

Axin is a scaffold protein that binds to various components of the Wnt signaling pathway including, β -catenin, GSK-3 β , CK1 α , APC, and Dvl. In the axin complex, GSK-3 β and CK1 α phosphorylate β -catenin efficiently to initiate degradation of this protein. Thus axin plays an important role to regulate intracellular levels of β -catenin and is thought to be a tumor suppressor. Mutations of the axin gene generate truncated forms that cannot play a role as a scaffold protein. These mutations of axin are found in hepatocellular carcinoma and other types of human cancers (20, 21). Axin2 (also called axil or conductin) is an axin homologue that shows 45% homology with axin, and the biochemical characteristics of axin2 are similar to those of axin. Whereas axin is a constitutively expressed component of the β -catenin degradation complex, axin2 is upregulated in response to increased β -catenin concentration and serves to limit the duration and intensity of the Wnt signaling. Mutations in the axin2 gene lead to an increase in β -catenin concentrations in colorectal cancers with defective mismatch repair system (22, 23). Moreover, it has been reported that tooth agenesis can also be caused by axin2 mutation (23). Both APC and axin have been reported to constantly shuttle between the nucleus and cytoplasm and move β -catenin out of the nucleus (24, 25). Therefore, APC and axin might play an important role to regulate β -catenin levels not only in the cytoplasm but also in the nucleus.

3-2. Other diseases

3-2-1. Bone diseases

The revelation that the same Wnt/ β -catenin signaling pathway which has been extensively studied in developmental and cancer models also participates in postnatal bone regulation is an exciting discovery giving us great advances in understanding skeletal biology. The Wnt/ β -catenin signaling pathway plays a critical role in the differentiation of osteoblasts and regulation of bone mass. Many components of the Wnt/ β -catenin signaling pathway have been studied in both in vitro and in vivo models of bone development.

Some Wnt proteins (Wnt1, Wnt2, and Wnt3a) have been reported to induce alkaline phosphatase, which is a well known marker during early osteoblast differentiation in osteoblast precursor cell lines (26, 27), while Dkk1, which inhibits the Wnt/ β -catenin signaling pathway, has been reported to reduce osteoblastogenesis

(28, 29). Moreover, it has been reported that inhibition of GSK-3 β activity with lithium chloride, which activates the Wnt/ β -catenin signaling pathway, stimulates precursor cells to differentiate into osteoblasts (29–31). Mutations in LRP5, co-receptor for Wnt proteins, have been identified that cause decreased bone mass in human and mice. In this case, LRP5 loses its function due to frame shift or missense mutations (32, 33). Conversely, a persistently active mutant for LRP5 results in increased bone mass (34, 35). This mutation is a single amino-acid substitution that makes LRP5 insensitive to Wnt signaling inhibition, resulting in the over-activation of the Wnt signaling pathway in bone. The increased bone mass phenotypes resulting from mutations in LRP5 have been clinically characterized under a number of different names including autosomal dominant osteopetrosis type I, Van Buchem disease, endosteal hyperostosis, osteosclerosis, and high bone mass (36, 37). In addition, the human disease osteoporosis-pseudoglioma syndrome (OPPG), which is characterized by a juvenile onset osteoporosis and congenital or early infancy-onset blindness, and familial exudative vitreoretinopathy (FEVR), which is a well-defined inherited disorder of retinal vessel development and can lead to partial or total retinal detachment, have been reported to be caused by the mutations in LRP5 (26, 38, 39). Thus, it has been clearly demonstrated that LRP5 and the Wnt/ β -catenin signaling pathway are key regulators in bone development in various levels and perturbations in this signaling pathway is involved in bone diseases.

3-2-2. Vascular calcification

Vascular calcification is an important manifestation of atherosclerosis. Its presence is a strong indicator of chronic inflammatory disease and relates to the atherosclerotic diseases (40, 41). However, the mechanisms for mineral deposition in arteries are not entirely understood. Recent evidence indicates that vascular calcification is a process similar to bone mineralization and many of the key regulators of bone mineralization are activated in cardiovascular calcification (42). Vascular smooth muscle cells in primary culture express bone proteins and are mineralized when treated with β -glycerophosphate, which serves as an inorganic phosphate donor in the presence of alkaline phosphatase. These cultured vascular smooth muscle cells lose expression of smooth muscle-specific proteins during osteogenic differentiation (43). It has been demonstrated using RT-PCR analysis and histomorphometry that osteoblast-like phenotype cells exist in calcified aortic valves removed on surgical valve replacement (44). Moreover, it has also been reported that cardiovascular calcification is promoted by the activation of the Wnt/ β -

catenin signaling pathway (45). These findings support the idea that the mineralization process in vascular smooth muscle cell is a consequence of abnormal activation of the Wnt/ β -catenin signaling pathway.

3-2-3. Cardiac hypertrophy

The adult myocardium undergoes hypertrophic growth in a variety of diseases including myocardial infarction, valvular disease, hypertension, endocrine disorders, and inherited mutations in components of cardiac sarcomere (46, 47). Since cardiomyocytes are terminally differentiated, they can increase only their size in response to the pathogenic stimuli. Although the hypertrophy may initially compensate heart function, prolonged and excessive hypertrophy is correlated with poor clinical prognosis and frequently leads to dilated cardiomyopathy and sudden death (48). Several signal transducers such as mitogen-activated protein kinases (MAPKs), Ca²⁺/calmodulin-dependent kinase (CaM kinase), and calcineurin (Ca²⁺/calmodulin-dependent phosphatase) have been implicated in cardiac hypertrophy as positive regulators. On the other hand, a number of endogenous molecules have been shown to negatively regulate cardiac hypertrophy (49). It has been suggested that GSK-3 β is one of these negative regulators, since the inhibition of GSK-3 β stimulates the hypertrophic response and overexpression of GSK-3 β inhibits the development of cardiac hypertrophy (50–52).

Components of the Wnt/ β -catenin signaling pathway revealed to be involved in human diseases are summarized in Table 1.

4. The Wnt signaling pathway as a therapeutic target

As evidence is accumulated that implicates the relationship between the Wnt/ β -catenin signaling pathway and human diseases, inhibitors of the Wnt/ β -catenin signaling pathway have been considered as candidates for drug development primarily for the treatment of cancers (53–55).

4-1. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, indomethacin, and sulindac, are widely used for the treatment of inflammation, fever, and pain. These drugs inhibit the activity of the cyclooxygenase (COX), a key enzyme in the arachidonic acid cascade, and thereby inhibit the synthesis of thromboxane A₂ and prostaglandins. However, recently, evidence suggests that NSAIDs can be used as anti-tumor agents. A number of experimental and epidemiological studies in humans suggested that aspirin and other NSAIDs show a chemopreventive effect against colon cancer (56–58).

Table 1. Human diseases related with the Wnt/ β -catenin signaling pathway

Disease	Wnt/ β -catenin signaling pathway component	Reference
Cancer	APC	18, 19
	β -catenin	15, 18
	axin1	20, 21
	axin2	22
FAP	APC	16, 17
Tooth agenesis	axin2	23
Bone disease (high or low bone mass)	LRP 5	33 – 37
OPPG	LRP 5	33
FEVR	LRP 5	38, 39
Vascular calcification	unknown	45
Cardiac hypertrophy	GSK-3 β	50 – 52

APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; LRP, low-density lipoprotein receptor-related protein; OPPG, osteoporosis-pseudoglioma syndrome; FEVR, familial exudative vitreoretinopathy; GSK-3 β , glycogen synthase kinase-3 β .

Several randomized trials have shown that the growth of polyps was inhibited and the number of existing polyps was decreased in patients with FAP who received sulindac (58, 59). Two randomized placebo-controlled studies have shown that aspirin reduced the recurrence risk of colorectal adenomas among patients previously suffering from colorectal cancer and adenoma, excluding FAP patients (60, 61). Regular use of NSAIDs has been also shown to have association with a reduced incidence of other cancers including breast and lung cancers (62). Possible cellular mechanisms underlying the chemopreventive effect of NSAIDs include the induction of apoptosis, cell-cycle arrest, and the inhibition of angiogenesis (57, 58). Suppression of elevated COX activity in cancer cells may be an important factor in the anti-cancer activity of NSAIDs, but it is not the only mechanism because NSAIDs also show the effect on tumor cells lacking COX activity (63). Moreover, NSAIDs have been reported to inhibit the Wnt/ β -catenin signaling pathway. Both aspirin and indomethacin attenuated the transcriptional activity of β -catenin/TCF-responsive genes (64). Sulindac also suppressed the Wnt/ β -catenin signaling pathway by inhibition of nuclear β -catenin localization and β -catenin/TCF regulated transcription of target genes (65).

There are two isoforms for COX. COX-1 is constitutively expressed in many tissues and is involved in maintaining biological homeostasis, such as cytoprotection of the gastric mucosa and platelet aggregation. In contrast, COX-2 is induced immediately in response to inflammatory stimuli including mitogens, growth factors, and cytokines. COX-2 levels are also elevated in several malignancies, such as colon, prostate, head and neck,

and skin cancer. The adverse effects of NSAIDs, such as damage of the gastric mucosa and bleeding due to anti-platelet effect, are caused by the inhibition of COX-1. This has prompted the development of COX-2 selective inhibitors such as celecoxib and rofecoxib. However, randomized trials have shown thrombotic cardiovascular events increased in patients treated with COX-2 selective inhibitors compared with the placebo control (66, 67), resulting in the withdrawal of rofecoxib. At present, celecoxib is the only NSAID approved by the Food and Drug Administration of the USA for the treatment of FAP patients. Treatment of colon cancer cell lines with celecoxib also has shown to inhibit the Wnt/ β -catenin signaling pathway (68, 69). Sakoguchi-Okada et al. reported that celecoxib inhibited the activity of TCF reporter plasmid and reporter gene driven by the human cyclin D1 promoter in the human colon cancer cell line HCT-116, suggesting that this compound inhibited the expression of Wnt/ β -catenin signaling target genes (69).

4-2. Vitamins

Vitamin A is converted in the body to a number of different metabolites collectively referred to as retinoids. Retinoids are potent regulators of cell proliferation and differentiation, applicable to cancer therapy and prevention (70). Retinoids have been shown to inhibit the function of the oncogenic AP-1 and Wnt/ β -catenin signaling pathway as well as stabilize components of the adherent junctions (71).

Vitamin D is an important factor to regulate calcium and phosphorus levels to maintain the body skeleton. An active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃, and its synthetic derivatives demonstrate a chemo-

preventive effect in animal models of colorectal and breast cancer, and there is an evidence that the inhibition of the Wnt/ β -catenin signaling pathway is a mechanism for the chemopreventive effect of vitamin D (72).

4-3. Imatinib mesylate and others

Imatinib mesylate, originally identified as an inhibitor of platelet-derived growth factor (PDGF) receptor, has been approved as a drug for the treatment of chronic myeloid leukemia. This chemical compound inhibits the Bcr-Abl kinase, a fusion protein constitutively activated in patients with chronic myeloid leukemia. Several studies suggested that various tyrosine kinases associated with epidermal growth factor (EGF) receptor and PDGF receptor phosphorylate Tyr⁶⁵⁴ in β -catenin, which regulate binding of β -catenin to E-cadherin. Phosphorylation of β -catenin releases E-cadherin, resulting in cell migration and tumor metastasis (73, 74). Imatinib mesylate inhibits tyrosine-phosphorylation of β -catenin (75), which may be one of the mechanisms for the chemopreventive effect of imatinib mesylate. However, imatinib mesylate has been reported to inhibit the Wnt/ β -catenin signaling in human colon cancer cell lines and thyroid carcinoma cell lines, suggesting that this chemical is also able to modulate the Wnt/ β -catenin signaling (75).

Chemical compounds other than those mentioned above, such as lithium (76), curcumin, and flavonoids, have also been reported to inhibit the Wnt/ β -catenin signaling pathway (53).

4-4. Potential drug targets

Although there is no selective inhibitor for the Wnt/ β -catenin signaling pathway available as a therapeutic agent at present, components of the Wnt/ β -catenin signaling pathway can be a target for new drug discovery. Fzs, receptors for Wnt ligand, are seven-span transmembrane receptors and structurally similar to G protein-coupled receptors (GPCRs). Today, more than 50% of chemically applicable drugs target GPCRs. However, in the case of the Wnt/ β -catenin signaling pathway, usage of the receptors as a drug target is complicated. As mentioned above, the Wnt family consists of at least 19 members and the Fz family consists of 10 members. Due to the abundance of members, little is known about the specificity for the ligand-receptor interaction. Moreover, the Wnt/ β -catenin signaling pathway requires co-receptors, LRP5 or LRP6, to transduce a Wnt signal into the cell. This adds further complexity, since numerous combinations of different receptors and co-receptors can transduce signals using different pathways. Among the downstream components, GSK-3 β could be a good target for

drug design (77). GSK-3 β was initially found as a key enzyme involved in glycogen metabolism, but now it is well known that GSK-3 β regulates a diverse array of cell functions (78). GSK-3 β is an essential component of the Wnt/ β -catenin signaling pathway and has been reported to be involved in many diseases including diabetes, bipolar disorder, Alzheimer's disease, heart failure, and cancer (50–52, 77).

5. Differentiation-inducing factor: a modulator of the Wnt/ β -catenin signaling pathway

The differentiation-inducing factors (DIFs), first identified in *Dictyostelium discoideum* as a putative morphogens required for stalk cell differentiation (79, 80), inhibit mammalian cell growth. In the DIF family (DIF-1, DIF-2, and DIF-3), DIF-3 is the most potent inhibitor of proliferation in mammalian cells (81). However, the target molecule of DIFs is unknown, even in *Dictyostelium*. In search of chemical substances applicable for the treatment of cancer and other proliferative disorders, we studied the signal transduction of DIFs in mammalian cells mainly using HeLa cells. We found that DIFs (DIF-1 and DIF-3) inhibited mammalian cell proliferation by suppressing the expression of cyclin D1 through the activation of GSK-3 β (82–85). Cyclin D1 is synthesized early in the G₁ phase and plays a key role in the initiation and progression of this phase. When cells enter the S phase, cyclin D1 is rapidly degraded by ubiquitin-proteasome-dependent proteolysis (86). GSK-3 β -induced phosphorylation of Thr²⁸⁶ of cyclin D1 has an important role in the initiation of cyclin D1 ubiquitination (87). Moreover, the cyclin D1 gene is one of the target genes for the Wnt/ β -catenin signaling pathway, and the degradation of β -catenin is initiated by GSK-3 β (88). Therefore, activation of GSK-3 β is expected to cause a reduction in both protein and mRNA levels of cyclin D1 through independent pathways. Indeed, DIFs induced phosphorylation of the Thr²⁸⁶ residue of cyclin D1 and β -catenin degradation, resulting in the reduction of cyclin D1 in the protein and mRNA levels (82, 84, 85). We also found that DIFs reduced the activity of a TCF reporter plasmid and a reporter gene driven by the human cyclin D1 promoter (83). These results suggest that DIFs inhibit the Wnt/ β -catenin signaling pathway.

Recently, Zou et al. (89) reported that dual-specificity tyrosine-phosphorylation regulated kinase 1B (DYRK1B), a member of the DYRK family (90), phosphorylated cyclin D1 on Thr²⁸⁸, resulting in its degradation. DYRK1B is highly expressed in normal skeletal muscle and certain carcinoma cell lines including HeLa cells, although it is not detectably expressed in

many normal tissues (91). There are some similarities between GSK-3 β and DYRK1B, since they phosphorylate the same substrates (glycogen synthase and cyclin D1) (89, 92). We revealed that not only GSK-3 β but also DYRK1B was involved in the phosphorylation of cyclin D1 induced by DIF-3 (85). Taken together, DIFs may be able to efficiently induce degradation of cyclin D1 using these kinases.

DIFs strongly reduced the expression of cyclin D1 in not only HeLa cells but also in human squamous cell carcinoma cell lines (SAS and NA) (83, 84), human colorectal carcinoma cell line (HCT-116), and human osteosarcoma cell line (SaOS-2) (author's unpublished observation). In tumor cells, genes regulating the cell cycle are often damaged. Among them, cyclin D1 is one of the genes strongly implicated in oncogenesis (93). Amplification of the gene encoding cyclin D1 and overexpression of cyclin D1 protein have frequently been shown in a number of human malignant neoplasms (94–97). It was also reported that cyclin D1-deficient mice are resistant to breast cancers (98), suggesting that cyclin D1 could be a good target to develop new

anticancer drugs.

We also analyzed the effect of DIF-1 on osteoblast differentiation using osteoblast-like cell lines, SaOS-2 and MC3T3-E1 (99). The expression of alkaline phosphatase, which is widely used as an osteoblast differentiation marker, was markedly suppressed by DIF-1-treatment in protein and mRNA levels. DIF-1 also suppressed the expression of other osteoblast differentiation markers, including core binding factor α 1, type I collagen, and osteocalcin, in protein and mRNA levels and inhibited osteoblast-mediated mineralization. We found that DIF-1 suppressed the expression of β -catenin protein and the activity of the reporter gene containing TCF consensus binding sites. DIF-1 significantly reduced the alkaline phosphatase reporter gene activity through the TCF binding site (–1023 /–1017 bp). Our data suggested that DIF-1 inhibits the Wnt/ β -catenin signaling, resulting in the suppression of alkaline phosphatase promoter activity in osteoblast-like cell lines (99).

As described above, DIFs act as an inhibitor of the Wnt/ β -catenin signaling pathway, whereas the target

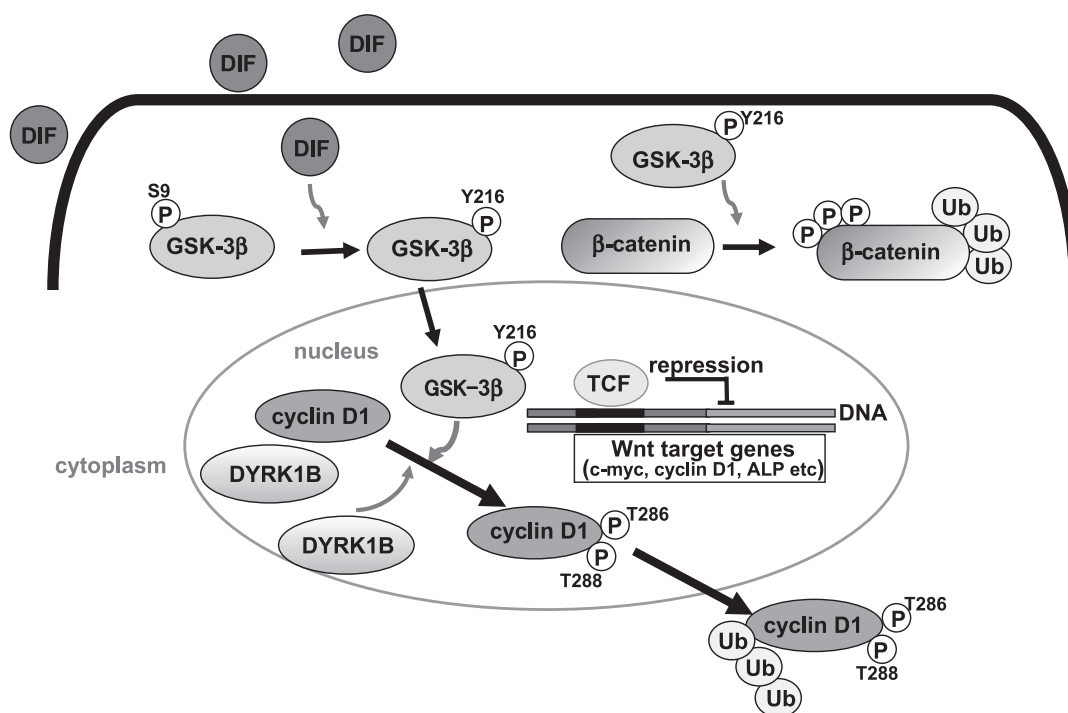


Fig. 2. DIF action and the Wnt/ β -catenin signaling pathway. DIFs enter into cell without receptor. Ser⁹ of GSK-3 β is dephosphorylated and Tyr²¹⁶ of GSK-3 β is phosphorylated by unknown mechanisms induced by DIFs, resulting in the activation of this kinase. Activated-GSK-3 β translocates into nucleus and phosphorylates Thr²⁸⁶ of cyclin D1. DIFs also activated DYRK1B, which is present in the nucleus, by an unknown mechanism and activated DYRK1B phosphorylates Thr²⁸⁸ of cyclin D1. Phosphorylated cyclin D1 is kicked out from the nucleus and degraded by the 26S proteasome system after ubiquitination. Activated-GSK-3 β also phosphorylates β -catenin in the cytoplasm. Phosphorylated- β -catenin is degraded, resulting in the inhibition of transcription of the target genes, such as cyclin D1 and c-myc. DYRK1B, dual-specificity tyrosine phosphorylation-regulated kinase 1B; ALP, alkaline phosphatase; β -TrCP, β -transducin repeat-containing protein; Ub, ubiquitin. (Quoted from ref. 100 with minor change).

molecule(s) and signaling pathway are not clarified (Fig. 2). DIFs could be potent antitumor agents and identification of the target molecule(s) for DIFs may offer ideas for the design of new anticancer drugs.

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