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Forum Minireview

Drug Development Targeting the Glycogen Synthase Kinase-3*β* (GSK-3*β*)-Mediated Signal Transduction Pathway: Inhibitors of the Wnt/*β*-Catenin Signaling Pathway as Novel Anticancer Drugs

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Abstract. Accumulating evidence suggests that the Wnt/*β*-catenin signaling pathway is often involved in oncogenesis and cancer development. Accordingly, a novel anticancer drug can be developed using inhibitors of this pathway. However, at present, there is no selective inhibitor of this pathway available as a therapeutic agent. Although all the components of the Wnt/*β*-catenin signaling pathway can be a target for drug development, glycogen synthase kinase-3*β* (GSK-3*β*), in particular, may be a good target because GSK-3*β* is an essential component of the pathway, and activation of this kinase results in the inhibition of the Wnt signaling pathway. We found that the differentiation-inducing factors (DIFs), putative morphogens for *Dictyostelium discoideum*, inhibit the Wnt/*β*-catenin signaling pathway via the activation of GSK-3*β*, resulting in the cell-cycle arrest of human cancer cell lines. In this review, we summarize our recent findings on the antiproliferative effect of DIFs and show the possibility for development of a novel anticancer drug from DIFs and their derivatives.

Keywords: Wnt/*β*-catenin signaling, cancer, glycogen synthase kinase-3*β* (GSK-3*β*), drug development, differentiation-inducing factor

The Wnt/*β*-catenin signaling pathway and GSK-3*β*

Cell signaling cascades activated by Wnt proteins (i.e., the Wnt signaling pathways) are well conserved through evolutionary processes across a variety of species. As well as regulating cellular processes such as proliferation, differentiation, motility, and survival /apoptosis, the Wnt signaling pathways play key roles in embryonic development and maintenance of homeostasis in mature tissues. Of four known Wnt signaling pathways, [the Wnt/*β*-catenin (canonical) pathway, the planar cell polarity (PCP) pathway, the Wnt/Ca^{2+} pathway, the protein kinase A pathway], the Wnt/*β*-catenin signaling pathway is best characterized (1 – 6).

The activity of the Wnt/*β*-catenin signaling pathway is dependent on the amount of *β*-catenin in the cytoplasm. Normally, the cytoplasmic *β*-catenin level is kept low through continuous ubiquitin-proteasome system–mediated degradation, which is regulated by a multiprotein complex containing axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3*β* (GSK-3*β*).

GSK-3*β* is a cytoplasmic serine/threonine protein kinase that is known to play central roles in a variety of biological processes including a number of signaling pathways such as the Wnt/*β*-catenin, Hedgehog, Notch, and insulin signaling pathways (7). The activity of GSK-3*β* is decreased by the phosphorylation of Ser^9 and several studies have shown that Ser^9 in GSK-3*β* is phosphorylated by Akt, a serine/threonine kinase that is activated by phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1), a protein kinase downstream of the mitogen-activated protein kinase (MAPK) cascade, and p70 ribosomal S6 kinase-1 (7 – 9).

In the Wnt/*β*-catenin signaling pathway, GSK-3*β* mediates the degradation of *β*-catenin molecules by phosphorylating specific amino acid residues, which marks the protein to trigger its degradation by the 26S

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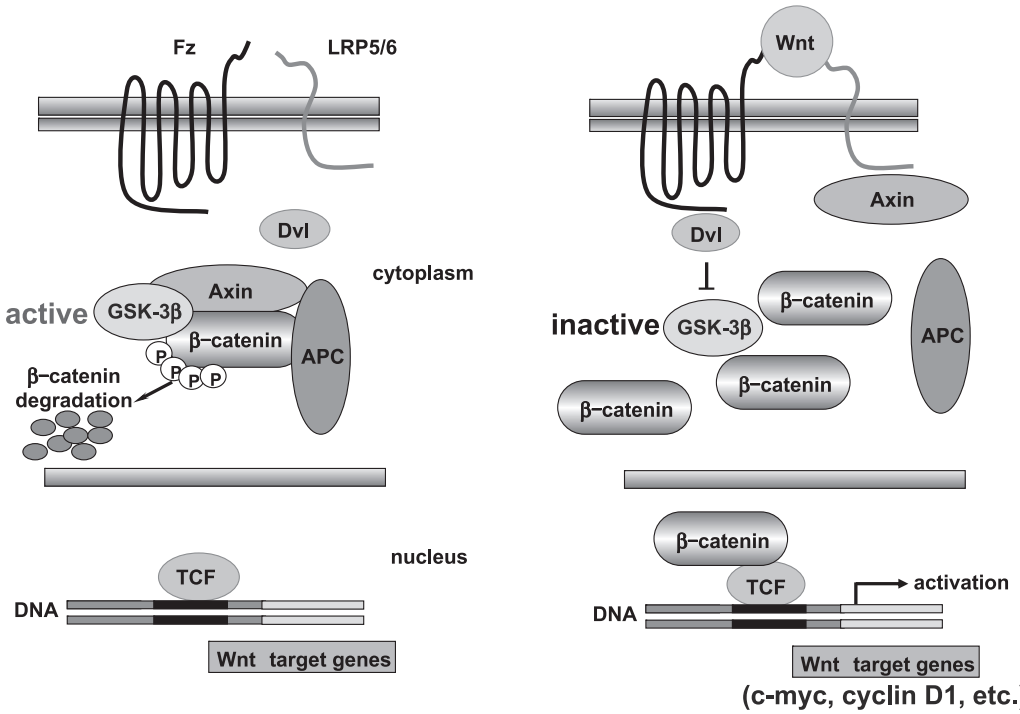

Fig. 1. The Wnt/*β*-catenin signaling pathway. In the absence of Wnt, *β*-catenin binds to the protein complex formed by axin, APC, and GSK-3*β* and then is phosphorylated by GSK-3*β*, resulting in its degradation by the 26S proteasome system (left). Wnt binds to the receptor Fz and the co-receptor LRP5/6, and these receptors mediate signal transduction in cells. GSK-3*β* and CK1*α* are inhibited by activated Dvl; thus *β*-catenin escapes phosphorylation. Unphosphorylated *β*-catenin accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, *β*-catenin activates the transcription of target genes together with TCF (right). Fz, Frizzled; LRP, low-density lipoprotein receptor–related protein; Dvl, disheveled; APC, adenomatous polyposis coli; GSK-3*β*, glycogen synthase kinase-3*β*; TCF, T-cell factor.

proteasome complex (10, 11). After the Wnt proteins bind to the receptor complex Frizzleds/low-density lipoprotein receptor–related protein (Fz/LRP), cytoplasmic disheveled (Dvl), a protein downstream of the receptor complex, is phosphorylated and inhibits GSK-3*β* by causing their retention at the scaffolding protein axin, resulting in the accumulation of non-phosphorylated *β*-catenin in the cytoplasm. Non-phosphorylated *β*-catenin avoids degradation and translocates into the nucleus. In the nucleus, *β*-catenin forms a complex with the transcription factor TCF and induces the transcription of downstream target genes (1 – 3). Thus GSK-3*β* plays a critical role in the regulation of Wnt/*β*-catenin target gene expression by controlling the level of cytoplasmic *β*-catenin (Fig. 1).

Cyclin D1 and GSK-3*β*

Since several oncogenes are included amongst the target genes, constitutive activation of the Wnt/*β*-catenin signaling pathway can lead to cancer (12). One oncogene, the cyclin D1 gene CCND1, is a well-known Wnt/*β*-catenin target gene.

The cell cycle progresses through four sequential phases, namely, gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M) phases. Passage through the cell cycle is strictly controlled by cyclin/cyclin-dependent kinase (CDK) complexes. During the G1 phase, cells need to decide whether to advance towards another division or withdraw from the cell cycle into the quiescence phase (G0) in response to extracellular signals. The point at which this decision is made is called the restriction point. Cyclin D (D1, D2, and D3) act as a mitogenic signal sensor and is expressed as a delayed early response to many mitogenic signals, which forces cells to enter the proliferative cycle from the G0 phase (13, 14). The cyclin D mRNA level is dramatically increased following mitogenic stimulation, and both mRNA and protein levels of cyclin D1 are strictly regulated after induction. Cyclin D forms a complex with and functions as a regulatory subunit of CDK4 or 6, the activity of which is required for the transition from the G1 phase to the S phase (Fig. 2).

In tumor cells, genes encoding the proteins that directly regulate the cell cycle are often quantitatively altered. Among these proteins, cyclin D1 is strongly

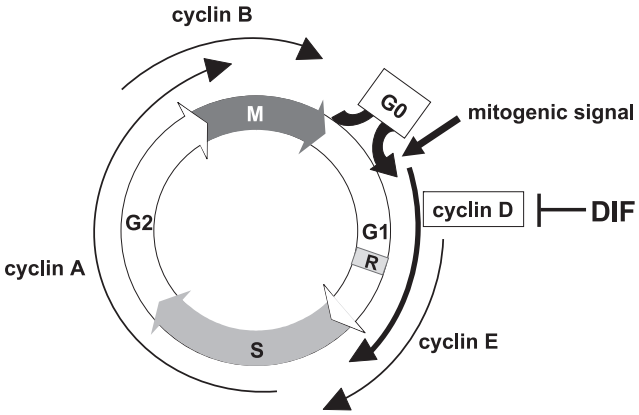

Fig. 2. Schematic representation of the mammalian cell cycle and its regulatory molecules. The cell cycle progresses through four sequential phases, gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M) phases. Passage through the cell cycle is controlled by cyclin /cyclin-dependent kinase complexes and each cyclin exhibits a characteristic pattern of expression and degradation. Among cyclins, cyclin D acts as a mitogenic signal sensor and is expressed as an early response to many mitogenic signals, which forces cells to enter the proliferative cycle from the G0 phase. DIFs inhibit mammalian cell proliferation by suppressing the expression of cyclin D1 mRNA and protein. R, restriction point.

implicated in oncogenesis (14). Amplification of the gene encoding cyclin D1 and overexpression of cyclin D1 protein are often found in several types of human malignant neoplasms (15 – 18). Thus cyclin D1 is particularly well known for its prominent role in driving tumorigenesis. Other members of the cyclin D family, cyclins D2 and D3, are also expressed in an overlapping and redundant fashion with cyclin D1 in all proliferating cell types and are overexpressed in human cancers, but much less commonly than cyclin D1 (19).

The level of the cyclin D1 protein is regulated by an ubiquitin-dependent mechanism throughout the progression of the cell cycle. Cyclin D1 is transported from the nucleus to the cytoplasm where it is degraded by the 26S proteasome. Although GSK-3β is a cytosolic protein, it is translocated into the nucleus when activated and phosphorylates cyclin D1 on Thr^{286}, thereby stimulating cyclin D1 turnover in response to mitogenic signals (7, 20, 21). Phosphorylation of cyclin D1 on Thr^{286} by GSK-3β facilitates its association with CRM1, which is a nuclear protein that mediates the nuclear export of proteins, resulting in the exclusion of cyclin D1 from the nucleus to initiate its proteasomal degradation (22).

As described above, cyclin D1 gene expression is activated by Wnt/β-catenin signaling, in which GSK-3β plays a critical role in its regulation, and cyclin D1 protein degradation is regulated by GSK-3β. Thus activation of GSK-3β is expected to lead to a reduction in the level of cyclin D1 mRNA at the transcriptional level and the protein at the degradation level. While many diseases, including diabetes mellitus and Alzheimer's disease, can be ameliorated by the use of GSK-3β inhibitors, cancers, especially cancers in which cyclin D1 is overexpressed, are likely to be more susceptible to pharmacological activation of GSK-3β.

Differentiation-inducing factors: modulators of the Wnt/β-catenin signaling pathway and potent anti-tumor agents

Differentiation-inducing factors (DIFs) were identified in *Dictyostelium discoideum* as the morphogens required for stalk cell differentiation (23). In the DIF family, DIF-1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone] was the first to be identified and DIF-3, the monochlorinated analog of DIF-1, is a natural metabolite of DIF-1 in *Dictyostelium* (24). However, the actions of DIFs are not limited to *Dictyostelium*. They also have strong effects on mammalian cells. DIF-1 and/or DIF-3 strongly inhibit proliferation and induce differentiation in several leukemia cells, including the murine erythroleukemia cell line B8, human leukemia cell line K562, and human myeloid leukemia cell line HL-60 (25, 26). DIF-3 has been reported to have the most potent antiproliferative effect on mammalian leukemia cells among the DIF analog examined to date (27).

However, the target molecule (receptor) of DIFs is unknown and it is not clear even in *Dictyostelium* how DIFs induce antiproliferative effects and cell differentiation. DIFs are small hydrophobic molecules and are therefore expected to be able to cross cell membranes without requiring channels or carriers. 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. Although the precise mechanisms underlying their antiproliferative effects are not yet known, we found that DIFs (DIF-1 and DIF-3) inhibited mammalian cell proliferation by suppressing the expression of cyclin D1 mRNA and protein through the activation of GSK-3β (28 – 31).

DIFs dephosphorylated Ser^{9} of GSK-3β by an unknown mechanism and thus activated this kinase. Activated GSK-3β by DIFs induced β-catenin degradation and suppressed β-catenin/TCF–dependent transcription activity, indicating that DIFs inhibit the Wnt/β-catenin signaling pathway. We also found that DIFs reduced the activity of a reporter gene driven by the human cyclin D1 promoter (+134/–961 bp) via a TCF binding site (–75/–81 bp) (29). This result suggests that DIFs inhibited cyclin D1 mRNA expression via the inhibition of β-catenin/TCF–dependent transcription

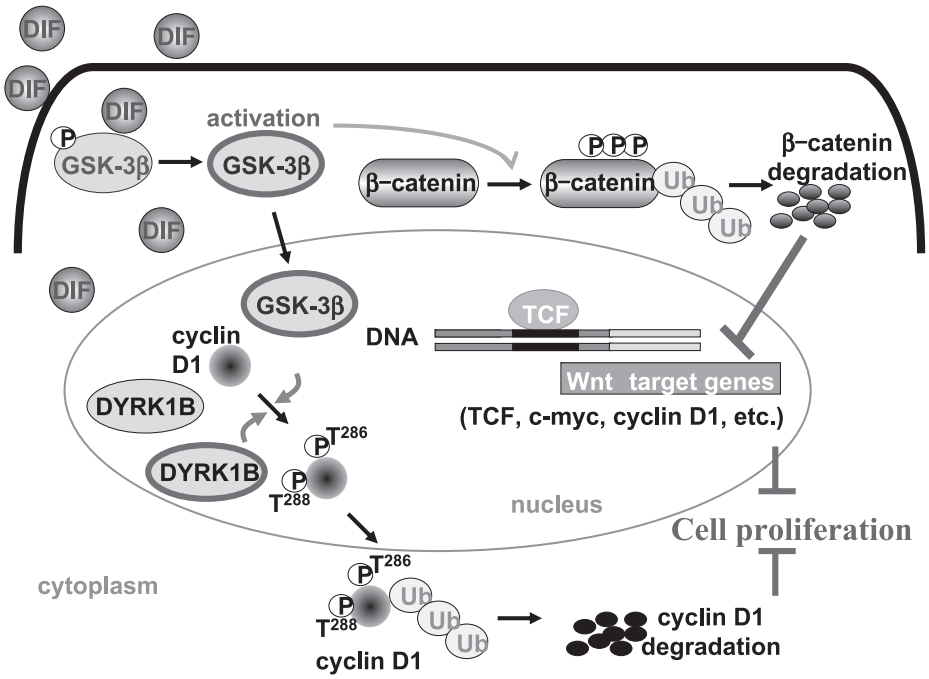

Fig. 3. DIFs action and the Wnt/*β*-catenin signaling pathway. DIFs enter into the cell and dephosphorylate GSK-3*β* at Ser^{9} by unknown mechanisms, resulting in the activation of this kinase. Activated-GSK-3*β* translocates into nucleus and phosphorylates Thr^{286} of cyclin D1. DIFs also activated DYRK1B, which is present in nucleus, by an unknown mechanism, and activated DYRK1B phosphorylates Thr^{288} of cyclin D1. Phosphorylated cyclin D1 is exported from the nucleus, resulting in its degradation 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. GSK-3*β*, glycogen synthase kinase-3*β*; DYRK1B, dual-specificity tyrosine phosphorylation-regulated kinase 1B; Ub, ubiquitin.

activity. On the other hand, we also found that the activated GSK-3*β* translocated to the nucleus and phosphorylated cyclin D1 on Thr^{286} to trigger the degradation of cyclin D1 by an ubiquitin-dependent mechanism (28, 30, 31). Correlated with the above observations, DIFs induced G0/G1 cell cycle arrest, which was rescued by the overexpression of cyclin D1 (28), suggesting that DIFs were likely to induce cell cycle arrest by reducing the expression of cyclin D1.

Cyclin D1 degradation is facilitated by the phosphorylation of specific threonine residues, not only 286 but also 288, according to previous reports (20, 21, 32). Zou et al. (32) reported that dual-specificity tyrosine-phosphorylation–regulated kinase 1B (DYRK1B), a member of the DYRK family, phosphorylates cyclin D1 on Thr^{288}, also resulting in cyclin D1 degradation. Therefore, the effect of DIF-3 on DYRK1B was examined and it was found that not only GSK-3*β* but also DYRK1B was involved in the phosphorylation of cyclin D1 to trigger its degradation (31). This may have an important implication in DIFs-induced cyclin D1 degradation because DIFs induce rapid and strong degradation of cyclin D1 (within 1 h). Clarified DIFs action is summarized in Fig. 3.

The antiproliferative effect of DIFs via strong reduction of the expression level of cyclin D1 is not limited to HeLa cells, but is also common to human squamous cell carcinoma cell lines (SAS and NA) (30), human colorectal carcinoma cell line (HCT-116), and human osteosarcoma cell line (SaOS-2) (author's unpublished observation). As described above, DIFs inhibit the Wnt/*β*-catenin signaling pathway via the activation of GSK-3*β*, whereas the target molecule is not clarified. Recently, Shimizu et al. reported that calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1) could be a pharmacological target molecule for DIF-1 (33). Although this protein might not be the molecule responsible for regulation of the antiproliferative effect of DIF-1 (4), some inhibitors for PDE1 are expected to be applicable to cancer (34, 35). Taken together, it seems likely that DIFs are potent antitumor agents, and identification of the target molecule(s) for DIFs may offer ideas for the design of new anticancer drugs.

Conclusions

Cyclin D1 is a positive regulator of the cell cycle and promotes transition from the G1phase to the S phase in cooperation with CDK4 or 6. Amplification of the gene encoding cyclin D1 and overexpression of the cyclin D1 protein are frequently found in several types of human malignant neoplasms. GSK-3*β* plays a critical role in the regulation of the amount of cyclin D1, as this kinase is involved in both cyclin D1 mRNA transcription and ubiquitin-dependent proteolysis. We found that DIFs act as an inhibitor of the Wnt/*β*-catenin signaling pathway via the activation of GSK-3*β*, whereas the target molecule is not clarified. Therefore, 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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