Protein-kinase-C-mediated β-catenin phosphorylation negatively regulates the Wnt/β-catenin pathway

Jungsug Gwak[1], Munju Cho[1], Soo-Jung Gong[2], Jaejoon Won[3], Dong-Eun Kim[2], Eun-Young Kim[4], Sang Sup Lee[5], Mina Kim[6], Tae Kook Kim[3], Jae-Gook Shin[4,5,*] and Sangtaek Oh[1,*]

[1]PharmcoGenomics Research Center, Inje University, Busan 614-735, Korea
[2]Department of Biotechnology and Bioengineering, Dong-Eui University, Busan 614-714, Korea
[3]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea
[4]Department of Clinical Pharmacology, Busan Paik Hospital, Inje University College of Medicine, Busan 614-735, Korea
[5]Department of Pharmacology, Inje University College of Medicine, Busan 614-735, Korea
[6]CGK Co. Ltd, Daejeon 305-701, Korea
*Authors for correspondence (e-mail: phshinjg@inje.ac.kr; ohsa@inje.ac.kr)

Accepted 4 September 2006
Journal of Cell Science 119, 4702-4709 Published by The Company of Biologists 2006
doi:10.1242/jcs.03256

Summary

Normally, the Wnt/β-catenin pathway controls developmental processes and homeostasis, but abnormal activation of this pathway is a frequent event during the development of cancer. The key mechanism in regulation of the Wnt/β-catenin pathway is the amino-terminal phosphorylation of β-catenin, marking it for proteasomal degradation. Here we present small-molecule-based identification of protein kinase C (PKC)-mediated β-catenin phosphorylation as a novel mechanism regulating the Wnt/β-catenin pathway. We used a cell-based chemical screen to identify A23187, which inhibits the Wnt/β-catenin pathway. PKC was activated by A23187 treatment and subsequently phosphorylated N-terminal serine (Ser) residues of β-catenin, which promoted β-catenin degradation. Moreover, the depletion of PKCα inhibited the phosphorylation and degradation of β-catenin. Therefore, our findings suggest that the PKC pathway negatively regulates the β-catenin level outside of the Wnt/β-catenin pathway.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/22/4702/DC1

Key words: Wnt/β-catenin pathway, Protein kinase C, Phosphorylation, Degradation

Introduction

Wnts are secreted glycoproteins that play important roles in cell proliferation, differentiation and oncogenesis (Korinek et al., 1997; Morin et al., 1997; Wodarz and Nusse, 1998; Polakis, 2000). The Wnt/β-catenin pathway is activated by Wnts (Wnt1, 3a and 8) interacting with Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein5/6 (LRP5/6) co-receptors (Giles et al., 2003). The signal is then transduced through the disheveled protein to negatively regulate glycogen synthase kinase-3β (GSK-3β), resulting in the accumulation of cytoplasmic β-catenin (Lee et al., 2003; Dominguez and Green, 2000). This β-catenin is translocated into the nucleus, where it forms a complex with the T cell factor/lymphocyte enhancer factor (TCF/LEF) families of transcription factors to activate the expression of Wnt/β-catenin responsive genes, such as c-*myc*, *cyclin D1* and *peroxisome proliferator-activated receptor-δ* (He et al., 1998; Tetsu and McCormick, 1999; He et al., 1999). In the absence of a Wnt signal, β-catenin forms a complex with adenomatous polyposis coli (APC)/Axin and is phosphorylated by a dual-kinase mechanism, which is catalyzed by casein kinase 1 (CK1) and GSK-3β, at Ser/Thr residues (Ser33, Ser37, Thr41 and Ser45) (Liu et al., 2002; Amit et al., 2002). These phospho-Ser/Thr residues are recognized by F-box β-transducin repeat-containing protein (β-TrCP), an F-box protein in the ubiquitin ligase complex, leading to the degradation of β-catenin (Latres et al., 1999). Thus, β-catenin phosphorylation is the key regulatory mechanism in the Wnt/β-catenin pathway. In this study, we used a small molecule-based approach to explore the mechanisms regulating the Wnt/β-catenin pathway. Our strategy was to use a cell-based chemical screen to identify a small molecule capable of modulating the Wnt/β-catenin pathway and to find the mode of action of this small molecule to determine the mechanism that regulates the Wnt/β-catenin pathway.

Results

Identification of a small-molecule inhibitor of the Wnt/β-catenin pathway

To screen for small molecules that modulate the Wnt/β-catenin pathway, we stably transfected HEK293 cells with the TOPFlash reporter plasmid and the human Frizzled-1 expression plasmid to create HEK293 reporter cells (Gwak et al., 2006; Park et al., 2006). When HEK293 reporter cells were incubated with Wnt3a-conditioned medium (Wnt3a-CM), TOPFlash reporter activity increased dramatically (data not shown). Using this system, we screened a library of small molecules that contained 960 bioactive compounds (Fig. 1A). One of the compounds identified was A23187, a calcium ionophore. As shown in Fig. 1B and Fig. S1 in supplementary material, treatment of HEK293 reporter cells with A23187 inhibited induction of β-catenin response transcription (CRT) by Wnt3a-CM, and the inhibition was dose- and time-dependent (IC_{50}=1.43±0.64 μM). By contrast, A23187 and Wnt3a-CM did not affect FOPFlash activity in HEK293

control cells (Park et al., 2006) (Fig. 1B). Under these conditions, A23187 exhibited no cytotoxic effects on the HEK293 reporter cells (Fig. S2 in supplementary material). These results indicate that A23187 specifically inhibits CRT stimulated by Wnt3a.

In the Wnt/β-catenin pathway, CRT is largely dependent on the level of cytoplasmic β-catenin, which stimulates target genes. Therefore, we investigated the effect of A23187 on cytoplasmic β-catenin levels by Western blotting. Consistent with a previous report (Shibamoto et al., 1998), Western blotting with anti-β-catenin antibody revealed that incubation with Wnt3a-CM increased the amount of β-catenin (Fig. 1C; Fig. S1 in supplementary material). Treatment with A23187 downregulated the β-catenin level (Fig. 1C; Fig. S1 in supplementary material), which is consistent with its effect on CRT.

Since the level of cytoplasmic β-catenin is regulated by the ubiquitin-dependent proteasome pathway (Latres et al., 1999), we next explored whether A23187-induced downregulation of β-catenin is mediated by proteasomes. When we used MG-132 to block proteasome-mediated protein degradation in HEK293 reporter cells, it abrogated the ability of A23187 to effect a consistent decrease in β-catenin (Fig. 1D), indicating that A23187 promotes the degradation of β-catenin in a proteasome-dependent manner. Taken together, these results indicate that A23187 inhibits the Wnt/β-catenin pathway by a mechanism involving degradation of intracellular β-catenin.

A23187-mediated β-catenin degradation requires the β-catenin N-terminus but not GSK-3β activity

Since the phosphorylation of β-catenin by GSK-3β and its subsequent association with β-TrCP leads to β-catenin degradation (Liu et al., 1999; Winston et al., 1999), we examined whether A23187-mediated inhibition of CRT requires GSK-3β activity. To this end, HEK293 reporter cells were incubated with A23187 and LiCl, an inhibitor of GSK-3β (Klein and Melton, 1996). As shown in Fig. 2A, A23187 suppressed LiCl-induced CRT. Furthermore, Western blot analysis using anti-β-catenin antibody consistently showed that A23187 reduced the level of β-catenin that accumulated with LiCl treatment (Fig. 2B), indicating that A23187-mediated inhibition of the Wnt/β-catenin pathway is independent of GSK-3β.

We next examined whether β-TrCP is necessary for induction of β-catenin degradation by A23187. Western blot analysis showed that a dominant negative mutant β-TrCP (ΔβTrCP), which interacts with phosphorylated β-catenin but is unable to form an $SCF^{\beta\text{-}TrCP}$-ubiquitin ligase complex (Hart et al., 1999), abrogated induction of β-catenin degradation by A23187 (Fig. 2C). Consistent with this result, A23187-mediated CRT suppression was also blocked by ΔβTrCP (Fig. 2D). These results indicate that A23187 induces the degradation of β-catenin through a β-TrCP-dependent mechanism.

We then examined the effect of A23187 treatment on HEK293 cells transfected with wild-type β-catenin or with a mutant β-catenin lacking the N-terminal phosphorylation motifs (Tetsu and McCormick, 1999). We found that wild-type β-catenin was efficiently downregulated in response to A23187 (Fig. 3A), whereas the level of Δβ-catenin was largely unaffected by A23187 treatment (Fig. 3B). In addition, A23187 did not affect the stability of another β-catenin mutant, S37A (Fig. 3C). Taken together, these results indicate that N-terminal residues of β-catenin are required for A23187-mediated β-catenin downregulation.

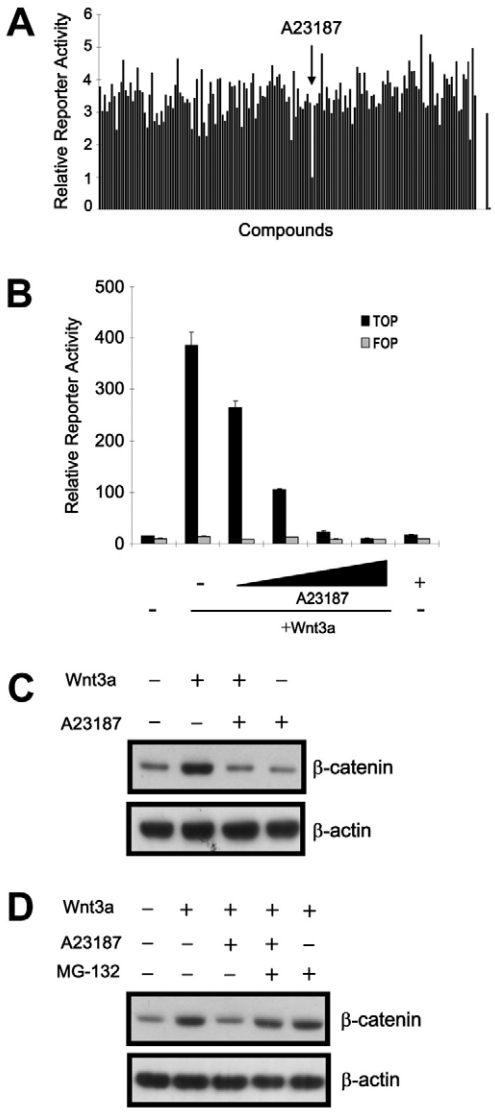

Fig. 1. Identification of A23187 as a small-molecule inhibitor of Wnt/β-catenin signaling. (A) Screening of compounds that inhibit Wnt/β-catenin signaling. (B) Dose-dependent inhibition of CRT induction plotted as increasing concentrations of A23187. HEK293 reporter and control cells were incubated with A23187 (0.625, 1.25, 2.5 and 5 μM) for 15 hours in the presence or absence of Wnt3a-CM, and luciferase activity was determined. The results shown are the average of three experiments; the bars indicate standard deviations. (C) Cytosolic proteins were prepared from HEK293 reporter cells treated with either vehicle (DMSO) or A23187 (2.5 μM) in the presence or absence of Wnt3a-CM and were then subjected to western blotting with anti-β-catenin antibody. (D) Cytosolic proteins prepared from HEK293 reporter cells were incubated with vehicle (DMSO) or A23187 (2.5 μM) in the presence or absence of Wnt3a-CM, exposed to MG-132 (20 μM) for 8 hours, and then subjected to western blotting with anti-β-catenin antibody. In C and D, the blots were reprobed with anti-actin antibody as a loading control.

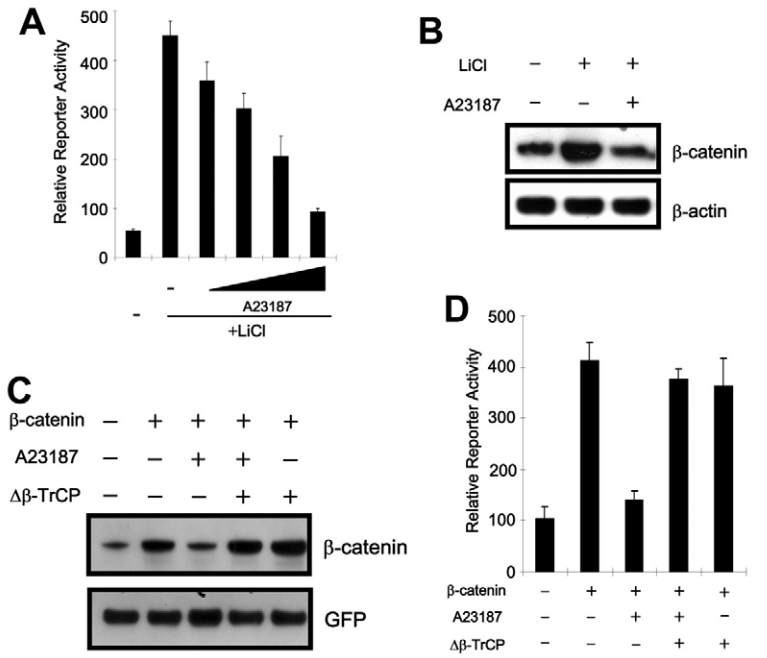

Fig. 2. A23187 promotes β-catenin degradation through GSK-3β-independent and β-TrCP-dependent mechanism. (A) HEK293 reporter cells were incubated with A23187 (0.625, 1.25, 2.5 and 5 μM) in the presence of 20 mM LiCl. After 15 hours, luciferase activity was determined. (B) Cytosolic proteins were prepared from HEK293 reporter cells treated with either vehicle (DMSO) or A23187 (2.5 μM) in the presence of 20 mM LiCl for 15 hours and then subjected to western blotting with β-catenin antibody. The blots were reprobed with anti-actin antibody as a loading control. (C) HEK293 cells were co-transfected with the indicated plasmids and then incubated with either the vehicle (DMSO) or A23187 (2.5 μM) for 15 hours. Cytosolic proteins were subjected to western blotting with β-catenin antibody. The blots were reprobed with anti-GFP antibody as a transfection control. (D) HEK293 reporter cells were co-transfected with the indicated plasmids and then incubated with either the vehicle (DMSO) or A23187 (2.5 μM) for 15 hours and then luciferase activity was measured. In A and D, the results are the average of three experiments, and the bars indicated standard deviation.

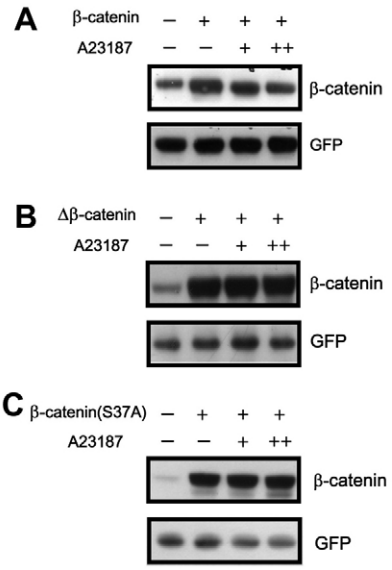

Fig. 3. The N-terminus of β-catenin is required for A23187-mediated β-catenin degradation. HEK293 cells were transfected with wild-type β-catenin (A), Δβ-catenin (B) or β-catenin (S37A) (C) plasmids, incubated with A23187 [1.25 (+) and 2.5 (++) μM] for 15 hours, and then cytosolic proteins were immunoblotted with anti-β-catenin antibody. The blots were reprobed with anti-GFP antibody as a transfection control.

A23187-mediated degradation of β-catenin requires protein kinase C activity

To better understand the mechanism by which A23187 acts to induce β-catenin degradation, we first examined its effect on the intracellular Ca^{2+} concentration. When we measured intracellular Ca^{2+} using the Ca^{2+} indicator fura-2 acetoxymethyl ester (Fura-2/AM), we found that treatment with A23187 dramatically increased the intracellular Ca^{2+} concentration in HEK293 reporter cells in the presence of Wnt3a (Fig. 4A).

An elevation in the intracellular Ca^{2+} concentration has been reported to activate Ca^{2+}-sensitive proteins, including protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and calcineurin (Saneyoshi et al., 2002; Sheldahl et al., 2003). To identify the protein required for inhibition of the Wnt/β-catenin pathway in response to an increase in intracellular Ca^{2+}, we tested the effects of specific pharmacological inhibitors of these proteins on the restoration of A23187-mediated inhibition of the Wnt/β-catenin pathway. Cyclosporin A (CsA) and KN-93, which inhibit CaMKII and calcineurin, respectively, did not rescue the CRT inhibition by A23187 (Fig. 4B), suggesting that CaMKII and calcineurin are not required for A23187-mediated inhibition of the Wnt/β-catenin pathway. By contrast, bisindoylmaleimide (BIM), a specific inhibitor of PKC (Martiny-Baron et al., 1993), abrogated the suppression of CRT by A23187 in a dose-dependent manner (Fig. 4B). Moreover, when incubated with HEK293 reporter cells, it inhibited the degradation of β-catenin induced by A23187 (Fig. 4C).

We also tested the effects of inhibitors of protein kinase A (PKA) and casein kinase1α (CK1α), which have been known to phosphorylate the Ser45 residue of β-catenin (Amit et al., 2002; Kang et al., 2002; Liu et al., 2002), on A23187-mediated CRT inhibition. These inhibitors did not abrogate A23187-mediated CRT inhibition (data not shown). Therefore, PKC, which is induced by an increase in intracellular Ca^{2+}, may be responsible for inhibiting the Wnt/β-catenin pathway.

PKC is essential for phosphorylation and degradation of β-catenin

We next examined whether PKC is activated by treatment with A23187 in HEK293 reporter cells. Since activated PKC translocates from the cytoplasm to the plasma membrane (Newton, 1995), we isolated the membrane fraction from A23187-treated and untreated cells and then measured the amount of PKC protein, using anti-PKCα antibody. Consistent with results reported for other cells (Chakraborti et al., 2004), A23187 treatment led to the translocation of PKCα to the membrane (Fig. 5A). We also observed A23187-induced membrane translocation of PKCα by immunofluorescence

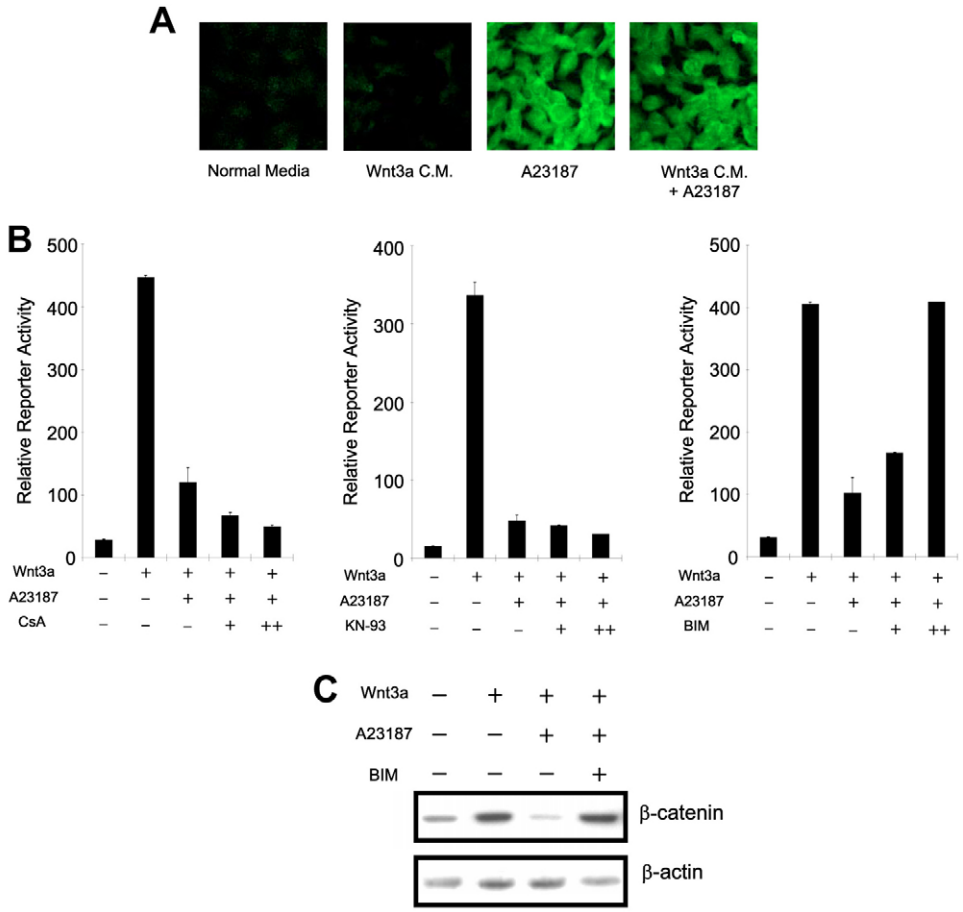

Fig. 4. PKC is required for A23187-mediated degradation of β-catenin. (A) A23187 increases the intracellular Ca^{2+} concentration. HEK293 reporter cells were incubated with vehicle (DMSO) or A23187 (2.5 μM) in the presence or absence of Wnt3a-CM for 15 hours. After fixation, the cells were stained with Fura-2/AM as described in Materials and Methods and observed at 400× magnification. (B) HEK293 reporter cells were incubated with A23187 (2.5 μM) or CsA [2.5 (+), 5 (++) μM], KN-93 [2.5 (+), 5 (++) μM] and BIM [2.5 (+), 5 (++) μM] for 15 hours in the presence or absence of Wnt3a-CM, and the luciferase activity was determined. The results are shown as the average of three experiments; the bars indicate standard deviations. (C) Cytosolic proteins were prepared from HEK293 reporter cells treated with A23187 (2.5 μM) or BIM (5 μM) in the absence or presence of Wnt3a-CM for western blotting with anti-β-catenin antibody. The blots were reprobed with anti-actin antibody as a loading control.

analysis (Fig. 5B). These results suggest that A23187 treatment activates PKCα.

Previous reports have demonstrated that phosphorylation at the N-terminal Ser residues (Ser33/Ser37/Ser45) of β-catenin plays an important role in its ubiquitin-dependent degradation (Liu et al., 2002; Amit et al., 2002). Therefore, to examine whether PKC directly phosphorylates these residues, we performed an in vitro kinase assay using purified β-catenin and PKC. Phosphorylation was analyzed using phospho-specific β-catenin antibodies. As shown in Fig. 6A, Ser33/Ser37/Ser45 phosphorylation was catalyzed by PKC in a dose-dependent manner. Moreover, BIM treatment inhibited this phosphorylation (Fig. 6B).

We also examined whether PKC activity is responsible for phosphorylation of β-catenin Ser residues in HEK293 reporter cells. In agreement with previous reports (Liu et al., 2002; Amit et al., 2002), western blot analysis showed that Wnt3a inhibited phosphorylation of β-catenin residues Ser33/Ser37/Ser45 (Fig. 7A). Moreover, A23187 induced Ser33/Ser37/Ser45 phosphorylation, and this phosphorylation was abrogated by the addition of BIM (Fig. 7A). These results suggest that PKC, which is activated by an increase in intracellular Ca^{2+}, phosphorylates N-terminal Ser residues of β-catenin, thereby promoting β-catenin degradation in vivo.

To confirm that PKC can mediate phosphorylation of Ser33/Ser37/Ser45 of β-catenin, we depleted the endogenous PKCα protein in HEK293 cells using small interference RNA (siRNA). PKCα siRNA significantly reduced the level of PKCα and inhibited Ser33/Ser37/Ser45 phosphorylation (Fig. 7B). Notably, PKCα siRNA also led to significant accumulation of β-catenin protein (Fig. 7B). Consistent with our western blotting results, CRT was markedly increased by transfection with PKCα siRNA (Fig. 7C).

PKC is partially involved in Wnt5a-mediated inhibition of the Wnt/β-catenin pathway

Since Wnt5a may trigger release of intracellular Ca^{2+} and

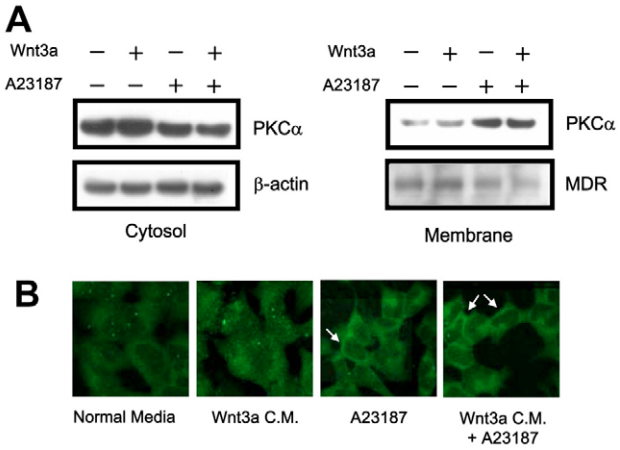

Fig. 5. A23187 induces the translocation of PKCα to the membrane. (A) Cytosolic and membrane fractions were prepared from HEK293 reporter cells treated with vehicle (DMSO) or A23187 (2.5 μM) in the presence or absence of Wnt3a-CM, for western blotting with anti-PKCα antibody. The blots were reprobed with anti-actin or anti-MDR antibodies as fractionation controls. (B) The cellular location of PKCα in HEK293 reporter cells was determined by immunofluorescence analysis. After fixation, the cells were stained with anti-PKCα antibody and observed at 400× magnification. Arrows indicate PKCα translocated to the membrane.

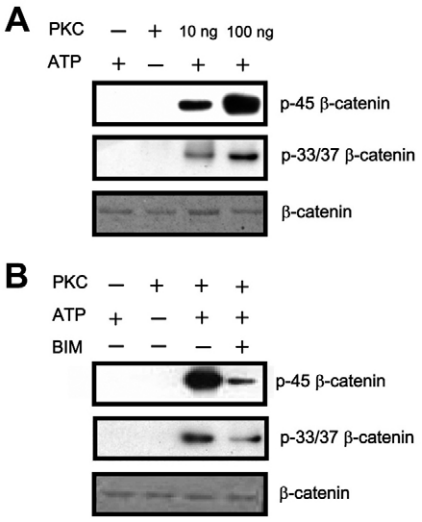

Fig. 6. PKC phosphorylates N-terminal Ser residues of β-catenin in vitro. GST-β-catenin (100 ng) was incubated with the indicated amount of purified PKC (A) or BIM (0.5 μM; B), and then the samples were analyzed by western blotting with anti-phospho-p45-β-catenin and anti-phospho-p33/37-β-catenin antibodies.

activate Ca^{2+}-responsive enzymes (Kuhl et al., 2000b), we examined whether Wnt5a also suppresses Wnt3a-induced CRT via a PKC-dependent mechanism. Consistent with a previous report (Topol et al., 2003), Wnt5a antagonized the Wnt/β-catenin pathway that is stimulated by Wnt3a (Fig. 8A). In addition, inhibition of PKC activity with BIM only partially abolished Wnt5a-mediated CRT inhibition (Fig. 8B). These results suggest that the inhibition of the Wnt/β-catenin pathway by Wnt5a may be mediated by a mechanism other than the PKC-dependent mechanism.

Discussion

The Wnt pathway regulates a variety of developmental processes, including cell proliferation and cell-fate specification (Korinek et al., 1997; Morin et al., 1997; Wodarz and Nusse, 1998). Inappropriate upregulation of this pathway is associated with the development of certain human cancers (Polakis, 2000). Central to the Wnt/β-catenin pathway is the level of cytosolic β-catenin, which is regulated by phosphorylation and subsequent ubiquitin-dependent proteolysis (Liu et al., 1999; Winston et al., 1999). In the present study, we exploited a small-molecule-based approach in demonstrating that PKC is a negative regulator of the Wnt/β-catenin pathway. The small molecule A23187 was found to inhibit the Wnt/β-catenin pathway. A23187 increased the intracellular Ca^{2+} level and subsequently stimulated PKC, which phosphorylated the N-terminal Ser residues of β-catenin, leading to the promotion of β-catenin degradation.

The level of intracellular β-catenin is regulated by multiple pathways. In the GSK-3β-dependent pathway, N-terminal serines of β-catenin are phosphorylated by a multi-protein complex composed of APC, Axin and GSK-3β (Hart et al., 1998; Ikeda et al., 1998). This phosphorylation marks the β-catenin protein for degradation by an ubiquitin-dependent mechanism (Aberle et al., 1997). In the Siah-1-dependent pathway, Siah-1 interacts with the carboxyl terminus of APC, recruits the ubiquitination complex, and promotes the degradation of β-catenin through a pathway independent of both GSK-3β and β-TrCP (Liu et al., 2001; Matsuzawa and Read, 2001). In addition to the two APC-dependent pathways, β-catenin is downregulated by a retinoid X receptor (RXR)-mediated degradation pathway that does not target the N-terminus of β-catenin (Xiao et al., 2003).

Several findings of the present study suggest that a novel degradation pathway, other than the above-described pathways, may mediate induction of β-catenin downregulation by A23187. First, A23187 was able to stimulate β-catenin degradation in the presence of the GSK-3β inhibitors Wnt3a and LiCl (Klein and Melton, 1996; Liu et al., 2002), demonstrating that A23187-mediated β-catenin degradation is GSK-3β-independent. Second, ΔβTrCP (F-box deletion mutant) nullified the effect of A23187 on the level of intracellular β-catenin, suggesting that A23187 promotes β-catenin degradation through a β-TrCP-dependent proteasome pathway. Third, since the N-terminus of β-catenin was indispensable for the downregulatory activity of A23187 (Fig. 3B), A23187 does not exert its effect through the RXR-mediated degradation pathway. Fourth, the β-catenin mutant S37A was not degraded in response to A23187, implying that phosphorylation of β-catenin at Ser37 may be essential for A23187-mediated β-catenin degradation.

Since A23187 is a Ca^{2+} ionophore that increases the intracellular Ca^{2+} level, it may activate one or more Ca^{2+}-dependent proteins, such as CaMKII, calcineurin or PKC (Sheldahl et al., 2003; Saneyoshi et al., 2002). Specific pharmacological inhibitors of CaMKII and calcineurin did not inhibit downregulation of β-catenin levels by A23187,

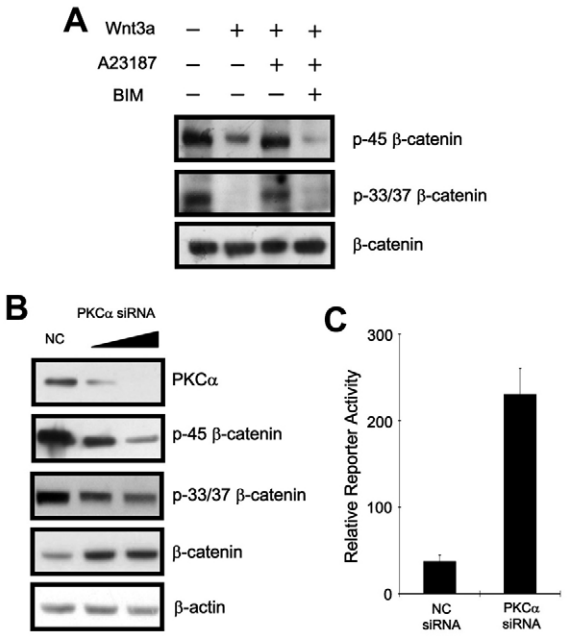

Fig. 7. PKC phosphorylates N-terminal Ser residues of β-catenin in vivo. (A) HEK293 reporter cells were incubated with A23187 (2.5 μM) and BIM (5 μM) for 15 h in the absence or presence of Wnt3a-CM. Cytosolic fractions were prepared and subjected to western blot analysis with anti-phospho-p45-β-catenin, anti-phospho-p33/37-β-catenin or anti-β-catenin antibody. The same amount of β-catenin was loaded in each lane. (B) HEK293 cells were transfected with negative control (NC) siRNA (40 μmol) and PKCα siRNA (20 and 40 μmol) for 48 hours and then cell lysates were subjected to western blot analysis with anti-PKCα, anti-β-catenin, anti-p45-β-catenin, anti-p33/37-β-catenin and anti-actin antibodies. (C) HEK293 reporter cells were transfected with negative control siRNA (40 μmol) and PKCα siRNA (40 μmol) for 48 hours and then luciferase activity was measured. The results are the average of three experiments, and the bars indicated standard deviation.

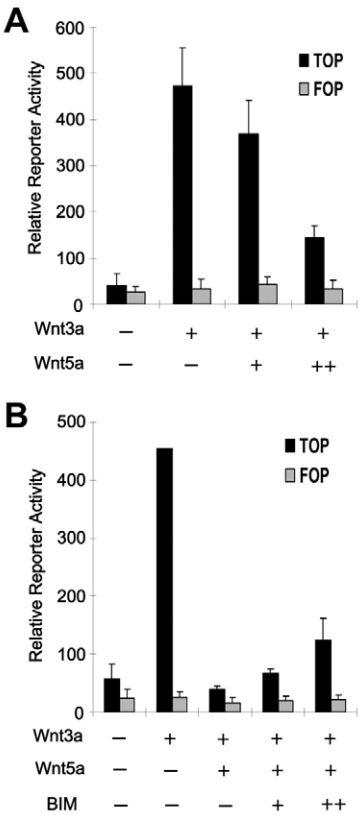

Fig. 8. Inhibition of PKC partially abolished the Wnt5a-mediated CRT inhibition. (A) HEK293 reporter and control cells were co-transfected with Wnt3a (50 ng) and Wnt5a [0.2 μg (+) and 1 μg (++)] plasmids for 48 hours, and then luciferase activity was determined. (B) HEK293 reporter and control cells were co-transfected with Wnt3a (50 ng) and Wnt5a (1 μg) plasmids, incubated with BIM [2.5 μM (+) and 5 μM (++)] for 15 hours, and then luciferase activity was measured. In A and B, the results shown are the average of three experiments; the bars indicated standard deviations.

revealing that activation of NF-AT or CaMKII is not required for the antagonistic activity of A32187 toward the Wnt/β-catenin pathway. By contrast, the specific PKC inhibitor BIM rescued induction of β-catenin degradation by A23187. Moreover, Western blot and immunofluorescence analysis showed that endogenous PKCα is activated by A23187 treatment. These results suggest that PKC may be involved in A23187-induced β-catenin degradation.

Previous studies have suggested a potential role for PKC in the Wnt/β-catenin pathway. Cook and colleagues reported that PKC inhibitors abrogate Wnt-mediated GSK-3β inhibition in mouse 10T1/2 fibroblasts (Cook et al., 1996), but they did not examine the effect of PKC inhibitors on the intracellular β-catenin level. Others have demonstrated that treatment with the PKC inhibitors cause β-catenin accumulation in human breast cell lines in the absence of Wnt activation (Orford et al., 1997). In the present study, an in vitro kinase assay showed that PKC directly phosphorylates residues Ser33/Ser37/Ser45 of β-catenin in the presence of Ca^{2+} and lipid activator. Most importantly, depletion of endogenous PKCα with siRNA prevented Ser33/Ser37/Ser45 phosphorylation and degradation, implying that PKCα suppresses the Wnt/β-catenin pathway in response to a change in the intracellular Ca^{2+} concentration through the phosphorylation and subsequent degradation of β-catenin.

Several studies have suggested a dual kinase mechanism in which phosphorylation of β-catenin at Ser45 by CK1 is essential for subsequent GSK-3β-mediated phosphorylation of the N-terminal Ser residues in a complex with APC and Axin (Amit et al., 2002; Liu et al., 2002). Parallel to GSK-3β-mediated β-catenin phosphorylation, PKC could mediate phosphorylation of β-catenin at both Ser33/37 and Ser 45 without requiring a priming kinase such as CK1α or PKA.

Wnt5a may also activate PKC by triggering the release of intracellular Ca^{2+} (Kuhl et al., 2000a). Moreover, Wnt/β-catenin is antagonized by the Wnt/Ca^{2+} pathways (Ishitani et al., 2003; Kremenevskaja et al., 2005; Kuhl et al., 2000b). Thus, Wnt5a might plausibly attenuate the Wnt/β-catenin pathway by activating PKC. However, we found that BIM could not completely rescue the Wnt5a-mediated suppression of the Wnt/β-catenin pathway, indicating that this suppression may be mediated by a mechanism distinct from the PKC-

dependent mechanism. This result can be explained by the earlier observation that Wnt5a promotes β-catenin degradation via a mechanism that is dependent on Siah/APC but not on Ca^{2+}-responsive enzymes (Topol et al., 2003).

In conclusion, we have evaluated a possible mechanism for Ca^{2+}-mediated inhibition of the Wnt/β-catenin pathway in mammals using cell-based small molecule screening. Since aberrant upregulation of β-catenin levels and subsequent activation of CRT is associated with the development of certain cancers, our findings may facilitate the development of new strategies to prevent tumorigenesis.

Materials and Methods

Cell culture, transfection, siRNA and luciferase assay

HEK293 and Wnt3a-secreting L cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 120 μg/ml penicillin, and 200 μg/ml streptomycin. For Wnt3a-conditioned medium (Wnt3a-CM), Wnt3a-secreting L cells were cultured in DMEM with 10% FBS for 4 days. The medium was harvested and sterilized using a 0.22-mm filter. Fresh medium was added, and the cells were cultured for another 3 days. The medium was collected and combined with the previous medium. Transfection was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. PKCα siRNA was designed as previously described (Chen et al., 2004) and synthesized by Invitrogen. Negative control siRNA (Silencer™) was purchased from Ambion. Luciferase assays were performed using the Dual luciferase assay kit (Promega).

Plasmid and recombinant proteins

Human Frizzled cDNA was cloned as described previously (Cho et al., 2005). The pTOPFlash and pFOPFlash reporter plasmids were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The dominant negative β-TrCP expression plasmid was kindly provided by Dr M. Davis (Hebrew University-Hadassah Medical School, Israel), and pGEX-4T-1-β-catenin plasmid was a gift from Dr W. Song (GIST, Korea). The recombinant protein was purified as previously described (Park et al., 2004).

Screening for small-molecule inhibitors of Wnt/β-catenin signaling

The HEK293 reporter cell line was established as previously described (Gwak et al., 2006; Park et al., 2006). The cells were inoculated into 96-well plates at 15,000 cells per well in duplicate and grown for 24 h. Next, Wnt3a-CM was added, and then the chemicals (Genesis Plus Collection, MicroSource Discovery Inc., Gaylordsville, CT) were added to the wells at a final concentration of 10 μM. After 15 hours, the plates were assayed for firefly luciferase activity and cell viability.

Western blot

The cytosolic fraction was prepared as previously described (Dignam et al., 1983). Proteins were separated using a 4-12% gradient of SDS-PAGE (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% nonfat milk and probed with anti-β-catenin (BD Transduction Laboratories), anti-phospho-β-catenin (Ser33/37; Sigma), anti-phospho-β-catenin (Ser45; Sigma), anti-PKCα (BD Transduction Laboratories), anti-GFP (Clonetech), and anti-actin antibodies (Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology) and visualized using the ECL system (Santa Cruz Biotechnology).

Preparing the membrane fraction

HEK293 reporter cells grown in 100-mm culture dishes were washed with ice-cold PBS. The cells were then scraped from the dish; suspended in 1 ml of ice-cold extraction buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, and 0.5 mM EGTA; homogenized using a syringe; and incubated on ice for 30 minutes. The homogenate was centrifuged at 13,400 ***g*** for 2 minutes at 4°C. The supernatant was centrifuged at 100,000 ***g*** for 30 minutes at 4°C in a 100Ti rotor (Beckman, USA). The pellet was suspended in 0.3% (w/v) Triton X-100. The membrane suspension was aliquoted and stored at –80°C.

In vitro kinase assay

Kinase assays were performed with a PKC assay kit (Upstate Cell Signaling Solutions) according to the manufacturer's instructions with minor modifications. Briefly, purified GST-β-catenin (100 ng) was incubated with PKC (Promega) at 30°C for 30 minutes in kinase assay buffer (20 mM Mops, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, PKA/CaMK inhibitor cocktail, PKC lipid activator, magnesium/ATP cocktail). The proteins were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The transferred proteins were analyzed using western blotting with anti-phospho-β-catenin antibody, and the membrane was exposed to X-ray film.

Measuring Ca^{2+} concentration

Ca^{2+} levels in the cells were determined using the fluorescent probe Fura-2 AM (Molecular Probes). HEK293 reporter cells were incubated in Wnt3a-CM medium containing 2.5 μM A23187. The cells were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature, washed three times with PBS, and then treated with 5 mM Fura-2 AM for 45 minutes at room temperature in the dark. The slides were washed three times with PBS and examined using confocal microscopy (Zeiss LSM510 Meta).

Immunofluorescence analysis

HEK293 reporter cells were cultured on glass chamber slides and then treated with DMSO or A23187 for 15 hour. After treatment, the cells were washed with PBS, fixed with 4% formaldehyde, permeabilized in 0.3% Triton X-100, and blocked in 4% bovine serum albumin for 1 hour. The cells were stained with anti-PKCα antibody and then analyzed by confocal microscopy using a Zeiss LSM510 Meta microscope.

We thank W. Song, E. Jho, and B. W. Vogelstein for providing plasmids. We also thank N. C. Ha for comments on the manuscript. This work was supported by a grant from Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A050307) (03-PJ10-PG13-GD01-0002).

References

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). β-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**, 3797-3804.

Amit, S., Hatzubai, A., Birman, Y., Andersen, J. S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y. and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of β-catenin at Ser 45, a molecular switch for the Wnt pathway. *Genes Dev.* **16**, 1066-1076.

Chakraborti, S., Michael, J. R. and Chakraborti, T. (2004). Role of an aprotinin-sensitive protease in protein kinase C α-mediated activation of cytosolic phospholipase A2 by calcium ionophore (A23187) in pulmonary endothelium. *Cell. Signal.* **16**, 751-762.

Chen, Y. W., Lang, M. L. and Wade, W. F. (2004). Protein kinase C-α and -δ are required for FcαR (CD89) trafficking to MHC class II compartments and FcαR-mediated antigen presentation. *Traffic* **5**, 577-594.

Cho, M., Gwak, J., Park, S., Won, J., Kim, D. E., Yea, S. S., Cha, I. J., Kim, T. K., Shin, J. G. and Oh, S. (2005). Diclofenac attenuates Wnt/β-catenin signaling in colon cancer cells by activation of NF-κB. *FEBS Lett.* **579**, 4213-4218.

Cook, D., Fry, M. J., Hughes, K., Sumathipala, R., Woodgett, J. R. and Dale, T. C. (1996). Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J.* **15**, 4526-4536.

Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475-1489.

Dominguez, I. and Green, J. B. (2000). Dorsal downregulation of GSK3β by a non-Wnt-like mechanism is an early molecular consequence of cortical rotation in early *Xenopus* embryos. *Development* **127**, 861-868.

Giles, R. H., van Es, J. H. and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim. Biophys. Acta* **1653**, 1-24.

Gwak, J., Park, S., Cho, M., Song, T., Cha, S. H., Kim, D. E., Jeon, Y. J., Shin, J. G. and Oh, S. (2006). *Polysiphonia japonica* extract suppresses the Wnt/β-catenin pathway in colon cancer cells by activation of NF-κB. *Int. J. Mol. Med.* **17**, 1005-1010.

Hart, M. J., del los Santos, R., Albert, I. N., Rubinfeld, B. and Polakis, P. (1998). Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3 β. *Curr. Biol.* **8**, 573-581.

Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R. et al. (1999). The F-box protein β-TrCP associates with phosphorylated β-catenin and regulates its activity in the cell. *Curr. Biol.* **9**, 207-210.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-1512.

He, T. C., Chan, T. A., Vogelstein, B. and Kinzler, K. W. (1999). PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* **99**, 335-345.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S. and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. *EMBO J.* **17**, 1371-1384.

Ishitani, T., Kishida, S., Hyodo-Miura, J., Ueno, N., Yasuda, J., Waterman, M., Shibuya, H., Moon, R. T., Ninomiya-Tsuji, J. and Matsumoto, K. (2003). The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca^{2+} pathway to antagonize Wnt/β-catenin signaling. *Mol. Cell. Biol.* **23**, 131-139.

Kang, D. E., Soriano, S., Xia, X., Eberhart, C. G., De Strooper, B., Zheng, H. and Koo, E. H. (2002). Presenilin couples the paired phosphorylation of β-catenin independent of axin: implications for β-catenin activation in tumorigenesis. *Cell* **110**, 751-762.

Klein, P. S. and Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93**, 8455-8459.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a β-catenin-Tcf complex in APC–/– colon carcinoma. *Science* **275**, 1784-1787.

Kremenevskaja, N., von Wasielewski, R., Rao, A. S., Schofl, C., Andersson, T. and Brabant, G. (2005). Wnt-5a has tumor suppressor activity in thyroid carcinoma. *Oncogene* **24**, 2144-2154.

Kuhl, M., Sheldahl, L. C., Malbon, C. C. and Moon, R. T. (2000a). Ca^{2+}/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. *J. Biol. Chem.* **275**, 12701-12711.

Kuhl, M., Sheldahl, L. C., Park, M., Miller, J. R. and Moon, R. T. (2000b). The Wnt/Ca^{2+} pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet.* **16**, 279-283.

Latres, E., Chiaur, D. S. and Pagano, M. (1999). The human F box protein β-Trcp associates with the Cul1/Skp1 complex and regulates the stability of β-catenin. *Oncogene* **18**, 849-854.

Lee, E., Salic, A., Kruger, R., Heinrich, R. and Kirschner, M. W. (2003). The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. *PLoS Biol*. **1**, 116-132.

Liu, C., Kato, Y., Zhang, Z., Do, V. M., Yankner, B. A. and He, X. (1999). β-Trcp couples β-catenin phosphorylation-degradation and regulates Xenopus axis formation. *Proc. Natl. Acad. Sci. USA* **96**, 6273-6278.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X. and He, X. (2002). Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108**, 837-847.

Liu, J., Stevens, J., Rote, C. A., Yost, H. J., Hu, Y., Neufeld, K. L., White, R. L. and Matsunami, N. (2001). Siah-1 mediates a novel β-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Mol. Cell* **7**, 927-936.

Martiny-Baron, G., Kazanietz, M. G., Mischack, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993). Selective inhibition of protein kinase C isozyme by the indolocarbazole Go 6976. *J. Biol. Chem.* **268**, 9194-9197.

Matsuzawa, S. I. and Reed, J. C. (2001). Siah-1, SIP, and Ebi collaborate in a novel pathway for β-catenin degradation linked to p53 responses. *Mol. Cell* **7**, 915-926.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* **275**, 1787-1790.

Newton, A. C. (1995). Protein kinase C: structure, function, and regulation. *J. Biol. Chem.* **270**, 28495-28498.

Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M. and Byers, S. W. (1997). Serine phosphorylation-regulated ubiquitination and degradation of β-catenin. *J. Biol. Chem.* **272**, 24735-24738.

Park, C. S., Kim, S. I., Lee, M. S., Youn, C. Y., Kim, D. J., Jho, E. H. and Song, W. K. (2004). Modulation of β-catenin phosphorylation/degradation by cyclin-dependent kinase 2. *J. Biol. Chem.* **279**, 19592-19599.

Park, S., Gawk, J., Cho, M., Song, T., Won, J., Kim, D.-E., Shin, J.-G. and Oh, S. (2006). Hexachlorophene inhibits Wnt/β-catenin pathway by promoting Siah-Mediated β-catenin degradation. *Mol. Pharmacol.* **70**, 960-966.

Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev.* **14**, 1837-1851.

Saneyoshi, T., Kume, S., Amasaki, Y. and Mikoshiba, K. (2002). The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. *Nature* **417**, 295-299.

Sheldahl, L. C., Slusarski, D. C., Pandur, P., Miller, J. R., Kuhl, M. and Moon, R. T. (2003). Dishevelled activates Ca^{2+} flux, PKC, and CamKII in vertebrate embryos. *J. Cell Biol.* **161**, 769-777.

Shibamoto, S., Higano, K., Takada, R., Ito, F., Takeichi, M. and Takada, S. (1998). Cytoskeletal reorganization by soluble Wnt-3a protein signalling. *Genes Cells* **3**, 659-670.

Tetsu, O. and McCormick, F. (1999). β-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-426.

Topol, L., Jiang, X., Choi, H., Garrett-Beal, L., Carolan, P. J. and Yang, Y. (2003). Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent β-catenin degradation. *J. Cell Biol.* **162**, 899-908.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J. and Harper, J. W. (1999). The $SCF^{\beta\text{-}TRCP}$-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination in vitro. *Genes Dev.* **13**, 270-283.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Xiao, J. H., Ghosn, C., Hinchman, C., Forbes, C., Wang, J., Snider, N., Cordrey, A., Zhao, Y. and Chandraratna, R. A. (2003). Adenomatous polyposis coli (APC)-independent regulation of β-catenin degradation via a retinoid X receptor-mediated pathway. *J. Biol. Chem.* **278**, 29954-29962.

Journal of Cell Science