Pin1-mediated Modification Prolongs the Nuclear Retention of β-Catenin in Wnt3a-induced Osteoblast Differentiation*§

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The canonical Wnt signaling pathway, in which β-catenin nuclear localization is a crucial step, plays an important role in osteoblast differentiation. Pin1, a prolyl isomerase, is also known as a key enzyme in osteogenesis. However, the role of Pin1 in canonical Wnt signal-induced osteoblast differentiation is poorly understood. We found that Pin1 deficiency caused osteopenia and reduction of β-catenin in bone lining cells. Similarly, Pin1 knockdown or treatment with Pin1 inhibitors strongly decreased the nuclear β-catenin level, TOP flash activity, and expression of bone marker genes induced by canonical Wnt activation and vice versa in Pin1 overexpression. Pin1 interacts directly with and isomerizes β-catenin in the nucleus. The isomerized β-catenin could not bind to nuclear adenomatous polyposis coli, which drives β-catenin out of the nucleus for proteasomal degradation, which consequently increases the retention of β-catenin in the nucleus and might explain the decrease of β-catenin ubiquitination. These results indicate that Pin1 could be a critical target to modulate β-catenin-mediated osteogenesis.

Wnt signaling is critical for the regulation of genes involved in normal embryonic development, cellular proliferation, and differentiation (1, 2). In the canonical pathway, in the absence of Wnt, β-catenin is subjected to proteasome-mediated degradation by a destruction complex that predominantly includes glycogen synthase kinase 3β (GSK3β), Axin, and the adenomatous polyposis coli (APC)2 protein (3). In the presence of Wnt signaling, there is reduced GSK3β kinase activity and increased accumulation of β-catenin in the nucleus, which is followed by stimulation of lymphoid enhancer factor/T cell factor (LEF/TCF) transcriptional activation (4–6). The well established canonical Wnt/β-catenin pathway is a key signaling pathway in bone development (7). For example, genetic studies in humans and mice have determined that low-density lipoprotein receptor-related protein 5 (LRP5)/Wnt signaling plays a major role in the control of bone mass (8–11).

The nuclear localization of β-catenin in response to Wnt is an essential event of the canonical Wnt signaling pathway for communicating with the transcription factor TCF/LEF (4, 12). Aberrant accumulation of β-catenin contributes to abnormal development and tumorigenesis. Therefore, it requires strict regulation. Previous reports have suggested that BCL9 (13), Smad3 (14, 15), and glucose-induced β-catenin and LEF1 interaction (16) actively import β-catenin to the nucleus, whereas APC (17–19) and Axin (20) export it to the cytoplasm. Specifically, APC has two major mechanisms by which it modulates the concentration of β-catenin in the nucleus. First, APC promotes the degradation of β-catenin by combining it with the degradation complex, and second, APC binds nuclear β-catenin and exports it to the cytoplasm for degradation (21).

The Ser(P)/TP motifs in certain proteins exist in either cis- or trans- conformation, and conversion between these states is catalyzed by the unique prolyl isomerase peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) (22, 23). Pin1 catalyzed, post-phosphorylation conformational regulation often functions as a molecular timer (24). We have found previously that the interaction of Pin1 with Runx2, the major transcription factor for osteoblast differentiation, is critical for bone development (25, 26). We showed that Pin1 hetero and KO mice exhibit reduced mineralization at birth. Pin1 mutation causes a short stature with hypomineralization throughout the skeleton. Pin1 is known to bind and modulate β-catenin stability and subcellular localization at the posttranslational level by regulating its interaction with APC in breast cancer (21) and neuronal differentiation (22). However, it is not known whether Pin1 is related to the regulation of osteoblast differentiation by β-catenin modulation or, if so, which detailed molecular mechanisms control this process.

In this study, we found that Pin1 is required for the transcriptional activity of β-catenin and demonstrated that interaction between Pin1 and β-catenin is critical in the osteogenic pathway. Furthermore, our data showed that Pin1-directed conformational changes in nuclear β-catenin are critical for the nuclear retention of β-catenin, which modulates the export of

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‡2 The abbreviations used are: APC, adenomatous polyposis coli; LEF, lymphoid enhancer factor; TCF, T cell factor; MEF, mouse embryonic fibroblast; alkaline phosphatase; IP, immunoprecipitation; CHX, cycloheximide; LMB, leptomycin B.
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β-catenin to the cytosol by regulating the interaction of β-catenin with APC in the nucleus. Taken together, our results reveal that Pin1 is a novel regulator that promotes osteoblast differentiation through structural modification and stabilization of β-catenin in the nucleus.

Materials and Methods

Animals—Pin1-deficient mice have been described previously (27) and were maintained under specific-pathogen-free conditions. Pin1 KO mice were generated from heterozygous matings as described previously (28). All animal studies were reviewed and approved by the Special Committee on Animal Welfare, Seoul National University, Seoul, Republic of Korea.

Antibodies and Reagents—The antibodies and reagents are described in detail in supplemental Tables 1 and 2.

DNA Construction and Site-directed Mutagenesis—Construction of the Pin1 (HA-pcDNA3.1-Pin1) WT and mutant (Y23A, W34A, and C113A) expression vectors has been described previously (25). Ds-Red Pin1 WT and mutant constructs have also been described previously (26). Full-length β-catenin cDNAs were generated by PCR and subcloned between BamH1 and Not1 sites in pcDNA3.1, respectively, to create FLAG epitope fusion proteins. All mutant constructs of Pin1 binding sites were generated by serial mutagenesis from the single and double binding site mutants with PCR, and the fragments were ligated using an In-Fusion HD cloning kit according to the user manual. PCR primers (supplemental Table 3) were synthesized and purchased from Integrated DNA Technology. PrimeSTAR DNA polymerase (Takara) was used, and PCR was performed using the protocol of the manufacturer.

Cell Culture and Nuclear-Cytoplasmic Fractionation—Mouse myogenic C2C12, HEK293, preosteoblast MC3T3-E1, multipotent ST2 mesenchymal progenitor, and mouse embryonic fibroblast (MEF) cells were cultured as described previously (25, 29). Nuclear-cytoplasmic fractionation was conducted using the NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific) according to the instructions of the manufacturer.

Alkaline Phosphatase (ALP) Staining—For ALP staining, cells were washed twice with phosphate-buffered saline, fixed with 2% paraformaldehyde, and stained for ALP-induced chrophomophores according to the instructions of the manufacturer (Sigma-Aldrich).

Luciferase Reporter Assay—After cell lysis with passive lysis buffer (Promega), luciferase activity was detected using a Bright-GloTM luciferase assay system (Promega) with a GloMax-Multi detection system reader (Promega).

Extraction of Total RNAs, RT-PCR, and Real-time PCR (Quantitative PCR)—Total RNA extraction from cultured cells, RT-PCR, quantitative PCR, and the primer sets for real-time PCR have been described previously (25, 28).

GST Pulldown Assay, Immunoprecipitation (IP), and Immunoblot Analyses—Cellular proteins and GST pulldown assays were prepared in lysis buffer as described previously (25). The GST pulldown assay, IP, and immunoblot analysis methods have been described previously (25).

Immunofluorescence and Immunohistochemistry—The detection of Pin1 and β-catenin by immunofluorescence and immunohistochemistry was performed as described previously (28, 29).

Knockdown Assay with siRNA and Transfection—To knock down Pin1 expression, siRNAs against Pin1 were purchased from Dharmacon (Lafayette, CO, siGENOME SMARTpool). siGENOME non-targeting siRNA 2 was used as a control (scramble siRNA). 40 pmol of siRNA was transfected. MC3T3-E1, ST2, and C2C12 cells were transfected by electroporation using the Neon transfection system (Invitrogen) according to the instructions of the manufacturer. Transfection of the HEK293 cells was performed using PolyJet reagent (SignaGen Laboratory) according to the instructions of the manufacturer.

Statistical Analysis—The results are presented as mean ± S.D. Each experiment was performed at least three times, and the results of one representative experiment are shown. The significance of the difference was evaluated using Student’s t test.

Results

Pin1 Controls β-Catenin Protein Stability and Wnt3a-induced Transactivation Activity—In this context, we aimed to characterize the role of Pin1 in adult bone metabolism through canonical Wnt signaling. For this purpose, the β-catenin protein level in the proximal tibial metaphysis of WT and Pin1 KO mice was determined by immunohistochemistry. Brown β-catenin expression spots were easily identified in osteoblasts of the WT, whereas spots were difficult to identify in the Pin1 KO counterpart (Fig. 1A). Because previous reports have indicated that Pin1 action influences substrate protein stability, we suspected that the decrease in β-catenin protein level in Pin1 KO mice was a result of decreased protein stability. Therefore, we confirmed that the mRNA level of β-catenin was not changed by Wnt3a treatment or no treatment in WT and Pin1 KO MF cells (Fig. 1B). We determined β-catenin stability after blocking de novo protein synthesis by treatment with cycloheximide (CHX). WT Pin1 overexpression strongly enhanced β-catenin stability, and the level was sustained 5 h after CHX treatment. However, overexpression of C113A Pin1, an enzyme defect mutant, did not enhance β-catenin stability (Fig. 1, C and D). TCF reporter gene (TOP Flash) activity in WT MEF cells was increased more than 5-fold by Wnt3a treatment, whereas in Pin1 KO MEF cells was increased about 2-fold (Fig. 1E). In addition, overexpression of Pin1 WT enhanced TOP Flash activity about 5-fold. On the other hand, overexpression of Pin1 mutants did not demonstrate a comparable enhancement of TOP Flash activity (Fig. 1F). Collectively, these results indicate that Pin1 action on β-catenin enhances its protein stability and Wnt3a-induced transactivation activity.

Pin1 Is Indispensable for Wnt3a-induced Osteoblast Differentiation—To understand the Pin1 contribution to Wnt3a-induced osteoblast differentiation, we performed cytological analysis of ALP activity and analysis of typical bone marker mRNA expression. Wnt3a-induced ALP staining was abrogated almost completely (Fig. 2A) by Pin1 knockdown (Fig. 2B) in MC3T3-E1 cells. On the contrary, ALP activity stimulated by minimal Wnt3a treatment was enhanced further by Pin1 over-
expression (Fig. 2D). Similarly, the Wnt3a-stimulated mRNA level of several bone marker genes was blocked almost completely by knockdown of Pin1 in the cells (Fig. 2, E–H). These results clearly indicate that Pin1 action is indispensable for Wnt3a-induced osteoblast differentiation. In addition, Pin1 knockdown also suppressed some bone marker gene expression and ALP staining even in the absence of Wnt3a treatment, suggesting a possibility of Pin1 involvement in β-catenin-independent osteogenic signaling pathways.

β-Catenin Is the Target of Pin1 in the Wnt3a Signaling Pathway—β-Catenin is the bottleneck molecule in the canonical Wnt signaling pathway, and the Wnt3a-induced signal suppresses GSK3-β activity, which, in turn, increases β-catenin levels in the cytosol and nucleus (3). Transcriptional activity such as TOP Flash (Fig. 3A) and ALP promoter (Fig. 3B) stimulated by β-catenin was enhanced further by overexpression of Pin1 in MC3T3-E1 cells. Because β-catenin was suspected as a candidate target for the Pin1 substrate, we tested the molecular interaction in MC3T3-E1 cells. Endogenously expressed β-catenin protein co-precipitated with GST-Pin1 recombinant protein. Moreover, β-catenin extracted from Wnt3a-treated cells showed greater binding than cells not treated with Wnt3a because nuclear β-catenin is increased by Wnt3a (Fig. 3C). In conclusion, Pin1 is indispensable for Wnt3a-induced osteoblast differentiation (Fig. 2, A–D). Similarly, the Wnt3a-stimulated mRNA level of several bone marker genes was blocked almost completely by knockdown of Pin1 in the cells (Fig. 2, E–H). These results clearly indicate that Pin1 action is indispensable for Wnt3a-induced osteoblast differentiation. In addition, Pin1 knockdown also suppressed some bone marker gene expression and ALP staining even in the absence of Wnt3a treatment, suggesting a possibility of Pin1 involvement in β-catenin-independent osteogenic signaling pathways.
Pin1 was localized to a nuclear substructure. We examined GST pulldown and IP of nuclear β-catenin with anti_Pin1 in the nuclear fraction (Fig. 3, E and F). Pin1 bound to nuclear β-catenin, and the interactions were increased with Wnt3a treatment. To confirm the biochemical interaction between these two molecules, MC3T3-E1 cells were transfected with Ds-Red Pin1 expression vectors, and endogenous β-catenin (green) and Ds-Red Pin1 (red) were analyzed using confocal microscopy. In the absence of Wnt3a treatment, most of the Pin1 and β-catenin molecules were localized independently in the nucleus. Only a small fraction of Pin1 molecules was co-localized with endogenous β-catenin (yellow) molecules (Fig. 3G, first row). With Wnt3a treatment, nuclear β-catenin increased, and co-localization with the Pin1 molecule also increased (Fig. 3G, second row).

The co-localization intensity was quantified and is shown in Fig. 3H. These results clearly demonstrate that the Pin1 target of the canonical Wnt signaling pathway is β-catenin and that the interaction of Pin1 and β-catenin becomes stronger following an increase in nuclear β-catenin by Wnt3a.

Pin1 Is Crucial for β-Catenin Accumulation in the Nucleus upon Stabilization by Wnt3a—To evaluate whether Pin1 regulates the subcellular distribution of endogenous β-catenin, we performed an immunofluorescence analysis in MC3T3-E1 cells. In the absence of Wnt3a, endogenous β-catenin staining was localized almost exclusively to the cell membrane (Fig. 4, A and B, first row). However, upon Wnt3a stimulation, β-catenin is accumulated in the nucleus, where it can stimulate the transcription of osteogenic marker genes (Fig. 4, A and B, second row).
Addition of Pin1 inhibitors, juglone or dipentamethylene thiuram monosulfide (DTM), with Wnt3a resulted in reduced accumulation of β-catenin in the nucleus. Knockdown of Pin1 also showed a reduction in nuclear β-catenin accumulation (Fig. 4B, third row). To confirm that Pin1 deficiency causes a strong decrease in nuclear β-catenin, we transfected WT or C113A (a dominant negative mutant) Pin1 constructs to examine the localization of endogenous β-catenin after treatment with Wnt3a. Almost all β-catenin was localized in the nucleus by Wnt3a (Fig. 4E, arrows). In particular, in WT Pin1-transfected cells, most of the β-catenin was observed in the nucleus (Fig. 4E, arrowhead). In C113A Pin1-transfected cells, the amount of nuclear β-catenin was reduced dramatically (Fig. 4F, arrowhead), and most β-catenin of untransfected cells was observed in the nucleus, which is consistent with the finding that Pin1 determines the localization of β-catenin (Fig. 4A and B). To biochemically confirm this localization pattern, an immunoblot of β-catenin was performed. SB216763, a potent inhibitor of GSK3 isozymes, is used to mimic the effect of Wnt3a (34). Treatment with dipentamethylene thiuram monosulfide abolished nuclear β-catenin that was increased by SB216763 (supplemental Fig. 1). We also determined whether β-catenin mislocalization can be rescued by overexpression of Pin1 in Pin1 KO primary osteoblast cells (Fig. 4H). Nuclear β-catenin was increased in WT osteoblast cells when treated with Wnt3a. However, in Pin KO osteoblasts, nuclear β-catenin was absent even after Wnt3a treatment. We determined the nuclear β-catenin localization change after Pin1 was overexpressed. In Pin1 KO primary osteoblasts, when Pin1 was overexpressed, nuclear β-catenin was increased slightly. Furthermore, when Pin1 was overexpressed in Pin1 KO primary osteoblasts, nuclear β-catenin was increased highly by Wnt3a. This shows that the defect of nuclear β-catenin in Pin1 KO primary osteoblasts can be rescued by reinstatement of Pin1. These results indicate that Pin1 is crucial for nuclear localization of β-catenin.
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A

Vehicle

Wnt3a

Wnt3a LMB

Wnt3a Juglone

Wnt3a LMB Juglone

B

Vehicle

Wnt3a

Wnt3a LMB

Wnt3a si Pin1

Wnt3a LMB si Pin1

C

Intensity/Area (Nucleus)

D

Intensity/Area (Nucleus)

E

Cytosol

Nucleus

IB: β-catenin

IB: Lamin A/C

IB: GAPDH

IB: Wnt3a

IB: LMB

IB: Juglone

IB: si Pin1

F

Nucleus

IP: β-catenin

IB: APC

IB: β-catenin

IB: APC

IB: GAPDH

IB: β-actin
Only β-Catenin Isomerized by Pin1 Can Be Retained in the Nucleus—Because Pin1 increases the accumulation of β-catenin in the nucleus, we assumed that there were two possibilities for Pin1-mediated nuclear β-catenin accumulation: that Pin1 enhances Wnt3a-induced nuclear translocation of β-catenin and that Pin1 inhibits the export of nuclear β-catenin to the cytosol. Wnt3a treatment induced nuclear translocation of β-catenin so that it was co-localized with Pin1 protein in the nucleus (Fig. 5, A and B, second row). Leptomycin B (LMB) is an inhibitor of CRM1 (chromosomal region maintenance)/exportin 1 protein (35), which is critical for the export of the protein containing a nuclear export sequence (36–38). LMB can be used to inhibit the export of β-catenin from the nucleus to the cytoplasm because β-catenin binds to APC, which has nuclear export sequences near the N terminus to export its partner proteins through the CRM1 export pathway (17). Wnt3a and LMB co-treatment maintained the nuclear co-localization of β-catenin and Pin1 (Fig. 5, A and B, third row). Wnt3a and juglone, which inhibit Pin1 activity, or siPin1, which reduces Pin1 expression, significantly decreased nuclear β-catenin (Fig. 5, A and B, fourth row). However, cells co-treated with Wnt3a, LMB, and juglone or siPin1 showed strong nuclear co-localization of β-catenin and Pin1 (Fig. 5, A and B, fifth row). These results indicate that, even in the absence of Pin1 activity or Pin1 expression, Wnt3a-induced nuclear translocation of β-catenin occurred. Juglone- or siPin1-mediated clearance of nuclear β-catenin was suppressed effectively by LMB, which is known to inhibit APC-mediated nuclear clearance of β-catenin (17, 19). We also quantified the intensity of nuclear β-catenin (Fig. 5, C and D). Consistent with the confocal images, Western blotting analysis also showed that the juglone-induced decrease of Wnt-induced nuclear accumulation of β-catenin was recovered completely by LMB (Fig. 5E). In vivo data showed that β-catenin expression in the WT tibia is more condensed in the nucleus compared with the Pin1 KO tibia (supplemental Fig. 2). In addition, we were more convinced by the high-resolution images showing that nuclear β-catenin retention is regulated by Pin1, not β-catenin translocation into the nucleus, because the nuclear β-catenin of the Pin1 KO tibia was also stained lightly (supplemental Fig. 2). Because APC protein is a critical chaperone of nuclear β-catenin to the cytosol (21), we investigated whether Pin1-mediated structural modification of β-catenin influences the interaction between β-catenin and APC in the nucleus. Wnt3a treatment strongly increased the nuclear β-catenin level (Fig. 5F), whereas it decreased the β-catenin-bound APC level (Fig. 5F). Inhibition of Pin1 increased the β-catenin-bound APC level, which was attenuated by LMB treatment both with and without Wnt3a treatment (Fig. 5F). On the basis of these results and the constitutive nuclear localization of Pin1 protein, we suggest that Pin1 is not actively involved in the nuclear translocation of β-catenin in response to canonical Wnt signaling but that it plays a crucial role in the APC-mediated cytosolic export of β-catenin.

β-Catenin as a Substrate of Pin1 Is Protected by Nuclear Localization—Pin1 only binds to peptide bonds between Ser(P)-Pro or Thr(P)-Pro substrates. In silico analysis of β-catenin indicated that there are three candidate target sites, Ser-191, Ser-246, and Ser-605 (Fig. 6A). We generated mutants with individual Ser-to-Ala substitutions, combinations of any two Ser-to-Ala substitution, or all three substitutions (3AP). We measured the biochemical molecular interaction by over-expression and GST pulldown analysis of FLAG-tagged β-catenin WT or mutants with Pin1 (Fig. 6B). Our analysis revealed that β-catenin mutants interact, except for the 3AP mutant (Fig. 6B). Compared with WT β-catenin, each single substitution mutant or combination of two substitution mutants did not significantly change TOP Flash activity (data not shown). Only 3AP β-catenin showed a dramatic decrease in the TOP Flash enhancing activity of β-catenin (Fig. 6C). Fig. 6 shows that structural modification by Pin1 is critical for β-catenin nuclear retention, so we compared the nuclear retention of WT β-catenin and 3AP. After 24-h overexpression of either WT or 3AP in MC3T3-E1 cells, each culture was treated with CHX for the indicated time, and then the intensity and localization of β-catenin were analyzed. Nuclear β-catenin was exported to the cytosol (Fig. 6D), and exported β-catenin is known to undergo proteasomal degradation (17). The rate of decay of the 3AP was faster than that of the WT (Fig. 6D). We quantified the intensity of WT and 3AP nuclear β-catenin (Fig. 6E). Nuclear β-catenin interacts with Lef1 and enhances its transcriptional activity to stimulate osteogenesis in canonical Wnt stimulation (4). We need to clarify whether the decrease of TOP Flash-enhancing activity of 3AP mutant β-catenin (Fig. 6C) resulted from its decreased stability or intractability with Lef1. The interaction between Lef1 and β-catenin WT or the 3AP mutant was determined by immunoprecipitation assay (Fig. 6F), which showed that the FLAG-tagged β-catenin level is quite proportional to the co-precipitated LEF1 level, indicating that its intractability with Lef1 was not abrogated by the 3AP substitution mutation. The input protein levels of 3AP β-catenin showed a strong decrease compared with WT β-catenin (Fig. 6F), indicating that the stability change is the main cause of decreased activity in the 3AP mutant. Over-expression of the 3AP mutation abrogated the stimulatory effect of β-catenin on osteogenic marker genes (Fig. 6, G–J). These results strongly indicate that Pin1 stabilizes β-catenin and prolongs its nuclear retention, which subsequently increases osteogenic marker gene expression.

FIGURE 5. Pin1 activity enhances the nuclear retention of β-catenin. A, MC3T3-E1 cells were pretreated without or with 10 μm juglone and/or 100 ng/ml LMB for 1 h and then treated with 50 ng/ml Wnt3a for 24 h. Endogenous β-catenin translocation was detected using cells immunostained with anti-β-catenin, Pin1, and DAPI. LMB blocks the export system. B, MC3T3-E1 cells were transfected with scramble (control) or siPin1 siRNA. After 24 h, the same procedure was conducted. C and D, endogenous nuclear β-catenin was quantified and is shown as a sigma plot (n = 50/group; **p < 0.01). E, MC3T3-E1 cells were pretreated without or with 2 μm juglone and/or 100 ng/ml LMB for 1 h. Cells were left untreated (top panel) or treated with (bottom panel) 50 ng/ml Wnt3a for 24 h. Cells were fractionated in hypotonic buffer into nuclear and cytoplasmic fractions, followed by immunoblot (IB) analysis with anti-β-catenin, anti-GAPDH, and anti-lamin A/C antibodies. Lamin A/C and GAPDH were used as loading controls for the nuclear and cytoplasmic fractions, respectively (n = 3). F, IP assay of APC and β-catenin. IP was performed with anti-β-catenin from dissociated nuclear extracts in HEK293 cells grown under the same conditions as in A. APC was analyzed using immunoblot analysis with anti-APC antibody (n = 3).
Discussion

Pin1 Is Critical for the Nuclear Retention of β-Catenin—In the presence of a Wnt signal, the inhibition of phosphorylation and subsequent ubiquitination of β-catenin results in its accumulation in the cytosol (1). However, the mechanisms controlling the nuclear localization of β-catenin, especially entrance into and exit from the nucleus, are poorly understood. Because β-catenin does not contain a nuclear localization signal or nuclear export signal (6), the efficient entry and exit of β-catenin to and from the nucleus are unknown. Even though previous reports have investigated the many molecules involved in the import and export of β-catenin (13, 17, 39, 40), the results are quite controversial. Rigorous control of β-catenin-mediated transcription is essential to prevent the tenacious activation of Wnt target genes in the canonical Wnt signaling pathway. Export of β-catenin from the nucleus completely terminates the transcriptional func-
Our data show that Pin1 is critical to keep β-catenin in the nucleus and not critical to translate β-catenin from the cytosol to the nucleus (Fig. 5). If Pin1 were involved in the nuclear translocation of β-catenin, then β-catenin would not be present in the nucleus when cells were treated with siPin1 or juglone and LMB because β-catenin would have already failed to enter the nucleus because of inhibited Pin1 expression and activity. We observed that nuclear β-catenin was still highly maintained (Fig. 5). This result is congruous with the results of earlier studies where Pin1-mediated conformational change determined the molecular localization in a cell (21, 41). Regulation of β-catenin retention by Pin1 affects to reductions in transcriptional activity and target gene expression when Pin1 was inhibited or absent (Figs. 1 and 2).

β-Catenin Interacts Directly with Pin1 in the Nucleus—Among the many prolyl isomerases, Pin1 shows the narrowest target specificity for Pro-directed phosphorylation sites in a subset of proteins (42). In silico analysis of β-catenin indicates that it has three candidate Pin1 binding sites, Ser-191, Ser-246, and Ser-605 (Fig. 6A). Previous reports have suggested that post-phosphorylation binding of Pin1 to the Ser-246 of β-catenin is vital for the stability and/or subcellular localization of β-catenin (21, 22). Another study stresses that Ser-191 and Ser-605 are more critical for β-catenin nuclear localization than Ser-246 (43). In this study, we did not find any significant difference in interaction (Fig. 6B), transcriptional activity, and localization between the WT and any Ser-to-Ala single or double substitution mutants in β-catenin. Only 3AP failed to bind to Pin1 (Fig. 6B) and showed a significant down-regulation of its transcriptional activity (Fig. 6C), indicating that all three candidate sites are targets of the Pin1 substrate. These results are different from those of previous reports in which the authors designated one or two specific sites for Pin1 interaction. We assume that this is because multiple signaling cascades converge on β-catenin via phosphorylation to control canonical Wnt signaling. Studies indicate that the intracellular molecular mechanisms of β-catenin are tightly controlled by multiple signaling pathways in different biological contexts. In fibroblasts, Wnt3a activates ERK and plays an important role in cell proliferation (44). In differentiated osteoblasts, Wnt3a prevents apoptosis through β-catenin-dependent signaling cascades involving Src/ERK and PI3K/AKT (45), and the TGFβ and Notch signaling pathways also maintain cross-talk with the Wnt signaling pathway (46–49). Pin1 target sequences are shared by several protein kinases, such as ERK, cyclin-dependent kinase, and GSK3α (26). Therefore, we suggest that the conformational change of each of the three different phosphorylation sites probably has different biological implications.

The Nuclear Interaction of β-Catenin and APC Is Regulated by Pin1—Our next question addressed the mechanism by which Pin1-modified β-catenin remains in the nucleus for long periods of time. Previous reports have indicated that a nuclear-cytoplasmic shuttling protein, APC, can function as a chaperone for β-catenin export from the nucleus (17, 19). Pin1 affects the interaction between β-catenin and APC (21). Therefore, we hypothesized that the conformational change of nuclear β-catenin by Pin1 does not allow for the interaction with APC, inhibiting the export of β-catenin to the cytosol from the nucleus and, consequently, enhancing the nuclear retention of β-catenin. We observed that the interaction between nuclear APC and β-catenin was generally reduced in the presence of Wnt ligand and increased...
when Pin1 activity was inhibited by juglone (Fig. 5F). Therefore, we suggest that the conformational change of nuclear β-catenin by Pin1 inhibits the ability of β-catenin to interact with nuclear APC and the subsequent APC-guided export of β-catenin to the cytosol (Fig. 7).

Regulation of Nuclear β-Catenin Retention by Pin1 Implicates It as a Drug Target for Bone Diseases and Cancer—The canonical Wnt signaling pathway is an important regulatory pathway in the osteogenic differentiation of mesenchymal stem cells (2, 50) and bone development (7). Induction of the Wnt signaling pathway promotes bone formation (8–10). Genetic studies have shown that LRP5 can control bone mass (8, 9), and conditional deletion of β-catenin in the embryonic limb and head mesenchyme results in the absence of mature osteoblasts in membranous bones (10, 11). Our previous studies have shown the importance of Pin1 in bone development (25, 26, 30). Considering this, Wnt signaling, including all components of the canonical pathway, and Pin1 are deemed essential for bone formation. We demonstrated that Pin1 increases Wnt-induced osteoblast differentiation. These results are consistent with those of previous reports showing that Pin1 KO mice had low bone mineral density (25, 51). These data identify, for the first time, a major function of Pin1 in osteoblast differentiation by regulating the interaction between β-catenin and APC. Therefore, Pin1 is a potential biological marker or therapeutic target that enhances Wnt-induced osteoblast differentiation. Both the Wnt signaling pathway and Pin1 have been studied in pathological human cancers (52–56). Pin1 plays a key role in the pathogenesis of other malignantities, such as breast, colon, and prostate cancer (32, 52, 57). In particular, unregulated activation of β-catenin is a major cause of tumorigenesis in cancers (31). In addition, Pin1 increases the transcription of several β-catenin target genes by inhibiting its interaction with APC (21) in cancer. Therefore, our novel finding that Pin1 is important for regulating the interaction between β-catenin and APC in the nucleus and the export of β-catenin to the cytosol (Fig. 5) can also be a useful tool in cancer therapeutics.

In conclusion, our results reveal a novel molecular mechanism whereby Pin1 promotes the nuclear retention of β-catenin through inhibition of the interaction between APC and β-catenin in the nucleus, which, in turn, up-regulates osteoblast differentiation. This mechanism is a new avenue for studying the translocation of nuclear β-catenin, and this knowledge leads to a deeper understanding of the importance of isomerized β-catenin in bone development. Although we examined the Pin1 and β-catenin relationship under physiological conditions such as in osteoblast differentiation, these results raise the possibility that the study of β-catenin and APC interaction by adjusting Pin1 activity in the pathological human colorectal cancer model can be very rewarding in order to utilize it as a cancer drug target.

Author Contributions—H. R. S., W. J. Y., and H. M. R. designed the study. H. R. S., T. L., H. S. B., and B. S. K. conducted the study. H. R. S., Y. D. C., K. M. W., J. H. B., and H. M. R. analyzed the data. H. R. S., Y. D. C., K. M. W., J. H. B., and H. M. R. drafted the manuscript.

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