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PTH/cAMP/PKA Signaling Facilitates Canonical Wnt Signaling Via Inactivation of Glycogen Synthase Kinase-3β in Osteoblastic Saos-2 Cells

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Abstract Although the intermittent administration of PTH is known to stimulate the bone formation, the underlying mechanisms are not fully understood. Here we investigated the crosstalk between PTH/cAMP signaling and canonical Wnt signaling using the human osteoblastic cell line Saos-2. Treatment with PTH or forskolin, an activator of adenylate cyclase, facilitated T-cell factor (TCF)-dependent transactivation in a dose-dependent manner, which was abolished by pre-treatment with a PKA inhibitor, H89. Wnt3a and forskolin synergistically increased the TCF-dependent transactivation. Interestingly, intermittent treatment with PTH enhanced the TCF-dependent transactivation more profoundly than continuous treatment. In addition to the effects on TCF-dependent reporter activity, treatment with PTH or forskolin resulted in the increased expression of endogenous targets of Wnts, *Wnt-induced secreted protein 2* (*WISP2*) and *naked cuticle 2* (*NKD2*). We then investigated the convergence point of PTH/cAMP signaling and the canonical Wnt pathway. Western blotting demonstrated that GSK-3β was rapidly phosphorylated at Ser^9 on treatment with PTH or forskolin, leading to its inactivation. Moreover, overexpression of a constitutively active mutant of GSK-3β abolished the TCF-dependent transactivation induced by forskolin. On the other hand, overexpression of the Wnt antagonist Dickkopf-1 (DKK1) failed to cancel the effects of forskolin on the canonical Wnt pathway. Interestingly, treatment with Wnt3a markedly reduced the forskolin-induced expression of *receptor activator of NF-κB ligand* (*RANKL*), a target gene of PTH/cAMP/PKA. These results suggest that cAMP/PKA signaling activates the canonical Wnt pathway through the inactivation of GSK-3β, whereas Wnt signaling might inhibit bone resorption through a negative impact on *RANKL* expression in osteoblasts. J. Cell. Biochem. 104: 304–317, 2008.

Key words: parathyroid hormone; canonical Wnt signaling; anabolic action; β-catenin; glycogen synthase kinase-3β; receptor activator of nuclear factor-κB ligand

The canonical Wnt signaling pathway, consisting of Wnt ligands, frizzled receptor (Fzd), low-density lipoprotein receptor-related protein 5 and 6 (Lrp5/6), β-catenin, and the T-cell factor (TCF)-lymphoid enhancer factor (LEF) family transcription factors, plays important roles both in embryonic development and in tumorigenesis [Nusse, 2005; Reya and Clevers, 2005]. In response to the binding of Wnt ligands to Fzd and Lrp5/6, cytoplasmic β-catenin is stabilized via the inhibition of glycogen synthase kinase-3β (GSK-3β) and casein kinase 1 (CK1), and subsequently translocated into the nucleus. Then, β-catenin interacts in the nucleus with TCF/LEF transcription factors to mediate many of the effects of Wnts on gene transcription [Zhong et al., 2006]. There are also many inhibitors for canonical Wnt/β-catenin signaling, including secreted frizzled-related proteins (sFRPs), dickkopfs (Dkks), and Wnt inhibitory factors (Wifs) [Kawano and Kypta, 2003] that function outside of the cell, and naked cuticle (nkd) which is an intracellular protein that inhibits Wnt signaling [Zeng et al.,

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2000; Yan et al., 2001]. These inhibitors also play important roles in many biological events.

Recently, several lines of evidence have established that the canonical Wnt signaling pathway is also involved in bone mineral accrual [Krishnan et al., 2006]. Notably, LRP5, which functions as a co-receptor for ligands including Wnt1, 2, 3a, 7b, and 10b, has been proved to play an important role in bone mineral metabolism genetically [Johnson et al., 2004]. Loss-of-function type mutations in the human *LRP5* gene lead to osteoporosis-pseudoglioma syndrome, which is characterized by low bone mineral content and persistence of fetal vitreous veins [Gong et al., 2001]. On the other hand, hypermorphic mutations in the gene cause a high-bone-mass phenotype [Boyden et al., 2002].

The critical role of the canonical Wnt signaling pathway in bone metabolism is also supported by studies in mice. Mice lacking the *Lrp5* gene exhibit a low-bone-mass phenotype [Kato et al., 2002]. In contrast, overexpression of β-catenin in osteoblasts leads to a high bone mass [Glass et al., 2005]. Moreover, we have demonstrated that *Lrp6* encoding the other co-receptor for Wnt ligands is a causative gene in a spontaneous mutant mouse named *ringelschwanz* with a reduced bone mass as well as skeletal dysplasia characterized by a short tail, spina bifida [Kokubu et al., 2004]. More recently, a loss-of-function type mutation in the human *LRP6* gene has been identified in an Iranian family with early coronary disease and metabolic risk factors, and osteoporosis was also found to be associated with the mutation [Mani et al., 2007].

In addition to its roles in bone formation as demonstrated by the manipulation of *Lrp5* in mice [Kato et al., 2002], the canonical Wnt signaling pathway may be involved in bone resorption [Glass et al., 2005]. It was demonstrated that mice in which an active form of β-catenin was specifically overexpressed in differentiated osteoblasts exhibited a high-bone-mass phenotype, while the targeted deletion of β-catenin in mature osteoblasts led to osteopenia, both of which were caused by dysregulated bone resorption [Glass et al., 2005]. Targeted deletion of adenomatous polyposis coli (*Apc*) in osteoblasts, which leads to the activation of β-catenin, has been reported to cause the impaired bone resorption associated with the decreased expression of *Rankl* and the increased expression of *Opg* [Holmen et al., 2005]. We also observed increased bone resorption due to the increased expression of *Rankl* in the *ringelschwanz* mouse with a mutation of the *Lrp6* gene [Kubota et al., 2005]. Thus, the actions of Wnts on bone are more complex than previously thought, and their molecular targets in osteoblasts and osteoclasts should be identified.

Parathyroid hormone (PTH) has a dual effect on bone, that is, activation of bone resorption and bone formation [Gensure et al., 2005; Potts, 2005]. Intermittent treatment with PTH is clinically recognized to be effective in patients with osteoporosis by promoting bone formation [Neer et al., 2001; Tashjian and Gagel, 2006]. When PTH stimulates bone formation rather than bone resorption, the net effect is anabolic. PTH increases the level of intracellular cAMP through its cognate receptor, PTHR1, and cAMP activates the protein kinase A, PKA. Then, PKA phosphorylates the transcription factor CREB (cAMP response element-binding protein) [Johannessen et al., 2004]. In addition, cAMP activates Epac, a guanine-nucleotide-exchange factor, and then the Raf/MEK/ERK pathway [Holz et al., 2006]. Although the major pathway of PTH signaling involved in bone metabolism is thought to be mediated by PKA, the Epac pathway is also reported to be involved [Fujita et al., 2002]. As to bone resorption, the target of PTH has been proved to be RANKL, which is essential for the differentiation and activation of osteoclasts [Tanaka et al., 2005]. On the other hand, the precise molecular mechanisms underlying its anabolic effects remain unclear. Considering the critical roles of canonical Wnt signaling in the determination of bone mass, it is reasonable to hypothesize that PTH exerts its anabolic action through effects on the Wnt signaling pathway. In this study, we have examined the effect of PTH/cAMP/PKA on the canonical Wnt pathway, and have found that the former facilitates the latter by the inactivation of GSK-3β. Moreover, Wnt in turn suppresses the PTH-induced expression of *RANKL*. Thus, the interaction between PTH and the canonical Wnt signaling pathway may contribute to the anabolic effect of PTH on bone.

MATERIALS AND METHODS

Cell Culture and Treatment

The human osteoblastic cell line Saos-2 was obtained from American Type Culture Collection

(ATCC, Manassas, VA), and maintained in alpha MEM (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS). Cells were starved in serum-free medium for 24 h before being treated with forskolin (Wako, Osaka, Japan) or hPTH[1–34] (Sigma, St. Louis, MO). In some experiments, cells were also treated with 50 μM of a PKA inhibitor, H89 (Seikagaku, Tokyo, Japan), for 1 h before the addition of forskolin. For transient transfections, FuGene6 reagent (Roche Diagnostics, GmbH, Mannheim, Germany) was utilized.

Preparation of Wnt1- or Wnt3a-Containing Conditioned Medium

Wnt3a-producing mouse L cells (L Wnt-3A cells) and the control L cells were obtained from ATCC. Wnt3a-containing conditioned medium (CM) was harvested from L Wnt-3A cells according to the protocol provided by ATCC. The control CM was collected from control L cells. As to the Wnt1-containing CM, Chinese hamster ovary (CHO) cells were transiently transfected with the Wnt1 expression plasmid pCAGGS-Wnt1, and the CM was harvested.

Reporter Assay

Saos-2 cells were transiently transfected with a luciferase reporter plasmid, Super(8x)TOPflash containing T-cell factor (TCF)-binding sites, or Super(8x)FOPflash with mutated TCF-binding sites (kindly provided by Dr. R.T. Moon, Washington University), together with a control Renilla luciferase plasmid phRL-TK (Promega, Madison, WI). Twenty-four hours after the transfection, the medium was changed to serum-free medium, and the cells were starved for 24 h before stimulants were added. After treatment with appropriate stimulants, cell lysates were harvested, and the reporter activity was assayed using a PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink, Tokyo, Japan). The firefly luciferase activity derived from Super(8x)TOPflash or Super(8x)FOPflash was standardized based on the Renilla luciferase activity of the same lysate. The luciferase reporter activity of Saos-TOPflash stable transfectants was assayed using a PicaGene Luminescence Kit (Toyo Ink) and standardized with respect to the protein content.

Generation of Saos-TOPflash Stable Transfectants

To generate the Saos-2 stable transfectants of the TCF-reporter plasmid (designated as Saos-TOPflash), Super(8x)TOPflash and pcDNA3.1-Zeo harboring a zeocin-resistant cassette (Invitrogen, Carlsbad, CA) were introduced into Saos-2 cells using FuGene6 (Roche), and the stable transfectants were selected in the presence of Zeocin (600 μg/ml; Invitrogen). Several clonal cell lines were expanded from single foci and were screened for the basal luciferase activity.

Intermittent and Continuous Exposure to PTH[1–34]

Saos-TOPflash stable transfectants were inoculated into six-well culture plates (1×10^5 cells/well) and cultured for 24 h in the absence of hPTH[1–34]. Then, they were divided into two groups according to the mode of exposure to PTH: the intermittent exposure group and the continuous exposure group. Intermittent exposure to PTH was carried out according to a previous report [Ishizuya et al., 1997]. In the intermittent exposure group, the cells were exposed to hPTH[1–34] for the first 6 h of each 48-h incubation cycle. Cell lysates were harvested for luciferase assays before and after each exposure to PTH. In the continuous exposure group, the cells were exposed to hPTH[1–34] throughout the experiment. During this period, the culture medium was replaced every 48 h.

Expression Plasmids

To construct an expression plasmid for human Dickkopf-1 (DKK1), the cDNA encoding human mature DKK1 was obtained by PCR using cDNA from Saos-2 cells as a template and the following primer set; EcoRI-hDKK1-F, 5′-GCGAATTCAACCTTGAACTCGGTTCTCAATTCC-3′, and EcoRI-hDKK1-R, 5′-GCGAATTCATTAGTGTCTCTGACAAGTGTGAA-3′. The PCR product was cloned into the vector pT7 Blue (Novagen, Madison, WI), and after the sequence was confirmed, the fragment excised by digestion with *EcoR*I was inserted into the vector pFLAG-CMV1 (Sigma). The resultant plasmid pFLAG-DKK1 encodes a FLAG®-tagged mature DKK1.

To construct an expression plasmid encoding the constitutively active form of GSK-3β, we obtained cDNA encoding human GSK-3β by PCR using Saos-2-derived cDNA and the following primers; EcoRI-hGSK3β-start, 5′-GCTGCAGAATTCGTTAACCTAACACCCCAACATAAAG-3′, and EcoRI-hGSK3β-End, 5′-CTGGCTG-

AATTCGACTGTTCAGGTGGAGTTGGAAGC-TG-3′. The product was cloned into the vector pGEM 11Zf (Promega) leading to pGEM-GSK-3β, and the sequence was confirmed. Then, mutagenesis was carried out to introduce into pGEM-GSK-3β a mutation that converts the Ser at position 9 into Ala (S9A) using the Gene Editor™ in vitro Site-directed Mutagenesis System (Promega). On confirmation that the mutagenesis was accomplished, the fragment excised from pGEM-GSK-3β or pGEM-GSK-3β[S9A] by digestion with *EcoR*I was cloned into pcDNA3.1-Zeo (Invitrogen). The resultant plasmids were named pcDNA-GSK-3β[WT] and pcDNA-GSK-3β[S9A], respectively.

Western Blotting

Western blotting was performed using the following antibodies; anti-β-catenin polyclonal antibody, anti-Dkk1 polyclonal antibody (Santa Cruz, Santa Cruz, CA), anti-FLAG® monoclonal antibody (M2; Sigma), anti-phospho-GSK3β (Ser^9) antibody, and anti-GSK3β antibody (Cell Signaling Technology, Inc., Beverly, MA).

To examine the accumulation of β-catenin in the nuclei, nuclear extracts were prepared. Cells were harvested in phosphate-buffered saline, and after centrifugation, the cell pellets were resuspended in NEA buffer [0.1 M HEPES-KOH (pH 7.8), 0.25 M KCl, 50 mM EDTA, 1% NP-40, 0.1 M DTT, and protease inhibitor cocktail Complete™ (Roche)]. Then, the suspension was centrifuged again, and the resultant pellets were resuspended in NEC buffer [0.5 M HEPES-KOH (pH 7.8), 1.25 M KCl, 250 mM EDTA, 0.5 M $MgCl_2$, 25% glycerol, 0.1 M DTT and Complete™]. The suspension was incubated on ice for 30 min, and centrifuged at 15,000*g* for 15 min. The supernatant was stored as the nuclear extract at −80°C until used. For immunoblotting with the other antibodies, whole cell extracts were harvested in RIPA buffer [1% Triton, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris−Cl (pH 7.4), 5 mM EDTA, 1 mM orthovanadate, and protease inhibitor cocktail Complete™ (Roche)]. Equal amounts of protein were separated by SDS−PAGE and transferred to PVDF membranes (BioRad, Helcules, CA). After blocking with Blocking One-P reagent (Nacalai Tesque, Kyoto, Japan) and incubation with a primary antibody, the membranes were washed and incubated with the corresponding HRP-conjugated secondary antibody, and the proteins were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

Measurement of Intracellular cAMP

The intracellular cAMP levels were determined using a BIOTRAK EIA kit (Amersham Biosciences) as recommended by the manufacturer.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real Time-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen), and treated with DNase (Qiagen K.K., Tokyo, Japan). The RNA (2.5 μg) was reverse-transcribed using Super-Script II (Invitrogen), and subjected to RT-PCR to examine the expression levels of Wnt signaling components (*WNT1*, *2*, *3*, *4*, *5a*, *7a*, *8b*, *10b*, *13*, *FRZ2*, *5*, *β-catenin*, *DKK1*, *3*, *DVL1*, *GSK-3β*) using TaKaRa Taq (Takara, Kyoto, Japan). The specific primers utilized in the RT-PCR are listed in Table I. The expression level of *RANKL* was determined by real time-PCR using Taq-Man® Gene Assay Hs00243519_m1 (Applied Biosystems, Foster City, CA) in a real-time PCR system 7300 (Applied Biosystems). As an internal control, the amount of 18S ribosomal RNA was also analyzed using the assay Hs99999901_s1.

Statistical Analyses

Data were statistically analyzed by one-way ANOVA.

RESULTS

PTH/cAMP/PKA Signaling Increases TCF/LEF-Dependent Transactivation

We first examined the effects of increased intracellular cAMP levels on TCF-dependent transactivation in a series of reporter assays. When Saos-2 cells were treated with 50 μM forskolin, the intracellular cAMP content reached a maximum within 15 min. Treatment with 10 nM hPTH[1−34] resulted in the intracellular accumulation of cAMP with a maximum reached within 60 min, while it took longer to reach a peak when the cells were treated with 1 nM hPTH[1−34] (Fig. 1A). We then examined the effects of forskolin and PTH on TCF-dependent transactivation in a series of reporter assays. Saos-2 cells were transfected with Super(8x)TOPflash or Super (8x)FOPflash together with a control Renilla

TABLE I. Primer Sets Utilized in RT-PCR

Gene	Primer sequence	Product size
WNT1		
Sense	5′-CACGACCTCGTCTACTTCGAC-3′	250 bp
Antisense	5′-ACAGACACTCGTGCAGTACGC-3′	
WNT2		
Sense	5′-CCAGCCTTTTGGCAGGGTC-3′	380 bp
Antisense	5′-GCATGTCCTGAGAGTCCATG-3′	
WNT3		
Sense	5′-TGAACAAGCACAACAACGAG-3′	439 bp
Antisense	5′-CAGTGGCATTTTTCCTTCC-3′	
WNT4		
Sense	5′-CCTTCTCACAGTCGTTTG-3′	429 bp
Antisense	5′-CACAGCCGTCGATGGCCTT-3′	
WNT5A		
Sense	5′-GGGAGGTTGGCTTGAACATG-3′	141 bp
Antisense	5′-GAATGGCACGCAATTACCTT-3′	
WNT7A		
Sense	5′-GCTGCCTGGGCCACCTCTTTCTCA-3′	411 bp
Antisense	5′-CCCGGTGGTACAGGCCTTGCTTCT-3′	
WNT8B		
Sense	5′-ATGTCTTTGGGGTTGGTTCCTAG-3′	272 bp
Antisense	5′-TTGCTAGGAGGAAGAAGGTCAG-3′	
WNT10B		
Sense	5′-GAATGCGAATCCACAACAACAG-3′	200 bp
Antisense	5′-TTGCGGTTGTGGGTATCAATGAA-3′	
WNT13		
Sense	5′-AAGATGGTGCCAACTTCACCG-3′	320 bp
Antisense	5′-CTGCCTTCTTGGGGGCTTTGC-3′	
FRZ2		
Sense	5′-CAGCGTCTTGCCCGACCAGATCCA-3′	386 bp
Antisense	5′-CTAGCGCCGCTCTTCGTGTACCTG-3′	
FRZ5		
Sense	5′-TTCATGTGCCTGGTGGTGGGC-3′	215 bp
Antisense	5′-TACACGTGCGACAGGGACACC-3′	
β-catenin		
Sense	5′-GGTGGGCTGCAGAAAATGGTT-3′	567 bp
Antisense	5′-GATGGCAGGCTCAGTGATGTCTTC-3′	
DKK1		
Sense	5′-AGGCGTGCAAATCTGTCTCG-3′	502 bp
Antisense	5′-TGCATTTGGATAGCTGGTTTAGTG-3′	
DKK3		
Sense	5′-GCAGCGGCTTGGGGCCACCCT-3′	1,064 bp
Antisense	5′-CTAAATCTCTTCCCCTCCCAG-3′	
DVL1		
Sense	5′-CGGGGCGGACGTGGTGGACTG-3′	532 bp
Antisense	5′-CTGGCCGGCCGGACGCTCTC-3′	
WISP2		
Sense	5′-CACGCTGCCTGGTCTGTCTGGATC-3′	352 bp
Antisense	5′-CACGCATAGGCTTGTATTCAGGAAC-3′	
NKD2		
Sense	5′-CACCCGTCCTAGCGCCACTG-3′	427 bp
Antisense	5′-CAAAGCCTCAGCTTTTCCATTCG-3′	
RANKL		
Sense	5′-ATGCGCCGCGCCAGCAGAGAC-3′	385 bp
Antisense	5′-TGCACAGCTCCTTGAAAGGC-3′	

luciferase plasmid, phRL-TK. After 24 h of serum-starvation, stimulants or corresponding vehicles were added to the cells, and cell lysates were sequentially harvested for luciferase assays. In the assay using Super(8x)TOPflash, treatment with forskolin increased the relative luciferase activity in a dose-dependent manner (Fig. 1B). About a threefold increase was observed in the relative reporter activity on treatment with 50 μM forskolin for 3–12 h. Longer treatment resulted in a reduction in the reporter activity to the basal level. When Super(8x)FOPflash containing mutated TCF/LEF response elements was utilized instead of Super(8x)TOPflash, treatment with forskolin did not enhance the reporter activity (Fig. 1B). Consistent with the results in the reporter assay, Western blotting confirmed the nuclear accumulation of β-catenin after the addition of forskolin (Fig. 1C). Treatment with hPTH[1–34] also significantly increased the Super(8x)TOPflash reporter activity in a dose-dependent manner (Fig. 1D), and caused nuclear accumulation of β-catenin (data not shown). To examine whether PKA is involved in the transactivation of Super(8x)TOPflash

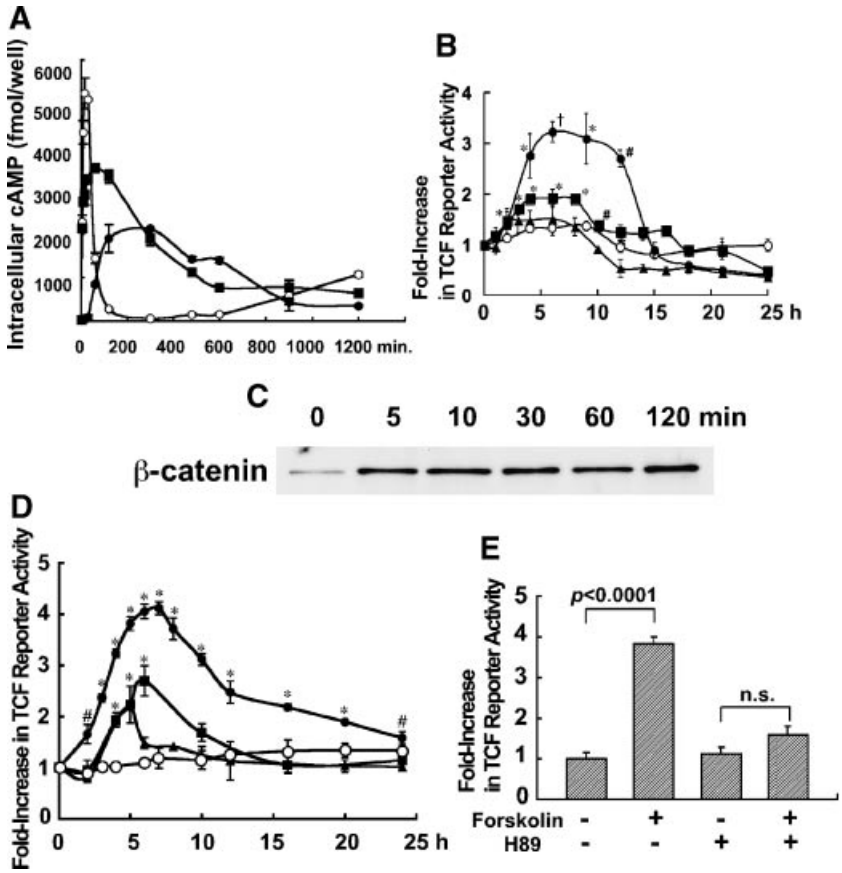

Fig. 1. PTH/cAMP signaling facilitates canonical Wnt signaling. **A**: Intracellular accumulation of cAMP after treatment with forskolin or PTH. Saos-2 cells were treated with 50 μM forskolin (open circles) or 1–10 nM hPTH[1–34] (1 nM; closed circles, 10 nM; closed squares) for the indicated period, and intracellular cAMP content was determined by EIA. The data are shown as the mean ± SD (n = 3). **B**: Effects of forskolin on the TCF-dependent transactivation. Super(8x)TOPflash (closed markers) or Super (8x)FOPflash (open circles) was introduced into Saos-2 cells together with a control Renilla luciferase reporter plasmid. Two days after the transfection, the cells were treated with various amounts of forskolin; closed and open circles, 50 μM; closed squares, 25 μM; closed triangles, 5 μM. The fold-increase in the relative luciferase activity was calculated referring to the activity in the untreated cells as 1. The data are described as the mean ± SEM (n = 3). $^{*,\#,\dagger}$ significantly different from the Super(8x)FOPflash control in the cells treated with forskolin for the same period ($^{*}P < 0.05$, $^{\#}P < 0.01$, $^{\dagger}P < 0.001$). **C**: Nuclear accumulation of β-catenin induced by forskolin. Saos-2 cells were treated with 50 μM forskolin for the indicated period, and the nuclear extracts were harvested and subjected to Western blotting using the antibody against β-catenin. **D**: The treatment with PTH also facilitated the TCF-dependent transactivation. Super(8x)TOPflash (closed markers) or Super(8x)FOPflash (open circles) was introduced into Saos-2 cells together with a control Renilla luciferase reporter plasmid. The cells were treated with various amounts of PTH; closed and open circles, 50 μM; closed squares, 25 μM; closed triangles, 5 μM. The fold-increase in the relative luciferase activity was calculated referring to the activity in the untreated cells as 1. The data are described as the mean ± SD (n = 4). $^{*,\#}$ significantly different from the Super(8x)FOPflash control in the cells treated with PTH for the same period ($^{*}P < 0.01$, $^{\#}P < 0.05$). **E**: Treatment with the PKA inhibitor H89 abolished the enhancing effects of forskolin on the TCF-dependent transactivation. Saos-2 cells transfected with Super(8x)TOPflash and a control Renilla reporter plasmid were pretreated with 1 μM H89 for 1 h, and then treated with 50 μM forskolin or vehicle for 6 h. The fold-increase in the relative luciferase activity was calculated referring to the activity in the vehicle-treated control cells as 1. The data are described as the mean ± SEM (n = 3).

caused by the forskolin- or PTH-induced intracellular cAMP accumulation, we treated the cells with H89, an inhibitor of PKA. Pre-treatment with 50 μM of H89 markedly decreased the reporter activity induced by forskolin (Fig. 1E), indicating that increased intracellular cAMP levels facilitated the TCF-dependent transactivation through the PKA pathway. Lower concentrations of H89 exhibited similar effects, although to less of an extent (data not shown).

Synergistic Effect of Forskolin and Wnt3a on the TCF-Dependent Transactivation

Then, to test the possibility of crosstalk between the PTH/cAMP and Wnt/β-catenin signaling pathways, we examined the effect of

simultaneous treatment with forskolin and canonical Wnt on the TCF-dependent transactivation. As expected, Wnt3a-containing conditioned media increased the Super(8x) TOPflash reporter activity in a dose-dependent manner. Simultaneous addition of forskolin synergistically enhanced the effect of Wnt on the reporter activity (Fig. 2), suggesting cross-talk between the PTH/cAMP and Wnt/β-catenin signaling pathways.

Intermittent Treatment Potentiates the Effects of PTH on the TCF-Dependent Transactivation

It has been established that PTH exerts bone-forming effects in vivo when administered intermittently [Gensure et al., 2005; Potts, 2005]. In addition, it has been reported that PTH exerts disparate effects on osteoblastic differentiation in vitro depending on the exposure time [Ishizuya et al., 1997]. Therefore, we next examined whether the mode of exposure influences the effects of PTH on the TCF-dependent transactivation. We treated the Saos-TOPflash stable transfectants with 1 nM hPTH[1–34] intermittently or continuously, and harvested cell lysates for luciferase assays as depicted in Figure 3A. Interestingly, the intermittent exposure potentiated the effects of PTH on the TCF-dependent transactivation compared with the continuous exposure (Fig. 3B).

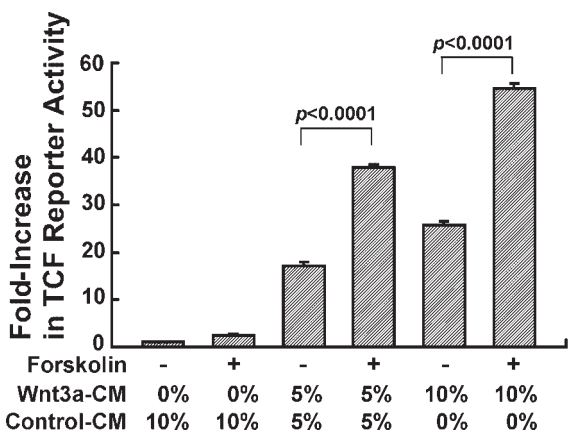

Fig. 2. Treatment with both forskolin and Wnt3a synergistically increased the TCF-dependent transactivation. Saos-2 cells transfected with Super(8x)TOPflash and a control Renilla reporter plasmid were treated with the indicated concentration of Wnt3a-conditioned medium (CM) or control-CM with or without 50 μM forskolin for 6 h. The fold-increase in the relative luciferase activity was calculated referring to the activity in the cells treated with neither Wnt3a nor forskolin as 1. The data are described as the mean ± SEM (n = 3).

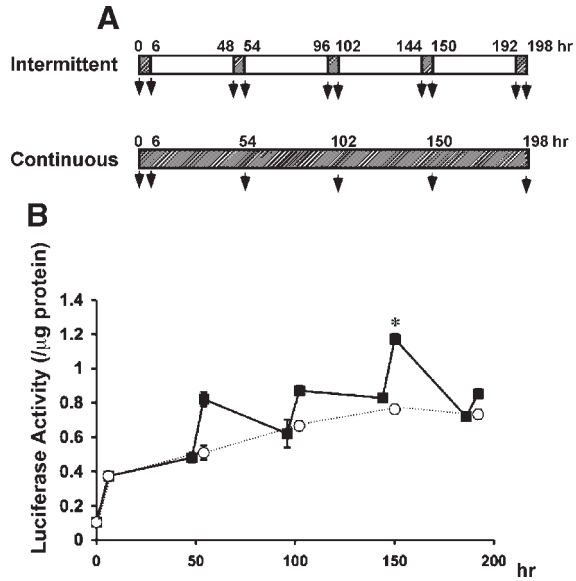

Fig. 3. Effect of intermittent and continuous treatment with PTH on TCF-dependent transactivation. **A**: Schematic representation of the experimental protocol for PTH exposure. Saos-TOPflash stable transfectants were inoculated into six-well culture plates (1×10^5cells/well). In the intermittent exposure group, the cells were exposed to hPTH[1–34] for the first 6 h of each 48-h incubation cycle. Cell lysates were harvested for luciferase assays before and after each exposure. In the continuous exposure group, the cells were exposed to hPTH[1–34] throughout the experiment. During this period, the culture medium was replaced every 48 h. The hatched bars indicate the periods of exposure. The arrows indicate the time-points of harvesting cells. **B**: TCF-reporter activity of the Saos-TOPflash stable transfectants. Cells were exposed to PTH[1–34] intermittently (closed squares) or continuously (open circles) as shown in (A). The data are shown as the mean ± SEM (n = 4). * significantly different from the value in the continuous exposure group at the same time-point ($P < 0.05$).

PTH/cAMP/PKA Signaling Increases the Expression of Endogenous Wnt Target Genes

Genes such as *WISP1*, *2*, *3*, *DLX2*, *3*, *5*, *Msx2*, *BMP4*, *NKD2* are reported to be regulated by canonical Wnt signaling [Parisi et al., 2006; Si et al., 2006; Willert and Jones, 2006]. To examine whether PTH/cAMP signaling actually influences the expression of endogenous targets of canonical Wnt signaling, we examined the effects of forskolin on the expression of *WISP2* and *NKD2*. As reported previously, addition of Wnt1-containing conditioned medium induced the expression of both *WISP2* and *NKD2* (Fig. 4A). An increase in the expression of *WISP2* was found on treatment with 50 μM forskolin in a time-dependent manner. Expression of *NKD2* was also induced on treatment with forskolin (Fig. 4B). Treatment with 5 μM

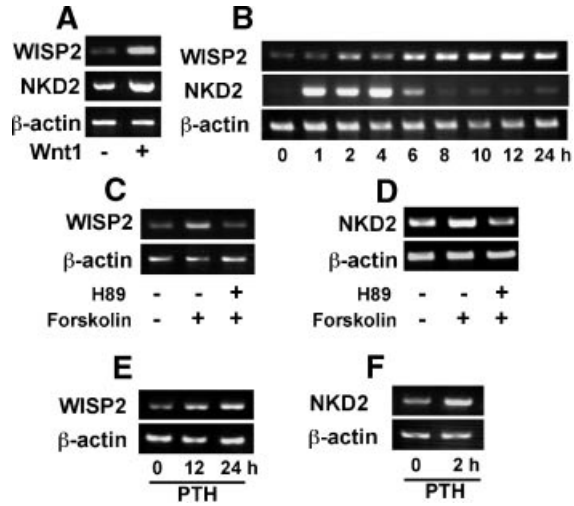

Fig. 4. The effects of forskolin on the expression of the endogenous target genes of Wnt were analyzed by RT-PCR. **A**: Induction of *WISP2* and *NKD2* expression by Wnt1. Saos-2 cells were treated with Wnt1-CM or control-CM (final concentration, 20%) for 6 h. The expression level of *β-actin* was also analyzed as an internal control. **B**: The expression of *WISP2* and *Naked Cuticle 2* (*NKD2*) was increased by treatment with forskolin. Saos-2 cells were treated with 50 μM forskolin for the indicated period. **C**,**D**: Forskolin induced the expression of *WISP2* (in C) and *NKD2* (in D) in a PKA-dependent manner. Saos-2 cells were pre-treated with 1 μM H89 or vehicle for 1 h, and then treated with 50 μM forskolin for 6 h (in C) or 2 h (in D). The pre-treatment with H89 suppressed the forskolin-induced expression of *WISP2* and *NKD2*. **E**,**F**: PTH induced the expression of *WISP2* (in E) and *NKD2* (in F). Saos-2 cells were treated with 1 μM PTH for the indicated period.

forskolin also induced the expression of *WISP2* and *NKD2*, although less effectively than the treatment with 50 μM forskolin (data not shown). Addition of H89 cancelled the effect of forskolin on the expression of *WISP2* and *NKD2*, suggesting the involvement of the cAMP/PKA pathway (Fig. 4C,D). Treatment with 1 μM hPTH[1–34] also increased the expression of *WISP2* and *NKD2* (Fig. 4E,F). Treatment with 100 nM PTH induced the expression of these genes as well, though to less of an extent (data not shown). These results indicate that PTH/cAMP/PKA increases the expression of endogenous Wnt target genes as well as the TCF/LEF-dependent reporter activity.

Reduced Expression of DKK1 was not Responsible for the TCF-Dependent Transactivation Induced by cAMP/PKA Signaling

As mentioned above, the results suggested that cAMP/PKA signaling exerts an influence on the canonical Wnt pathway. To clarify the underlying molecular mechanisms, we next examined the effects of forskolin on the expression of the components of the Wnt signaling pathway. The expression of *WNT1*, *2*, *3*, *4*, *5a*, *7a*, *8b*, *10b*, *13*, *β-catenin*, *FRZ2*, *5*, *DKK1*, *3*, *DVL1*, and *GSK-3β* was examined. The expression of *DKK1* was markedly suppressed by the treatment with forskolin (Fig. 5A). The expression of the other genes examined was not obviously changed (data not shown). Addition of H89 abolished the effect of forskolin on the expression of *DKK1*, indicating the involvement of PKA (Fig. 5B). Since DKK1 is known to antagonize the canonical Wnt signaling pathway by down-regulating the expression of LRP5/6 from the cell surface, it is plausible that the reduction in *DKK1* expression leads to the enhanced Wnt signaling in response to the increase in intracellular cAMP. Therefore, we introduced the expression plasmid encoding DKK1 into Saos-2 cells to cancel the forskolin-induced down-regulation of *DKK1* expression. As expected, the overexpression of *DKK1* reduced the basal reporter activity from Super (8x)TOPflash. However, the enhancement of the reporter activity by forskolin was retained with the overexpression of *DKK1* (Fig. 5C,D). These results suggest that down-regulation of *DKK1* expression is unlikely to be responsible for the TCF-dependent transactivation induced by cAMP/PKA signaling.

Involvement of GSK-3β Inactivation in the TCF-dependent Transactivation Induced by PTH/cAMP/PKA Signaling

There is a study suggesting that PKA potentially phosphorylates GSK-3β at the amino acid Ser^9, by which GSK-3β is inactivated and the degradation of β-catenin is avoided [McManus et al., 2005]. Therefore, we examined the involvement of GSK-3β's inactivation in the TCF-dependent transactivation induced by PTH/cAMP/PKA signaling in Saos-2 cells. Western blotting demonstrated that GSK-3β was phosphorylated at Ser^9 within 5 min in response to the addition of 50 μM forskolin (Fig. 6A). Treatment with hPTH[1–34] also resulted in the phosphorylation of GSK-3β at Ser^9, although the time to reach the maximum varied depending on the concentration of PTH (Fig. 6B). Since the phosphorylation at this site leads to the inactivation of GSK-3β, we assume that both forskolin and PTH exerted the

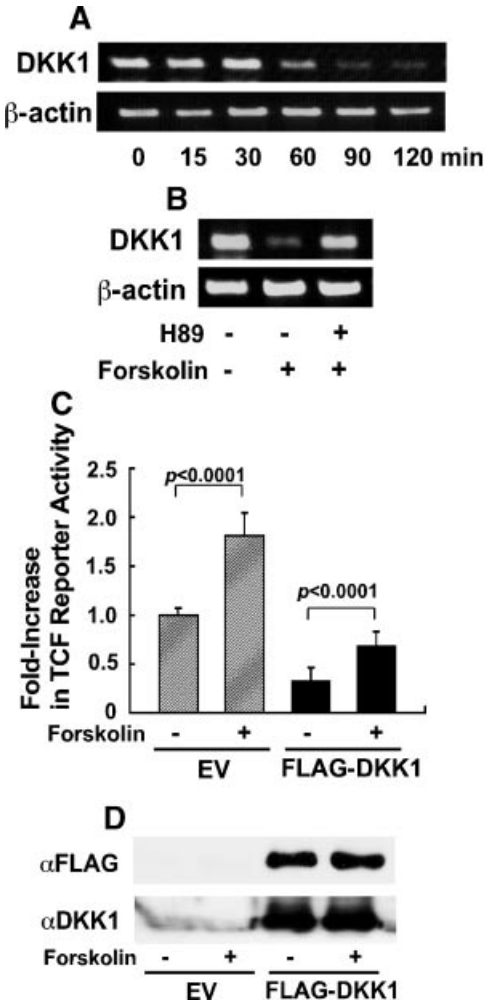

Fig. 5. The down-regulation of *DKK1* expression little contributes to the TCF-dependent transactivation induced by forskolin. **A**: The down-regulation of *DKK1* expression on treatment with forskolin. Saos-2 cells were treated with 50 μM forskolin for the indicated period, and subjected to RT-PCR. **B**: Forskolin down-regulated the expression of *DKK1* in a PKA-dependent manner. Saos-2 cells were pre-treated with 1 μM H89 or vehicle for 1 h, and then treated with 50 μM forskolin for 2 h. The pre-treatment with H89 cancelled the forskolin-induced down-regulation of *DKK1* expression. **C**: Overexpression of *DKK1* failed to abolish the TCF-dependent transactivation induced by forskolin. Super(8x)TOPflash and a control Renilla reporter plasmid were introduced into Saos-2 cells together with pFLAG-DKK1 (FLAG-DKK1) or pFLAG-CMV1 empty vector (EV). After 24 h of serum-starvation, the cells were treated with 50 μM forskolin or vehicle for 6 h, and cell lysates were harvested to determine the reporter activity. The fold-increase in the relative luciferase activity was calculated referring to the activity in the vehicle-treated EV-transfected cells as 1. The data are described as the mean ± SEM (n = 3). **D**: The overexpression of *DKK1* in (C) was confirmed by Western blotting using antibodies against FLAG® and DKK1.

positive effects on canonical Wnt signaling via phosphorylation of GSK-3β at Ser^9 by PKA. Indeed, co-treatment with H89 abolished the phosphorylation of GSK-3β at Ser^9 (Fig. 6C).

To confirm that forskolin and PTH facilitate canonical Wnt signaling through the inactivation of GSK-3β by PKA, a constitutively active form of GSK-3β which is resistant to phosphorylation by PKA, GSK-3β[S9A], was introduced into Saos-2 cells. The expression of GSK-3β[S9A] suppressed the basal reporter activity from Super(8x)TOPflash, and more importantly, abolished the induction of the reporter activity by forskolin, indicating that GSK-3β is the convergence point of this cross-talk (Fig. 6D,E).

Wnt3a Suppressed the Forskolin-Induced Expression of RANKL

As mentioned above, our results have indicated that PTH/cAMP/PKA signaling exerts positive effects on canonical Wnt signaling through the inactivation of GSK-3β. Therefore, we next examined whether Wnt signaling has an influence conversely on PTH/cAMP signaling. To address this issue, the expression of *RANKL* was analyzed, which is one of the target genes of PTH/cAMP signaling in osteoblasts. The expression of *RANKL* was up-regulated by addition of forskolin, and simultaneous treatment with Wnt3a suppressed the expression (Fig. 7), indicating that Wnt signaling exerts an influence on PTH/cAMP signaling.

DISCUSSION

In the present study, we have demonstrated in osteoblastic Saos-2 cells that the intracellular accumulation of cAMP, induced by either PTH or forskolin, facilitates canonical Wnt signaling in association with the activation of PKA. The reporter activity from Super(8x)TOPflash with multiple TCF/LEF responsive elements was clearly increased by treatment with PTH or forskolin, while that from Super (8x)FOPflash was not (Fig. 1B,D). In addition, PTH or forskolin facilitated the accumulation of β-catenin in the nucleus (Fig. 1C). Nuclear accumulation of β-catenin induced by PTH has also been reported in murine osteoblastic MC3T3-E1 cells [Tobimatsu et al., 2006]. Moreover, we have found that treatment with PTH or forskolin induced the expression of endogenous targets of the canonical Wnt signaling pathway

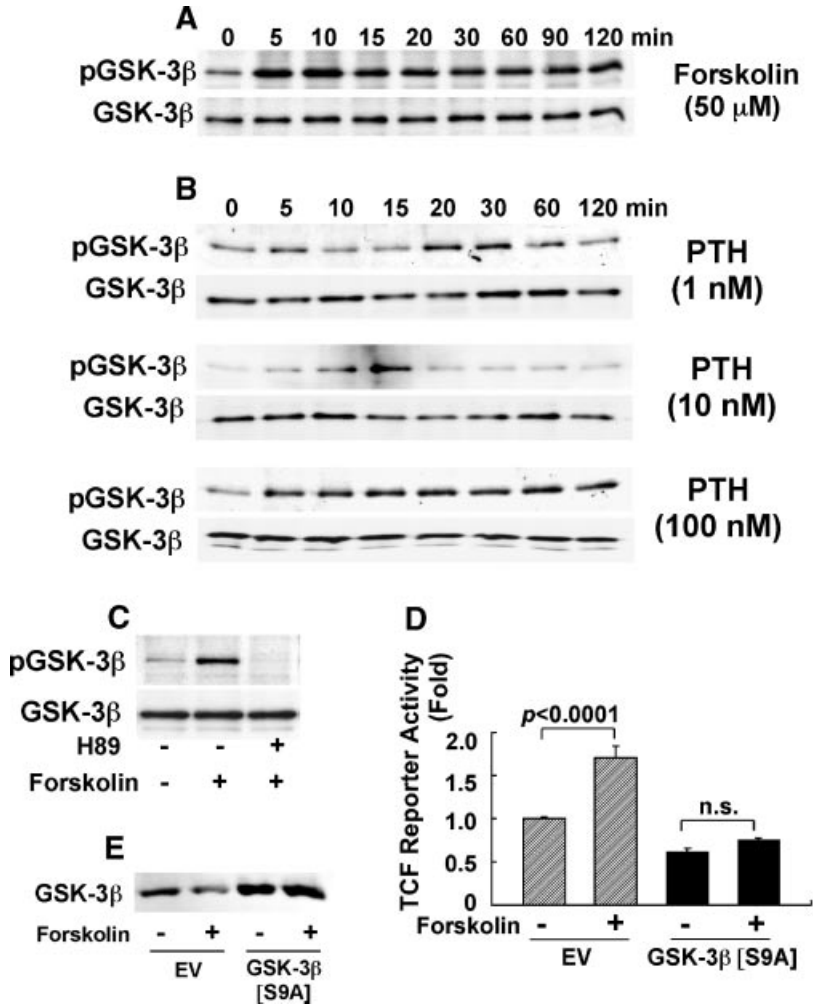

Fig. 6. The phosphorylation of GSK-3β at Ser[9] is involved in the facilitated canonical Wnt signaling induced by PTH/cAMP signaling. **A**,**B**: Treatment with 50 μM forskolin (A) or PTH (B) induced the phospholylation of GSK-3β at Ser[9]. Saos-2 cells were serum-starved overnight, and then treated with 50 μM forskolin (in A) or 1–100 nM PTH (in B) for the indicated period. Cell lysates were harvested and subjected to Western blotting. Both forskolin and PTH induced phosphorylation of GSK-3β at Ser[9]. **C**: Forskolin-induced phosphorylation of GSK-3β at Ser[9] was PKA-dependent. Saos-2 cells were pre-treated with 1 μM H89 for 1 h, and then treated with 50 μM forskolin for 5 min. Pre-treatment with H89 cancelled the phosphorylation of GSK-3β at Ser[9]. **D**: Overexpression of constitutively active form of GSK-3β abolished the TCF-dependent transactivation induced by treatment with forskolin. Super(8x)TOPFlash and a control Renilla reporter plasmid were introduced into Saos-2 cells together with pcDNA-GSK-3β[S9A] or pcDNA empty vector (EV). After 24 h of serum-starvation, the cells were treated with 50 μM forskolin or vehicle for 6 h, and cell lysates were harvested to determine the reporter activity. The fold-increase in the relative luciferase activity was calculated referring to the activity in the vehicle-treated EV-transfected cells as 1. The data are described as the mean ± SEM (n = 3). **E**: The overexpression of GSK-3β[S9A] in (D) was confirmed by Western blotting using the antibody against GSK-3β.

such as *WISP2* and *NKD2*, although the time course differed between these two genes (Fig. 4B,E,F). The enhancing effects of increased cAMP levels on TCF-reporter activity and the expression of endogenous target genes of Wnt signaling were cancelled by pretreatment with H89, indicating the involvement of PKA (Figs. 1E and 4C,D). The regulation of endogenous target genes of Wnts by cAMP/PKA signaling suggests the physiological significance of the effects of the PTH/cAMP/PKA pathway on canonical Wnt signaling. In mature osteoblasts, *WISP2* may activate osteoblastic function, because it belongs to the CCN family whose members are reported to promote osteoblastic activity [Parisi et al., 2006; Si et al., 2006]. Since the canonical Wnt signaling pathway contributes to the promotion of osteoblastic differentiation of mesenchymal stem cells, the activation of osteoblasts, and bone mineral

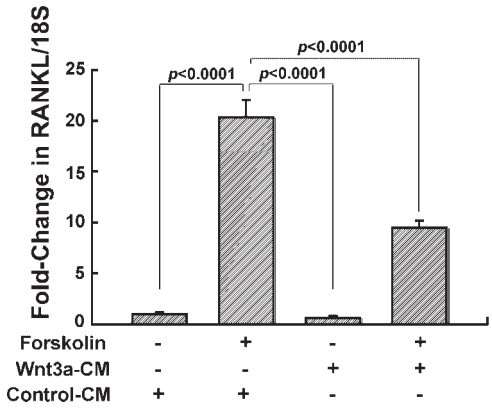

Fig. 7. Wnt3a suppressed the forskolin-induced *RANKL* expression. Saos-2 cells were treated with 20% Wnt3a-CM or control CM in the presence or absence of 50 μM forskolin for 24 h, and total RNA was extracted for real-time PCR for *RANKL*. The expression level of *RANKL* was standardized based on the amount of 18S ribosomal RNA, and the fold-change in the relative *RANKL* expression was calculated referring to the value in the cells incubated in the absence of forskolin and Wnt3a-CM as 1. The data are described as the mean ± SEM (n = 3).

accrual [Day et al., 2005; Glass et al., 2005; Jackson et al., 2005], the anabolic effect of PTH on bone might be explained, at least partly, by the crosstalk between the PTH and Wnt signaling pathways [Kulkarni et al., 2005; Martin et al., 2006].

The previous studies using intact and osteopenic animals have demonstrated that intermittent administration of PTH increases bone mass, whereas continuous infusion causes a decrease [Gensure et al., 2005; Potts, 2005]. Ishizuya et al. [1997] attempted to reproduce the intermittent administration of PTH in vitro, and reported that PTH exerted disparate effects on osteoblastic differentiation depending on exposure time in rat osteoblastic cells. They reported that a 6-h exposure to PTH in each 48-h incubation cycle efficiently induces osteoblastic differentiation of rat osteoblastic cells with an increase in the expression of osteoblastic markers including alkaline phosphatase and osteocalcin, while continuous treatment with PTH inhibited osteoblastic differentiation. They also demonstrated the involvement of IGF-1 in the osteoblastic differentiation induced by intermittent exposure to PTH [Ishizuya et al., 1997]. In the present study, we examined the effects of intermittent and continuous exposure to PTH on the TCF-dependent transactivation in the Saos-TOPflash stable transfectants following their protocol. Interestingly, the intermittent exposure potentiated the effects of PTH on the TCF-dependent transactivation compared with the continuous exposure (Fig. 3), suggesting that the effects of PTH on the canonical Wnt signaling might be strengthened by the intermittent administration, although the precise mechanisms are unclear.

Based on the results shown in Figure 6, PTH/cAMP signaling is likely to influence the canonical Wnt pathway via the phosphorylation of GSK-3β at Ser^9 by PKA, which leads to its inactivation. The inactivation of GSK-3β results in the stabilization and nuclear accumulation of β-catenin. In fact, overexpression of the constitutively active form of GSK-3β abolished the activation of TCF/LEF-dependent transcription by forskolin (Fig. 6D), providing strong evidence that the convergence point of these signaling pathways is GSK-3β. Consistent with our results, several reports describe the modulation of GSK-3β activity by PKA in other cell types [Yusta et al., 2002; Jensen et al., 2006]. In contrast, Hino et al. [2005] reported the direct phosphorylation of β-catenin by PKA as the mechanism of crosstalk between the canonical Wnt pathway and cAMP/PKA. They reported that PKA phosphorylates a different serine residue of β-catenin from GSK-3β, which leads to the stabilization of β-catenin. It is plausible that PKA acts both on GSK-3β and on β-catenin. However, phosphorylation of GSK-3β by PKA seems to be more important in osteoblastic cells, because the overexpression of the constitutively active GSK-3β, which functions upstream of β-catenin, abolished the effects of forskolin on TCF-dependent transactivation in the present study (Fig. 6D). Interestingly, glucocorticoid also affects the activity of GSK-3β in osteoblasts, leading to deterioration of the canonical Wnt signaling and osteoblastic function [Smith and Frenkel, 2005].

Recently, it has been reported that PTH stimulates bone formation in mice deficient in *Lrp5*, suggesting that *Lrp5* is not essential for the stimulatory effects of PTH on bone formation [Iwaniec et al., 2007]. Our results have indicated that PTH/cAMP signaling facilitates canonical Wnt signaling through the inactivation of GSK-3β by PKA (Fig. 6), which is consistent with the dispensability of *Lrp5* for the stimulatory effects of PTH on bone formation, because the inactivation of GSK-3β is an intracellular event downstream of Lrp5.

Another possible mechanism for the effects of PTH/cAMP/PKA signaling on the canonical

Wnt pathway is the decreased expression of *DKK1*, encoding a soluble inhibitor of canonical Wnt signaling, by cAMP [Kulkarni et al., 2005]. Actually, the expression of *DKK1* was reduced in Saos-2 cells by treatment with forskolin (Fig. 5A). However, it took 1 h before the expression of *DKK1* was decreased by the addition of 50 μM forskolin, while the nuclear accumulation of β-catenin was observed 5 min after the addition (Figs. 1C and 5A). To examine whether the down-regulation of *DKK1* expression by cAMP plays a significant role in the crosstalk between PTH/cAMP/PKA and the canonical Wnt pathway, an expression plasmid encoding *DKK1* was introduced into Saos-2 cells. The overexpression of *DKK1* failed to abolish the stimulatory effect of forskolin on the TCF-dependent transactivation in Saos-2 cells, although the basal reporter activity from super(8x)TOPflash was reduced (Fig. 5C). The basal reporter activity was decreased by the overexpression of *DKK1*, probably due to the extracellular antagonism of the endogenous Wnt expressed by Saos-2 cells. However, the overexpression of *DKK1* failed to abolish the effects of increased cAMP on the canonical Wnt pathway. Although we cannot exclude the possibility that the expression level of *DKK1* was not sufficient, we prefer to propose an intracellular convergence point of these two pathways rather than a reduction in extracellular antagonism by DKK1. Indeed, our finding shown in Figure 6 strongly suggests that GSK-3β provides an intracellular convergence point between PTH/cAMP/PKA signaling and Wnt singaling. Since the down-regulation of the expression of *DKK1* is reported to be important to the function of osteoblasts at a late stage, it might mediate long-term effects of Wnt signaling in bone [van der Horst et al., 2005].

In contrast to the facilitating effect of PTH/cAMP/PKA signaling on the canonical Wnt pathway, the Wnt signaling suppressed the expression of *RANKL* induced by PTH or forskolin. Since RANKL plays a pivotal role in osteoclastic differentiation and activation, the suppressive effect of canonical Wnt signaling on *RANKL* expression may reduce the bone resorption activated by PTH. Considering the positive interaction in bone formation and the negative one in bone resorption between the PTH/cAMP/PKA and canonical Wnt pathways, anabolic effects of PTH on bone may be achieved. However, the crosstalk point between them in the *RANKL* induction remains to be established. It has been reported that PTH induces the expression of *RANKL* via distal multiple cAMP-responsive elements [Fu et al., 2006; Kim et al., 2007]. On the other hand, several lines of evidence show that the canonical Wnt signaling alone suppresses *RANKL* expression [Shin et al., 2005].

The crosstalk with the canonical Wnt pathway is not unique to PTH/cAMP/PKA signaling. Other signaling pathways involving BMP [Nakashima et al., 2005], TGF-β [Warner et al., 2005], and nuclear receptors [Mulholland et al., 2005] are also reported to interact with the canonical Wnt pathway. The canonical Wnt pathway may mediate the function of these signaling molecules in bone as well as that of PTH.

In conclusion, PTH/cAMP/PKA signaling enhances the canonical Wnt pathway by the inhibition of GSK-3β. Moreover, Wnt signaling in turn suppresses PTH-induced *RANKL* expression. Thus, the interaction between the PTH and Wnt signaling pathways may at least partially account for the anabolic effect of PTH on bone.

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