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Original

The Comparison between Single vs Repeated Administration of Wnt3A of HPDL Cells

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Abstract: Developmental studies indicate the Wnt/ β-catenin pathway biphasically regulates tissue differentiation. Bone morphogenetic protein (BMP) and Wnt signaling reported function in osteoblast differentiation, but few studies have investigated these complex mechanisms. We hypothesized that Wnt/β-catenin signaling acts, either promoting or inhibiting osteogenesis of human periodontal ligament (HPDL) cells depending on times of treatment of Wnt3A. HPDL cells were treated with single or repeated administration (12- or 24-h intervals) of 10 ng/ml of Wnt3A and cultured for 3 days. Single Wnt3A administration increased alkaline phosphatase (ALP) activity. Conversely, repeated Wnt3A administration significantly decreased the expression of osteoblast marker genes. Single Wnt3A administration resulted in Smad1/5 and Akt phosphorylation; however, repeated Wnt3A administration inhibited it. BMP2/7 or IGF-1 treatment was sufficient to reverse the inhibitory effects of repeated Wnt3A treatment. Single administration of a glycogen synthase kinase 3 inhibitors, CHIR99021, increased *ALP* expression, but repeated CHIR99021 administration significantly decreased *ALP* expression and ALP activity compared with single administration. These findings suggest that repeated activation of Wnt signaling inhibits osteoblast differentiation by suppressing the BMP2/7–Smad1/5 and phosphatidylinositide 3-kinase (PI3K) /Akt pathways in HPDL cells. This indicated that crosstalk between the Wnt, Smad1/5, and PI3K/Akt pathways is important for osteoblastic differentiation of HPDL cells.

Key words: Transforming Growth Factors, Periodontal Ligament, Osteogenesis, Wnt Proteins

Introduction

The human periodontal ligament (HPDL) cells exhibit the potential to differentiate into cells of multiple lineages, including osteoblasts, *in vitro*[1,2], and provide the machinery to regenerate periodontal tissues, including alveolar bone, whereas growth factors such as cytokines found in periodontal tissues modulate cellular activities and induce cell differentiation. We previously reported an experimental model that allows the investigation of inhibitory effects of transforming growth factor-β1 (TGF-β1) on osteoblast differentiation and found that repeated or high-dose administration of TGF-β1 inhibited osteoblast differentiation of HPDL cells by decreasing *insulin-like growth factor-1* (*IGF-1*) expression and subsequently downregulating phosphatidylinositide 3-kinase (PI3K)/Akt signaling[3,4]. However, many aspects of the mechanisms by which HPDL cells differentiate into osteoblasts remain unclear.

Wnt signaling is a critical regulator of skeletal physiology. Wnt signaling activation enhances osteoblastogenesis and suppresses adipogenesis by inhibiting peroxisome proliferator-activated receptor γ (PPARγ)[5]. Wnt signaling draw many attentions recently,because neutralizing antibodies against Wnt antagonists are promising new drugs for bone diseases. But recent evidences indicated that Wnt signaling exerts a pivotal biphasic and dosage-dependent regulation of osteoclastogenesis[6,7]. Therefore, although Wnt signaling is known as potent enhancer of osteoblast differentiations, these eviedences still indicate that Wnt signaling might have biphasic function in osteoblast differentiations. Based on these facts and our previous results, we hypothesized that Wnt3A might have biphasically effects on osteoblastic differentiation of HPDL cells like our reported of TGF-β1.

Wnt signaling plays essential roles in osteogenesis by directly stimulating *runt-related transcription factor 2* (*RUNX2*) gene expression, promoting osteogenesis[8]. Recently, a previous study involving the knockout of inhibitors of Wnt signaling revealed the importance of Wnt signaling in bone formation and metabolism[9]. The *sclerostin* (*SOST*) gene encodes the SOST protein, which antagonizes Wnts by binding to LRP5/6 identically to Dkk1; *SOST* mutations prevent osteocytes from secreting sufficient levels of fully functional SOST[10]. The bone-generating

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Table 1. Primers used for quantitative real time PCR

Gene symbol	GenBank™ accession no.	Primsequence:sense/antisense	Probe no.	Amplicon
RUNX2	NM_001024630.2	5'-gtgcctaggcgcatttca-3'	#29	78bp
		5'-gctcttcttactgagagtggaagg-3'		
ALP	NM_000478.3	5'-caaccctggggaggagac-3'	#19	78bp
		5'-gcattggtgttgtacgtcttg-3'		
BSP	NM_004967.3	5'-caatctgtgccactcactgc-3'	#38	74bp
		5'-tcattttggtgattgcttcct-3'		
COL1A1	NM-000088.3	5'-gggattccctggacctaaag-3'	#67	63bp
		5'-ggaaacctcgctctcca-3'		
IGF-1	NM_000618.3	5'-tgtggagacaggggctttta-3'	#67	84bp
		5'-atccacgatgcctgtctga-3'		
MSX-2	NM_002449.4	5'-tcggaaaattcagaagatgga-3'	#70	86bp
		5'-caggtggtagggctcatatgtc-3'		
18 S rRNA	M11188.1	5'-cggacaggattgacagattg-3'	#77	78bp
		5'-cgctccaccaactaagaacg-3'		

effects of targeting functional antagonists of Wnt signaling have yielded new therapeutic tools to neutralize Wnt antagonists for the treatment of selected bone diseases and skeletal conditions.

Wnt3A is a major activator of the canonical Wnt pathway[11]. Wnt3a is involved in bone formation and maintenance during growth and in the adult skeleton, and it reportedly plays an important role in the metabolism of bone mass[12-14]. However, crosstalk between canonical Wnt signaling and other known regulators of osteogenesis such as the Bone morphogenetic protein (BMP)/Smad and PI3K/Akt, is only undergoing investigation[15-19]. In a recent report, genetic ablation of a BMP receptor was unexpectedly shown to enhance osteogenesis by downregulating Wnt antagonists[20]. In this study, we show that repeated Wnt3A treatment negatively regulates the osteoblastic differentiation of HPDL cells. We found that BMP2/7 or IGF-1 effectively compensate for the inhibitory effects of repeated Wnt3A treatment. Interestingly, IGF-1 exhibited a more potent compensatory effect. Furthermore, our results suggested that the BMP/Smad and PI3K/Akt pathways play important roles in Wnt3A signaling in HPDL cells and during their early-phase osteoblastic differentiation.

Materials and Methods

Cell culture and osteogenic differentiation

Normal HPDL cells were purchased from Lonza Group AG (Basel, Switzerland) and cultured in BulletKit™ Stromal Cell Growth Medium (Lonza Group AG). HPDL Cells at passages 5–8 were seeded at a density of 1×10^5 cells/cm^2 in α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA), with or without 10 ng/ml of rhWnt3A (R&D Systems, Minneapolis, MN, USA) added the following day. Some cells were treated with 3-μM CHIR99021 (R&D Systems), a GSK3β inhibitor. Some cells were also treated with 200 ng/ml IGF-1 (Wako Pure Chemical Industries, Ltd.) or 100 ng/ml BMP2/7 (R&D Systems). In the repeated administration group, the medium was replaced with fresh α-MEM containing Wnt3A or Wnt3A and IGF-1 or Wnt3A and BMP2/7, every 12 or 24 h, for 3 days. Control cells were cultured in α-MEM.

ALP activity assay

Three days after stimulation, cells were washed two times with phosphate-buffered saline (Invitrogen), fixed with 4 % paraformaldehyde for 5 min at room temperature, and washed three times with distilled water. For staining, an ALP substrate solution (Roche Diagnostics, Basel, Switzerland) was added to the fixed cells for 60 min at room temperature. After staining, cells were washed three times with distilled water, and images were analyzed.

Real-time reverse transcription-polymerase chain reaction

Total cellular RNA was extracted using QIAzol® Lysis Reagent (Qiagen NV, Venlo, Limburg, The Netherlands) according to the manufacturer's instructions. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA). Real-time reverse transcription-polymerase chain reaction (rRT-PCR) was performed on a 7500 Real-Time PCR System (Applied Biosystems, Inc.) using the Premix Ex Taq™ reagent (Takara Bio Inc., Shiga, Japan). The target genes were *ALP*, *bone sialoprotein* (*BSP*), *IGF-1*, *RUNX2*, *msh homeobox 2* (*MSX2*), and *collagen type I alpha 1* (*COL1A1*). All primers and probes are presented in Table 1 and were designed using Probefinder (http://qpcr.probefinder.com/organism.jsp). The expression of each gene was normalized to that of 18S rRNA.

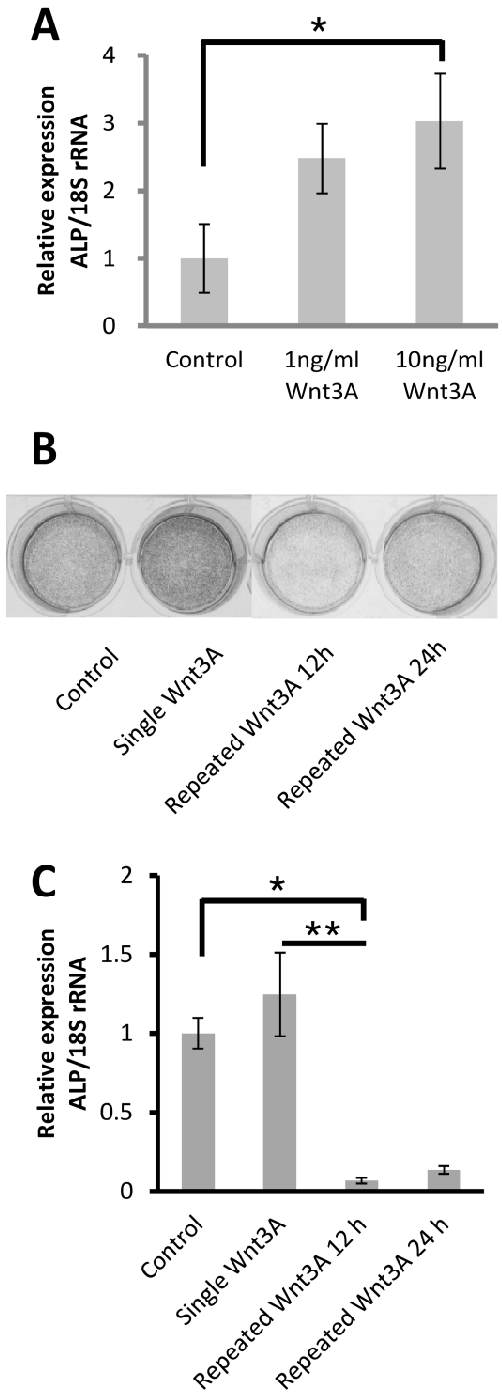

Figure 1. Single Wnt3A administration activates *alkaline phosphatase* expression in a concentration-dependent manner in HPDL cells.
(A) mRNA expression levels of ALP in HPDL cells, treated with a single administration of 1, or 10 ng/ml of Wnt3A and cultured in α-MEM for 3 days, were examined by rRT-PCR. (n = 4). (**P < 0.01, *P < 0.05). (B) ALP activity assay of single or repeated administration of Wnt3A and untreated (Control). (C) mRNA expression levels of ALP treated with single or repeated administration of Wnt3A were examined by rRT-PCR. (n = 5). (**P < 0.01, *P < 0.05).

The relative expressions of genes of interest were estimated by the ΔΔ*Ct* method. Each experiment was performed in triplicate, and the data represent the means ± standard deviation (**P < 0.01, *P < 0.05).

Protein extraction and immunoblotting

Cells were lysed in lysis buffer [10-mM Tris-HCl (pH 7.5), 150-mM NaCl, complete protease inhibitor mixture, 1-mM sodium orthovanadate, and 1 % Nonidet P-40], and protein concentrations were measured using the DC™ Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equivalent amounts of protein were separated by electrophoresis on NuPAGE® 4 %–12 % Bis-Tris gels (Invitrogen) and transferred on to polyvinylidene difluoride membranes. Membranes were probed with anti-Akt/anti-phosphorylated Akt (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-Smad1/anti-phosphorylated Smad1/5 (1:1000; Cell Signaling Technology, Inc.) antibodies, followed by horseradish peroxidase-conjugated goat anti-rabbit Immunoglobulin G antibody (MP Biomedicals, Illkirch-Graffenstaden, France). Bound antibodies were visualized using a chemiluminescent substrate (ECL™ Prime Western Blotting Detection Reagent; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and ImageQuant LAS 4000 Mini (GE Healthcare).

Statistical analysis

Each experiment was repeated three times. All data are expressed as means ±standard error of the mean. Differences were assessed using the Kruskal–Wallis test with the Tukey–Kramer *post-hoc* test.

Results

ALP expression of HPDL cells following Wnt3A treatment

Single Wnt3A treatment (1 or 10 ng/ml) increased *ALP* expression in HPDL cells cultured in α-MEM in a dose-responsive manner (Fig. 1A).

Repeated Wnt3A administration inhibits ALP activity of HPDL cells

Single administration of 10 ng/ml of Wnt3A increased ALP activity significantly, as assessed by ALP cell staining (Fig. 1B). Conversely, repeated Wnt3A administration decreased ALP activity (Fig. 1B). ALP activity exhibited by cells decreased as the number of Wnt3A treatments increased. Single Wnt3A administration increased *ALP* expression; however, repeated Wnt3A administration reduced *ALP* expression by almost 90 % in HPDL cells (Fig. 1C).

Expressions of osteoblast markers and related genes in HPDL cells

As shown in Fig. 2A-E, repeated Wnt3A administration caused a significant decrease in the expressions of osteoblast

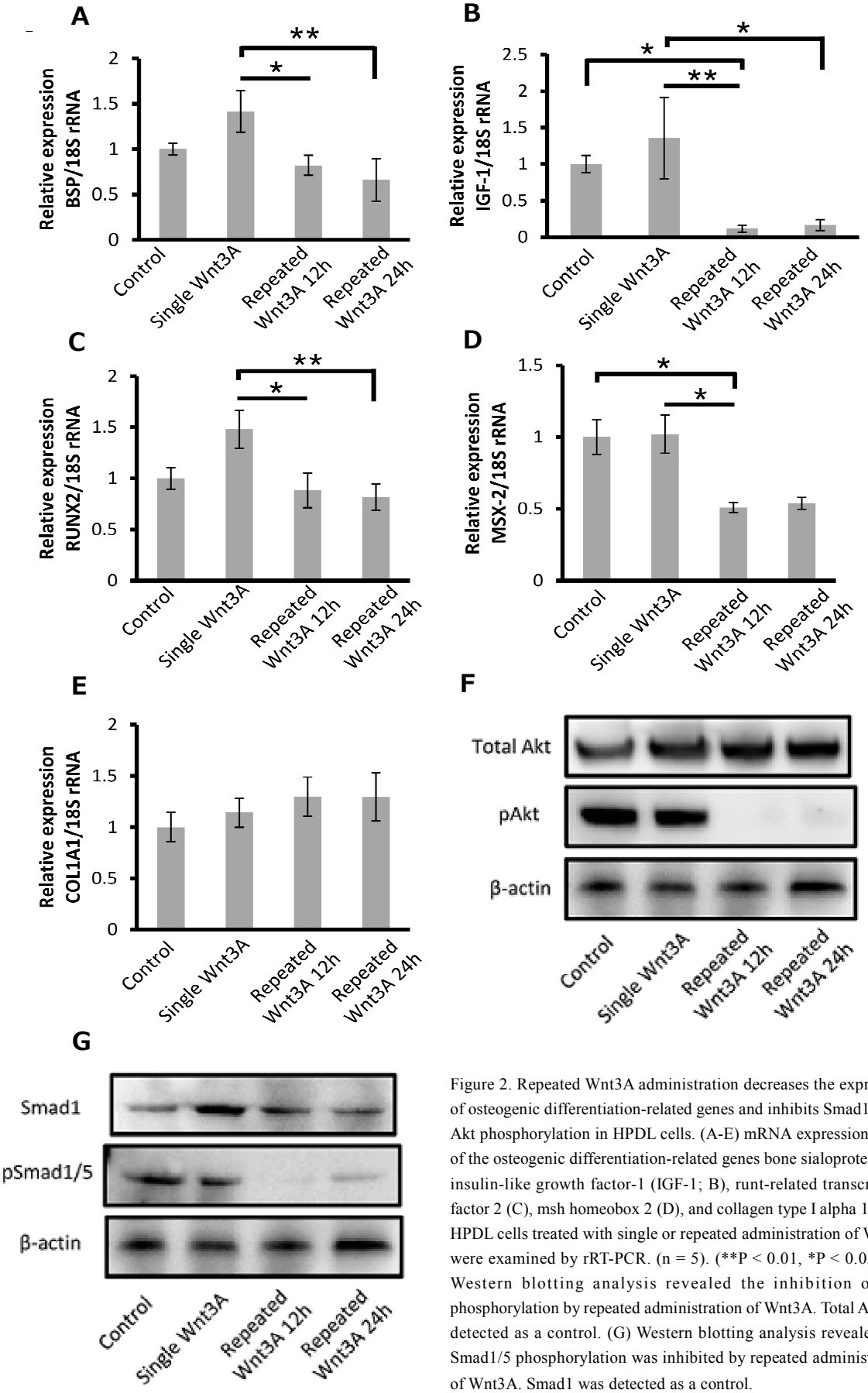

Figure 2. Repeated Wnt3A administration decreases the expression of osteogenic differentiation-related genes and inhibits Smad1/5 and Akt phosphorylation in HPDL cells. (A-E) mRNA expression levels of the osteogenic differentiation-related genes bone sialoprotein (A), insulin-like growth factor-1 (IGF-1; B), runt-related transcription factor 2 (C), msh homeobox 2 (D), and collagen type I alpha 1 (E) in HPDL cells treated with single or repeated administration of Wnt3A were examined by rRT-PCR. (n = 5). (**P < 0.01, *P < 0.05). (F) Western blotting analysis revealed the inhibition of Akt phosphorylation by repeated administration of Wnt3A. Total Akt was detected as a control. (G) Western blotting analysis revealed that Smad1/5 phosphorylation was inhibited by repeated administration of Wnt3A. Smad1 was detected as a control.

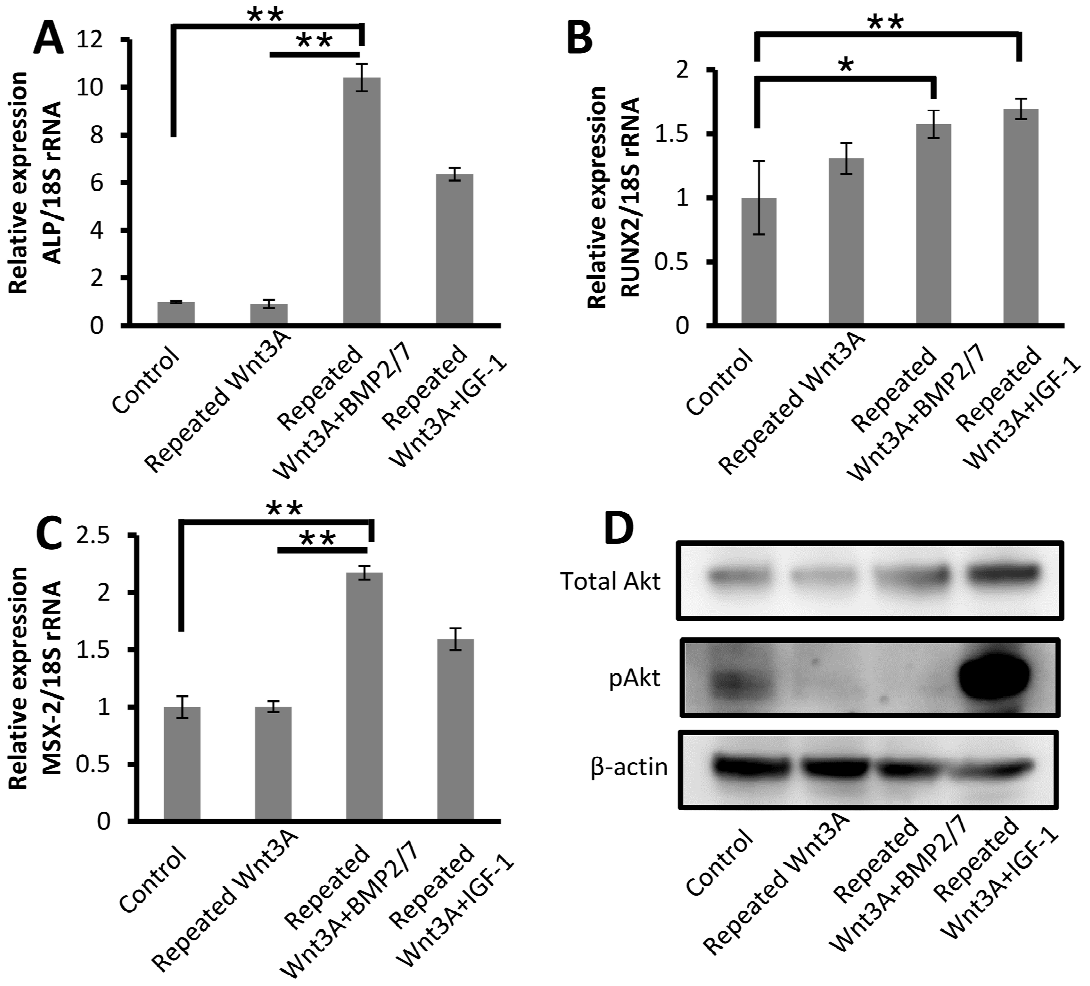

Figure 3. Exogenous IGF-1 and BMP2/7 reversed the inhibition of osteogenic differentiation by repeated Wnt3A administration. (A) mRNA expression levels of ALP treated with repeated administration of Wnt3A and IGF-1, or repeated administrations of Wnt3A and BMP2/7 were examined by rRT-PCR. (n = 5). (**P < 0.01, *P < 0.05). (B and C) mRNA expression levels of the osteogenic differentiation-related genes RUNX2 (B) and MSX-2 (C) in HPDL cells were examined by rRT-PCR (n = 5). (**P < 0.01). (D) Western blotting analysis revealed that the inhibition of Akt phosphorylation caused by repeated Wnt3A administration was reversed by IGF-1. Total Akt was detected as a control.

differentiation-related genes (*BSP*, *IGF-1*, and *RUNX2)*. Expression of *MSX2*, which is known to regulate osteoblast differentiation under the control of BMP2, also significantly decreased following repeated Wnt3A administration. We observed no significant difference in *COL1A1* expression following single and repeated Wnt3A administrations (Fig. 2E).

Effect of repeated Wnt3A administration on the Smad and PI3K/Akt pathways in HPDL cells

Previously, we reported that repeated TGF-β1 treatment reduced IGF-1 secretion, which subsequently downregulated PI3K signaling[3]. We examined whether the inhibitory effects caused by repeated Wnt3A treatment were reversed by IGF-1 administration. Next we investigated Akt activation and found that repeated Wnt3A treatment significantly reduced Akt phosphorylation (Fig. 2F). Because BMP2 reportedly regulates osteoblast differentiation through MSX-2 and RUNX2, we examined BMP/Smad1/5 activation[21]. Repeated Wnt3A administration inhibited phosphorylation of Smad1/5 (Fig. 2G). In controls, we observed normal phosphorylation of Smad1/5 and Akt (Fig. 2F and G).

Exogenous IGF-1 and BMP2/7 reversed the inhibition of osteogenic differentiation by repeated Wnt3A administration

The 100-ng/ml BMP2/7 treatment significantly induced *ALP* expression even with the repeated Wnt3A administration (Fig. 3A). The reversal also caused increased *ALP* expression in the IGF-1 addition group (Fig. 3A) and was accompanied by increased Akt phosphorylation (Fig. 3D). We observed significant difference in *RUNX2* expression when cells were treated with Wnt3A and BMP2/7 repeatedly (Fig. 3B). Exogenous IGF-1 treatment significantly increased *RUNX2* expression (Fig, 3B). Exogenous

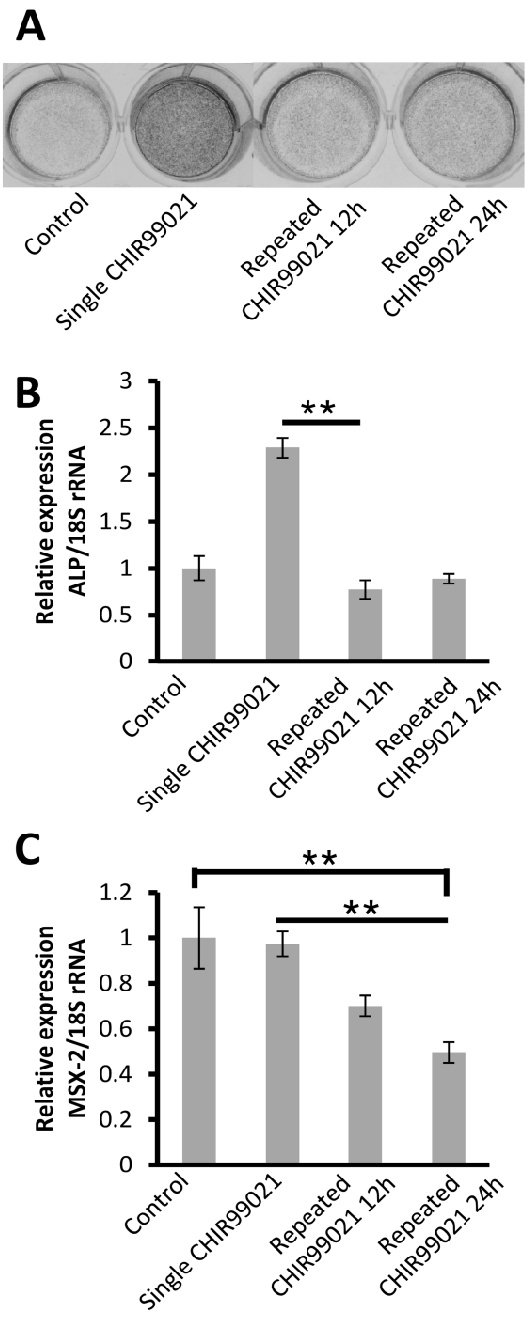

Figure 4. The effect of single or repeated CHIR99021 administration on the osteogenic differentiation of HPDL cells. (A) ALP activity assay of single or repeated administration of CHIR99021 and untreated (Control). (B and C) mRNA expression levels of ALP (B) and MSX-2 (C) in HPDL cells treated with single or repeated administration of CHIR99021 were examined by rRT-PCR (n = 5). (**P < 0.01).

BMP2/7 significantly increased *MSX2* expression (Fig. 3C).

Effect of CHIR99021 administration on the expressions of osteoblast markers and differentiation-related genes in HPDL cells

We investigated the effects of single or repeated administration of CHIR99021, a specific GSK3β inhibitor, on osteoblast differentiation of HPDL cells. GSK3β is an inhibitor of β-catenin translocation into the nucleus; therefore, CHIR99021 administration activates the canonical Wnt/β-catenin pathway. Single CHIR99021 administration increased ALP activity and expression (Fig. 4A and B). Repeated CHIR99021 administration significantly decreased ALP activity and expression, as evident after repeated Wnt 3A administration (Fig. 4A and B). Repeated CHIR99021 administration every 24 h significantly decreased *MSX2* expression compared with single CHIR99021 administration (Fig. 4C).

Discussion

In this study, we report two important findings. First, repeated Wnt3A treatment downregulates *ALP* expression and early-phase osteoblast differentiation of HPDL cells. Previously, we reported that repeated TGF-β1 treatment to HPDL cells downregulates *ALP* expression[4]. We observed subsequent decreased *IGF-1* expression and inhibition of PI3K/Akt signaling[3]. In this study, we found that repeated Wnt3A treatment caused both decreased *IGF-1* expression and decreased Akt phosphorylation, also previously observed following repeated TGF-β1 administration[3].

Wnts trigger multiple signals essential for tissue regeneration and embryonic development[22]. It is established that Wnt3A functions in osteoblast differentiation[23]. As shown in Figure 1B, single Wnt3A administration induced ALP activation in HPDL cells. Wnts are essential during many stages of osteoblast lineage development and maturation; however, their signaling mechanisms are complex. Genetic analyses involving mutations in Wnt receptors and their antagonists implicate Wnt signaling in bone disease[9]. A fast-growing field of Wnt signaling implies aberrant Wnt signaling underlies a wide range of bone diseases[24]. Wnt3A and Wnt5A mutations are embryonic lethal; however, heterozygous Wnt3A and Wnt5A mouse mutants exhibit decreased bone mineral density[25]. Therefore, even marginal alterations in the amplitude and duration of Wnt signaling affect skeletal formation and bone remodeling[13]. Thus, prolonged activation of Wnt signaling may cause biphasically effects through excessive activation of feedback mechanisms.

We found that repeated Wnt3A administration could suppress *ALP* expression (Fig. 1B and C), similar to repeated TGF-β1 administration, as we previously reported [4]. Expressions of other osteoblast markers and differentiation-related genes such as *BSP*, *IGF-1*, *RUNX2*, and *MSX2* are also decreased following repeated Wnt3A administration (Fig. 2A-D). We found that repeated Wnt3A administration remarkably decreased *IGF-1* expression and, consequently, Akt phosphorylation (Fig. 2B and F). IGF-1 plays an important role in cell growth, differentiation, survival, cell cycle progression and regulation of bone homeostasis[26]. *IGF-1* knockout mice reportedly exhibit short-limb dwarfism, delays in mineralization, and increased chondrocyte apoptosis[27]. Our recent

report revealed that the inhibition of autocrine *IGF-1* expression may inhibit osteogenic differentiation[3]. We found that repeated Wnt3A administration suppressed both *IGF-1* expression and Akt phosphorylation, indicating the inhibition of the IGF-1/PI3K pathway. However, exogenous IGF-1 reversed this inhibition, suggesting that pathways downstream of IGF-1/PI3K signaling were not blocked by repeated Wnt3A treatment.

Our second important finding was the importance of the BMP/Smad pathway in Wnt signaling. Repeated Wnt3A treatment decreased *MSX2* expression (Fig. 2D). BMP2 reportedly induces *MSX2* and *RUNX2*, which in turn induce *Osterix*[21]. It is possible that reduced *MSX2* expression downregulates BMP signaling of HPDL cells. Other indications of crosstalk between Wnt and BMP2 signaling are evident in BMP-receptor knockout mice. Osteoblast-specific conditional knockout mice for BMP receptor type IA reportedly exhibit increased bone mass during embryonic development due to the suppression of the Wnt inhibitor SOST[20]. These findings suggest that BMP2/7 treatment induces Wnt inhibitors, enhancing the inhibition of osteoblast differentiation. Contrary to our expectations, BMP2/7 could remarkably increase *ALP* expression even following repeated Wnt3A treatment (Fig. 3A). We found that repeated Wnt3A treatment decreased Smad1/5 phosphorylation (Fig. 2G). The addition of BMP2/7 to HPDL cells undergoing repeated Wnt3A treatment significantly increased *MSX2* expression (Fig. 3C). These facts sugeested that prolonged Wnt3A treatment causes downregulation of the BMP/Smad cascade. It is established that the PI3K/Akt signaling pathway is enhanced by DNA binding of Runx2 and Runx2-dependent transcription[28]. Runx2 increases ALP activity, expression of *BMP* genes, and mineralization in immature mesenchymal cells and osteoblastic cells *in vitro*[13,28-30]. Thus, repeated Wnt3A treatment coordinately inhibits the BMP and IGF-1/PI3K pathways. According to these results, we speculated that repeated Wnt3A administration would decrease the expression of *MSX2*, a direct target of the Smad pathway, which in turn would inhibit early-phase osteogenic differentiation of HPDL cells.

A previous study reported that CHIR99021, an inhibitor of GSK3β, induced osteogenesis, as indicated by elevated ALP activity, in ST2 mice mesenchymal cells[31]. Our results showed that single CHIR99021 administration increased ALP activation and expression, whereas repeated CHIR99021 administration significantly decreased ALP activation and expression compared with single CHIR99021 administration (Fig. 4A and B). In addition, repeated CHIR99021 administration decreased *MSX2* expression (Fig. 4C). A previous report showed that the inhibition or stimulation of GSK3β activity resulted in induction or suppression of ALP activity, respectively, through a GSK3β-dependent mechanism[32]. *Gsk3β* knockout mice do not survive embryogenesis; however, *Gsk3β* $^{+/-}$mice exhibit higher trabecular bone volume density, more osteoblasts per area of bone surface, and increased bone formation rates[33]. These results are consistent with those of previous studies showing decreased bone mineralization in targeted heterozygous Wnt3A or Wnt5A mice[25].

These biphasic function of Wnt signaling might have a clinical significance, because Wnt activation is a promising therapeutic strategy for treating bone diseases based on its currently known bone formation-stimulating anabolic effects. For example, a neutralizing monoclonal antibody (Ab) against the Wnt antagonist sclerostin, which is secreted specifically from osteocytes[34], has been shown to markedly increase bone formation and reverse estrogen deficiency-induced bone loss[35]. Currently, SOST-antibody is being developed as a new anabolic treatment for bone disorders, such as postmenopausal osteoporosis[35]. Other findings provide evidences that Wnt activation also inhibits osteoclast differentiation and bone resorption[7]. Consistent with this notion, in a recent first-in-humans study, a sclerostin monoclonal antibody not only increased bone formation marker but also decreased bone resorption marker in a dose-dependent manner. Therefore, bone-specific activators of Wnt/β-catenin signaling may promise an exciting new class of drugs that can more effectively prevent and treat skeletal fragility. But our evidences indicate that Wnt signaling might not be that simple. Developmental studies indicate that the Wnt canonical pathway biphasically regulates tissue differentiation[7]. Even BMP2 pathway could negatively regulate Wnt signaling by inducing *SOST* [20].

In summary, it is possible that sufficient Wnt signaling is required for proper osteoblast differentiation. Excess or insufficient Wnt3A may inhibit osteoblast differentiation, i.e., alterations in the intensity, amplitude, and duration of Wnt signaling affects the osteogenesis of HPDL cells positively and negatively. There is a great deal of complexity about bone remodeling, as detailed in many reports and in the results of this study. Thus, further studies are warranted to clarify its complicated mechanisms.

In conclusion, Wnt3A plays an important role of osteogenic differentiation of HPDL cells by activation of not only Wnt signaling but also of the Smad and PI3K/Akt pathways. Conversely, repeated Wnt3A administration has a negative effect on osteogenic differentiation of HPDL cells. This negative effect of Wnt3A was reversed by IGF-1 or BMP2/7. Thus, crosstalk between Wnt3A, IGF-1, and BMP2/7 signaling is necessary for the regulation of osteoblast differentiation. We believe that a better understanding of these complex mechanisms will lead to find new way to study about the role of Wnt sgnaling in pathogenesis of periodontal diseases and eventually will lead to find a new clue for bone regeneration of periodontal tissues.

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