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Wnt3A positively and negatively regulates osteoblast differentiation of human periodontal ligament cells

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ABSTRACT

Despite accumulated knowledge of various cellular signalings regulating bone formation, the complex molecular network has not been revealed sufficiently to lead to clinical application of periodontal therapies. We previously reported an experimental model to study the inhibitory mechanism of TGF-β1 in osteoblast differentiation, and our findings confirmed that TGF-β1 has a dual effect in osteogenesis, promotion and suppression in human periodontal ligament (HPDL) cells. This study investigated that the effect of multiple repeated Wnt3A administration in osteogenic differentiation of HPDL cells. HPDL cells were treated with single or multiple (12 h or 24 h intervals) 10 ng/ml Wnt3A for 3 days. Single Wnt3A administration increased ALP activities as well as *ALP* expression compared with repeated Wnt3A administration. Repeated Wnt3A administration caused significant decrease of osteoblast differentiation-related genes expressions (*BSP*, *IGF-1*, *RUNX2* and *MSX-2*). Single Wnt3A administration caused Smad1/5 and Akt phosphorylation, however repeated Wnt3A administration strongly inhibited phosphorylation of Smad1/5 and Akt. Single CHIR99021 administration increased *ALP* expression, but repeated CHIR99021 administration significantly decreased *ALP* expression and activity. Repeated CHIR99021 administration showed

significant decrease of *MSX-2* expression compared with single CHIR99021 administration. This study indicated the possibility of dual effects of Wnt3A on osteogenesis in HPDL cells. It is also suggested that Wnt signaling has a crosstalk with Smad pathway and PI3K/Akt pathway in HPDL cells. We found that Smad pathway and PI3K/Akt pathway play important roles for this adverse effect of Wnt3A.

INTRODUCTION

The human periodontal ligament (HPDL) is a connective tissue consisting of heterogeneous population which includes undifferentiated mesenchymal cells, osteoprogenitors, and fibroblasts (1). HPDL cells exhibit the potential to differentiate into multi-lineage cells including osteoblasts *in vitro* (2). Therefore, HPDL cells are featured as a useful source of regenerative engineering of periodontal therapy.

We previously reported an experimental model to study the inhibitory mechanism of transforming growth factor-β1 (TGF-β1) in osteoblast differentiation and found that a single low dose TGF-β1 administration significantly promoted osteoblast differentiation, but its repeated or high dose administration inhibited osteoblast differentiation in HPDL cells (3). We also reported that TGF-β1 inhibits osteoblast differentiation through suppression of insulin-like growth factor-1 (IGF-1) expression and subsequent down-regulation of the PI3K/Akt pathway (4). However, there are many unclear points of the mechanisms leading to the differentiation into osteoblasts in HPDL cells.

Wnt signaling comprises a family of 19 secreted glycoproteins that have functions related to cell specification, formation of the body plan, cell growth, differentiation and apoptosis (5). When Wnt signaling is active, glycogen synthase

kinase 3β (GSK3β) is inhibited. This loss of GSK3β activity allows cytosolic β-catenin to accumulate and translocate to the nucleus where it binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and activates transcription of Wnt-regulated target genes (5). It was previously found that canonical Wnt signaling plays essential roles in osteogenesis by directly stimulating runt-related transcription factor 2 (*Runx2*) gene expression promoting osteogenesis (6). Recent several evidences revealed the importance of Wnt signaling in bone formation and bone metabolism by knockout experiments of Wnt signaling inhibitors (7). For example, dickkopfs 1 (Dkk1) is a well-characterized secreted Wnt signaling inhibitor. *Dkk1*$^{+/-}$ mice have increased bone formation and bone mass without a compensatory change in bone resorption (8). Secreted frizzled-related proteins 1 (Sfrp1) antagonizes Wnts through direct binding. Targeted disruption of *Sfrp1* increased trabecular bone but not cortical bone mineral density to a similar extent as parathyroid hormone (9). *SOST* gene encodes the protein sclerostin which is antagonist of Wnts, the mutations introduce premature transcriptional stop codons, interfere with splicing, or delete regulatory elements in *SOST*, thereby preventing osteocytes from secreting sufficient levels of fully functional sclerostin (10). From these findings, functional antagonists of Wnt signaling may offer new therapeutic tools for select bone diseases and skeletal conditions.

Wnt3A is a secreted cysteine-rich glycoprotein, and a major canonical Wnt pathway activator (11). Wnt3a is involved in bone formation and maintenance in growth process and adult skeleton, it was reported that Wnt3a plays an important role in the metabolism of bone mass (12, 13). Wnt3a-null mouse showed the severe low bone mass (14). Other articles reported that C3H10T1/2 with overexpression of Wnt3a or a stabilized form of β-catenin cells show increased expression of bone-specific alkaline phosphatase (*ALP*) (15). Because Wnt signaling has been drawing many attentions in recent years, the crosstalk between Wnt signaling and other osteogenic factors, such as BMP/Smad pathway, PI3K/Akt pathway and Src/ERK pathway, have been investigated (16-20). Recent reports described that genetical ablation of BMP receptor unexpectedly enhanced osteogenesis due to down-regulation of Wnt antagonists (21). Because Wnt signaling has many redundant and complicated regulatoly mechanisms, we are still miles away from fully understanding relations between Wnts and osteogenesis. Here we show that multiple repeated Wnt3A treatment inversely regulate osteogenesis of HPDL cells. We found that Smad pathway and PI3K/Akt pathway play important roles for this adversed effects.

MATERIALS & METHODS

Cell culture and osteogenic differentiation of Wnt3A and GSK3β inhibitor

Normal HPDL cells were purchased from Lonza (Basel, Switzerland) and cultured in BulletKit® Stromal cell growth medium (SCGM, Lonza). The cells of passage number 5 to 8 were seeded at a density of 1×10^5 cells/cm^2 in α-MEM (Life Technologies, MD, USA), with or without 10 ng/ml rhWnt3A (R&D Systems, MN, USA) which was added the following day. The cells were also treated with or without 3 μM CHIR99021 (Funakoshi, Tokyo, Japan), a specifically inhibitor of GSK3β. The cells were re-fed every 24 h except those with repetitive Wnt3A treatment. Control cells were treated with only α-MEM.

Assay of ALP activity

Cells were washed twice with phosphate-buffered saline (PBS, pH7.4, Life Technologies), fixed with 4% paraformaldehyde for 5 min at room temperature, and washed three times with distilled water. An ALP substrate solution (Roche Diagnostics,

Basel, Switzerland) was added to the fixed cells and incubated for 60 min at room temperature. After staining, cells were washed three times with distilled water, and images were scored.

Real Time Quantitative RT-PCR (qRT-PCR)

Total cellular RNA was extracted using QIAzol reagent (Qiagen Inc., CA, USA) according to the manufacturer's instructions. cDNA was prepared using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). Real-time PCR was performed on 7500 Real-Time PCR System (Applied Biosystems) with the Premix Ex Taq^{TM} reagent (Takara Bio Inc., Shiga, Japan). The target genes were *ALP*, bone sialoprotein (*BSP*), *IGF-1*, *RUNX2*, msh homeobox 2 (*MSX-2*), and collagen type I alpha 1 (*Col1A1*). These genes expressions were normalized to 18 S rRNA expression in each sample. All primers and probes are presented in Table. Relative expression of genes of interest was estimated using $\Delta\Delta Ct$ method.

Protein extraction and Immunoblotting

Cells were lysed with 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 the protein concentrations were measured using a DC protein assay kit (Bio-Rad, Marnes-la-Coquette, France). Equivalent amount of protein were fractionated on electrophoresis on NuPAGE 4–12% Bis Tris gels (Life Technologies) and transferred onto a PVDF membrane. The membranes were probed with anti-Akt/anti-phosphorylated Akt (1:1000; Cell Signaling Technology Inc., MA, USA), anti-Smad1/anti-phosphorylated Smad1/5 (1:1000; Cell Signaling Technology) antibodies, followed by HRP-conjugated goat anti-rabbit IgG. Bound antibodies were visualized using a chemiluminescent substrate (ECL^{TM} Prime Western Blotting Detection Reagent; GE Healthcare, UK Ltd., Buckinghamshire) and ImageQuant LAS 4000 mini (GE Healthcare).

Statistical analysis

Each experiment was repeated three times. All data are expressed as mean ± S.E. Differences were assessed by Kruskal-Wallis with Tukey-Kramer post hoc test.

RESULTS

Repeated Wnt3A administration inhibits ALP activity in HPDL cells

Single administration of 10 ng/ml Wnt3A increased the number of ALP positive cells (Fig.1A). Repeated Wnt3A administration contrastingly decreased ALP activity (Fig.1A). Single Wnt3A administration significantly increased *ALP* expression compared with repeated Wnt3A administration in HPDL cells (Fig. 1B). Repeated Wnt3A administration at every 12 h significantly decreased *ALP* expression compared with only α-MEM (Fig. 1B).

The effect of repeated Wnt3A administration on the expression of osteoblast differentiation-related genes in HPDL cells

As shown in Fig. 2, repeated Wnt3A administration caused significant decrease of osteoblast differentiation-related genes expressions (*BSP*, *IGF-1*, *RUNX2* and *MSX-2*). We observed no significant differences of *COL1A1* expression.

The effect of repeated Wnt3A administration on Smad pathway and PI3K/Akt pathway in HPDL cells

Next we examined other osteogenic signaling pathways. Single Wnt3A administration caused Smad1/5 and Akt phosphorylation (Fig. 3A and B). However, repeated Wnt3A administration strongly inhibited phosphorylation of Smad1/5 (Fig. 3A). Repeated Wnt3A administration strongly inhibited phosphorylation of Akt (Fig. 3B). In controls we observed normal phosphorylation of Smad1/5 and Akt (Fig. 3A and B).

The effect of CHIR99021 administration on osteoblast differentiation-related genes in HPDL cells

We investigated the effects of single or repeated CHIR99021 administration on osteoblast differentiation. Single CHIR99021 administration increased *ALP* expression and activity compared with control (Fig. 4A and B). Repeated CHIR99021 administration significant decreased *ALP* expression and activity (Fig. 4A and B). Repeated CHIR99021 administration at every 24 h showed significant decrease of *MSX-2* expression compared with single CHIR99021 administration (Fig. 4C).

DISCUSSION

The key finding of this study is contradictory effects of Wnt3A on osteogenesis. Wnts are a large family of 19 secreted glycoproteins that trigger multiple signals essential for tissue regenerations and embryonic development (5). Mutations in several Wnt pathway components play a role in causing inherited disorder of skeletal systems (7). Germline deletion of Wnt3A causes early embryonic lethality; however, heterozygotic Wnt3A males exhibited bone loss, with decreases in bone mineral density and trabecular number (22). Recent researches revealed that polymorphisms in Wnt pathway components were linked to altered bone mineral density (8). Therefore even marginal alterations in the amplitude and durations of Wnt signaling pathways affect skeletal formation as well as bone remodeling (13).

Wnt3A induces the proliferation of mesenchymal precursor cells (23), and prevents apoptosis of cells (20). Wnt3A keep undifferentiated state of stem cells, which has been attracting attention in induced pluripotent stem cells study (24). Thus, Wnt3A has diverse and critical function in undifferentiated cells.

Wnt3A was effective in osteogenic differentiation with a single Wnt3A administration in HPDL cells as inferred from result shown in Fig. 1A. It has been well

known that Wnts are essential for many stages of osteoblast lineage development and maturation. Because Wnt3A is commercially available, numerous studies showed that Wnt3A is supposed to be involved in osteoblast function (25). Our results supported these result showing that Wnt3A could be essential for osteogenic development of cells derived from periodontal tissues. Repeated Wnt3A administration could suppress expression of *ALP* (Fig. 1B) as is the case with TGF-β1 we previously reported. Other osteoblast differentiation-related genes were reduced the expression, such as *BSP*, *IGF-1*, *RUNX2* and *MSX-2* (Fig. 2A-D). Most notably, we found that repeated Wnt3A administration remarkably decreased *IGF-1* expression and subsequently Akt phosphorylation (Fig. 2B and 3B). IGF-1 is an important regulator of bone homeostasis. The IGF-1 gene knock-out mice had short limb dwarfism, delays in mineralization and increased chondrocyte apoptosis (26). Our recent reports described that the inhibition of autocrine *IGF-1* expression may cause inhibition of osteogenic differentiation (5). It is known that PI3K/Akt signaling pathway greatly enhanced DNA binding of Runx2 and Runx2-dependent transcription (27). Runx2 increased ALP activity, expression of BMP genes, and mineralization in immature mesenchymal cells and osteoblastic cells *in vitro* (28-30).

Because RUNX2 is a direct target molecule of BMPs-Smads signaling (31), we

determined to examine whether Smad pathway is involved in the inhibition of osteogenesis of HPDL cells by repeated Wnt3A administration, our results showed that Smad1/5 phosphorylation was inhibited (Fig. 3A). Several previous studies indicated that Smad pathway is a key process in osteogenesis. Overexpression of Smad3 in MC3T3-E1 cells enhanced ALP activity and mineralization (32). Mice with a targeted deletion of Smad3 were osteopenic, had decreased bone mineral density and a decreased rate of bone formation (33). The other report showed that GSK3β down-regulates Smad1/5/8 signal by phosphorylation of Smad1/5/8 (34). It is reasonable to speculate that repeated Wnt3A administration decreased *MSX-2* expression, one of the direct target molecule of Smad pathway, which in turn inhibited osteogenic differentiation (Fig. 2D). These results suggest that Smad pathway and Wnt pathway influence each other reciprocally and plays an important role in osteogenic differentiation of HPDL cells.

A previous study reported that CHIR99021, an inhibitor of GSK3β, induces osteogenesis as indicated by elevated ALP activity in ST2 mice mesenchymal cells (35). It has long been known that GSK3β is a negative regulator of canonical Wnt signaling (13). GSK3β suppression by genetic deletion or pharmacological inhibition enhances bone density. GSK3β knock-out mice do not survive embryogenesis, however, $Gsk3\beta^{+/-}$ mice have higher trabecular bone volume density, more osteoblasts per bone surface and

increased bone formation rates (36). Our results showed that single CHIR99021 administration increased *ALP* expression and activation, while repeated CHIR99021 administration significant decreased *ALP* expression and activation compared with single CHIR99021 administration (Fig. 4A and B). In addition, repeated CHIR99021 administration significant decreased mRNA expression of *MSX-2* compared with single CHIR99021 administration (Fig. 4C). We also observed that repeated Wnt3A administration down-regulate *RUNX2* expression (Fig. 2C). Therefore, canonical Wnt pathway was not quite active in our experiments due to some feedback mechanisms. The previous report show that inhibition or stimulation of GSK3β activity resulted in either stimulation or suppression of ALP activity, through a GSK3β-dependent but β-catenin-independent mechanism (37). Our results indicated that alterations in the intensity, amplitude, and durations of Wnt signaling affects osteogenesis of HPDL cells positively and negatively. There is a great deal of complexity about bone remodeling observed in many reports along with the results shown above. Even bone morphogenic cytokines, such as TGF-β and BMPs have dual action on bone formation. BMPs have mostly positive effects on bone formation. Thus, it is not quite right that BMPs have dual effect on bone formation. But, its receptors are different, genetic deletion of one of BMP receptor type 1A revealed significant excessive bone formation. This excessive

bone formation could be related to up-regulation of Wnt signaling because *SOST* and *Dkk1* expressions were remarkably low in those experiments (22). This evidence clearly showed that BMP signaling could negatively affect on bone formation. Thus, BMP signaling could have dual effects on bone formation. TGF-β1 plays a pivotal role in connective tissue regeneration and bone remodeling. A previous report demonstrated that TGF-β1 has significant effects in osteogenic differentiation and bone formation (38). However, many evidences also indicated that TGF-β1 could negatively regulate osteogenesis. We previously reported an experimental model to study the inhibitory mechanism of TGF-β1 in osteoblast differentiation, our findings confirmed that TGF-β1 has a dual effect in osteogenesis, promotion and suppression in HPDL cells (4). Present study suggested that Wnt3A also has a dual effect in osteogenesis by the number of administration of Wnt3A in HPDL cells (Fig. 1A and B).

In conclusion, Wnt3A promotes osteogenic differentiation of HPDL cells by activation not only Wnt cascade but also Smad pathway and PI3K/Akt pathway. Repeated Wnt3A administration could affect inversely. This opposite effects of Wnt3A on osteogenic differentiation of HPDL cells might be caused by down-regulation of IGF-1/PI3K/Akt signaling cascade. Further studies are necessary to elucidate clear mechanisms. But adverse effects of Wnt signaling on osteogenesis will contribute to a

better understanding of the pathological mechanism of periodontitis that will lead to new periodontal therapies.

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FIGURE LEGENDS

Fig. 1

Repeated Wnt3A administration inhibits ALP expression and activity of HPDL cells.

(A) Confluent HPDL cells were cultured in α-MEM with 10 ng/ml Wnt3A single or repeated administration, or untreated (Control) for three days and immunohistochemically stained to assay ALP activity.

(B) The mRNA expression levels of the osteogenic differentiation genes ALP in HPDL cells treated with 10 ng/ml Wnt3A single or repeated administration for three days, were examined by qRT-PCR.

In the process of multiple administrations with Wnt3A, α-MEM containing fresh Wnt3A was changed every 12 h or every 24 h, or untreated (Control).

The ratio of the expression of each gene to that of 18 S rRNA was calculated. Each experiment was performed in triplicate, and the data represent the means ±SD. (n=5). (**P<0.01, *P<0.05, Kruskal-Wallis with Tukey-Kramer post hoc test).

Fig. 2

Repeated Wnt3A administration decrease osteogenic differentiation-related genes of HPDL cells.

(A-E) The mRNA expression levels of the osteogenic differentiation-related genes BSP (A), IGF-1 (B), RUNX2 (C), MSX-2 (D), COL1A1 (E) in HPDL cells treated with 10 ng/ml Wnt3A single or repeated administration.

The ratio of the expression of each gene to that of 18 S rRNA was calculated. Each experiment was performed in triplicate, and the data represent the means ±SD. (n=5). (**P<0.01, *P<0.05, Kruskal-Wallis with Tukey-Kramer post hoc test).

Fig. 3

Repeated administration of Wnt3A inhibits Smad1/5 and Akt phosphorylation in HPDL cells.

(A) Western blot analysis revealed that inhibition of Smad1/5 phosphorylation by repeated Wnt3A administration. Smad1 was detected as a control.

(B) Western blot analysis revealed that inhibition of Akt phosphorylation by repeated Wnt3A administration. Total Akt was detected as a control.

Fig. 4

Single or repeated CHIR99021 administrations effect on osteogenic differentiation of HPDL cells.

(A) Confluent HPDL cells were cultured in α-MEM with 3 μM CHIR99021 single or repeated administration for three days and immunohistochemically stained to assay ALP activity.

(B, C) The mRNA expression levels of the osteogenic differentiation-related genes ALP (B) and MSX-2 (C) treated with 3 μM CHIR99021 single or repeated administration for three days.

In the process of multiple administrations with CHIR99021, α-MEM containing fresh CHIR99021 was changed every 12 h or every 24 h, or untreated (Control), were examined by qRT-PCR.

The ratio of the expression of each gene to that of 18 S rRNA was calculated. Each experiment was performed in triplicate, and the data represent the means ±SD. (n=5). (**P<0.01, Kruskal-Wallis with Tukey-Kramer post hoc test).

Table

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' 5'-gctcttcttactgagagtggaagg-3'	#29	78bp
ALP	NM_000478.3	5'-caaccctggggaggagac-3' 5'-gcattggtgttgtacgtcttg-3'	#19	78bp
BSP	NM_004967.3	5'-caatctgtgccactcactgc-3' 5'-tcattttggtgattgcttcct-3'	#38	74bp
COL1A1	NM-000088.3	5'-gggattccctggacctaaag-3' 5'-ggaaacctcgctctcca-3'	#67	63bp
IGF-1	NM_000618.3	5'-tgtggagacaggggctttta-3' 5'-atccacgatgcctgtctga-3'	#67	84bp
MSX-2	NM_002449.4	5'-tcggaaaattcagaagatgga-3' 5'-caggtggtagggctcatatgtc-3'	#70	86bp
18 S rRNA	M11188.1	5'-cggacaggattgacagattg-3' 5'-cgctccaccaactaagaacg-3'	#77	78bp

Figure 1

A

Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h

B

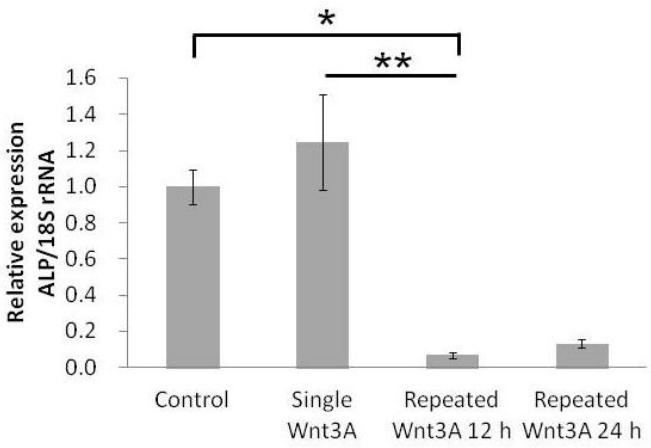

*
**
Relative expression ALP/18S rRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h

Figure 2

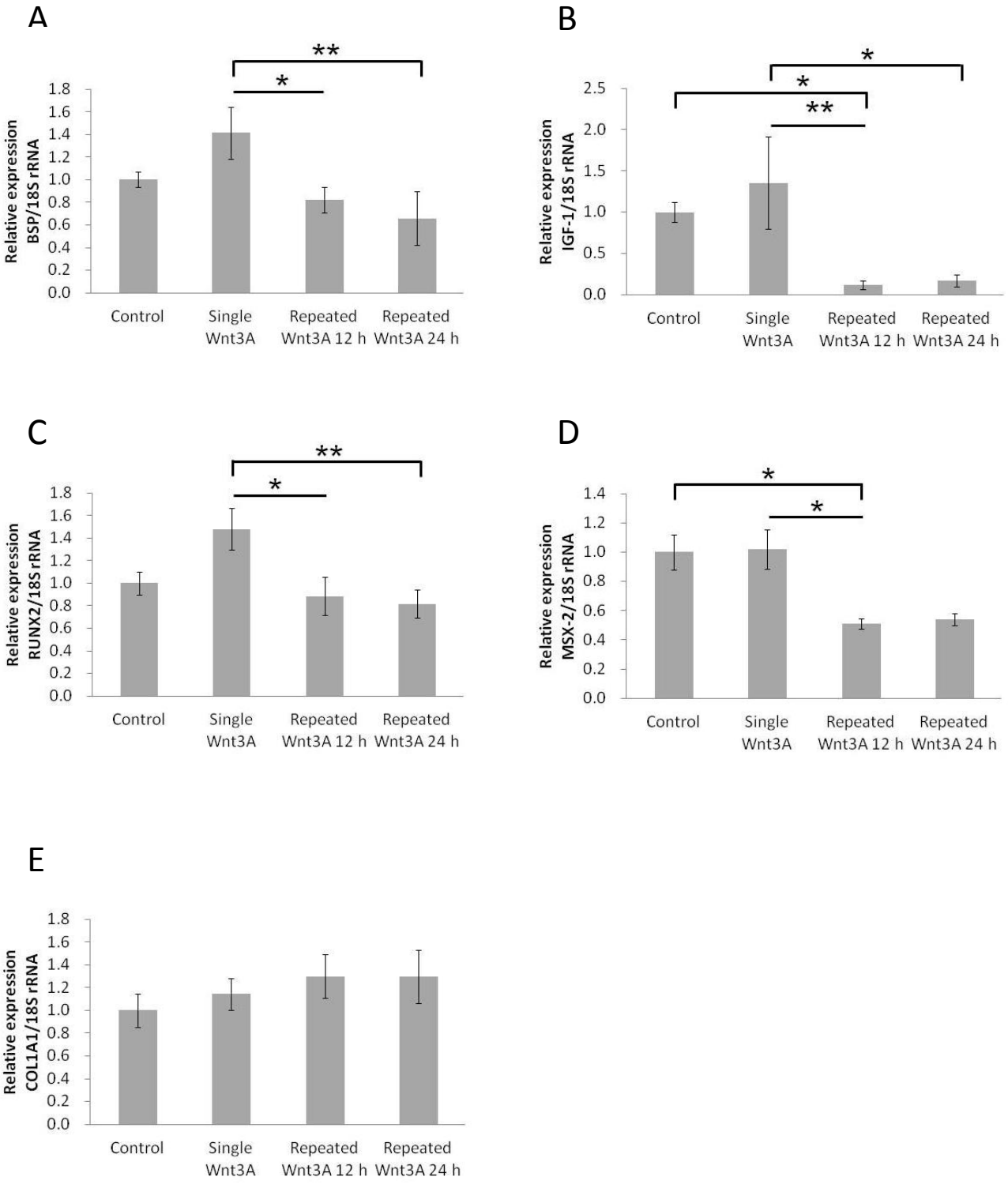

A
Relative expression BSP/18S rRNA
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h
B
Relative expression IGF-1/18S rRNA
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h
C
Relative expression RUNX2/18S rRNA
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h
D
Relative expression MSX-2/18S rRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h
E
Relative expression COL1A1/18S rRNA
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h

Figure 3

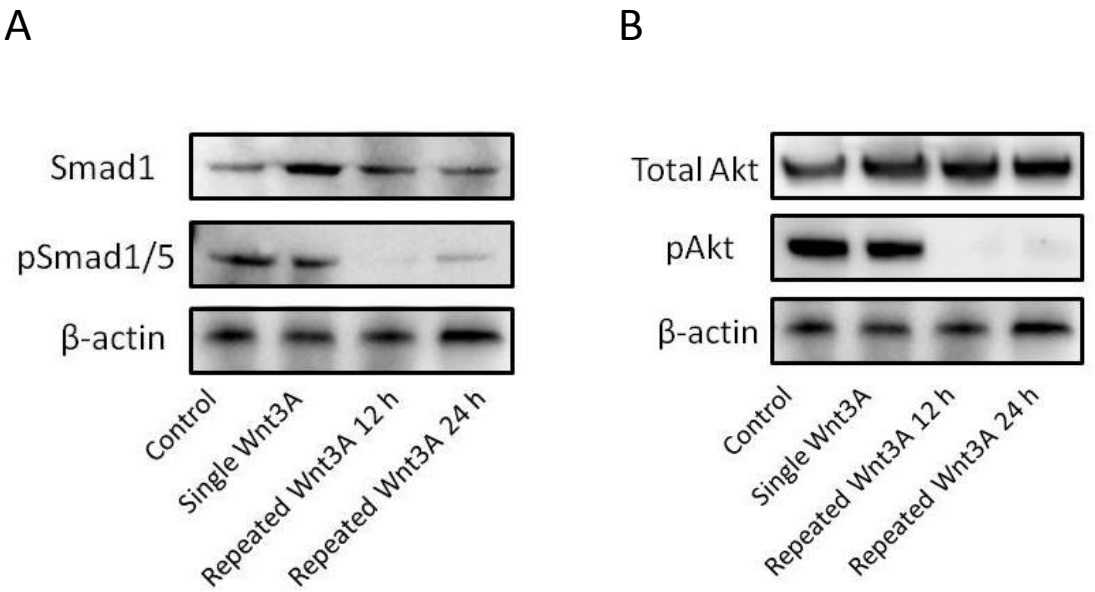
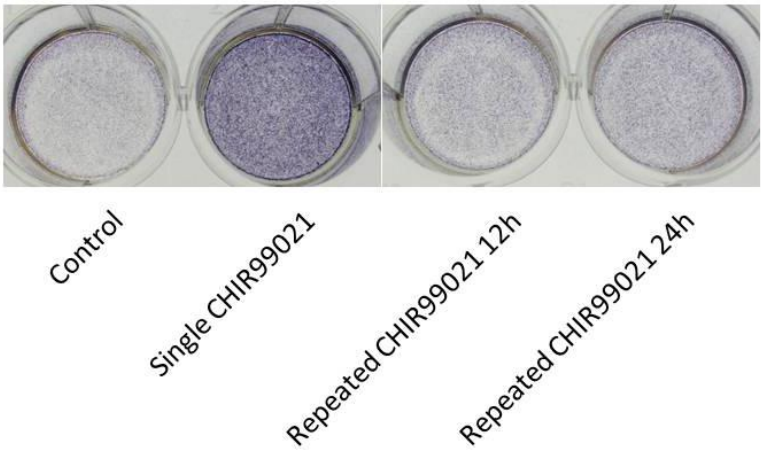
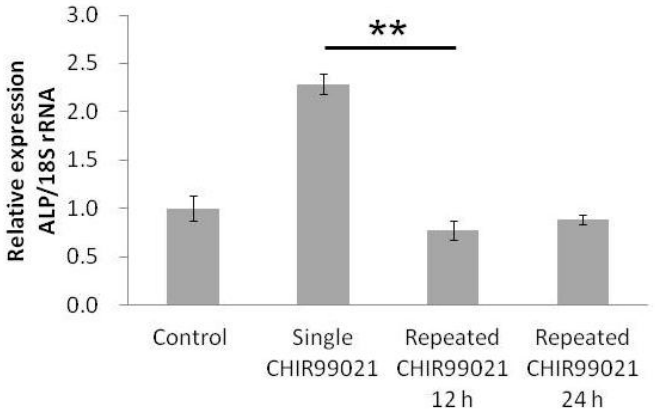

A
Smad1
pSmad1/5
β-actin
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h
B
Total Akt
pAkt
β-actin
Control
Single Wnt3A
Repeated Wnt3A 12 h
Repeated Wnt3A 24 h

Figure 4

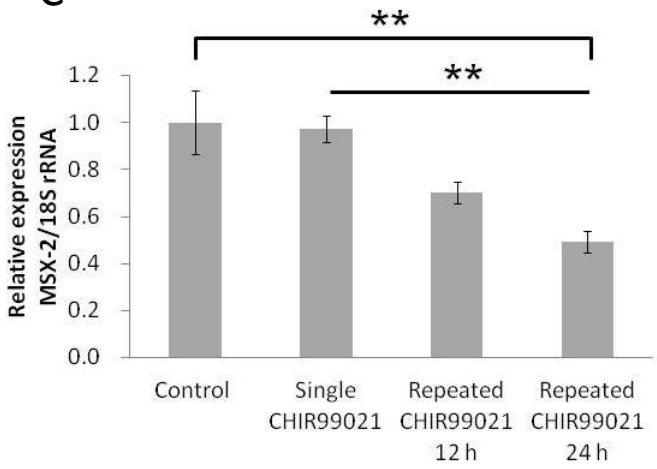
A


Control
Single CHIR99021
Repeated CHIR99021 12h
Repeated CHIR99021 24h

B


Relative expression ALP/18S rRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
Single CHIR99021
Repeated CHIR99021 12 h
Repeated CHIR99021 24 h

C


Relative expression MSX-2/18S rRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
Control
Single CHIR99021
Repeated CHIR99021 12 h
Repeated CHIR99021 24 h