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Intermittent compressive stress regulates Notch target gene expression via transforming growth factor-β signaling in murine pre-osteoblast cell line

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ABSTRACT

Objective: Different mechanical stimuli regulate behaviors of various cell types, including osteoblasts, osteocytes, and periodontal ligament fibroblasts. Notch signaling participates in the mechanical stress-regulated cell responses. The present study investigated the regulation of Notch target gene and sclerostin (*Sost*) expression in murine pre-osteoblast cell line (MC3T3-E1) under intermittent compressive stress.

Methods: MC3T3-E1 were subjected to the intermittent compressive force under the computerized controlled machine. In some experiments, cells were pretreated with chemical inhibitors for Notch and transforming growth factor (TGF)-β signaling prior to mechanical stimuli. To evaluate role of Notch signaling in MC3T3-E1 cells under unloaded condition, cells were seeded on indirect immobilized Notch ligand (Jagged1). Gene expression was determined using real-time quantitative polymerase chain reaction.

Results: The intermittent compressive stress significantly upregulated Notch target gene expression (Hes Family BHLH transcription factor 1; *Hes1* and Hairy/enhancer-of-split related with YRPW motif protein1; *Hey1*). The intermittent stress-induced *Hes1* and *Hey1* mRNA expression could be inhibited by a γ-secretase inhibitor (DAPT) or a TGF-β superfamily type I activing receptor-like kinase receptors inhibitor (SB431542). The results imply that intermittent compressive stress regulates Notch signaling via TGF-β pathway. Further, the intermittent compressive stress reduced *Sost* mRNA expression and this phenomenon could be rescued by a DAPT pretreatment, implying the involvement of Notch signaling. However, activation of Notch signaling under the unloaded condition resulted in the increase of *Sost* expression and the reduction of osteogenic marker genes.

Conclusions: These results imply the involvement of Notch signaling in the homeostasis maintaining of osteogenic cells under mechanical stress stimuli.

1. Introduction

Alveolar bone and periodontal ligament consistently receive a cyclic compressive force during normal mastication. Influence of mechanical force on osteoblast and periodontal ligament cell has widely been reported. Various publications demonstrated that cells response differently to different force types (Basso & Heersche, 2002; Cardwell et al., 2015). In this regard, it has been shown that intermittent mechanical force treatment promoted higher receptor activator of nuclear factor kappa-B ligand (RANKL) expression in periodontal ligament cells than those treated with continuous force treatment (Nakao et al., 2007). Continuous shear stress significantly enhanced in vitro osteogenic differentiation than intermittent shear stress in rat calvarial derived osteoblasts as determined by the increase of alkaline phosphatase expression and mineralization (Ban et al., 2011). Previous studies demonstrated that intermittent compressive stress induced insulin-like growth factor-1 (*IGF-1*), periostin (*POSTN*), transforming growth factor-β (*TGF-β*), and sclerostin (*SOST*) mRNA expression in human periodontal ligament cells (hPDLs) (Manokawinchoke et al., 2015; Pumklin, Manokawinchoke, Bhalang, & Pavasant, 2015). Nevertheless, the static continuous force and intermittent force regulated different cell responses (Manokawinchoke, Sumrejkanchanakij, Pavasant, & Osathanon, 2017). The effect of intermittent compressive stress on periodontal ligament cells and osteoblasts remains limited.

Mechanical stress regulates Notch signaling and further influence cell's behaviors. Cyclic strain increased Notch receptor expression and Notch subsequently promoted network formation by endothelial cells (Morrow, Cullen, Cahill, & Redmond, 2007). On the contrary, cyclic strain inhibited Notch receptor expression in vascular smooth muscle cells and the reduction of Notch signaling, in part, attenuated cell growth and enhanced apoptosis (Morrow et al., 2005). Notch signaling also participated in the intermittent regulated SOST expression in

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hPDLs (Manokawinchoke et al., 2017). In this regard, Notch signaling inhibition resulted in the reduction of intermittent stress-induced SOST expression by hPDLs (Manokawinchoke et al., 2017). Together, these evidences suggest the participation of Notch signaling in the mechanical force-regulated cell response.

Although, there are some similar responses between periodontal ligament cells and osteoblasts. Several distinct regulations of mechanical stimuli on these two cell types were reported (Nettelhoff et al., 2016; Ngan et al., 1990). For example, compressive force treatment enhanced osteopontin expression in human osteoblasts but not in hPDLs (Nettelhoff et al., 2016). Thus, an investigation regarding the specific role of intermittent stress on specific aspect for these cells is indeed necessitated. The present study aimed to investigate the regulation of Notch target gene expression in murine pre-osteoblast cell line (MC3T3-E1) under intermittent compressive stress *in vitro*.

2. Methods

2.1. Cell culture and treatment

Murine osteoblastic cells, MC3T3-E1, were maintained in Minimum Essential Medium (MEM/EBSS, Hyclone, Logan, Utah, USA) containing 10% fetal bovine serum (Hyclone, Cramlington, Northumberland, UK), 100 unit/ml penicillin and 100 μg/ml streptomycin, 250 ng/ml amphotericin B, and 2 mM L-glutamine (Gibco BRL, Carlsbad, CA, USA). Cells were maintained in humidified atmosphere and 5% CO_2, at 37 °C. The medium was changed every 2 days. For osteogenic induction, the growth medium was supplemented with 50 μg/ml L-ascorbic acid and 5 mM β-glycerophosphate.

An intermittent compressive stress treatment was performed according to our previous published protocol (Manokawinchoke et al., 2015). Briefly, cells were seeded in 6-wells tissue culture plate at density of 37,500 cells/cm^2. Serum starvation was performed for 8 h and cells were subsequently subjected to the intermittent compressive stress treatment using a computerized controlled apparatus at a force of 1.5 g/cm^2 with 14 cycles per minute.

For Notch signaling treatment, cells were seeded on Jagged-1 immobilized surface according to our previous publications (Osathanon, Nowwarote, Manokawinchoke, & Pavasant, 2013; Osathanon, Ritprajak et al., 2013; Sukarawan, Peetiakarawach, Pavasant, & Osathanon, 2016). Briefly, tissue culture surfaces were incubated with recombinant protein G (50 μg/mL, Invitrogen, USA), bovine serum albumin (10 mg/ml, Invitrogen, USA), and recombinant human Jagged-1/Fc (R & D systems, USA), respectively. The surfaces were rinsed three times with sterile PBS between each step. Human IgG, Fc fragment (hFc, Jackson ImmunoResearch Laboratory, USA) was used as the control.

In some experiments, cells were pre-treated with the following inhibitors for 30 min prior to treatment. The agents used in the present study were 4 μM SB431542 (a TGF-β inhibitor; Sigma-Aldrich Chemical, St. Louis, MO, USA) and 20 μM N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester (DAPT, Sigma-Aldrich Chemical). To confirm the influence of Tgf-**β**1, MC3T3-E1 cells were treated with recombinant human Tgf-**β**1 at concentration of 10 ng/ml in serum-free culture condition.

2.2. Polymerase chain reaction (PCR)

RNA isolation was done using RiboEx total RNA isolation solution (GeneAll, Seoul, Korea). One microgram of RNA was converted to cDNA using a reverse transcriptase kit (Promega, Madison, WI, USA). Quantitative real-time PCR was performed using FastStart® Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA) in a Lightcycler Nano RT-PCR machine (Roche Applied Science). Expression levels were normalized to the reference gene (*18S*) and the control. The primer sequences are shown in Table 1.

Table 1
Primer sequences.

Gene	Sequences (F; forward, R; reverse)		Reference
18s	F	5′CTTAGAGGGACAAGTGGCG3′	NR_003278.3
	R	5′ACGCTGAGCCAGTCAGTGTA3′	
Runx2	F	5′CGGGCTACCTGCCATCAC3′	NM_001145920.1
	R	5′GGCCAGAGGCAGAAGTCAGA3′	
Osx	F	5′CTCGTCTGACTGCCTGCCTAG3′	NM_130458.3
	R	5′GCGTGGATGCCTGCCTTGTA3′	
Alp	F	5′GCCCTCTCCAAGACATATA3′	NM_007431.2
	R	5′CCATGATCACGTCGATATCC3′	
Sost	F	5′GGAATGATGCCACAGAGGTCAT3′	NM_024449.6
	R	5′CCCGGTTCATGGTCTGGTT3′	
Hes1	F	5′GCAGACATTCTGGAAATGACTGTGA3′	NM_008235.2
	R	5′GAGTGCGCACCTCGGTGTTA3′	
Hey1	F	5′GCAGGAGGGAAAGGTTATTTTGA3′	NM_010423.2
	R	5′CGAAACCCCAAACTCCGATAG3′	
Dlx5	F	5′GCCCCTACCACCAGTACG3′	NM_010056.3
	R	5′TCACCATCCTCACCTCTG3′	
Tgf-β1	F	5′GCCCTCGGGAGCCACAAACC3′	NM_011577.2
	R	5′GCAGCAGGAGTCGCGGTGAG3′	

2.3. Mineralization assay

Samples were fixed with cold methanol and rinsed with deionized water. Further, samples were incubated in 1% Alizarin red S solution (Sigma-Aldrich Chemical) and the excess staining was washed with deionized water. Mineral deposition was quantified by solubilizing alizarin red staining in 10% cetylpyridinium chloride monohydrate. The solution's absorbance was examined at 570 nm.

2.4. Statistical analysis

Results are demonstrated as box and whisker plots. Nonparametric statistical analysis (IBM SPSS Statistics for Mac, Version 22, Armonk, NY, USA) was employed to evaluate a statistically significant difference at the p value < 0.05.

3. Results

3.1. Intermittent compressive stress regulated Notch target gene expression via Tgf-β

Cells were treated with intermittent compressive stress for 24 h. The results demonstrated that intermittent compressive stress significantly upregulated Notch target genes, Hes Family BHLH transcription factor 1 (*Hes1)* and Hairy/enhancer-of-split related with YRPW motif protein1 (*Hey1*), mRNA expression in MC3T3-E1 cells (Fig. 1A and B). When cells were pretreated with a γ-secretase inhibitor (DAPT) prior to the stimulation of intermittent compressive force. The stress-induced *Hes1* and *Hey1* mRNA expression was attenuated (Fig. 1C and D).

Previous study reported that intermittent compressive stress upregulated TGF-β1 expression in hPDLs and this mechanism participated in the regulation of Notch signaling under intermittent compressive stress (Manokawinchoke et al., 2017). MC3T3-E1 cells were treated with intermittent compressive stress for 24 h. The significant upregulation of *Tgf-β1* was observed (Fig. 2A). Inhibition of Tgf-β pathway by SB431542 attenuated the intermittent compressive stress-induced *Hes1* and *Hey1* mRNA expression (Fig. 2B and C). Correspondingly, the addition of exogenous rhTgf-β1 resulted in the significant increase of *Hes1* and *Hey1* mRNA levels at 24 h (Fig. 2D and C). Taken all evidences together, the intermittent compressive stress induced Notch target gene expression via Tgf-β signaling pathway.