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Nephronectin expression is regulated by SMAD signaling in osteoblast-like MC3T3-E1 cells

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ABSTRACT

Nephronectin (Npnt) is an extracellular matrix protein known to be a ligand for the integrin α8β1. We previously demonstrated that *Npnt* expression was suppressed by TGF-β through ERK1/2 and JNK in osteoblasts. In this study, we found that inhibition of a TGF-β type I receptor (TGF-β R1, Alk5) by a specific inhibitor {2-[3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl]-1,5-naphthyridine} strongly induced *Npnt* expression in osteoblast-like MC3T3-E1 cells. The Alk5 inhibitor-induced increase of *Npnt* expression occurred in both time- and dose-dependent manners, while that expression was also induced by introduction of an siRNA for Smad2, a central intracellular mediator of TGF-β signaling. These results suggest that the expression of *Npnt* is regulated by the Alk5-SMAD signaling pathway in osteoblasts.

1. Introduction

Nephronectin (Npnt), also known as POEM, is an extracellular matrix protein considered to play critical roles in the development and functions of various tissues [1,2]. Npnt is associated with integrin α8β1, and possesses strong cell adhesion, spreading, and survival-promoting activities [1,2]. Previous studies demonstrated that Npnt promotes osteoblast differentiation through its own epidermal growth factor-like repeats (EGF repeats) and 3′-untranslated region [3,4].

TGF-β is a secreted factor abundantly present in bone, and plays a key role in osteoblast differentiation as well as bone development and remodeling [5]. TGF-β signaling is initiated by ligand binding to a cell-surface receptor complex comprised of two types of transmembrane serine/threonine kinase receptors, termed TGF-β type I receptor [TGF-β R1, activin-like receptor kinase 5 (Alk5)] and TGF-β type II receptor (TGF-β R2), leading to activation of intracellular signaling molecules, such as SMADs, MAPKs, and other molecules [6].

In our previous study, we found that *Npnt* expression was strongly down-regulated by TGF-β through ERK1/2 and JNK in MC3T3-E1, a mouse calvaria-derived osteoblast-like cell line [7]. Although inhibition of ERK1/2 and JNK activation partially inhibited the down-regulation of *Npnt* gene expression by TGF-β, we were not able to exclude the possibility that this expression is also regulated by SMAD signaling. In the present study, we found that canonical TGF-β signaling through Smad2 regulates *Npnt* gene expression in osteoblasts.

2. Materials and methods

2.1. Reagents

TGF-β RI kinase inhibitor II (2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine) was purchased from Merck and used as the TGF-β type I receptor (Alk5) inhibitor. The antibody against Smad2 was purchased from Cell Signaling (D43B4) and that against β-actin from Sigma–Aldrich (A5060). A horseradish peroxidase-conjugated secondary antibody was purchased from GE Healthcare (NA934 V).

2.2. Cell culture

MC3T3-E1 cells (RIKEN BioResource Center) were cultured in MEMα (Wako Pure Chemical Industries, Ltd.) supplemented with 10% FBS and penicillin–streptomycin (Life Technologies). MC3T3-E1 cells were treated with 0–10 μM of Alk5 inhibitor for 0–24 h for dose- and time-dependent effects of the Alk5 inhibitor on *Npnt* gene expression.

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2.3. ALP activity

Cells were fixed with 10% formalin in PBS, then ALP activity was visualized after incubation for 20 min with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma–Aldrich), 0.6 mg/ml Fast Blue BB salt (Sigma), 2 mM $MgCl_2$, and 0.5% N,N,-dimethylformamide in 0.1 M Tris–HCl, pH 8.5. For quantification of ALP activity, the cells were disrupted by sonication on ice in 50 mM Tris–HCl (pH 7.5) containing 0.1% Triton X-100. ALP activity in the lysates was determined following incubation with the substrate p-nitrophenylphosphate (Wako), in buffer (pH 10) containing 0.1 M 2-amino-2-methyl-1-propanol and 2 mM $MgCl_2$ [8].

2.4. RT-PCR

All RNA samples were extracted with TRIzol reagent (Life Technologies), then reverse-transcribed using SuperScript III (Life Technologies). Semi-quantitative PCR was performed using the following primer sets: *Tgf-β1*, GCAACAATTCCTGGCGTTACC and CCCTGTATTCCGTCTCCTTGGT; *TGF-β2*, TCCCGAATAAAAGCGAAGAGC and GGTGCCATCAATACCTGCAAA; *TGF-β3*, CCAGATACTTCGACCGGATGA and TGACATCGAAAGACAGCCATTC. Quantitative real-time PCR was performed using a SYBR Green Fast PCR system (Life Technologies). The primer sequences were as follows: *Alp*, GGGACTGGTACTCGGATAACGA and CTGATATGCGATGTCCTTGCA; *Npnt*, CACGAGTAATTACGGTTGACAACAG and CTGCCGTGGAATGAACACAT; *Smad2*, CTCCTCATCCCATTCCTGTTC and TGCCCACACAAACCTTTCC; and *Gapdh*, AAATGGTGAAGGTCGGTGTG and TGAAGGGGTCGTTGATGG.

2.5. Introduction of siRNA

MC3T3-E1 cells (50% confluence) were seeded and transfected with a 10 pmol/cm^2 culture surface area of the *Smad2* siRNAs (Stealth siRNA, Smad2MSS206405–07, Life Technologies) using Lipofectamine RNAi MAX reagent (Life Technologies) in OPTI-MEM (Life Technologies).

3. Results and discussion

To investigate the mechanisms governing regulation of Npnt expression via the TGF-β type I receptor (Alk5) in osteoblasts, MC3T3-E1 cells were treated with TGF-β RI kinase inhibitor II (2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine), known to be a specific inhibitor of Alk5 kinase activity [9]. TGF-β1, -β2, and -β3, expected ligands for TGF-β receptors, were expressed in MC3T3-E1 cells (Suppl. Fig. S1). When MC3T3-E1 cells were treated with different concentrations of the Alk5 inhibitor for 24 h, a slight increase in *Npnt* mRNA levels was observed at a concentration of 0.1 μM (Fig. 1A). Induction by the Alk5 inhibitor appeared to plateau at 1 μM. The time-dependent effect on induction of *Npnt* expression was further examined using a fixed Alk5 inhibitor concentration of 10 μM. A significant increase in *Npnt* mRNA was detected 12 h after its addition (Fig. 1B). These results suggest that Alk5 inhibitor-induced up-regulation of *Npnt* gene expression occurs in a time- and dose-dependent manner.

Alk5 is known to phosphorylate and activate Smad2/3 in canonical TGF-β signaling. Smad2/3 then form complexes with Smad4, which allows them to translocate into the nucleus for target gene expression regulation [6,10,11]. To determine whether canonical TGF-β-Smad2/3 signaling is involved in the regulation of *Npnt* gene expression, MC3T3-E1 cells were treated with a small interfering RNA (siRNA) targeting Smad2. First, we checked mRNA and protein levels of Smad2 when treated with Smad2 siRNA. Treatment with Smad2 siRNA caused a significant decrease in levels of Smad2

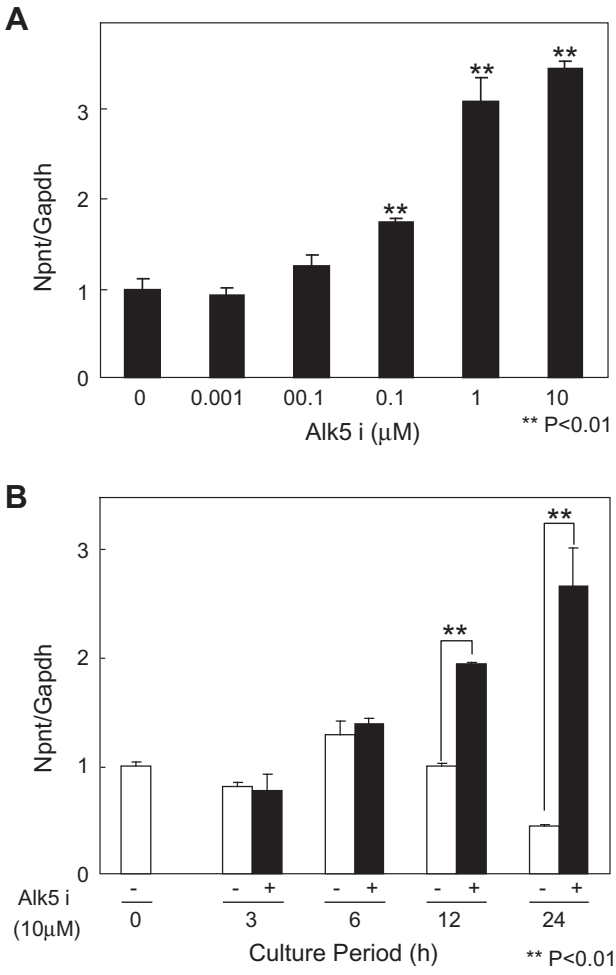

Fig. 1. Dose- and time-dependent up-regulation of *Npnt* mRNA expression by Alk5 inhibitor. Total cellular RNA was extracted, and mRNA samples for *Npnt* and *Gapdh* were examined using real-time PCR. (A) Dose-dependent effects of Alk5 inhibitor on *Npnt* mRNA expression. MC3T3-E1 cells were treated with 0, 0.001, 0.01, 0.1, 1, or 10 μM of Alk5 inhibitor for 24 h. $^{**}P < 0.01$, Student's *t* test, relative to level without Alk5 inhibitor. (B) Time-dependent effects of Alk5 inhibitor on *Npnt* mRNA expression. MC3T3-E1 cells were treated with 10 μM of Alk5 inhibitor for 3, 6, 12, or 24 h. $^{**}P < 0.01$, Student's *t* test, relative to level without Alk5 inhibitor at 12 and 24 h.

mRNA and protein (Fig. 2A). In addition, ablation of Smad2 in MC3T3-E1 cells enhanced *Npnt* gene expression (Fig. 2B). These results suggest that *Npnt* gene expression is regulated by canonical TGF-β-SMAD signaling.

Previously, we showed that TGF-β inhibits *Npnt* gene expression through ERK1/2 and JNK in osteoblasts [7]. In the present study, treatment with the Alk5 inhibitor and Smad2 siRNA dramatically induced *Npnt* gene expression. Interestingly, our results demonstrated the presence of inhibitory molecules that decrease *Npnt* gene expression by binding to the TGF-β receptor complex in the culture medium. This phenomenon was also shown in the down-regulation of expression and activity of Alkaline phosphatase (Alp), one of the markers for osteoblast differentiation by using the Alk5 inhibitor (Suppl. Fig. S1). Although the exact molecules are not known, it is possible that through autocrine secretion from MC3T3-E1 cells, TGF-β1, β2, and β3 could inhibit *Npnt* gene expression in the culture (Suppl. Fig. S2). Furthermore, our results also suggest that inhibitory factors towards osteoblast differentiation existed in the present culture medium, which contained serum.

Ehnert et al. demonstrated that canonical TGF-β-SMAD signaling regulates expression of osteoblast marker genes including *Alp*

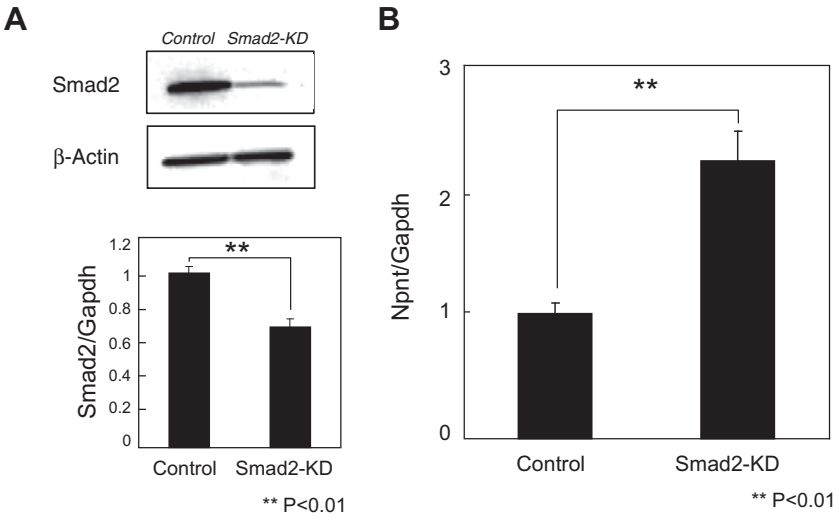

Fig. 2. Introducing siRNA from Smad2 induced *Npnt* gene expression in osteoblasts. MC3T3-E1 cells introduced with Smad2 siRNA were incubated for 48 h. After extracting total cellular RNA and protein, mRNA samples for *Smad2*, *Npnt*, and *Gapdh* were examined using real-time PCR, and protein samples for Smad2 and β-actin were examined by western blotting. (A) Expression of Smad2 was suppressed by introducing Smad2siRNA. (B) *Npnt* gene expression was increased by introducing Smad2 siRNA. $^{**}P < 0.01$, Student's *t* test, relative to level without Alk5 inhibitor.

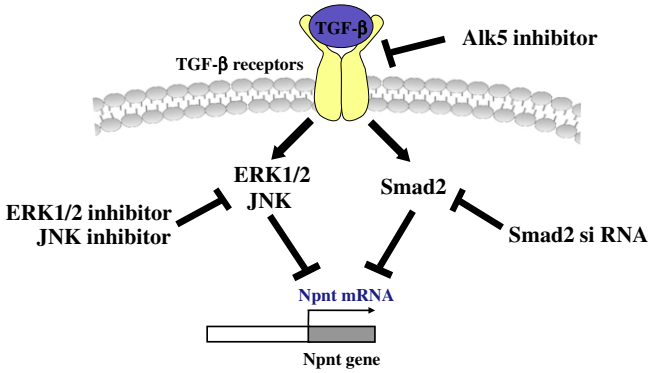

Fig. 3. Model of *Npnt* gene expression through canonical and non-canonical TGF-*β* signaling in osteoblasts. Alk5 inhibitor inhibited both the MAPK and SMAD signaling pathways, resulting in up-regulation of *Npnt* gene expression.

[12]. Alp expression and activity were down-regulated by TGF-β through Alk5-Smad2/3 signaling, which is similar to the regulation of *Npnt* gene expression. The exact mechanisms of *Npnt* gene expression by TGF-β-SMAD signaling are unclear, though Hoot et al. successfully revealed TGF-β signaling in HGF transcriptional regulation, which was largely dependent on the ratio of Smad2 and Smad4 [13].

In summary, MAPK and SMAD signaling pathways are currently thought to be responsible for *Npnt* gene regulation, though it remains to be determined whether other pathways are also involved in the regulation of *Npnt* gene expression (Fig. 3).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.106.

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