

## CD47 regulates the TGF- $\beta$ signaling pathway in osteoblasts and is distributed in Meckel's cartilage

Koichi Shimada<sup>1,3)</sup>, Akira Nakajima<sup>2,3)</sup>, Kyoko Ikeda<sup>1)</sup>, Kazuya Ishibashi<sup>1)</sup>,  
Noriyoshi Shimizu<sup>2,3)</sup> and Koichi Ito<sup>1,3)</sup>

<sup>1)</sup>Department of Periodontology, Nihon University School of Dentistry, Tokyo, Japan

<sup>2)</sup>Department of Orthodontics, Nihon University School of Dentistry, Tokyo, Japan

<sup>3)</sup>Division of Advanced Dental Treatment, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan

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**Abstract:** Previously, CD47 gene expression has been shown to increase during mandible development using a micro array technique. To determine the function of CD47 in osteoblasts, CD47 was silenced using siRNA *in vitro*. The TGF- $\beta$ 1 and phosphorylated-Smad2 levels and transcription factor genes related to bone metabolism increased dose-dependently with CD47 silencing. Furthermore, we determined the distribution of CD47 in mouse embryonic E13 and E15 *in vivo*. The CD47-positive cells were localized in Meckel's cartilage and antenatal mandibular bone. These results suggest that TGF- $\beta$ 1 signaling and mandible development might be regulated by CD47. (J Oral Sci 53, 169-175, 2011)

**Keywords:** CD47; TGF- $\beta$ ; osteoblasts; Meckel's cartilage; mandibular development.

### Introduction

The transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily including bone morphogenetic proteins (BMPs) has important functions in osteoblast differentiation and maturation *in vitro* and in bone formation *in vivo*. TGF- $\beta$  binds to TGF- $\beta$  types I and II serine/threonine kinase

receptors. Upon ligand binding and activation, TGF- $\beta$  type II receptor (TGF $\beta$ -IIR) kinase phosphorylates the TGF- $\beta$  type I receptor (TGF $\beta$ -IR), which in turn phosphorylates the receptor-regulated Smads (R-Smads), including Smad2 and Smad3. In contrast, the binding of BMP to BMP type I/II receptors activates Smad1, Smad5, and Smad8. The phosphorylated R-Smads form complexes with the common-partner Smad (Co-Smad, Smad4 only in mammals), which then act as signal transducers in both the TGF- $\beta$  and BMP pathways (1-5). These complexes translocate to and accumulate in the nucleus, where they regulate the transcription of various target genes such as transcription factors closely related to osteoblast differentiation and maturation and bone development. The main transcription factors are runt-related transcription factor 2 (Runx2), distal-less homeobox 5 (Dlx5), msh homeobox homolog 2 (Msx2), and trans-acting transcription factor 7 (Sp7, also called Osterix) (6-12).

A previous micro array study (13) showed that the gene expression of CD47 (integrin-associated protein) increased significantly in a time-dependent manner during the period of mandible development. CD47<sup>-/-</sup> mice increased bone mass and defective osteoclast function *in vivo* and *in vitro* (14,15). CD47 is involved in increasing the intracellular calcium concentrations after a cell has adhered to the extracellular matrix (16), and it plays a role of receptor for integrin  $\alpha$ v $\beta$ 3,  $\alpha$ IIb $\beta$ 3,  $\alpha$ 2 $\beta$ 1, and  $\alpha$ 4 $\beta$ 1 (17-20). Statin, which might offer protection against osteonecrosis, enhances osteogenic genes such as alkaline phosphatase, type I collagen, osteocalcin, and CD47 in osteoblasts (21,22). These studies suggest that the CD47 might be

Correspondence to Dr. Koichi Shimada, Department of Periodontology, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan  
Tel: +81-3-3219-8107  
Fax: +81-3-3219-8349  
E-mail: shimada-ki@dent.nihon-u.ac.jp

involved in bone metabolism.

To date, however, no report has addressed the association between CD47 and the TGF- $\beta$ /BMP signaling pathway in osteoblasts. Herein, to determine the role of CD47 in this pathway, we have used short-interfering RNA (siRNA) to silence CD47 expression in a mouse osteoblast cell line, MC3T3-E1. We then examined the protein levels of components in the pathway and the expression of the transcription factors. Furthermore, the distribution of CD47 was also analyzed immunohistochemically at the late embryonic stage.

## Materials and Methods

### Cell culture

Mouse osteoblast-like MC3T3-E1 cells were cultured in  $\alpha$ -minimum essential medium (Gibco BRL, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lexena, KS, USA) and 1% penicillin-streptomycin solution (Sigma-Aldrich, St Louis, MO, USA). The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### CD47 gene silencing

The endogenous transcription of CD47 was disrupted in MC3T3-E1 cells using a 21-nucleotide siRNA, r(GGAAUGACCUCUUUCACCA) dTdT (Sigma-Genosys., The Woodland, TX, USA). The cells were transfected with AllStars Negative Control siRNA (Qiagen, Tokyo, Japan) as a negative control or with 20 or 100 nM of CD47 siRNA using Lipofectamine™ 2000 (Life Technologies., Rockville, MD, USA) according to the manufacturer's protocol.

### Real-time PCR

Total RNA was isolated from cultured MC3T3-E1 cells at 24 h after transfection with CD47 siRNA using a FastPure® RNA Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Aliquots containing equal amounts of mRNA were subjected to reverse transcription and real-time PCR analysis. The RNA (1.0  $\mu$ g/reaction) was reverse-transcribed at 42°C for 60 min using a T-Primed First-Strand Kit (GE Healthcare., Milwaukee, WI, USA). Quantitative real-time PCR (qRT-PCR) was performed using 1.0  $\mu$ l of cDNA template, TaqMan® Universal PCR Master Mix (Life Technologies), and primer/probe sets (Life Technologies) for the target genes *Cd47*, *Bmp2*, *Tgfb1*, and transcription factors such as *runx2*, *dlx5*, *msx2* or *sp7*. *Gapdh* was used as an internal control. PCR amplifications were performed in a capped 96-well optical plate and incubated as follows: 5 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at

95°C and 1 min at 60°C. The gene-specific PCR products were measured continuously using an ABI PRISM 7700 detection system (Life Technologies). Samples were normalized against the internal control, and the results were expressed relative to the data for the transfection of negative siRNA. The data represent the mean  $\pm$  standard deviation from three independent experiments. Statistical significance was determined using Bonferroni's modification of Student's *t*-test, and  $P < 0.05$  was considered to be statistically significant.

### Enzyme-linked immunosorbent assay (ELISA)

At 48 h after the cells were transfected with 20 or 100 nM of CD47 siRNA or with negative control siRNA, the concentrations of BMP-2 and TGF- $\beta$ 1 in the medium were measured using commercial ELISA kits (R&D Systems., Minneapolis, MN, USA) according to the manufacturer's instructions. Quadruplicate assays were performed for each sample, and Bonferroni's modification of Student's *t*-test was used as a statistical test.

### SDS-PAGE and Western blotting of whole-cell extracts

Cells were transfected with 20 or 100 nM of CD47 siRNA or with negative control siRNA and incubated for 48 h. To obtain whole-cell extracts, the cells were rinsed with phosphate-buffered saline, suspended in lysis buffer, and sonicated as above. Aliquots containing equal amounts of protein in SDS-PAGE sample buffer were subjected to SDS-PAGE and Western blotting. Western blotting was performed using the following primary antibodies: rabbit polyclonal anti-CD47, rabbit anti-phospho-Smad1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Smad2 (BD Transduction Laboratories, Lexington, KY, USA), rabbit anti-phospho-Smad2 (Cell Signaling, Beverly, MA, USA), and mouse monoclonal anti- $\beta$ -actin (Santa Cruz Biotechnology), all with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare for mouse- and rabbit-IgG). Immunoreactive proteins were visualized using a chemiluminescence kit (ECL Western Blotting Reagent Kit; GE Healthcare) and exposed to X-ray film.

### Immunohistochemical analysis of CD47 in mouse embryos

Pregnant Swiss-Webster mice were used for this study. The females were mated for 2 h and the presence of vaginal plug was used to determine E0. To examine the Meckel's cartilage immunohistochemistry, tissues were dissected at E13 and E15. They were sectioned in the transverse plane at 7  $\mu$ m on a freezing Cryostat and immunostained

according to the avidin-biotin peroxidase method. The sections were blocked in Serum Blocking Solution (Zymed., San Francisco, CA, USA) for 30 min, followed by overnight incubation with CD47 primary antibody at room temperature. The slides were laid in the chamber, and the secondary antibody (Zymed) was added for 10 min, followed by the enzyme conjugate for 10 min. Finally, the substrate mixture was added for approximately 15 min. The tissue was stained additionally by hematoxylin as a counterstain. The mount slides were covered with aquamount and cover slips.

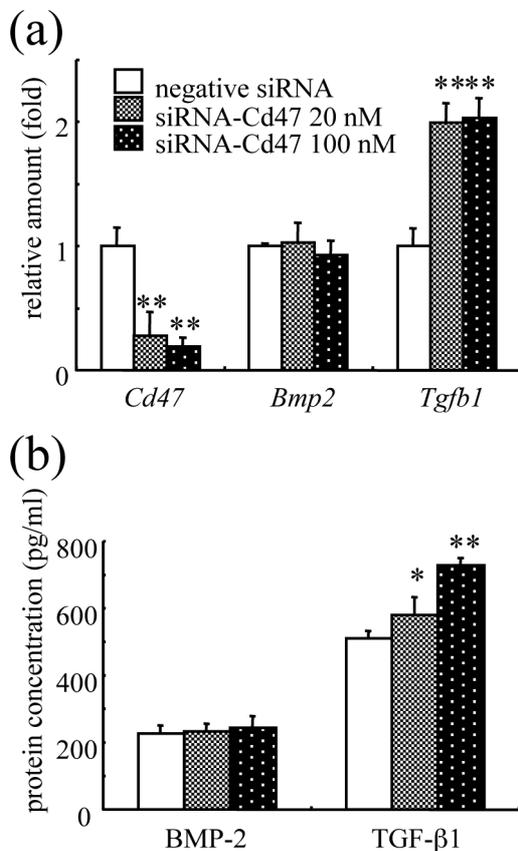


Fig. 1 CD47 silencing increases the expression of TGF-β1. MC3T3-E1 osteoblasts were transfected with 20 or 100 nM CD47 siRNA or with negative control siRNA for 24 or 48 h. (a) Gene expression of *Bmp2* and *Tgfb1* in the presence of CD47 siRNA. Total RNA was isolated after 24 h, and qRT-PCR was performed. Gene expression was quantified relative to the internal control (*Gapdh*) and is represented as multiples of the expression level compared to the negative control siRNA ( $n = 3$ ). (b) BMP-2 and TGF-β1 protein levels in the supernatant. The proteins were detected using a commercial ELISA kit ( $n = 5$ ). \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

## Results

### CD47 silencing increases TGF-β1 expression

To determine the function of CD47 in osteoblasts, CD47 was silenced in MC3T3-E1 cells using siRNA, and the protein in the medium and the levels of expression for BMP-2 and TGF-β1 were determined by qRT-PCR and ELISA. The protein and gene expression levels for TGF-β1 increased dose-dependently ( $P < 0.05$ ), but the protein and gene levels for BMP-2 did not change significantly (Fig. 1a, b).

Next, the protein expression levels of CD47, phospho-Smad1, phospho-Smad2, and Smad2 were determined by Western blotting. CD47 protein expression was silenced in a dose-dependent manner, and the phospho-Smad2 level increased dose-dependently by the siRNA to the same levels as TGF-β1. Smad2 and β-actin protein expression did not change (Fig. 2). These results suggest that CD47 down-regulates the TGF-β1 signaling pathway in osteoblasts.

### CD47 silencing increases the gene expression of transcription factors

The gene expression levels of *runx2*, *dlx5*, *msx2*, and *sp7* were determined by qRT-PCR. The levels of all

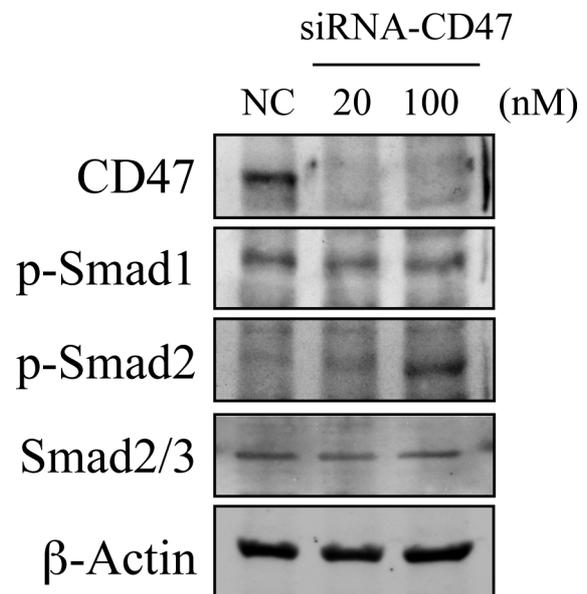


Fig. 2 CD47 silencing increases the levels of phospho-Smad2 proteins. The phosphorylation of Smad1 and Smad2 in the presence of CD47 siRNA. Whole-cell lysates were prepared after 48 h. The samples were normalized by the total protein concentration, subjected to SDS-PAGE, and immunoblotted with anti-CD47, anti-phospho-Smad1, anti-phospho-Smad2, or anti-Smad2/3 antibodies. β-Actin served as a control.

transcription factors increased with CD47 siRNA treatment in a dose-dependent manner (Fig. 3,  $P < 0.05$ ), suggesting that CD47 down-regulates these transcription factors, which are related to bone metabolism and maturation.

### Localized distribution of CD47 in mouse embryo

To determine the distribution of CD47 *in vivo*, we conducted an immunohistological analysis using mouse embryonic E13 and E15. The CD47-positive cells were chondrocyte-like cells and were localized below the tongue at E13 and E15 (Figs. 4a, c), suggesting that the CD47-positive cells might be Meckel's cartilage. A patch of CD47-positive cells was also localized at the side of the cartilage at E15 (Figs. 4a, b). Therefore, the CD47-positive cells might be the origin of mandible.

### Discussion

The silencing of the CD47 gene increased the levels of TGF- $\beta$ 1 and phospho-Smad2, but not BMP-2 and phospho-Smad1, and the gene expressions of transcription factors related to osteoblastic differentiation and maturation increased significantly in osteoblasts. Furthermore, we demonstrated that CD47 was distributed in Meckel's cartilage at mouse stages E13 and E15 and in the embryonic mandible at the E15 stage. These results suggest for the first time that CD47 might regulate the TGF- $\beta$  signaling pathway and influence the development of the mandible.

Normally, TGF- $\beta$ 1 is secreted as a complex of three proteins, including the bioactive peptide of TGF- $\beta$ 1, a latency-associated peptide  $\beta$ 1 (LAP- $\beta$ 1), and a latent TGF-

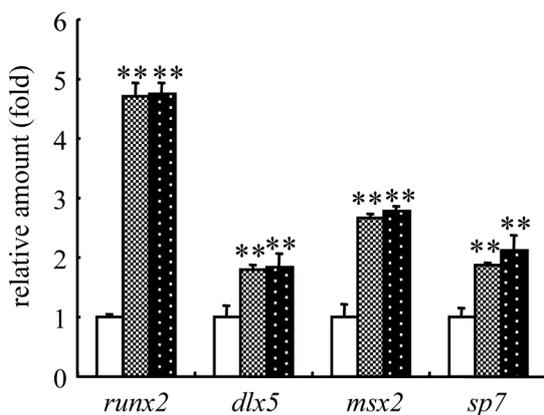


Fig. 3 Gene expression of transcription factors, which is related to bone metabolism, in the presence of CD47 siRNA. Total RNA was isolated after 24 h, and qRT-PCR was performed. Gene expression was quantified relative to the internal control (*Gapdh*) and is shown as multiples of the expression level compared to the negative control siRNA ( $n = 3$ ). \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

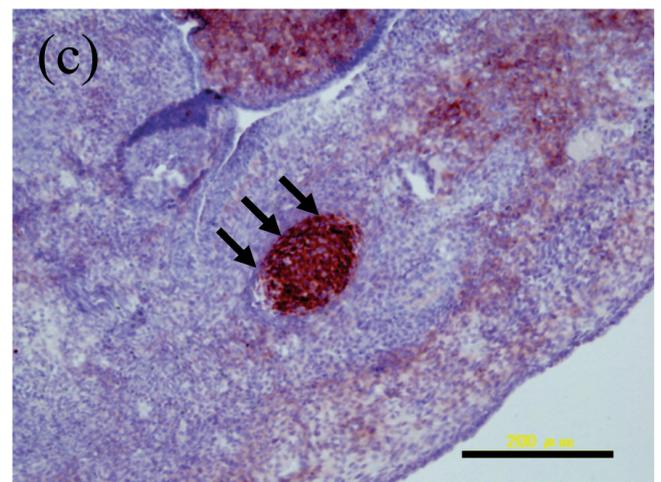
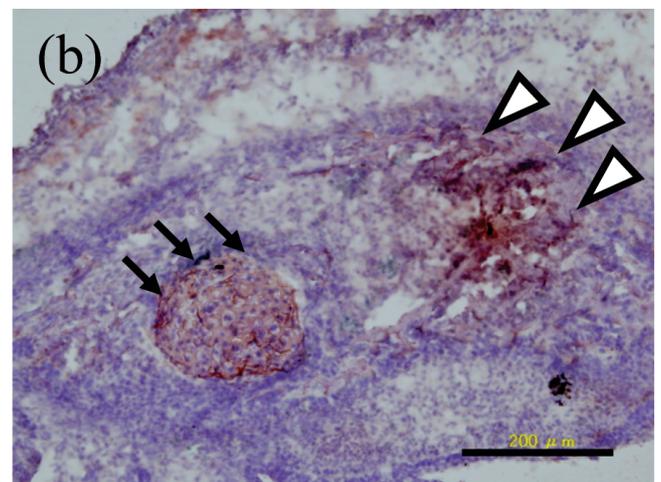
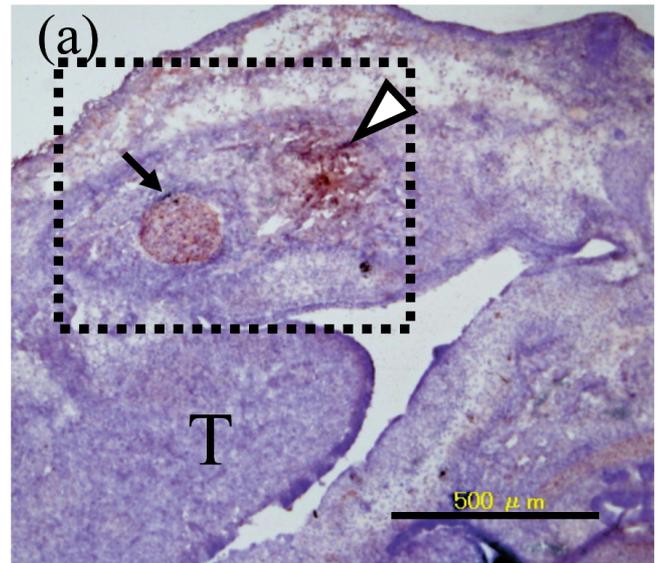


Fig. 4 The distribution of CD47 in mouse embryonic mandible. Mouse embryonic mandible was analyzed immunohistochemically. (a, b) CD47-positive chondrocyte-like cells in Meckel's cartilage (black arrows) and antenatal mandible bone (white arrow heads) at E15 and (c) in Meckel's cartilage at E13. T: tongue, bars: (a) 500  $\mu$ m, (b, c) 200  $\mu$ m.

$\beta$  binding protein-1 (23). LAP- $\beta$ 1 is recognized by  $\alpha$ v-containing integrins such as  $\alpha$ v $\beta$ 1,  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5,  $\alpha$ v $\beta$ 6, and  $\alpha$ v $\beta$ 8 (24-27). Furthermore, TGF- $\beta$ 1 enhances the cell surface expression of  $\alpha$ v,  $\beta$ 1, or  $\beta$ 3 integrin subunits in osteoclasts or human breast cancer cells (28,29). CD47 and TGF- $\beta$ 1 might be associated through the  $\alpha$ v integrin subunit because CD47 is a receptor for integrin  $\alpha$ v $\beta$ 3 (16,20). Our data also suggest that CD47 might be able to regulate the TGF signaling pathway because the CD47 silencing increased dramatically the protein expression of TGF- $\beta$ 1 and the phosphorylation of Smad2. To understand the cross-talk mechanism in detail, further study is needed.

CD47<sup>-/-</sup> mice displayed increased bone mass and diminished osteoclast function (14,15). There is no knowledge of the osteoblast function in CD47<sup>-/-</sup> mice, but our data demonstrated that the transcription factors for bone differentiation and maturation of osteoblasts increased upon CD47 silencing using siRNA. The function of osteoblasts in CD47<sup>-/-</sup> mice may be enhanced *in vivo* because intercellular communication exists between osteoblasts and osteoclasts (30).

Smad2 KO mutant embryos fail to form an organized egg cylinder and lack mesoderm. Interestingly, some of the Smad2 heterozygous embryos have severely defective mandibles or eyes, suggesting that the dosage of Smad2 is critical for normal craniofacial development (31). Although both TGF- $\beta$  and BMPs specific Smads are present in cartilage, different pathway-specific Smads may have different functions in regulating chondrogenesis (32). Here, we focused on CD47 and TGF- $\beta$  including the Smads pathway. Collectively, our analysis suggests that CD47 and TGF- $\beta$  signaling may play important regulatory roles during the development of Meckel's cartilage. The function of CD47 in bone formation, including Meckel's cartilage development, and the delay and disposition of the initial mandible formation might be due to malformation of Meckel's cartilage (primordium for mandibular development) and may account for the malformation of mandible as reported in Smad2 heterozygous mutant mice (33). A direct correlation was observed between the expression level of Smad2 and the delay in Meckel's cartilage formation, and the Smad2 heterozygous mutant mandibular explants with at least half (or more than half) of the normal Smad2 expression level did not show any alteration of Meckel's cartilage development (34). Exogenous TGF- $\beta$ 1 was added to Smad2 heterozygous mutant mandibular explants culture medium and did not rescue the development of Meckel's cartilage, indicating that an adequate level of Smad2 expression is critical in regulating TGF- $\beta$ -mediated chondrogenesis (31,34,35). Thus, we identified that CD47 is strongly associated with

TGF- $\beta$  signaling following Meckel's cartilage development in mouse embryo.

In conclusion, our data suggest that CD47 down-regulates the TGF- $\beta$  signaling pathway and the transcription factors of bone differentiation and maturation *in vitro* and that CD47 distributes in antenatal Meckel's cartilage *in vivo*. CD47 may be a key factor in the cross-talk between TGF- $\beta$  and the integrin signaling pathway, although further studies will be needed.

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