

# The expression of TGFβ1 mRNA in the early stage of the midpalatal suture cartilage expansion

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## Abstract

**Introduction:** The application of orthodontic expansion force induces bone formation at the midpalatal suture because of cell proliferation and differentiation. Expansion forces may stimulate the production of osteoinductive cytokines, such as transforming growth factor β1 (TGFβ1), in the progenitor cells. **Objectives:** This study determined the role of TGFβ1 in the early stage of midpalatal suture cartilage expansion. **Methods:** An orthodontic appliance was placed between the right and left upper molars of 4-week-old rats. The initial expansion force was 50 g. Animals in the control and experimental groups were sacrificed on days 0, 2, and 5 and 6 μmm thick sections were prepared for an in situ hybridization technique. **Results:** Two days after the application of force, prechondroblastic and undifferentiated mesenchymal cells distributed along the inner side of the cartilaginous tissue had high levels of TGFβ1 transcription. On day 5, the TGFβ1 transcription was found in osteocytes and osteoblastic cells on the surface of newly formed bone. Immunohistochemistry using Osteocalcin-Pro (OC-Pro) confirmed osteoblastic activity. **Conclusions:** Results suggest that the expansion of midpalatal suture cartilage induces differentiation of osteochondroprogenitor cells into osteoblasts after stimulation by cytokine production.

**Keywords:** Transforming growth factor β1. Proliferation. Differentiation. Osteoblasts. "In-situ" Hybridization.

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## INTRODUCTION

The midpalatal suture cartilage of growing rats is composed of layers of precartilaginous cells located in the central part of the suture, and of mature cartilaginous cells layers on both sides of the precartilaginous layers. The precartilaginous cells layers are filled with prechondroblastic and undifferentiated mesenchymal cells with a high capacity to proliferate and differentiate into chondrocytes and osteoblasts.

Bone formation at the midpalatal suture cartilage initiates from the outer side of the cartilaginous tissue by means of endochondral ossification. However, when an orthodontic expansion force is applied to the suture, new bone formation is initiated on the inner side of the cartilaginous tissue by means of intramembranous ossification.<sup>7,18</sup> This process involves the proliferation of undifferentiated mesenchymal cells and their differentiation into osteoblasts.

Kobayashi et al<sup>7</sup> described the early cell response caused by the induction of orthodontic forces, which increase the expression of proliferating cell nuclear antigen (PCNA), a specific cell proliferation marker, and many other proteins of the bone matrix in the inner side of the cartilaginous tissue. Their results showed that mechanical stress is an important mediator of proliferation and differentiation of osteochondrogenitor cells into osteoblasts.

However, no studies have definitively explained the molecular mechanism of cell response mediated by orthodontic expansion forces that leads to proliferation and differentiation of the progenitor cells into osteoblasts.

Both *in vivo*<sup>11,12,14</sup> and *in vitro*<sup>4,8,9</sup> studies have demonstrated the participation of transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), a cytokine that belongs to the TGF $\beta$  superfamily, in bone formation.

This study was performed using an *in situ* hybridization technique to evaluate the transcription level of TGF $\beta$ 1, a cytokine with high

osteogenic capacity, after an orthodontic expansion force was applied to the midpalatal suture cartilage of growing rats.

## MATERIALS AND METHODS

### Expansion of the midpalatal suture

Four-week-old male Wistar rats (Charles River Corporation, Kanagawa, Japan) weighing 67-83g were housed at the animal laboratory and fed a standard pellet chow (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. All experimental procedures were approved by the Animal Welfare Committee of Nagasaki University, Japan.

An orthodontic expansion appliance (0.014 inch Co-Cr wire, green Elgiloy Semi-Resilient wire; Rocky Mountain Morita Corporation, Denver, CO, USA) was placed between the maxillary right and left molars, as described by Kobayashi et al.<sup>7</sup>

A strain gauge (Tomy International Co., Tokyo, Japan) was used to adjust the initial expansion force to 50 g. The animals in the control and experimental groups were sacrificed on days 0, 2, and 5. Each group was composed of 3 animals.

### Tissue preparation for immunohistochemistry

The maxillary bone was surgically removed and fixed by immersion in 4% paraformaldehyde overnight at 4°C. After fixation, the maxilla was demineralized in 10% ethylenediaminetetraacetic acid (EDTA) for 10 days at 4°C, and then dehydrated using an increasing ethanol series. The specimens was embedded in paraffin, cut into 6  $\mu$ m thick serial frontal sections at the mesial root of the maxillary first molar, and mounted on 3-aminopropyltriethoxysilane coated slides.

### Tissue preparation for *in situ* hybridization

Sections for *in situ* hybridization were prepared in the same way as for immunohisto-

chemical staining. All solutions were free of RNase due to the addition of 0.1% diethyl pyrocarbonate (DEPC) to H<sub>2</sub>O.

### Preparation of cRNA digoxigenin-labeled probes for in situ hybridization

The plasmid containing TGFβ1 cDNA was transferred into *Escherichia coli* to amplify cDNA. TGFβ1 cDNA was cut at the BamHI/HindIII site, subcloned into Bluescript KS+ vector, and then used as a model for cRNA production. Single strand RNA antisense (complementary) and sense (non complementary) digoxigenin-labeled probes were prepared according to the instructions supplied with the DIG-RNA labeling kit (Boehringer Mannheim, Germany). Transcriptions were performed using T3 or T7 RNA polymerase. Labeling with digoxigenin was confirmed using a hybridization filter. Each probe reacted only with a corresponding RNA reverse strand.

### In situ hybridization

In situ hybridization was performed according to the method described by Nakase et al.<sup>13</sup> After blocking the alkaline phosphatase activity with acid, the sections were incubated with RNA DIG-UTP (1.5 mg/ml) label probes at 55°C overnight, and then washed extensively and treated for RNase. The DIG-labeled probes were detected using an anti-DIG antibody conjugated with alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate as a substrate and developed using a DIG nucleic acid detection kit (Boehringer Mannheim).

Controls were: (a) hybridization with sense (mRNA) probe; (b) hybridization with non probe.

### Immunohistochemical and histochemical staining

Immunohistochemistry was performed using the peroxidase-anti-peroxidase method as described by Sakai et al.<sup>16</sup> Briefly, the sections were pretreated with first antibody. Rabbit an-

tiseria against rat Cathepsin K (CK)<sup>3</sup> and rat Osteocalcin-Pro peptide (OC-pro)<sup>2</sup> were diluted at 1:200 and 1:100 and kept in blocking buffer overnight at 4°C.

On the following day, the sections were washed and incubated with the second antibody (goat anti-rabbit IgG).

The immunoreactivity sites were visualized using peroxidase-anti-peroxidase and reacted with 3,3 diaminobenzidine to produce a brown benzidine staining precipitation.<sup>17</sup> For Proliferating Cell Nuclear Antigen (PCNA) detection, specimens were kept overnight at 4°C in mouse monoclonal antibody (clone PC10, DAKO, Tokyo, Japan) at 1:50 dilution as the first antibody.

The sections were stained with streptavidin-biotin peroxidase (Histofine ABC kit-Nichirei Co. Ltd., Tokyo) according to the manufacturer's instructions. Negative control immunoreactivity was evaluated using normal rabbit serum (1:100 dilution) or normal mouse IgG (100 mg/ml). The histochemical tests for hematoxylin and eosin were performed using the method described by Lyon.<sup>10</sup>

## RESULTS

### Histological changes during midpalatal suture cartilage expansion

On day 0, the central area of the suture cartilage was filled with a cartilaginous cell layer composed of undifferentiated mesenchymal at the center, and prechondroblastic cells. Around this area, the cells exhibited features of mature chondroblasts and/or chondrocytes (Fig 1A).

On day 2, the mature cartilaginous cell layers were displaced laterally, and the central part of the suture still had immature prechondroblastic and mesenchymal cells. In addition, a cell cluster was observed at the border of prechondroblastic and chondroblastic cells (Fig 1B).

New trabecular bone formation was first seen 5 days after the application of an expansion force (Fig 1C).

### In situ hybridization and immunohistochemistry

In this study, *in situ* hybridization technique was carried out using cRNA-DIG-labeled probes to evaluate the expression of TGFβ1 mRNA localized in the midpalatal suture cartilage. On day 0 (Fig 2A), a positive TGFβ1 mRNA (a) transcription level was detected in the mature osteoblasts located in the periphery of trabecular bone, laterally to the layer of cells compatible with chondroblasts, as shown by arrows in Figure 2A.

Positive PCNA immunoreactivity (Fig 2B) was found in the prechondroblastic cells in the central part of the suture and in the mature and hypertrophying cartilage cells located in the periphery of the suture. Intense cathepsin (CK) (Fig 2C) immunoreactivity was observed in the outer side of the cartilaginous cell layers, in the

transition to bone tissue.

On day 2 (Fig 3A), an intense positive TGFβ1 mRNA (a) transcription was seen at the border of prechondroblastic and chondroblastic cells.

Strong PCNA (Fig 3B) immunoreactivity was seen in the same area. The positive immunoreactivity pattern for CK found in the control group (day 0) was also seen in the outer side of cartilaginous tissue, which was indicative of osteoclastic activity.

During the following days (Fig 4A), an intense specific signal for TGFβ1 mRNA (a) was observed in osteocytes (open arrowheads) and osteoblasts (filled arrowheads) inside and on the surface of newly formed bone (day 5).

Osteoblastic activity was confirmed by immunohistochemistry using Osteocalcin-Pro (OC-Pro) (Fig 4B). The pattern of osteoclastic activity (Fig 4C) was the same found on day 0 (control group).

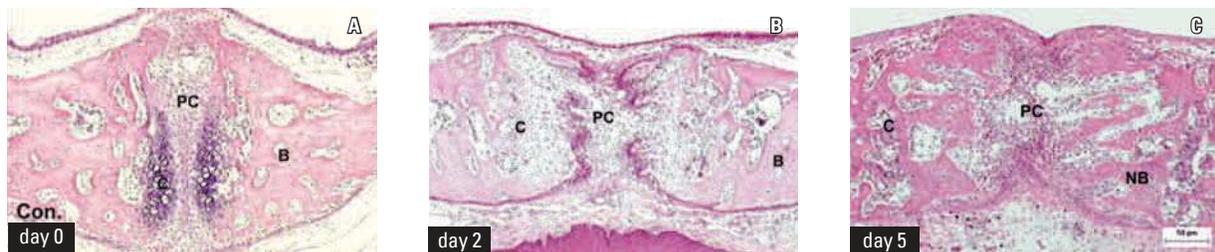


FIGURE 1 - Sequence of histological changes in midpalatal suture after application of expansion force. Con: control; PC: precartilaginous cells; c: cartilaginous cells; B: bone; NB: newly formed bone. Bars A, B and C = 50 μm.

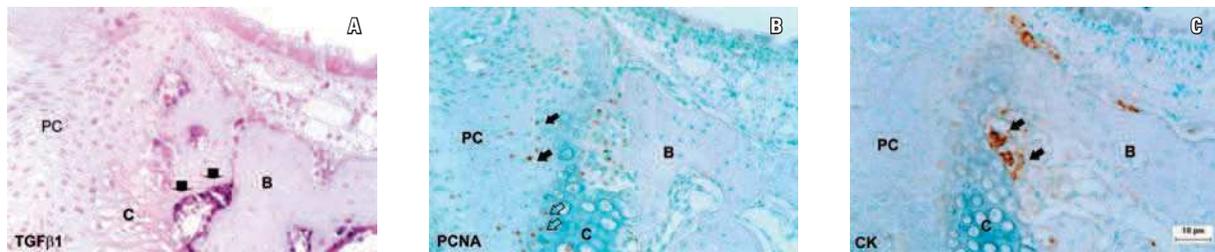


FIGURE 2 - Expression of TGFβ1 mRNA (A), PCNA (B) and CK (C) on day 0. TGFβ1: transforming growth factor β1; PCNA: proliferating cell nuclear antigen; CK: cathepsin K. Bars A, B and C = 10 μm.

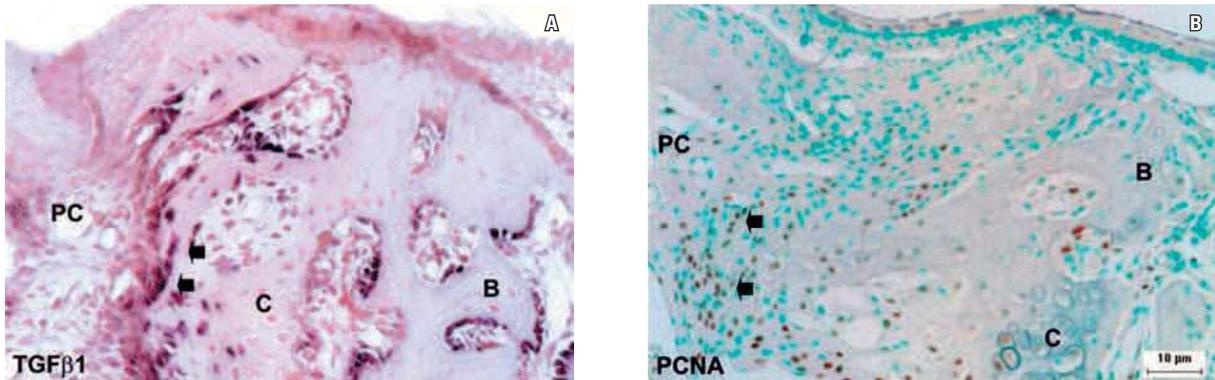


FIGURE 3 - Expression of TGFβ1 mRNA (A) and PCNA (B) on day 2. Bars A and B = 10 μm.

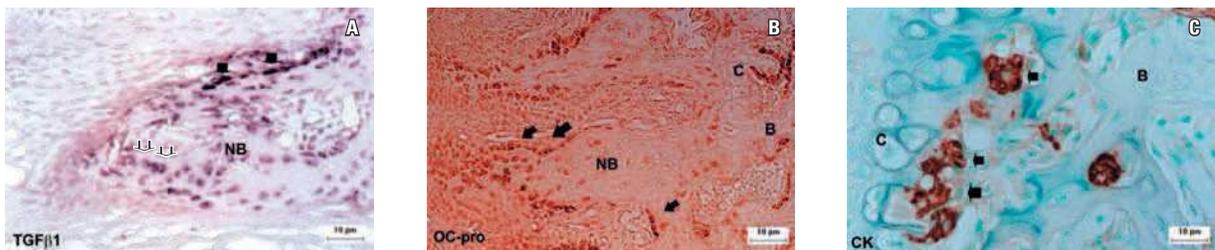


FIGURE 4 - Expression of TGFβ1 mRNA (A), OC-Pro (B) and CK (C) on day 5. OC-Pro: Osteocalcin-Pro. Bars A, B, and C = 10 μm.

## DISCUSSION

On day 0, positive PCNA immunoreactivity was expressed in the prechondroblastic cells located in the central area of the midpalatal suture cartilage and in some mature and hypertrophying cartilage cells, which was indicative of their proliferative activity. PCNA is a protein found in the cell nucleus that acts as a DNA polymerase delta cofactor during the DNA synthesis stage.<sup>1</sup> It is used to determine the level of proliferative activity. At this stage, proliferative activity may be associated with normal cross-sectional development of the palate.<sup>7</sup>

On day 2, the expression of PCNA immunoreactivity increased substantially in the border of the prechondroblastic and chondroblastic layers after the orthodontic expansion force was applied.

Previously to our study<sup>7</sup>, positive immu-

noreactivity for osteocalcin (OCN), a specific marker for osteoblasts, and alkaline phosphatase (ALPase) activity were found in the same stage and area, which suggests that osteochondroprogenitor cells differentiate into osteoblasts in response to the expansion force. Accordingly, high TGFβ1 mRNA transcription levels were expressed in the same region on day 2, as well as in mature osteoblasts on day 0.

TGFβ1 expression associated with newly formed bone has been investigated by many authors. Noda et al<sup>14</sup> reported the occurrence of bone formation after TGFβ1 injection in the calvarium of newborn rats.

In addition, the role of TGFβ1 in osteoblastic differentiation from undifferentiated mesenchymal cells was been investigated by Joyce et al,<sup>5</sup> who reported that TGFβ1 induces differentiation of mesenchymal-like cells into osteoblasts

by stimulating proliferation and extracellular matrix protein production.

TGFβ1 may mediate osteogenesis because of its chemotactic effect on the osteoblastic precursor cells as it recruits those cells to the region to start the process of bone formation.<sup>15</sup>

In the late stage of the treatment (day 5), new bone formation continued and developed a columnar bone structure that grew from the center of the suture.

Positive immunoreactivity for Osteocalcin-Pro (OC-Pro), a specific osteoblastic marker, confirmed osteoblastic activity on the surface of the newly formed bone in this region.

TGF1 transcription was detected in osteocytes and osteoblasts on the surface of newly formed bone, which suggests that those cytokines participate in the regulation of the differentiation of mesenchymal cells into osteoblasts.

At all experimental time points (day 0, 2, 5), CK immunoreactivity was expressed exclusively in the outer side of the cartilaginous cell layers, following the normal pathway of calcified cartilage matrix absorption by osteoclastic cells. This protease is involved in the degradation of type I and type II collagen and osteonectin by osteoclasts.<sup>3</sup>

However, there was no osteoclastic activity in the inner side of the cartilaginous tissue, although there were blood vessels that promoted the migration of precursor osteoclasts to this region. Osteoclastic activity may change due to the

high TGFβ1 expression, which can inhibit the differentiation of precursor osteoclasts and also the absorptive activity of mature osteoclasts.<sup>6</sup>

## CONCLUSIONS

The results of this study suggest that:

The expansion of the midpalatal suture increases TGFβ1 transcription in the cells in the border of precartilaginous and cartilaginous cell layers and in osteocytes and osteoblasts on the surface of newly formed bone.

The expression of TGFβ1, osteocalcin (OCN), and alkaline phosphatase (ALPase) in the border of the precartilaginous and cartilaginous cell layers on day 2, was an indicative of the beginning of osteochondroprogenitor cells differentiation into osteoblasts.

New bone formation by means of intramembranous ossification was induced in the inner side of the cartilaginous layers.

The absence of osteoclastic activity in the inner side of the expanded cartilaginous tissue may be associated with the high level of TGFβ1 transcription.

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