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Periostin-like-factor-induced bone formation within orthopedic maxillary expansion

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Structured Abstract

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Objective – To examine whether periostin-like factor (PLF) stimulation of the expanded palatal suture would accelerate mineral formation of bone.

Materials and Methods – Expanded palates of 6-week-old male C57BL/6 mice were maintained in an organ culture system, and tissue was either unstimulated or stimulated with PLF or BMP-7 for 8 days. Bone mineral formation was assessed by Raman microspectroscopy analysis and histological examination.

Results – Raman microspectroscopy analysis demonstrated that unstimulated palates maintained their bone mineral concentration within the palatal suture over 8 days (%increase = 11.29 ± 2.34). In comparison, palates exposed to either PLF (%increase = 29.33 ± 1.61, $p = 0.012$) or BMP-7 (% increase = 25.49 ± 1.09, $p = 0.016$) formed significantly more bone at the osteogenic fronts of the palatal suture compared with unstimulated samples at day 8. Alkaline phosphatase activity along the bone edges was markedly greater in the PLF and BMP-7 groups compared with that in unstimulated groups at day 0 and day 8. The levels of osteocalcin proteins in the palatal suture tissues of the PLF and BMP-7 groups were significantly higher than those in the unstimulated group.

Conclusion – PLF can increase bone mineral formation within the expanded palatal suture.

Key words: bone formation; mechanical force; palatal suture; periostin-like factor

Introduction

Transverse skeletal deficiency of the maxilla is a common clinical problem in orthodontics and dentofacial orthopedics. Transverse maxillary deficiency, isolated or associated with other dentofacial deformities, results in esthetic and functional impairment (1). Orthopedic maxillary expansion is the preferred treatment approach to increase the maxillary transverse dimension in young patients by a widening of the palatal suture. A lateral shift of the two horizontal processes of the maxilla is usually forced, which triggers proliferation, osteoblastic differentiation of the osteoprogenitor cells within the suture and new bone deposition in the palatal suture (2).

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However, even after a retention period, the expanded maxillary arch has a strong tendency to rebound to its previous form (3, 4). Although the reason for relapse is not fully elucidated, the rate and quality of bone deposited in the palatal suture during expansion may influence post-treatment relapse (5, 6). For a successful treatment, the expanded tissue must be stabilized by accelerating bone formation within the expanded area, thus preventing relapse of the skeletal base and shortening time of the retention period (7). To expand the palatal suture more effectively, the method of accelerated bone formation in the palatal suture in response to expansion must be developed further.

Several studies have shown the biological effects of TGF-β (6), BMP-7 and Nell-1(8) on inducing bone formation in an expanding suture. Owing to the limited success of these growth factors in clinical settings (9) or medical complications (10), researchers continue to identify other osteogenic growth factors for use in bone formation. Periostin-like factor (PLF) has been found to play an important role in osteogenesis (11). PLF is a secreted matricellular protein, which is expressed in bone (12) and promotes osteoblast proliferation and differentiation (11). In a model of fracture healing, PLF expression is robustly upregulated in callus osteoblasts at post-fracture days 7 and 14 (11). PLF can induce bone formation. These facts make it a potential candidate for stimulating bone formation within palatal sutures.

In the current study, we surveyed the effect of PLF stimulation on bone formation within a mouse palate organ culture system after orthopedics expansion. The actions of PLF were compared with those of BMP-7, which has successfully induced both chondrocyte proliferation and differentiation within the expanded palatal sutures of rats (8). BMP-7 is known to recruit and stimulate differentiation of local osteoprogenitor cells and promote bone formation (13). The data showed that both PLF- and BMP-7-stimulated sutures significantly increased bone formation within the palatal suture compared with that in unstimulated sutures. The ability of PLF to successfully accelerate mineral formation of bone within the palate suture will help researchers to understand the mechanism of bone formation in palatal sutures in response to expansion and facilitate the development of more effective expansion methods.

Materials and methods

Animals

A total of 60 six-week-old male C57BL/6 mice (Shanghai SLAC Laboratory Animal Co. Ltd, Songjiang, Shanghai, China) were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Rapidly expanded palatal sutures *in vivo*

To evaluate the intrinsic expression of PLF during suture expansion, 12 mice were allocated randomly to four experimental groups that were treated with an applied expansion force according to the method of Hou et al.(2) at different time courses: 0, 1, 4 or 8 days. Three samples were used to put into trizol and stored at −70°C for isolation RNA to conduct QPCR.

Rapidly expanded palatal sutures *ex vivo*

Forty-eight mice were euthanized with carbon dioxide, and the palate was dissected with razor blades for organ culture. Harvested tissues consisted of the middle and posterior regions of the palate including the periosteum and palatal bone.

A total of 48 palates were randomly assigned to four groups: 1) day 0 unstimulated samples (n = 12); 2) day 8 (expansion unstimulated samples, n = 12); 3) expansion samples stimulated with 200 ng/ml BMP-7 (Sigma-Aldrich, USA, (n = 12)); 4) expansion samples stimulated with 200 ng/ml PLF (the full-length PLF cDNA was cloned as described (11),expressed in *Escherichia coli*, purified protein *in vitro* by Genovel Biotechnology, Shanghai, China (n = 12)). BMP-7 or PLP was supplemented the culture media duration for 8 days. Media were changed on every other day.

The force expansion systems were based on the description by Cowan et al. (8). Expansion force was applied to each palatal suture using a helical spring made of stainless steel orthodontic wire (0.012-in; GACInternational Inc., Bohemia, NY, USA). Samples were put within 6-well culture plates. Tissues were incubated in 75% TC-medium BGJ and 25% fetal bovine serum, supplemented with 100 U/ml penicillin–streptomycin, 12 mM L-glutamine, and 50 μg/ml L-ascorbic acid at 37°C with 5% CO_2.

After an initial 24-h culture period, 36 samples of sutures were expanded using a helical spring with

needles attached at two points that were located one-third of the width from the tissue edges (Fig. 1). Needles remained in the tissue throughout the experiment. To calibrate the amount of force produced by the activation of the opening loop, the F/Δ rates of the appliance were determined. The initial force magnitude used in the experiments was 0.3 N. The average force was about 0.1 N at day 8.

In each group, six samples were fixed in 4% (w/v) paraformaldehyde and stored at 4°C before being used for staining of hematoxylin–eosin (H&E) and for alkaline phosphatase (ALP) activity. Three samples were used for Raman analysis. In addition, three samples were used to extract protein and stored at −70°C for western blotting.

RNA isolation and quantitative real-time PCR

Quantitative real-time RT-PCR (qPCR) was performed on pooled RNA samples (n = 3 animals at 0, 1, 4, 8 days) from mid-palatal sure expanded. Tissues were harvested in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA USA). Total RNA was isolated as per the manufacturer's instructions (Invitrogen) and treated with RNase-free DNase. Reverse transcription reaction was performed on 1 μg of RNA using the First Strand Synthesis Kit (Invitrogen). Relative transcript levels were measured by quantitative PCR in 25 μl reaction volume using ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA), following the recommended protocol for SYBR-Green, and normalized with GAPDH levels (Applied Biosystems).

For the amplification of PLF, primers located on either side of exon 17 were used (11):

(Forward: 5′ GATAAAATACATCCAAATCAAGTTTGCTCG,
Reverse: 5′CGTGGATCACTTCTGTCACCGTTTCGC).
GAPDH:
Forward: 5′ ACCACAGTCCATGCCATCAC
Reverse: 5′ TCCACCACCCTGTTGCTGTA.

Experiments were performed three times, and the results are reported as mean ± SD.

Histological analysis

Six harvested samples of each group were processed and fixed overnight with 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline solution. The fixed specimens were demineralized in 0.5 M ethylenediaminetetraacetic acid (EDTA) for 14 days at 4°C, and then, three samples were embedded in paraffin, sectioned at 7-μm intervals, and stained with hematoxylin–eosin (H&E) before microphotographs were taken. For ALP, another three specimens were embedded in OCT (Tissue Tek; Miles Laboratories, Naperville, IL, USA) for frozen sections instead of embedment in paraffin. To observe osteoblasts, representative sections were stained for ALP using an ALP substrate kit (Sigma Diagnostics, St. Louis, MO, USA).

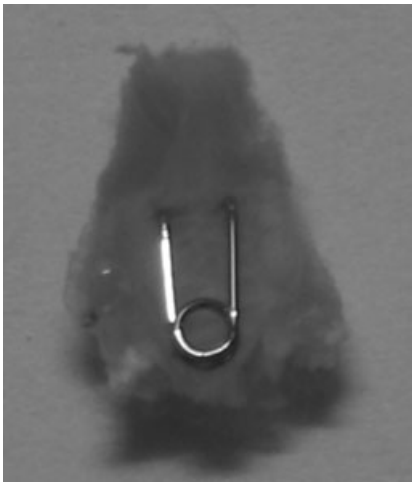

Fig. 1. Rapidly expanded palatal sutures *ex vivo*. The palatal suture was expanded using helical spring needles at two points that were located one-third of the width from the tissue edges.

Raman spectroscopic imaging

An HR-800 Raman spectrophotometer was used, as described previously (14, 15), to evaluate organic and inorganic matrix composition of the middle of the palatine bone tissue and suture. The palatal suture areas were cut perpendicular to the long axis of the suture into 1-mm sections, turned on their side, and placed on a quartz slide for Raman imaging. The dimensions of the scanned areas were 100 μm × 30 μm. The HR-800 Raman spectrophotometer uses monochromatic radiation emitted by a HeNe laser (632.8 nm) operating at 20 mW with a 0.5-μm spot size. Two-dimensional maps were created using a computer-controlled translation stage to move the specimen in 1-μm increments. Spectra were acquired for the phosphate (PO_4^{3-}/amide I) band envelope (900–995/cm) and for the amide I band envelope (1600–1700/cm) to measure organic content. The phosphate

$v1$ band is used as a marker of mineral content of bone at approximately 960/cm, and such a position of $v1$ assigned to P-O stretching vibrations is characteristic for carbonate-rich Hydroapatite (HAP). Bone mineral concentration information was obtained from the PO_4^{3-}/amide I intensity ratio. An increasing PO_4^{3-}/amide I intensity ratio indicates a more mineralized matrix.

The score images presented in Fig. 3 represent the relative amount and the location of either the mineral or the matrix. Lighter colors represent higher Raman peaks. Color scales indicate the bone matrix mineral concentration. Red areas represent high mineral and low collagen content, whereas blue areas represent low mineral and normal collagen content.

Western blot analysis

Because osteocalcin was produced by mature osteoblasts and primarily deposited in the ECM of skeletal tissue and its levels reflected the rate of bone formation (16), it was chosen for western blot analysis. Western blotting was performed as described previously (17). β-Actin was used as a loading control. The separated proteins were incubated overnight with primary anti-OCN antibodies (Santa Cruz Biotechnology, CA, USA). The blots were then incubated with peroxidase-conjugated secondary antibody (1:2000). Protein bands were visualized using an enhanced chemiluminescence system (Supersignal West Pico Trial Kit; Pierce, Rockford, IL, USA), and the density of each band was quantified with a Fluor-S MultiImager (Bio-Rad). The experiment was performed with samples from three mice in each group, and the mean expression level for each protein was calculated accordingly.

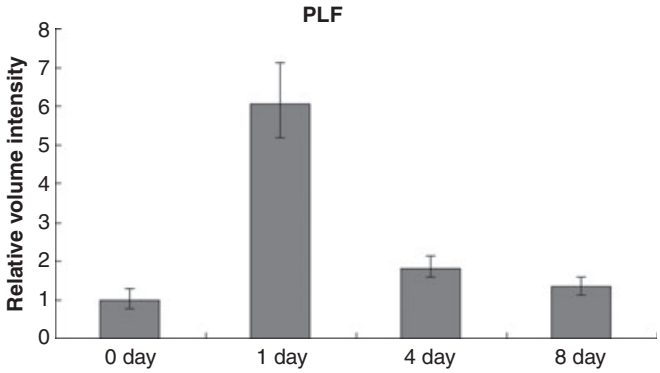

Fig. 2. QPCR of total RNA from the palatal suture using Periostin-like factor primers. Data obtained from an average of three runs. GAPDH was used as a housekeeping gene to normalize the data.

Statistical analysis

All experiments were repeated at least three times. A Student's t test was used to determine data homogeneity, and Dunnet's test was used to detect significant differences. Values of $p < 0.05$ were considered statistically significant.

Results

Expression of PLF during palatal suture expansion *in vivo*

Figure 2 shows that mechanical force induces the secretion of PLF, and PLF was expressed at low levels on day 0, at significantly higher levels on day 1, and then sharply decreased during palatal suture expansion *ex vivo*. This result indicates the necessity of applying PLF to enhance bone formation.

Accelerated mineral formation of bone

Results of the Raman microspectroscopy are shown in Fig. 3. Relative amounts and the location of either the mineral or the matrix are indicated and reveal considerable mineral formation of bone in the PLF and BMP-7 groups.

Raman microspectroscopy analysis further demonstrated that unstimulated palates maintained their

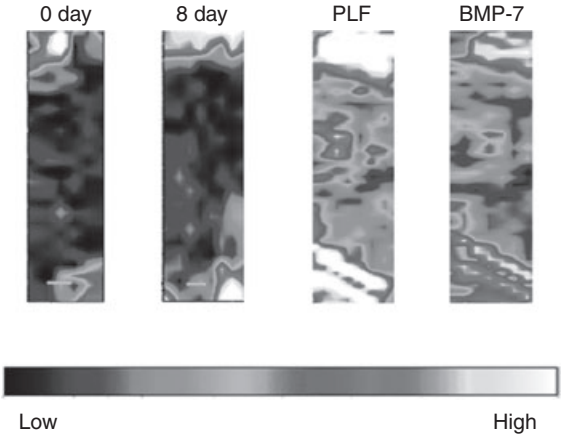

Fig. 3. Raman microspectroscopy mapping of bone mineral composition reveals that either periostin-like factor (PLF) or BMP-7 resulted in significantly greater bone formation at the osteogenic fronts. Lighter colors represent higher Raman peaks. Color scales indicate the bone matrix mineral concentration. Areas with high mineral and low collagen content (red) had an increased elastic modulus, whereas the elastic modulus was reduced in areas with low mineral and normal collagen content (blue). PLF-stimulated suture obviously displayed areas of bone mineral concentration within the palatal suture. Scale bar = 5 μm.

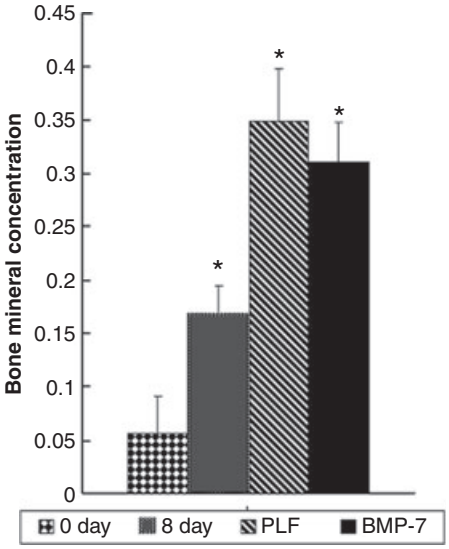

Fig. 4. Effect of periostin-like factor (PLF) or BMP-7 on bone mineral concentration. Quantitative analysis of Raman data showed increase in mineral concentration by PLF or BMP-7(*$p < 0.05$).

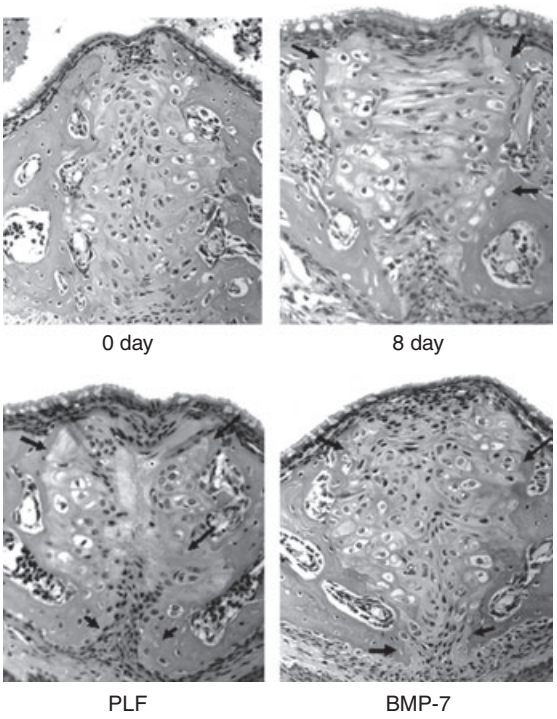

Fig. 5. Effects of periostin-like factor (PLF) on palatal bone formation. Hematoxylin and eosin staining of frontal sections of mid-palatal sutures stimulated with control (day 0, day 8), PLF, or BMP-7 for 8 days. Arrows point to areas of newly formed bone at the edges of palatal bones. Bone formation was observed at the edges of the palatal bones at the PLF group. Newly formed bone was covered with several layers of chondrocytes with a structure similar to the cartilage layers of the original suture. The PLF-stimulated suture underwent minor changes related to BMP-7-stimulated suture with a decrease in the number of chondrocytes and an increase in the amount of bone formation. Scale bar = 50 μm.

bone mineral concentration within the palatal suture over 8 days (%increase = 11.29 ± 2.34). In comparison, palates exposed to either PLF (%increase = 29.33 ± 1.61, p = 0.012) or BMP-7 (%increase = 25.49 ± 1.09, p = 0.016) formed significantly more bone at the osteogenic fronts (OFs) of the palatal suture compared with unstimulated samples at day 0 (Fig. 4). Only PLF-stimulated sutures obviously displayed areas of bone mineral concentration within the palatal suture. These results indicate that PLF accelerated the progression of bone formation within the palatal suture.

H&E staining provided detailed information on the morphology of the tissues within the sutures (Fig. 5). Expanded palatal sutures were widened, and the suture structure was reorganized. Endochondral bone formation was noticed in the *in vitro* suture expansion model, and more bone formation occurred at the edges of the palatal bones in sutures stimulated with PLF or BMP-7 as compared with unstimulated sutures. The PLF-stimulated sutures underwent minor changes as compared to BMP-7-stimulated sutures with a decrease

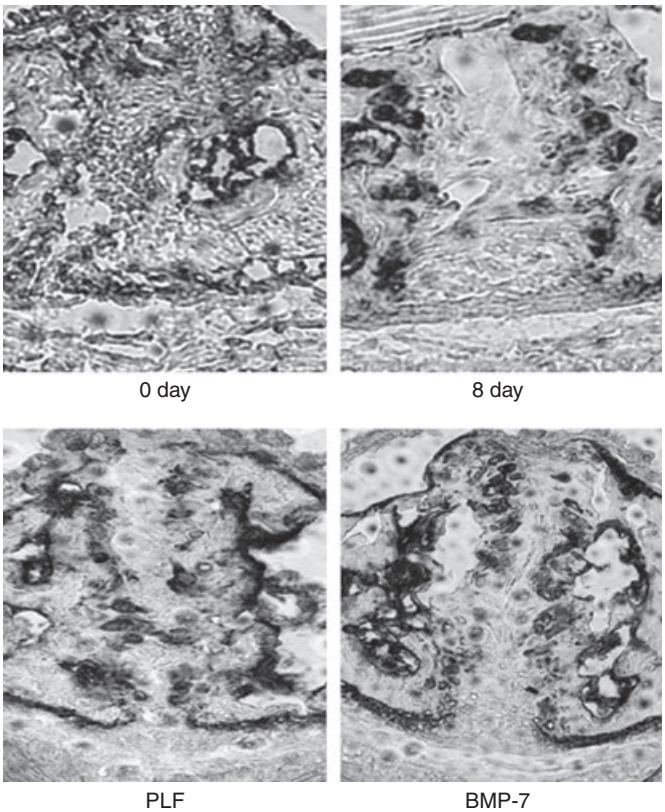

Fig. 6. Alkaline phosphatase (ALP) staining in the suture stimulated with control (day 0, day 8), periostin-like factor (PLF), or BMP-7 for 8 days. Staining intensity of ALP activity was markedly increased by PLF or BMP-7 compared with the corresponding controls (day 0 and day 8). Scale bar = 100 μm.

in the number of chondrocytes and an increase in the amount of bone formation.

Histochemistry

In the unstimulated groups, weak ALP activity was found along the bone edges that were regarded as the osteoblastic zone of the suture. Strong ALP staining was observed in periosteal cells in close proximity to palatal bone in the expansion groups. ALP activity along the bone edges was markedly greater in the PLF and BMP-7 groups compared with that in the 8-day unstimulated group (Fig. 6).

Western blot analysis

As illustrated in Figs 7 and 8, the levels of osteocalcin proteins in the palatal suture tissues of the PLF and BMP-7 groups were confirmed to be significantly higher than those in the control group ($p < 0.01$).

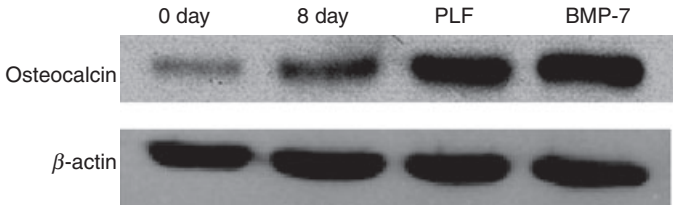

Fig. 7 Representative western blots for the expression analyses of osteocalcin. β-Actin was used as the loading control.

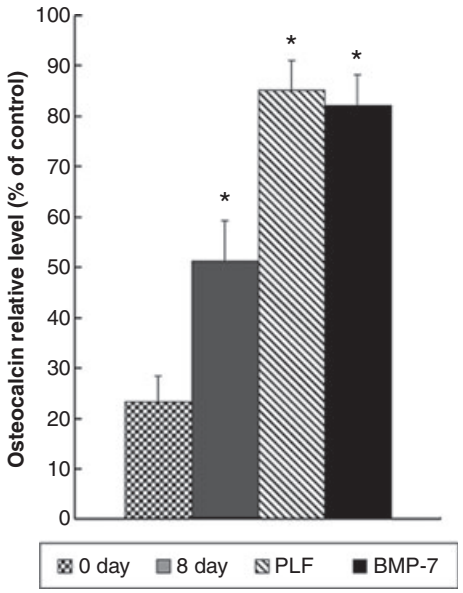

Fig. 8. Osteocalcin protein levels relative to β-actin protein levels were assessed by densitometric analysis and expressed in the periostin-like factor (PLF) and BMP-7 groups relative to the same proteins in the control group. (*The level in PLF group was significantly higher than that in the respective controls, $p < 0.01$).

Discussion

In clinical dentofacial orthopedics, relapse of the expanded maxillary arch during treatment is regarded as a cumbersome problem (3–7). The main benefit of accelerating bone formation in the palatal suture during and after expansion is to shorten the time required for bone remodeling, shorten the retention period, and impede relapse of the skeletal base. Based on these considerations, it is of great significance to explore effective approaches for achieving accelerated bone formation as a potential key determinant in preventing orthopedic relapse.

Using a mouse palate organ culture system after orthopedic expansion, applying PLF to rapidly expanded palatal sutures, and using a high-resolution approach, we showed the ability of PLF to accelerate bone formation within the expanding palate suture. These findings extend our understanding of the role of PLF as a regulator of bone growth.

Raman microspectroscopy and histological analyses were used to examine the specific sites of bone and cartilage formation and to quantify the new bone volume. While histology has been long regarded as the gold standard for analyzing sutures, Raman microspectroscopy has recently gained ground as a promising alternative method with the added benefits of increased accuracy, resolution, and reproducibility (14, 15). Raman microprobe analysis was used to gather information about the tissue chemistry in microstructural compartments of expansive force–induced newly formed bone. Raman spectroscopy has excellent spatial resolution (up to 0.5 μm) and spectral resolution (1–2/cm). Raman spectroscopy is particularly well suited to study bone (18–20). We found that in the Raman mineral score image, increased amounts of OFs areas were more clearly seen in the PLF-stimulated specimens than in unstimulated specimens, suggesting marked new growth and dramatically increased mineral deposition at the OFs in the stimulated group. Periostin-like factor can enhance mineral concentration. Increased mineral crystallinity of the newly formed bone suggests that the newly formed bone was consolidated into a dense and well-mineralized tissue. In the PLF groups, the mineralization rate of bone matrix

increased, which may be consistent with ALP activity. The effect of this rate increase on ALP activity by mechanical stimuli was significantly greater in the PLF groups compared with that in the unstimulated group. Alkaline phosphatase is considered to be a differentiation marker of osteoblastic cells, and its activity increases during the mineralization process of the bone matrix. High ALP activity may reflect high bone matrix mineralization or more mineralized bone. Alkaline phosphatase activity in the PLF group increased notably. Similarly, osteocalcin expression levels reflected the rate of bone formation. In the PLF group, osteocalcin expression levels were obviously upregulated compared with those in the unstimulated group. Together, these observations supported our hypothesis that PLF can efficiently accelerate the progression of bone formation within the orthopedic maxillary expansion. Preventing post-treatment relapse to some extent depends on the rate and quality of bone formation in the palatal suture during and after expansion (5, 6). Reorganization of hard tissue in the suture begins by the end of the active treatment phase (21). The complete mineralization of the suture margins takes 60–90 day (21). Periostin-like factor signaling in force-induced new bone increases functional parameters of bone quality, including bone mass and mineral concentration. These may be beneficial in shortening the retention period and preventing post-treatment relapse.

The histological analysis showed that PLF-stimulated suture underwent endochondral bone formation during orthopedic maxillary expansion. The mechanism of PLF-accelerated endochondral bone formation remains to be determined. Periostin-like factor play an important role in endochondral bone formation and promotes the proliferation and differentiation of chondrocytes (22). β1-Integrins are highly expressed on chondrocytes and mediate adhesion to cartilage matrix proteins (23). It has been shown that an expansive force inhibits chondrogenesis probably mediated by β1-integrin and cell–extracellular matrix interactions (24). Periostin-like factor interacts with β1-integrin in cardiac myocytes (25). Periostin-like factor interaction with β1-integrin may also mediate intercellular and cell–matrix interactions in chondrocytes and osteoblasts. Added exogenous PLF may strengthen the function of β1-integrin and promote differentiation of chondrocytes. Thus, it is possible that PLF may directly interact with β1-integrin, accelerate osteochondral differentiation, decrease chondrocytes proliferation, inhibit chondrogenesis, and promote endochondral bone formation. The mechanism of applying PLF-induced bone formation within this model should be further investigated.

The way of bone formation in the palatal suture is endochondral ossification during embryonic skeletogenesis, postnatal bone growth, and fracture repair (26). Our data showed that the PLF-stimulated suture underwent minor changes as compared to the BMP-7-stimulated suture with a decrease in the number of chondrocytes and an increase in the amount of bone formation. BMP-7 may increase the number of chondrocytes through proliferation. This will not directly benefit the stabilization of the expansion suture. Periostin-like factor -stimulated samples displayed areas of new bone formation within the center of the fibrous suture, presumably through endochondral ossification. These results indicate the potency of PLF in palatal suture bone formation and a strong potential for clinical translation while eliminating many of the side effects noted during BMP delivery (10).

PLF has been shown to promote osteoblast proliferation and differentiation (6, 11). It regulates the expression of osteoblastic gene makers such as osteocalcin, osteopontin, and collagen I, each of which can affect matrix mineralization (11). Periostin-like factor in force-induced new bone enhances functional parameters of bone quality, including bone mass and mineral concentration. Manipulation of PLF levels by gene therapy provides an attractive new approach for stimulating bone formation within palatal sutures. Therefore, PLF as a therapeutic target during orthopedic maxillary expansion *in vivo* deserves further research.

Conclusion

The findings suggest that PLF can increase bone formation within the palatal suture. PLF may be beneficial in preventing orthopedic relapse.

Clinical relevance

Transverse skeletal deficiency of the maxilla is a common clinical problem in orthodontics. Maxillary expansion is the preferred treatment approach to

increase the maxillary transverse dimension. However, even after a retention period, the expanded maxillary arch has a strong tendency to rebound to its previous form. The rate and quality of bone deposited in the palatal suture during expansion may affect post-treatment relapse. Periostin-like factor -accelerated bone formation within a mouse expansion palate organ culture system will help clinicians to understand the mechanism of bone formation in palatal sutures in response to expansion and may be beneficial in preventing post-treatment relapse.

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