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Featured Original Research

Biphasic sutural response is key to palatal expansion

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ABSTRACT

Introduction: It is assumed that transverse force physically open maxillary sutures and induce tensile stress that directly stimulates bone formation. However, orthopedic tensile stress is static, which cannot directly stimulate bone formation. We hypothesize that the anabolic response to transverse forces is indirect, the result of inflammation-induced osteoclast activation followed by a transition into osteogenesis. To test our hypothesis, we evaluated tissue, cellular, and molecular responses in the sutures during maxillary expansion.

Materials and methods: Sprague-Dawley rats (n = 95) were divided into four groups (n = 5 rats/group/time point, except for the expansion group, which did not have a day 0 sample): untreated control (C), sham (S), expansion (Exp), and expansion with nonsteroidal anti-inflammatory medication (Exp + NSAID). Maxillae were collected 0, 1, 3, 7, 14, and 28 days postexpansion for micro–computed tomography, light microscopy, gene expression, protein, and immunohistochemistry analysis.

Results: Compared with the sham group, the Exp group showed early expression of cytokines in the mid-palatal suture, osteoclast activation, and bone resorption resulting in widening of the suture. Anabolic bone formation was delayed, occurring after this initial catabolic phase. NSAIDs significantly decreased sutural widening, bone formation, and skeletal and dental expansion. During the transition from catabolic to anabolic phase, expression of osteoclast-osteoblast communicator molecules increased significantly.

Conclusion: Transverse force stimulates two distinct phases in the mid-palatal suture. An early catabolic phase, characterized by inflammation, osteoclast recruitment, and activity, results in bone resorption and sutural widening. Then osteoclasts activate osteoblasts resulting in an anabolic phase, during which the integrity of the skeleton is reestablished.

1. Introduction

It is generally accepted in orthodontics that static tensile force, applied to the craniofacial skeleton during orthopedic and orthodontic treatment, is osteogenic. However, previous studies have demonstrated that direct application of static force on long bones or alveolar bone does not have any significant effect on the skeleton [1,2]. How can we then explain bone formation due to orthopedic and orthodontic force?

A closer look at the physical distribution of force in the craniofacial skeleton shows that loading is largely indirect with the intermediary connective tissue between the point of force application and the bone transmitting the force. In the case of the upper jaw (maxilla), this connective tissue is sutures between the maxilla and the rest of the skull. In the case of the teeth, the connective tissue is the periodontal ligament (PDL), which is embedded in the cementum covering the entire tooth root and the alveolar bone. These examples of indirect loading are in contrast to weight-bearing bones, which are exposed primarily to direct loading.

Recently, we among others [3–6], have shown that application of static orthodontic force stimulates an inflammatory reaction throughout the PDL. Based on these studies, we concluded that

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alveolar bone resorption observed during orthodontic tooth movement does not result directly from orthodontic force on the bone. Rather, orthodontic force first induces inflammatory cytokine and chemokine release throughout the PDL, which then recruits and activates osteoclasts, resulting in bone resorption around the entire root. This singular catabolic phase stands in distinct contrast to the conventional model of compression-induced bone resorption on one side of the tooth with a simultaneous tension-induced bone formation on the opposite side of the tooth. Once the force dissipates, the catabolic phase is complete and the anabolic phase of bone formation around the entire root begins. We refer to this new model of orthodontic tooth movement as the biphasic theory of tooth movement [7].

The studies presented here question the widely held belief that tensile force directly stimulates bone formation during orthopedic treatment. Is it possible that similar to what we observed in the PDL, bone formation in response to tensile orthopedic force is just a delayed reaction and not a direct effect of this force on the bone? One would expect that in response to tensile stress, similar to compressive stress, the osteoclasts would appear in the area of trauma during application of orthopedic force. Because it has been shown that osteoclasts play a role in activating osteoblasts [8], is it possible that activating osteoclasts is a prerequisite for the osteoblast activity observed during orthopedic treatment?

To test our hypothesis, we systematically examined the effect of maxillary expansion force on the mid-palatal suture in rats. Specifically, we examined the skeletal, cellular, and molecular changes that occur during the first 28 days of expansion treatment.

2. Materials and methods

2.1. Study design

Growing Sprague-Dawley male rats (n = 95, average weight 60 g, 21 days old) were treated according to the protocol approved by the New York University Institutional Animal Care and Use Committee.

Rats at this stage are in a growth phase and their first and second maxillary molars are fully erupted. Animals were randomly divided into four groups: untreated control (C), sham (S), expansion (Exp), and expansion with nonsteroidal anti-inflammatory drug (Exp+NSAID). Control group animals did not receive any appliance. The expansion group received a calibrated custom-designed expansion spring that delivered a 100 cN transverse force to the first and second molars [9]. The springs were calibrated to produce 100 cN force (50 cN on each side) using a digital force gauge. This force was selected based on previous studies demonstrating that 100 cN maximally induces cellular activity in maxillary sutures [10]. Considering the rats would have normal vertical chewing force averaging 54 to 76 N [11], this force is not considered excessive. The appliance was connected to the first molars with ligature wires and fixed with composite. The sham group received a similar spring that was not activated and did not produce any force. To ensure that the sham group did not receive an activated spring, we compared sham results with the control group. We did not find any differences between the two groups in any experiments (data not shown). Therefore, all expansion groups are compared with the sham group in each experiment. The expansion with NSAID group received a similar active spring as the expansion group and a daily dose of a diclofenac (5 mg/kg) administered intramuscularly (injection sites were changed at each injection to prevent additional discomfort to the animals). Animals were weighed daily to accurately calculate the dose of the medication for each animal.

Bone labeling was performed by means of an intraperitoneal injection of calcein (15 mg/kg) on day 0, xylenol orange (25 mg/kg) at day 12 and doxycycline (25 mg/kg) at day 26. The health status and body weight of the rats were evaluated daily, and no significant differences were observed among groups. Animals were euthanized by CO_2 narcosis on days 0, 1, 3, 7, 14, and 28. Samples were collected for micro–computed tomography analysis (μCT), histology, and immunohistochemistry (5 animals per time point, per group, total of 25), fluorescence microscopy (5 animals per group killed on day 28, total of 20), mRNA (5 animals per time point, per group, total of 25) and protein analysis (5 animals per time point, per group, total of 25).

2.2. Tensile transverse force application

On day 0, animals from sham, expansion, and expansion + NSAIDs groups were anesthetized with intraperitoneal injection of ketamine and xylazine (0.09 mL/100 g). All groups were fit with custom-designed expansion springs as described before [2]. Tensile transverse force was applied for 0 to 28 days.

2.3. Specimen preparation

2.3.1. μCT, histology and immunohistochemistry analyses

The whole skull was dissected and fixed for 72 hours with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, followed with storage in 70% ethanol. μCT analysis was then performed, after which all samples were demineralized in 14% ethylenediaminetetraacetic acid for 3 to 4 weeks at 4°C, and dehydrated in ethanol gradients and xylene prior to embedding in paraffin.

2.3.2. Fluorescence microscopy

After fixation in 100% methanol for 3 days at 4°C, samples were placed in acetone for another 2 days at room temperature. Specimens were dehydrated in alcohol, cleared in xylene, and embedded in methyl methacrylate [12].

2.4. μCT analysis

Before decalcification, the whole maxilla was scanned by μCT on a Skyscan1172 system (Bruker microCT, Kontich, Belgium). The specimens were scanned in 70% ETOH at 13.55 μm voxel size, 100 KV, 0.300° rotation step (192.30° angular range), and a 1910-ms exposure. Results were analyzed utilizing the NRecon software (1.6.9.16 version; Bruker microCT) for three-dimensional (3D) reconstruction and viewing of images, and superimpositions were performed using Amira 6.0.0 software (FEI Houston Inc, Hillsboro, OR).

Three measurements were obtained from the μCT images at the level of the mid-coronal plane of the maxillary first molar: 1) width of the mid-palatal suture (distance between the borders of the suture); 2) width of palate (distance between the palatal walls at the level of intersection between palate and alveolar), and 3) interdental width (distance between height of contour of first molars) (Fig. 1).

Two examiners, who were blinded to the samples being evaluated, completed all μCT quantifications. Both intraobserver and interobserver errors were evaluated. Intraobserver error was evaluated by individual investigators who measured five μCT twice at least 2 weeks apart. Interobserver error was evaluated using the same set of five μCT measured by a second investigator. The Dahlberg formula [13] was applied to estimate the random errors and the paired *t*-test was applied to identify systematic errors according to Houston [14]. Random error for intraobserver evaluation was 0.015 mm and 0.017 mm for the interobserver evaluation, and not statistically significant. Systematic errors were also small and not statistically significant ($P = 0.87$ for intraobserver and $P = 0.84$ for interobserver).

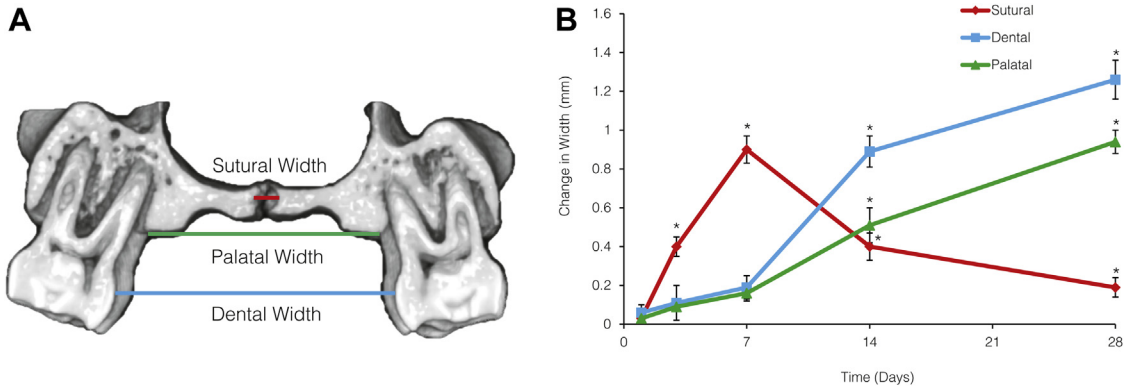

Fig. 1. Changes in sutural, palatal, and dental widths over time. (A) Palatal, dental, and mid-palatal suture widths were measured using μCT 3D reconstructed images, and sections at the level of the mid-coronal plane of the maxillary first molar. Red line shows width of the mid-palatal suture (distance between the borders of the suture), green line shows the width of the palate (distance between the palatal walls at the level of intersection between palate and alveolar walls), and blue line shows the dental width (distance between height of contour of first molars). (B) Mid-palatal suture, palatal, and dental widths were measured over time in the expansion (lines shown in graph) and sham maxillae. Data expressed as the mean ± standard deviation (SD) of distances in millimeters. Each number represents the average of five samples. Widths in the sham group did not change (data not shown). *Expansion width significantly different from sham, $P < 0.05$. (C) Three-dimensional μCT reconstructed images of rat maxilla showing mid-palatal suture changes over time in both expansion and sham maxillae. (D) Immunohistochemistry for TRAP was performed in paraffin sections of both expansion and sham maxilla to identify active osteoclasts in the area. Light microphotographs show TRAP-positive osteoclasts in mid-palatal suture and surrounding bone at different time points. All images were collected at the suture area between first and second molars. Magnification ×10. Osteoclasts are stained as multinucleated red cells (*black arrows*, magnification ×10).

2.5. Histology and immunohistochemistry

Fixed demineralized maxillae were embedded in paraffin, and cut into 5-μm occlusal sections, using Leica Biosystems RM2265 Fully Automated Rotary Microtome (Leica Biosystems Inc., Buffalo Grove, IL). Some sections were stained with hematoxylin and eosin (H&E) to view the general cellular and tissue structure. Consecutive sections were stained with TRAP (tartrate-resistant acid phosphatase) to identify osteoclasts. Osteoclasts were defined as TRAP-positive multinucleated bright red cells in the mid-palatal suture or adjacent bone. Sections were scanned on a Scan Scope GL series optical microscope (Aperio, Bristol, UK) at ×20 magnification.

2.6. Reverse transcriptase–polymerase chain reaction analysis

For gene expression studies, five animals from each group were euthanized by CO_2 narcosis at days 0, 1, 3, 7, 14, and 28. The palatal bone extending 0.5 mm from each side of the mid-palatal suture was dissected and frozen in liquid nitrogen. The alveolar bone surrounding the molars was also dissected and frozen for PDL studies. Total mRNA was isolated as described previously [7]. Cytokines, osteoclast markers (cathepsin K [CtsK] and receptor activator of nuclear factor kappa-β ligand [RANKL]), osteoblast markers (alkaline phosphatase [ALP] and osteopontin), and bone simulating factors (bone morphogenetic protein 6 [BMP6] and transforming growth factor [TGF]-β), were analyzed with a QuantiTect SYBR Green reverse transcriptase–polymerase chain reaction (RT-PCR) kit and rat-specific primers (both Qiagen, Valencia, CA) on a DNA Engine Optican 2 System (MJ Research, Waltham, MA). Each mRNA specimen was tested three times. Relative levels of mRNA were calculated and normalized to the level of GAPDH and acidic ribosomal protein mRNA.

2.7. Protein analysis

Levels of inflammatory markers were measured by enzyme-linked immunosorbent assay (ELISA). Five palatal bones extending 0.5 mm from each side of the mid-palatal suture were dissected, frozen in liquid nitrogen, and pulverized. Lysates were prepared and total protein quantitated using a BCA protein assay kit (Pierce, Rockford, IL). Concentration of interleukin (IL)-1β (Thermo, Rockford, IL) and tumor necrosis factor alpha (TNF-α) (Thermo) was determined by ELISA. Data were analyzed in comparison with standard curves specific to each inflammatory marker.

2.8. Statistical analysis

After confirming normal distribution of samples by the Shapiro-Wilk test, group comparisons were assessed by analysis of variance. Pairwise multiple comparison analysis was performed with Tukey's post hoc test. In some experiments, paired *t* tests were used to compare the two groups. Two-tailed *P* values were calculated; $P < 0.05$ was set as the level of statistical significance.

3. Results

3.1. Mid-palatal suture widening in response to transverse force occurred before dental and skeletal changes

To understand the morphological changes observed in the mid-palatal suture in response to transverse force, we measured the suture, the palate, and the dental widths (Fig. 1A) in 3D μCT images. If the effect of transverse force is purely physical, then there should be a linear relationship between the magnitude of suture widening and the increase in palatal and dental width. The mid-palatal suture width increased significantly on days 3, 7, 14, and 28 in the expansion group compared with the sham group ($P < 0.05$) (Fig. 1B, Table 1). The mid-palatal suture returned to its original width by day 28 (Fig. 1B and 1C, Table 1).

Surprisingly, the dental and palatal widths in the expansion group significantly increased only on day 14 (0.89-mm and 0.54-mm increase, respectively) and day 28 (1.26 mm and 0.92 mm, respectively) ($P < 0.05$) compared with the sham group. At both time points, dental changes were significantly greater than skeletal changes ($P < 0.05$) (Table 1). On days 1, 3, and 7, the skeletal and dental changes were not significant ($P > 0.05$), whereas the mid-palatal suture was significantly wider on days 3 and 7 ($P < 0.05$).

3.2. Transverse force increased osteoclast number and activity in the mid-palatal suture

To correlate the morphological changes with cellular changes in the mid-palatal suture following expansion, we

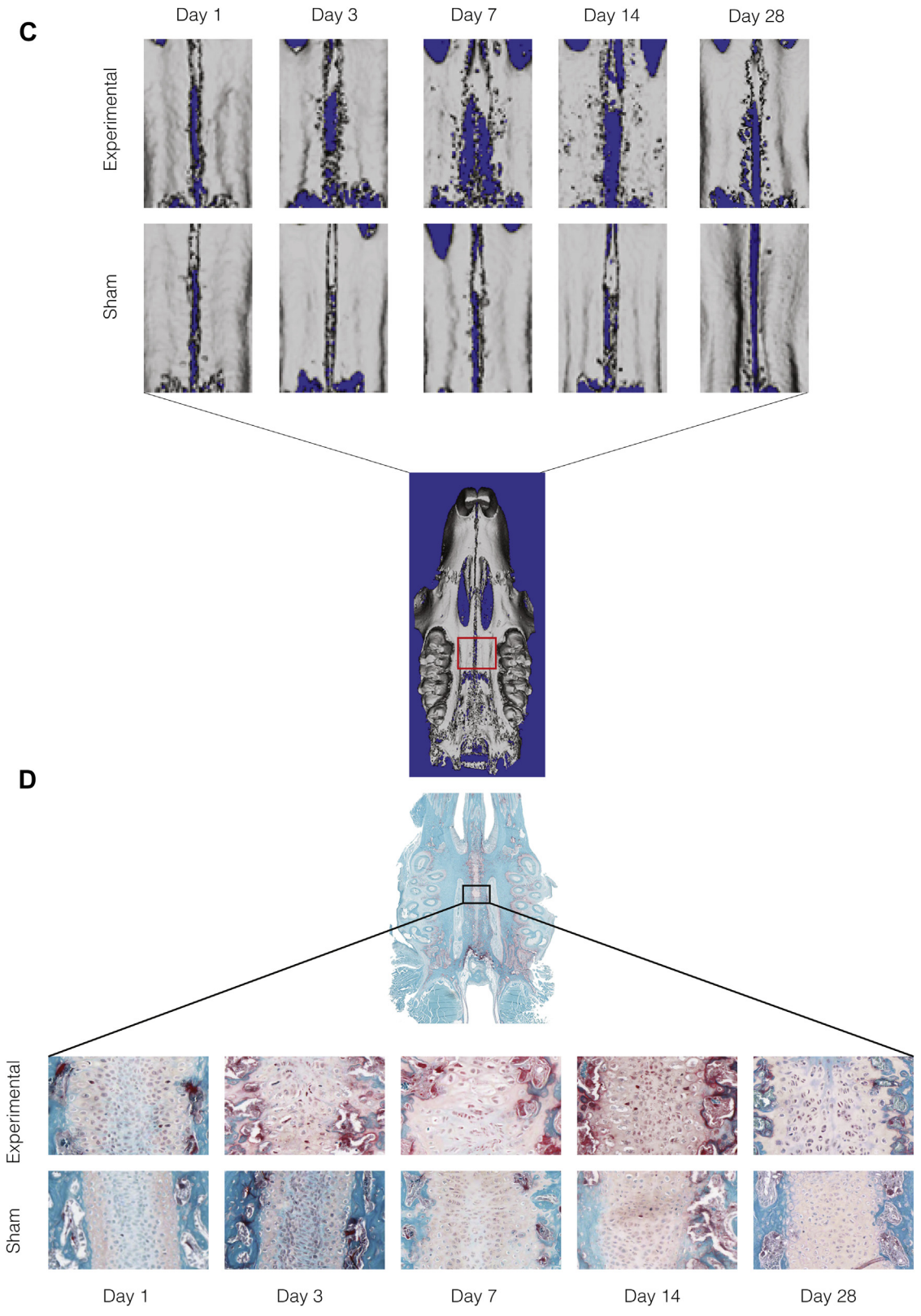

Figure 1. (*continued*).

investigated the presence of osteoclast by TRAP staining. Compared with the sham group, the expansion group developed a noticeable change in the suture's cellular organization on days 7 and 14 (Fig. 1D), which correlated with the greatest sutural widening observed in Figure 1C and Table 1. We observed an increase in the number of TRAP-positive osteoclasts in the mid-palatal suture and adjacent bone, especially at days 3, 7, and 14 (Fig. 1D).

Table 1
Changes in palatal, dental, and sutural widths over 28 days of maxillary expansion

	Day 0	Day 1	Day 3	Day 7	Day 14	Day 28
Palatal width						
Sham	1.89 ± 0.03	1.89 ± 0.02	1.99 ± 0.04	2.06 ± 0.03	2.33 ± 0.05	2.73 ± 0.04
Expansion		1.92 ± 0.04	2.08 ± 0.03	2.22 ± 0.04	2.87 ± 0.05[a]	3.65 ± 0.05[a]
Dental width						
Sham	3.12 ± 0.02	3.12 ± 0.02	3.84 ± 0.02	4.24 ± 0.03	4.3 ± 0.04	4.42 ± 0.03
Expansion		3.18 ± 0.04	3.97 ± 0.04	4.43 ± 0.04	5.19 ± 0.04[a]	5.68 ± 0.05[a]
Sutural width						
Sham	0.08 ± 0.02	0.08 ± 0.02	0.1 ± 0.03	0.11± 0.04	0.1 ± 0.03	0.1 ± 0.03
Expansion		0.11 ± 0.02	0.53 ± 0.04[a]	1.2 ± 0.03[a]	0.64 ± 0.02[a]	0.29 ± 0.04[a]

Data presented as mean ± SEM of five samples.
[a] Significantly different from sham.

To further evaluate osteoclast activity in the mid-palatal suture, expression of osteoclast markers RANKL and CtsK was evaluated at different time points (Fig. 2A). RANKL expression was significantly higher ($P < 0.05$) on days 1, 3, 7, and 14 (2.6-, 4.8-, 5.4-, 3.9-fold, respectively) in the expansion group compared with the sham group. Likewise, CtsK expression increased significantly ($P < 0.05$) at days 1, 3, 7, and 14 (2.3-, 3.4-, 6.2-, and 5.1-fold, respectively) in expansion animals compared with sham animals.

3.3. Increased and sustained expression of inflammatory markers during expansion

Having observed that transverse, tensile force increased osteoclast number and activation (rather than decrease them, as the compression-tension model would predict), we hypothesized that the local environment in the mid-palatal suture immediately after force application, similar to the PDL after orthodontic force application, promotes osteoclastogenesis through inflammatory mediators.

To determine the pattern of inflammatory marker expression during maxillary expansion, the levels of the cytokines IL-1β and TNF-α in the mid-palatal suture were followed for 28 days after applying transverse force (Fig. 2B). IL-1β and TNF-α levels peaked twice in the mid-palatal suture, once on day 1 and again on day 14 before returning to baseline by day 28.

3.4. Blocking inflammation and osteoclast activation with NSAIDs prevented skeletal changes in response to transverse force

To evaluate how inhibiting inflammation and osteoclast activity affect the skeletal response to transverse force, expansion was performed in the presence or absence of NSAIDs. Expression of prominent inflammatory markers CCL2 and IL-1β was measured in the mid-palatal suture of the sham, expansion, and expansion + NSAID groups 3 days after applying transverse force (Fig. 3A). Expression of CCL2 in the expansion group increased 3.6-fold compared with the sham group; however, this increase was blocked with NSAIDs (1.3-fold increase compared with the sham group). IL-1β expression in the expansion group increased 3.2-fold compared with the sham group. As with CCL2, the same force in the expansion + NSAID group did not stimulate IL-1β expression in the suture (1.1-fold change compared with the sham group). The decrease in IL-1β and CCL2 expression in response to NSAIDs was statistically significant ($P < 0.05$).

To evaluate how anti-inflammatory medication affects osteoclast number in the mid-palatal suture, H&E and TRAP staining were performed in sections from the mid-palatal suture of sham, expansion, and expansion + NSAID groups at the peak of osteoclast activation (Fig. 3B). On day 7, transverse force in the expansion group resulted in suture widening and marked resorption of the suture's bony borders. TRAP staining in the expansion group revealed a significant increase in the number of osteoclasts. However, in the expansion + NSAID group, no widening or bone resorption was observed. TRAP staining in the expansion + NSAID group was significantly reduced. To further confirm the reduction in osteoclastic activity in the suture, the expression of RANKL and CtsK was measured in the suture 7 days after applying transverse force (Fig. 3C). Anti-inflammatory medication decreased RANKL expression significantly from 4.7-fold in the expansion group to 1.4-fold in the expansion + NSAID group ($P < 0.05$). Similarly, CtsK expression decreased significantly from 5.8- to 1.6-fold in response to the medication ($P < 0.05$).

To investigate how osteoclast inhibition with NSAIDs affects maxillary expansion, we performed μCT analysis of sham, expansion, and expansion + NSAID maxilla. As clearly demonstrated, there was no increase in either mid-palatal or dental widths after applying transverse force in the expansion + NSAID group compared with the sham group (Fig. 3D). Differences in mid-palatal and dental widths between expansion and expansion + NSAID groups were statistically significant ($P < 0.05$) (data not shown).

3.5. Bone formation at the mid-palatal suture was delayed in response to transverse force and required osteoclast activation

Because the mid-palatal suture width returned to baseline by day 28 (Fig. 1B), we next investigated the osteogenic activity in the suture in response to transverse force. We examined the expression of the osteogenic markers ALP and osteopontin at days 1, 3, 7, 14, and 28 after applying transverse force (Fig. 4A). ALP levels increased significantly in the expansion group at day 3 (3.3- and 2.4-fold, respectively), peaked at day 7 (11.4- and 4.3-fold, respectively) and stayed elevated at days 14 (9.8- and 4.2-fold, respectively) and 28 (4.8- and 2.4-fold, respectively), compared with the sham group ($P < 0.05$). Osteopontin increased significantly only at days 7, 14, and 28 (3.2-, 3.8-, and 2.7-fold, respectively) compared with the sham group ($P < 0.05$) (Fig. 4A).

Expression of the osteogenic markers ALP, osteopontin, and osteocalcin in the expansion + NSAID group was significantly lower (1.9-, 1.4-, and 1.2-fold, respectively) on day 14 compared with the expansion group, but was not significantly different from the levels in the sham group ($P < 0.05$) (Fig. 4B).

It has been suggested that osteoclasts may activate osteoblasts through TGF-β, BMP6, WNT10, and SP1 pathways [15–17]. To further study the relation between osteoclasts and osteoblasts, the expression of TGF-β and BMP6 in the mid-palatal suture was studied at days 1, 3, 7, 14, and 28 after the application of transverse force (Fig. 4A). Compared with the sham group, TGF-β expression in the expansion group was highest at days 3 and 7 and returned to baseline by day 28. BMP6 peaked at day 1 and dropped throughout the remainder of the study. All increases were statistically significant compared with the sham group ($P < 0.05$).

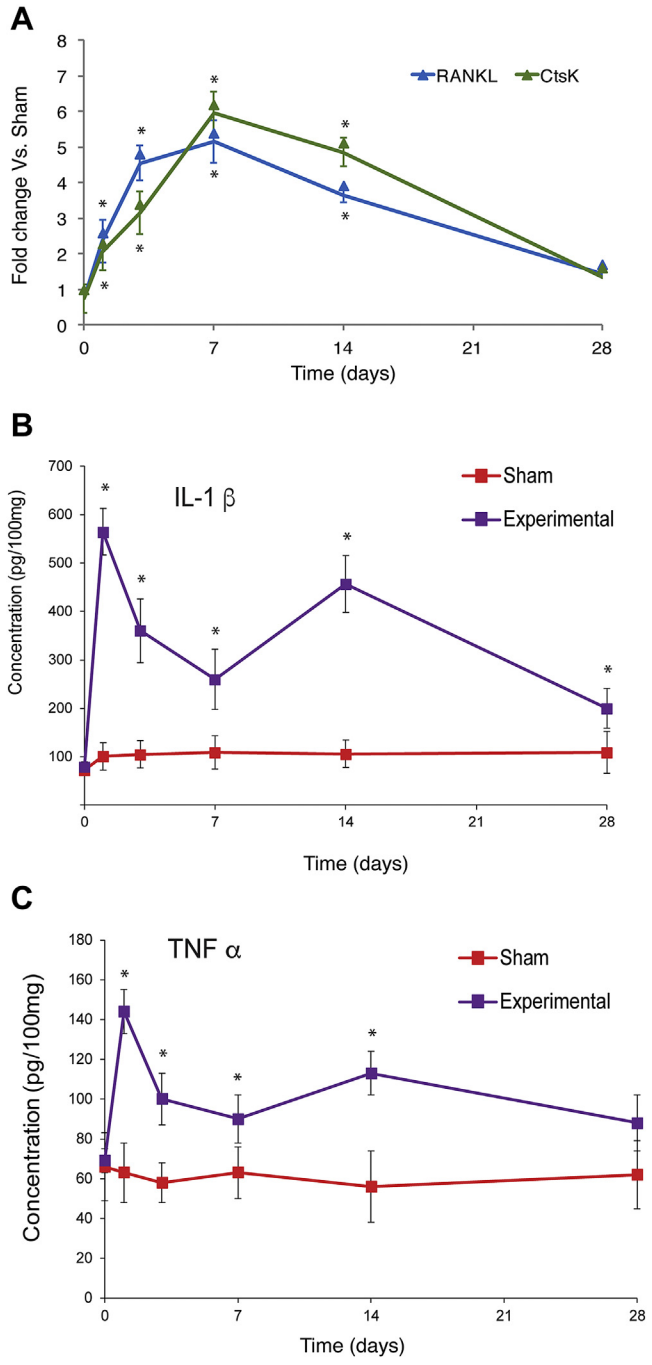

Fig. 2. Increase in expression of osteoclast markers, and inflammatory mediators during sutural response to transverse forces. (A) Gene expression of RANKL and CtsK in the mid-palatal suture was measured by RT-PCR from day 1 to day 28. Data expressed as the mean ± SD fold change in comparison with the sham group. Each value represents the average of five samples. *Significantly different from sham group, $P < 0.05$. (B) Mean concentration of IL-1β and (C) TNF-α, in the mid-palatal suture at different time points was evaluated by ELISA. Data expressed as the mean ± SD of concentration in picograms per 100 mg of tissue. Each number represents the average of five samples. *Significantly different from sham; $P < 0.05$.

4. Discussion

4.1. Sutural opening in response to transverse force is a biological phenomenon

Application of transverse force to the maxilla has been traditionally advocated for orthopedic correction in patients with maxillary constriction [18,19]. There are two general assumption behind these treatments: 1) heavy force separates the two hemi-maxillae creating tensile stress in the mid-palatal suture, and 2) tensile stress has a direct osteogenic effect on the mid-palatal suture [20–24]. Based on the first part of this assumption, opening of the mid-palatal suture has been considered a direct result of hemi-maxillae displacement in response to transverse force and, therefore, a physical phenomenon rather than the result of cellular activity. This concept is so dominant in orthodontics that most research in this field has focused on stress and strain distribution using dry skulls or finite element analysis [25,26]. However, if a biological response is not only a part of the process, but is the main part of the skeletal response to transverse force, then the predictions from dried skulls that have no biological response misrepresent the clinical reality, which could explain the conflicting results between in vitro and clinical studies [27–29].

Indeed, our findings show that physical displacement of the maxilla is not the first event in response to transverse force, and suture widening occurs ahead of skeletal changes. Our experiments demonstrate that although there is an immediate skeletal and dental displacement in response to continuous transverse force, these movements are minuscule and halt quickly. In fact, the first significant increase in palatal and dental width did not occur until a few days after maximum sutural widening. In addition, the magnitude of widening of the mid-palatal suture was not linear with the magnitude of skeletal displacement. These results contradict the general understanding that expansion is an immediate response to applied force and suture widening is the direct result of skeletal displacement. Our histological studies confirmed these findings by demonstrating appearance of TRAP-positive osteoclasts in the mid-palatal suture early after the application of transverse force, which coincided with suture opening in the μCT images, ahead of the skeletal changes. From this aspect, maxillary expansion is very similar to tooth movement, where movement does not occur until osteoclasts have been activated to resorb the bone in the path of movement, ahead of the movement.

In support of our conclusion that a catabolic phase is an important part of skeletal changes in response to tensile force, our results demonstrate that application of anti-inflammatory medication significantly decreased the skeletal changes in response to transverse force, highlighting the requirement for osteoclast activation in those skeletal changes. If these changes were just a physical phenomenon, anti-inflammatory medication would not have significantly affected the skeletal changes.

Our finding is also in agreement with previous observations that incremental application of high-magnitude force in a dry skull (where there is no biological reaction) causes accumulation of stress at the connection of the maxilla to adjacent bones that ultimately may cause the skull to fracture [30]. On the other hand, in vivo stress generated in response to application of incremental force is relieved gradually (if there is enough time between force applications) [22], thereby preventing damage to adjacent bones. Indeed, the rapid delivery of high-magnitude force that bypasses this biological prerequisite for expansion can cause fracture of adjacent skeletal structures [31] and can be dangerous to patients. These studies emphasize the vital role of a biological reaction in response to transverse force not only as a necessary step before maxillary expansion, but also as a natural safety mechanism to prevent stress build up around vital structures and organs.

4.2. Inflammatory and osteoclast markers mediated the transverse maxillary force response

Because osteoclastogenesis is largely regulated by inflammatory markers, we investigated if transverse maxillary force increases osteoclasts through activation of inflammatory markers. Many of the inflammatory cytokines, chemokines, and their receptors

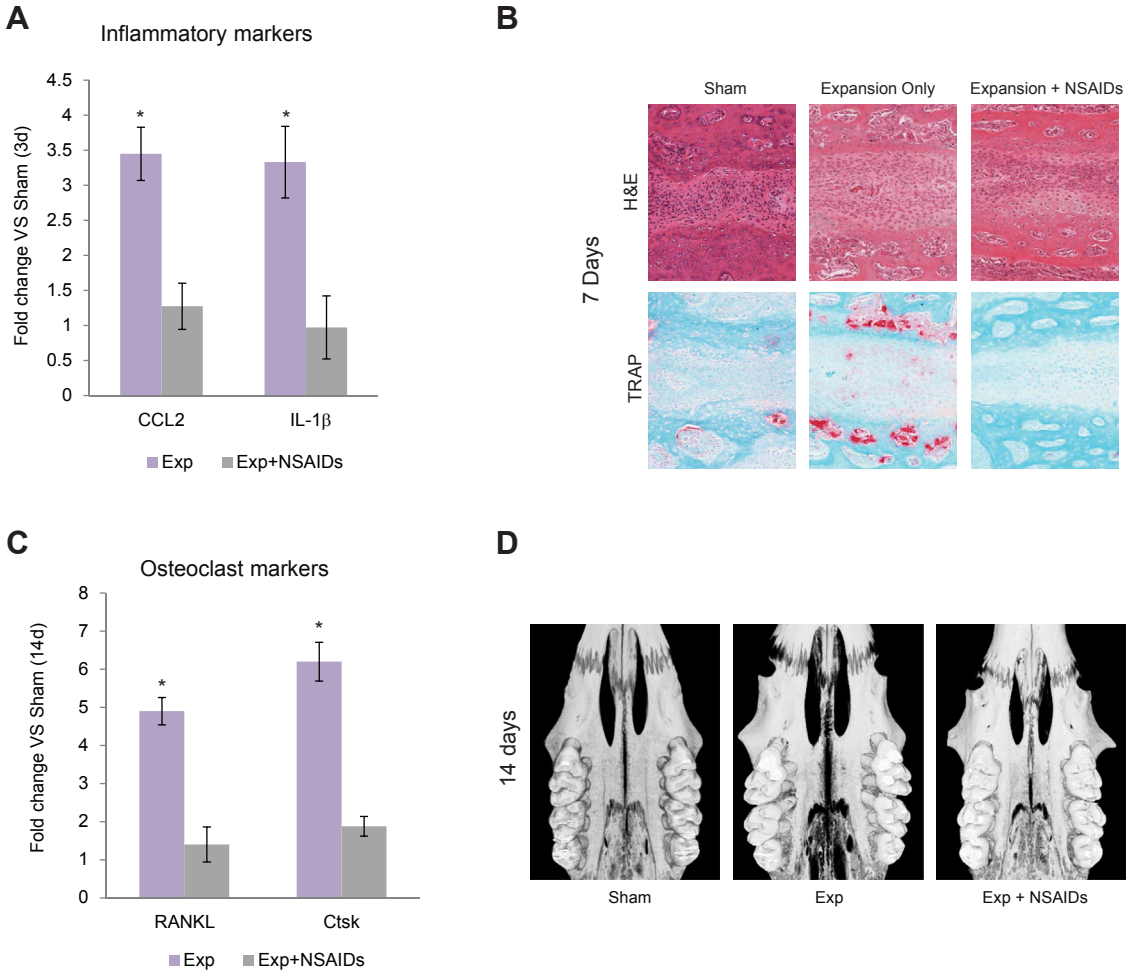

Fig. 3. Anti-inflammatory medication blocked the catabolic effect of transverse forces and expansion. Change in expression of (A) IL-1β and CCL2, in mid-palatal suture in the presence and absence of NSAIDs was measured by RT- PCR at day 3. Data are expressed as mean ± SD fold change in expression in comparison with sham. Each value represents the average of five samples. *Significantly different from sham group, $P < 0.05$. (B) H&E and TRAP staining of histological sections taken from mid-palatal suture in area of contact between first and second molars in sham, expansion, and expansion + NSAID maxilla after 7 days of transverse force application. Osteoclasts appear as red multinucleated cells (black arrows). (C) RANKL and CtsK expression in the mid-palatal suture in the presence and absence of NSAID was measured by RT- PCR at day 7. Data are expressed as mean ± SD fold change in expression in comparison with sham. Each value represents the average of five samples. *Significantly different from sham group, $P < 0.05$. (D) μCT images of representative maxillae from sham, expansion, and expansion + NSAID groups after 14 days of force application.

significantly increased in the mid-palatal suture 24 hours after transverse force application. Our data show that expression of inflammatory markers in the suture coincided with increased expression of osteoclast markers, such as CtsK and RANKL, which signal the start of catabolic changes at the suture. The fact that blocking the inflammatory markers with anti-inflammatory medication inhibited expression of osteoclast markers, strongly supports the role of inflammation in osteoclastogenesis and initiation of bone resorption, in agreement with previous reports [32–35].

When we followed two of these genes over the course of the experiment, each demonstrated two peaks of activation in suture. Although the first peak increase in expression of inflammatory markers in suture can be related to immediate microtrauma induced by force, the second peak of inflammatory markers in the mid-palatal suture coincided with skeletal movement that can reexpose the mid-palatal suture to further microtrauma from tensile stress.

4.3. Molecular and cellular changes mark the transition from catabolic to anabolic phases to tensile force

As we discussed earlier, it is widely held among orthodontists that tensile stress directly stimulates bone formation. Is that true? One would expect that if tensile stress directly triggers osteogenesis, then it should start immediately. To our surprise, changes in bone formation marker levels (i.e., the anabolic phase) were not observed until late in the catabolic phase. Based on this observation, one would wonder if the catabolic phase is a prerequisite for the anabolic phase? Our results demonstrate that in the presence of anti-inflammatory medication, which significantly inhibited the catabolic phase, the expression of osteogenic markers was also significantly reduced. One could argue that the decrease in the anabolic phase is due to the direct effect of anti-inflammatory medication on osteoblasts. Research in this area is not clear. Although some reported inhibitory effects of high-dose anti-inflammatory medication on osteoblasts, others have shown that low-dose anti-inflammatory medication, similar to what has been used in the experiments presented here, does not affect osteoblast activity [36–38]. On the other hand, a decrease in osteoclast number and activity induced by transverse force in the presence of low-dose anti-inflammatory medication coincided with a decrease in osteoblast activity, which suggests a possible role for osteoclasts in the activation of osteoblasts in agreement with previous studies [15–17].

Indeed, the final piece of evidence to support our conclusion that inflammatory bone resorption must precede the anabolic response

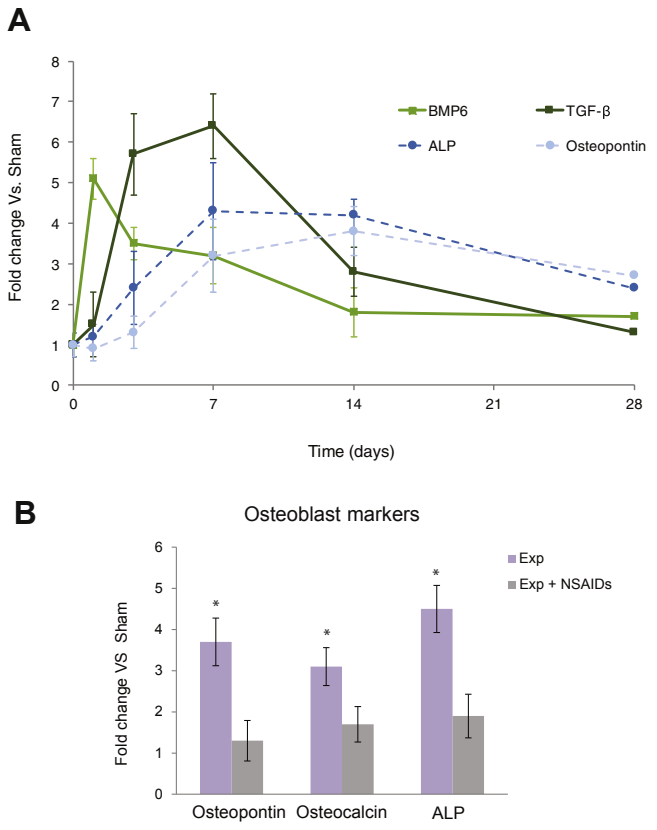

Fig. 4. Expression of osteoblast markers and transition molecules in the suture suggest a delayed anabolic response to expansion forces. (A) Gene expression of BMP-6, TGF-β (transitional molecules), and ALP, osteopontin, and osteocalcin (osteoblast markers) in the mid-palatal suture was measured by RT-PCR at different time points. Data expressed as the mean ± SD fold change in comparison with sham group. Each value represents the average of five samples. *Significantly different from sham group, $P < 0.05$. (B) Change in expression of ALP, osteopontin, and osteocalcin in the mid-palatal suture in the presence and absence of NSAID was measured by RT-PCR at day 14. Data are expressed as the mean ± SD fold change in expression in comparison with sham. Each value represents the average of five samples. *Significantly different from expansion + NSAID group and from sham group, $P < 0.05$.

to tensile stress is the temporal profile of genes that drive the transition of the bone remodeling machinery from the catabolic to the anabolic phase.

It has been shown that osteoclasts may activate osteoblasts by the release of diffusible paracrine factors such as sphingosine 1-phosphate, BMP6, and Wnt10b [15]. Our experiments show the presence of TGF-β and BMP6 in the suture at the time points coinciding with the transition phase is marked by an increase in osteoblastic markers and a decrease in osteoclastic markers. It should be emphasized that there are at least two other modes of osteoclast-osteoblast communication that were not investigated in this study: 1) direct contact between these two cell types allowing membrane-bound ligands and receptors to interact and initiate intercellular signaling, and 2) during bone resorption, osteoclasts may liberate molecules deposited in bone matrix, such as TGF-β or insulin like growth factor-1, which attract and activate osteoblasts [15–17]. Further research in this area is necessary. Therefore, considering the osteogenic effect of transverse force, the direct effect of tensile stress on the bone is an oversimplified and misleading model.

4.4. Clinical implications of sutural biological response to transverse force

The overall understanding that sutural opening is a cellular reaction and not just a physical phenomenon has a significant impact on clinical decisions, on selecting the magnitude of transverse force and rate of application of force during treatment of patients with maxillary constriction. If separation of the hemi-maxillae is a purely physical phenomenon, then high force to overcome sutural resistance is justified, especially in adults. A faster rate of force delivery would achieve skeletal changes before dental changes occur, considering skeletal changes will be a physical phenomenon with a rapid rate of movement and dental changes would be a biological phenomenon with slow rate of movement. On the other hand, if the separation of hemi-maxillae is a biological response to mechanical stimulation, the optimum force should be defined based on optimum biological response and high force not only is unnecessary but may even be dangerous, especially in adults, as it has been previously reported to cause severe damage to the skull [39]. Indeed, if the rate of expansion depends on the rate of osteoclast activity, application of higher force does not increase the rate but mostly causes accumulation of stress and bending of structures. This hypothesis can explain why previous studies did not find significant differences in magnitude of expansion between heavy and light force [31].

If skeletal changes are a biological reaction similar to dental changes, the magnitude of orthopedic force should differ by age. In children, due to a wider sutural width, it is possible to accomplish some skeletal displacement before full activation of osteoclasts. That can explain why children are more tolerant of rapid activation. In adults, because the initial sutural width is smaller, application of

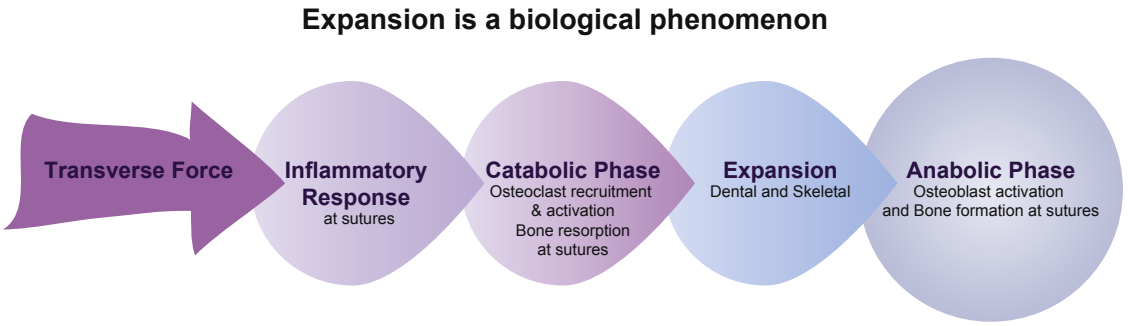

Fig. 5. Model of events in sutures in response to orthopedic transverse tensile forces. As an initial response to tensile forces, a robust and sustained inflammatory response recruits osteoclast precursors into the mid-palatal suture and adjacent bone. As a result of osteoclast activity and bone resorption, the catabolic phase begins with a significant reduction in the bone density in the area and the suture width is visibly increased. After the catabolic phase there is a transition to the anabolic phase, which begins with stimulation of bone formation via coupling factors including diffusible factors, membrane-bound molecules, and factors embedded in bone matrix. During this period and only after the catabolic phase, do the palatal and dental widths significantly increase, and physical expansion finally occurs. The anabolic phase then continues with robust osteoblast stimulation that ensures that the resorbed bone is replaced with new bone, reestablishing the integrity of the suture in the framework of the new skeletal dimensions.

heavy force in a short period is contraindicated. As our data show, the expression of inflammatory markers and activation of osteoclasts in the suture is a prerequisite for skeletal changes in adults and, therefore, a longer interval between force applications is required.

In general, the catabolic phase of expansion will define the magnitude of skeletal changes. In addition, if osteoclasts play a significant role in activating osteoblasts, perhaps our treatment should maximize osteoclast activity in the early phase of expansion for improved osteogenic response. Therefore, from a biological standpoint, the clinician should consider osteoclasts the main target of orthopedic treatment.

Based on our results, the target of orthopedic treatment by application of static force is not the bone but the suture. In this regard, any type of stress on the suture can induce microtrauma that will produce a mild inflammatory reaction that in turn activates osteoclasts in the suture. Osteoclast activation is a necessary first step for osteoblast activation that follows. Based on these results, orthopedic treatment in the craniofacial area has two consecutive phases, a catabolic phase defined by sutural opening, and an anabolic phase as a delayed reaction that reestablishes the sutural width (Fig. 5). The catabolic phase defines the magnitude of the maxillary movement and therefore should be optimized to achieve maximum orthopedic correction, whereas the anabolic phase, contributes to the stability and integrity of the skeleton after maxillary displacement. The goal of orthopedic research, therefore, should be optimizing the force regimen that maximize the catabolic phase during movement and the anabolic phase during retention. The optimized force regimen for these two phases is not the same and should be further investigated.

5. Conclusion

We have proven our hypothesis that, similar to the PDL, tensile force on the mid-palatal suture stimulates the release of inflammatory markers resulting in osteoclast recruitment and activation, causing widening of sutures during the catabolic phase. The catabolic phase is followed by skeletal and dental widening and osteoblast activation that reestablishes the bone integrity at the sutures during the anabolic phase. Together, these studies and those supporting the biphasic theory of tooth movement, overturn the conventional compression-tension theory, and clearly show that catabolic and anabolic responses to force occur simultaneously on the same structure (e.g., tooth root, craniofacial sutures).

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