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Requirement of alveolar bone formation for eruption of rat molars

Gary E. Wise, Hongzhi He, Dina L. Gutierrez, Sherry Ring and Shaomian Yao

Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, USA

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Tooth eruption is a localized event that requires a dental follicle (DF) to regulate the resorption of alveolar bone to form an eruption pathway. During the intra-osseous phase of eruption, the tooth moves through this pathway. The mechanism or motive force that propels the tooth through this pathway is controversial but many studies have shown that alveolar bone growth at the base of the crypt occurs during eruption. To determine if this bone growth (osteogenesis) was causal, experiments were designed in which the expression of an osteogenic gene in the DF, bone morphogenetic protein-6 (*Bmp6*), was inhibited by injection of the first mandibular molar of the rat with a small interfering RNA (siRNA) targeted against *Bmp6*. The injection was followed by electroporation to promote uptake of the siRNA. In 45 first molars injected, eruption was either delayed or completely inhibited (seven molars). In the impacted molars, an eruption pathway formed but bone growth at the base of the crypt was greatly reduced compared with the erupted first-molar controls. These studies show that alveolar bone growth at the base of the crypt is required for tooth eruption and that *Bmp6* may be essential for promoting this growth.

Gary E. Wise, Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, 70803 USA

Telefax: +1–225–5789895
E-mail: gwise@vetmed.lsu.edu

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It has been well established that teeth of limited eruption require the presence of a dental follicle (DF), which, in turn, regulates the osteoclastogenesis needed for the formation of an eruption pathway in the alveolar bone (1, 2). Specifically, teeth do not erupt when the DF is surgically removed (3) and teeth do not erupt in osteopetrotic rats and in mice devoid of osteoclasts (4–6). Reducing osteoclast activity by injection of a bisphosphonate, such as pamidronate, also delays tooth eruption (7). The molecular regulation by the DF for the osteoclastogenesis and subsequent bone resorption to form an eruption pathway has previously been detailed (1, 2, 8).

With the above in mind, what is required during the intra-osseous phase of eruption such that the tooth can move out of its alveolar bony socket? Work in our laboratory and by others has suggested that alveolar bone growth (osteogenesis) at the base of the crypt lifts the tooth out of its bony socket (i.e. this bone growth is the motive force of the intra-osseous phase of eruption). For example, using scanning electron microscopy we have shown that during the intra-osseous phase of eruption of the rat first mandibular molar, alveolar bone growth in the base of the crypt begins at postnatal day 3 and proceeds rapidly from postnatal day 9 onwards until the first molar erupts (pierces the gingiva) at postnatal day 18 (9).

The requirement of the DF for this alveolar bone growth during eruption was first demonstrated in the dog by Marks and Cahill (10). They demonstrated that removal of the basal half of the DF of the fourth mandibular premolar of the dog before the onset of eruption inhibited alveolar bone formation at the base of the crypt and resulted in no tooth eruption, despite the fact that an eruption pathway formed. With this in mind, we examined the expression of an osteogenic gene, bone morphogenetic protein-2 (*Bmp2*), and determined that, starting at postnatal day 3 it was expressed more strongly in the basal half of the DF than in the coronal half (11). In preliminary studies, we also discovered that at least seven osteogenic genes are expressed more strongly in the basal half of the DF than in the coronal half from postnatal days 3 through 11. In particular, the expression of the bone morphogenetic protein-6 gene (*Bmp6*) is dramatically increased in the basal half of the DF at postnatal day 11.

To determine if inhibition of expression of an osteogenic gene that is strongly expressed in the basal half of the DF might inhibit alveolar bone growth and subsequent tooth eruption, experiments were designed in which small interfering RNAs (siRNAs) targeted against *Bmp6* were injected into the DF followed by electroporation to enhance uptake of the siRNAs into the DF cells. The siRNAs are short, double-stranded molecules (20–25 nucleotides in length) that degrade the mRNA of a specific gene and thus inhibit the effect of that gene. The effect of this inhibition of expression of *Bmp6* on tooth eruption and alveolar bone formation at the base of the crypt was then determined.

Material and methods

In vivo expression of *Bmp6* in the DF

To determine the chronological gene expression of *Bmp6* in the DFs, rat pups were killed on postnatal days 1, 3, 5, 7, 9,

and 11, and the DFs were surgically isolated from the first mandibular molars. Total RNA was extracted from the DFs and treated with DNase I. The RNA concentration was measured using a spectrophotometer and 3 μg of total RNA was reverse transcribed into cDNA. Real-time PCR was conducted with *Bmp6* gene-specific primers (forward: CTTACAGGAGCATCAGCACAGA, and reverse: GTCACCACCCACAGATTGCTA) to determine the cycle threshold (C_T) values. Relative gene expression (RGE) was calculated using the Delta C_T method.

Laser capture microdissection was used to collect the coronal and basal halves of the DFs from rat pups on postnatal day 11 using procedures described previously (11). In brief, frozen sections were cut, fixed, and stained before regions of the DF were excised from the section using a PALM Laser Capture Microdissection system (P.A.L.M. Microlaser Technologies, Bernried, Germany). RNA was extracted using the Arcturus PicoPure RNA isolation kit (MDS Analytical Technologies, Sunnyvale, CA, USA) and quantified using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). About 100 ng of RNA was reverse transcribed into cDNA and subsequent real-time PCR was conducted to determine RGE (basal vs. coronal). All experiments were repeated three times, and all procedures involving animals were approved by the Institutional Animal Care and Use Committee of Louisiana State University.

Bmp6 siRNA for gene knockdown

The siRNAs for targeting *Bmp6* were purchased from Dharmacon (Boulder, CO, USA). The control siRNA was self-designed (sequence: UAAGGCUAUGAAGAGAUA C) and synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Dental follicle cells were isolated and cultured using methods previously described (12). To determine the knockdown efficiency of *Bmp6* siRNA, DF cells were transfected with *Bmp6* siRNA using the Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, lipofectamine was mixed with Opti-MEM Invitrogen medium and siRNA, and then incubated at room temperature for 15–20 min to allow formation of the transfection complex. Next, the mixture was added into each well with the final concentration of siRNA at 50 nM. Cells were collected to determine the knockdown efficiency after transfection.

Gene knockdown efficiency of *Bmp6* siRNA was determined by RT-PCR 48 h after transfection. In brief, total RNA from transfected cells was extracted and purified using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). Two micrograms of total RNA of each sample was reverse transcribed into cDNA. The cDNA was used as template for conventional PCR to determine gene expression using gene-specific primers (*Bmp6*: forward, CGACA CCACAAGGAGTTCAA and reverse, ACCTCGCTCAC CTTGAAGAA; beta-actin: forward, GAGAGGGAAA TCGTGCGTGAC and reverse, CATCTGCTGGAAG GTGGACA).

To assess the knockdown effect of *Bmp6* siRNA on BMP6 protein levels, cells were collected 24–72 h after transfection, and total protein was extracted. The protein concentration was quantified with Pierce bicinchoninic acid (BCA) (Thermo Fisher Scientific), and samples containing 7.5 μg of total protein each were electrophoresed on 10% SDS–polyacrylamide gels. Next, the proteins on each gel were transferred to a polyvinylidene difluoride (PVDF) membrane and hybridized with mouse anti-BMP6 or anti-β-actin IgG1 at 4°C overnight. After washing three times with phosphate buffered saline - Tween, the membrane was hybridized with secondary antibody conjugated to horseradish peroxidase (HRP). After washing with phosphate buffered saline - Tween, the membrane was developed using a chemiluminescence kit (Thermo Scientific) and the BMP6 and β-actin signals were captured with a ChemiDoc instrument (BioRad, Hercules, CA, USA).

In vivo injection and electroporation

For *in vivo* transfection, siRNAs were first dissolved in 1 × siRNA buffer and then diluted to 10 μM with nuclease-free water plus saline. Twenty microlitres of siRNA solution was injected into the first mandibular molars of the rat pups at postnatal days 5 and 7 or at postnatal days 7 and 11 using a 31-G syringe with a stopper attached to the needle to ensure an injection depth of 1.8 mm, the optimal depth to reach the DF (13). The rat pups were anaesthetized with isoflurane before injection. As controls, the first molars of pups were injected either with siRNA buffer plus saline or with scrambled siRNA. Immediately after injection, the given mandibles were clamped with forceps electrodes and the electroporation (four pulses at 120 V, 20 ms per pulse, at 1-s intervals) was carried out using a BTX ECM850 Electro Square Porator (Harvard Apparatus, Holliston, MA, USA) (13). The pups were evaluated for the eruption of the first mandibular molar twice daily from postnatal day 16 until the day that the molars erupted. Animals with impacted molars and comparable controls were killed at various time-points after the molars in the control rats had erupted, and the mandibles were surgically collected.

Histological analysis

The collected mandibles were fixed with neutral-buffered formalin and then decalcified with Cal-EX (Fisher Scientific, Waltham, MA, USA) for 12 d before being embedded in paraffin. Tissues were cut longitudinally into 5-μm-thick sections. The sections were mounted on glass slides and stained either with Masson's trichrome or haematoxylin and eosin (H&E) before examination by light microscopy. The thickness of the bone in the basal part of the crypt was measured using OLYMPUS QCOLOUR-3 software (Olympus America Center, Valley, PA, USA) to compare the bone formation between the impacted molars and the erupted molars. In brief, for a given age (postnatal day 20, 27 or 30), the mandibles were longitudinally sectioned until the distal and mesial roots appeared. Then, the amount of alveolar bone under the roots was measured in 10 serial sections for both the impacted first molar and its erupted counterpart.

Statistical analysis

Three litters of rat pups were used to determine the RGE of *Bmp6* at different postnatal days. The experiments were conducted in a completely randomized block design with litters as blocks. ANOVA and least significant difference (LSD) were used to compare RGE. The mean values were separated by LSD at $P \leq 0.05$, and a Student's *t*-test was conducted to compare the RGE of *Bmp6* in the basal DF vs. the coronal DF.

Results

Chronological expression of *Bmp6* in the DF was analyzed using real-time RT-PCR (Fig. 1). A continuous increase of *Bmp6* expression was seen from postnatal days 1–11. On average, the expression of *Bmp6* on day 11 had increased by more than sixfold compared with its expression on day 1 (Fig. 1). Further study of its expression in the coronal half and the basal half of the DF was conducted by coupling laser capture microdissection and real-time RT-PCR. Real-time RT-PCR showed that the expression of *Bmp6* in the basal half of the DF was some eightfold higher than in the coronal half of the DF on day 11, a time of rapid alveolar bone growth in the base of the crypt. The difference was statistically significant ($P = 0.022$). Conventional RT-PCR demonstrated that siRNA targeted against *Bmp6* inhibited the expression of *Bmp6* in DF cells, whereas the scrambled siRNA control did not (Fig. 2A). Western blotting showed that the knockdown effect on BMP6 protein levels lasted for at least 72 h after transfection, with the maximum knockdown seen 48 h after transfection (Fig. 2B).

The effect of *in vivo* injection and electroporation of siRNA targeted against *Bmp6* into the region of the DF in the first mandibular molar of the rat was examined in 45 molars. In seven (15.5%) molars, eruption was inhibited such that the tooth was impacted. Eruption of the remaining 38 molars was delayed by 0.8 d, a value that was statistically significant at $P \leq 0.05$ using the Student's *t*-test. For all seven-first mandibular molars that were impacted, the eruption pathway formed (Fig. 3) but the tooth did not erupt. Histological analyses of the impacted teeth showed a reduction in the amount of alveolar bone in the crypt at the base of the impacted first molar, in comparison with the adjacent second molar that erupted (Fig. 4A–C) and in comparison with the erupted first-molar control on the contralateral side (Fig. 5A–D). In many instances, the alveolar bone in the bony crypt at the base of the erupted molar also

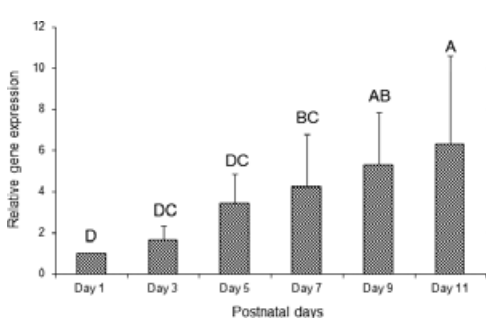

Fig. 1. In vivo expression of bone morphogenetic protein 6 (*Bmp6*) in the dental follicles (DFs) of the first mandibular molars of postnatal rats, as determined by real-time RT-PCR. Chronologically increased expression of *Bmp6* was seen at the postnatal days examined. Different letters above the bars indicate that the means were significantly different at $P \leq 0.05$ in ANOVA and least significant difference (LSD) tests. Bars that contain the same letter are not significantly different, for example, day 11 (A) and day 9 (AB) are not significantly different.

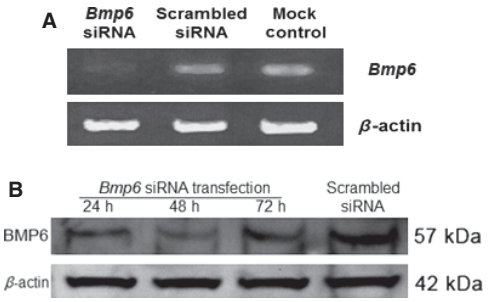

Fig. 2. Gene-knockdown efficiency of small interfering RNA (siRNA) against the bone morphogenetic protein 6 (*Bmp6*) gene (*Bmp6* siRNA) was determined by conventional RT-PCR and Western blotting. (A) RT-PCR indicated that expression of the *Bmp6* gene in *Bmp6*-siRNA-transfected cells was reduced to an almost undetectable level at 48 h after transfection, whereas no notable reduction of *Bmp6* expression was seen in the cells transfected with scrambled siRNA compared with the untreated controls. In addition, siRNA transfection appeared to have no effect on expression of the β-actin gene. (B) Western blotting analysis showed that the maximum knockdown effect on BMP6 protein was at 48 h after transfection, but the knockdown effect lasted for at least 72 h after transfection.

appeared more compact than the comparable alveolar bone of the impacted molar (Fig. 5B). Measurements of a minimum of 10 sections of three different impacted first molars and their counterpart erupted first molars showed that on postnatal day 20 the depth of the alveolar bone at the base of the impacted molar was 0.44 ± 0.05 mm, whereas the depth of the alveolar bone at the base of the erupted first molar was 1.22 ± 0.02 mm. On postnatal day 27, the depth of the alveolar bone at the base of the impacted molar was 0.89 ± 0.11 mm, and the depth of the alveolar bone at the base of the erupted molar was 1.34 ± 0.03 mm. On postnatal day 30, the depth of the alveolar bone at the base of the impacted molar was 0.45 ± 0.05 mm, and the depth of the alveolar bone at the base of the erupted molar was 1.56 ± 0.03 mm.

Discussion

That alveolar bone formation at the base of the crypt might be involved in tooth eruption was suggested by the studies of CAHILL (14), in which he used transmandibular wires to temporarily impact the unerupted fourth premandibular molar of the dog. An eruption pathway formed above the tooth and, when the wires were removed, the tooth erupted rapidly accompanied by a rapid growth of alveolar bone under the tooth at the base of the socket. Although not proof that this bone growth was causal, it was a strong correlation between bone growth and the intra-osseous phase of eruption. As mentioned in the Introduction, such bone growth is also seen during eruption of the first mandibular molar of the rat (9). Moreover, the regulation of such alveolar bone growth is probably regulated by the basal half of the DF: this is based on surgical studies in the dog, in which either the basal half or the coronal half of the DF was removed (10), and by gene-expression studies showing

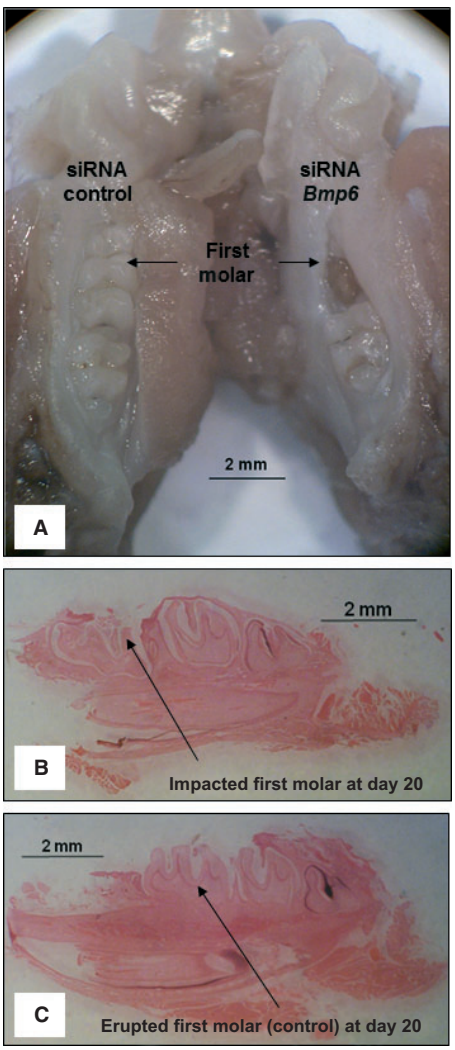

Fig. 3. Transfection of dental follicle (DF) with small interfering RNA (siRNA) bone morphogenetic protein 6 (*Bmp6*) resulted in tooth impaction. (A) Mandible showing the impacted and erupted first molars on day 30. Note that the eruption pathway still formed above the impacted molar. (B, C) Histology sections also show the eruption pathway above the impacted molar (B) and that there was much less alveolar bone at the base of the impacted molar (B) than at the base of the erupted molar (C).

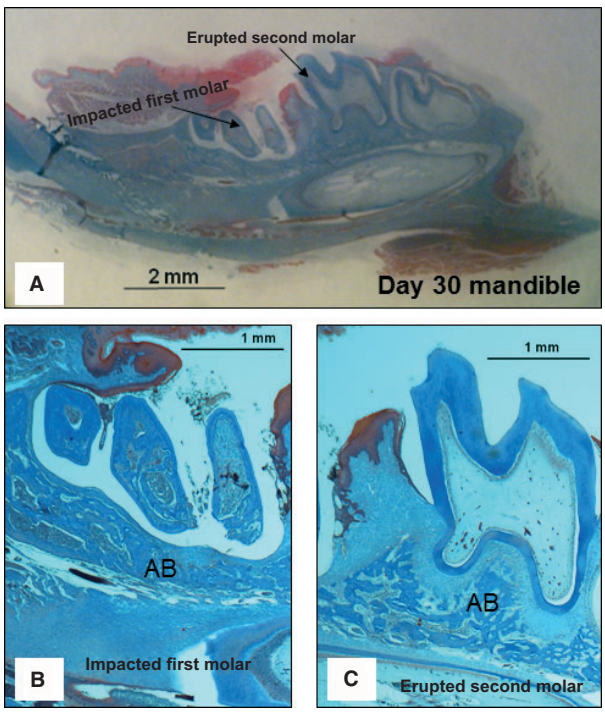

Fig. 4. (A) Comparison of the alveolar bone between the impacted first molar and the adjacent erupted second molar on day 30 showed that there was less bone at the base of the impacted first molar than at the base of the erupted second molar. (B, C) High-power magnifications of the same area illustrate the differences in the quantity of the alveolar bone (AB) at the base of these molars (i.e. less bone under the first molar).

that an osteogenic gene, such as *Bmp2*, is expressed more in the basal half of the DF than in the coronal half (11).

This study demonstrated that local injection of an siRNA targeted against *Bmp6* can inhibit alveolar bone formation and subsequent tooth eruption despite the fact that an eruption pathway is formed. That alveolar bone formation is reduced in the impacted teeth is seen in Figure 4, in which less alveolar bone is present at the base of the crypt of the impacted first mandibular molar compared with the amount of bone under the erupted second mandibular molar. This is particularly dramatic given that the second molar erupts later (day 25) than the first molar (day 18) and thus the second molar crypt started alveolar bone formation later than the first molar. Comparing the alveolar bone under the impacted first molar with its companion erupted first molar also showed less bone under the impacted molar compared with the erupted control (Fig. 5).

The reason why not every first molar is impacted by injection of siRNA targeted against *Bmp6* is because the small size of the tooth and surrounding DF makes it impossible to hit the target (the DF of the first molar) precisely every time. As demonstrated in a previous study, in which we injected a plasmid with a *lacZ* reporter gene, introduced the plasmid into the DF by electroporation, and subsequently analyzed the tissues by histology and X-Gal staining, success in transfecting the DF ranged from about 43% to 86% for a given litter transfected (13). However, in that study many of the DFs transfected did not show staining in the basal half and therefore, in this study, the numbers of DFs that received siRNA in the basal half was probably much $< 43\%$. Even so, the first molar was impacted in seven (15.5%) of 45 rats injected with siRNA against *Bmp6*. Precise injection of siRNA into the DF is important to achieve the desired effect on tooth eruption. We attempted an injection regimen at postnatal days 5 and 7 in one litter, but precise injection of rat pups at postnatal day 5 was difficult because of their smaller size. Considering that more rapid bone growth at the base of the tooth crypt begins on postnatal day 9 (9), the injection regimen of postnatal days 7 and 11 was more appropriate.

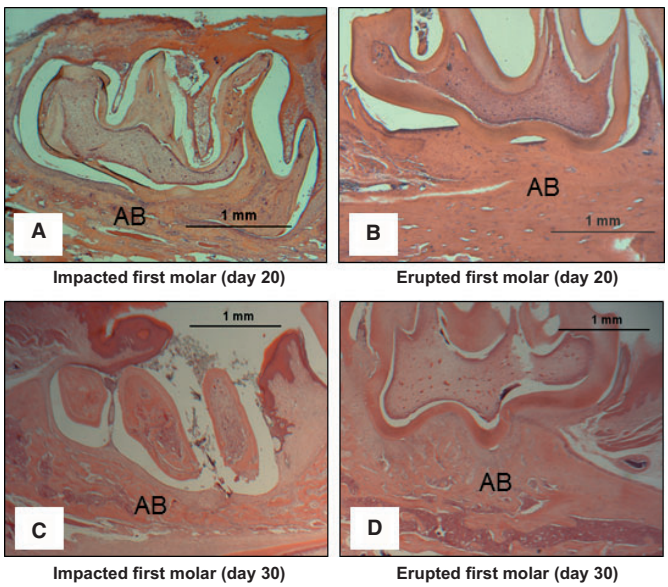

Fig. 5. Comparison of the impacted and erupted first molars at different postnatal days. A significant reduction of the amount of alveolar bone (AB) was seen at the base of the impacted first molars from pups at postnatal days 20 and 30 (A, C) vs. the large amount of bone seen at the base of the erupted first molars at the same time-points (B, D). Note that the alveolar bone (AB) at the base of the erupted molars appeared to be more compact than the bone in the comparable part of the impacted molars (A vs. B; C vs. D).

The impaction is probably not caused by damage of the DF as a result of electroporation because the controls were also electroporated. Furthermore, the eruption pathway formed normally, suggesting that electroporation results in no substantial damage to the DF. To eliminate the possibility of the off-target effect of the *Bmp6* siRNA, we screened the expression of other osteogenesis genes, including several *Bmp* genes, in the *Bmp6* siRNA-transfected cell population containing DF cells and DF stem cells. The results indicate that the *Bmp6* siRNA is quite specific, and no significant off-target effect appears (data not shown). Thus, BMP6 appears to be a key molecule in eruption that is needed to promote osteogenesis of the alveolar bone at the base of the tooth. BMP6 is a member of the transforming growth factor-β (TGF-β) super-family and it has long been known to induce osteoprogenitor cells to differentiate into osteoblasts (15). It is present in a specific zone of the growth plate (16) and if its expression is inhibited, as in BMP6 null mice, growth plate function is impaired (17). Of particular interest is that BMP6 expression is up-regulated in human periodontal ligament (PDL) under cyclic tensile strain (18). Given that the PDL is derived from the DF, the role of BMP6 in osteogenesis is consistent. In future studies, we will determine if other osteogenic genes that are expressed more in the basal half of the DF affect osteogenesis and eruption.

It should be noted that one early report in the literature indicated that *Bmp6* knockout mice are viable, and the only skeletal defect seen was delayed ossification of the sternum (19). This is probably a result of functional redundancy, common among the BMPs, in which one gene has substituted for another. In that vein, the genes for *Bmp6*, *Bmp5*, and *Bmp7* show 80–90% sequence homology (20, 21). Although mice null for each of these genes are viable (22), mice with the *Bmp5*/*Bmp7* double knockout are not viable, suggesting that BMP5 and BMP7 can compensate for each other at early stages of embryonic development (23). Mice with the *Bmp6*/*Bmp5* double knockout display numerous skeletal defects (19). Moreover, in the developing mouse teeth, there is an extensive overlap of BMPs in many tissues, suggesting a functional redundancy of the genes to ensure that early development occurs (24).

Regarding the above, it is critical to note that in our experiments, inhibition of *Bmp6* expression occurred postnatally (days 7 and 11), not during embryogenesis. Thus, it is likely that the redundant gene(s) that might replace the function of *Bmp6* in the DF were permanently turned off and there was no signal to up-regulate them. Tooth eruption is a genetically programmed localized event directed by the DF (1) and inhibiting a gene in the programme at the time it is to be expressed postnatally probably does not allow time for any reprogramming to occur. Regardless of the degree to which BMP6 regulates the alveolar bone growth, it is evident in this study that alveolar bone growth was inhibited at the base of the crypt in the impacted molars after injection of siRNA targeted against *Bmp6*. As a consequence, when alveolar bone formation at the base of the crypt is inhibited, tooth eruption does not occur. Another requirement for eruption, a tooth-eruption pathway, does form, indicating that the inhibition of eruption is indeed a result of the reduced osteogenesis and not reduced osteoclastogenesis.

Finally, this osteogenesis needed for eruption is compatible with many other studies of eruption. For example, the PDL is not involved in pulling the tooth

during the intra-osseous phase of eruption because it is not organized and attached to the alveolar bone until the tooth has erupted (9). Moreover, in the dog, an inert metal replica of a tooth can be substituted for the tooth and the replica will erupt (25). This latter study also eliminated vascular pressure changes in the pulp of the tooth as a motive force because there is no pulp. It also eliminated root growth as a force of eruption, which confirmed Gowgiel's findings that rootless teeth can erupt (26). Thus, we conclude that the motive force of tooth eruption during the intra-osseous phase appears to be alveolar bone formation at the base of the crypt and that this osteogenesis at the base of the crypt is required for tooth eruption.

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Conflicts of interest – The authors declare no conflict of interest.

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