Contribution of the PDL to Osteotomy Repair and Implant Osseointegration

Journal of Dental Research
2017, Vol. 96(8) 909–916
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for Dental Research 2017
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DOI: 10.1177/0022034517707513
journals.sagepub.com/home/jdr

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Abstract

Our objective was to clarify the fate of the periodontal ligament (PDL) retained in the socket after tooth extraction, then determine if this tissue contributed to the osseointegration of “immediate” implants placed in these fresh extraction sockets. Mice underwent maxillary first molar extraction, the residual PDL was removed by an osteotomy, and titanium implants were placed. The osteotomy was created in such a way that the palatal surface was devoid of PDL remnants while the buccal, mesial, and distal surfaces retained PDL fibers. At multiple time points after surgery, tissues were analyzed using a battery of molecular, cellular, and histomorphometrical assays. We found that PDL remnants mineralized and directly contributed to new bone formation in the extraction site. Compared with regions of an extraction site where the PDL was removed by osteotomy, regions that retained PDL fibers had produced significantly more new bone. Around immediate implants, the retained PDL remnants directly contributed to new bone formation and osseointegration. Thus, we conclude that PDL remnants are inherently osteogenic, and if the tissue is healthy, it is reasonable to conclude that curetting out an extraction socket prior to immediate implant placement should be avoided. This recommendation aligns with contemporary trends toward minimally invasive surgical manipulations of the extraction socket prior to immediate implant placement.

Keywords: extraction socket, bone, regeneration, periostin, immediate implant, histology

Introduction

The placement of implants immediately after tooth extraction is an increasingly common surgical approach to replace missing teeth. “Immediate” implants, as they are sometimes called, were introduced as an alternative to implants that are placed into healed extraction sites (Albrektsson et al. 1981). Detractors often point out that placement of a cylindrical implant into an extraction socket leaves peri-implant voids (Ferrus et al. 2010) that may not heal spontaneously and that the risk of implant bed infection—and therefore implant failure—may be higher (reviewed in Susarla et al. 2008; Urban et al. 2012). Proponents of immediate implants argue that the technique has several advantages, including a reduction in the number of surgeries and the rapid return of a patient to function with minimal risk (reviewed in Lazzara 1989; Evans and Chen 2008).

Whether immediate implants osseointegrate through the same process as implants placed into healed sites is not altogether clear. Certainly, some clinical observations indicate that osseointegration appears to be equal between the 2 types of implants (Barzilay et al. 1996; Cordaro et al. 2009), but only a few studies have histologically assessed osseointegration, and then usually only a single time point was examined (Karabuda et al. 1999). Therefore, it is not possible to conclude that the osseointegration of an implant placed into a fresh extraction socket is directly comparable to that of an implant placed into an osteotomy (Becker et al. 1998). Understanding the biological and mechanical factors that control early bone formation

around implants is key to improving existing clinical techniques and to developing new approaches to stimulate osseointegration.

Here, our intent was to first understand if there were significant differences between extraction socket healing and osteotomy healing. If there were salient, distinguishing features, our second goal was to determine how these differences affected the osseointegration of implants. Protocols established in larger animals (Botticelli et al. 2006; Vignoletti et al. 2009) were adapted to mice. The use of a small animal model allowed us to carry out a detailed molecular/cellular analysis of how extraction sites and osteotomies healed and how these healing programs contributed to implant osseointegration. We discovered that the presence of healthy periodontal ligament (PDL)

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A supplemental appendix to this article is available online.

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remnants made a significant contribution to new bone formation, and thus extraction site healing is significantly faster than osteotomy site healing. We also found that implants placed into fresh extraction sockets take advantage of this inherent bone-forming potential and show robust new bone formation specifically in those sites where PDL remnants are retained.

Materials and Methods

Animals

Stanford Committee on Animal Research approved all protocols (#13146). Wild-type mice (Jackson Laboratory; #003291) were housed in a temperature-controlled environment with 12-h light/dark cycles. After maxillary first molar (M1) extraction and/or osteotomy site preparation, animals were fed a soft food diet. Water was supplied *ad libitum*.

Experimental Groups

Animals were randomly assigned to 1 of 3 experimental groups (Appendix Table 1). In group 1, bilateral M1 extraction was followed by sacrifice at postextraction days (PEDs) 0, 1, 2, 3, 4, 7, and 14. In group 2, bilateral M1 extraction was followed by the creation of an osteotomy in the palatal root socket (see below for details), which specifically removed alveolar bone and its associated PDL on the palatal portion of the palatal root socket. Animals were sacrificed in PEDs and postosteotomy days (PODs) 0, 3, and 7. In group 3, bilateral M1 extraction was followed by the creation of an osteotomy in the palatal root socket. This osteotomy removed alveolar bone and its associated PDL specifically on the palatal portion of the palatal root socket. A similar technique has been employed by others to remove the PDL (Cardaropoli et al. 2005). Immediately thereafter, implants (manufactured by Nobel Biocare from CP Titanium Grade 4 with a TiUnite surface, with an outer diameter = 0.5 mm, a thread core diameter = 0.4 mm, a thread pitch = 0.15 mm, and the length of threaded portion of the implant = 1.6 mm) were self-tapped into place. Animals were sacrificed on PODs 0, 3, 14, and 21.

Tooth Extraction

Inhalation (2% isoflurane) anesthesia was followed by an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). Subcutaneous injections of buprenorphine (0.1 mg/kg) followed. The mouth was rinsed and bilateral M1 extractions were performed. Bleeding was controlled by local pressure; daily monitoring revealed no evidence of prolonged inflammation during healing.

Osteotomy Site Preparation

In experiments comparing osteotomy site healing with extraction socket healing (groups 2 and 3), M1 extraction was followed by creation of an osteotomy made using a low-speed

handpiece (drill diameter = 0.48 mm; 1,000 rpm, with saline irrigation).

Implant Placement

As stated above, the implant had an outer diameter of 0.5 mm and an inner core diameter of 0.4 mm. The osteotomy (see above) was made with a drill with a diameter of 0.48 mm; therefore, the threads of the implant engaged in bone ~200 μ m, and around the core there existed a gap of 800 μ m that in some cases was occupied by the residual PDL. As stated previously, implants were placed in osteotomies created in the palatal root socket immediately following tooth extraction; consequently, no flap was raised to gain access to the osteotomy site. All implants were placed transmucosally, for example, at the height of the gingiva. The vertical distance from the implant to the crestal bone was ~150 to 200 μ m, that is, the width of the gingiva. Tissue adhesive (VetClose; Henry Schein) was used to approximate the soft tissues. Daily monitoring revealed no evidence of prolonged inflammation during implant placement.

Cellular Assays

Histologic stains, alkaline phosphatase (ALP) activity and tartrate-resistant acid phosphatase (TRAP) activity, and picosirius red staining were performed as described (Leucht et al. 2007). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche) was performed as described by the manufacturer. Immunohistochemistry for osterix and periostin was carried out as described (Minear et al. 2010; Huang et al. 2016).

Histology and Histomorphometry

Histology staining was performed (Appendix Table 2). Histomorphometric measurements were performed using ImageJ software 1.4 (image processing and analysis in Java freeware; National Institutes of Health) software. To quantify the amount of new bone formed in the extraction site as a function of time, a minimum of 4 extraction sites was analyzed for each time point. For each extraction site, a minimum of 6 aniline blue-stained histologic sections that spanned the distance from the furcation to the root apex were used to quantify new bone formation. Each section was photographed using a Leica digital image system at 20 $\times$ magnification. The percentage of aniline blue-positive pixels corresponding to new bone (%NB) was the numerator, and the total number of pixels within the defined region of interest was the denominator; the resulting value was expressed as percent of new bone (%NB).

Statistical Analyses

Results are presented as the mean $\pm$ standard error values of independent replicates. Student's *t* test was used to quantify differences described in this article. *P* $\leq$ 0.05 was significant.

This study complies with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Figure 1. Molecular and cellular events characterizing extraction socket healing. (A) Representative transverse tissue section through the mouse maxilla stained with aniline blue, illustrating the position of the first molar (M1), its periodontal ligament (PDL), and surrounding alveolar bone. Black bracket indicates the cementum. (B) Aniline blue staining of the extraction socket; in all panels, the asterisks identify PDL remnants attached to the bundle bone. (C) On postextraction day (PED) 1, sagittal tissue section immunostained with periostin. (D) On PED2, tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) activate in the socket. (E) On PED3, the residual PDL expresses osterix. (F) Pentachrome staining of sagittal sections through the extraction socket on PED1, (G) PED4, (H) PED7, and (I) PED14. ab, alveolar bone; bb, bundle bone; c, cementum; d, dentin; es, extraction socket; pdl, periodontal ligament. Scale bars = 100 μ m.

Results

The Physiology of Extraction Socket Healing Is Analogous to Wound Healing

Our first objective was to analyze extraction socket healing, using the intact state as a starting point. Aniline blue staining of a transverse tissue section through the M1 palatal root illustrated mineralized alveolar bone; nonmineralized, fibrous PDL; the cementum (bracket); dentin; and pulp (Fig. 1A). After M1 removal on PED1, a similar tissue section through

the palatal root socket revealed remnants of the PDL (asterisk) still attached to the alveolar bone and the fibrin clot-filled extraction socket (Fig. 1B). A sagittal tissue section immunostained to detect periostin (Horiuchi et al. 1999) verified the tissue remnants as PDL (Fig. 1C).

Tooth extraction triggers remodeling of the alveolar bone socket, and this was readily detectable on PED2 by staining for osteoclast activity (shown by TRAP) and osteoblast activity (shown by ALP) (Fig. 1D). Based on the distribution of TRAP and ALP, it appeared as if regions of the socket with retained PDL fragments were sites of less resorption and more bone

Figure 2. The periodontal ligament (PDL) retained in the extraction socket undergoes mineralization. **(A)** Representative sagittal tissue section stained with pentachrome, illustrating the intact M1 PDL. **(B)** Adjacent tissue section stained with picrosirius red to identify the organization of collagen in the PDL. **(C)** Periostin immunostaining identifies the intact PDL. On postextraction day (PED) 1, **(D)** pentachrome staining of the PDL and **(E)** picrosirius red shows the change in collagen organization after tooth extraction. **(F)** PDL remnants retain the periostin-positive status. On PED7, the structure of the PDL is notably altered as shown by **(G)** pentachrome staining. **(H)** PDL remnants still express periostin; in addition, **(I)** cells in the PDL remnants strongly express osterix. On PED14, **(J)** pentachrome, **(K)** picrosirius red staining, and **(L)** osterix immunostaining do not differentiate between the mineralized PDL remnants and adjacent alveolar bone. Asterisks indicate PDL remnants. ab, alveolar bone; c, cementum; d, dentin. Scale bars = 50 μ m.

formation (asterisks, Fig. 1D). By PED3, immunostaining for the osteogenic marker osterix (Nakashima et al. 2002) confirmed that cells in the PDL remnants were undergoing osteogenic differentiation (Fig. 1E).

PDL Remnants Mineralize and Contribute to Extraction Socket Healing

Mineralization of these PDL remnants coincided with sites of new bone formation. In the first 12 h following tooth extraction, the PDL remnants could be easily distinguished from the surrounding alveolar bone (Fig. 1F), but by PED4, it was difficult to discriminate between PDL remnants and new bone (Fig. 1G). By PED7, the socket was filled with bone (Fig. 1H), and by PED14, the healed extraction site appeared similar to adjacent, intact alveolar bone (Fig. 1I). These data indicated that new bone formation in the extraction socket corresponded to sites where the PDL was retained.

In vitro, cells from the PDL can differentiate into osteoblasts (Lin et al. 1994), but whether the PDL gives rise to new bone in vivo is not known. We carried out a stepwise analysis to address this question. The intact state was again used as a starting point: PDL fibers (Fig. 2A) were readily visualized by picrosirius red staining (Fig. 2B) and periostin immunostaining (Fig. 2C). Immediately after tooth extraction (Fig. 2D), the PDL lost its linear organization (Fig. 2E), although it remained

immunopositive for periostin (Fig. 2F). By PED7, the PDL structure had changed notably (Fig. 2G): the tissue remained periostin positive (Fig. 2H), but at this point it was also strongly osterix positive (Fig. 2I). By PED14, the mineralized PDL was indistinguishable from adjacent alveolar bone (Fig. 2J, K), and periostin immunostaining no longer was detectable (Fig. 2L). These analyses demonstrated that PDL remnants had converted into a mineralized tissue.

Extraction Sockets Heal Faster Than Osteotomies

Remnants of the PDL mineralize, suggesting that an extraction site containing residual PDL fragments should heal faster than an equivalent size osteotomy. To eliminate potential bias based on differences in vascularity or other anatomical variations, we tested this hypothesis within a single extraction socket. Bilateral M1 extractions were followed by the creation of an osteotomy, which was made in the palatal root socket of M1. This osteotomy technique, which has been used by others in large animals (Cardaropoli et al. 2005), effectively removed alveolar bone and its associated PDL only on the palatal portion of the palatal root socket (Fig. 3A). Tissues were then analyzed on PEDs/PODs 0, 3, and 7. As before, alveolar bone with attached PDL (i.e., bundle bone; Fig. 3B, C) was easily distinguished from the smooth-edged osteotomy sites that were devoid of a PDL (Fig. 3D, E).

Figure 3. Periodontal ligament (PDL) remnants contribute to new bone formation. **(A)** Schematic of the experimental design. On the day of extraction, a representative transverse tissue section stained with **(B)** picrosirius red to detect the collagenous PDL remnants, and an adjacent section stained with **(C)** pentachrome staining shows the relationship between PDL remnants the underlying bundle bone after tooth extraction. **(D)** Removal of the PDL by osteotomy site preparation leaves the alveolar bone smooth and devoid of a **(E)** collagenous PDL. In all panels, the dotted white line indicates the demarcation between alveolar bone and PDL remnants; the dashed white line indicates the osteotomy edge. **(F)** Tartrate-resistant acid phosphatase (TRAP) staining and **(G)** alkaline phosphatase (ALP) activity in the extraction socket on postextraction day (PED) 3. **(H)** TRAP staining and **(I)** ALP activity in the osteotomy on postosteotomy day 3 (POD3). **(J)** Quantification of new bone formation in an extraction socket versus an osteotomy. **(M, N)** In a single extraction socket, an osteotomy was made, which specifically removed alveolar bone on the palatal aspect but retained the PDL on the mesial, distal, and buccal aspects. Three days later, picrosirius red staining identifies regions **(K)** with and **(O)** without PDL remnants. **(L, P)** The same type of site, evaluated on day 7. Asterisks indicate $P < 0.05$. ab, alveolar bone; bb, bundle bone; es, extraction socket; ost, osteotomy; pdl, periodontal ligament; PR, picrosirius red staining. Scale bars = 50 μ m.

On PED3, extraction sites were undergoing bone remodeling, but the underlying bundle bone had little, if any, TRAP staining (Fig. 3F and see Araújo and Lindhe 2005). ALP activity and new bone formation, on the other hand, were evident in both the bundle bone and the mineralizing PDL (Fig. 3G). Conversely, osteotomies showed no ALP activity, and TRAP staining was minimal (Fig. 3H, I). Histomorphometric analyses confirmed new bone formation was faster in extraction sockets compared with osteotomies (Fig. 3J).

There was a size difference between the osteotomy and the palatal root socket, which could have contributed to the difference in bone-healing rates. Therefore, we undertook a second series of experiments to demonstrate a direct, causal link between the presence of PDL remnants and faster bone healing. We created an osteotomy in the M1 palatal root socket and positioned it in such a way that the drilling once again removed alveolar bone and PDL only from the palatal surface of the socket (Fig. 3K, L, M, N, O, P). Picrosirius red staining was used to distinguish those sites with a residual PDL (Fig. 3K) from sites where the PDL had been removed by the osteotomy (Fig. 3O). By PED/POD7, it was clear that osterix immunostaining and new bone were restricted to those regions of the

extraction socket that had PDL remnants (Fig. 3L) and were not yet evident in regions that had been prepared by the osteotomy (Fig. 3P). Thus, we conclude that extraction sockets heal significantly faster than osteotomies and that the reason for this was the presence of PDL remnants that directly contributed to new bone formation.

The PDL in an Extraction Socket Contributes Directly to Implant Osseointegration

We were now in a position to directly test whether PDL remnants contributed to the osseointegration of implants placed in fresh extraction sockets. In group 3, bilateral M1 extractions were followed by osteotomies in the palatal root socket, which removed alveolar bone and PDL specifically on the palatal portion of the palatal root sockets. Immediately thereafter, implants (see Materials and Methods) were placed (Fig. 4A). All implants were below the level of the occlusal plane, at the height of the gingiva. Animals were sacrificed at various time points after implant placement, and tissues were sectioned in the transverse plane as indicated (Fig. 4B).

Figure 4. Periodontal ligament (PDL) remnants contribute directly to implant osseointegration. **(A)** Schematic of the experimental design. **(B)** Schematic of the orientation of the implant in the palatal root and plane of section used to analyze regions of the implant in direct contact with bone, as well as in regions with an intervening PDL. **(C)** On the day of extraction, a representative transverse tissue section stained with pentachrome in a region with an intervening PDL and **(D)** a region with direct bone/implant contact. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)/4',6-diamidino-2-phenylindole (DAPI) staining of **(E)** an intervening PDL region and **(F)** a direct bone/implant contact region. On postimplant day (PID) 3, tartrate-resistant acid phosphatase (TRAP) staining of a region with **(G)** an intervening PDL and **(H)** one with direct bone/implant contact. **(I, J)** Equivalent regions, stained to detect alkaline phosphatase (ALP) activity. On PID14, **(K)** bone is in direct contact with the implant in regions that previously had an intervening PDL. **(L)** In regions that had previously had a direct contact between bone and implant now had a small fibrous envelope. ab, alveolar bone; bb, bundle bone; BIC, bone-implant contact; im, implant; pdl, periodontal ligament.

On postimplant day 0 (PID0), pentachrome staining identified interfacial regions where PDL remnants existed (Fig. 4C) and interfacial regions where PDL remnants were removed and the alveolar bone and implant were in contact (Fig. 4D). On PID3, the extent of programmed cell death was evaluated: in cases where an intervening PDL was evident, TUNEL staining of osteocytes was minimal (Fig. 4E). In cases where an osteotomy had been created, TUNEL-positive osteocytes were found throughout the interfacial bone (arrowheads, Fig. 4F).

TRAP activity confirmed that interfacial gaps with PDL remnants showed little bone resorption (Fig. 4G). In contrast, interfacial bone was in the process of being resorbed (Fig. 4H). ALP activity was robust in the mineralizing PDL and adjacent bundle bone (Fig. 4I). At this early stage, no ALP activity was detectable in interfacial bone (Fig. 4J).

On PID14, aniline blue staining confirmed that osseointegration was well under way, and on mesial, buccal, and distal sites, a mineralized PDL could be distinguished from alveolar bone (red arrows; Fig. 4K). In other regions where the implant was in contact with bone, a thin band of interfacial connective tissue was evident (Fig. 4L), which occurs when osteotomy site preparation triggers bone resorption (Cha et al. 2015). A

similar nonmineralized soft tissue has been observed around immediate implants placed in dogs (Botticelli et al. 2003a).

Discussion

The precipitous rise in the use of “immediate” implants was the driving force behind this study. We hypothesized that since these implants are placed immediately after tooth extraction, the rate, timing, and robustness of extraction socket healing would have a direct impact on their osseointegration. Here, we used a mouse model of tooth extraction, osteotomy site preparation, and implant placement to address this hypothesis.

It is generally agreed that extraction socket healing in humans and other mammals is analogous (Claflin 1936), but species-specific variances in bone healing also have been attributed to mechanobiological differences (Checa et al. 2011). We do not find such differences: our results in rodents align very well with experimental results from large animal studies, including dogs (Botticelli et al. 2003b). For example, implants placed in fresh extraction sockets of beagle dogs were analyzed after 7 mo, and histologic data showed that new bone filled the gaps that had initially existed between the implant

and extraction socket wall (Scipioni et al. 1997; Botticelli et al. 2003b). In 1 study, investigators also reported that nonmineralized soft tissue transiently occupied those regions where the implant had initially been in direct contact with bone (Botticelli et al. 2003b). Identical results were observed in our rodent model (Fig. 4). Extensive comparisons, however, cannot be made between dog studies and our study because most studies in large animals have small sample sizes and very few analytical endpoints. Although the size of the mouse can present a technical challenge for implant placement, with experience, a talented surgeon can re-create clinically relevant healing scenarios with ease (Mouraret et al. 2013; Mouraret et al. 2014; Cha et al. 2015; Yin et al. 2016). There are also clear advantages to the use of rodents, including the availability of molecular and cellular tools that permit detailed evaluations of clinically relevant procedures, including extraction socket healing, osteotomy healing, and implant osseointegration.

PDL Remnants Mineralize and Contribute to New Bone Formation

PDL fibers are retained in the extraction socket after tooth removal (Fig. 1), and a series of analyses showed that this residual PDL mineralized and gave rise to new bone that contributed to extraction socket healing (Fig. 2). Compared with osteotomies, extraction sockets healed faster specifically because they contained PDL remnants (Fig. 3). These same observations could be observed in a single extraction socket, where 1 wall of the socket was stripped of its PDL by osteotomy site preparation (Fig. 3).

Collectively, these data suggest that the PDL has a direct role in osseointegration of implants placed in fresh extraction sockets. We show that the sites of new bone formation around immediate implants correspond to the regions where PDL remnants remain (Fig. 4). Conversely, sites that have been denuded of the PDL show less bone formation (Fig. 4). These data suggest that curetting out an extraction socket prior to immediate implant placement should be avoided. This recommendation aligns with contemporary trends toward minimally invasive surgical manipulations of the extraction socket prior to implant placement (Becker 2006). What if the PDL is diseased? Human studies are inconclusive regarding this point; some suggest that extraction sockets associated with diseased teeth heal slower (Ahn and Shin 2008), while others argue that there is little/no effect of disease state on osseointegration of immediate implants (Villa and Rangert 2005). Our experiments did not address the extent to which a diseased PDL contributes to bone healing, but this is obviously an important question because the clear majority of teeth extracted to make way for an immediate implant are diseased in some way.

A Case for the Use of “Immediate” Implants

Although once considered risky (Brånemark et al. 1977), the use of “immediate” implants is now one of the standard practices

in implant dentistry. Our data provide an explanation for the success of immediate implants. We demonstrate that PDL remnants make a significant contribution to bone formation around these implants. There is, however, a variable that was not introduced into this study—namely, mechanical loading. Immediate implants achieve primary stability because regions of the implant are in direct contact with bone. The gaps around the implants exist because of the mismatch between implant shape and socket geometry. The question comes down to whether new bone formation in the gap regions is sufficiently robust—both in quantity and timing—that the implant will obtain secondary stability.

There is no simple answer to this question. Surely, our data demonstrate that in regions where healthy PDL remnants exist, they mineralize and thus contribute to peri-implant bone. But what if most of the implant surface comprises a gap interface without remnants of a PDL, or the gap is especially large? These conditions represent real clinical challenges, and ongoing work in our (J.A.H.) laboratory is focused on developing a WNT-based approach to accelerating bone formation around implants placed in fresh extraction sockets.

Author Contributions

X. Pei, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; L. Wang, C. Chen, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; X. Yuan, contributed to conception and design, critically revised the manuscript; Q. Wan, contributed to data interpretation, critically revised the manuscript; J. Helms, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This research project was supported by a grant from the National Institutes of Health (R01DE024000-11, J.A.H. and J.B.), National Natural Science Foundation of China (81601613, X.P. and Q.W.; 81600910, L.W.), and the Science & Technology Support Program of Sichuan Province (2016FZ0085, X.P. and Q.W.). We thank Yan Wu for her invaluable suggestions on establishing a mouse model, Serdar Tulu and Bo Liu for invaluable aid in performing histology and data analysis, and John Brunski for his critical reading of the manuscript. Our thanks also to Nobel Biocare for providing implants for this study. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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