Title
Efficacy of Slow-Release Long-Term Osteoprotegerin Delivery in Post-Orthodontic Retention

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Efficacy of Slow-Release Long-Term Osteoprotegerin Delivery in Post-Orthodontic Retention

by
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of the
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Abstract

Efficacy of Slow-Release Long-Term Osteoprotegerin Delivery in Post-Orthodontic Retention

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Post-orthodontic relapse is the phenomenon in which teeth move back towards their original position after orthodontic tooth movement. This provides a challenge for clinicians and patients in maintaining results and benefits from orthodontic treatment. The exact mechanism of relapse is unclear, but bone resorption is required to allow teeth to move. Current relapse prevention relies on patient compliance with wearing appliances. However, past studies have looked at methods that target biological pathways associated with bone remodeling. Osteoprotegerin fusion protein (OPG-Fc) has been shown to reduce relapse with limited systemic effects. A past study found that a single, localized injection could reduce relapse to just 30% of initial tooth movement. However, bolus OPG-Fc has a limited half-life (6-7 days), and with a single dose, the effects of OPG-Fc decrease significantly within a week. These studies raise the question of whether OPG-Fc could be even more effective with long-term, sustained release. Our study examined the efficacy of a slow- release long-term delivery system to deliver OPG-Fc. This study tested polyethylene glycol dimethylacrylate (PEGDMA) microparticles as a sustained delivery system. First, OPG-Fc-loaded PEGDMA microspheres were shown to deliver 1.6µg of OPG-Fc over 24 days *in vitro*. The particles were then tested in an *in vivo* rat model of orthodontic tooth movement. Effectiveness of this slow-release long-term delivery system were inconclusive due to lack of statistical power.

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List of Abbreviations

BMD – bone mineral density

BVF – bone volume fraction

Tb Th – trabecular bone thickness

Tb Sp – trabecular bone spacing

TMD – tissue mineral density

OPG – osteoprotegerin

OPG-FC – osteoprotegerin fusion protein

PBS – phosphate buffered saline

PEG - polyethylene glycol

PEGDMA – polyethylene glycol dimethylacrylate

A: Introduction

A1: Background

One of the challenges faced by clinicians and patients after the completion of orthodontic treatment is the natural tendency for teeth to move towards their original positions. This phenomenon where teeth tend to move back toward their pretreatment positions is called orthodontic relapse. Current methods of preventing relapse rely on long term patient compliance with orthodontic appliances. The exact mechanism of relapse is still poorly understood, but bone quality and maturity are theorized to be major components [1].

A2: Relapse Biology

To improve therapies to prevent relapse, it is important to better understand the biology of orthodontic tooth movement and relapse. One widely accepted theory of orthodontic tooth movement is the “pressure-tension” theory. In this theory, the periodontal ligament (PDL) is compressed on the side of the direction the tooth is moving, the “pressure” side. When the PDL is compressed, a cytokine cascade is signaled that activates osteoclast precursor cells to differentiate into mature osteoclasts. Osteoclasts then resorb the bone in the direction of the force application, which is known as osteoclast mediated resorption [2] [3]. On the other side, the “tension” side, the PDL is stretched, and osteoblast mediated bone deposition occurs in the area from where the tooth has moved. This combination of bone resorption and deposition results in tooth movement. Thus, for relapse to occur and a tooth to move back towards its original position, the newly formed and relatively immature bone adjacent to the tooth must be resorbed. To prevent relapse, biologics or drugs that contribute

to enhanced bone synthesis and maturation or minimize bone resorption by osteoclasts could have high therapeutic value in reducing relapse and increasing post-orthodontic stability of teeth [4].

A3: Relapse Prevention

Several biologics that work by either primarily increasing bone synthesis or decreasing bone resorption have been used to inhibit tooth movement in animal models, including bisphosphonates [5], nitric oxide synthase inhibitor [6], echistatin [7], MMP inhibitor [7] [8], and osteoprotegerin (OPG) gene [9] or OPG protein [10] [11] [12] [13]. However, several have adverse effects. In contrast, OPG is a well-studied factor and has been shown to limit relapse without significant systemic effects on long bones. In osteoclast activation, RANKL binds to the RANK receptor found on osteoclasts precursor cells to induce differentiation of precursor cells into osteoclasts [3]. OPG is a decoy receptor for RANKL and acts as a competitive inhibitor of RANK, thus inhibiting osteoclastogenesis and subsequent bone resorption [3]. OPG was first used to reduce bone resorption in a study on postmenopausal women [15].

A4: OPG-Fc Reduces Orthodontic Relapse

Previous studies have looked at the use of modulating orthodontic tooth movement in an animal model [10] [11] [12] [13]. Two studies have specifically focused on evaluating the use of OPG-Fc for altering tooth movement in a relapse model. OPG-Fc was shown to reduce relapse in an *in vivo* rat model when repeatedly administered at either a high dose of 5 mg/kg or low dose of 1 mg/kg [11]. Subsequently it was found that a single injection of 1 mg/kg OPG-Fc resulted in a 60%, reduction in relapse [12]. These proof-of-concept studies showed reduced relapse but did not completely eliminate relapse. Additionally, bolus OPG-Fc has limited *in vivo*

activity with a half-life of only six to seven days [16]. These findings suggests that a drug delivery system that allows for sustained, controlled release may be needed to further enhance the effects of OPG-Fc in preventing orthodontic relapse.

A5: Polyetheylene Glycol (PEG)

Polyethylene glycol (PEG) based microparticles offer controlled release dynamics for sustained release of drugs [17] [18] [19]. PEG-based microparticles are an ideal delivery system because they can be made into an injectable form, are biocompatible, and are relatively simple to modify for drug, protein, and cell encapsulation [20] [21] [22] [23]. Proteins encapsulated in PEG microspheres exhibit sustained drug release kinetics with release ranging from hours to months [17] [18] [24]. Polyethylene glycol dimethylacrylate (PEGDMA) microparticles were determined as the ideal drug delivery system for the delivery of OPG-Fc in this study.

A6: Hypothesis and Aims

Hypothesis: Sustained slow-release delivery of OPG-Fc through PEGDMA microspheres enhances post-orthodontic stability relative to immediate-release of these agents and vehicle controls

Aim 1. Optimize PEGDMA microspheres delivery system design for desired release dynamics and doses of OPG-Fc *in vitro*.

Aim 2. Use the optimized slow-release drug delivery device (PEGDMA) to administer the desired sustained doses of OPG-Fc and determine the efficacy in mitigating post-orthodontic relapse in an orthodontic tooth movement rat model.

B: Methods and Materials

B1: *In Vitro* Study Methods and Materials

Microspheres were fabricated and optimized *in vitro* to address Aim 1, optimizing PEGDMA microspheres delivery system designs for desired release dynamics and doses of OPG-Fc *in vitro*.

B1.a: Microsphere Fabrication and Selection

The hydrogel monomer solution was created by first mixing PEGDMA (750 MW) mixed with 3% (w/v) PEGDMA (250 MW). To create OPG-loaded microspheres, the monomer solution was mixed with OPG-Fc in PBS (18.85 µg/mL; Amgen, Thousand Oaks, CA, USA) at a ratio of 1:1 (v/v). For the final hydrogel monomer solution, 10% 2,2-Dimethoxy-2-phenylacetophenone (DMPA) in 1-Vinyl-2-pyrrolidinone 2 was made and added to 1:1 PEG or PEG/OPG-Fc solution at a ratio of 1:10 (v/v).

500 µL of final hydrogel monomer solution with or without protein was mixed with 500 µL HFE 7500 oil with 2% surfactant (008-FluoroSurfactant; RanBiotechnologies, Beverly, MA, USA) in a 2 mL centrifuge tube. An emulsion was formed by passing the oil/aqueous mixture 5 times through an 18G syringe needle. The size of the emulsion particles was measured by microscope. The emulsion was exposed to UV light for 5 minutes (Blak-Ray XX-15L UV light source; Analytik Jena, Jena, Germany) to polymerize.

The emulsion was centrifuged at 1000 RCF for 1 minute and the bottom oil layer was removed by using a gel loading tip. 200 µL of 20% PFO (1H,1H,2H,2H-perfluoro-1-octanol, Sigma, 370533) and 500 µL of PBST buffer (0.4% tween 20 in PBS) were added into the emulsion. The mixture was vortexed at maximum speed for 1 minute to break the emulsion and

centrifuged at 1000 RCF for 5 minutes. Any remaining oil was removed by pipetting through a gel-loading tip.

Differential velocity centrifugation was performed to select the hydrogel beads within the diameter between 10 to 30 µm (Figure 1). The hydrogel beads were resuspended in 15 mL of PBS with 0.4% tween 20 and centrifuged at 100 RCF for 5 minutes to pellet large gels. The supernatant containing small sized gel beads with diameters less than 10 µm was removed. This process was repeated 3 times to ensure complete removal of the small beads. The pellet was resuspended in 1 mL PBS with 0.4% tween 20 and stored at 4°C.

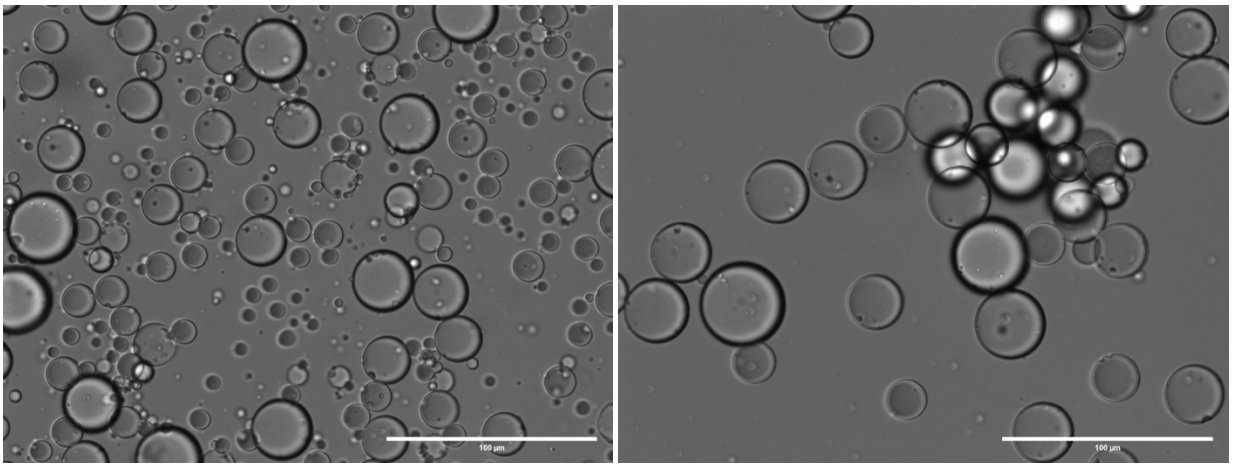

Figure 1 PEGDMA microspheres before (left) and after (right) size selection. Image taken at 40x.

Hydrogel beads for use *in vivo* were aliquoted into 50 µL aliquots containing either 875,000 or 125,000 microspheres in 1x PBS. Aliquots were flash frozen in liquid nitrogen and stored at -80°C. For *in vitro* OPG-Fc release quantification, 875,000 microspheres were aliquoted and suspended in 350µL of 1xPBS and were incubated at 37°C with slight agitation to begin collecting elutions.

B1.b: *In Vitro* Quantification of OPG-Fc Release

3 samples of 875,000 microspheres were suspended in 350 µL 1x PBS and were incubated at 37°C with slight agitation. Daily supernatants were collected by centrifuging the

spheres at 8,000 RPM for 5 minutes at 37°C. Supernatants were stored in Eppendorf tubes and flash frozen in liquid nitrogen. Microspheres were resuspended in 350 µL 1x PBS and were returned to incubation at 37°C with slight agitation

To quantify OPG-Fc release, elution collections were diluted at a ratio of 1:1700. The release was quantified according to the manufacturer's instructions using a rat osteoprotegerin enzyme-linked immunosorbent assay (ELISA) kit (ab255723; Abcam, Waltham, MA, USA).

B2: *In Vivo* Study Methods and Materials

An *in vivo* rat model was utilized to address Aim 2, testing the optimized slow-release drug delivery microparticles to administer the desired sustained doses of OPG-Fc and determine the efficacy in mitigating post-orthodontic relapse in an orthodontic tooth movement rat model. The methods of this study were modified from previous orthodontic relapse models [11] [12].

B2.a: Animals

Experiments were approved by the UCSF Institutional Animal Care and Use Program (protocol AN177331) and performed at the UCSF Laboratory Animal Resource Center.

Thirty-one young adult, male Sprague-Dawley rats (CD rat; Charles Rivers Laboratories, Willmington, MA, USA) approximately 12 to 14 weeks old and weighing approximately 400 g were used. The procedure was divided into two phases: a 28-day orthodontic tooth movement phase followed by a 24-day relapse phase (Figure 2). 24 hours prior to orthodontic tooth movement appliance removal, biologics were delivered into the palatal mucosa near the mesiopalatal and distopalatal surfaces of the maxillary first molar using 33-gauge microneedles (Hamilton Company, Reno, NV, USA).

Twenty-three rats underwent orthodontic tooth movement and were randomly divided into four groups and given 25 µL injections bilaterally. Based upon previous studies [11] [12], it was determined that a sample size of 6 for each group was needed to yield 80% power. Seven rats received an experimental injection of 437,500 OPG-Fc-loaded PEGDMA microspheres in 25 µL 1x PBS, six rats were administered a control 437,500 empty PEGDMA microspheres in 25 µL 1x PBS, and six rats were administered a volumetrically equivalent control of 25 µL 1x PBS. An additional 4 rats received a positive control injection of 25 µL OPG-Fc in PBS (18.85 ug/mL) for an optimal dosage of about 1mg/kg OPG-Fc as described in a previous study [12]. An additional eight rats were used as baseline controls; 4 rats did not undergo orthodontic tooth movement and 4 rats underwent orthodontic tooth movement but no relapse.

To measure tooth movement, polyvinylsiloxane impressions were taken of maxillary teeth to fabricate tooth models. Tooth movement was measured prior to appliance placement, at appliance removal, and then every 4 days during the relapse phase. Blood was also collected for future serum analysis at appliance placement, at appliance removal, and then every 12 days during the relapse phase.

For orthodontic appliance placement procedures, the rats were anesthetized by intraperitoneal administration of ketamine (87 mg/kg) and xylazine (10 mg/kg) supplemented with inhaled isoflurane. For all other survival procedures, including injection, appliance removal, maxillary teeth impressions, and blood draw, the rats were anesthetized with inhaled isoflurane only. Animals receiving injections were euthanized at day 24 of relapse for microCT analysis by CO_2 inhalation with bilateral thoracotomy.

Animals were weighed and springs were checked daily while appliances were in place during the initial tooth movement phase and then monitored and weighed every four days during the relapse phase.

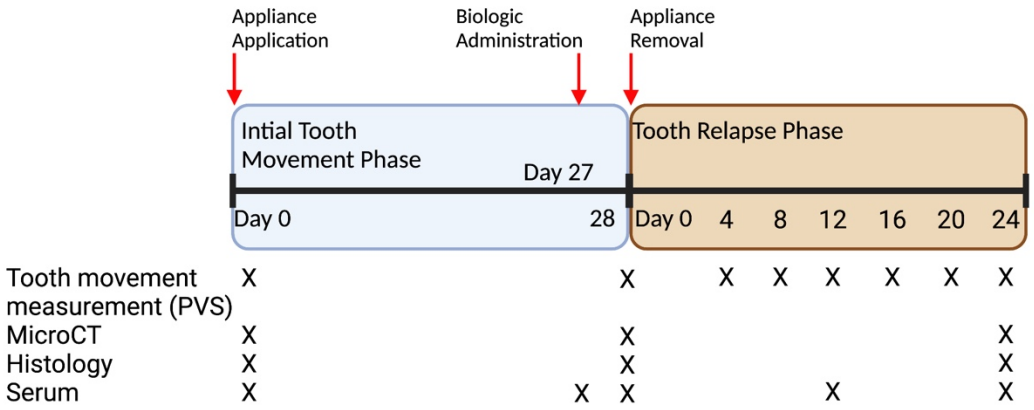

Figure 2 Schematic of animal procedure timeline. Created with BioRender.com.

B2.b: Orthodontic Tooth Movement and Relapse Rat Model

After a week of acclimation to powdered food diet, orthodontic appliances were placed bilaterally. Retentive grooves were notched into the mesial of the maxillary first molars and circumferentially at the gingival margin of the maxillary incisors. A 60g force 12mm NiTi closed coil spring (12mm Closed Coil Adjustable Force Coil Spring with eyelet; American Orthodontics, Sheboygan, WI, USA) was fastened from the maxillary first molar to the ipsilateral incisor with 0.010" stainless teel ligature ties (Figure 3). Ligatures were secured by bonding to the teeth with flowable composite (L-Pop Self-Etch Adhesive and Transbond Supreme LV Low Viscosity

Light Cure Adhesive; 3M/ESPE, St. Paul, MN, USA). To prevent appliance breakage, lower incisors were trimmed weekly over the 28-day initial tooth movement phase.

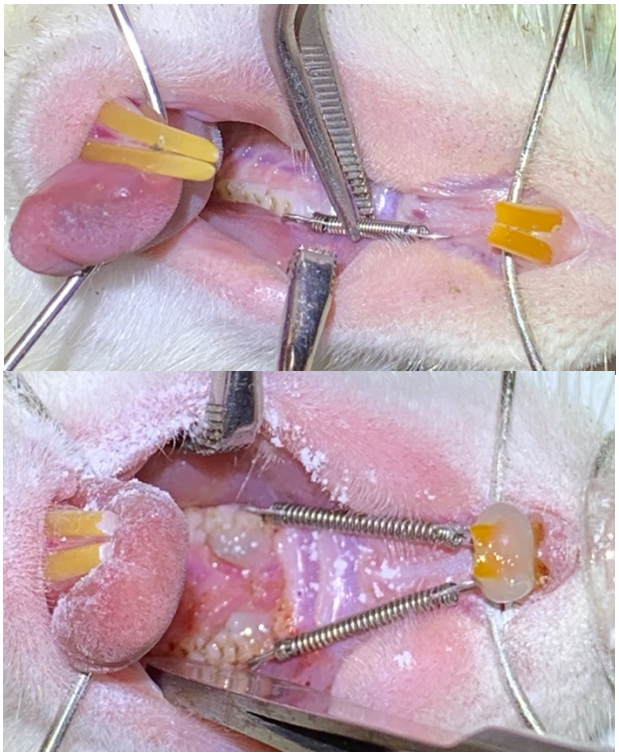

Figure 3 Images of appliance placement with spring ligated to molar before activation (above) and after activation and completion of procedure (below).

B2.c: Measurement of Tooth Movement

Tooth movement was measured as previously described [11] [12] with minor modifications. Tooth position was measured prior to appliance placement, at appliance removal and then every 4 days of the relapse phase. Impressions were taken of the maxillary teeth using polyvinylsiloxane (Dimension Garant 2L Quick; 3M/ESPE, St. Paul, MN, USA). The impressions were used to fabricate precise stone models (Hydrock Yellow Stone; Kerr Dental, Brea, CA, USA). The stone models were trimmed to allow for a flat occlusal plane and occlusal surfaces were scanned (Brother MFCL2700DW; Brother USA, Bridgewater Township, NJ, USA) at 1200 dpi adjacent to a 100 mm ruler. A single, blinded, calibrated examiner measured at each time point on images magnified by 100X using the ImageJ measuring tool (ImageJ; NIH, Washington,

D.C., USA). Measurements for molar tooth movement were taken from the distal surface of the maxillary third molar to the distal groove of the maxillary first molar and incisor tooth movement from the distal surface of the maxillary third molar to the lingual surface of the ipsilateral incisor at the gingival margin. Measurements were recorded to the nearest 0.01 mm.

B2.d: Tissue Harvest and Micro-Computed Tomography

Animals were euthanized using CO_2 followed by bilateral thoracotomy. After sacrificing, hemimaxillae and femurs were harvested, fixed in 10% formalin overnight, washed 3 times with 1x PBS, and stored in 70% ethanol.

Specimens were placed in a 19 mm diameter specimen holder and scanned using a microCT system (µCT100; Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 18 µm, 70 kVp, 114 µA, 0.5 mm AL filter, and integration time 500 ms. Analysis was performed using the manufacturer's evaluation software, and a fixed global threshold of 22% (220 on a grayscale of 0–1000) for maxillary interradicular bone, 30% (300) for femoral trabecular bone, and 23% (230) for femoral cortical bone was used to segment bone from non-bone. For maxillary analysis, a 1.4mm region (80 slice) beginning at the separation of the roots under the furcation was used (Figure 4). For femur analysis, a 0.9 mm (50 slice) region of trabecular bone was analyzed immediately below the growth plate, and a 1.8 mm (100 slice) cortical region was analyzed around the midpoint of the femur. Measurements of bone volume fraction (BVF), bone mineral density (BMD), trabecular bone thickness (Tb Th), and trabecular bone spacing (Tb Sp) were recorded. Tissue mineral density (TMD) was also recorded for the cortical region of the femur.

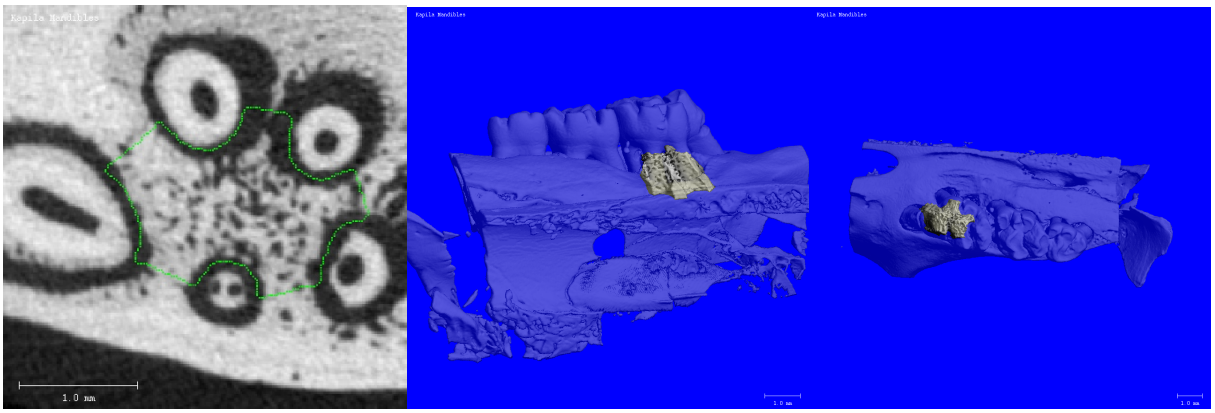

Figure 4 Views of maxillary alveolar microCT region of interest (ROI): coronal microCT section (left), buccal view of 3D reconstruction (center), occlusal view of 3D reconstruction (right).

B2.e: Statistical Analysis

Each animal was treated as one unit, and bilateral measurements were averaged to give a single measure for each animal at a given time point. If samples were broken or lost during sample harvesting or processing, the remaining unilateral side was used as the measurement for the animal. Descriptive statistics (means and standard deviations) were calculated for all measurements in all animals. Tooth movement by group was compared using two-way repeated measures analysis of variance (ANOVA). MicroCT measurements between groups were compared using one-way ANOVA. Inter-group differences were analyzed using Tukey's post-hoc analysis procedure. Differences with $P < 0.05$ were considered significant. Data was analyzed using Prism 9 statistical software (Prism 9; GraphPad Software, San Diego, CA, USA).

C: Results

C1: *In Vitro* Study Results

The 875,000 OPG-Fc-loaded microspheres demonstrated an initial burst release over the first three days with 1670.71 ng of OPG-Fc (SD ±275.86), 432.69 ng (SD ±153.45), and 231.91 ng

(SD ±131.99) on days 1, 2, and 3, respectively. After the burst release, the particles had a sustained release rate between 14.22 to 28.55 ng/day between days 9 to 35. This trend of a burst release followed by sustained release demonstrates first order kinetics. By day 23, the 875,000 microspheres had cumulatively released 3199.92 ng (SD ±1047.90).

When calculated for release from 437,500 microspheres, the number of microspheres injected on each side, 1599.96 ng of OPG-Fc (SD ±523.95) was cumulatively released by day 23 (Figure 5) and 10.43 ng/day of OPG-Fc (SD ±5.88) was still being released from the microspheres (Figure 6). By the end of elution collection on day 35, the 437,500 microspheres were still releasing 7.11 ng/day of OPG-Fc (SD ±1.81).

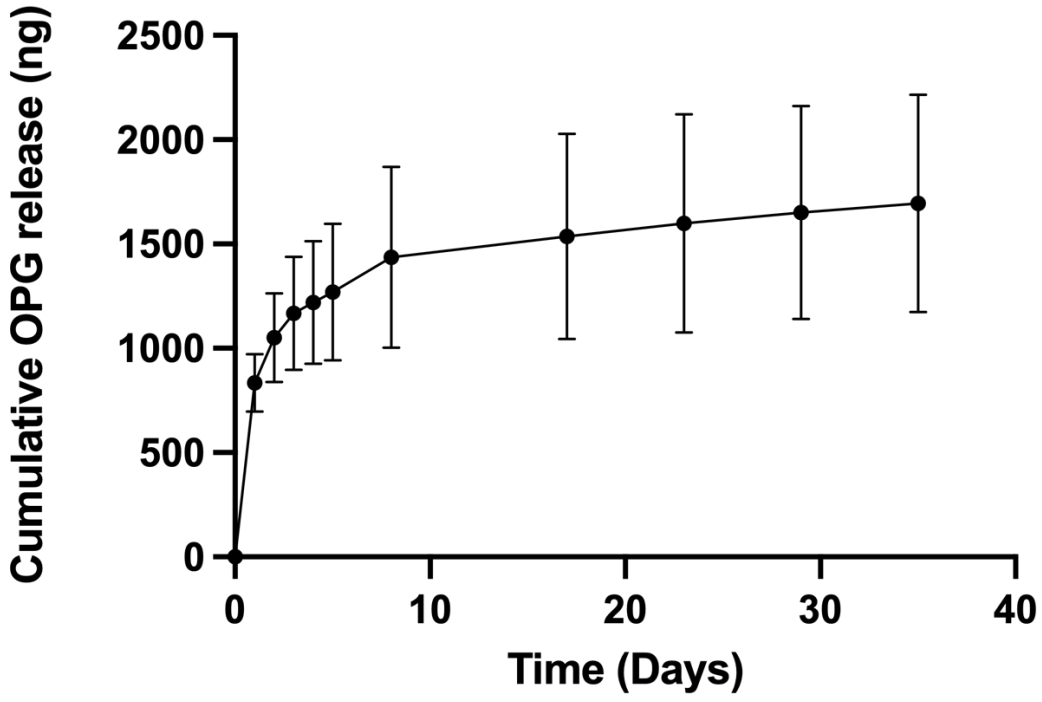

Figure 5 Graph of cumulative release of OPG from 437,500 OPG-Fc-loaded microspheres over 35 days.

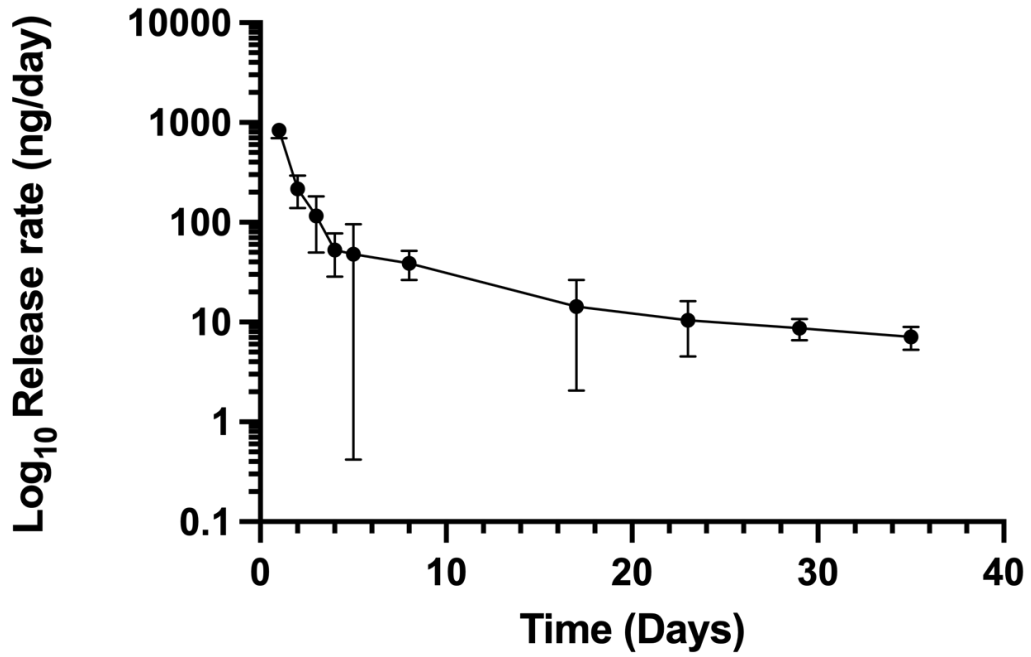

Figure 6 Graph of release rate of OPG from 437,500 OPG-Fc-loaded microspheres over 35 days.

C2: *In Vivo* Study Results

C2.a: Animal Status

One animal had an incisor fracture and two animals died from anesthetic complications during the appliance placement procedure. As no tooth movement occurred, these three animals were used in the baseline no tooth movement group. All other animals tolerated the procedures. One animal had a spring break on the day 22 of tooth movement, and the spring was replaced within 24 hours. All animals that were administered a biologic survived all 24 days of relapse.

Average initial weight was 403 g (SD ±15), and average weight at appliance removal 486 g (SD ±26) (Figure 7). There were no significant differences between treatment groups in weight

and weight gain throughout the relapse phase (p>0.09). Average weight at end of the relapse phase was 531 g (SD ±26).

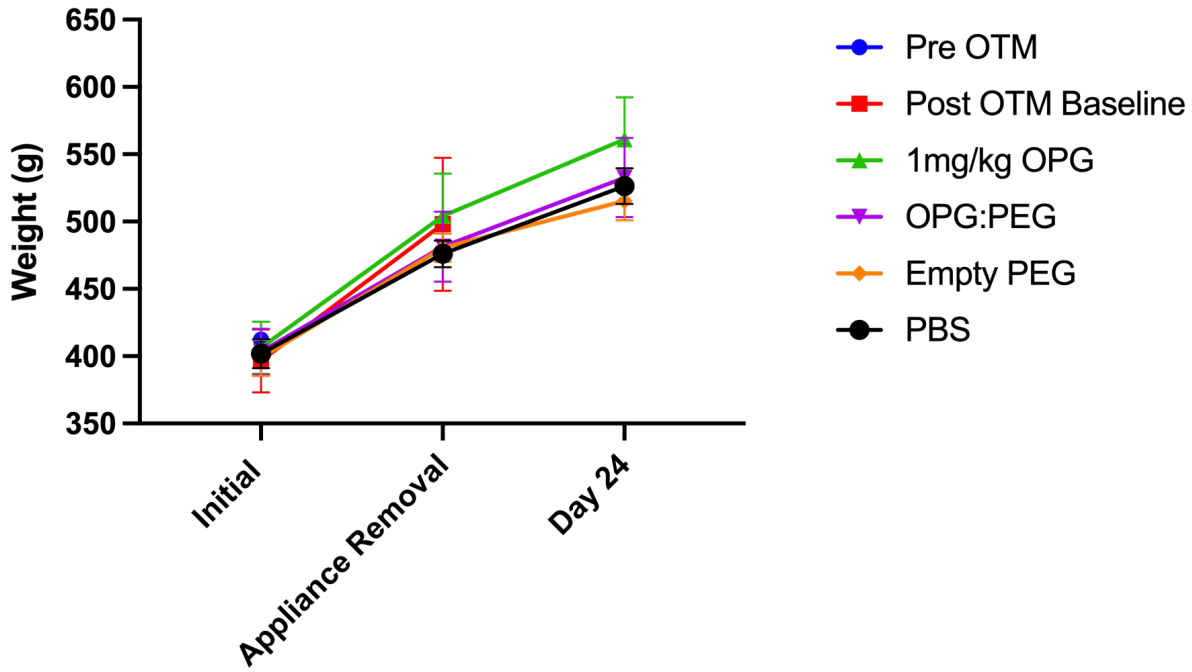

Figure 7 Average weight (with SD) of animals in each group throughout in vivo *experiment*

C2.b: Molar Tooth Movement

There were no statistically significant differences in molar tooth movement between groups. When comparing molar tooth movement distance, all treatment groups had similar initial molar tooth movement, which was similar to the post-orthodontic tooth movement baseline group (p>0.38, 0.86±0.06 mm for post-orthodontic tooth movement baseline, 0.86±0.23 mm for 1 mg/kg OPG-Fc, 1.08±0.26 mm for OPG-Fc-loaded PEGDMA microspheres, 0.87±0.31 mm for empty PEGDMA, and 0.91±0.09 mm for PBS). In Figure 8, all groups appear to follow a similar relapse trend, but 1mg/kg OPG-Fc starts to deviate slightly at Day 4 of relapse, and empty PEGDMA starts to deviate slightly from PBS at day 12 of relapse, although these differences were not statistically significant.

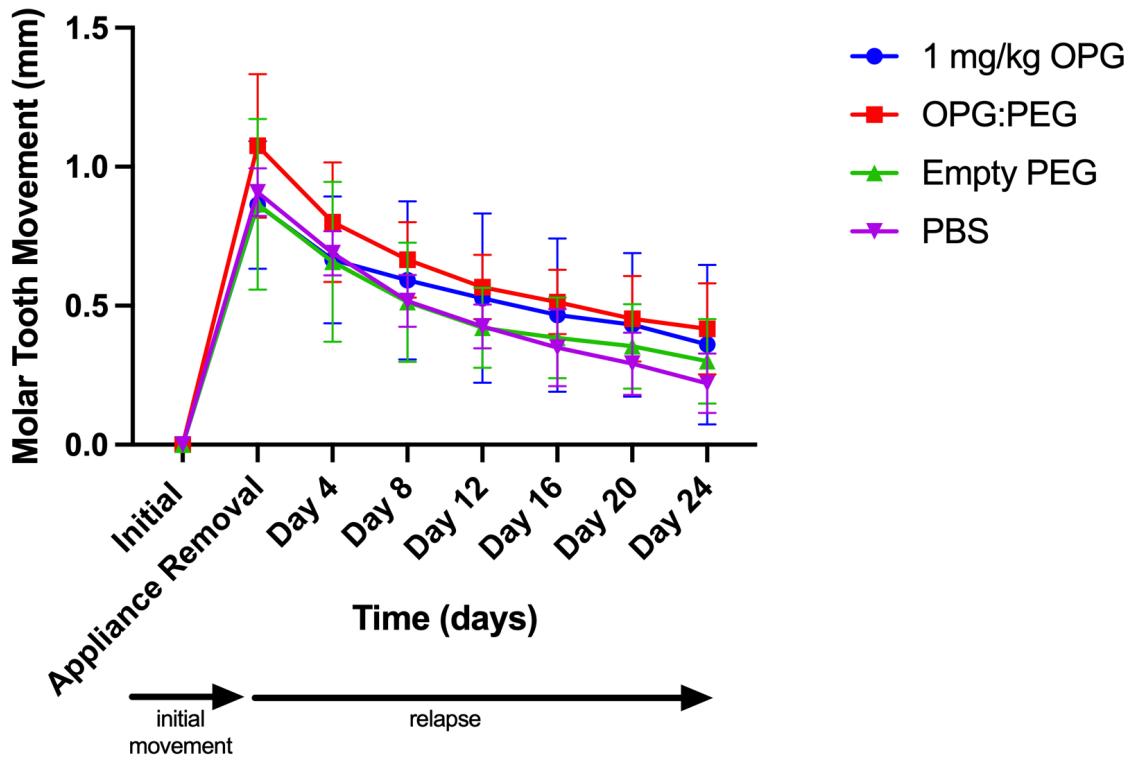

Figure 8 Graph of average (with SD) molar tooth movement distance for each treatment group throughout the experiment.

When molar tooth movement relapse was calculated as a percent of initial movement, there was no statistically significant difference in final relapse percent ($p > 0.64$) (Figure 9). However, at the end of the relapse, the 1 mg/kg OPG-Fc had the least relapse with 62.35% (SD ±25.20%), OPG-Fc-loaded PEGDMA microspheres with 65.50% (SD ±8.91%) had similar relapse to empty PEGDMA microspheres with 66.74% (SD ±15.66%), and all three groups had less relapse than the PBS group 76.55% (SD ±10.96%).

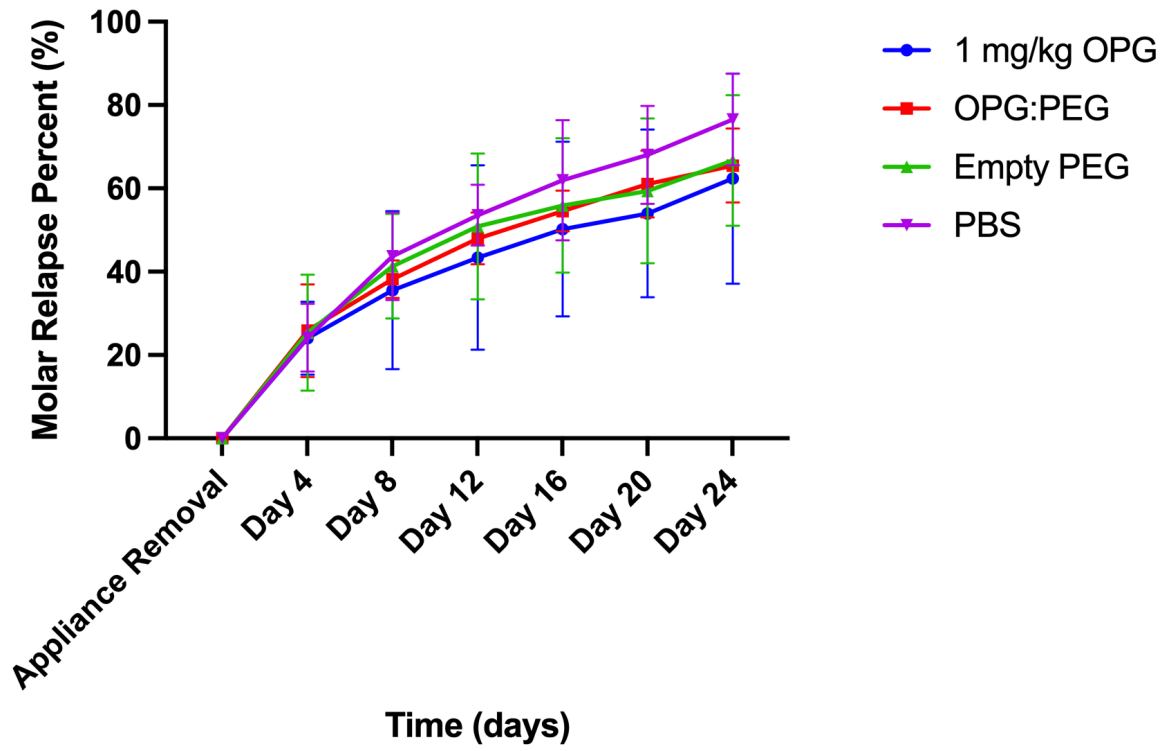

Figure 9 Graph of average (with SD) percent molar relapse of initial tooth for each treatment group throughout the experiment.

Molar tooth relapse rate, calculated as relapse distance divided by number of days, between appliance removal and Day 4 of relapse were similar between treatment groups (0.05±0.02 mm/day for 1 mg/kg OPG-Fc, 0.07±0.03 mm/day for OPG-Fc-loaded PEGDMA microspheres, 0.05±0.3 mm/day for empty PEGDMA, and 0.06±0.02 mm/day for PBS) (Figure 10). Though not statistically significant, molar tooth relapse rate between relapse phase days 4 and 8 were less for the 1 mg/kg OPG-Fc group (0.02±0.02 mm/day) than the other groups (0.03±0.03 mm/day for OPG-Fc-loaded PEGDMA microspheres, 0.04±0.02 mm/day for empty PEGDMA, and 0.04±0.01 mm/day for PBS). Between day 8 and the end of the relapse phase, relapse remained between 0.01 to 0.02 mm/day for all groups. However, it is worth noting that these relapse rate measurements are small and fall within the standard deviation.

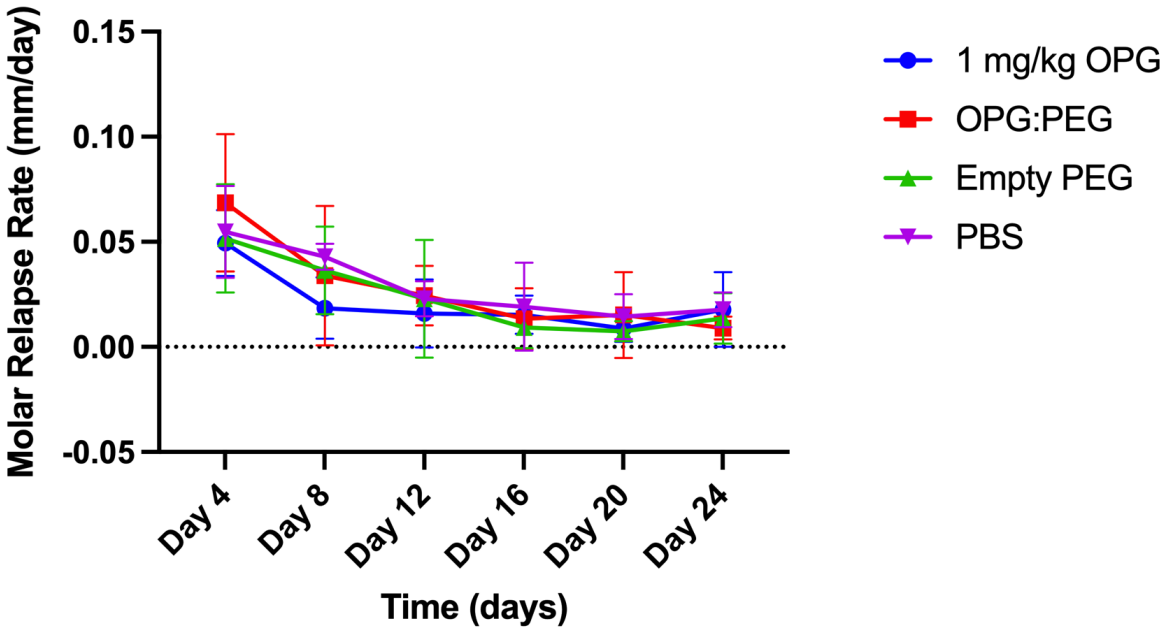

Figure 10 Graph of average (with SD) molar relapse rate for each treatment group throughout the experiment.

For molar tooth movement measurements, there was variability between subjects within treatment groups and within subjects. When measurements are shown for all individual side, there is a wide range within treatment groups, and each treatment group has some measurements outside of the interquartile range (Figure 11). However, no subjects could be determined as true outliers due to averaging data between sides for the individual, and no sides could be determined as true outliers due to the small sample size.

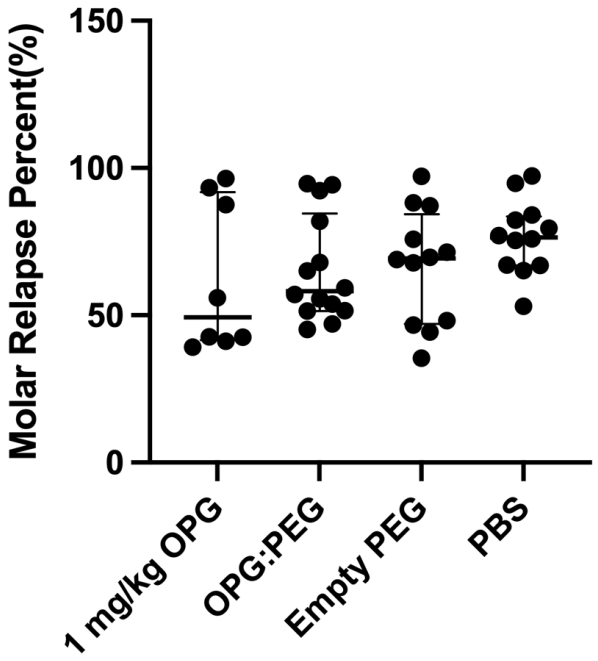

Figure 11 Scatter plot (median and IQR) of percent molar relapse of initial tooth movement measurements for indvidual sides of animals in each treatment group on Day 24 of relapse.

C2.c: Incisor Relapse

Incisor initial movement and relapse were similar with no statistically significant differences across all groups ($p>0.65$) (Figure 12). All groups had initial tooth movement between 1.20 to 1.45mm at appliance removal (1.45±0.42 mm for 1 mg/kg OPG, 1.20±0.30 mm for OPG-Fc-loaded PEGDMA microspheres, 1.27±0.28 for empty PEGDMA, and 1.23±0.29 for PBS). All groups relapsed around 70% of initial orthodontic tooth movement (74.92±13.29% for 1 mg/kg OPG-Fc, 62.87±16.55% for OPG-Fc-loaded PEGDMA microspheres, 72.21±14.28% for empty PEGDMA, and 66.61±16.00% for PBS).

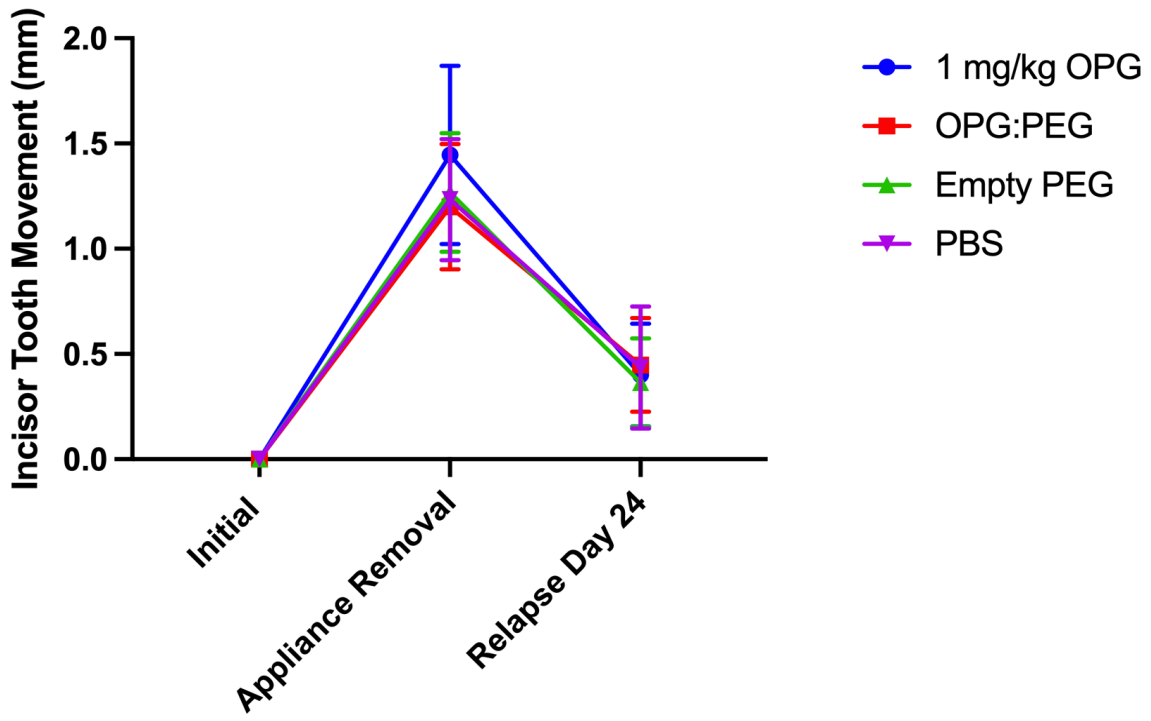

Figure 12 Graph of average (with SD) incisor tooth movement for each treatment group throughout the experiment.

C2.d: Alveolar Bone Micro-Computed Tomography

Only the post orthodontic tooth movement baseline control group differed significantly from the other groups in bone parameter measurements. For bone volume fraction, bone mineral density, and trabecular bone thickness, the post-orthodontic tooth movement baseline control group was lower than all other groups with statistical significance (Figure 13).

The post-orthodontic tooth movement group had a BVF of 0.49 (SD ±0.06) and was lower than all other groups ($p<0.007$, 0.73±0.01 for pre-orthodontic tooth movement baseline, 0.68±0.08 for 1 mg/kg OPG-Fc, 0.66±0.11 for OPG-Fc-loaded PEGDMA microspheres, 0.69±0.04 for empty PEGDMA, and 0.69±0.04 for PBS). The post-orthodontic tooth movement group had a BMD of 488.9 mg/cc (SD ±58.66) and was lower than all other groups ($p<0.016$, 726.1±13.02 mg/cc for pre-orthodontic tooth movement baseline, 669.3±94.93 mg/cc for 1 mg/kg OPG-Fc,

643.1±101.6 mg/cc for OPG-Fc-loaded PEGDMA microspheres, 664.7±50.17 mg/cc for empty PEGDMA, and 664.2±33.42 mg/cc for PBS). The post-orthodontic tooth movement group had a Tb Th of 0.18 mm (SD ±0.04) and was lower than all other groups ($p<0.011$, 0.32±0.01mm for pre-orthodontic tooth movement baseline, 0.31±0.07 mm for 1 mg/kg OPG-Fc, 0.31±0.05 mm for OPG-Fc-loaded PEGDMA microspheres, 0.30±0.05 mm for empty PEGDMA, and 0.29±0.04 mm for PBS).

Tb Sp was similar across all groups ($p>0.16$, 0.14±0.01 mm for pre-orthodontic tooth movement baseline, 0.16±0.05 mm for post-orthodontic tooth movement baseline, 0.10±0.01 mm for 1 mg/kg OPG-Fc, 0.20±0.15 mm for OPG-Fc-loaded PEGDMA microspheres, 0.10±0.02 mm for empty PEGDMA, and 0.10±0.02 mm for PBS).

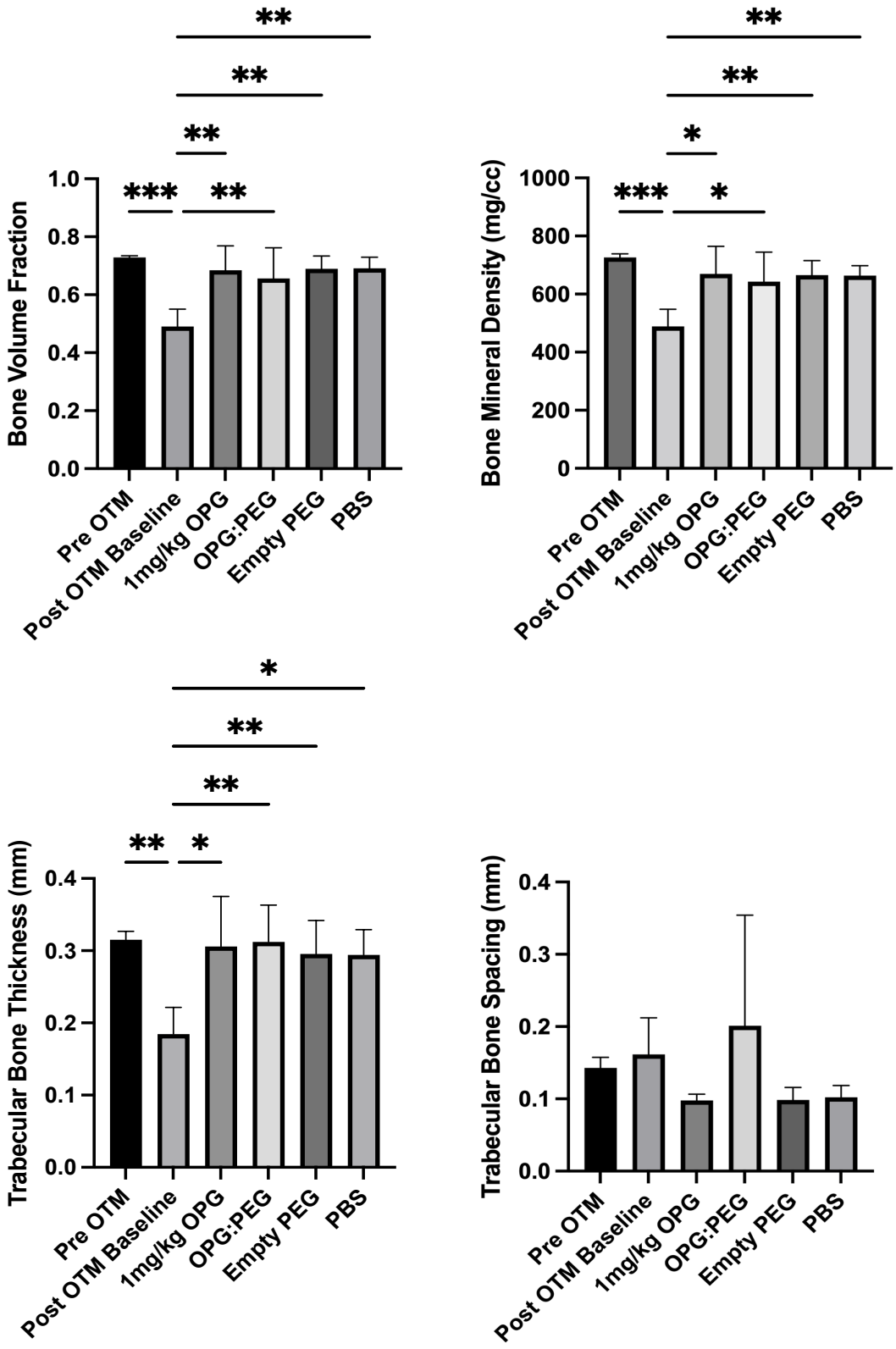

Figure 13 Histogram graphs of average (with SD) measurements from maxillary alveolar microCT scans: BVF (upper left), BMD (upper right), Tb Th (lower left) and Tb Sp (lower right).

C2.e: Femur Bone Micro-Computed Tomography

In the trabecular bone of the femur, only the post-orthodontic tooth movement baseline group had less bone volume fraction ($p<0.029$) and trabecular bone thickness ($p<0.017$) than the pre-orthodontic tooth movement baseline group that was statistically significant (Figure 14). Though not statistically significant, all groups appear to have less BVF, BMD, and Tb Th and more Tb Sp than the pre-orthodontic tooth movement baseline group.

In the cortical bone, statistically significant differences were seen in TMD, BMD, and BVF. For TMD, the 1mg/kg OPG group had greater cortical bone TMD than both the PBS and pre-orthodontic tooth movement baseline groups ($p<0.029$) (Figure 15). The pre-orthodontic tooth movement baseline group has less cortical bone TMD than all groups ($p<0.044$). For BMD, the OPG-loaded PEGDMA treatment group had greater BMD than the pre-orthodontic tooth movement baseline, post-orthodontic tooth movement baseline, and PBS groups ($p<0.0014$). The pre-orthodontic tooth movement baseline the 1mg/kg OPG-Fc, OPG-Fc-loaded PEGDMA, and empty PEGDMA treatment groups ($p<0.003$). For BVF, the OPG-Fc-loaded PEGDMA treatment group had a BVF of 0.65 (SD ±0.02), which was greater than all groups ($p<0.030$) except the empty PEGDMA treatment group.

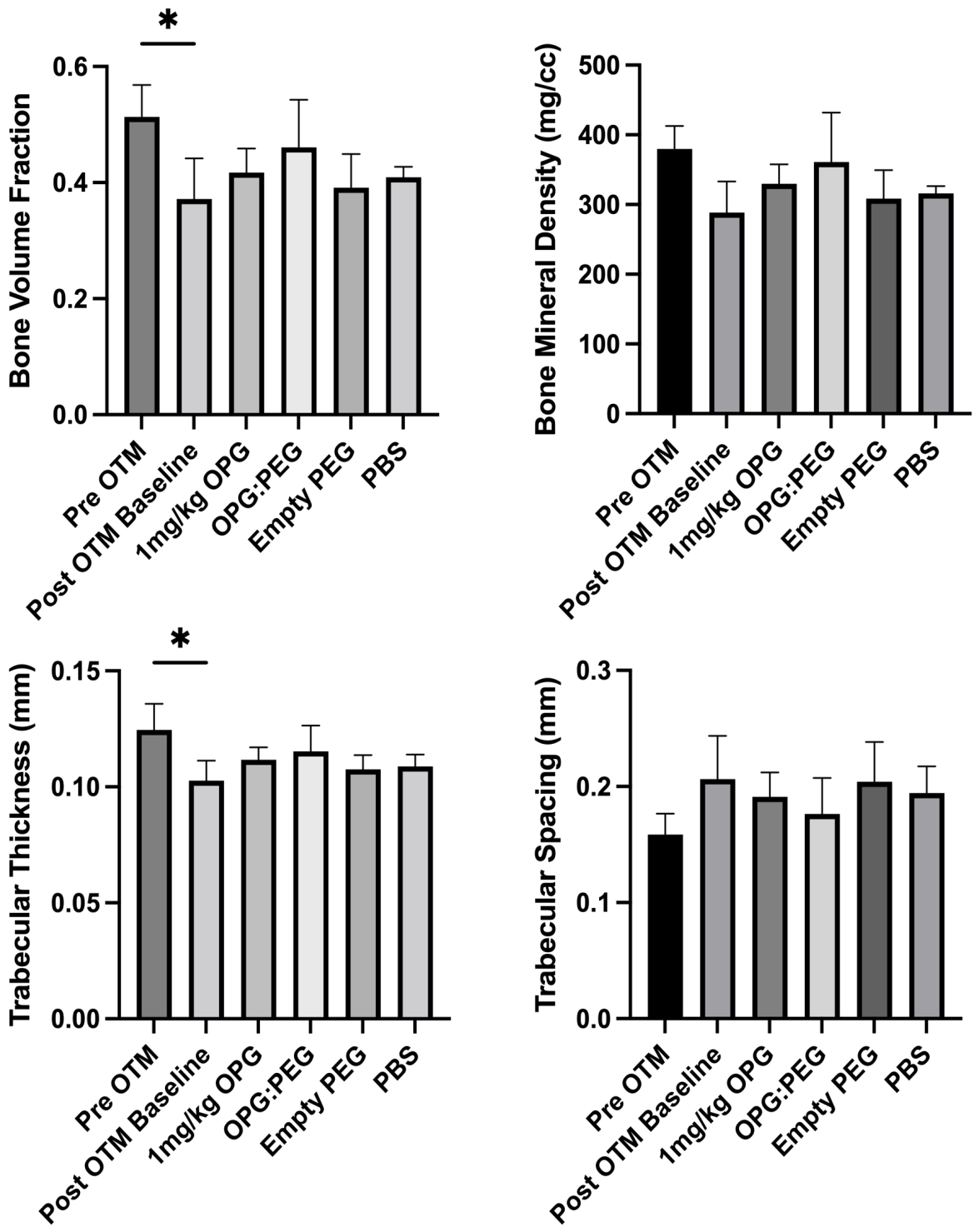

Figure 14 Histogram graphs of average (with SD) measurements from femoral trabecular bone microCT scans: BVF (upper left), BMD (upper right), Tb Th (lower left) and Tb Sp (lower right).

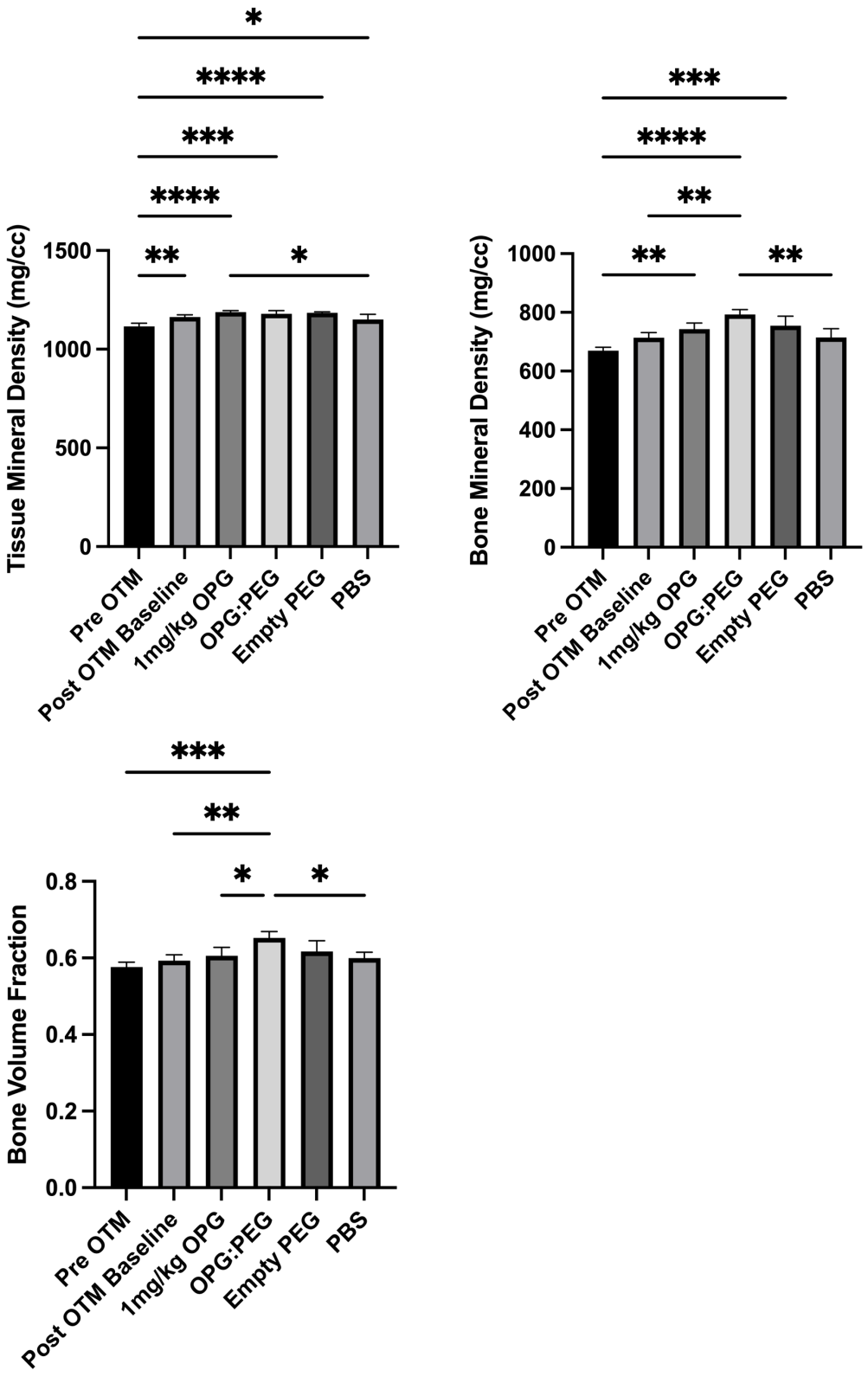

Figure 15 Histogram graphs of average (with SD) measurements from femoral cortical bone microCT scans: BVF (upper left), BMD (upper right), Tb Th (lower left) and Tb Sp (lower right).

D. Discussion

Although there were not many statistically significant results to allow for conclusive findings, there are some interesting potential points of discussion.

This study developed a low-dose, slow-release drug delivery system for OPG-Fc and tested it in a rat orthodontic relapse model. Based on previous studies, the initial goal was to release 1mg/kg over 24 days. However, our 875,000 OPG-Fc-loaded PEGDMA microspheres only released a total of 3199.92 ng (SD ±523.95) over 23 days in the *in vitro* study. However, at Day 23, the spheres were still releasing 20.86 ng/day (SD ±11.76) in 350mL for a concentration of 59.6 ng/mL. 59.6 ng/mL is much higher 1ng/mL, the IC50 for OPG [25], so we would expect this concentration to inhibit osteoclast differentiation *in vitro*. Thus, the *in vitro* data suggest that even with a dose lower than initially targeted, the spheres released a concentration that may have the potential to be effective in inhibiting osteoclastogenesis.

From the *in vivo* study, OPG-Fc released from the OPG-Fc-loaded PEGDMA microspheres is likely active and may be somewhat effective in preventing relapse. Within the OPG-Fc-loaded PEGDMA microspheres group, there was a wide range of relapse measurements (40 to 90%), indicating that the OPG-Fc being released is active, and OPG-Fc may be effective at reducing relapse at much lower levels than previously administered. However, we cannot draw any conclusions because of lack of statistical significance. The wide variation in relapse measurements suggests that more investigation into *in vivo* drug delivery and release dynamics is needed. Location and volume of injection can influence diffusion and delivery as was previously shown for other drugs such as local anesthetic palatal injections that require a lower volume to take effect but also have a much smaller area of diffusion [26]. Thus, adjusting

injection site may lead to more consistent effects from the OPG-Fc-loaded PEGDMA microspheres.

The molar tooth movement relapse in the OPG-Fc at a dose of 1mg/kg and the PBS control groups differed from the previous study. Schneider et al. found that a single injection of OPG-Fc at a dose of 1mg/kg could limit relapse to about 30% of initial tooth movement at day 24 [12]. However, in our study, the group receiving a single injection of 1mg/kg OPG-Fc relapsed to about 60% of the initial tooth movement. However, when looking at our samples, there was a wide variety of relapse ranging from 30 to 90% relapse of initial tooth movement. With a larger sample size, we may expect to see relapse inhibition more similar to the previous study. Interestingly, our PBS control also exhibited greater percent relapse compared to Schneider et al., 70% and 50%, respectively, which could indicate subject differences contributing to greater relapse across all groups [12].

The OPG-Fc-loaded PEGDMA microspheres and the empty PEGDMA microspheres had similar percent relapse of initial molar movement. Both OPG-loaded PEGDMA microspheres and empty PEGDMA group both had less relapse than PBS group, though not statistically significant. These data may suggest that the PEG itself may play a role in reducing relapse. The particles themselves may act as a physical barrier or PEG byproducts during degradation may play a role in preventing relapse [19] [27].

Incisor movements was similar across all groups at 70%, suggesting that effects were local for all treatment groups receiving biologics. This is also consistent with incisor tooth movement relapse from past studies [11] [12].

One other area of interest is that the post orthodontic tooth movement baseline group was the only group with statistically different maxillary alveolar bone microCT measurements. All animals receiving biologics were euthanized at day 24 of the relapse period. However, Hudson et al. found that with OPG-Fc, the bone showed a high level of organization as early as day 8.[10] Thus, there may be differences in bone quality and quantity not seen due to the selected time point. Remodeling may be happening at a much earlier time point during the relapse phase, and it may be beneficial to have additional microCT analysis at earlier time points in future studies. Additionally, maxillary alveolar bone microCT measurements were only taken at one region of interest, so we may differential remodeling in other areas.

Lastly, in the femoral microCTs, the animals receiving OPG-Fc-loaded PEGDMA injections had higher femoral cortical bone BVF, TMD, and BMD but no differences in femoral trabecular bone. While this finding may indicate systemic effects from released OPG, it may also be due to individual animal differences based on activity or bone-loading [28]. Analysis of OPG levels in circulating serum would be needed to determine if the released OPG-Fc had systemic effects.

D1: Future Directions

Further analysis of existing samples is a future direction. Serum was collected and could be analyzed for evaluation of systemic OPG and TRAP-5b levels and to determine if the OPG released from the microspheres contributed to the increase of femoral cortical bone BVF, BMD, and TMD. Hemimaxillae could be prepared for histology to examine differences in bone organization between groups as well as look at the location of microsphere injection and deposition. Adding additional samples would help increase the power, determine true outliers, and may allow for statistically significant findings.

Another direction would be to look at the effects of empty PEGDMA and examine if the drug delivery vehicle (PEG) may be having an effect.

Though we could not draw any conclusions from our data, some samples showed a reduction in relapse from sustained low-dose delivery of OPG-Fc. A future direction could be to continue to optimize and analyze OPG-Fc release dynamics from PEGDMA microspheres or examine other low-dose sustained release delivery systems *in vitro* and *in vivo*.

D2: Limitations

The greatest limitations in this study were small sample size and variability within the data. Due to the small sample size, outliers could not be determined, and statistically supported conclusions could not be drawn.

There were also limitations in the orthodontic tooth movement rat model. Both molar and incisor movement occurred, thus potentially limiting the full potential movement of the molar. Additionally, though calibrated, the spring could have manufacturing variability in force application during molar movement. Furthermore, some measurements were small and our accuracy in measuring tooth movement in the range of 0.1-0.2mm was limited.

Efficacy of injection technique and drug delivery was also questionable, and it was a potential source of the high variability in the study. Additional preliminary studies *in vitro* and *in vivo* may optimize OPG-Fc drug delivery and release, providing more robust data on effect of OPG-Fc on orthodontic tooth relapse.

E. Conclusions

1. An *in vivo* rat orthodontic relapse model was established at UCSF.

2. PEGDMA microspheres were successfully loaded with OPG-Fc and demonstrated a first order release curve.

3. PEG itself may have a role in reducing relapse and/or a role in bone remodeling.

4. Administration of vehicle is an important factor when designing a drug delivery system.

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