Inhibition of RANKL and Sema4d Improves Residual Ridge Resorption in Mice.

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Title

Inhibition of RANKL and Sema4d improves residual ridge resorption in mice

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20 **Abstract**

21 Residual ridge resorption (RRR) is a chronic and progressive bone resorption following
22 tooth loss. It causes deterioration of the oral environments and leads to the pathogenesis
23 of various systemic diseases. However, the molecular mechanisms and risk factors for
24 RRR progression are still unclear and controversial. In this study, we developed a tooth
25 extraction model using mice for analyzing long-term morphological and gene expression
26 changes in the alveolar bone. We further applied ovariectomy to this model to elucidate
27 the effects of osteoporosis on RRR progression. As a result, the alveolar bone loss was
28 biphasic and consisted of rapid loss in the early stages and subsequently slow and
29 sustained bone loss over a long period. Gene expression analysis indicated that
30 ovariectomy increased the expression of pro-inflammatory cytokines in the alveolar bone
31 and prolonged the activation of osteoclasts same as histological analysis. Furthermore,
32 the expressions of *Tnfsf11* and *Sema4d* kept increasing for a long time in OVX mice.
33 Administration of neutralization antibodies for receptor activator of NF- κ B ligand
34 (RANKL) effectively suppressed RRR. Similarly, inhibition of Semaphorin 4d (Sema4d)
35 also improved alveolar bone loss. This study demonstrated that osteoporosis is a risk
36 factor for RRR and that RANKL and Sema4d suppression are potential treatments.

37

38 **Introduction**

39 Residual ridge resorption (RRR) is a continuous, often lifelong, alveolar bone resorption
40 occurring after tooth loss. Losing teeth in adult is the result of injury or disease, such as
41 dental avulsion, tooth decay, and periodontal disease. The proportion of people who lost
42 their teeth increases with age. In Japan, 40% of people in their late 40s and 60% of people
43 in their early 50s have lost at least one tooth¹. In the United States, 26% of adults aged 65
44 or older have eight or fewer teeth and about 17% in them have lost all of their teeth²⁷.
45 RRR therefore can occur in anyone with a high probability.

46 RRR follows initial wound healing, which includes epithelial integrity
47 restoration accompanied by bone formation within the extraction socket and bone
48 resorption at the edge of the socket. After the initial rapid healing stage, bone resorption
49 slows down but sometimes persists for a long time. As RRR progresses, dentures become
50 unstable, and dental implant placement becomes difficult, thus worsening the oral health-
51 related quality of life and social activity. Several studies have suggested that prolonged
52 alveolar bone resorption is caused by aging, excessive mechanical stress, and
53 periodontitis³⁻⁵. However, the risk factors of RRR progression are still controversial.

54 Osteoporosis is a metabolic disease that affects postmenopausal women. This
55 disease is commonly characterized by low bone mass and bone tissue deterioration, which

can lead to increased risk of bone fractures. However, not all individuals with osteoporosis develop RRR. Some animal studies have demonstrated positive correlations between alveolar bone loss or delayed healing of extraction sockets and systemic osteoporosis^{6,7}. Contrarily, others have shown a weak relationship or no relevance at all^{8,9}. Thus, the relationship between osteoporosis and jawbone resorption has not been elucidated.

The treatment for osteoporosis mainly involves two drug types. Most of the currently available drugs for osteoporosis are bisphosphonates or anti-RANKL monoclonal antibody drugs. These drugs inhibit osteoclastic bone resorption; however, they can sometimes, but not necessarily, induce drug-related osteonecrosis of the jaw (ARONJ) following tooth extraction^{10,11}. Another type of drug promoting osteoblastic bone formation is parathyroid hormone (PTH). Intermittent administration of PTH can treat osteoporosis of long bones and vertebrae¹², and some studies have indicated that intermittent PTH therapy could increase the mineral density of the jawbone in animal models^{13,14}. However, intermittent PTH treatment only initially increases bone formation and promotes bone resorption; therefore, the treatment period is limited. A Sost-specific antibody treatment, which promotes bone formation and inhibits bone resorption, was developed and used clinically. An animal study suggested that sclerostin inhibition

increased the alveolar bone volume and architecture in rats with alveolar bone loss¹⁵.

Semaphorin 4d (Sema4d) is expressed by osteoclasts and is a mediator of osteoclast–osteoblast communication. Moreover, it inhibits osteoblastic bone formation.

Injection of Sema4d-specific antibodies into ovariectomized mice was shown to promote osteoblastic bone formation without affecting osteoclastic bone resorption in the femur¹⁶.

However, there have only been a few reports on the therapeutic effect of Sema4d inhibition on jawbone loss following tooth extraction.

This study aimed to 1) elucidate the factor of alveolar bone resorption using a murine tooth extraction model and 2) investigate whether injection of RANKL-specific antibodies or Sema4d-specific antibodies could be a useful approach to the suppression of alveolar bone resorption in an ovariectomized mouse model of postmenopausal osteoporosis.

RESULTS

Maxillary teeth extractions in mice does not induce long-term loss of maxillary alveolar bone volume

RRR in humans is characterized by sustained loss of the mandible and maxillary bone mass for prolonged periods following tooth extraction. However, there is little information about bone morphological changes after extraction in other mammals, including rodents. Therefore, we investigated the morphological changes of the maxillary alveolar bones of mice following teeth extractions using μ CT imaging. At 16 weeks after teeth extractions, the maxillary bone was absorbed on the maxillary sinus side and the maxilla alveolar crest side but was only absorbed slightly on the buccal side (Supplementary Fig. S1 online).

We examined the long-period changes of BV on the extracted and non-extracted sides of the maxillary bone from day 0 to 24 weeks following teeth extractions. The BV of the extracted side rapidly decreased to $73.6\% \pm 1.51\%$ (mean $\pm$ standard deviation) by 9 weeks post-extraction (Fig. 1a). Subsequently, although the alveolar BV slightly decreased, there was no significant difference between 9 and 24 weeks ($P = 0.27$ calculated using Student's t -test). This result suggested that alveolar bone resorption following teeth extractions is rarely long-lasting in healthy mice.

The CT value at an extraction socket of the first molar recovered to $80.0\% \pm 0.06\%$ of the amount of the adjacent alveolar bone of the buccal side at 5 weeks post-extraction, indicating that the extraction socket of the first molar was almost filled with new bone by 5 weeks post-extraction (Fig. 1b).

These results indicated that the sharp decrease in alveolar BV in the weeks following teeth extractions was related to the activation of bone remodeling during healing of the extraction socket^{17, 18}.

Ovariectomy promotes long-term resorption of the maxillary bone after teeth extractions

Postmenopausal osteoporosis could be one of the possible risk factors for ridge resorption¹⁹. We analyzed bone morphology following teeth extractions using OVX mice as an animal model for osteoporosis. The images obtained *via* superimposition of CT-3D images revealed that the height and width of the alveolar bone were reduced after 16 weeks post-extraction in OVX mice (Fig. 2a).

The temporal observation of alveolar BV with μ CT indicated that the BV in OVX mice rapidly decreased to $69.7\% \pm 2.60\%$ at 5 weeks post-extraction and subsequently decreased gradually to $55.3\% \pm 2.63\%$ by 17 weeks following teeth

extractions. This result indicated that alveolar bone resorption in OVX mice continues over the long term. Meanwhile, the BV in the sham mice decreased to $84.5\% \pm 0.30\%$ at 5 weeks and then slightly decreased, but was not statistically significant ($P = 0.267$ calculated using Student's *t*-test), to $79.5\% \pm 0.49\%$ at 17 weeks (Fig. 2b). This kinetic BV change in the sham mice was almost identical to the non-treated mice (Fig. 1a).

The ratio of the CT values in the extraction socket of sham mice recovered to $84.2\% \pm 3.05\%$ at 5 weeks after extraction and reached $96.7\% \pm 0.89\%$ at 17 weeks, whereas the CT value in that of OVX mice only recovered to $55.5\% \pm 1.33\%$ at five weeks and subsequently remained almost unchanged during our observation period up to 17 weeks ($66.1\% \pm 3.06\%$) following teeth extractions (Fig. 2c). This result indicated that the extraction socket was not entirely restored in the OVX mice.

Prolonged activation of bone remodeling during the restoration of extraction sockets in OVX mice

We examined the temporal distribution of osteoclasts during healing of the extraction sockets using enzymatic histochemistry of TRAP, which demonstrated that osteoclasts increased around the extraction sockets immediately after teeth extractions (Fig. 3a). High TRAP activity persisted in the tooth extraction area, especially on the surface of the

buccal side of the alveolar bone of OVX mice even at 12 weeks following teeth extractions. Quantitative image analysis revealed that there was prolonged activation of osteoclasts around the tooth extraction site of OVX mice (Fig. 3b).

Furthermore, quantitative PCR analysis confirmed that the increased expressions of gene expression encoding the osteoclast-related molecules—*Acp5*, *Mmp9*, and *Ctsk*, *Ccl2*—were sustained up to 12 weeks following teeth extractions in the alveolar bone of OVX mice (Fig. 4). These data suggested that osteoclast activities were sustained for a long time after teeth extractions in the alveolar bone of OVX mice.

We detected persistent increases in the expressions of genes encoding osteoblast-related molecules, including *Alpl*, *Bglap*, *Tnfsf11*, *Dkk1*, *Slc2a1*, *Sema4d*, *Sost*, and *Runx2*, except for *Tnfrsf11b*, in OVX mice (Fig. 4). Similar increased expressions in OVX mice were observed for genes encoding proinflammatory cytokines, including *Tnf*, *Ifng*, and *Il1a*, except for *Il17* (Fig. 4).

Neutralization of RANKL or Sema4d can prevent bone resorption of alveolar bone

RANKL is expressed on the surface of osteoblasts and enhances osteoclastogenesis²⁰. Sema4d derived from osteoclasts inhibits osteoblastic bone formation¹⁶. Consequently, both molecules may promote bone resorption. We confirmed that the increased

expressions of genes *Tnfsf11* and *Sema4d* encoding RANKL and Sema4d, respectively,
in alveolar bone were sustained at least until 12 weeks after teeth extractions (Fig. 4).

We examined the effects of antibodies for RANKL and Sema4d. As a result, both
neutralizing antibodies increased the volumes of alveolar bone after teeth extractions; the
BV was increased to 126.5% and 130.0% at 12 weeks after teeth extractions of OVX mice
administered with anti-RANKL and anti-Sema4d antibodies, respectively, compared with
that of the control experiment (Fig. 5). These data indicated the possibility that
neutralization of RANKL or Sema4d inhibits long-time bone resorption and sustainably
increases bone formation in alveolar bone after teeth extractions.

Discussion

Bone loss in the mandibular or maxillary bones, which lasts long following tooth loss, is characteristic of RRR in humans. In this study, we used mice as an experimental model to observe long-term maxillary BV for up to 24 weeks after teeth extractions. We found that, in healthy mice, a sharp decrease in BV was observed in the short period after teeth extractions, and subsequently, the loss of BV was slight or almost none. Conversely, ovariectomized mice, used as animal model of postmenopausal osteoporosis, had long-lasting gradual loss of BV after a short and sharp decrease. The decrease in BV observed in ovariectomized mice was similar to that during RRR in humans, which shows a biphasic decline, with a sharp reduction in the early stage and a gradual long-lasting decrease thereafter²¹. The long-lasting loss of maxilla BV after teeth extractions may be promoted by risk factors such as osteoporosis but is not observed in healthy conditions.

Postmenopausal osteoporosis leads to micro-architectural deterioration of bone tissue and low bone mineral density²². Some clinical studies reported that the RRR increased in osteoporotic edentulous patients^{23, 24}. However, other studies demonstrated that there was no statistical relationship between edentulous jaw resorption and osteoporosis^{25, 26}. Thus, the relationship between RRR and osteoporosis is still controversial and has not been sufficiently elucidated. Our experimental study on a novel

animal model revealed the possibility that postmenopausal osteoporosis due to reduced ovarian function exacerbates RRR.

Ovariectomy increases osteoclast formation and the lifespan by producing osteoclastogenic cytokines and stimulating bone resorption, resulting in rapid bone loss. We found transient increases in several osteoclastogenic cytokines after teeth extractions. In healthy mice, these transient increases immediately returned to near their original level. Contrarily, in OVX mice, higher levels of expression of osteoclastogenic cytokines, including *Tnfsf11*, *Tnfrsf11b*, *Sema4d*, and *Tnf*, were found to persist for more extended periods, at least until 12 weeks after teeth extractions. Enzymatic histochemistry revealed that the activity of osteoclasts persists in the maxilla of OVX mice after teeth extractions. Our results indicated that osteoclast activation by ovariectomy has a persistent and short-term effect on RRR.

We focused on *Sema4d*, which functions as an inhibitor of bone formation by suppressing osteoblast differentiation and modulating osteoblast mobility^{27, 28}. *Sema4d* derived from osteoclasts played a role as an inhibitor of bone formation, and mice with targeted deletion of gene encoding *Sema4d* or its receptor, *Plexin-B1*, had increased BV. We further demonstrated that administration of a neutralization antibody for *Sema4d* efficiently prevented femur bone loss in ovariectomized mice¹⁶. In our experimental

205 model, administration of Sema4d neutralization antibodies into OVX mice significantly
206 suppressed the loss of alveolar BV after teeth extractions. This result suggested that
207 Sema4d is a factor involved in the promotion of bone resorption after tooth extraction and
208 that inhibition of Sema4d may be a useful treatment for RRR. Consistent with our study,
209 a recent study reported the therapeutic effects of small interfering RNA for silencing
210 Sema4d mRNA, which increased the BV over the total volume of the mandibular bone
211 and prevented alveolar bone height loss in an osteoporotic model²⁹.

212 RANKL is a tumor necrosis factor (TNF) cytokine family and functions as a key
213 factor for osteoclast differentiation and activation. Human anti-RANKL neutralizing
214 antibodies are utilized for the treatment of osteoporosis and cancer-induced bone diseases.
215 Administration of RANKL antibodies into OVX mice also suppressed long-lasting
216 alveolar bone resorption after teeth extractions in our experimental model. It has been
217 pointed out that RANKL-neutralizing antibodies have the risk of causing antiresorptive
218 agent-related osteonecrosis of the jaw (ARONJ), which is local osteonecrosis of the
219 maxilla and mandible with no symptoms in other bones^{30, 31}. In our experimental model,
220 however, no significant signs of maxilla bone necrosis were found. This result indicated
221 that some additional factors, such as chronic inflammation caused by periodontal disease

or excessive stimulation from dentures, may be required for the onset of ARONJ in addition to the suppression of osteoclasts by inhibiting RANKL function.

With long-term observation of the maxillary bone in this study, we demonstrated that suppression of bone resorption by administration of anti-RANKL or anti-Sema4d antibodies could improve long-lasting alveolar bone resorption following teeth extractions. The mechanism underlying the development of RRR is still unclear. Our experimental model, therefore, will be a good tool for studying ridge resorption and developing therapeutic drugs.

In conclusion, 1) bone resorption after tooth extraction did not progress without a risk factor in our murine model; 2) postmenopausal osteoporosis delays the healing of extraction sockets and can be a risk factor for RRR; and 3) administration of anti-RANKL antibodies or anti-Sema4d antibodies may be a good therapeutic method to delay bone loss by RRR.

240 **Materials and methods**

241 **Animals**

242 BALB/cAJcl female mice were used in all experiments and were purchased from the
243 CLEA Japan, Inc. The mice were maintained in conventional conditions under standard
244 condition of 12/12 hours of light/dark cycle at temperature $25^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and 35% to 60%
245 humidity at the animal facility of Graduate School of Medicine, Hokkaido University.
246 One-week acclimation period was provided before the start of the experiment. All animal
247 experiments followed ARRIVE guidelines and approved by the animal care guidelines
248 for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of
249 Medicine, Japan (approval number: 150139).

250

251 **The teeth extractions of mice**

252 The left maxillary first molar (M1) and second molar (M2) were extracted from 7-week-
253 old mice under anesthesia *via* intraperitoneal injection of an anesthesia cocktail of 0.75
254 mg/kg medetomidine (Nippon Zenyaku Kogyo Co., Ltd.), 4 mg/kg midazolam (Maruishi
255 Pharmaceutical Co., Ltd.), and 5 mg/kg butorphanol (Meiji Seika Pharma Co., Ltd.;
256 supplementary Fig. S2a online). After the teeth extractions, the mice were awakened *via*
257 injection of 0.75 mg/kg atipamezole (Nippon Zenyaku Kogyo Co., Ltd.), which is an

antagonistic reagent for medetomidine. The group of mice without teeth extractions were used as an experimental control. Three independent experiments were performed using three mice in each experimental group. The heads of mice at day 0 to 24 weeks post-extraction were scanned using micro x-ray computed tomography (μ CT; Latheta LC-200, HITACHI, Japan) at 24- μ m voxel resolution with an energy level of 50 kV under anesthesia (supplementary Fig. S2b online). We weighed the mice daily to confirm that no significant weight loss occurred.

The μ CT images were imported into the ImageJ software and then processed into three dimensional images to measure the maxillary alveolar bone volume (BV) and CT value. The BVs were compared with the non-extracted sides. The measurement range of BV was between the M1 mesial buccal root and M2 distal buccal root (Fig. 1a). The CT value of the extraction socket of M1 was compared with the alveolar bone of the buccal side of the same side. The region of interest (ROI)-1 was placed in the maxilla alveolar bone of the buccal side, and ROI-2 was placed in the extraction socket (Fig. 1b). Mice were sacrificed at 24 weeks post-extraction.

Changes in the morphology of maxillary alveolar bones after teeth extractions in OVX mice

Five-week-old female mice were divided into two groups, sham-operated and ovariectomized (OVX), with each group consisting of three mice. Two independent experiments were performed. Ovariectomies were performed under anesthesia *via* intraperitoneal injection with a cocktail of 0.75 mg/kg medetomidine, 4 mg/kg midazolam, and 5 mg/kg butorphanol. Ovaries were removed using small bilateral dorsal flank incisions after the hair was shaved. Sham-operated mice received similar incisions under anesthesia without ovary removal and used as an experimental control. The maxillary molars (M1 and M2) of the sham mice and OVX mice were extracted at 2 weeks following surgery. The changes in the maxillary bone by μ CT were assessed, and the BV and CT values of the maxillary extraction sockets were measured. We monitored the weight of the mice daily to confirm that no significant weight loss occurred. Mice were sacrificed and maxillary bones were harvested at 16 weeks post-extraction.

Tissue preparation for enzymatic histochemistry of tartrate-resistant acid phosphatase (TRAP)

OVX and sham-operated mice were euthanized by intraperitoneal injection with an overdose of pentobarbital sodium 1 day, 1 week, 3 weeks, 5 weeks, and 12 weeks after tooth extraction. They were perfused with physiological saline through the heart, followed

294 by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The heads of the mice were
295 removed, immersed in the same fixative for 24 h, and decalcified with 5% EDTA for 4
296 weeks at 4°C. The decalcified tissues were dipped in 30% sucrose solution overnight at
297 4°C, embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in
298 liquid nitrogen. Frozen sections, about 16 µm in thickness, were mounted on MAS-coated
299 glass slides.

300 Histological sections were stained with TRAP Staining Kit in accordance with
301 the manufacturer's protocol (Wako, Japan). The nuclei were stained with hematoxylin.
302 The stained sections were observed and captured by a light microscope with digital
303 camera (BX51 with DP-80, Olympus, Tokyo, Japan). The ratio of the total area of TRAP-
304 positive cells to the surface area of the bone on the extracted side in both sham mice and
305 OVX mice was calculated using the ImageJ software.

306

307 **Quantitative PCR analysis**

308 For the RNA preparation from the maxillary bone, the tissues were snap-frozen in liquid
309 nitrogen and homogenized in liquid nitrogen using a mortar and pestle to powderize it.
310 TRIzol® Reagent (Life Technologies) was added to the homogenized tissues, and the total
311 RNA was purified with a high salt solution (Nippon Gene). First-strand cDNA synthesis

was completed using ReverTraAce[®] (TOYOBO). Quantitative PCR reactions were conducted using Rotor-Gene 6000 equipment (Qiagen) or StepOnePLUS[™] (Thermo Fisher Scientific K.K.) using KAPA SYBR[®] Green Fast PCR Kit (KAPA Biosystems). Most of the specific primers were designed by Primer Bank³² and are presented in Supplementary Table S online.

Administration of neutralized antibodies into OVX mice

The molars (M1 and M2) were extracted at 2 weeks after ovariectomy. Ovariectomized 7-week-old mice were divided into three groups (n = 3 per group). OVX mice were intraperitoneally administered PBS or 1 mg/kg anti-Sema4d antibody (clone BMA-12, BioLegend) once every 3 days for 8 weeks or were injected with 5 mg/kg anti-RANKL antibodies (clone OYC1, ORIENTAL YEAST CO., LTD.) only once on the next day following teeth extractions. The mice were subjected to μ CT analysis, and the BV and CT values were measured at 12 weeks following teeth extractions. Mice were sacrificed and maxillary bones were harvested at 12 weeks post-extraction.

Statistics

Statistical analyses in certain time point were conducted using Student's *t* test. Differences between groups in time-dependent changes were analyzed statistically using two-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni's multiple comparisons test by calculating with Prism software. The results were expressed as mean $\pm$ S.D.

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479

480 **Author Contributions**

481 M.H. and S.K. contributed to conception, design, data acquisition, analysis, and
482 interpretation, performed statistical analysis, drafted and critically revised the manuscript.
483 K.I. contributed to conception, data analysis, and critically revised the manuscript. T.I.
484 contributed to conception, data analysis, and interpretation, and critically revised the
485 manuscript. A.Y. contributed to conception and design, and interpretation of data, and
486 critically revised the manuscript.

487 All authors gave their final approve and agree to be accountable for all aspects of the
488 work.

489

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492

493 **Declarations**

494 **Ethical approval**

495 The experimental protocols used in the present study were approved by the animal care

496 guidelines for the Care and Use of Laboratory Animals in Hokkaido University Graduate

497 School of Medicine, Japan (approval number: 150139).

498 **Conflict of interest**

499 The authors have no conflicts of interest to declare that are relevant to the content of this

500 article.

501

Figure legends

Fig. 1

Resorption of the maxillary alveolar bone after teeth extractions in mice.

(a) Bone volumes of both the extracted and non-extracted sides were determined with μ CT analysis. The measurement range of bone volume is indicated in purple in the μ CT-3D image of the upper images, which is between the M1 mesial buccal root and M2 distal buccal root. M1, M2, and M3 denote the first molar, second molar, and third molar, respectively. The graph in the lower panel shows the time-dependent changes in the bone volumes of the teeth-extracted and the non-extracted sides. Data are expressed as mean $\pm$ S.D. ($n = 3$). The data shown in the graphs are representative of three independent experiments. (b) The selected regions of interest (ROIs) in or areas near the extraction socket of the first molar are displayed in the circled areas of the μ CT image in the upper panel. The graph in the lower panel shows the time-dependent changes in the ratio of the CT values of the maxilla alveolar bone of the extracted region (ROI-2) to the non-extracted buccal side (ROI-1). Each symbol represents an individual mouse. The data shown in the graphs are representative of three independent experiments. *** $P < 0.005$; calculated using two-way ANOVA

Fig. 2

Ovariectomy prolongs maxillary bone resorption after teeth extractions.

(a) Ventral views (upper images) and lateral views (lower images) of μ CT-3D images of the maxilla at day 0 (red) and 16 weeks (green) post-extraction of OVX mice are shown in the left and the middle images, respectively. The right image shows a superimposed illustration of day 0 and 16 weeks post-extraction. The arrows indicate decreased regions of the alveolar bone after 16 weeks post-extraction. (b) The graph shows the time-dependent changes in the bone volumes of the teeth-extracted and the non-extracted sides as described in Figure 1a. Data are expressed as mean $\pm$ S.D. ($n = 3$). (c) The graph shows the time-dependent changes in the ratio of the CT values of the maxilla alveolar bone of the extracted region (ROI-2) to the non-extracted buccal side (ROI-1), as described in Figure 1b. The data shown in the graphs are representative of two independent experiments. *** $P < 0.005$; calculated using two-way ANOVA

Fig. 3

Prolonged increase of osteoclasts in the maxillary bone of OVX mice.

(a) Enzymatic histochemistry staining for TRAP (red) on the decalcified maxillary alveolar bone at day 1, 3 weeks, and 12 weeks of sham or OVX mice following teeth

extractions. The arrows indicate TRAP-positive signals inside and around the extraction socket. The arrowheads indicate TRAP-positive signals on the buccal side of the maxillary alveolar bone. Bars: 100 μ m. (b) The graph shows the time-dependent changes in the ratio of the total area of TRAP-positive cells to the surface area of the maxilla alveolar bone of the extracted side in sham or OVX mice. The bone of the extracted side was around M2. Data are expressed as mean $\pm$ S.D. (sham mice: n = 4–5. OVX mice: n = 4–6), *** P < 0.005; calculated using two-way ANOVA followed by Bonferroni's multiple comparisons test

Fig. 4

Ovariectomy sustains prolonged high expression of several mRNAs encoding bone metabolism and proinflammatory cytokines after teeth extractions.

The expressions of mRNA encoding bone metabolism and proinflammatory cytokines in the maxillary alveolar bone were examined *via* quantitative PCR in both OVX mice and sham mice. The heatmap shows logFC normalized to the expression level of day 0 of the sham mice. Three mice were used in each group

Fig. 5

556 **Administration of neutralization antibodies for RANKL or Sema4d can prevent**
557 **prolonged bone resorption of the maxillary alveolar bone of OVX mice.**

558 The graphs show maxillary alveolar ridge volumes at 12 weeks after teeth extractions of
559 OVX mice administrated with anti-RANKL antibodies (a) or anti-Sema4d antibodies (b).

560 The bone volume was measured as described in Figure 1a. The data shown in the graphs
561 are representative of two independent experiments. * $P < 0.05$, ** $P < 0.01$ (calculated
562 using Student's *t*-test)

563

564

Figures

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