Effects of Usag-1 and Bmp7 deficiencies on murine tooth morphogenesis

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Abstract

Background: Wnt5a and Mfrzb1 genes are involved in the regulation of tooth size, and their expression levels are similar to that of Bmp7 during morphogenesis, including during the cap and early bell stages of tooth formation. We previously reported that Usag-1-deficient mice form supernumerary maxillary incisors. Thus, we hypothesized that Bmp7 and USAG-1 signaling molecules may play important roles in tooth morphogenesis. In this study, we established double genetically modified mice to examine the in vivo inter-relationships between Bmp7 and Usag-1.

Results: We measured the volume and cross-sectional areas of the mandibular incisors using micro-computed tomography (micro-CT) in adult Bmp7- and Usag-1-LacZ knock-in mice and their F2 generation upon interbreeding. The mandibular incisors of adult Bmp7+/− mice were significantly larger than those of wild-type (WT) mice. The mandibular incisors of adult Usag-1−/− mice were the largest of all genotypes examined. In the F2 generation, the effects of these genes were additive; Bmp7+/− was most strongly associated with the increase in tooth size using generalized linear models, and the total area of mandibular supernumerary incisors of Usag-1−/−/Bmp7+/− mice was significantly larger than that of Usag-1−/−/Bmp7+/+/ mice. At embryonic day 15 (E15), BrdU assays demonstrated that the labeling index of Bmp7+/− embryos was significantly higher than that of WT embryos in the cervical loop. Additionally, the labeling index of Usag-1−/− embryos was significantly the highest of all genotypes examined in dental papilla.

Conclusions: Bmp7 heterozygous mice exhibited significantly increased tooth sizes, suggesting that tooth size was controlled by specific gene expression. Our findings may be useful in applications of regenerative medicine and dentistry.

Keywords: Tooth size, Bmp7, Usag-1, Mouse model, Tooth volume, Tooth morphogenesis

Background

Development of the dentition is regulated by time- and position-specific reciprocal epithelial-mesenchymal interactions [1–4]. These odontogenic interactions are directed and coordinated by transcription factors, growth and signaling factors, their cognate receptors, and extracellular matrix constituents [5]. Collectively, various combinations of molecules within these interactions determine when or where teeth develop and modulate the specifications for tooth size and shape.

Noggin, Wnt5a, and Mfrzb1 genes have been shown to regulate tooth size [6–9]. Cai et al., using hetero-specific tissue recombination from rat molar tooth organs, demonstrated that tooth size is determined not by dental papilla mesenchymal cell number, but by intrinsic tissue-specific dental papilla mesenchymal factors [10]. In addition, exogenous treatment with bone morphogenic protein (BMP) 4, Noggin, fibroblast growth factor (FGF) 3, and FGF10 does not affect tooth size, despite the observation that BMP2/4, FGF3/10, LEF1, and WNT5a/5b are expressed in the dental mesenchyme during the cap and early bell stages of tooth morphogenesis [6, 11–15]. The crown width of a bioengineered molar reconstructed with dissociated epithelial and mesenchymal cells through the organ germ method is correlated with the length of the contact area between the epithelial and mesenchymal cell layers [16].
In conditional Bmp7-deficient embryos, the maxillary incisor tooth organs are either missing or hypoplastic, and the development of the first molar tooth organs is delayed, malformed, or missing [17]. Therefore, Bmp7 is assumed to be an essential growth factor for tooth morphogenesis. BMP7 is a 35-kDa homodimeric protein that is associated with a variety of signaling pathways, including the canonical SMAD pathway, mitogen-activated protein kinase (MAPK)-related pathways, and the phosphoinositol 3-kinase (PI3K)/Akt pathway [18–21]. Uterine sensitization-associated gene-1 (USAG-1; also known as sclerostin domain-containing protein 1 [SOSTDC1], ectodin, and Wise) is a heavily glycosylated 28–30 kDa secretory protein that functions as a monomer to induce signal transduction. USAG-1 binds to the Wnt coreceptors low-density lipoprotein receptor-related protein (LRP) 5 and LRP6 and inhibits Wnt signaling [22, 23]. LRP4 modulates and integrates BMP and canonical Wnt signaling during tooth morphogenesis by binding to secreted USAG-1 [24]. USAG-1 expression is further restricted to the distal renal tubules, in a pattern similar to the localization of BMP7. USAG-1 is a BMP antagonist; it interacts with BMP7 in the developing and adult kidney and directly binds to BMP2/4/7, as assayed using co-immunoprecipitation studies [25]. Moreover, we previously reported that Usag-1-deficient mice exhibit supernumerary maxillary incisors in response to enhanced BMP signaling and that BMP signaling is modulated by Wnt signaling in Usag-1-deficient mice [26, 27].

Based on these observations and findings, we hypothesized that BMP7 and USAG-1 play important roles in tooth morphogenesis. Therefore, we previously established double genetically modified mice to analyze the in vivo inter-relationships between BMP7 and USAG-1. Using this model, we demonstrated that USAG-1 inhibits BMP7 signaling, leading to apoptosis and degeneration of rudimentary tooth germs in maxillary supernumerary incisor formation [28]. Moreover, we found that the tooth size in mandibular incisors of Bmp7 heterozygous mice was altered. Accordingly, in this study, we examined the effects of BMP7 and USAG-1 signaling on tooth size in mandibular incisors using the F2 generation of mice.

Methods

Production and analysis of Usag-1- and Bmp7-LacZ knock-in mice

Bmp7-LacZ knock-in (ICR) mice and Usag1-LacZ/LacZ knock-in (C57BL/6) mice were produced as previously described [29, 30]. Bmp7-deficient mice were embryonic lethal. Day E0 was established as midnight prior to finding a vaginal plug.

Polymerase chain reaction (PCR) amplification was performed using KOD FX NEO polymerase (TOYOOBO, Osaka, Japan) and specific primers for genotyping.

X-gal staining

The bacterial LacZ (β-galactosidase) gene in Bmp7LacZ/LacZ (ICR) and Usag1LacZ/LacZ (C57BL/6) mice was knocked into the gene of interest [29, 30]. Frozen sections of embryos were fixed in 4% paraformaldehyde for 2 min and stained with X-Gal (Wako) twice as whole mounts and frozen sections, followed by counterstaining with Nuclear Fast Red (Kernechtrot). For X-gal staining, embryos were incubated at 37 °C in buffer (20 mg/mL X-gal/dimethylformamide, 35 mM K3Fe(CN)6, 35 mM K4Fe(CN)6, 2 mM MgCl2, 0.02 % NP-40, 0.01 % sodium deoxycholate, 1x phosphate-buffered saline [PBS]). We observed mandibular incisors in Bmp7+/– (ICR) and Usag-1+/– (C57BL/6) mice and mandibular molars in Usag-1+/– (C57BL/6) mice at E14 and E15.

Analysis of the adult tooth phenotype

Three-dimensional (3D) computed tomography (CT) scans (SMX-100XT-SV3; Shimadzu, Kyoto, Japan) were performed using the heads of adult mice. We converted CB files to TIFF files. CB files had 512 × 512 pixels, 8 bits, and a voxel size of x:y:z = 1:1:1 (approximately 0.06 mm per side). Next, 3D images were reconstructed and analyzed with computer imaging software (INTAGE Realia and Volume Player software; KGT Inc, Tokyo, Japan) [31]. The mandibular incisor volume was measured using the imaging software; then, to measure the cross-sectional area, mandibular incisors were cut vertically to the tooth axes at the uppermost point on the incisor alveolar rim (at the bone-tooth junction), so as to avoid the influence of environmental factors, such as dental occlusion and tooth attrition. The cross-sectional area of the incisors was measured using ImageJ software. We analyzed a total of 22 wild-type (WT) and 20 Bmp7+/– samples in adult Bmp7-LacZ knock-in (ICR) mice at 2 months after birth, a total of 21 individual genotypes in adult Usag1LacZ knock-in (C57BL/6) mice at 3 months after birth, and nine Usag-1+/+Bmp7+/+, 12 Usag-1+/-Bmp7/+, 18 Usag-1+/-−Bmp7/+, 14 Usag-1+/-−Bmp7−/−, 14 Usag-1−/−Bmp7−/−, 28 Usag-1−/−Bmp7−/−, and 17 Usag-1+/-Bmp7+/− adult F2 generation mice at 4 months after birth. Data for volume and cross-sectional area obtained from the right and left incisors were summed.

We measured the cross-sectional area of mandibular supernumerary incisors of three Usag-1−/−Bmp7+/+ and six Usag-1−/−Bmp7−/− adult F2 generation mice. We used two Usag-1−/−Bmp7+/+ and six Usag-1−/−Bmp7−/− mice at 1 month after birth and one Usag-1−/−Bmp7+/+ mouse at 4 months after birth. The total area was measured in cases of multiple supernumerary teeth.

To evaluate the effects of Bmp7 and Usag-1 on the total dentition, we also measured mandibular molars.
We obtained photographs of mandibular three molars of the F2 generation from directly above the occlusal view under a stereomicroscope and measured the projected area with ImageJ software using an image of a ruler taken at the same magnification. The distances between the lens and specimen were fixed. We examined unilateral mandibular molars from seven Usag-1/+∗Bmp7+/+, 16 Usag-1+/−Bmp7+/+, 16 Usag-1+/+Bmp7+−/−, and 31 Usag-1−/+Bmp7+/− adult F2 generation mice at 1 month after birth. Because molar form Usag-1−/− mice fused [24, 32, 33], we did not measure these molars.

**BrdU immunostaining**

Cell proliferation was detected by BrdU immunostaining (BrdU Solution and BD Pharmingen BrdU In-situ Detection Kit; BD Biosciences) according to the manufacturer’s specifications. We made a working solution of BrdU in PBS at 1 mg/mL and injected the mice intraperitoneally with 1 mL of the BrdU solution. After 2 h, the mice were sacrificed, and the heads of the embryos were removed and fixed in 4 % paraformaldehyde overnight. Paraﬁn sections (7 μm) were then created. Background tissue was stained with hematoxylin for 5 s. We counted approximately 500–1000 nuclei under a light microscope, and the labeling index was determined as (BrdU+ cells/total nuclei)/100.

**Detection of apoptosis**

Apoptotic cells in situ were detected by the TUNEL method using an apopTag Plus In Situ Apoptosis Detection Kit-Fluorescein (Chemicon International) according to the specifications of the manufacturer.

For paraffin-embedded sections, embryos were fixed in 4 % paraformaldehyde in PBS overnight. After fluorescent staining, the sections were counterstained with 4′,6-diamidino-2-phenylindole nuclear staining (Dapi Fluoromount-G; Southern Biotech).

**Statistical analysis**

Data are presented as the means ± standard deviations. Statistical significance was assessed by analysis of variance (ANOVA) with the statistical program R. Gaussian distributions were determined by the Anderson Darling normality test.

In adult Bmp7−/−LacZ knock-in mice, 22 WT samples and 20 Bmp7+/− samples collected from mice at 2 months after birth were analyzed. We calculated the mean volume and area of the right and left incisors. Statistical significance was determined by unpaired one-tailed t-tests for volume and by Mann–Whitney U tests for cross-sectional area.

In adult Usag1−/−LacZ knock-in mice, 21 samples of individual genotypes at 3 months after birth were analyzed as stated above. Statistical significance was determined using Kruskal–Wallis and Steel-Dwass tests for multiple comparisons in volume and by one-way ANOVA using a Games Howell test for multiple comparisons in cross-sectional area.

In the adult F2 generation, we examined nine Usag-1+/+Bmp7+/+, 12 Usag-1+/−Bmp7+/+, 18 Usag-1−/−Bmp7+/+, 14 Usag-1−/−Bmp7+/−, 28 Usag-1+/−Bmp7+/−, and 17 Usag-1+/+Bmp7+−/− mice, as mentioned above. Statistical significance was determined by one-way ANOVA using a Games Howell test for multiple comparisons.

In the analysis of mandibular molars in the adult F2 generation, statistical significance was determined by unpaired two-tailed t-tests (except for M1 and M2 in Usag-1−/− mice, for which Welch’s tests [M1] or Mann–Whitney U tests [M2] were used).

Finally, for analysis of cell proliferation using BrdU staining in the mandibular incisors in Bmp7−/−LacZ knock-in mice at E15 and analysis of mandibular supernumerary incisors in the adult F2 generation, statistical significance was determined using Mann–Whitney U tests. For analysis of cell proliferation using BrdU staining in the mandibular incisors in Usag1−/−LacZ knock-in mice at E15, statistical significance was determined using Kruskal–Wallis and Steel-Dwass tests for multiple comparisons.

**Results**

**Expression of Bmp7 and Usag-1 at E14 and E15**

Sections from Bmp7+/− mice indicated that Bmp7 was expressed (blue) in the mesenchyme around the tooth germ (Fig. 1a, d, g), rudimentary incisor (Fig. 1a, d), and enamel knot (Fig. 1b, e). Sections from Usag-1+/− mice indicated that Usag-1 was expressed (blue) in a small portion of the epithelia, excluding the enamel knot and mesenchyme around the tooth germ at E15, during the early bell stage (Fig. 1f, h) (Additional file 1: Figure S1B, C).

**Micro-CT analysis of the mandibular incisors in adult Bmp7-LacZ knock-in mice**

The volume and cross-sectional area of the mandibular incisors were larger in Bmp7+/− (ICR) mice than in WT mice (Fig. 2a). Volumes of Bmp7+/− mice were significantly larger than those of WT mice (Fig. 2e). The cross-sectional areas of Bmp7+/− mice were significantly larger than those of WT mice (Fig. 2f).

**Micro-CT analysis of the mandibular incisors in adult Usag1-LacZ knock-in mice**

The volume and cross-sectional area of the mandibular incisors in Usag-1LacZ knock-in (C57BL/6) mice were larger than those in any other diplotype (Fig. 2b). The volume and cross-sectional area in Usag-1+/− mice were significantly larger than those in all other diplotype (Fig. 2e, f). The volume and cross-sectional area in
Usag-1−/− mice were significantly smaller than those in WT mice (Fig. 2e, f).

Few mice exhibited malocclusion among Usag-1−/− mice. Mice that had malocclusion, taking into account feeding disorders and changes in tooth size, were excluded from the statistical analyses. Two of 23 Usag-1−/− mice were excluded (Additional file 2: Figure S2C).

Analysis of the projected areas of the occlusal surface of mandibular molars in adult F2 mice

The areas of the occlusal surface of the mandibular molars in Bmp7+/− mice were significantly larger than those in WT mice for both the three individual molars and the total (Fig. 3a, b). The areas of the occlusal surface of the mandibular molars in Usag-1+/− mice were significantly smaller than those in WT mice, except for M1 (Fig. 3a, c).

We used a generalized linear model (GLM) to examine the effects of respective genes and genotypes on tooth size. The model formula of the GLM was “Tooth Size = Usag-1 + Bmp7”. We did not consider interactions. From this analysis, we found that Bmp7+/− was associated with an increase in tooth size, whereas Usag-1+/− was associated with a decrease in area (Table 1). The error structure of the response variable followed a Gaussian distribution. The link function was a linear model. The explanatory variable was categorical data. We used genotypes as the categorical data.

Micro-CT analysis of the mandibular incisors in the adult F2 generation

The volume and cross-sectional area of the mandibular incisors of Usag-1−/−Bmp7+/− mice were larger than those of all other genotypes (Fig. 2c). This analysis clarified that the effects of each diplotype of Bmp7 and Usag-1 were additive for volume and cross-sectional area (Fig. 2g, h).

Next, a GLM was used, as described in the Methods. Usag-1−/− and Bmp-7+/− were associated with increased tooth size, whereas Usag-1+/− was associated with decreased tooth size (Table 2). This analysis confirmed that Bmp7+/− was most strongly associated with an increase in tooth size.

Two of 16 Usag-1−/−Bmp7+/− mice exhibited malocclusion was excluded (Additional file 2: Figure S2D).

Analysis of cell proliferation using BrdU staining of the mandibular incisors at E15

In murine incisors and molars, tooth eruption and tooth root formation are completed by about P20 or P21. The
cap stage and early bell stage are considered equal to the morphogenetic phase. During the early bell stage, only the labial epithelium gives rise to the enamel-forming ameloblasts in mandibular incisors [34]. Murine incisors are continuously growing; however, Bmp7 and Usag-1 are not expressed in enamel epithelial stem cells in adult mice [35].

Therefore, we investigated cell proliferation by BrdU staining in the mandibular incisors at E15, during the early bell stage. The tissues of the mandibular incisors at the early bell stage were categorized as vestibular lamina, stellate reticulum, dental papilla, labial mesenchyme, lingual mesenchyme, labial cervical loop, and lingual cervical loop (Fig. 4a, b). The labeling index of Bmp7+/− embryos was significantly higher than that of WT embryos in the cervical loop (Fig. 4c–f, j). Additionally, the labeling index of Usag-1−/− embryos was significantly higher than that of WT and Usag-1+/− embryos in dental papilla (Fig. 4g–i, k). The labeling index of WT embryos was significantly higher than that of Usag-1+/− embryos in stellate reticulum (Fig. 4k).

![Fig. 2 Analysis of the adult incisor phenotype.](image-url)

**Fig. 2** Analysis of the adult incisor phenotype. a Micro-CT analysis of mandibular incisors at 2 months after birth. Left to right: image from a stereomicroscope, three-dimensional image, and cross-section. b Micro-CT analysis of mandibular incisors at 3 months after birth. Left to right: image from a stereomicroscope, three-dimensional image, and cross-section. c Left to right: image from a stereomicroscope, three-dimensional image, and cross-section. d Upper images are from a stereomicroscope. Left image is the lingual side. Right image is the buccal side. Lower images show the cross-sectional area of mandibular supernumerary teeth (arrowheads) and mandibular incisors. a–d Scale bars: 1 mm. e, f Volume and cross-sectional area of adult mandibular incisors. Numbers indicate respective incisors. Data in (e) are the mean volume ± standard deviation (SD) of mandibular incisors at 2 months (left) or 3 months (right) after birth. Data in (f) are the mean cross-sectional area ± SD of mandibular incisors at 2 months (left) or 3 months (right) after birth. g, h Volume and cross-sectional area of mandibular incisors in the adult F2 generation at 4 months after birth. Data in (g) are the mean volume ± SD of mandibular incisors. Data in (h) are the mean cross section ± SD of mandibular incisors. i Statistical analysis of cross-sectional area of mandibular supernumerary incisors in the adult F2 generation at 1 month. Error bars: SDs. j Images of trimmed and extracted mandibular incisors in a, b, and c. Whole computed tomography images of a mouse skull bone and an incisor with soft tissue removed using INTAGE Realia are shown (a, b, and c). We opened the image in Volume Player software (KGT Inc., Tokyo, Japan) (d), created .bmp files, and opened the files in Image J software (e). *P < 0.05, **P < 0.01, ***P < 0.001
TUNEL staining in the mandibular incisors at E15

Next, we investigated the occurrence of apoptosis in cells in the mandibular incisors of Bmp7+/− (ICR), Usag-1−/− (C57BL/6), Usag-1+/−Bmp7+/- (F2 generation), and Usag-1+/− (C57BL/6) embryos at E15 (Fig. 4l). Apoptotic cells were detected near Meckel’s cartilage, the developing bone and cartilage, the epithelium near the oral cavity, and the mesenchyme near the tooth germ (Fig. 4l). Few apoptotic cells were observed in the tooth germ for all genotypes. In the early bell stage, apoptosis was not associated with the determination of tooth size.

Analyzing mandibular supernumerary incisors in the adult F2 generation

In the F2 generation, the size of supernumerary incisors of Usag-1−/−Bmp7+/- mice was larger than that of Usag-1−/−Bmp7+/+ mice (Fig. 2d). Moreover, the total area of mandibular supernumerary incisors of Usag-1−/−Bmp7+/- mice was significantly larger than that of Usag-1−/−Bmp7+/+ mice (Fig. 2l).

Discussion

The expression levels of Usag-1 and Bmp7 are opposing in the region near the rudimentary incisor tooth primordia between the maxilla and mandible [28]. Moreover, in several types of genetically modified mice, the dental phenotype differs between the maxilla and mandible [13, 36, 37]. For example, in Usag-1-deficient mice, supernumerary teeth are observed in 100 % of the maxillary incisor regions, whereas partial penetrance is observed in the mandible [26]. Thus, the genetic controls for tooth organ size and shape play critical roles in

| Table 1 | A generalized linear model to analyze adult tooth size. The response variable is the total area of the occlusal surface of the molars of the F2 generation at 1 month after birth. |
|----------|----------------------------------------------------------------------------------|
| estimate | std.error | t value | p value |
| Usag-1+/− | −0.05311 | 0.02503 | −2.122 | 0.03755 |
| Bmp7+/− | 0.09814 | 0.02503 | 3.921 | 0.00021 |
### Table 2
A generalized linear model to analyze adult tooth size. The response variables are volume and cross-section of the mandibular incisors of the F<sup>2</sup> generation at 4 months after birth.

|                | Volume       | Area        |
|----------------|--------------|-------------|
|                | Estimate     | Std.Error   | T value | P value  | Estimate     | Std.Error   | T value | P value  |
| Usag-1+/−      | −0.08545     | 0.11291     | −0.757  | 0.45107  | −0.012964   | 0.008759   | −1.480  | 0.142169 |
| Usag-1−/−      | 0.20793      | 0.12003     | 1.732   | 0.08651  | 0.019976    | 0.009311   | 2.145   | 0.034498 |
| Bmp7+/−        | 0.29578      | 0.09514     | 3.109   | 0.00249  | 0.027771    | 0.007380   | 3.763   | 0.000292 |

**Fig. 4** BrdU immunostaining and detection of apoptosis in mandibular incisors at E15. **a-j** Analysis of proliferation using BrdU staining in mandibular incisors at E15. **a** Sagittal section of mandibular incisors (200x magnification) of WT mice. **b** Schematic diagram. VL: vestibular lamina, SR: stellate reticulum, DP: dental papilla, LaM: labial mesenchyme, LiM: lingual mesenchyme, LaC: labial cervical loop, LiC: lingual cervical loop. **c-f** Sagittal section of cervical loop (1000x magnification). **g-i** Sagittal section of dental papilla (1000x magnification). **j** Analysis of cell proliferation using BrdU staining in mandibular incisors of WT and Bmp7+/− (ICR) mice at E15. Percent labeling index in respective tissues of WT and Bmp7+/− E15 embryos are shown. Labeled cells were counted under 1000x magnification. We counted approximately 500 nuclei under a light microscope and determined the labeling index as: (BrdU+ cells / total nuclei) / 100. Data are the mean ± standard deviation (SD). ns: not significant. *P*<0.05 by Mann–Whitney U test. **k** Analysis of cell proliferation using BrdU staining in mandibular incisors of Usag-1+/+, Usag-1+/− and Usag-1−/− (C57BL/6) mice at E15. Percent labeling index of mandibular incisors of Usag-1+/+, Usag-1+/− and Usag-1−/− embryos at E15. Data are the mean ± SD. ns: not significant. *P*<0.05 by Steel Dwass test. **l** TUNEL staining in mandibular incisors of Bmp7+/− (ICR), Usag-1+/− (C57BL/6), Usag−−/−Bmp7+/− (F<sub>2</sub>), and Usag−−/− (C57BL/6) mice at E15. Under 100x magnification, the developing bone and cartilage (white arrow) are shown. Under 200x magnification, Meckel’s cartilage (white arrowhead), epithelium near the oral cavity (green arrowheads), and the mesenchyme near the tooth germ (red arrowheads) are shown. All bars: 100 μm.
tooth regeneration. In this report, we demonstrated that the sizes of mandibular incisor and molar tooth organs in Bmp7+/− mice were significantly larger than those of WT mice. In contrast, the sizes of incisors and molars were significantly smaller in Usag-1+/− mice than in WT mice, and the volume and cross-sectional areas in Usag-1−/− mice were significantly larger than those of all other genotypes. Thus, our results demonstrated, for the first time, that Bmp7 and Usag-1 heterozygous mice exhibited changes in tooth size.

In conditional Bmp7-deficient embryos, the maxillary incisors have been shown to be missing or hypoplastic [17]. Thus, the phenotypes of Usag-1 heterozygous mice and Bmp7 heterozygous mice were opposite those of Usag-1-deficient mice and Bmp7-deficient mice, respectively. Our results suggested that the levels of Bmp7 and Usag-1 expression in heterozygotes did not reach the threshold level necessary for normal tooth morphogenesis, that is, the embryos exhibited haploinsufficiency. Furthermore, heterozygotes may exhibit changes in downstream gene expression, resulting in changes in tooth size. Global analysis of gene expression in heterozygotes, such as microarray analysis and next-generation sequencing, is needed in order to elucidate the molecular mechanism involved in these processes.

The expression level of Bmp7 was similar to those of Wnt5a and Mefzb1 and was localized adjacent to Usag-1 expression in the dental mesenchyme. USAG-1 is a BMP7 antagonist; however, the effects of BMP7 and USAG-1 were additive within the mandibular incisors of the F2 generation. BrdU assays confirmed that cell proliferation was increased within the cervical loop in association with larger sized mandibular incisors in Bmp7+/− embryos. Furthermore, we confirmed that the increased cell proliferation of dental papilla mesenchymal cells was associated with larger mandibular incisors in Usag-1−/− embryos. Importantly, apoptosis was not associated with tooth size at E15. These data indicated that there was a difference in the mechanisms of action of BMP7 and USAG-1 during the cap or bell stages of tooth morphogenesis. Continuous growth and enamel deposition in incisors can be modulated by the levels of FGF3/10, activin, and BMP2/4/7 mesenchymally expressed in the epithelial stem cell niche [38]. Thus, we concluded that elongation of the cervical loop in Bmp7+/− embryos at E15 enlarged the incisors and that BMP7 expressed in the mesenchyme around the cervical loop had distinct local effects on the loop.

The volume and cross-sectional area were largest in Usag-1−/− mice, and the labeling index of the dental papilla in Usag-1−/− mice was highest of all genotypes examined. We have also reported that phosphorylated SMAD1/5/8 levels are increased and that β-catenin is localized in the nucleus in odontogenic mesenchymal cells within the maxillary rudimentary incisor tooth organ in Usag-1-deficient embryos [27]. Using organ culture of WT and Usag-1-deficient mandibular incisors, Munne et al. demonstrated that Usag-1 expression is limited to the mesenchyme and that the dental mesenchyme may limit supernumerary tooth induction resulting from activated Wnt signaling, thereby minimizing the amount of mesenchymal tissue surrounding the incisor tooth germs prior to culture [39]. USAG-1 is downstream of sonic hedgehog (Shh) signaling; therefore, a Wnt-Shh-SOSTDC1 negative feedback loop may control the spatial patterning of teeth, and Wnt, Shh, and SOSTDC1 may act as the activator, mediator, and inhibitor, respectively, in reaction–diffusion models [32]. Consistent with this, patients with supernumerary teeth have larger teeth than controls in humans [40]. Thus, these findings, combined with our new results, suggest that enhanced Bmp- and Wnt-mediated signal transduction in the dental mesenchyme of Usag-1−/− mice may increase the proliferation of cells in the dental mesenchyme at the cap or early bell stages, resulting in increased tooth size and formation of supernumerary teeth.

The size of a tooth is determined not only by genes related to tooth development but also by the development of the mandible. The size of mandibular incisors is altered by craniosynostosis in the Apert Fgr2S252W mouse model [41]. Importantly, in our study, we found no significant difference in the linear distance of the anteroposterior diameter and altitude between respective genotypes in Bmp7-LacZ knock-in mice at 2 months of age (Additional file 3: Figure S3B). However, at 3 months of age, Usag-1−/− mice exhibited a significantly longer linear distance of the anteroposterior diameter and altitude compared with that in WT and Usag-1+/− mice (Additional file 3: Figure S3C). The development of the mandible in the context of Usag-1 deficiency may be associated with changes in the size of the mandibular incisor and molar in Usag-1−/− mice. These results suggested that we may be able to control the size of teeth by regulating the gene expression level locally in humans when dental epithelial stem cells, such as third dentitions and outer enamel epithelium, are used for tooth regeneration in vivo.

**Conclusion**

Our findings showed that Bmp7 heterozygous mice exhibited dramatic increases in tooth size and that tooth size was controlled by the expression levels of specific genes.

**Ethics approval and consent to participate**

The study protocol and procedures were approved by the Animal Research Committee of Kyoto University (reference number: Med Kyo 11518) and the Recombinant
DNA Experiment Safety Committee of Kyoto University. All experiments were carried out in accordance with the approved guidelines.

Consent for publication
Not applicable.

Additional files

Additional file 1: Figure S1. X-gal staining in mandibular molars of Usag-1+/− (C57BL/6) mice at E14 and E15. Tissue sections from mandibular molars of Usag-1+/− (C57BL/6) mice at E14 and E15 were stained with X-gal. Scale bar: 100 μm. (A, B) Sagittal sections. (C) Coronial sections. (B, C) Usag-1 was expressed (blue) in a small portion of epithelia, except for the enamel knot and the mesenchyme near the tooth germ. (TIF 16106 kb)

Additional file 2: Figure S2. Malocclusion observed in Usag-1−/− (C57BL/6) mice and Usag-1+/− Bmp7+/− (F2 generation) mice. (A) Normal occlusion. A Usag-1−/− female mouse in the C57BL/6 background at 3 months after birth. These data were added in the analysis of the lower incisors. Scale bar: 1 mm. (B) Normal occlusion. A Usag-1−/− Bmp7+/− male mouse in the F2 generation at 4 months after birth. These data were added in the analysis of the lower incisors. (C) Malocclusion. A Usag-1+/− female mouse in C57BL/6 background at 3 months after birth. The mouse was excluded from the analysis of the lower incisors. (D) Malocclusion. A Usag-1−/− Bmp7+/− female mouse in the F2 generation at 4 months after birth. The mouse was excluded from the analysis of the lower incisors. (TIF 14671 kb)

Additional file 3: Figure S3. Difference in mandibular morphology between respective genotypes in Bmp7−/− or Usag-1 LacZ knock-in mice. (A) Wild-type mouse (ICR) mandible at 2 months after birth with locations of landmarks used to analyze the morphological differences between respective genotypes. Linear distances between the identifiable landmarks were measured. a: inferior-most point on the incisor alveolar rim, b: anterior point on the molar alveolar rim, c: inferior-most point on border of the ramus inferior to incisor alveolar, d: mandibular angle. (B) Difference in mandibular morphology between WT and Bmp7−/− mice. We analyzed a total of 6 WT and 6 Bmp7−/− samples in adult Bmp7−/− LacZ knock-in (ICR) mice at 2 months after birth. Statistical significance was determined by the Mann–Whitney U test. (C) Difference in the mandibular morphology among WT, Usag-1+/−, and Usag-1−/− mice. We analyzed a total of 6 individual genotypes in adult Usag-1 LacZ knock-in (C57BL/6) mice at 3 months after birth. Statistical significance was determined using a Kruskal-Wallis test and a Steel-Dwass test for multiple comparisons. *P < 0.05. (TIF 3550 kb)

Abbreviations
−/−: Knockout genotype; +/-: Heterozygous genotype; +/-: Wild-type; LacZ/LacZ: Knockout genotype in LacZ knock-in mice; WT: Wild-type.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
KS, KT, KB, MA, and MM contributed to the conception and design of the experiment. KS, HK, YT, and HT contributed to acquisition of the data. MA carried out measurement and analysis of the molars. KS drafted the manuscript. KT, MA, BH, HCS, MS, and AS revised the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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