BMP-2 Regulates the Formation of Oral Sulcus in Mouse Tongue by Altering the Balance Between TIMP-1 and MMP-13

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BMP-2 Regulates the Formation of Oral Sulcus in Mouse Tongue by Altering the Balance Between TIMP-1 and MMP-13

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ABSTRACT

The aim of this study is to investigate whether BMP-2 regulates the oral sulcus formation of mouse embryonic tongue by modifying the expression of TIMP and MMP. The BMP-2 siRNA induced a 180% increase in the depth of oral sulcus cavity ($P < 0.01$) by stimulating the invagination of oral sulcus into the mesenchymal tissues consisting of tongue floor, whereas the recombinant BMP-2 suppressed the process in the organ culture system of mouse embryonic tongue. The BMP-2 siRNA induced a 60% decrease in the expression of TIMP-1 mRNA ($P < 0.05$) and a drastic decline in TIMP-1 protein was observed around the oral sulcus in the BMP-2 siRNA treated mandibles. The recombinant BMP-2 induced a 220% increases in the expression of TIMP-1 mRNA and the area of the immunostaining for TIMP-1 around the oral sulcus was larger in the mandibles treated with the recombinant BMP-2 than the vehicle. The BMP-2 siRNA induced a 60% increase in the expression of MMP-13 protein and a marked increase in the staining intensity for MMP-13 was observed in the epithelial region of the BMP-2 siRNA treated mandibles. The recombinant BMP-2 induced a 70% decrease in the expression of MMP-13 mRNA and the decrease was mainly observed in the tissues around oral sulcus. The expressions of BMP-2, TIMP-1, and MMP-13 were verified in the tissues around *in vivo* developing oral sulcus at E11, 12, and 13 by immunohistochemistry. These results suggest that BMP-2 regulates the formation of oral sulcus by altering the balance between TIMP-1 and MMP-13. Anat Rec, 293:1408–1415, 2010.

Key words: BMP-2; TIMP-1; MMP-13; oral sulcus; tongue; mouse

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGFβ) super family, and comprise a highly conserved and expanding family of 15 genes critical to various developmental events, such as the formation of bone, cartilage, and teeth (Cobourne and Sharpe, 2003; Chen et al., 2004; Wan and Cao, 2005). BMP signals are mediated through serine/threonine kinase receptors, which are classified into Types I and II. BMP ligands initiate signaling by first directly binding Type II receptors, which leads to the recruitment of appropriate Type I receptors (Massague and Chen, 2000; Chen et al., 2004; Miyazono et al., 2005). Both BMPs and their receptors are expressed in the developing mouse tongue (Suga et al., 2007a,b).

The matrix metalloproteinases comprise a family of at least 28 zinc-dependent endopeptidase collectively capable of processing and degrading various extracellular matrix. MMP-13 (collagenase-3) is the third member of the collagenase with distinct properties compared with the other collagenases MMP-1 (interstitial collagenase) and MMP-3 (neutrophil collagenase) and it has key roles in modulating extracellular matrix degradation through its direct matrix degradation and activation of other MMPs (Leeman et al., 2002). Tissue inhibitors of metalloproteinases (TIMPs) are the major cellular inhibitors of the MMPs, and four subtypes of TIMPs have been cloned, purified, and characterized. They are implicated in various kinds of biological events such as cell proliferation, apoptosis, and angiogenesis, by modifying the activity of MMPs (Baker et al., 2002). Both TIMPs and MMPs play a role in the regulation of morphogenesis of mouse embryonic tongue (Chin and Werb, 1997), and BMP-2 alters the expression of TIMP-1, 3 and MMP-13 in bone and cartilage cells (Varghese and Canalis, 1997; Frenkel et al., 2000).

In mouse, the tongue bud originates as two lateral swellings along the midsection of the first branchial arch at around embryonic day (E) E11. The two swellings of tongue bud grow larger and fuse at around E12. The epithelium at the outer edges of tongue bud proliferates and invaginates into the mesenchymal tissues consisting of tongue floor, forming a groove (oral sulcus) at E13 that frees the tongue from the floor of the mouth (Kaufman, 1992). Since the tongue can obtain the ability of free movements in the three-dimension by the formation of oral sulcus, the formation of oral sulcus is an essential morphogenetic process for the function of tongue. It is reported that an inhibitor of MMP suppresses the formation of oral sulcus, suggesting that MMPs is involved in the formation of oral sulcus (Chin and Werb, 1997).

On the basis of these findings, we hypothesized that BMP-2 regulates the morphogenesis of mouse embryonic tongue, especially the formation of oral sulcus, by modifying the expressions of TIMP-1–4 and MMP-13. Since the null mutation in the BMP-2 gene leads to embryonic lethality between E7.5 and E9.0 (Zhang and Bradley, 1996) and the development of mouse tongue including oral sulcus initiates after E11 (Kaufman, 1992), we are not able to study the development of tongue using BMP-2 null mutation mice model. In this study, thus, to test the above hypothesis, we used an organ culture system of the mandibles and, furthermore, to verify the results of organ culture system, we analyzed the immunolocalization of TIMP-1 and MMP-13 during the normal development of oral sulcus *in vivo*.

MATERIALS AND METHODS

Animals

Pregnant ICR mice were purchased from Nippon Clea (Tokyo, Japan) and killed by cervical dislocation at E10, E11, E12, and E13. Embryos were isolated from uterine deciduas and were removed from their membranes under a dissection microscope. The first branchial arches of the E10 embryos were used for organ culture. The mandibles of E11–E13 embryos were carefully dissected out and were fixed in 4% paraformaldehyde fixative for immunohistochemical analysis.

Organ Culture

The explants obtained from E10 embryos were supported by membrane filters having a 0.8-μm pore size (type AABP, Millipore, Bedford, MA) on steel rafts and were cultured in BGJb medium (Life Technologies, Rockville, MD). Cultures were maintained for 8 days at 37°C in an atmosphere of 5% carbon dioxide and 95% air with medium changes every 2 days. A cocktail comprising four kinds of BMP-2 siRNA (GAACACAGCUGGUCACA GAUU, GCAGCCAACUUGAAAUUUCUU, GCAAGAGA CACCCUUUGUAUU, and CCACAGAGCUCAGCGCAA UUU) and nontarget control RNA (NTC) was purchased from GE Healthcare UK (Buckinghamshire, England), was mixed with a cationic agent, oligofectamine (Invitrogen, Carlsbad, CA), and was supplemented to the culture BGJb medium at a final concentration of 250 nM. Human recombinant BMP-2 (R&D Systems, Minneapolis, MN) in the vehicle (4 mM HCl, 0.15% bovine serum albumin) or the vehicle only was supplemented to the culture BGJb medium at a final concentration of 4 μg/mL of human recombinant BMP-2. Experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine.

Analysis of Oral Sulcus in the Organ Culture

Six mandibles in BMP2 siRNA or NTC group each were cultured in one dish each and, after 8 days in the culture, the explants were fixed in 4% paraformaldehyde fixative. Serial transverse sections at around the middle portions of tongues on the mandibular explants were prepared at a 10-μm thickness with a cryostat. The serial sections were stained with hematoxylin and eosin, and visualized with microscope (PCM2000, Nikon, Tokyo, Japan). The visualized image was taken by a digital camera (AxioCam, Carl Zeiss Japan, Tokyo, Japan), imported into a personal computer and printed out pictures. The depth of oral sulcus cavity was measured on the pictures in the right and left sides of tongue (Fig. 2A) and the values of the right and left sides were averaged to obtain the mean value for each cultured mandible. The depth of oral sulcus was analyzed at around same position along the longitudinal axis of all tongues. We did not measure the depth of oral sulcus in the mandibles treated with the recombinant BMP-2, because it was not able to determine the reference mark point due to the marked suppression of oral sulcus formation by the recombinant BMP-2.

Immunohistochemistry

Immunofluorescent and immunoenzyme stainings were performed as previously described (Yamane et al., 2000b; Urushiyama et al., 2004). The rabbit and goat antibodies against TIMP-1 and BMP-2, and the goat antibody against MMP-13 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon International (Temecula, CA), respectively. For immunofluorescent staining, FITC-, TRITC-, or Cy3-conjugated secondary donkey antibodies against goat and rabbit IgGs were purchased from Jackson ImmunoResearch (West Grove, PA), respectively. For immunoenzyme staining, Vector Elite Immunodetection Kit (Vector Laboratories, Burlingame, CA) was used. For control staining, the primary antibodies were replaced with PBS or heat-denatured primary antibodies.

Quantitative RT-PCR

Total RNA extraction, treatment with deoxyribonuclease I, and reverse transcription were performed as previously described (Ohnuki et al., 2000; Yamane et al., 2000a). Briefly, total RNA was isolated from individual mandible samples according to the manufacturer's specifications (Trizol, Life Technologies, Gaithersburg, MD). The isolated total RNA was treated with deoxyribonuclease I, then the RNA (1.5 μg) was reverse-transcribed to cDNA.

SYBR Green real-time PCR was performed on the ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA) using the following cycle parameters: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec for denaturation and 55°C for 15 sec for annealing and extension. PCR amplification was performed using SYBR Green qPCR Super Mix for ABI PRISM (Applied Biosystems, Foster City, CA). The mRNA quantities of TIMP-1, 2, 3, 4, MMP-13, BMP-2, and GAPDH, a house keeping gene, were calculated using a standard curve of the known concentrations of cDNA of each gene. The quantity of each target mRNA was normalized by the quantity of GAPDH mRNA. The sequences of primers for TIMPs and MMP-13 were as follows: TIMP-1, FW: 5′-ACC ACC TTA TAC CAG CGT TA-3′ and BW: 5′-ACC ACC TTA TAC CAG CGT TA-3′; TIMP-2, FW: 5′-GGT CTC GCT GGA CAT TGG AGG AA AG-3′ and BW: 5′-GGT CTC GCT GGA CGT TGG AGG AA AG-3′; TIMP-3, FW: 5′-TGC AAG ATC AAG TCC TGC T-3′ and BW: 5′-GGT GAG GTG GGG CAG GTC T-3′; TIMP-4; FW: 5′-ATC TGT TTG ATT TCA TAC CGG-3′ and BW: 5′-CAC CCC CAG CAG CAC TTC TG-3′; MMP-13, FW: 5′-CAT TCA GCT ATC CTG GCC ACC TTC-3′ and BW: 5′-AAA GAT TGC ATT TCT CGG AGC CTG-3′. The sequences of primers for BMP-2 and GAPDH were described before (Ohnuki et al., 2000; Suga et al., 2007a).

Western Blot Analysis

The tissues of mandibles were homogenized in 2% SDS, 62.5 mM Tris-HCl (pH 6.8) and 10% glycerol. The homogenate was centrifuged at 8,000 rpm for 15 min and the supernatant was stored at −20°C until use. The protein concentration of the each supernatant was measured using the BCM protein assay (Pierce, Rockford, IL). After β-mercaptoethanol was added to the supernatant (final concentration, 5%), the supernatant was heated at 100°C for 5 min. The samples containing 10-μg total protein were subjected to 5–20% gradient SDS-PAGE. After electrophoresis, the proteins were transferred onto a PVDF membrane (Hybond-P PVDF Membrane, Amersham Biosciences, Piscataway, NJ). The membranes were then treated with Casein solution (Vector Laboratories, Burlingame, CA) for 3 hr at 25°C, and incubated overnight at 4°C with the primary antibody, goat polyclonal antibody against BMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or MMP-13 (Chemicon International, Temecula, CA). Immunoreactions were made visible using the Vectastain Elite ABC Kit and 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories, Burlingame, CA) and images were acquired through a digital camera (FinePix, Fujifilm, Tokyo, Japan) and loaded into a personal computer. The intensity in the bands was measured using Densitograph (ATTO, Tokyo, Japan). βIII-tubulin was used as a loading control to confirm that the same amount of protein was loaded to each lane of SDS-PAGE.

Statistical Analyses

Tukey's method or Mann-Whitney U test was used to compare the mean values between two groups.

RESULTS

To evaluate the toxic effects of siRNA on the cultured mandible, we observed the gross morphology of E10 mandibles cultured for 8 days in the BGJb medium containing NTC and BMP-2 siRNA (Fig. 1A). No significant difference in the shape and size of the cultured mandibles was observed between the NTC and BMP-2 siRNA treated mandibles. To estimate the whole tissue volume of the cultured mandibles, the quantity of GAPDH, a house keeping gene, mRNA was measured (Fig. 1B). No marked difference in the quantity of GAPDH among BGJb, NTC, and BMP-2 siRNA was found, suggesting that the siRNA had no toxic effect on the cultured mandible. Although the treatment with BMP-2 siRNA induced 37% and 35% suppressions in the mRNA (Fig. 1C) and protein (Fig. 1D) of BMP-2 in the whole cultured mandibles, respectively ($P < 0.05$), the marked decrease was observed in the epithelial region of BMP-2 siRNA-treated mandible where the oral sulcus was formed (white arrows in Fig. 1F).

To evaluate the formation of oral sulcus, we observed hematoxylin and eosin staining images of E10 mandibles cultured for 8 days in the BGJb medium containing NTC, BMP-2 siRNA, vehicle or recombinant BMP-2 and measured the depth of oral sulcus cavity (Fig. 2). The treatment of BMP-2 siRNA induced ∼2.8-fold increase in the depth of oral sulucs cavity ($P < 0.01$) (Fig. 2B). On the other hand, since the depth of oral sulcus was very small in the mandible treated with 4 mg/mL of recombinant BMP-2 (arrow in the right picture of Fig. 2C), we were not able to measure the depth.

To determine whether BMP-2 regulates the formation of the oral sulcus cavity by altering the expression of TIMPs, we analyzed the mRNA expression levels of TIMP-1–4 in the mandible treated with NTC and BMP-2 siRNA (Fig. 3A–D). The mRNA expression of only TIMP-1 in the BMP-2 siRNA treated mandibles was ∼60% less

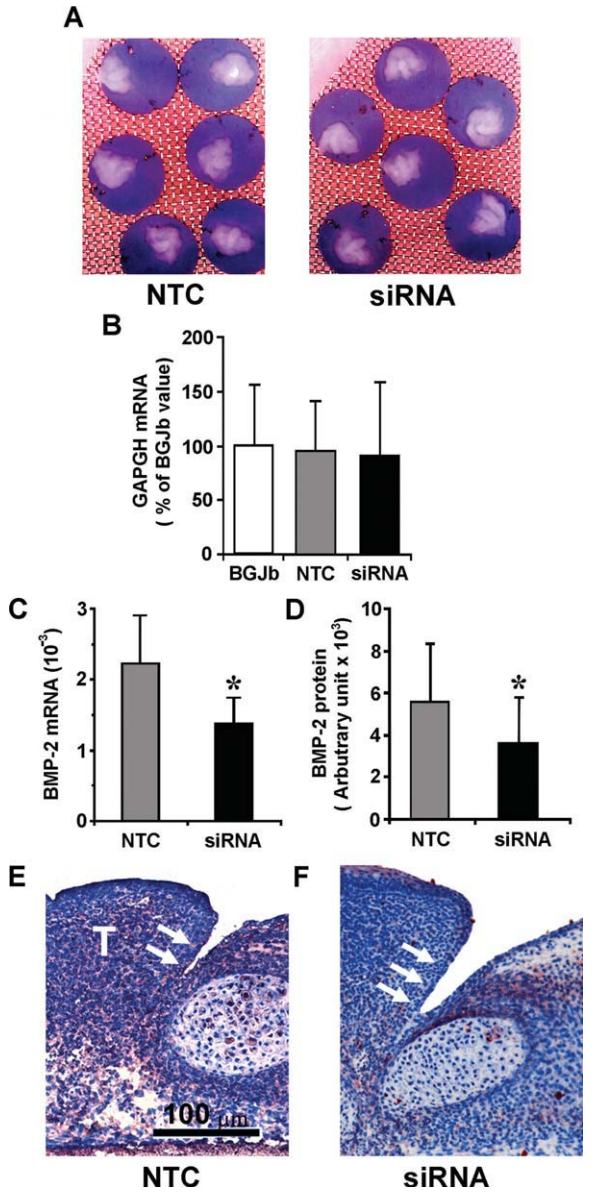

Fig. 1. Gross morphology of E10 mandibles cultured for 8 days in BGJb medium containing NTC and BMP-2 siRNA (**A**). Expression levels of GAPDH (**B**) and BMP-2 (**C**) mRNAs, and BMP-2 (**D**) protein in whole E10 mandibles cultured for 8 days. Significant difference between the NTC and BMP-2 siRNA mandibles, $^{*}P < 0.05$. Each column and vertical bar represent the mean + one SD of six cultured mandibles. Immunostaining images for BMP-2 in the epithelial region of E10 mandibles cultured for 8 days in BGJb medium containing NTC (**E**) and BMP-2 siRNA (**F**). T in (E) and white arrows in (E) and (F) indicate tongue and oral sulcus, respectively. The same magnification was used for (E) and (F). Although the treatment with BMP-2 siRNA induced ~40% suppression in the mRNA and protein expressions of BMP-2 in the whole cultured mandible, a marked decrease was found in the epithelial region.

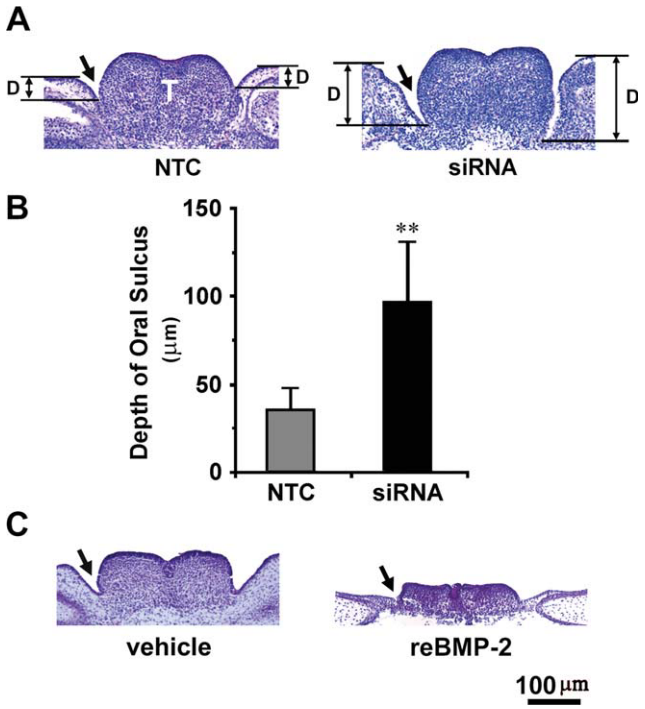

Fig. 2. Hematoxylin and eosin staining images of the transverse sections of the middle portion of tongues of E10 mandibles cultured for 8 days in BGJb medium containing NTC and BMP-2 siRNA (**A**), and vehicle and recombinant BMP-2 (**C**). T in (A) indicates the tongue. D in (A) was measured as an indicator of the depth of oral sulcus cavity. (**B**) The depth of the oral sulcus cavity. Significant difference between the NTC and BMP-2 siRNA mandibles, $^{**}P < 0.01$. Each column and vertical bar represent the mean + one SD of six cultured mandibles. The treatment of BMP-2 siRNA induced ~2.8-fold increase in the depth of oral sulcus, whereas the depth of oral sulcus was very small and not measurable in the mandible treated with 4 mg/mL of recombinant BMP-2.

than that in those treated with NTC ($P < 0.05$) (Fig. 3A), whereas there was no significant differences in the mRNA expression levels of the other three TIMPs between the two any treatment groups (Fig. 3B–D). To identify whether the expression of TIMP-1 is inhibited around the oral sulcus cavity by treatment with BMP-2 siRNA, we analyzed the immunolocalization TIMP-1 in the mandibles treated with NTC and BMP-2 siRNA (Fig. 3E–H). The immunostaining for TIMP-1 was detected in the tissues around oral sulcus cavity in the mandibles treated with NTC (arrow in Fig. 3F), but the immunostaining mostly disappeared in the mandibles treated with BMP-2 siRNA (Fig. 3H). No marked differences in the immunolocalizations for TIMP-2, -3, and -4 were found between the NTC and BMP-2 siRNA-treated mandibles (data not shown).

Furthermore, we analyzed the mRNA expression levels of TIMP-1–4 in the mandible treated with vehicle and recombinant BMP-2 (Fig. 4A–D). The treatment with recombinant BMP-2 induced ~220% and 42% increases in the mRNA expressions of TIMP-1 and TIMP-4, respectively ($P < 0.05$–0.01) (Fig. 3A,D), but there was no significant differences in the mRNA expression levels of TIMP-2 and 3 between the recombinant BMP-2 and vehicle treated groups (Fig. 4B,C). To verify whether recombinant BMP-2 induced an increase in the expression of TIMP-1 around the oral sulcus cavity, we immunolocalized the expression of TIMP-1 in the mandibles treated with both the vehicle and recombinant BMP-2 (Fig. 4E–H). The immunostaining for

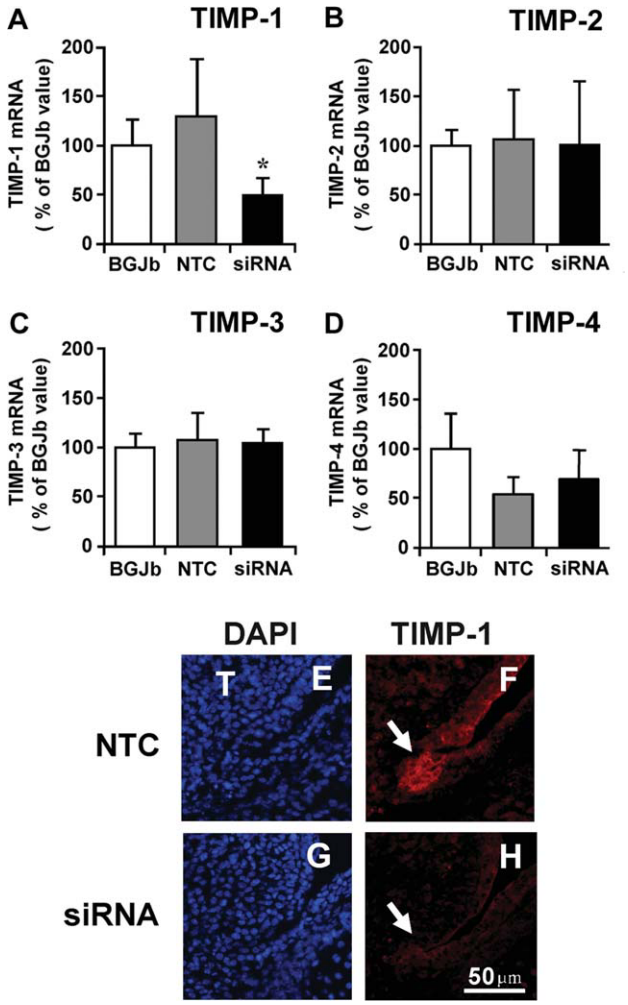

Fig. 3. Expression levels of TIMP-1 (**A**), 2 (**B**), 3 (**C**), and 4 (**D**) mRNAs in the E10 mandibles cultured for 8 days in the BGJb medium containing NTC and BMP-2 siRNA. Each column and vertical bar represent the mean + one SD of six cultured mandibles. The vertical axis is expressed as a percentage of the mean BGJb value set at 100. Significant difference between the NTC and BMP-2 siRNA treated mandibles, $^{*}P < 0.05$. The treatment with BMP-2 siRNA induced ~60% suppression in the expression of TIMP-1. DNA staining image by DAPI (**E**, **G**) and the immunostaining image for TIMP-1 (**F**, **H**) in E10 mandibles cultured for 8 days in BGJb medium containing NTC (E, F) and BMP-2 siRNA (G, H). White arrows in (F) and (H) indicate immunostainings for TIMP-1 around the oral sulcus cavity. Images (E–G) used the same magnification as (H).

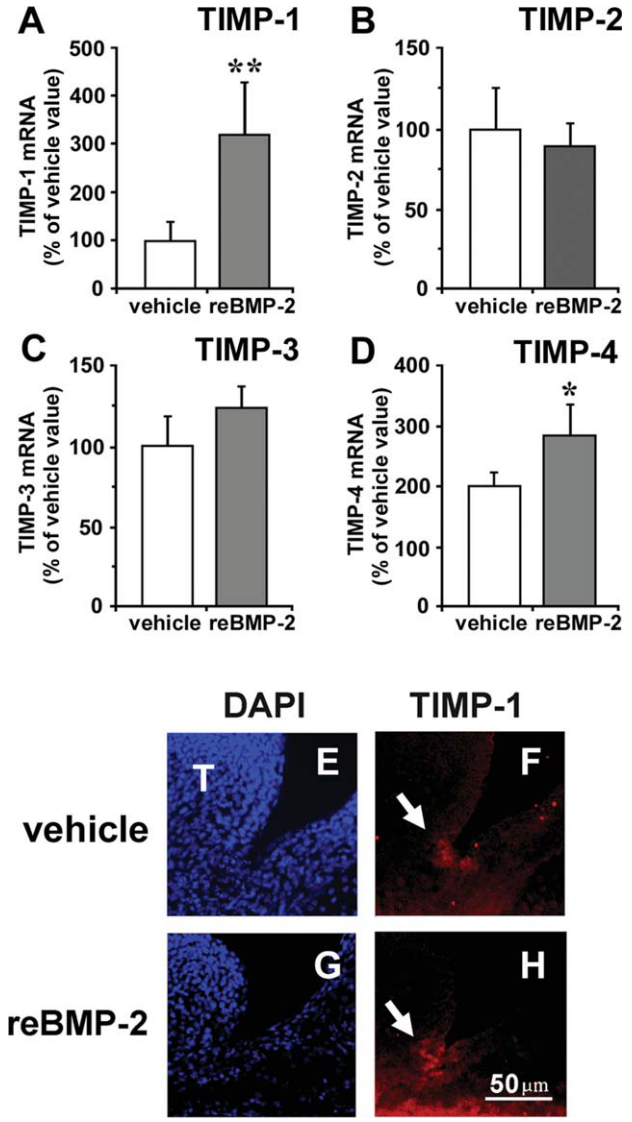

Fig. 4. Expression levels of TIMP-1 (**A**), 2 (**B**), 3 (**C**), and 4 (**D**) mRNAs in the E10 mandibles cultured for 8 days in the BGJb medium containing vehicle and 4 mg/mL of recombinant BMP-2. Each column and vertical bar represent the mean + one SD of six cultured mandibles. The vertical axis is expressed as a percentage of the mean vehicle value set at 100. Significant difference between the vehicle and recombinant BMP-2 treated mandibles, $^{*}P < 0.05$, $^{**}P < 0.01$. The treatment with recombinant BMP-2 induced ~220% and 42% increases in the mRNA expressions of TIMP-1 and TIMP-4, respectively. DNA staining image by DAPI (**E**, **G**) and the immunostaining image for TIMP-1 (**F**, **H**) in E10 mandibles cultured for 8 days in BGJb medium containing vehicle (E, F) and recombinant BMP-2 (G, H). White arrows in (F) and (H) indicate immunostainings for TIMP-1 around the oral sulcus cavity. The area of the immunostaining appeared to be larger in apical region of oral sulcus in the mandibles treated with recombinant BMP-2 [arrow in (H)] than the vehicle [arrow in (F)]. Images (E–G) used the same magnification as (H).

TIMP-1 was detected around the apical region of the oral sulcus cavity in the mandibles treated with vehicle and recombinant BMP-2, but the area of the immunostaining appeared to be larger in the mandibles treated with recombinant BMP-2 (arrow in Fig. 4H) than the vehicle (arrow in Fig. 4F). No marked differences in the immunolocalizations for TIMP-2, -3, and -4 were found between the vehicle and recombinant BMP-2 treated mandibles (data not shown).

We analyzed the expression and the immunolocalization of MMP-13 protein in the mandibles treated with either NTC or BMP-2 siRNA. The treatment with BMP-2 siRNA induced ~60% increase in the expression of MMP-13 protein in the whole cultured mandible (Fig. 5A) and the increase in MMP-13 was mainly observed in the epithelial tissue of the oral sulcus (white arrows in Fig. 5E).

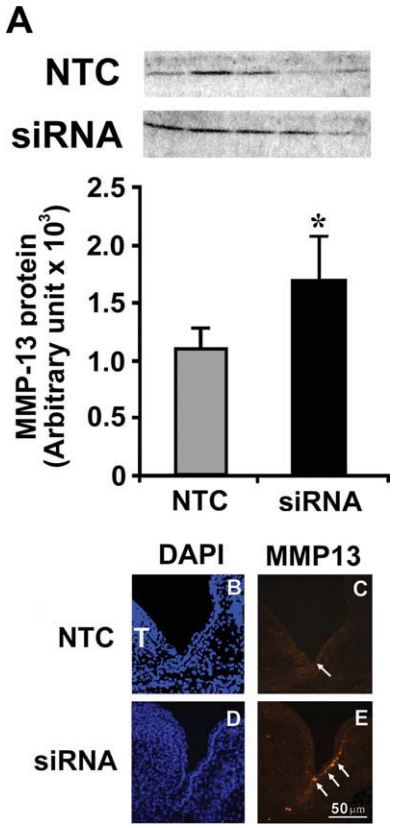

Fig. 5. Representative western blotting patterns and the expression levels of MMP-13 protein in the E10 mandibles cultured for 8 days in the BGJb medium containing NTC and BMP-2 siRNA (**A**). The western blotting patterns show the result of five NTC [upper picture in (A)] and five BMP-2 siRNA [lower picture in (A)] treated cultured mandible. Each column and vertical bar represent the mean + one SD of five cultured mandibles. Significant difference between the NTC and BMP-2 siRNA mandibles, $*P < 0.05$. The treatment with BMP-2 siRNA induced ~60% elevation in the expression of MMP-13. DNA staining image by DAPI (**B**, **D**) and the immunostaining image for MMP-13 (**C**, **E**) in E10 mandibles cultured for 8 days in BGJb medium containing NTC (B, C) and BMP-2 siRNA (D, E). White arrows in (C) and (E) indicate immunostainings for MMP-13 in the tissues around oral sulcus. T in (**B**) indicates tongue. Images (B–D) used the same magnification as (E).

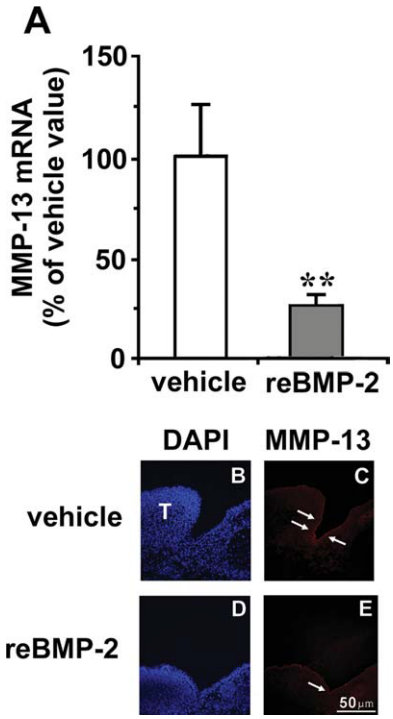

Fig. 6. The expression levels of MMP-13 mRNA in the E10 mandibles cultured for 8 days in the BGJb medium containing vehicle and 4 mg/mL of recombinant BMP-2 (**A**). Each column and vertical bar represent the mean + one SD of five cultured mandibles. Significant difference between the vehicle and recombinant BMP-2 treated mandibles, $**P < 0.01$. The treatment with BMP-2 siRNA induced ~70% decrease in the expression of MMP-13 mRNA. DNA staining image by DAPI (**B**, **D**) and the immunostaining image for MMP-13 (**C**, **E**) in E10 mandibles cultured for 8 days in BGJb medium containing NTC (B, C) and BMP-2 siRNA (D, E). White arrows in (C) and (E) indicate immunostainings for MMP-13 in the epithelium of oral sulcus. T in (B) indicates tongue. Images (B–D) used the same magnification as (E).

Furthermore, we analyzed the mRNA expression and the immunolocalization of MMP-13 in the mandibles treated with either vehicle or recombinant BMP-2 (Fig. 6). The treatment with recombinant BMP-2 induced ~70% decrease in the mRNA expression of MMP-13 in the whole cultured mandible (Fig. 6A). Slight immunostaining for MMP-13 was observed in the tissues around the oral sulcus in the mandible treated with the vehicle (arrows in Fig. 6C), but it was hardly detected in the mandible treated with the recombinant BMP-2 (Fig. 6E).

To study the function of BMP-2, TIMP-1, and MMP-13 during normal development of oral sulcus *in vivo*, we analyzed their localization around the developing oral sulcus of embryos at E11, 12, and 13 by immunohistochemistry (Fig. 7). The immunostaining for BMP-2 was found at E11 in tissues around the developing oral sulcus (Fig. 7A) and, thereafter, the intensity of immunostaining in tissues around the developing oral sulcus gradually decreased until E13 (Fig. 7D,G). Immunostaining for MMP-13 was found in tissues around the developing oral sulcus at E11–E13 (Fig. 7B,E,H) and the intensity of staining appeared to be most strong at E12 (Fig. 7E). The slight immunostaining for TIMP-1 was found in the tissues around the developing oral sulcus at E11 (Fig. 7C) and its intensity became intense at E12 and E13 (Fig. 7F,I).

To further understand the mechanism for the oral sulcus formation, we analyzed the immunolocalization of PCNA, a maker of cell proliferation, and caspase 3, a marker of apoptosis, in the tissues around the oral sulcus of the cultured mandibles treated with vehicle, recombinant BMP-2, NTC, or BMP-2 siRNA (Supporting Information Fig. 1). The area of immunostaining for PCNA in the epithelial region of oral sulcus appeared to be larger in the mandible treated with BMP-2 siRNA (Supporting Information Fig. 1K) than with NTC (Supporting Information Fig. 1H), but be smaller in the mandible treated with the recombinant BMP-2 (Supporting Information Fig. 1E) than with vehicle (Supporting Information Fig. 1B). No marked difference in the expression of caspase-3 was found in the tissues around the oral sulcus between the vehicle and recombinant BMP-2 treated

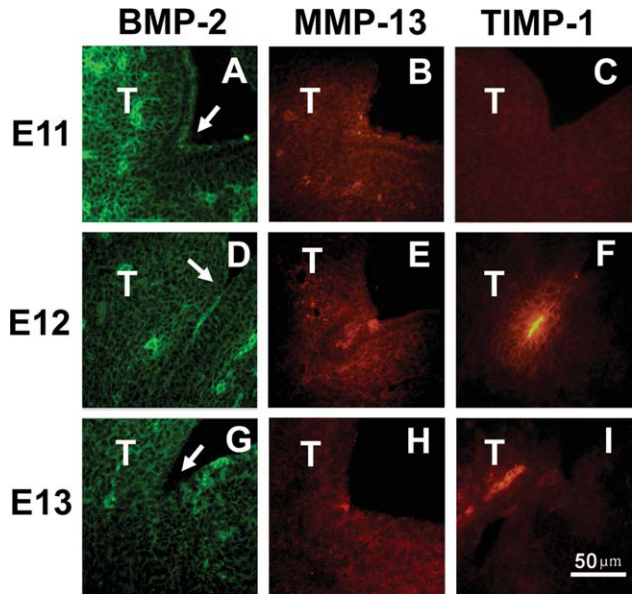

Fig. 7. The immunostaining image for BMP-2 (**A**, **D**, **G**), MMP-13 (**B**, **E**, **H**) and TIMP-1 (**C**, **F**, **I**) in the tissues around the developing oral sulcus cavity of E11 (A–C), E12 (D–F), and E13 (G–I) mouse embryos. T and arrows indicate tongue and oral sulcus, respectively. (A–H) used the same magnification as (I).

mandibles, and between NTC and BMP-2 siRNA treated mandibles (Supporting Information Fig. 1C,F,I,L).

DISCUSSION

In this study, we found that the treatment with BMP-2 siRNA promoted the formation of oral sulcus by stimulating the invagination of the oral sulcus into the mesenchymal tissues consisting of tongue floor, whereas that with recombinant BMP-2 suppressed the process. BMP-2 siRNA suppressed the expression of TIMP-1 and stimulated the expression of MMP-13 in the tissues around the oral sulcus in the cultured mandibles, whereas recombinant BMP-2 has opposite effects of BMP-2 siRNA on the expressions of TIMP-1 and MMP-13. During the normal development of mouse tongue *in vivo*, the localization for BMP-2, TIMP-1, and MMP-13 was observed in tissues around the developing oral sulcus. These results suggest that BMP-2, TIMP-1, and MMP-13 play a role in the formation of the oral sulcus cavity.

MMP-13 cleaves extracellular collagens (Types I, II, and III) into typical C-terminal and N-terminal polypeptide fragments and has a central position in the MMP activation cascade; MMP-13 activates MMP-2 and 9, whereas MMP-13 is activated by MT1-MMP, MMP-2 and 3 (Leeman et al., 2002). MMP-13 is one of the targets of TIMP-1 and inactivated by the binding of TIMP-1 to its catalytic region (Baker et al., 2002; Nagase et al., 2006). The balance between MMPs and TIMPs is known to play a key role in the regulation of many developmental processes, including branching morphogenesis, angiogenesis, wound healing, and extracellular matrix degradation (Vu and Werb, 2000). Thus, we suggest that BMP-2 siRNA induced an increased in the depth of oral sulcus, but recombinant BMP-2 suppressed the depth of oral sulcus in the cultured mandible by altering the extracellular matrix degradation via the expressions of MMP-13 and TIMP-1.

No obvious craniofacial anomalies were reported in TIMP-1 gene inactivated mice (Baker et al., 2002; Nagase et al., 2006), which appears to be inconsistent with our present study. However, a similar example is already reported; although the TIMP-1 gene inactivated mice exhibited no marked change in the renal and hepatic fibrosis, the expression level of TIMP-1 in various fibrotic diseases is upregulated (Kim et al., 2001; Vaillant et al., 2001). Probably, compensations of the function of TIMP-1 with other protease inhibitors such as TIMP-2, 3, and 4, and/or plasminogen activator inhibitor are attributable to no obvious craniofacial anomalies in TIMP-1 gene inactivated mice.

In this study, recombinant BMP-2 was reported to suppress the expression of MMP-13 and stimulate those of TIMP-1 and 3 in osteoblasts isolated from fetal rat calvaria (Varghese and Canalis, 1997). In this study, recombinant BMP-2 suppressed the expression of MMP-13 and stimulated those of TIMP-1 and 4 in the whole cultured mandible. Our present findings of MMP-13 and TIMP-1 are consistent with the previous results, but those of TIMP-3 and 4 are inconsistent. In the previous study, the expressions of TIMPs were analyzed in the osteoblasts isolated from fetal rat calvaria (Varghese and Canalis, 1997), but in this study, they were analyzed in the cultured mandible of mouse embryo. The difference in the species of tissues and animals may be attributable to the inconsistency of TIMP expression between the previous and present studies.

Although the treatment with BMP-2 siRNA induced $<40\%$ suppression in the mRNA and protein expressions of BMP-2 in the whole cultured mandible, it still stimulated the formation of the oral sulcus cavity. This limited suppression of the expression of BMP-2 by the siRNA is probably due to the difficulty of siRNA infiltration inside the cultured mandibles. However, the treatment with BMP-2 siRNA markedly suppressed the expression of BMP-2 in the epithelial region of cultured mandibles because more amount of BMP-2 siRNA seems to reach the epithelial region than inside regions. Thus, the treatment seems able to stimulate the formation of the oral sulcus cavity.

It is well known that BMP-2 and BMP-4 have functional redundancy in the morphogenesis of several kinds of tissues (Bandyopadhyay et al., 2006). In this study, the expression of BMP-4 was not affected by the treatment with BMP-2 siRNA (data not shown). However, we previously reported that the expression level of BMP-4 is much less than that of BMP-2 during the development of the mouse tongue (Suga et al., 2007a). Thus, it seems that BMP-2 plays a more important role in the formation of the oral sulcus cavity in comparison with BMP-4, and that the functional redundancy of BMP-2 with BMP-4 is quite limited in the formation of the oral sulcus cavity.

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