The expression and function of thymosin beta 10 in tooth germ development

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ABSTRACT This study presents the expression pattern and functions of thymosin beta 10 (Tβ10), a Tβ4 homologue during the development of mouse lower first molars. An in situ signal of Tβ10 was detected on embryonic day 10.5 (E10.5)-E15.5 mainly in dental mesenchymal cells as well as in dental epithelial cells, while Tβ4 was expressed in dental epithelial cells. In the late bell stage, preodontoblasts with strong Tβ10 expression and preameloblasts with strong Tβ4 expression exhibited face-to-face localization, suggesting that an intimate cell-cell interaction might exist between preodontoblasts and preameloblasts to form dentin and enamel matrices. A strong Tβ10 signal was found in odontoblasts in the lateral side of the dental pulp and in Hertwig’s epithelial root sheath, thus suggesting that Tβ10 participates in the formation of the outline of the tooth root. An inhibition assay using Tβ10-siRNA in E11.0 mandibles showed significant growth inhibition in the tooth germ. The Tβ10-siRNA-treated E15.0 tooth germ also showed significant developmental arrest. The number of Ki67-positive cells significantly decreased in the Tβ10-siRNA-treated mandibles. The cellular proliferative activity was also significantly suppressed in Tβ10-siRNA-treated cultured mouse dental pulpal and epithelial cells. These results indicate that developmental arrest of the tooth germ might be caused by a reduction in cell proliferative activity. The stage-specific temporal and spatial expression pattern of Tβ10 in the developing tooth germ is indicative of multiple functions of Tβ10 in the developmental course from initiation to root formation of the tooth germ.

KEY WORDS: thymosin beta 10, thymosin beta 4, tooth germ, development, knockdown assay

Introduction

Mammalian tooth development is mediated through sequential and reciprocal epithelial-mesenchymal interactions similar to those observed in other organs, e.g. the hair, glands, lungs, kidneys, etc. A complex multi-step process of gene expression is involved in the early stage of tooth development (Pispa et al., 2003). Tooth germ development occurs via coordinated multi-step molecular interactions between the ectomesenchymal and ectodermal cells (Thesleff 2003). Tooth germ development also occurs via coordinated multi-step molecular interactions between endomesodermal and ectodermal cells (Mina 2001). There are many reports regarding the expression of various genes related to tooth morphogenesis (Cobourne and Sharpe 2003; Thesleff 2003; Chen et al., 2009). However, the precise molecular signaling pathways related to the initiation, growth and differentiation of the tooth germ have not yet been fully elucidated.

Previous studies have demonstrated that thymosin beta 4 (Tβ4) is closely involved in the tooth germ development of the mouse lower first molar (Akhter et al., 2005; Ookuma et al., 2013). Tβ10, a Tβ4 homologue, showed a quite different expression pattern in the developing tooth germ on embryonic day 15.5 (E15.5). An

Abbreviations used in this paper: DE, dental epithelium; DM, dental mesenchyme; DP, dental papilla; E, embryonic day; EO, enamel organ; HERS, Hertwig’s epithelial root sheath; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P, postnatal day; SM, surrounding mesenchyme; Tβ4, thymosin beta 4; Tβ10, thymosin beta 10; TUNEL, terminal deoxynucleotidyl transferase-dUTP nick end labeling.

# Note: The indicated authors have contributed equally to this paper.

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intense expression of Tβ10 was observed in the dental papilla, while the expression of Tβ4 was localized in the odontogenic epithelial cells (Akhter et al., 2005), thus suggesting different functional roles for Tβ10 and Tβ4 in the developmental course of the tooth germ. Although both Tβ10 and Tβ4 are actin monomer-sequestering proteins (Yu et al., 1993), accumulated evidence demonstrates that Tβ10 exerts different effects on the cellular function in comparison to Tβ4. Tβ10 suppresses angiogenesis by inhibiting vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation via its interaction with Ras (Lee et al., 2005), whereas Tβ4 is a potent enhancer of angiogenesis (Philp et al., 2004). An overexpression of Tβ10 decreases cell growth and induces apoptosis in ovarian cancer cell lines (Kim et al., 2012), while Tβ4 mediates the prevention of apoptotic cell death induced in cardiomyocytes and endothelial progenitor cells (Bock-Marquette et al., 2004; Zhao et al., 2011). Tβ10 inhibits the cell migration of human endothelial cells (Mu et al., 2006). In contrast, Tβ4 promotes cell migration by producing matrix metalloproteinase and downregulating E-cadherin (Wang et al., 2003; Ookuma et al., 2013). In addition, Tβ10 is expressed in embryonic organs and modulates embryological development (Lin et al., 1990; Hall 1991; Gerosa et al., 2010; Fanni et al., 2011). Although Tβ10 appears to have various functions in different cells, including embryonic cells, there is little knowledge regarding the detailed expression pattern, possible roles and mechanisms of Tβ10 in tooth germ development.

In this study, the expression pattern of Tβ10 in the course of tooth development from initiation to root formation was examined to elucidate the possible functional roles of Tβ10 in comparison to Tβ4. Furthermore, a Tβ10 knockdown assay was performed in cultured E11.0 mandibles and E15.0 tooth germs using treatment with siRNA, to confirm whether Tβ10 actually participates in tooth germ development.

**Fig. 1. In situ expression of Tβ10 and Tβ4 in the tooth germ at fetal stages.** The in situ Tβ10 expression was temporally and spatially stage-specific during the tooth germ development (left panels: A,C,E,G,I,K,M,O). The expression pattern was different from that of Tβ4 (right panels: B,D,F,H,J,L,N,P). (A,C) An expression of Tβ10 was diffusely observed in the subepithelial mesenchymal cells. Strong Tβ10-positive mesenchymal cells were observed to aggregate beneath the mucosal epithelium (arrow) on E10.5 and E12.0. (B,D) On E10.5 and E12.0, a strong Tβ4 expression was seen in the oral mucosal epithelial layer at the site where the tooth germ would form (arrow). (E) On E13.5, the Tβ10 expression was diffusely found in the enamel organ and the surrounding mesenchymal cells (arrow). (G) On E14.0, a strong Tβ10 expression was seen in the mesenchymal cells surrounding the epithelial tooth bud (arrow), and a weak Tβ10 expression was observed in the epithelial cells in the central area of the tooth bud. (F,H) The Tβ4 expression was localized in the epithelial tooth bud (arrow) and the mucosal epithelial layer on E13.5 and E14.0. (I,K) On E14.5 and E15.5, a strong Tβ10 expression was localized in the dental papilla (asterisk) and the outer enamel epithelium cells of the enamel organ (arrow). (J,L) A strong Tβ4 expression was demonstrated in the outer enamel epithelium (arrow) on E14.5 and E16.5. The Tβ4 expression in the primary enamel knot (arrowheads) observed on E14.5 had disappeared by E15.5. (M,O) On E16.5 and E18.0, a strong Tβ10 expression was detected in the dental papilla at the presumptive cusps (asterisks), and a weak expression was also observed in the inner enamel epithelium (arrowheads). (N,P) On E16.5 and E18.0, a strong Tβ4 signal was partly expressed in the outer enamel epithelium (arrow), and a weak Tβ4 expression was found in the inner enamel epithelial cells (arrowheads). Li; lingual side, Bu; buccal side. Scale bars, 100 μm.
Results

In situ hybridization of Tβ10 and Tβ4 in the developing tooth germ

Embryonic stages

Initiation (E10.5)

Thickening of the oral mucosal epithelium to form the tooth bud was not apparent on this embryonic day.

A strong expression of Tβ10 was diffusely observed in the subepithelial mesenchymal cells. A weak Tβ10 expression was also detected in the oral mucosal epithelial cells (Fig. 1A).

In contrast, a strong Tβ4 expression was detected in the oral mucosal epithelial cells localized at the site where the tooth bud had formed. A weak expression of Tβ4 was observed in the blood vessels (Fig. 1B).

Thickening of the dental epithelium (E12.0)

Local epithelial thickening was observed in the oral mucosal epithelium, indicating the tooth bud formation.

An in situ expression of Tβ10 was diffusely observed in the mesenchymal tissue (Fig. 1C). Strong Tβ10-positive mesenchymal cells were to aggregate around the thickened mucosal epithelium (Fig. 1C, arrow). A weak Tβ10 expression was also detected in the oral mucosal epithelial cells.

The Tβ4 expression was observed in the oral mucosal epithelial cells, including those located at the site of the presumptive tooth bud. The Tβ4 expression was also detected in the blood vessels in the mesenchymal tissue (Fig. 1D).

Bud stage (E13.5 - E14.0)

The thickened dental epithelium invaginated into the mesenchyme and formed the tooth bud.

A strong Tβ10 expression was primarily observed in the mesenchymal cells that surrounded the invaginated epithelial tooth bud in the E13.5 mandible. A weak expression was noted in the mesenchymal cells that were diffusely distributed in the mandibular tissue. A weak Tβ10 expression was also observed in the epithelial...
cells in the central area of the tooth bud (Fig. 1E).

In contrast, an in situ expression of Tβ4 was localized in the invaginated epithelial tooth bud as well as in the mucosal epithelial layer. The Tβ4 expression was hardly observed in the mesenchymal cells located around the tooth bud, with the exception of the blood vessels (Fig. 1F).

The in situ expression patterns of both Tβ10 and Tβ4 in the E14.0 mandible were coincident to those observed in the E13.5 mandible (Fig. 1 G,H).

Cap stage (E14.5 - E15.5)

The enamel organ showed a cap-shaped structure at this stage. A strong in situ expression of Tβ10 was observed in the dental papilla cells in both the E14.5 (Fig. 1I) and E15.5 (Fig. 1K) mandibles. A weak in situ signal was detected in the mesenchymal cells of the dental sac. The Tβ10 expression was also observed in the outer enamel epithelium and inner cells of the enamel organ. No signals were detected in the primary enamel knot (Fig. 1I).

A strong in situ expression of Tβ4 was demonstrated in the outer enamel epithelium and primary enamel knot. A strong signal

was also noted in the dental lamina and oral mucosal epithelium. A weak in situ signal was detected in the inner enamel epithelium (Fig. 1J).

The expression patterns of both Tβ10 and Tβ4 in the E15.5 mandible (Fig. 1 K,L) were almost identical to those at E14.5. However, a higher positive intensity of the in situ Tβ10 signal was detected in the dental papilla cells (Fig. 1K). No Tβ4 expression was found in the enamel knot (Fig. 1L).

Bell stage (E16.5 - E18.0)

The periphery of the enamel organ extended to the mesenchymal tissue, resulting in the shape of a bell in the enamel organ.

On E16.5, a strong Tβ10 expression was demonstrated in the dental papilla localized at the site where the cusps had formed (Fig. 1M). A weak Tβ10 expression was also observed in the inner enamel epithelium and the dental sac, while there was an undetectable level of the Tβ10 expression in the oral mucosal epithelium and the outer enamel epithelium in which the Tβ10 expression was detected in the bud (Fig. 1 C,E) and cap stages (Fig. 1 I,K).

Tβ4 was strongly expressed in the dental lamina and the outer enamel epithelium on E16.5. A weak Tβ4 expression appeared in the inner enamel epithelium. No apparent Tβ4 expression was detected in the dental papilla, except for in the blood vessels (Fig. 1N).

The expression patterns of Tβ10 and Tβ4 on E18.0 (Fig. 1 O,P) were coincident with those observed in the E16.5 mandible.

Postnatal stages

Late bell stage (P0 - P3)

The dental pulp cells facing the inner enamel epithelium differentiated into preodontoblasts. Neither enamel nor dentin matrices had formed on postnatal day 0 (P0), while the formation of both enamel and dentin matrices between ameloblasts and odontoblasts was seen at the presumptive cusp site in the P1 tooth germ. The cells in the stage just before matrix formation are termed preamloblasts and preodontoblasts, respectively (Byers et al., 1990).

On P0, Tβ10 was expressed in the preodontoblasts localized at the presumptive cusp sites of the dental pulp. An in situ signal was also detected in the inner and outer enamel epithelia (Fig. 2A). A strong Tβ4 expression was detected throughout the inner enamel epithelium on P0. The outer enamel epithelium also exhibited weak positive signals (Fig. 2B).

On P1, strong Tβ10 expression was observed in the preodontoblasts and preameloblasts localized at the presumptive buccal cusp site and the central groove region (Fig. 2 C,F,G,L,M). Meanwhile, the Tβ10 signal intensity was reduced in the odontoblasts and ameloblasts in the presumptive lingual cusp site where matrix formation was detected (Fig. 2 E,K).

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**Fig. 3. In situ expression of Tβ10 and Tβ4 in the developing tooth germ at the tooth root formation stage.** The HE staining sections of tooth germs on P5, P7 and P14 are shown (A,D,G). The Tβ10 and Tβ4 expression patterns are shown in (B,E,H) and (C,F,I), respectively. (B) On P5, the Tβ10 expression was observed in HERS cells (arrows). (C) No Tβ4 expression was detected in either ameloblasts or odontoblasts at this stage. (E, F) On P7, the Tβ10 and Tβ4 expression patterns were similar to those observed on P5. (H) On P14, the Tβ10 expression was observed in HERS cells, dental pulp cells facing the HERS and periodontal ligament cells (arrows). (I) No Tβ4 expression was detected at this stage. Li; lingual side, Bu; buccal side. Scale bars, (A-F) 100 μm; (G-I) 50 μm.

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**Table 1**

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Developmental arrest of tooth germ was significantly demonstrated in Tβ10 in comparison to Ut and C (**P<0.01). Ut: untreated explants, C: explants treated with universal negative control siRNA, Tβ10: explants treated with siRNA for Tβ10. Bud: bud-like stage, Cap: cap-like stage.

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The effects of Tβ10 inhibition on the development of the tooth germ in cultured E11.0 mandibles

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Byers et al., 1990.

**TABLE 1**

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The periphery of the enamel organ extended to the mesenchymal tissue, resulting in the shape of a bell in the enamel organ. On E16.5, a strong Tβ10 expression was demonstrated in the dental papilla localized at the site where the cusps had formed (Fig. 1M). A weak Tβ10 expression was also observed in the inner enamel epithelium and the dental sac, while there was an undetectable level of the Tβ10 expression in the oral mucosal epithelium and the outer enamel epithelium in which the Tβ10 expression was detected in the bud (Fig. 1 C,E) and cap stages (Fig. 1 I,K).

Tβ4 was strongly expressed in the dental lamina and the outer enamel epithelium on E16.5. A weak Tβ4 expression appeared in the inner enamel epithelium. No apparent Tβ4 expression was detected in the dental papilla, except for in the blood vessels (Fig. 1N).

The expression patterns of Tβ10 and Tβ4 on E18.0 (Fig. 1 O,P) were coincident with those observed in the E16.5 mandible.

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The expression patterns of Tβ10 and Tβ4 on E18.0 (Fig. 1 O,P) were coincident with those observed in the E16.5 mandible.

Postnatal stages

Late bell stage (P0 - P3)

The dental pulp cells facing the inner enamel epithelium differentiated into preodontoblasts. Neither enamel nor dentin matrices had formed on postnatal day 0 (P0), while the formation of both enamel and dentin matrices between ameloblasts and odontoblasts was seen at the presumptive cusp site in the P1 tooth germ. The cells in the stage just before matrix formation are termed preamloblasts and preodontoblasts, respectively (Byers et al., 1990).

On P0, Tβ10 was expressed in the preodontoblasts localized at the presumptive cusp sites of the dental pulp. An in situ signal was also detected in the inner and outer enamel epithelia (Fig. 2A). A strong Tβ4 expression was detected throughout the inner enamel epithelium on P0. The outer enamel epithelium also exhibited weak positive signals (Fig. 2B).

On P1, strong Tβ10 expression was observed in the preodontoblasts and preameloblasts localized at the presumptive buccal cusp site and the central groove region (Fig. 2 C,F,G,L,M). Meanwhile, the Tβ10 signal intensity was reduced in the odontoblasts and ameloblasts in the presumptive lingual cusp site where matrix formation was detected (Fig. 2 E,K).
The different expression patterns of B-preodontoblasts located at the presumptive cusps and the occlusal lateral sides of the dental pulp. A weak expression was found in the layer (Fig. 2N). A highly strong expression was detected in the preodontoblasts or odontoblasts on P1 (Fig. 2 D,H,I,J).

No expression was observed in the central groove sites where the strong Tb10 expression faced each other in the tooth germ at the late bell stage. The Tb10 expression is indicated by red dots. The yellow zone indicates the enamel and dentin matrices.

An in situ signal of Tp4 was observed through the inner enamel epithelial layer (Fig. 2D). A strong Tp4 expression was detected in the preameloblasts localized at the presumptive buccal cusp and the central groove sites where the strong Tb10 expression was exhibited (Fig. 2 I,J). A weak Tp4 expression was observed in the ameloblasts located under the formation of the enamel matrix at the presumptive lingual cusp site (Fig. 2 H,K). No in situ signals were found in preodontoblasts or odontoblasts on P1 (Fig. 2 D,H,I,J).

On P2, the Tb10 expression was observed in the odontoblastic layer (Fig. 2N). A highly strong expression was detected in the lateral sides of the dental pulp. A weak expression was found in the preodontoblasts located at the presumptive cusps and the occlusal groove sites. A strong expression was also demonstrated in the inner and outer enamel epithelia at the lateral sides of the enamel organ. As a result, preodontoblasts and preameloblasts exhibiting a strong Tp4 expression faced each other at the lateral side of the tooth germ (Fig. 2N).

A strong Tp4 expression was noted in the preameloblasts located in the lateral enamel organ on P2 (Fig. 2O). A weak Tp4 expression was also seen in the preameloblasts and ameloblasts in the inner enamel epithelium.

On P3, the expressions of both Tb10 and Tp4 were observed in limited areas. Preodontoblasts and preameloblasts exhibiting strong Tb10 signals were localized in a limited area of the lateral edge of the tooth germ (Fig. 2P).

The Tp4 expression was observed in the preameloblasts localized at the lateral edges of the inner enamel epithelium only. No in situ signals of Tp4 were detected in the preodontoblasts or odontoblasts (Fig. 2Q).

**Tooth root formation stage (P5 - P14)**

Enamel matrix formation was terminated by this stage. The free edge of the enamel organ on the developing tooth germ extended toward the mesenchymal tissue, thus forming a Hertwig's epithelial root sheath (HERS). Dentin matrix formation further progressed at this stage.

On P5 and P7, a strong Tb10 signal was demonstrated in preodontoblasts in a limited area of the lateral side of the tooth germ (Fig. 3 B,E). These preodontoblasts were considered to have originated before dentin matrix formation, as confirmed by observations of serial sections stained with hematoxylin and eosin (HE) (Fig. 3 A,D). In addition, a strong expression was especially seen in the HERS (Fig. 3 B,E).

There was almost no Tp4 expression in the crown at this stage (Fig. 3 C,F).

On P14, the root formation was almost completed (Fig. 3G). The Tb10 expression was observed only in the HERS (Fig. 3H). The Tp4 expression was found in the dental pulp cells facing the Tb10-positive HERS. Periodontal ligament cells were also shown.

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**Fig. 4. Schematic illustration of Tb10 and Tp4 expression patterns observed during tooth development.** The different expression patterns of Tb10 and Tp4 observed during tooth development are summarized. An in situ Tb10 signal from the initiation stage to the cap stage was detected primarily in dental mesenchymal cells, while Tp4 was expressed in dental epithelial cells. Interestingly, Tb10-expressing preodontoblasts and Tp4-expressing preameloblasts faced each other in the tooth germ at the late bell stage. The Tb10 expression is indicated by blue dots, while the Tp4 expression is indicated by red dots. The yellow zone indicates the enamel and dentin matrices.

**Fig. 5 (Left). Increases in Tb10 and Tp4 mRNA levels in the mandible at E12.0 compared with those observed at E10.5.** A real-time PCR analysis showed that the expression of Tb10 (A) and Tp4 (B) mRNAs were significantly increased in the mandible on E12.0 in comparison to those observed on E10.5. The data represent the mean ± S.D. of more than three samples. **P<0.01; versus E10.5 mandibles.

**Fig. 6 (Right). Inhibition of Tb10 gene expression using siRNA treatment.** The Tb10 mRNA expression was significantly decreased in the E11.0 mandible (A) and the E15.0 tooth germ (B) explants treated with Tb10-siRNA (Tb10-siRNA) in comparison to that observed in the untreated explants (Ut), or explants treated with universal negative control siRNA (Cont). The data represent the mean ± S.D. of more than three samples. *P<0.05, **P<0.01.
positive signals. No Tβ4 expression was found in the crown and root (Fig. 3f). The characteristic expression patterns of both Tβ10 and Tβ4 in each stage are illustrated in Fig. 4. Quantification of Tβ10 and Tβ4 mRNA levels in the mandible using real-time PCR

The mRNA level of Tβ10 in the mandibles on E12.0 was increased approximately 4-fold in comparison to that observed on E10.5 (Fig. 5A, p=0.0000013). Similarly, a significant increase in the level of Tβ4 mRNA in the mandibles on E12.0 was demonstrated in comparison to that observed on E10.5 (Fig. 5B, p=0.0000091), thus confirming the findings of previous study (Yamaza et al., 2001).

Inhibition assay for Tβ10 gene expression induced by siRNA treatment

The effects of siRNA on the expression of Tβ10 were examined using semi-quantitative real-time PCR methods in the cultured E11.0 mandible and the E15.0 tooth germ on the 8th culture day. The Tβ10 mRNA expression levels in both the E11.0 mandible and the E15.0 tooth germ explants treated with Tβ10-siRNA were significantly decreased to approximately 50% compared with those observed in the untreated explants and the explants treated with universal negative control siRNA (Fig. 6A, p=0.000035 by one-way ANOVA, for each p<0.01 by Tukey-Kramer test; Fig. 6B, p=0.019 by one-way ANOVA, for each p<0.05 by Tukey-Kramer test).

Histological analysis of cultured E11.0 mandibles and E15.0 tooth germs treated with Tβ10-siRNA

In the controls, the epithelium appeared to form a cap-shaped structure on the 8th culture day. In addition, both dental papilla and dental follicle formed by the surrounding mesenchymal cells were observed (Fig. 7 A,B). The mandibles treated with Tβ10-siRNA exhibited a developmental arrest of the enamel organ and continued to show the bud-like stage on the 8th culture day (Fig. 7C). The development of the tooth germ was significantly suppressed in the E11.0 mandibles treated with Tβ10-siRNA compared with that observed in the controls (p=0.00059, Table 1).

Tooth germs obtained from E15.0 embryos were also cultured for eight days with siRNA treatment. In the controls, the tooth germs appeared to form a bell-shaped structure on the 8th culture day. The formation of a predentin matrix was observed (Fig. 7 D,E). Meanwhile, significant suppression of tooth germ growth was demonstrated in the explants treated with Tβ10-siRNA in comparison to that observed in the controls (p=0.013, Table 2, Fig. 7 D-F). However, treatment with Tβ10-siRNA did not influence the morphology or arrangement of ameloblasts and odontoblasts. Predentin matrix formation was also observed in all of the controls and explants treated with Tβ10-siRNA (Fig. 7 G-I). These findings suggest that Tβ10 may not affect the differentiation of ameloblasts or odontoblasts.

Ki67 immunohistochemical and TUNEL staining in cultured E11.0 mandibles and E15.0 tooth germs treated with Tβ10-siRNA

Since the development of the tooth germs in the E11.0 mandibles and the E15.0 tooth germs was disturbed by treatment with Tβ10-siRNA, cell proliferation and cell death were examined in the tissue specimens.

In the cultured E11.0 mandibles, the numbers of Ki67- or Table 2

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Developmental arrest of dental papilla was significantly demonstrated in Tβ10 in comparison to Ut and C (∗p<0.05). Ut: untreated explants, C: explants treated with universal negative control siRNA, Tβ10: explants treated with siRNA for Tβ10, +: positive findings, -: negative findings.

Fig. 7. Suppressed morphogenesis of the tooth germ in cultured E11.0 mandibles and E15.0 tooth germs treated with Tβ10-siRNA. An inhibitory assay for Tβ10 was performed using Tβ10-siRNA in cultured E11.0 mandibles (A,B,C) and E15.0 tooth germs (D,E,F) for eight days. (A,B) In the E11.0 mandibles, untreated explants (Ut) and explants with universal negative control siRNA (Cont) revealed a cap-like formation of the tooth germ. (C) No further development of the tooth germ was observed in the Tβ10-siRNA-treated explants (Tβ10-siRNA). (D,E) In the E15.0 tooth germs, Ut and Cont revealed tooth germs with a bell-like appearance, ameloblast and odontoblast differentiation and predentin matrix formation. (F) Some Tβ10-siRNAs did not show tooth germs with a bell-like appearance. (G,H,I) Each image shows a higher magnification of the boxed area in (D,E,F) respectively. No apparent differences were observed in the morphology or arrangement of ameloblasts and odontoblasts, or the formation of predentin among Ut, Cont and Tβ10-siRNA. DE: dental epithelium, DM: dental mesenchyme, SM: surrounding mesenchyme, DP: dental papilla, EO: enamel organ, am: ameloblasts, pd: predentin, od: odontoblasts. Scale bars, (A-F) 100 μm; (G-I) 50 μm.
terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL)-positive cells and the total cell numbers were counted in three objective areas: the “dental epithelium (DE)”, the “dental mesenchyme (DM)” and the “surrounding mesenchyme (SM)”. The DM was either the “DP and follicle” in the cultured explants showing the normal cap-like tooth germ or the “odontogenic ectomesenchyme” in the explants exhibiting an inhibition of tooth germ development (Figs. 7 and 8) (Takahashi et al., 2010; Ookuma et al., 2013).

The localization of Tβ10-expressing cells corresponded well with that of the Ki67-positive cells in the cultured mandible and tooth germ of the control and/or untreated samples. Furthermore, the immunohistochemical reactivity for Ki67 in the E11.0 mandibles revealed a significant decrease in cell proliferation activity in the DE, DM and SM of the Tβ10-siRNA-treated explants in comparison to that observed in the controls. TUNEL-positive cells were also observed in both the controls and the explants treated with Tβ10-siRNA. However, there were no significant differences in apoptotic cells among the examined groups.

The cultured E15.0 tooth germs were composed primarily of two areas: the “enamel organ (EO)” and the “dental papilla (DP)” (Figs. 7 and 8). The numbers of cells in these areas were counted. Immunohistochemical reactivity for Ki67 in the E11.0 tooth germs revealed a significant decrease in the cell proliferation activity of the EO and DP in the Tβ10-siRNA-treated explants in comparison to that observed in the controls. In the TUNEL staining, there were no significant differences in apoptotic cells among the examined groups.

Fig. 8. Cell proliferation and cell death activity in cultured E11.0 mandibles and E15.0 tooth germs treated with Tβ10-siRNA. A cell proliferation assay was performed to analyze the involvement of Tβ10 in the morphogenesis of the cultured E11.0 mandibles (A-D) and E15.0 tooth germs (E-H) on the 8th day. (A,E) Ki67 immunostaining (lower panel) and HE staining (upper panel) are presented in the untreated explants (Ut) and explants treated with universal negative control siRNA (Cont) or Tβ10-siRNA (Tβ10-siRNA). (C,G) TUNEL staining (lower panel) and HE staining (upper panel) are presented in the Ut, Cont and Tβ10-siRNA. (B) Significant decreases in the ratios of the number of Ki67-positive cells to the total cell number (ratio of Ki67 staining) were observed in the DE, DM and SM areas of the Tβ10-siRNA in comparison to those observed in the Ut and Cont. (C) TUNEL-positive cells (green) were primarily observed in the primary enamel knot and scattered in both the epithelium and mesenchyme of the Ut and Cont. (D) There were no significant differences in the ratios of the number of TUNEL-positive cells to the total cell number (ratio of TUNEL staining) in the objective areas among the Ut, Cont and Tβ10-siRNA. (F) Significant decreases in the ratios of Ki67 staining were observed in the EO and DP areas of the Tβ10-siRNA in comparison to those observed in the Ut and Cont. (G) TUNEL-positive cells were scattered in both the epithelium and mesenchyme of the examined explants of Ut, Cont and Tβ10-siRNA. (H) There were no significant differences in the ratios of TUNEL staining in the objective areas among the Ut, Cont and Tβ10-siRNA. DE; dental epithelium, DM; dental mesenchyme, SM; surrounding mesenchyme, DP; dental papilla, EO; enamel organ. Scale bars: 100 μm. *P<0.05, **P<0.01.
On the other hand, Tβ10-siRNA treatment showed no effects on the expressions of dentin matrix protein-1 (Dmp-1) or dentin sialophosphoprotein (Dsp) in the mDP cells (Supplementary Fig. 1 A,B). The expression of amelogenin (Amel) in the mDE6 cells was not affected by Tβ10-siRNA treatment (Supplementary Fig. 1C).

Since both Tβ10 and Tβ4 are known to be actin monomer-sequestering proteins (Yu et al., 1993), the influence of Tβ10-siRNA treatment on the change in the ratio of G- to F-actin in the mDP and mDE6 cells was evaluated. Treatment with Tβ10-siRNA for 48 h decreased the ratio of G-/F-actin in the mDP cells, while the ratio of G-/F-actin modestly changed in the mDE6 cells (Supplementary Fig. 2 A,B). The Tβ10 expression level in the mDP cells was higher than that observed in the mDE6 cells, although real-time PCR revealed that there was no significant difference between the cells. Meanwhile, the Tβ4 expression level in the mDP cells was less than one-third of that observed in the mDE6 cells. A significant difference was noted between these values (Supplementary Fig. 2 C,D).

These results indicate the possibility that Tβ10-siRNA treatment induces the arrest of tooth germ development caused by the decreased cell proliferative activity rather than regulating the cell differentiation of both dental epithelial and mesenchymal cells.

Discussion

Several studies have reported the existence of a close relationship between the expression of Tβ10 and organogenesis (Lin et al., 1990; Hall 1991; Gerosa et al., 2010; Fanni et al., 2011). However, there are no articles describing the relationship between tooth germ development and the Tβ10 expression.

Previous studies (Akhter et al., 2005; Ookuma et al., 2013) demonstrated that Tβ4, another member of the beta-thymosin family, participates in the morphogenesis of the tooth germ. However, the in situ expression pattern of Tβ10 apparently differs from that of Tβ4. In this study, the expression of Tβ4 was primarily observed in the epithelial elements. On the other hand, Tβ10 was expressed in the dental papilla in the E15.5 tooth germ (Akhter et al., 2005).

We reconfirmed that the in situ expression pattern of Tβ10 from the early to the middle developmental stage (E10.5 to E18.0) apparently differs from that of Tβ4. Carpintero et al. (1996) reported that the in situ expression of Tβ10 in the E9.5 mouse embryo is detected primarily in the mesenchymal tissue, including the maxilla and mandible. This expression pattern is coincident with that observed in our present study in which Tβ10 mRNA was primarily detected in the mesenchymal tissue of the mandible on E10.5. The Tβ10 expression has also been reported to exist in the developing renal proximal and distal tubules (Gerosa et al., 2010), and the acinar and ductal epithelial cells of developing salivary gland (Fanni et al., 2011). Therefore, it may be reasonable to consider that Tβ10 may relate to the growth of mesenchymal tissue as well as epithelial cells in the organogenesis and development of the tooth germ. Indeed, a knockdown assay for Tβ10 in the E11.0 mandibles and the E15.0 tooth germs using siRNA resulted in the developmental arrest of the tooth germ. We further examined whether the developmental arrest of the tooth germ was caused by a disturbance in cell proliferation or an increase in cell death. Ki67 immunostaining showed that treatment with siRNA in the organ cultured E11.0 mandibles resulted in a significantly decreased ratio of Ki67 staining in the dental mesenchymal cells. The cell proliferative activity level evaluated with Ki67 immunostaining was suppressed in the

Effects of Tβ10-siRNA on the proliferative activity of mDP and mDE6 cells

To confirm the effects of Tβ10-siRNA on cell proliferation activity, a cell growth assay was performed using mDP and mDE6 cells, which are mouse dental pulpal and epithelial cell lines established from tooth germs, respectively (Tsubakimoto et al., 2007; Yoshizaki et al., 2008).

A significant reduction in the Tβ10-mRNA expression level was observed in the mDP cells with respect to Tβ10-siRNA-treated explants in comparison with that observed in the controls (Fig. 9A). The cell proliferation activity level was significantly lower in the mDP cells treated with Tβ10-siRNA than in the controls at both 24- and 48-hour culture periods, thus indicating that Tβ10-siRNA treatment affects the cell proliferative activity of tooth germ development (Fig. 9B). Similar effects of Tβ10-siRNA on the mRNA expression were observed in the mDE6 cells (Fig. 9C). Significant suppression of the cell proliferation activity at both 24 and 48 h was also noted in the Tβ10-siRNA-treated mDE6 cells in comparison to that observed in the controls (Fig. 9D).

Fig. 9. Effects of Tβ10-siRNA on the cell proliferation of tooth germ-derived cells. A cell proliferation assay of tooth germ-derived cells, mDP (A,B) and mDE6 (C,D) cells, was performed with or without Tβ10-siRNA treatment. (A) The level of Tβ10 transcripts in the mDP cells treated with Tβ10-siRNA (Tβ10-siRNA) were significantly reduced in comparison to those observed in the untreated explants (Ut) and explants treated with universal negative control siRNA (Cont) (P<0.01). (B) At 24 and 48 h after transfection with siRNA, the cell numbers of Tβ10-siRNA were significantly lower than those of Ut (P<0.05, **P<0.01) or Cont (P<0.05, ##P<0.01). (C) Significant decreases in the levels of Tβ10 transcripts were also observed in the mDE6 cells treated with Tβ10-siRNA (**P<0.01). (D) In the mDE6 cells, similar effects of Tβ10-siRNA on the cell proliferation were observed at 24 and 48 h after transfection with siRNA (P<0.05, **P<0.01; versus Ut, #P<0.05, ##P<0.01; versus Cont).
The characteristic expression pattern of Tβ10-expressing areas corresponded to the localization of Ki67-positive cells. Therefore, treatment with Tβ10-siRNA appears to effectively suppress tooth germ development in cultured organs, thus resulting in the developmental arrest of the tooth germ in the E11.0 mandible and E15.0 tooth germ. Furthermore, treatment with Tβ10-siRNA also showed decreased proliferative activity in the cultured mDP and mDE6 cells. Meanwhile, no significant increases in the number of TUNEL-positive cells were observed in the cultured E11.0 mandibular following treatment with Tβ10-siRNA. Therefore, the developmental arrest of the tooth germ observed in the cultured E11.0 mandibles might have been caused by a reduced cell proliferation activity rather than by cell death. These results indicate that Tβ10 may play important roles in cell proliferation in odontogenic epithelial and mesenchymal cells in the initiation and development of the tooth germ.

Tβ10 is an actin monomer-sequestering protein (Yu et al., 1993), and therefore changes in the ratio of G-/F-actin were examined. Treatment with Tβ10-siRNA changed the ratio of G-/F-actin in the mDP cells. Tβ10-siRNA treatment might effectively suppress actin sequestering in the mDP cells due to the lower Tβ4 expression in the mDP cells (Supplementary Fig. 2). Santelli et al., (2002) showed that blocking Tβ10 using an antisense methodology in human thyroid carcinoma cells significantly decreases anchorage-independent growth and improves actin filament organization. Thus, Tβ10 may affect cell proliferation via the regulation of actin assembly. In the future study, it is necessary to clarify the detailed mechanisms underlying the actin arrangement and cell proliferation regulated by Tβ10 during tooth development.

Interestingly, the expression patterns of both Tβ10 and Tβ4 just before matrix formation were quite different in this study. As shown in Fig. 2, a strong expression of Tβ10 was observed in preodontoblasts as well as in preameloblasts. In contrast, Tβ4 was strongly expressed in preameloblasts only. Furthermore, preodontoblasts with a strong Tβ10 expression and preameloblasts with a strong Tβ4 expression exhibited face-to-face localization in the tooth germ. These findings suggest the presence of an intimate cell-cell interaction between Tβ10-expressing preodontoblasts and Tβ4-expressing preameloblasts mediated by two different thymosins in the formation of dentin and enamel matrices. Therefore, other new functions of Tβ10 and Tβ4 might exist that are not related to the development or differentiation of the tooth germ. The peculiar expression patterns of both Tβ10 and Tβ4 observed in the matrix formation stage indicate that these unknown functions are important for the growth of the tooth germ. Further examinations are needed to clarify the true functions of Tβ10 and Tβ4 in tooth germ development at this stage.

Both Tβ10 and Tβ4 also showed peculiar expression patterns in the root formation stage. In this stage, the expression of Tβ10 was found in preodontoblasts at the lateral side of the tooth germ. In particular, the expression of Tβ10 was localized in the dental epithelial cells and the dental mesenchymal cells of the dental pulp at the apex site of the enamel organ that formed the HERS after P5. The characteristic expression pattern of Tβ10 observed at the root formation stage suggests that Tβ10 may participate in forming the outline of the tooth root and extending the HERS.

In conclusion, Tβ10 and Tβ4 exhibit developmental stage-specific temporal and spatial expression patterns in the tooth germ. These findings suggested that Tβ10 and Tβ4 might play different roles in mouse tooth germ development. Previous studies have shown that the immunolocalization of Tβ10 is altered cell by cell in the developing kidneys and salivary glands (Gerosa et al., 2010; Fanni et al., 2011). Fanni et al., (2011) reported the existence of a close relationship between the immunohistochemical expression of Tβ10 and salivary gland development, and speculated that alternative immunoreactivity is associated with the organogenesis of the salivary glands. Our present study and previous reports indicate that Tβ10 and Tβ4 could potentially perform multiple functional roles in tooth germ development. However, the signal transduction pathways both up- and down-streams of Tβ10 and Tβ4 in tooth germ development remain unknown. Further examinations are needed to elucidate the complete functional roles of these beta-thymosins and the interactions between beta-thymosin-associated factors in tooth germ development.

Materials and Methods

Animals
At least three BALB/c embryos and postnatal mice at each developmental stage were used in this study. The examined developmental stages included E10.5, 11.0, 12.0, 13.5, 14.0, 14.5, 15.0, 15.5, 16.5 and 18.0 after gestation and P0, 1, 2, 3, 5, 7 and 14. Adult BALB/c mice were obtained from Charles River Japan, Inc. (Yokohama, Japan). All experimental procedures using mice were performed in accordance with the guidelines of the Animal Care and Use Review Committee of Kyushu University (Fukuoka, Japan). Adult female mice (10-20 weeks) were caged together with male mice. Successful insemination was determined based on the presence of a postcopulatory plug in the vagina 12 h later. The embryonic day E0.5 was defined by the presence of the vaginal plug (Takahashi et al., 2010; Okuma et al., 2013).

In situ hybridization
To confirm the specificity and sensitivity of the antsense RNA probes for Tβ10 and Tβ4 mRNAs, membrane hybridization was performed. Membrane hybridization and in situ hybridization methods were carried out according to the protocol described in previous studies (Yamaza et al., 2001). Specific probes for Tβ10 and Tβ4 mRNAs were designed according to the NCBI Reference Sequences (accession number Tβ10: NM_025284.4, Tβ4: NM_021278.2) (Akhter et al., 2005). Strong and weak signals were used to describe for the relative evaluation of the signal intensity observed in the same tissue section. The developmental tooth germ process in the embryonic mandible was defined according to previous studies (Akhter et al., 2005; Takahashi et al., 2010).

Semi-quantitative real-time PCR
Total RNA was isolated from mandibles on E10.5 and E12.0, and from tooth germs on E15.0, E18.0, P1 and P5 using the SV Total RNA Isolation System (Promega, WI, USA). Total RNA was also isolated from cultured E11.0 mandibles, E15.0 tooth germs and mDP and mDE6 cells. cDNA was synthesized using the SuperScript™ VILO™ cDNA Synthesis System (Invitrogen, CA, USA) according to the manufacturer’s instructions. Real-time PCR was performed using a Thermal Cycler Dice™ Real Time System with the SYBR Premix Ex Taq™ II (TaKaRa Bio, Shiga, Japan) (Takahashi et al., 2010; Okuma et al., 2013). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous reference gene. The gene-specific primers for Tβ10, Tβ4 and GAPDH were as follows: Tβ10, 5'-AAC GAG AGT GGG AGC ACC TG-3' and 5'-AGC TTG GCC TTA CGG AAG CTG-3'; Tβ4, 5'-CTG ACA AAC CCG TGG CTG A-3' and 5'-ACG ATT CGC CAG CTT GCT TC-3'; GAPDH, 5'-TGT GTC CGT CGT GGA TCT GA-3' and 5'-TTG CTG TTG AAG TCG CAG GAG-3'. The relative expression levels of each targeted gene were normalized using the delta-delta cycle threshold (ΔΔCT) comparative method based on the reference gene values. The specificity of the PCR products was confirmed using a
melting curve and/or gel electrophoresis (Takahashi et al., 2010; Naher et al., 2012; Ookuma et al., 2013).

**Inhibition assay for Tβ10 using siRNA on organ culture**

The mandibles dissected from the E11.0 embryos and the tooth germs dissected from the E15.0 embryos were cultured for eight days. The culture period of both the E11.0 mandibles and E15.0 tooth germs was determined based on previous studies (Kobayashi et al., 2006; Xie et al., 2007; Ookuma et al., 2013). The protocols for organ culture were identical to those used in these studies. In order to inhibit the functions of Tβ10 during tooth germ development, cultured explants were treated with siRNAs supplemented in the culture media according to the manufacturer’s protocol of Lipofectamine RNAiMAX (Invitrogen)(Naher et al., 2012). siRNA for murine Tβ10 (Mm_Tmsb10_0122) and a universal negative control siRNA (Sigma-Aldrich, St. Louis, MO, USA) were used as a target and negative control, respectively. 30 pmol of siRNA and 3 μl of Lipofectamine RNAiMAX were contained in 1-ml culture media (siRNA final conc. 30 nM). The culture medium was changed every 24 h.

**Histological analysis of the cultured explants**

The cultured explants were fixed with 4% paraformaldehyde for 24 h at 4°C and then embedded in paraffin. Paraffin-embedded explants were cut into 5-μm thick sections in the anteroposterior direction, and HE staining was performed. The explants were prepared for the histological analysis as previously described (Kobayashi et al., 2006; Xie et al., 2007; Ookuma et al., 2013).

**Cell proliferation and cell death assays in the cultured organs**

In order to evaluate the effects of Tβ10 on cell proliferation during tooth development, immunohistochemistry using a rabbit polyclonal antibody to Ki67 (Abcam, Cambridge UK) was performed. These sections were nuclear-counterstained with hematoxylin.

TUNEL was applied to detect apoptotic cells using the *in situ* Apoptosis Detection Kit (TaKaRa). Briefly, terminal deoxynucleotidyl transferase (TdT) enzymes were used on the sections of the cultured organs treated with siRNA, and then the sections were labeled with FITC on the nick sites in the DNA. Propidium iodide was then used for nucleus staining.

The percentages of Ki67-positive or FITC-positive cells were evaluated in the DE, DM and SM areas in the cultured E11.0 mandibles. In the cultured E15.0 tooth germs, the numbers of cells in the EO and DP areas were counted. More than one hundred cells were examined as a population of each area in at least three different explants. The number of stained cells was divided by the total number of stained and non-stained target cells to calculate the ratio of Ki67 or FITC staining. The analyses of cellular proliferation and cell death were carried out as described in previous studies (Takahashi et al., 2010; Ookuma et al., 2013).

**Cell lines and cell culture**

Mouse dental epithelial and pulpal cell lines established from tooth germs (named mD6 and mDP, respectively) were kindly provided by Professor Satoshi Fukimoto (Tohoku University, Japan) and Associate Professor Masahiro Saito (Tokyo University of Science, Japan) (Tsubakimoto et al., 2012). Both types of cells were maintained in D-MEM/F-12 (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, Filttron, Brooklyn, Australia), 100 U/m of penicillin and 100 μg/ml of streptomycin (Invitrogen) in a humidified atmosphere of 5% CO2 at 37°C.

**Cell growth assays of dental epithelial and mesenchymal cells treated with Tβ10 using siRNA in cell culture**

Tβ10-siRNA (final conc. 124 nM) was simultaneously transfected into mDP and mD6 cells using hemagglutinating virus of Japan (HVJ)-liposomes (GenomOne, Ishihara Sangyo Kaisha, Osaka, Japan) when the cells were seeded in triplicate onto 3 x 104 cells/well in 6-well plates. The HVJ-liposome complex was prepared according to the manufacturer’s instructions.

At 24 and 48 h after treatment with siRNA, the numbers of cells in four different microscopic fields of each well were counted. The average cell number was calculated in triplicate. At least three independent experiments were performed in triplicate.

**Statistical analysis**

All experiments were independently repeated at least three times. One-way ANOVA with the Tukey-Kramer comparison test or Student’s *t*-test was used to determine the presence of significant differences in the real-time PCR data, immunostaining-positive ratios and cell growth values. *p*-value (*p*<0.05 or *p*<0.01) was considered to indicate statistically significant differences. Significant differences in the numbers of explants were assessed using the chi-square test for independence.

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