Expression and role of *Lhx8* in murine tooth development*

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Summary. We examined the expression and possible functions of *Lhx8*, a member of the LIM-homeobox gene family, during tooth morphogenesis of the mouse. *Lhx8* was expressed in the dental mesenchyme between the bud and early bell stage of the molar tooth germ. Tooth germ explants from embryonic day 12.5 mice treated for 5 to 7 days with antisense-oligodeoxynucleotides (AS-ODN) against *Lhx8* showed a marked decrease in the number of mesenchymal cells. The explants treated with AS-ODN for 11 to 14 days were filled with a large number of undifferentiated epithelial cells and a limited number of undifferentiated mesenchymal cells, but did not contain a tooth germ. Treatment of explants with AS-ODN for 7 days suppressed the proliferation of dental mesenchymal cells and induced apoptosis; the latter was confirmed by histochemical and ultrastructural examinations. Moreover, the expression of *Lhx6*, *Msx1*, *Msx2*, *Bmp4* and *Gsc*, which are also known to be involved in tooth morphogenesis, were suppressed after the application of AS-ODN against *Lhx8* for 7 days. The present results suggest that *Lhx8* plays an important role in the survival of mesenchymal cells of the tooth germ during development.

Introduction

The development of tooth germs reportedly entails such events as ecto-mesenchymal interaction and association of various cytokines including fibroblast growth factor (FGF), bone morphogenetic protein (BMP), sonic hedgehog (SHH), and WNT (Maas and Bei, 1997; Weiss *et al.*, 1998a, b; Peters and Balling, 1999; Tucker and Sharpe, 1999). Moreover, transcription factors, such as *Msx1*, *Msx2*, *Lef1*, and *Barx1* are expressed spatiotemporally in the developing tooth germs, and are essential for tooth morphogenesis (Jernvall and Thesleff, 2000).

Homeobox genes encoding proteins with DNA-binding homeodomains control different modes of development, and molecules containing such homeodomains function as transcription factors. Several homeobox-containing genes have been identified in *Drosophila* (Bopp *et al.*, 1986; Gehring, 1987). Most of these genes are required for segment formation or identity and are also expressed in specific cell types or sets of cells. In vertebrates, some homeobox genes are expressed in the nervous system and other tissues (Jackson *et al.*, 1985; Awgulewitsch *et al.*, 1986; Simeone *et al.*, 1986; Wolgemuth *et al.*, 1986). Homeobox genes have been classified into several subfamilies according to similarities in the amino acid sequences of their homeodomain and the additional domains encoded by them, and each is likely to play a distinct role during development (Rubenstein and Puelles, 1994). The LIM domain, found in a number of invertebrate and mammalian proteins, was first identified in the protein products of three genes, namely, *lin-11* (Freyd *et al.*, 1990), *isl-1* (Karlsson *et al.*, 1990), and *mec-3* (Way and Chalfie, 1988). The LIM family of proteins is defined by the presence of one to three repeats of a 52-residue segment containing two adjacent zinc fingers separated by a two-residue linker. There have been reports

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suggesting the involvement of LIM proteins in various protein-protein interactions (Feuerstein *et al.*, 1994; German *et al.*, 1992; Leonard *et al.*, 1992; Schmeichel and Beckerle, 1994; Xue *et al.*, 1993). The LIM-domain is conserved among highly diverged species, suggesting that the LIM region corresponds to a functionally important protein domain seemingly involved in a variety of differentiation processes.

The *Lhx8* protein consists of 367 amino acids and is encoded in the *Lim homeodomain 3 (L3)* (Matsumoto *et al.*, 1996a, b); for this reason, *Lhx8* is called *L3* (Matsumoto *et al.*, 1996b). *Lhx7*, another member of the LIM-homeobox gene family, has a 97% homology with *Lhx8*, and these two genes are thought of as identical. *Lhx8* has been localized on mouse chromosome 3, H3-4 (Kitanaka *et al.*, 1998), and homologs of genes in that region have been mapped to human chromosome 4, q25-31m, a region that has been linked to craniofacial clefting (Mitchell *et al.*, 1995). During mouse embryogenesis, *Lhx8* is expressed in the first branchial arch including the maxillary and mandibular processes – where tooth germs develop – as well as in some other regions of the head, including the ventral forebrain (Matsumoto *et al.*, 1996a, b; Grigoriou *et al.*, 1998). In addition, *Lhx6*, another member of the LIM-homeodomain gene family exhibiting 86% homology with *Lhx8*, is uniformly expressed in the odontogenic mesenchyme of the first branchial arch during early development, but is restricted to the mesenchyme participating in the formation of individual teeth during odontogenesis (Grigoriou *et al.*, 1998). *Lhx8* signals are detected in the palatal mesenchyme but not in the epithelium, and *Lhx6* is expressed in both the mesenchyme and epithelium (Zhang *et al.*, 2002). These data suggest that, like other transcription factors including *Msx1*, *Msx2*, *Lef1* and *Barx1*, *Lhx8* (or *Lhx7*) and *Lhx6* are associated with tooth morphogenesis, in the odontogenic mesenchyme of the first branchial arch. Moreover, a homeobox-containing *Goosecoid (Gsc)* is localized in the caudal mesenchyme in a pattern that complements that of *Lhx6* and *Lhx7* in the rostral (Tucker *et al.*, 1999), indicating that *Gsc* might also be associated with primary tooth morphogenesis-associated genes. This evidence suggests that *Lhx8* may be involved in tooth morphogenesis, but the expression and function of *Lhx8* during tooth development have yet to be investigated. The present study therefore examined the spatiotemporal distribution of *Lhx8* in developing mouse molar tooth germs *in vivo* by using *in situ* hybridization histochemistry. Moreover, the role of *Lhx8* during tooth development was analyzed by use of an antisense (AS)-oligodeoxynucleotides (ODN) method. Our results suggest that *Lhx8* is essential for the survival of dental mesenchyme cells during tooth development.

Materials and Methods

Animals

Pregnant time-mated ICR mice were purchased from Nippon SLC Ltd. (Shizuoka). The day that a vaginal plug appeared was designated as embryonic day 0 (E0), and the day of birth was designated as postnatal day 0 (P0). All experiments were reviewed and approved by the Institutional Animal Use and Care Committee of Osaka University Graduate School of Dentistry prior to the experiments.

In situ hybridization

A cDNA (1.1kb) of *Lhx8* containing the 5' end open reading frame was cloned into a pBS-KS plasmid vector (Stratagene, La Jolla, USA). After linearization using BamHI (New England Biolabs, Beverly, USA), a ³⁵S-labeled antisense RNA probe was synthesized by using T₇ polymerase (Boehringer Mannheim, Mannheim, Germany). *In situ* hybridization histochemistry was performed as previously described (Wanaka *et al.*, 1990). Briefly, the mandibles containing molar tooth germs from E13.5, E14.5, E16.5, and E18.5 embryos and P0.5 mice (n=5 each) were fixed in 4% paraformaldehyde, embedded into paraffin, and paraffin sections (8 µm) were mounted onto silane-coated slide glasses. After deparaffinization, pre-hybridization treatment was performed with anhydride with 10 µg/ml proteinase K (TaKaRa, Kusatsu) for 10 min and then with 0.25% acetic anhydride. The specimens were hybridized with approximately 8000 cpm/µl of the ³⁵S-labeled probes in moist chambers at 55°C overnight. After washing in the 2 × saline sodium citrate (SSC) containing 50% formamide and 0.1 M DTT solution for 30 min at 65°C, specimens were treated with a 10 mM Tris-HCl buffer containing 1 mM EDTA, 0.5 M NaCl, and 1 µg/ml RNase A (Sigma, St. Louis, USA) for 30 min at 37°C. Washings with 2 × SSC and 0.1 × SSC were performed for 10 min each at room temperature. Slides were covered with NBT-2 emulsion (EASTMAN Kodak, Rochester, USA), exposed for 14 days at 4°C, and developed. Some sections were counterstained with hematoxylin-eosin (HE).

Organ culturing and translation arrest by AS-ODN

The protocol for the organ culturing of tooth germs was almost identical to that of our previous studies (Tabata *et al.*, 1996; Liu *et al.*, 2000). Briefly, explants containing bud stage tooth germs of the mandibular first molar were removed gently from E12.5 embryos and cultured under conditions of 100% humidity in an atmosphere containing 5% CO₂ and 95% air at 37°C in a modified Trowell's system for a period of up to 14 days. The explants were cultured in a BGJb medium (Fitton-Jackson's modified BGJ;

Gibco BRL, Gaithersburg, USA) containing 100 µg/ml ascorbic acid (Nakai Chemical, Kyoto), 100 U/ml penicillin-streptomycin (Gibco BRL), and 10% fetal bovine serum (Sigma). Initial pH was adjusted to 7.4, and the medium was changed every two days.

For the translation arrest of *Lhx8*, we designed AS-ODN against *Lhx8* (Lhx8-AS-ODN) as 5'-GCT CTT CCA ATA CAT-3' for a 15 mer cDNA sequence of *Lhx8* (GenBank AB007588 - AB007596) directly downstream from the initiation codon. The corresponding sense (S)-ODN (5'-ATG TAT TGG AAG AGC-3') and random sequence (RS)-ODN (5'-GAT AGC GTC ACG CTA-3') were used for control experiments. Lhx6-AS-ODN was designed as 5'-GGC TCG GTG GGC GTC AC-3'. All ODN, as synthesized by Hokkaido System Science (Sapporo, Japan), were dissolved in double-distilled water and quantified by optical density at 260 nm. Each ODN was added at a final concentration of 20 µM to the culturing medium, and the medium was changed every two days. The optimal concentration of the AS-ODN, 20 µM, was determined after testing various concentrations (10, 20, 30 µM) of it, on the basis of the morphology of the cultured explants. As control experiments, an equal volume of double-distilled water was added.

After a culturing period of 4 to 14 days, the specimens were fixed with 4% paraformaldehyde overnight. Paraffin sections of 10 µm thickness were prepared and stained with HE or silver impregnation.

Total RNA preparation and RT-PCR

First-strand cDNA was synthesized from 1 µg of RNA obtained from explants of the mandibular first molar tooth

germs removed from E12.5 mice and cultured for 4 and 7 days; Superscript II reverse transcriptase (Gibco BRL) and random hexanucleotide primers for reverse-transcription-polymerase chain reaction (RT-PCR) were used (Liu *et al.*, 2000). Primers for the detection of mouse *Lhx8*, *Lhx6*, *Msx1*, *Msx2*, *Bmp4*, *Gsc* and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), a constitutively expressed house-keeping gene, were used (Table 1). Twenty-nine cycles of amplification were carried out for 15 sec at 95°C and 1 min at 63°C for a cycle by using Elongase (Gibco BRL) (Enomoto-Iwamoto *et al.*, 1998). The products were analyzed by 1.5% agarose gel electrophoresis.

Cell proliferation assay

For cell proliferation assay, a 5-bromo-2'-deoxyuridine (BrdU) labeling method was applied to tooth germ explants using a commercially available cell proliferation kit (Amersham Pharmacia Biotech, Tokyo) as described elsewhere (Tabata *et al.*, 1996). Briefly, the tooth germ explants removed from E12.5 mice and cultured for 7 days were incubated with BrdU (1 µg/ml) for 4 h, and rinsed in 0.01M phosphate-buffered saline (PBS) for 15 min. They were then cryosectioned at 12 µm, mounted onto slide glasses, and fixed in 10% neutralized buffered formalin for 30 min at room temperature. After the blockage of non-specific binding sites with 3% normal goat serum for 30 min, nuclease/anti-BrdU antibody solution (1:200) was applied for 2 h, followed by the application of horseradish peroxidase (HRP)-conjugated anti-mouse IgG solution and polymerizing 3,3'-diaminobenzidine tetrahydrochloride (DAB) in the

Table 1. Primers used for RT-PCR of mouse *Lhx8*, *Lhx6*, *Msx1*, *Msx2*, *Bmp4*, *Gsc*, *G3PDH*.

Gene	Length of cDNA (bp)	Primers
m <i>Lhx6</i>	350	5'-TCG TTG AGG AGA AGG TGT TTT GTC G-3' 5'-ACT GGG TGC TGC GGT GTA TGC TT-3'
m <i>Lhx8</i>	502	5'-TCA TGT CGG AGG AGT GCG GG-3' 5'-TAC CGT TCT CCA CTT CTC TC-3'
m <i>Msx1</i>	717	5'-GGA GCT GGA GAA GCT GAA GAT G-3' 5'-GCC AAA GCG CTT AGA GTT TGA C-3'
m <i>Msx2</i>	413	5'-CGC AAT TTC CGC CAG AAA CAG T-3' 5'-AGA GGG AGG GGG ATG CTT TTA TG-3'
m <i>Bmp4</i>	522	5'-CCC AGA CTC CCA TGG GTA TTT-3' 5'-CAG GCT CCA AGA CGG TTT AGA C-3'
m <i>Gsc</i>	389	5'-CAC CAG ATG CTG CCC TAC ATG A-3' 5'-CCG AGT CCA AAT CGC TTT TAC CT-3'
m <i>G3PDH</i>	322	5'-CCA TCA CCA TCT TCC AGG AG-3' 5'-GCA TGG ACT GTG GTC ATG AG-3'

presence of cobalt and nickel. At the end of each step, specimens were rinsed in PBS containing 0.05% Tween 20 (TPBS) for 5 min. Eosin was used for counterstaining.

TdT-mediated dUTP nick end labeling (TUNEL)

Cryosections (12 μ m) were prepared from the tooth germ explants of mandibular first molars removed from E12.5 mice and cultured for 7 days, and were fixed in 10% neutralized buffered formalin for 30 min at room temperature. After treatment with a terminal deoxynucleotidyl transferase (TdT) buffer consisting of 200 mM potassium cacodylate, 25 mM Tris-HCl, and 1 mM cobalt chloride, a TdT solution consisting of 0.15 U/ μ l TdT Recombinant (Gibco BRL) and 20 μ M digoxigenin (DIG)-11d-2'-deoxyuridine-5'-triphosphate(-UTP) (Boehringer Mannheim, Mannheim, Germany) was applied to the specimens for 1 h at 37°C. They were rinsed in a buffer consisting of 300 mM sodium chloride and 30 mM sodium citrate at room temperature for 15 min, and applied with 2% H₂O₂ in TPBS for 5 min at room temperature. Sections were incubated with an HRP-conjugated sheep anti-DIG-antibody (Boehringer Mannheim), developed with DAB, and slightly stained with methyl green.

Ultrastructural observation

Tooth germ explants removed from E12.5 mice and cultured for 4 to 7 days with or without ODN were fixed with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in a 0.1M cacodylate buffer (pH 7.4) for 2 h at 4°C, washed in a 0.1 M cacodylate buffer, and then post-fixed in 1% OsO₄ for 1 h. After the washing in the 0.1 M cacodylate buffer and dehydration by subsequent passage through the ethanol series, the specimens were embedded in Epon 812. Ultra-thin sections were prepared, stained with 1% uranyl acetate and 1% lead citrate, and examined with a HITACHI H-7500 transmission electron microscope.

Results

Expression of Lhx8 in developing murine molar tooth germs in vivo

Observation of the E13.5 embryo showed the bud stage

tooth germs of mandibular and maxillary first molars (Fig. 1a). *Lhx8* was strongly expressed in the dental mesenchyme and the mesenchyme beneath the oral epithelium around the tooth germs at this stage (Fig. 1b). At E14.5, tooth germs of the mandibular first molar had developed to the cap stage (Fig. 1c). The gene was clearly expressed in the dental papilla and slightly so in the dental sac of cap stage tooth germs. The signal for *Lhx8* at the mesenchyme beneath the oral epithelium was weak (Fig. 1d). At E16.5, the early bell stage tooth germ of the mandibular first molar could be recognized (Fig. 1e). The expression of *Lhx8* was downregulated in the dental papilla at this stage, and was hardly recognized in the mesenchyme beneath the oral epithelium (Fig. 1f). At E18.5, late bell stage tooth germs of the mandibular first molar (Fig. 1g) and cap to early bell stage of the mandibular second molar (Fig. 1i) were observed. The gene was not expressed in the late bell stage tooth germs of mandibular first molars (Fig. 1h), but it was clearly detected in the dental papilla of mandibular second molars which were at the early bell stage (Fig. 1j), as was observed at the same developmental stage of the first molar (Fig. 1d, f). No gene expression was detectable in the tooth germs of the mandibular first molars of P0.5 mice (data not shown). These results indicated that *Lhx8* was only expressed in mesenchymal but not in epithelial cells of tooth germs during the bud to early bell stages.

RT-PCR

RT-PCR products of *Lhx6*, *Lhx8*, *Msx1*, *Msx2* and *Bmp4* were not affected in tooth germ explants cultured with or without AS-ODNs against *Lhx6* and *Lhx8* for four days, but the expression of *Gsc* slightly increased following treatment with *Lhx6*-AS-ODN or *Lhx8*-AS-ODN for four days (Fig. 2). In tooth germs cultured with *Lhx6*-AS-ODN or *Lhx8*-AS-ODN for seven days, both *Lhx6* and *Lhx8* were downregulated. The product levels of *Msx1*, *Msx2*, *Bmp4*, and *Gsc* were reduced in tooth germs explants cultured with *Lhx8*-AS-ODN for seven days, but were not affected when without ODN, or with RS-ODN and *Lhx6*-AS-ODN. The expression of *G3PDH* was not affected by any of the treatments at any time.

Fig. 1. Expression of *Lhx8* in developing mouse molar tooth germs *in vivo*. Micrographs of HE-staining sections (left column) and dark-field micrographs (right column) of corresponding adjacent sections processed for *in situ* hybridization are shown. Sections show the bud stage tooth germs of mandibular and maxillary first molars at E13.5 embryo (a, b), the cap stage tooth germ of the mandibular molar at E14.5 (c, d), an early bell stage tooth germ at E16.5 (e, f), a late bell stage tooth germ of the mandibular first molar at E18.5 (g, h), and the early bell stage tooth germ of the mandibular second molar in the same section of Figure g, h (i, j). Note that genes are expressed in mesenchymal cells during tooth development. Arrowheads: *Lhx8* expression, EO: enamel organ, DP: dental papilla. Bars: 200 μ m

Fig. 1. Legend on the opposite page.

Fig. 2. Gel electrophoresis of RT-PCR products. Gel electrophoresis patterns of the RT-PCR products of *Lhx6*, *Lhx8*, *Msx1*, *Msx2*, *Bmp4*, *Gsc* and *G3PDH* in tooth germs, cultured for four or seven days, of E12.5 explants controls without ODN (C), or with RS, Lhx6-AS, Lhx8-AS, are shown. RT-PCR products of *Lhx6*, *Lhx8*, *Msx1*, *Msx2*, *Bmp4* and *Gsc* are not affected in the tooth germ explants cultured with or without ODN for four days. RT-PCR products of *Lhx6* and *Lhx8* are markedly reduced after Lhx6-AS-ODN and Lhx8-AS-ODN treatments, respectively, in tooth germs cultured for seven days compared with levels in those cultured without ODN or with RS-ODN. The product levels of *Msx1*, *Msx2*, *Bmp4* and *Gsc* are reduced in tooth germs explants cultured with Lhx8-AS-ODN for seven days, but are not affected if without ODN or with RS-ODN or Lhx6-AS-ODN. The expression of *G3PDH* is not affected by any of the treatments at any time.

Time-course of the effect of AS-ODN for *Lhx8* on tooth germ morphology

Tooth germ explants removed from E12.5 embryos and cultured without ODN (Fig. 3a) or with AS-ODN (Fig. 3b) for four days contained the cap stage tooth germ consisting of the dental papilla and the enamel organ as observed *in vivo*, indicating that, at least at the morphological level, AS-ODN did not affect tooth development during the first four days of culture. Although explants cultured without ODN for seven days contained the early bell stage tooth germ (Fig. 3c), explants cultured with AS-ODN for this same period showed an impaired development of the dental papilla with low numbers of mesenchymal cells and reduced volumes of mesenchyme (Fig. 3d). Tooth germ explants removed from E12.5 mice and cultured without ODN (Fig. 4a) or with RS-ODN for 14 days (Fig. 4e) were observed *in vivo* to contain well-developed tooth germs at the late bell stage; these consisted of the dental papilla and the enamel organ. On the other hand, explants cultured with AS-ODN consisted of bulky cystic tissue full of undifferentiated epithelial cells, and no tooth germ-like structure was recognized (Fig. 4b). Although the explants cultured without ODN contained a normal tooth germ consisting of the enamel organ (epithelium) and the dental papilla (mesenchyme) as observed *in vivo* (Fig. 4c), silver impregnation staining of the sections revealed that those cultured with AS-ODN consisted of large numbers of undifferentiated epithelial cells, limited numbers of undifferentiated mesenchymal cells, and a lack of any tooth germ-like structure (Fig. 4d). These observations indicated that the Lhx8-AS-ODN-treatment of tooth germs selectively suppressed the proliferation of the mesenchymal cells of the tooth germs. The results of this experiment are summarized in Table 2.

Table 2. Effect of ODN-treatment on tooth germ explants.

Treatment	No. of specimens	Development of tooth germs	
		Normal	Irregular ^a
AS-ODN	59	0	59
RS-ODN	16	16	0
Without ODN	54	54	0

AS: antisense, ODN: oligodeoxynucleotides, RS: random sequence. ^aTooth germ explants showed an irregular cystic structure, and no tooth germ-like structure was found. ^{**}The incidence of irregular bulky tissues was significantly greater ($p < 0.001$, χ test) in AS-ODN-treated explants than in explants treated with RS-ODN or without ODN.

Fig. 3. Effect of *Lhx8*-AS-ODN on the development of tooth germs cultured for four and seven days. HE-stained sections of tooth germs explanted from E12.5 mice and cultured without ODN (left column) or with AS-ODN (right column) for four (**a**, **b**), or seven (**c**, **d**) days are shown. Note that, although the effect of AS-ODN-treatment on the morphology of tooth germs is not obvious in explants cultured for four days (**b**), the development of dental papilla is markedly suppressed in an explant treated for seven days (**d**). DP: dental papilla, EO: enamel organ. Bars: 100 μ m

Effect of Lhx8-AS-ODN-treatment on the cell proliferation of tooth germs

In the tooth germs in explants removed from E12.5 mice and cultured for seven days without ODN, BrdU-labeled cells were observed in the dental papilla and in the inner and outer enamel epithelium (Fig. 5a). However, in the tooth germs of explants cultured with *Lhx8*-AS-ODN, although numerous BrdU-labeled cells were found in the

inner and outer enamel epithelium, few labeled cells were observed in the dental papilla (Fig. 5b).

Effect of Lhx8-AS-ODN-treatment on apoptosis in tooth germs

TUNEL-positive cells were scarce in tooth germs of explants cultured without ODN (Fig. 6a). However, in

Fig. 4. Effect of Lhx8-AS-ODN-treatment for 14 days on the development of cultured tooth germs. HE- (**a**, **b**, **e**) and silver impregnation (**c**, **d**) stained sections of tooth germ explants from E12.5 mice cultured for 14 days are shown. Note that explants cultured without ODN contain normal tooth germs at the late bell stage (**a**), whereas a tooth germ explant cultured with AS-ODN contains bulky cystic ectoderm consisting of undifferentiated epithelial cells and a limited number of undifferentiated mesenchymal cells, though no tooth germ has formed (**b**). **c** and **d**. Darkly stained mesenchyme is easily distinguished from the faintly stained ectoderm by silver impregnation staining. Note that the control explant contains a normal tooth germ (**c**) but that the AS-ODN-treated explant consists of a large amount of clear ectoderm and a small amount of mesenchyme (arrows) with no tooth germ-like structure (**d**). **e**. Tooth germ cultured with RS-ODN is not affected and shows a well developed late bell stage. DP: dental papilla, EO: enamel organ. Bars: 200 μ m

Fig. 5. Effect of *Lhx8*-AS-ODN-treatment on BrdU labeling in tooth germ explants removed from E12.5 mice and cultured for seven days. Explants removed from E12.5 mice and cultured for seven days without ODN (**a**) or with AS-ODN against *Lhx8* (**b**) were labeled with BrdU. Although the cells of dental papilla in the explant treated without ODN are heavily labeled (**a**), those of the AS-ODN treated explant are scarcely labeled (**b**). DP: dental papilla. EO: enamel organ. Bars: 100 μ m

Fig. 6. Effect of *Lhx8*-AS-ODN-treatment on apoptosis in tooth germs. TUNEL analysis was done on the tooth germ explants removed from E12.5 mice and cultured without ODN (**a**) or with AS-ODN against *Lhx8* for 7 days (**b**). It can be seen that, although very few TUNEL-positive cells are detected in the explant cultured without ODN (**a**), a large number of TUNEL-positive cells are found in the dental papilla (**b**). DP: dental papilla, EO: enamel organ. Bars: 100 μ m

tooth germs of explants treated with AS-ODN for seven days, numerous TUNEL-positive cells were observed in the dental papilla (Fig. 6b). Although TUNEL-positive cells were found in the enamel organ of tooth germs of explants cultured both without ODN (Fig. 6a) and with AS-ODN (Fig. 6b), the numbers of positive cells in enamel organs in both groups of explants were limited.

Effect of Lhx8-AS-ODN on the ultrastructures of tooth germ explants

The cells of dental papilla of explants cultured without ODN (Fig. 7a) or with AS-ODN (Fig. 7b) for four days were normal. Some cells of dental papilla of explants cultured with AS-ODN for five (Fig. 7c) or seven (Fig. 7d) days showed pyknotic features. However, epithelial cells,

Fig. 7. TEM examination of the effects of Lhx8-AS-ODN. Tooth germ explants removed from E12.5 embryos cultured for four (a, b), five (c) or seven days (d, e, f) without AS-ODN (a, f) or with AS-ODN (b, c, d, e). Note the presence of apoptotic mesenchymal cells (*) in the dental papilla of explants cultured for five (c) or seven days (d) with AS-ODN. Such cells are hardly observed in the mesenchyme of tooth germs cultured for four days with AS-ODN (b), in the epithelium (e) of tooth germs cultured for seven days with AS-ODN or in the mesenchyme (a, f) of tooth germs cultured without AS-ODN for four or seven days, respectively. Bars: 2 μm (a, f), 3 μm (b, e), 1 μm (c, d)

even in the explants cultured with AS-ODN for seven days, showed normal structures (Fig. 7e). Cells of dental papilla of control explants cultured for seven days without ODN were normal (Fig. 7f).

Discussion

Our *in situ* hybridization study on the expression of *Lhx8* in tooth germs of mouse molars revealed that *Lhx8* was expressed in the dental mesenchyme of tooth germs during the bud to early bell stage, but not in the dental epithelium. The expression of *Lhx6* and *Lhx7* is almost identical during tooth development (Grigoriou *et al.*, 1998); they are first detectable in a subdomain of the first branchial arch of E9.5, and are restricted to the neural crest-derived mesenchyme throughout the odontogenesis period. *Lhx7*, thought to be similar to or the same as *Lhx8*, is also expressed in the mesenchyme of the first branchial arch (Tucker *et al.*, 1999). Since *Lhx6* and *Lhx7* have respective homologies of 86% and 97% with *Lhx8*, it is reasonable that *Lhx6* and *Lhx7* show similar expression patterns with that of *Lhx8* during the early stages of tooth development. Although *Lhx6* and *Lhx7* are expressed in the dental papilla at the late bell stage (Grigoriou *et al.*, 1998), *Lhx8* disappears from the dental papilla. These results suggest that *Lhx8* regulates the genes expressed in the cells of dental mesenchyme before the secretion of dentin and enamel.

To clarify the possible function(s) of *Lhx8* during tooth morphogenesis, we used an organ culturing system using AS-ODN. RT-PCR analysis showed that, although the treatment of tooth germ explants with *Lhx8*-AS-ODN for four days had very little or no effect on the levels of *Lhx6*, *Lhx8*, *Msx1*, *Msx2* and *Bmp4*, prolonged treatment, i.e. treatment for seven days, suppressed the expression of these genes. The histological observations that the mesenchyme was specifically affected morphologically by the application of *Lhx8*-AS-ODN onto the cultured tooth germs suggest that *Lhx8* regulates the expression of genes in the mesenchymal cells that participate in cell survival and proliferation. Although AS-ODN-treatment did not affect proliferation of the dental epithelium, it did prevent its differentiation in accordance with the suppression of *Lhx6*, *Msx1*, *Msx2*, *Bmp4* and *Gsc*. The need for reiterative signaling and patterning between the ectoderm and mesenchyme during mammalian tooth morphogenesis (Jernvall and Thesleff, 2000) may explain the defect in the differentiation of the dental epithelium after the remarkable reduction in the amount of dental mesenchyme caused by the AS-ODN-treatment.

Morphologically, *Lhx8*-AS-ODN application onto the cultured tooth germ caused defects in tooth germs by the

incomplete development of mesenchyme. The TUNEL method and ultrastructural observations revealed the existence of many apoptotic cells in the dental mesenchyme of explants cultured with AS-ODN. During normal tooth development, some apoptotic cells are observed: In cap stage tooth germs, apoptotic cells are concentrated in the primary enamel knot, a temporary cell mass, but they soon disappear because of phagocytosis by neighboring cells (Nozue, 1971; Viriot *et al.*, 1997). Apoptosis is also observed in ameloblasts and cells of the papillary layer, in stellate reticulum cells, and in stratum intermedium cells in late bell stage tooth germs (Nishikawa and Sasaki, 1995; Vaahtokari *et al.*, 1996). These reports reveal that, in normal tooth development, apoptosis occurs predominantly in the epithelium, but seldom in the mesenchyme of tooth germs. Our results therefore suggest that *Lhx8* regulates the genes involved in the apoptosis of mesenchymal cells. It has been suggested that *Msx2*, epidermal growth factor (EGF), FGF4, and BMP4 are involved in apoptosis during tooth development; here, EGF and FGF4 have been shown to suppress the apoptosis of dental mesenchyme *in vitro* (Vaahtokari *et al.*, 1996).

Application of *Lhx6*-AS-ODN or *Lhx8*-AS-ODN onto the cultured tooth germs reduced the expression of both *Lhx6* and *Lhx8*. The reduced expression by an application of AS-ODN may derive from the high homology between *Lhx6* and *Lhx8* (Matsumoto *et al.*, 1996b). Moreover, *Lhx8*-AS-ODN treatment onto cultured tooth germs for seven days reduced the expression of *Msx1*, *Msx2*, *Bmp4* and *Gsc*. No downstream of *Lhx8* has been detected, but the reduction of *Msx1*, *Msx2*, *Bmp4* and *Gsc* expression may also work together in irregular tooth morphogenesis after the application of *Lhx8*-AS-ODN onto cultured tooth germs. When bud stage tooth germs removed from E12.5 mice were cultured with *Lhx8*-AS-ODN for four days, they developed to cap stage teeth just as did those cultured without ODN. The epithelium of the tooth germs from E9.5 to E12.0 can signal to induce development, but this capability then shifts to the mesenchyme at the early bud stage (Kollar and Baird, 1969; Mina and Kollar, 1987; Lumsden, 1988). This shifting of the ability of induction from the ectoderm to the mesenchyme has been thought to be induction by BMP4, which is stimulated by *Msx1* in the mesenchyme (Chen *et al.*, 1996; Bei *et al.*, 2000). These reports and our data suggest that *Lhx8* is not involved in the signaling system of BMP4 during the bud to cap stage period.

RT-PCR revealed that the application of *Lhx6*-AS-ODN or *Lhx8*-AS-ODN onto cultured tooth germs resulted in an upregulation of *Gsc* expression. *Lhx7* is expressed prior to *Gsc*, and *Gsc* is repressed by *Lhx7* (Tucker *et al.*, 2000). Thus, the applications of both *Lhx6*-AS-ODN and *Lhx8*-AS-ODN to the cultured tooth germs caused the suppres-

sion of *Lhx6* and *Lhx8*, which have a high homology with *Lhx7*, and may result in the upregulation of *Gsc*.

The existence of transient putative tooth anlage in the prospective mouse upper diastemal region has been documented in morphological studies (Peterkova, 1983). Although *Msx2* is expressed in the incisor and molar tooth germs, it is not expressed in the diastemal dental rudiments after the epithelial thickening stage (Tureckova *et al.*, 1995). These data suggest that the deficiency of *Msx2* products may play a role in retarding the growth of diastemal dental primordia, resulting in their subsequent involution. *Msx2* deficient mice exhibit the inhibition of apoptosis in the stellate reticulum and stratum intermedium of the enamel organ and the arrest of tooth development (Satokata *et al.*, 2000), suggesting that *Msx2* induces apoptosis in tooth development. Moreover, an *in vitro* study suggested that BMP4 induces programmed cell death via an *Msx2* mediated pathway (Marazzi, *et al.*, 1997). *BMP4* is expressed in the primary enamel knot, which soon disappears due to apoptosis (Tucker *et al.*, 2000). These findings strongly suggest the involvement of BMP4 in apoptosis during tooth development. Furthermore, antagonistic effects of FGF4 on apoptosis induced by BMP4 in chick limb buds have been reported (Buckland *et al.*, 1998). Therefore, expression of *Msx2* and *BMP4* may be reduced after cultures with Lhx8-AS-ODN for seven days, and Lhx8 may inhibit apoptosis by inducing EGF and FGF4, or by repressing BMP4 and *Msx2*.

Recently, Zhao *et al.* (1999) reported on *Lhx8* knockout mice in which a cleft palate was induced by impaired contact and fusion of the palatal shelves due to a reduction in their size caused by a reduction in cell proliferation; tooth development, however was not affected. This indicates that the loss of function due to *Lhx8* deficiency is thought to be compensated by other genes. In the present study, the treatment of explants with Lhx8-AS-ODN induced abnormal tooth germ development. At present, the difference in phenotypes of tooth development between total deficiency in *Lhx8* and its mRNA suppression by antisense ODN remains unknown. The present data showing that *Lhx8* mRNA, was suppressed in explants only when they were treated for seven days may indicate that the spatiotemporal suppression of *Lhx8* does not allow other genes to compensate for the function of the gene.

In conclusion, *Lhx8* expression was detected in the mesenchyme of mouse molar tooth germs during the bud to early bell stages, and the treatment of tooth germs with antisense ODN against this gene induced a marked reduction in the number of dental mesenchymal cells due to apoptosis. These results suggest that the expression of *Lhx8* is essential for the proliferation and survival of dental mesenchymal cells during tooth development.

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