Cell Tissue Res (2002) 310:201–212
DOI 10.1007/s00441-002-0611-2

REGULAR ARTICLE

**Makoto Abe · Yoshihiro Tamamura
Hiroyuki Yamagishi · Takashi Maeda · Joji Kato
Makoto J. Tabata · Deepak Srivastava
Satoshi Wakisaka · Kojiro Kurisu**

Tooth-type specific expression of dHAND/Hand2: possible involvement in murine lower incisor morphogenesis

Received: 16 January 2002 / Accepted: 19 June 2002 / Published online: 14 September 2002

Abstract dHAND/Hand2 is a basic helix-loop-helix transcription factor required for the development of the heart, pharyngeal arches, and vasculature and is expressed during embryogenesis. However, there are no reports on the involvement of the *dHAND* gene in tooth development. In the present study, the expression of *dHAND* was examined in developing tooth germs of mice. The *dHAND* gene was expressed in the mesenchyme of the presumptive incisor region of the lower jaw at an early stage and in the mesenchyme of the lower incisor tooth germ at a later stage. However, the *dHAND* gene was not expressed in the upper incisor region or the upper and lower molar regions during jaw development. Treatment of tooth germ explants of lower incisors with antisense oligodeoxinucleotide (ODN) against *dHAND* prevented the differentiation of tooth germ cells, including ameloblasts and odontoblasts, the formation of dentin and enamel, and the proliferation of tooth germ cells and increased the apoptosis of tooth germ cells, suggesting that *dHAND* is essential for these cells during development. On the other hand, the treatment of tooth germ explants of upper incisor and upper or lower molars did not induce severe effects on their development. Treatment of the explants with basic fibroblast growth factor in association with antisense ODN partially rescued them from the effects of antisense ODN. The present results suggest that the *dHAND* gene plays important roles in type-specific development of lower incisors, and that basic fibroblast growth factor is involved downstream of the dHAND pathway in tooth germ cells.

Keywords *dHAND* · Tooth germ · Incisor · Basic fibroblast growth factor · Antisense oligodeoxinucleotide · Mouse (ICR)

M. Abe (✉) · Y. Tamamura · J. Kato · M.J. Tabata
S. Wakisaka · K. Kurisu
Department of Oral Anatomy and Developmental Biology,
Osaka University Graduate School of Dentistry, 1-8, Yamadaoka,
Suita, Osaka 565-0871, Japan
e-mail: makoto@dent.osaka-u.ac.jp
Tel.: +81-6-68792874, Fax: +81-6-68792875

T. Maeda
Department of Radiology and Radiation Oncology,
Osaka University Graduate School of Dentistry, 1-8, Yamadaoka,
Suita, Osaka 565-0871, Japan

H. Yamagishi · D. Srivastava
Department of Pediatrics and Molecular Biology,
University of Texas Southwestern Medical Center,
6000 Harry Hines Blvd., Dallas, TX 75390-9148, USA

K. Kurisu
Yukioka School of Allied Health Professions, 2-2-11, Ukita,
Kita-ku, Osaka 530–0021, Japan

Introduction

Murine teeth develop through a series of morphologically distinct stages, and reciprocal epithelial-mesenchymal interactions are essential for the development of teeth and branchial arches. Many regulatory molecules, such as sonic hedgehog (SHH), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and WNTs participate in tooth development (for reviews, see Thesleff and Nieminen 1996; Maas and Bei 1997; Peters and Balling 1999; Jernavall and Thesleff 2000). Furthermore, the products of non-Hox-type homeobox genes, such as *Dlx*, *Msx*, and *Barx*, are involved in these processes (Jowett et al. 1993; Mackenzie et al. 1991; Thomas et al. 1997; Tissier-Seta et al. 1995).

Mammalian dentitions are highly patterned, e.g., incisors (single-cusped teeth) are present in the distal region, whereas molars (multi-cusped teeth) are found in the proximal region. In rodents, the development of incisors is different from that of molars in that (1) incisors rotate proximo-distally after the epithelial bud forms, (2) enamel formation is observed only on the labial side, and (3) incisors continuously erupt throughout the animal's life by virtue of stem cells distributed to the labial apex of the incisor (Smith and Warshawsky 1975a, 1975b, 1976; Harada et al. 1999). Several candidate genes have been proposed as determining the type of teeth. *Dlx* and

Msx genes are expressed in the restricted region of the branchial arch at a very early stage, viz., embryonic days (E) 9.5–10.5, and are subsequently expressed in both incisors and molars where they may play a role in cell differentiation (Thomas et al. 1997; Jowett et al. 1993; MacKenzie et al. 1991, 1992; Zhao et al. 2000). To date, *Barx1* is the only gene known to be expressed in a region-specific or tooth-type-specific fashion, being expressed in the proximal region of the branchial arches at an early stage but being confined to molar tooth germs in a later stage (Tissier-Seta et al. 1995). Double-knockout *Dlx-1* and *Dlx-2* mice only show developmental defects in the upper molars, indicating that these two genes are essential for the specific development of the upper molar region, and that dentition is determined in the early stage of branchial arch development (Thomas et al. 1997; Weiss et al. 1998). *Activin-beta-A*-null embryos exhibit developmental arrest of the upper and lower incisors and lower molars but show normal development of upper molars (Ferguson et al. 1998, 2001). Moreover, the involvement of BMPs in dentition has been demonstrated in experiments by using Noggin, an effective inhibitor of BMP signaling: the exposure of early branchial arch (E9–10) to Noggin results in transformations of incisors to molars evaluated from cusp morphology (Tucker et al. 1998). These results suggest that dentitions are determined in an early stage of branchial arch development under the control of several genes.

dHAND, also known as Hand2/Thing2, is a basic helix-loop-helix (bHLH) transcription factor expressed during embryogenesis and is required for the development of several organs (Srivastava et al. 1995, 1997; Hollenberg et al. 1995; Thomas et al. 1998; Yamagishi et al. 2000). During mouse development, the *dHAND* gene is expressed in the heart, neural-crest-derived mesenchymal cells in the pharyngeal arches, and limb buds (Hollenberg et al. 1995; Srivastava et al. 1995, 1997; Fernandez-Teran et al. 2000; Charité et al. 2000). *dHAND* is predominantly expressed in the right ventricle region of the heart and in the posterior region of limb buds. Mice lacking *dHAND* display hypoplasia of the right ventricular region, and transgenic mice misexpressing *dHAND* in the anterior region of the limb bud exhibit pre-axial polydactyly with duplication of posterior skeletal elements, suggesting that region-specific expression of *dHAND* is important for normal development of the heart and limbs (Srivastava et al. 1997; Fernandez-Teran et al. 2000; Charité et al. 2000). Pharyngeal arch defects and vascular defects have also been described in *dHAND*-null embryos (Thomas et al. 1998; Yamagishi et al. 2000). Moreover, the expression of *Msx-1*, which is considered to be one of the key molecules in tooth development (Satokata and Maas 1994), is downregulated in the neural-crest-derived mesenchyme of *dHAND*-null branchial arches, suggesting that Msx-1 is downstream of the dHAND signaling pathway in early branchial arch development (Thomas et al. 1998; Mina 2001). Dentition is determined at the early stage of branchial arch development when *dHAND* is expressed, and since *Msx-1* is thought to be downstream of the dHAND signaling pathway, it is speculated that the *dHAND* gene also participates in the formation of dentition and the morphogenesis of tooth germs.

In the present study, we first present the expression patterns of *dHAND* in the mandibular arch and tooth germs of mice and compare its expression pattern with *Msx-1*. Secondly, to investigate the role of *dHAND* in the development of tooth germs, we examine the effect of antisense oligodeoxinucleotide (ODN) against *dHAND* in arresting the translation of dHAND on the cultured incisor tooth germs of lower incisors. Finally, in order to search for factors participating downstream of the dHAND signaling pathway, the effects of exogenous regulatory molecules, such as basic FGF (bFGF), SHH and BMP4 on the antisense ODN-treated incisor tooth germs are examined.

Materials and methods

Animals

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

In situ hybridization

Radioactive in situ hybridization was carried out as described previously (Maeda et al. 2001). Briefly, animals at E12.5–P0.5 were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and embedded in paraffin. They were sectioned at a thickness of 7–9 μm and mounted on 3-aminopropyltriethoxysilane-coated glass slides (Muto Pure Chemicals, Tokyo, Japan). Paraffin sections were deparaffinized and rehydrated through a descending ethanol series, treated with proteinase K (TaKaRa, Ohtsu, Japan), and post-fixed in 4% paraformaldehyde. Hybridization was performed (1×10^5 cpm/slide) at 56°C for 16 h. After hybridization, the sections were washed with 5×SSC (saline sodium citrate; 1×SSC=150 m NaCl, 15 mM sodium citrate, pH 7.0) containing 10 mM dithiothreitol (DTT) at 50°C for 30 min, SF solution (2×SSC, 50% formamide, 20 mM DTT) at 65°C for 30 min, and NTE buffer (0.5 M NaCl, 10 mM TRIS-HCl, 1 mM EDTA) at 37°C for 30 min, treated with 20 mg/ml RNase A (Wako Pure Chemicals, Osaka, Japan) at 37°C for 30 min, and then washed with 2×SSC and 0.1×SSC at room temperature. The sections were dehydrated through graded series of ethanol, air-dried, coated with NTB-2 emulsion (Eastman Kodak) and exposed for 2 weeks in the dark. Microphotographs were taken by both bright- and dark-field optics. Several dark-field images were incorporated by the Adobe Photoshop (version 5.5) with red images. The full-length *dHAND* probe used in this study was as described previously (Srivastava et al. 1997), and the *Msx1* probe (900 bp in pSP72: a gift from Dr. Masahiro Iwamoto, Osaka University Graduate School of Dentistry) was used for sense and antisense cRNA preparation.

Total RNA preparation and reverse transcription/polymerase chain reaction

Total RNA preparation and reverse transcription/polymerase chain reaction (RT-PCR) were performed as described previously (Maeda et al. 2000). Total RNAs from tooth germs of E16.5 mice and

E14.5 incisor tooth germs cultured for 3 days with or without ODNs were prepared by using the RNeasy mini kit (Qiagen, Hilden, Germany), digested with RNase-free DNase I (Promega, USA), and then reverse transcribed with 200 U Moloney murine leukemia virus reverse transcriptase (Wako Pure Chemicals) on a 20-µl scale. Aliquots of 1 µl reverse-transcribed samples were used for the following PCR. Thirty cycles of denaturation (95°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 45 s) were carried out in the DNA thermal cycler PERSONA (TaKaRa). The primer sequences for mouse *dHAND* were 5'TACCAGCTACA TCGCCTACCT3' and 5'TCACTGCTTGAGCTCCAGGG3', generating a 242-bp fragment. The primer sequences for the mouse glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) were 5'AAGCAACATAGACGTTGTCGC3' and 5'AATCAACACCTT CTTCGCACC3', generating a 286-bp fragment. The amplified products were analyzed by 1.5% agarose gel electrophoresis.

Organ culture of incisor tooth germs of mice

Explants containing incisor tooth germs of the lower incisor were obtained from mandibles of E14.5 mice and cultured in a modified Trowell's system for periods of up to 11 days as described elsewhere (Tabata et al. 1996; Liu et al. 2000). Explants were cultured in BGJb medium (Fitton-Jackson's modified BGJ; Gibco BRL, Gaithersburg, USA), containing 100 µg/ml ascorbic acid (Nakai Chemical, Kyoto, Japan) and 100 U/ml penicillin-streptomycin (Gibco BRL). The initial pH was adjusted to 7.4, and the explants were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. The medium was changed every 2 days. For the control experiment, tooth germs of the upper incisor and upper and lower molars were dissected from the same E14.5 mice from which tooth germs of the lower incisors were dissected. They were cultured by the same method.

Translation arrest of *dHAND* gene expression by antisense ODN

For the translation arrest of *dHAND*, we designed antisense-phosphorothionate-ODN (5'ACTCGGGGCTGTAGGAC3'; for 17-mer of nucleotides 191–207 of mouse dHAND: GenBank accession no. AJ131846), which is exactly the same as that used by Srivastava et al. (1995) and Howard et al. (1999). The corresponding sense (5'GTCCTACAGCCCCGAGT3') and random sequence ODN (5'GATAGCGTCACGCTAGG3') were designed for control experiments. A homology search was performed by BLAST. The ODNs were synthesized by Hokkaido System Science (Sapporo, Japan). They were added to the culture medium at a final concentration of 30 µM, and the culture medium was changed every 2 days. The optimal concentration of antisense ODN, 30 µM, was determined after testing various concentrations (5 nM–30 µM) on the basis of the morphology of the cultured explants. In the rescue experiment, 30 µM antisense ODN together with 100–200 ng/ml bFGF (R and D Chemicals, Minneapolis, USA), 100–200 ng/ml BMP4 (Genzyme, Mass., USA) or 100–200 ng/ml SHH (R and D Chemicals) were added to the culture simultaneously. The explants were cultured for 3 or 6 days, fixed in 10% neutralized buffered formalin, embedded in paraffin, and sectioned at a thickness of 7 µm. They were stained with hematoxylin-eosin and observed under the light microscope.

Van Gieson staining, keratin immunohistochemistry, proliferation assay, TUNEL staining, and alkaline phosphatase activity detection

For van Gieson staining of whole-mount tooth germs, tissues cultured for 6 days were fixed with 10% neutralized-buffered formalin, stained with van Gieson solution (1.25% 2,4,6-trinitrophenol, 0.15% acidic fuchsin in distilled water), dehydrated with ethanol, and finally cleared with xylene.

For keratin immunohistochemistry, tooth germ explants of incisors treated either with antisense or sense ODN were freshly embedded in OCT compound. Sections were cut at a thickness of 14 µm on a cryostat and thaw-mounted onto poly-L-lysine-subbed glass slides. Sections were dried, fixed with 10% neutralized-buffered formalin, and treated in 0.1 M sodium citrate buffer (pH 6.0) for 10 min in a microwave oven for antigen retrieval (Cattoretti et al. 1992). They were incubated with monoclonal mouse anti-keratin (1:200; clone K8. 13; Sigma, St. Louis, USA) overnight at room temperature. This monoclonal antibody recognizes epitopes of cytokeratins 1, 5–8, 10, 11, and 13 (Gigi-Leitner et al. 1986). After being rinsed in phosphate-buffered saline, sections were incubated with fluorescein-isothiocyanate-conjugated horse anti-mouse IgG (1:100; Vector). These sections were coverslipped with PermaFluor (Immunon, Pittsburgh, PA, USA) and examined under an Olympus fluorescence microscope equipped with an appropriated exciting filter.

Proliferation assay was performed by using a cell proliferation kit (RPN20; Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's protocols. Tooth germ explants of lower incisors cultured for 3 days were labeled with labeling reagent for 4 h at 37°C and then fresh-frozen in OCT compound. Cryosections were made at a thickness of 14 µm and fixed by 10% neutralized-buffered formalin for 15 min at room temperature. Immunodetection of the labeled cells was performed according to the protocol of the manufacturer.

Adjacent sections were used for detection of alkaline phosphatase activity. After fixation with 10% neutralized buffered formalin and several rinses in distilled water, sections were treated with 0.05% Fast Ted BB salt (Sigma), 0.05% naphthol AS-MX phosphate (Sigma) in 0.1 M NaCl, 0.1 M TRIS-HCl (pH 9.5) and 50 mM $MgCl_2$. The reaction was stopped by rinsing in tap water.

TUNEL staining was performed in paraffin sections as described elsewhere (Thomas et al. 1998). Paraffin-embedded sections were deparaffinized and treated with 10 µg/ml proteinase K (TaKaRa) and then with 0.3 U/ml terminal transferase (Roche Molecular, Indianapolis, USA). The sections were subsequently labeled with digoxigenin-11-dUTP at 37°C for 60 min. Labeled cells were detected with alkaline-phosphatase-conjugated anti-digoxigenin Fab fragment (Roche Molecular, 1:1000) and followed by nitroblue tetrazolium chlotide/5-bromo-4-chloro-3-indolylphosphatate *p*-toluidine salt colorization in darkness.

Statistical analysis

To assess the effects of ODNs and exogenous molecules added to organ cultures of tooth germs, significance of the mean differences was tested by using the unpaired Student's *t* test. Samples in which normal cellular morphology was observed were counted as either normal or rescued. Differences were considered significant at the probability level of $P<0.05$ in all analyses. The statistical analysis of all data was performed on a Macintosh computer with a standard statistical package (StatView version 4.5, Abacus Concepts, Berkeley, USA).

Results

Expression of *dHAND* and *Msx-1* in orofacial region of embryonic mouse

In E12.5 mice at the initiation stage of tooth development, *dHAND* was first expressed in the distal (presumptive incisor region) mesenchyme of the mandible (Fig. 1A, C), whereas it was not expressed in the proximal (presumptive molar region) mesenchyme of the mandible (Fig. 1A). In E14.5–P0.5 mice, *dHAND* expression was detected in the dental papilla of the mandibular incisor (Figs. 1D, 2A, C, E–G), but not in tooth germs of the lower molars (Figs. 1F, 2B), suggesting that

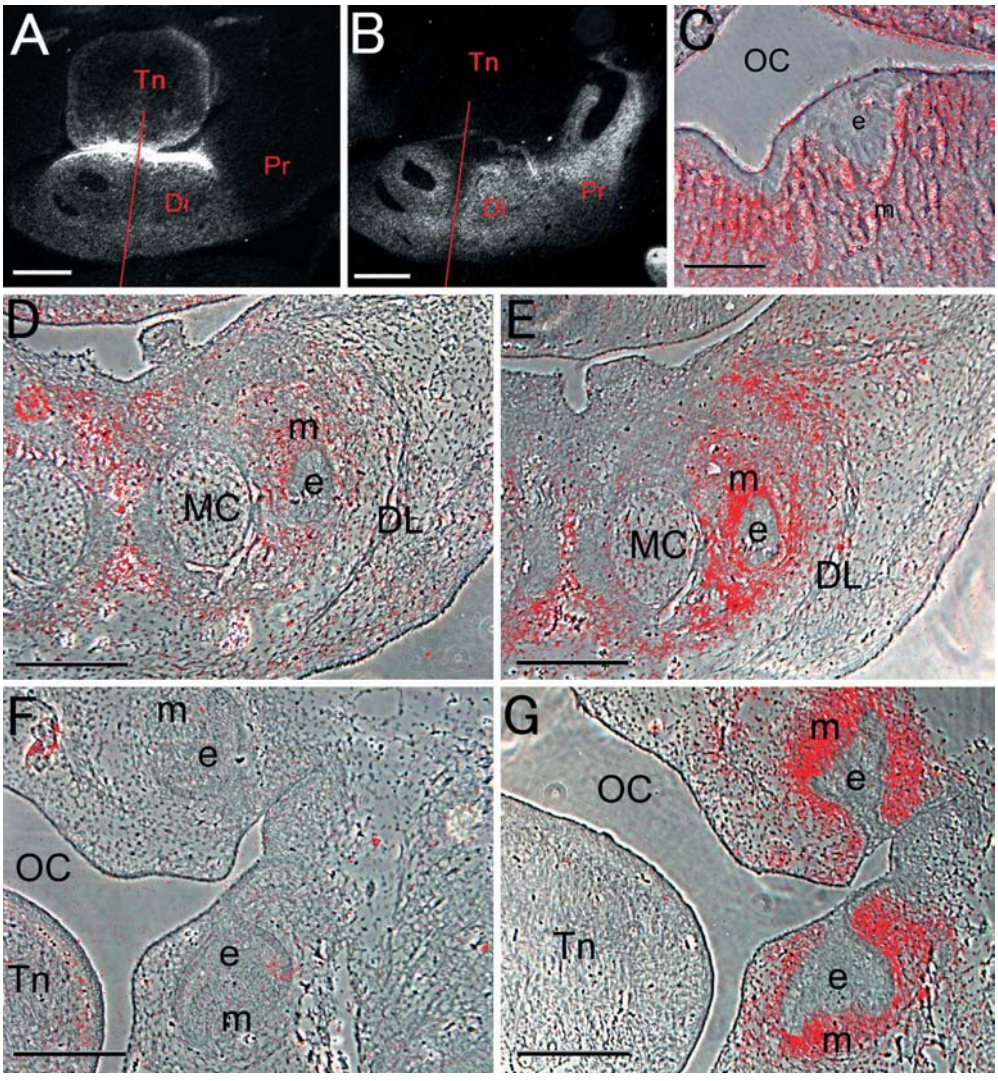

Fig. 1 In situ hybridization of expression of *dHAND* (**A, C, D, F**) and *Msx1* (**B, E, G**) in the mandibles of E12.5 (**A–C**) and E14.5 mice (**D–G**). *Red lines* in **A** and **B** indicate the median line of the mandible (*Tn* tongue, *Pr* proximal region, *Di* distal region, *OC* oral cavity, *e* dental epithelium, *m* dental mesenchyme, *MC* Meckel's cartilage, *DL* dental lamina). Dark-field images of *dHAND* (**A**) and *Msx1* (**B**) expression in the frontal sections of the orofacial region of E12.5 mouse. **C** Expression of *dHAND* in a sagittal section of mandible of E12.5 mouse is superimposed on the bright-field image as red spots. Expression of *dHAND* (**D, F**) and *Msx1* (**E, G**) of incisor (**D, E**) and molar (**F, G**) tooth germs of E14.5 mouse. *dHAND* expression is detected in dental mesenchyme of incisor but not in molar tooth germs, whereas *Msx1* expression is detected in dental mesenchyme of both incisor and molar. *Bars* 300 μm (**A, B**), 100 μm (**C**), 200 μm (**D–G**)

dHAND is expressed tooth-type specifically. Interestingly, *dHAND* expression was not detected in tooth germs of the maxilla (Fig. 1F), including the tooth germs of the upper incisor (data not shown). The intensity of *dHAND* expression coincided with the differentiation of odontoblasts in the lower incisor (Fig. 2E–G). Dental papilla cells other than odontoblasts of the lower incisor expressed *dHAND* weakly (Fig. 2E–G). In contrast, *Msx1,* which has been reported to be expressed in the dental mesenchyme, was expressed in both incisor and molar tooth germs of both the maxilla and mandible (Figs. 1B, E, G, 2D). By RT-PCR analysis, *dHAND* gene expression was detected only in lower incisor tooth germs but not in other tooth germs including the upper incisor (Fig. 3).

Morphology of cultured tooth germs of lower incisors

In explants cultured for 3 days (Fig. 4A–C), an apical loop-like structure on one side of the developing epithelium (Fig. 4B), differentiation of the outer layer cells of the dental papilla to odontoblasts (Fig. 4C), and the formation of dentin matrix (Fig. 4C) were observed and persisted during the culture period (Fig. 4E, F, H, I). Differentiation of the inner enamel epithelium to preameloblasts and ameloblasts was seen in explants cultured for 6 days (Fig. 4D, F) and 11 days (Fig. 4G, I). Formation of enamel matrix was detected in explants cultured for 11 days (Fig. 4I).

Effect of antisense ODN against *dHAND* on cultured tooth germs of the lower incisor

To examine the effect of antisense ODN treatment on gene expression, we performed RT-PCR analysis by using mRNA isolated from explants cultured with or without ODNs. The level of amplified PCR products derived from antisense ODN-treated explants was low compared with those derived from control and sense ODN-treated explants (Fig. 5). The products for

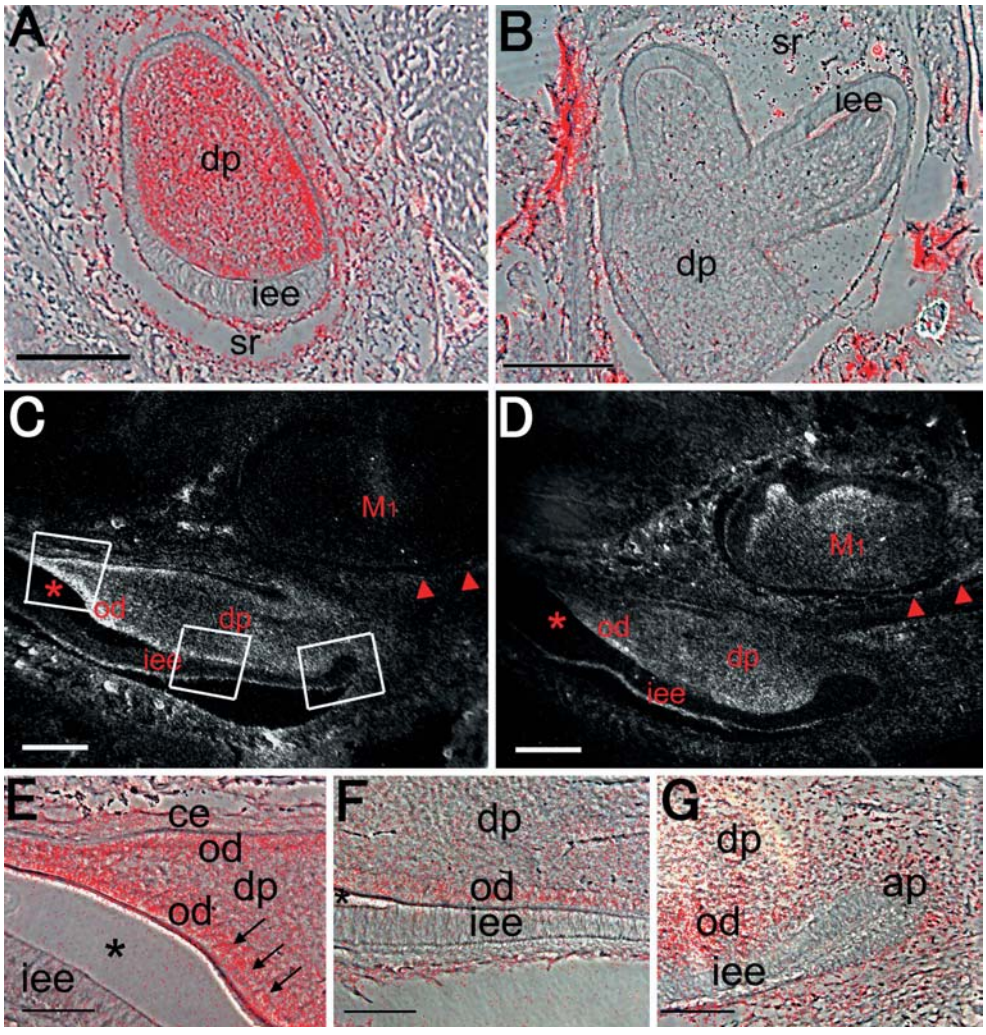

Fig. 2 In situ hybridization on the expression of *dHAND* (**A–C, E–G**) and *Msx1* (**D**) in mandibular tooth germs of E18.5 and P0.5 mice (M_1 tooth germ of first molar, *iee* inner enamel epithelium, *od* odontoblast layer, *dp* dental papilla, *asterisk* dentin matrix, *ce* cementoblast layer, *ap* apical loop). Expression of *dHAND* in incisor (**A**) and molar (**B**) tooth germs in frontal sections of the orofacial region of E18.5 mouse. Expression of *dHAND* (**C**) and *Msx1* (**D**) in sagittal section of mandible of P0.5 mouse. *dHAND* is expressed the dental papilla of incisor tooth germs but not in molar tooth germs, whereas *Msx1* is expressed in the dental papilla and surrounding mesenchymal cells of both incisor and molar tooth germs. Higher magnifications of an incisor tooth germ in **C** (*squares*) at the tip (**E**), middle (**F**), and apical (**G**) regions. Heavy labeling is detected in the differentiated odontoblasts secreting dentin matrix (*arrows* in **E**), whereas preodontoblasts (*od* in **F**) and undifferentiated dental papilla cells (*dp* in **G**) show moderate labeling. *Bars* 100 μm (**A**), 200 μm (**B**), 300 μm (**C, D**), 100 μm (**E, F, G**)

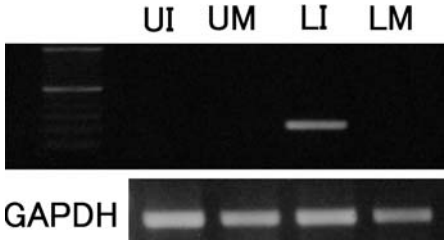

Fig. 3 Expression of *dHAND* (*top*) and *GAPDH* (*bottom*) genes in tooth germs of E16 mice as determined by RT-PCR. Expression of *dHAND* is detected only in tooth germs of the lower incisor (*LI*), but not in tooth germs of upper incisor (*UI*), lower molar (*LM*), or upper molar (*UM*)

GAPDH were used as an internal marker control and were detected in all samples including the antisense ODN treated explants (Fig. 5). After 3 days culture, although the size of tooth germ was slightly reduced and the development of the apical loop structure was poor in antisense ODN-treated explants, there was no obvious histological difference between tooth germ explants treated with sense ODN and those treated with antisense ODN (data not shown). Treatment of lower incisor explants with antisense ODN for 6 days reduced the size of the explants (Fig. 6A), whereas treatment with sense ODN showed no such effect (Fig. 6B). Treatment of the lower molar (Fig. 6C) and upper incisor (Fig. 6D) for 6 days with antisense ODN produced no obvious effect on tissue size compared with sense ODN treatment (data not shown). Histologically, in the tooth germ explants of lower incisors cultured for 6 days with antisense ODN, cells of the inner enamel epithelium and dental papilla did not differentiate, the size of the tooth germ rudiment was small, and no dentin matrix was observed (Fig. 6E), whereas the explants treated with sense ODN showed normal development (Fig. 6F) as observed in non-treated explants. Most of the explants of lower molars (Fig. 6G), upper incisors (Fig. 6H), and upper molars (data not shown) treated with antisense ODN showed normal development when they were dissected from the same embryos from which the explants of lower incisors were dissected. The effect of antisense ODN treatment on dentin formation was clearly demonstrated by van Gieson staining of the explants. The explants of the lower incisors treated

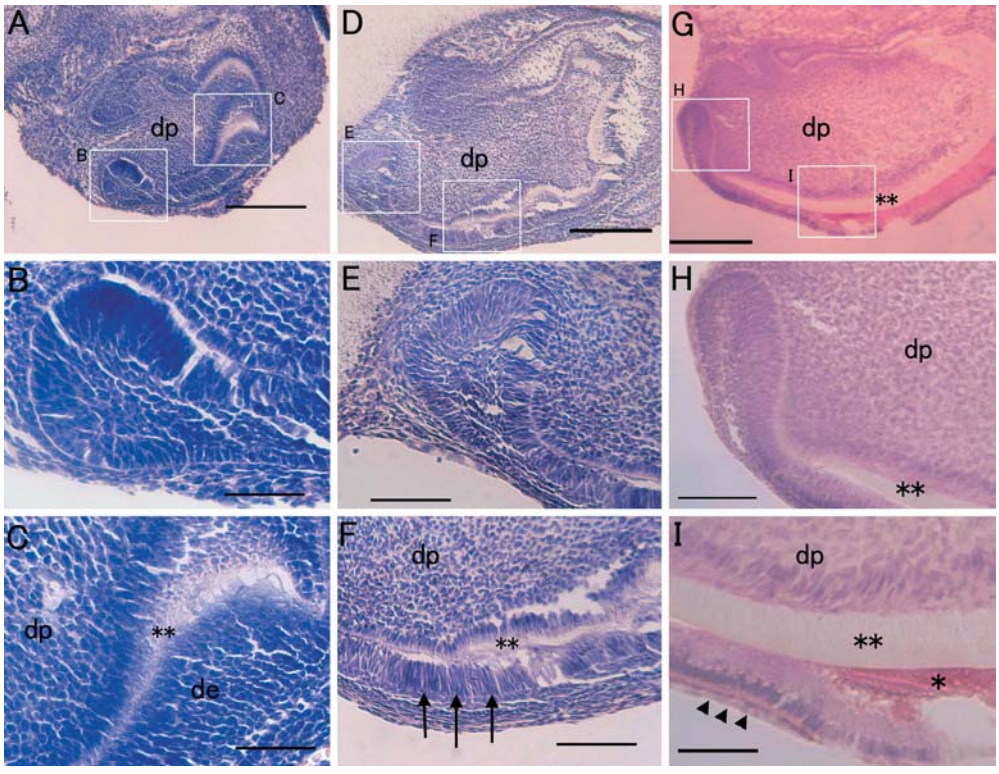

Fig. 4A–I Morphology of tooth germs of lower incisors cultured in control conditions (*dp* dental papilla, *double asterisks* dentin, *asterisk* enamel, *arrows* preameloblasts, *arrowheads* ameloblasts). Tooth germs of lower incisors removed from E14.5 mice were cultured for 3 (**A–C**), 6 (**D–F**) and 11 (**G–I**) days. **B, C** Higher magnification of indicated areas in **A**. **E, F** Higher magnification of indicated areas in **D**. **H, I** Higher magnification of indicated areas in **G**. Formation of apical loop structure is maintained (**B, E, H**); the differentiation of odontoblasts (**C, F, I**) and ameloblasts (**F, I**), and the formation of dentin (**C, F, H, I**) and enamel (**I**) are observed in this culture system. *Bars* 200 μm (**A, D, G**), 50 μm (**B, C**), 100 μm (**E, F, H, I**)

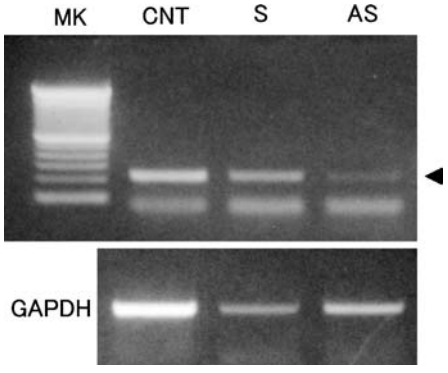

Fig. 5 Effect of antisense ODN against *dHAND* on *dHAND* gene expression in cultured tooth germs of lower incisors. Expression of *dHAND* (*top*) and *GAPDH* (*bottom*) genes in tooth germ explants from E14.5 mice cultured for 3 days without ODNs (*CNT*), or with sense (*S*) or antisense (*AS*) ODN for *dHAND* mRNA was determined by RT-PCR (*MK* molecular standard). Note that the antisense ODN treatment induced the selective suppression of the *dHAND* gene expression (*arrowhead*)

Table 1 Numbers of normal tooth germs observed after 6 days culture with antisense ODN treatment

Lower incisor	Lower molar	Upper incisor	Upper molar
0/18[a]	17/18[b]	16/18[b]	16/18[b]

[a]All of the samples showed reduction in tissue size, and normal cellular differentiation was not observed
[b]The samples that showed modest reduction in size were counted as abnormal tooth germs, but cellular differentiation was normal in all these samples at the histological level

with antisense ODN were negative for van Gieson staining (Fig. 6I), whereas positive staining, which represented dentin, was detected in the explants treated with sense ODN (Fig. 6J). Although some cultured explants of lower and upper molars and upper incisors showed a reduction in size, the differentiation of the cells was normal in all cases (Table 1). To confirm the effect of antisense ODN treatment on tissue differentiation of tooth germ explants, we performed immunostaining for keratin, which clearly discriminates epithelial tissue from mesenchyme in tooth germ. Differentiation of enamel organ was not detected in the explants treated with antisense ODN (Fig. 6K), whereas normal enamel organs were observed in the explants treated with sense ODN (Fig. 6L). Explants cultured with random sequence ODN (data not shown) showed normal structure, as observed in those cultured without ODN (see Fig. 4D–F).

Antisense ODN treatment for 3 days greatly reduced the numbers of BrdU-positive cells in tooth germ explants of lower incisors (Fig. 7G, H) compared with that following sense ODN treatment (Fig. 7A, B). Although TUNEL-positive cells were scarcely detected in tooth germs of sense ODN-treated explants (Fig. 7C, D), large numbers of TUNEL-positive cells were observed in dental papilla and dental epithelium of antisense ODN-treated explants (Fig. 7I, J). Antisense ODN treatment for 3 days reduced the alkaline phosphatase activity in dental papilla cells but up-regulated that in dental epithelial

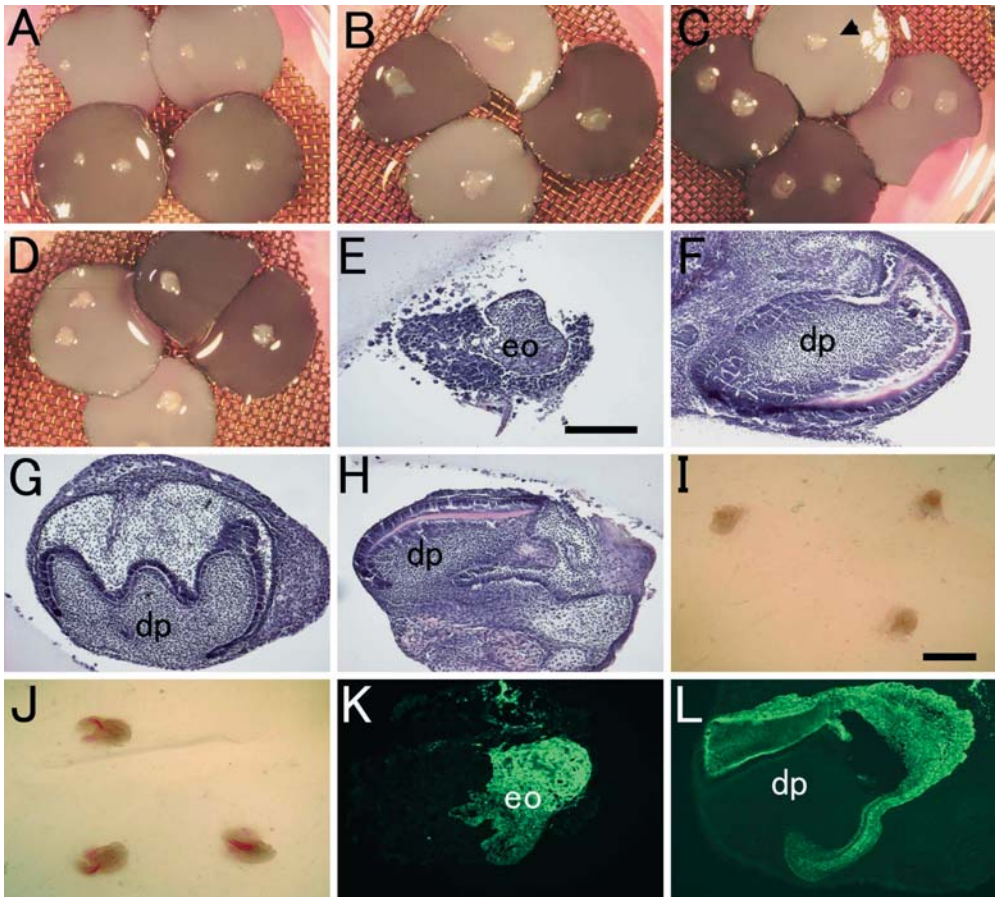

Fig. 6 Effect of ODNs on the development of cultured tooth germ explants of lower incisors (**A, B, E, F, I–L**), lower molars (**C, G**), and upper incisors (**D, H**). The tooth germ explants removed from E14.5 mice were cultured with antisense (**A, C–E, G–I, K**) or sense (**B, F, J, L**) ODN for 6 days. The explants were examined at the stereomicroscopic (**A–D, I, J**) and histological (**E–H, K, L**) levels (*dp* dental papilla, *eo* enamel organ-like structure). The lower incisor explants treated with antisense ODN show an obvious reduction of tissue size (**A**) compared with those treated with sense ODN (**B**). On the other hand, the explants of lower molar (**C**) or upper incisor (**D**) treated with antisense ODN do not show such an effect. No histodifferentiation of tooth germ is observed, and dentin is not formed in the lower incisor explants treated with antisense ODN (**E**), whereas normal differentiation is observed in the explants treated with sense ODN (**F**). Note that the antisense ODN treatment of explants of lower molar (**G**) and upper incisor (**H**) removed from the same embryo from which the lower incisor explants were removed induces normal differentiation of tooth germs. Lower incisor explants treated with antisense ODN are negative for van Gieson staining (**I**), whereas those treated with sense ODN show red positive staining (**J**). The lower incisor explants treated with antisense ODN show inhibition of the differentiation of the enamel organ, which is immunopositive for keratin (**K**), whereas those treated with sense ODN exhibit normal differentiation of this organ (**L**). *Bars* 200 μm (**E**, also applies to **F–H, K, L**), 1 mm (**I**, also applies to **J**)

Table 2 Histological analysis of the effect of ODN treatment with or without bFGF on the tooth germ explants of lower incisors from E14.5 mice cultured of 6 days (*numbers* in *parentheses* represent percentages)

Histology	Without ODNs	Sense	Antisense	Antisense+bFGF
Differentiation of ameloblasts	12/12 (100)	6/6 (100)	0/15 (0)[a]	4/12 (33)[b]
Dentin formation	12/12 (100)	6/6 (100)	0/15 (0)[a]	5/12 (41)[c]
Normal apical loop structure	12/12 (100)	6/6 (100)	0/15 (0)[a]	0/12 (0)

[a]Incidence is significantly different from that of the sense ODN-treated group at $P<0.001$
[b]Incidence is significantly different from that of antisense ODN-treated group at $P<0.05$
[c]Incidence is significantly different from that of antisense ODN-treated group at $P<0.01$

cells (Fig. 7K, L) compared with that of sense ODN-treated cells (Fig. 7E, F). Antisense ODN treatment of explants for 3 days reduced the size of tooth germs (Fig. 7H, J, L) compared with sense ODN treatment (Fig. 7B, D, F).

Effect of bFGF on antisense ODN-treated tooth germs of lower incisors

In order to investigate the role of *dHAND* in the development of incisors, we examined the effect of SHH, BMP4

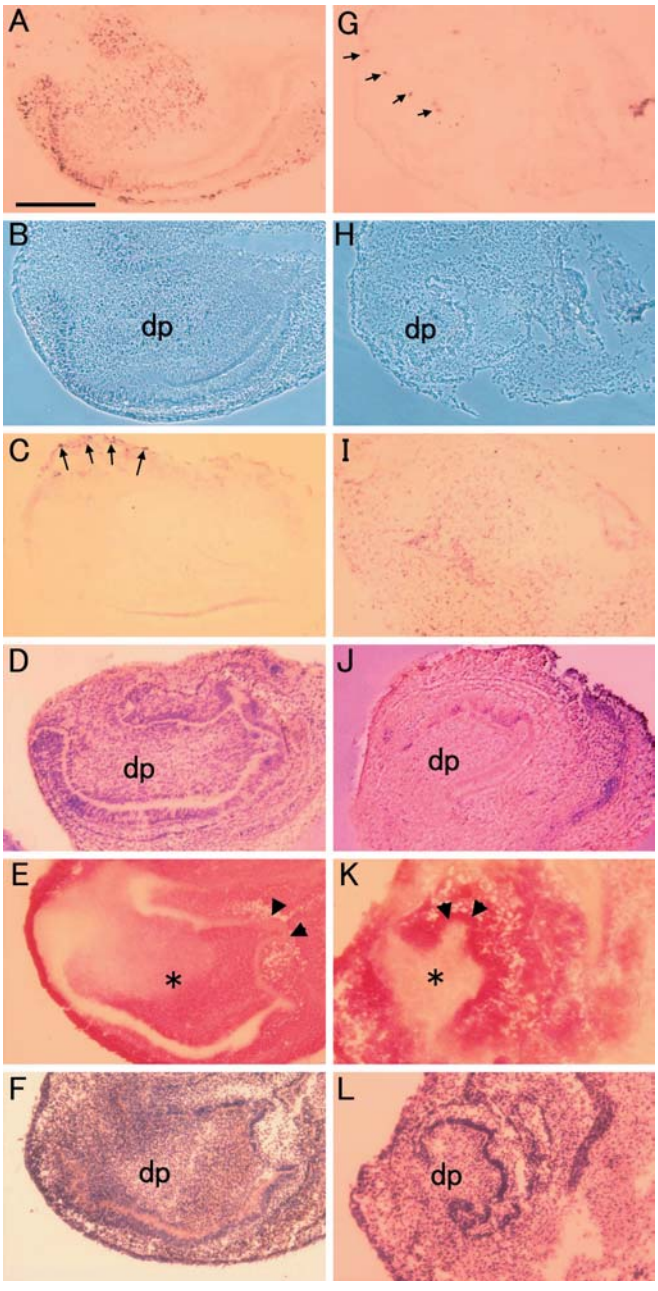

Fig. 7A–L Effect of antisense ODN treatment on the proliferation, apoptosis, and alkaline phosphatase activity of cultured tooth germ explants of the lower incisor (*dp* dental papilla). The explants removed from E14.5 mice were cultured with sense (**A–F**) or antisense ODN for *dHAND* (**G–L**) for 3 days and processed for proliferation assay (**A, G**), TUNEL analysis (**C, I**), or alkaline phosphatase histochemistry (**E, K**). **B, H** Phase-contrast images of **A** and **G**, respectively. **D, J, F, L** Hematoxylin-stained images of adjacent sections of **C, I, E**, and **K**, respectively. BrdU incorporation is greatly reduced in antisense ODN-treated tooth germs (**G**) compared with the sense ODN-treated tooth germs (**A**). TUNEL-positive cells are abundantly observed in the tooth germ in dental epithelium and dental papilla of antisense ODN-treated explants (**I**) compared with sense ODN-treated explants (**C**, *arrows*). Alkaline phosphatase activity of dental papilla cells of antisense ODN-treated explants (*asterisk* in **K**) is greatly reduced compared with sense ODN-treated explants (*asterisk* in **E**), whereas that of dental epithelial cells is up-regulated in antisense ODN-treated explants (*arrowheads* in **K**) compared with sense ODN-treated explants (*arrowheads* in **E**). In comparison with sense ODN-treated tooth germs (**B, D, F**), antisense ODN-treated ones (**H, J, L**) show a reduction in size. *Bar* 200 μm (**A**, applies also **B–L**)

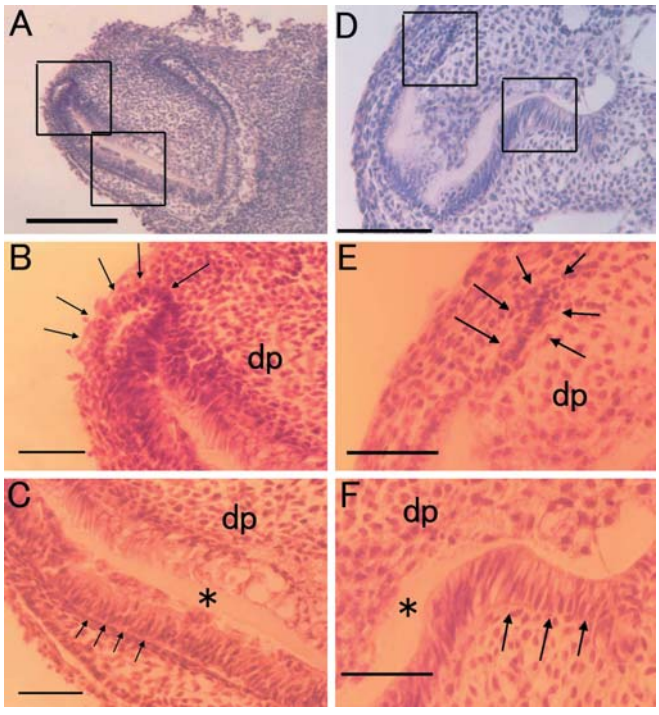

Fig. 8A–F Effect of bFGF on tooth germ explants of lower incisors treated with antisense ODN for *dHAND*. The explants removed from E14.5 mice were cultured with antisense ODN in combination with 200 ng/ml bFGF for 3 (**A–C**) or 6 (**D–F**) days. (**B, C, E, F**). *Squares* Higher magnifications of **A** in **B** and **C**, and of **D** in **E** and **F**. At 3 days of culture, the cell differentiation of pre-ameloblasts and dental papilla cells is normal (*arrows* in **C**), but the apical loop structure is somewhat irregular (*arrows* in **B**). At 6 days of culture, dentin formation (*asterisks* in **C, F**) and polarization of pre-ameloblasts (*arrows* in **F**) are observed, whereas the formation of the apical loop is poor (*arrows* in **E**). *Bars* 100 μm (**A, D**), 50 μm (**B, C, E, F**)

and bFGF, which are known to be expressed in tooth germs during the expression of the *dHAND* gene, on the tooth germ explants of lower incisor treated with antisense ODN against *dHAND*. Of the molecules examined, bFGF showed partial rescue effects. When 100 ng/ml bFGF was added with antisense ODN to the culture for 6 days, dentin formation was observed in a few explants, although the differentiation of ameloblasts and the formation of an apical loop were not detected (data not shown). When 200 ng/ml bFGF was added with antisense ODN for 3 days, relatively normal development of tooth germs was observed (Fig. 8A, C) as in the control explants (Fig. 6G), although the apical loop structure was poorly developed (Fig. 8B). When 200 ng/ml bFGF was added with antisense ODN for 6 days, dentin formation (41% of explants, Table 2) and the differentiation of ameloblasts (33% of explants) were observed (Fig. 8D, F), although the tooth germs were relatively small, and the apical loop structure was very poor (Fig. 8E).

Discussion

In the present study, we have demonstrated, by using in situ hybridization, that the expression of the *dHAND* gene can be detected in the mesenchyme of the presumptive region of incisor development at an early stage of mandibular development and in incisor tooth germs at a later stage, but that it is undetectable in other tooth germs of both jaws including the tooth germs of the upper incisors. These observations have further been confirmed by RT-PCR analysis by using tooth germs removed from E16.5 mice. The treatment of tooth germ explants of lower incisors removed from E14.5 mice for 6 days with antisense ODN against *dHAND* inhibits the differentiation of ameloblasts and odontoblasts and the formation of dentin matrix, whereas treatment without ODNs or with sense ODN induces no effect on the explants. On the other hand, antisense ODN treatment induces no remarkable effects on most of the tooth germ explants of upper incisors and upper and lower molars. These results suggest that the *dHAND* gene plays important roles in type-specific development of lower incisor.

Mammalian dentitions are highly patterned, with different types of teeth being positioned in different regions of the jaws. In their in situ hybridization study on the expression of homeobox genes in the first branchial arches of mice, Thomas and Sharpe (1998) have demonstrated that *Msx-1* and *Msx-2* are expressed within the mesenchyme of the distal region, the presumptive region of incisor development, whereas *Barx-1* and *Dlx-2* are expressed within the mesenchyme in a more proximal aspect of the arches, the presumptive region of molar development. Within the ectoderm, both *Dlx-2* and *Msx-2* expression is seen in the most ventral (incisor) aspects of the maxilla and mandible. The inhibition of BMP signaling in mandibular development by the addition of exogenous Noggin protein, which binds BMP4 with high affinity and can abolish BMP4 activity by blocking binding to cell surface receptors, results in ectopic *Barx-1* expression in the distal presumptive incisor mesenchyme and a transformation of tooth identity from incisor to molar (Tucker et al. 1998). *Msx-1* and *Msx-2*, however, are expressed even in the molar tooth germ (Jowett et al. 1993). These observations indicate that *Msx-1* and *Msx-2* are not incisor-specific genes. Logan et al. (1998) have reported that T-cell leukemia translocation homeobox gene, *Tlx-1/Hox11*, is detectable in tongue, salivary gland, and several components of the nervous system, and in incisor tooth germs. They, however, do not indicate whether *Tlx-1* is expressed in tooth germs of maxillary incisors. In the present study, we demonstrate that *dHAND* is the first gene specifically expressed in the tooth germ of a single type of tooth, the lower incisor. Several developmental differences between upper and lower incisors have been reported. Ferguson et al. (2000) have reported that ectomesenchymal cells of the mandibular and maxillary primordia appear to be intrinsically different in their response to the epithelial signal, FGF8, by using *Dlx-2* for the maxillary marker and *Dlx-5* for the mandibular marker. Upper incisors are formed from the nasal processes, not from maxillary processes where the upper molars are formed, whereas lower incisors are formed from mandibular processes where lower molars are formed. Moreover, fate mapping in avian and mouse embryos shows that the mandible is mainly composed of cranial neural crest cells that migrate from the midbrain with some contribution from rhombomeres 1 and 2 (Imai et al. 1996; Kontges and Lumsden 1996), whereas the maxillary ectomesenchymal cells are derived from cells migrating from both the midbrain and forebrain (Osumi-Yamashita et al. 1994). The difference of origin of the upper and lower incisor cells may cause the difference of expression of *dHAND* gene in these teeth. Further investigation is needed to confirm this hypothesis.

The null mutation mouse is a powerful tool for investigating gene function. However, it cannot be used to examine the role of *dHAND* in tooth development, because *dHAND* (–/–) mice are embryonic-lethal because of cardiac failure at E10.5, a point when tooth germs have not yet developed (Srivastava et al. 1997). Therefore, we have used the antisense technique and examined cultured tooth germs of incisors to analyze the function of *dHAND* in incisor development. The antisense technique has been shown to be an effective method for investigating the function of molecules involved in tooth development, including epidermal gowth factor (Kronmiller et al. 1991; Shum et al. 1993), amelogenin (Diekwisch et al. 1993), transforming growth factor-2 (Chai et al. 1994), hepatocyte growth factor (Tabata et al. 1996), and parathyroid hormone-related peptide (Liu et al. 2000). Using an *in ovo* culture system, Srivastava et al. (1995) have shown that treatment with antisense ODNs against *dHAND* and *eHAND* simultaneously affect heart looping. Organ culture of incisor tooth germs has been employed to examine the effects of various molecules on development (Schwartz and Snead 1982; Bloch-Zupan et al. 1994; Meyer et al. 1995; Harada et al. 1999). Although care needs to be taken in the interpretation of the results of such experiments, the previous reports described above and our present observations demonstrate that antisense ODNs selectively suppress gene expression when the appropriate target sequence and experimental conditions are selected. Treatment with antisense ODN against *dHAND* inhibits the development of the lower incisor but not that of the upper incisor and upper and lower molars, even if these tooth germs are removed from the same embryo. This result provides a good control for the antisense technique, as the *dHAND* gene is only expressed in the lower incisor. Although similar development occurs in the upper and lower incisors in vivo, *dHAND* gene activity has not been detected in the upper incisor, and antisense ODN against *dHAND* does not disturb its morphogenesis in organ culture system.

In this study, the tooth germ explants have been removed from E14.5 mice, because (1) the incisor tooth germ is at the cap stage in which dentin matrix is not yet secreted, and (2) the tooth germs are easily separated from neighboring Meckel's cartilage, which disturbs the development of tooth germs in vitro. Since *dHAND* expression is initially detected in the first branchial arch of

E12.5 mice, we have tried, in the preliminary study, to use explants of tooth germs or mandibles from embryos earlier than E14.5, e.g., incisor tooth germs of E13.5 mice or first branchial arches of E10.5 mice. However, for technical reasons, we have not been able to obtain reproducible results using such materials. Therefore, we have performed the present study on explants of incisor tooth germs from E14.5 mice. The expression pattern of *dHAND* gene in tooth development indicates that the gene works in different ways at different developmental stages. Therefore, the information obtained in our study on the role of the *dHAND* gene by using tooth germ explants from E14.5 mice is restricted to the middle or later stage of tooth development. Further studies with other methods, e.g., transgenic or mis-expression techniques, will be needed to investigate the role of *dHAND* gene in earlier stages of tooth development.

Although the treatment of tooth germ explants of lower incisors removed from E14.5 mice with sense ODNs, random sequence ODNs, or without ODNs induces almost normal development of tooth germ, that with antisense ODN against *dHAND* prevents the differentiation of tooth germ cells, including ameloblasts and odontoblasts, and the formation of dentin and enamel. These results are highly reproducible. Histological observation of antisense ODN-treated tooth germs suggests that the antisense ODN treatment suppresses the proliferation and differentiation of tooth germ cells. However, the severe effect of antisense ODN observed in explants cultured for 6 days prevents a precise analysis of the effects of antisense ODN on tooth germs. Therefore, we have used explants cultured for 3 days with antisense ODN. Proliferation assays of cultured tooth germs have revealed that antisense ODN treatment of explants inhibits the proliferation of tooth germ cells. The TUNEL method has demonstrated that apoptosis is induced in tooth germ cells by the antisense ODN treatment. This suggests that *dHAND* is essential for the survival of tooth germ cells. Tooth germ explants of the lower incisor treated with antisense ODN show a decrease in alkaline phosphatase activity in the dental papilla. However, the reason that alkaline phosphatase activity is up-regulated in the dental epithelium of antisense ODN-treated tooth germs is unclear.

It has been suggested that SHH is involved downstream of the dHAND signaling pathway in limb development (Fernandez-Teran et al. 2000; Charité et al. 2000), and that BMPs participate upstream of dHAND during sympathetic ganglia development and in early heart development (Howard et al. 2000; Schlange et al. 2000). In order to search for the candidate molecules involved downstream of dHAND in tooth development, we have explored the effects of molecules that can rescue the inhibitory effect of antisense ODN against *dHAND* on the development of mouse tooth germs of lower incisors. Of several growth factors examined, only bFGF shows a partial rescue effect on the inhibition of tooth development induced by antisense ODN, suggesting that bFGF is involved downstream of the dHAND pathway. We cannot however exclude the possibility that bFGF stimulates the proliferation of mesenchymal and epithelial cells by a pathway different from that of dHAND (Jernvall et al. 1994; Shiba et al. 1995; Kettunen and Thesleff 1998; Kettunen et al. 1998). bFGF is confined to odontoblasts in tooth germs at a later tooth developmental stage, suggesting that bFGF plays an important role in dentin formation (Russo et al. 1998). Therefore, bFGF might induce the dentin formation of incisor tooth germs. Further investigation is however necessary to clarify these issues.

Heart looping is affected when the expression of dHAND and eHAND, which can form heterodimers in vitro (Firulli et al. 2000), are inhibited at the same time (Srivastava et al. 1995), suggesting that these two molecules work in concert. In tooth development, however, *dHAND* is expressed in the tooth germs of mandibular incisors, whereas *eHAND* is not expressed in tooth germs but in the median area of the mandible until at least E18.5 (unpublished observation), suggesting that *eHAND* does not cooperate with *dHAND* in tooth development.

Koyama et al. (1996) have reported that, when bud and late bell-stage tooth germs of incisors and molars of embryonic mice are grafted into the anterior margin of wing buds of chick embryos, supernumerary digits are induced, whereas, when the tooth germs from postnatal mice are so grafted, only incisor tooth germs induce supernumerary digits. These results indicate that incisor and molar tooth germs of embryonic mice and incisor tooth germs of postnatal mice have polarizing activity. This may be attributable not only to the presence of SHH in the graft, but also to *dHAND* in the postnatal incisor mesenchyme, which also possesses polarizing activity (Fernandez-Teran et al. 2000; Charité et al. 2000).

In conclusion, *dHAND* gene is expressed exclusively in the mesenchyme of tooth germs of mandibular incisors in embryonic mice, suggesting that it plays a role in type-specific development of lower incisors. The treatment of tooth germ explants of lower incisors from embryonic mouse mandibles with antisense ODN against *dHAND* inhibits the development of tooth germs of the lower incisor. The treatment of explants with bFGF in association with antisense ODN partially rescues the inhibitory effect of antisense ODN on tooth development, suggesting that bFGF is involved downstream of dHAND.

Acknowledgements We thank Dr. Masahiro Iwamoto of Osaka University Graduate School of Dentistry for his generous gift of the *Msx1* probe and for his useful comments and critical reading of the manuscript.

References

Bloch-Zupan A. Mark MP, Weber B, Ruch JV (1994) In vitro effects of retinoic acid on mouse incisor development. Arch Oral Biol 39:891–900

Cattoretti G, Becker MH, Key G, Durchrow M, Schluter C, Galle J, Gerdes J (1992) Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 168:357–363

Chai Y, Mah A, Crohin C, Groff S, Bringas Jr P, Le T, Santos V, Slavkin HC (1994) Specific transforming growth-beta subtypes regulate embryonic mouse Meckel's cartilage and tooth development. Dev Biol 162:85–103

Charité J, McFadden DG, Olson EN (2000) The bHLH transcription factor dHAND controls sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. Development 127:2461–2470

Diekwisch T, David S, Bringas Jr P, Santos V, Slavkin HC (1993) Antisense inhibition of AMEL translation demonstrates supramolecular controls for enamel HAP crystal growth during embryonic mouse molar development. Development 117:471–482

Ferguson CA, Tucker AS, Christensen L, Lau AL, Matzuk MM, Sharpe PT (1998) Activin is an essential early mesenchymal signal in tooth development that is required for patterning of the murine dentition. Genes Dev 12:2636–2649

Ferguson CA, Tucker AS, Sharpe PT (2000) Temporospatial cell interactions regulating mandibular and maxillary arch patterning. Development 127:403–412

Ferguson CA, Tucker AS, Heikinheimo K, Nomura M, Oh P, Li E, Sharpe PT (2001) The role of effectors of the activin signaling pathway, activin receptors IIA and IIB, and Smad2, in patterning of tooth development. Development 128:4605–4613

Fernandez-Teran M, Piedra ME, Kathiriya IS, Srivastava D, Rodriguez-Rey JC, Ros MA (2000) Role of dHAND in the anterior-posterior polarization of the limb bud: implications for the sonic hedgehog pathway. Development 127:2133–2142

Firulli BA, Hadzic DB, McDaid JR, Firulli AB (2000) The basic helix-loop-helix transcription factors dHAND and eHAND exhibit dimerization characteristics that suggest complex regulation of function. J Biol Chem 275:33567–33573

Gigi-Leitner O, Geiger B, Czernobilsky B (1986) Cytokeratin expression in squamous metaplasia of the human uterine cervix. Differentiation 31:191–205

Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I (1999) Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. J Cell Biol 147:105–120

Hollenberg SM, Sternglanz R, Cheng PF, Weintraub H. (1995) Identification of a new family of tissue-specific basic helix-loop-helix proteins with two hybrid system. Mol Cell Biol 15: 3813–3822

Howard M, Foster DN, Cserjesi P (1999) Expression of HAND gene products may be sufficient for the differentiation of avian neural crest-derived cells into catecholaminergic neurons in culture. Dev Biol 215:62–77

Howard MJ, Stanke M, Schneider C, Wu X, Rohrer H (2000) The transcription factor dHAND is a downstream effector of BMPs in sympathetic neuron specification. Development 127: 4073–4081

Imai H, Osumi-Yamashita N, Ninomiya Y, Eto K (1996) Contribution of early-emigrating midbrain midbrain crest cells to the dental mesenchyme of mandibular molar teeth in rat embryos. Dev Biol 176:151–165

Jernavall J, Thesleff I (2000) Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 92:19–29

Jernavall J, Kettunen P, Karavanova I, Martin LB, Thesleff I (1994) Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating Fgf-4 gene. Int J Dev Biol 38:463–469

Jowett AK, Vainio S, Ferguson MWJ, Sharpe PT, Thesleff I (1993) Epithelial-mesenchymal interactions are required for *msx1* and *msx2* gene expression in the developing murine molar tooth. Development 117:461–470

Kettunen P, Thesleff I (1998) Expression and function of FGFs-4, -8, and -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. Dev Dyn 211: 256–268

Kettunen P, Karavanova I, Thesleff I (1998) Responsiveness of developing dental tissues to fibroblast growth factors: expression of splicing alternatives of FGFR1, -2, -3, and of FGFR4; and stimulation of cell proliferation by FGF-2, -4, -8, and -9. Dev Genet 22:374–385

Kontges T, Lumsden A (1996) Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development 122:3229–3242

Koyama E, Yamaai T, Iseki S, Ohuchi H, Nohno T, Yoshioka H, Hayashi Y, Leatherman JL, Golden EB, Noji S, Pacifici M (1996) Polarizing activity, sonic hedgehog, and tooth development in embryonic and postnatal mouse. Dev Dyn 206:59–72

Kronmiller JE, Upholt WB, Kollar EJ (1991) EGF antisense oligodeoxynucleotides block murine odontogenesis in vitro. Dev Biol 147:485–488

Liu JG, Tabata MJ, Fujii T, Ohmori T, Abe M, Ohsaki Y, Kato J, Wakisaka S, Iwamoto M, Kurisu K (2000) Parathyroid hormone-related peptide is involved in protection against invasion of tooth germs by bone via promoting the differentiation of osteoclasts during tooth development. Mech Dev 95:189–200

Logan C, Wingate RJT, McKay IJ, Lumsden A (1998) *Tlx-1* and *Tlx-3* homeobox gene expression in cranial sensory ganglia and hindbrain of the chick embryo: markers of patterned connectivity. J Neurosci 18:5389–5402

Maas R, Bei M (1997) The genetic control of early tooth development. Crit Rev Oral Biol Med 8:4–39

MacKenzie A, Leeming GL, Jowett AK, Ferguson MW, Sharpe PT (1991) The homeobox gene *Hox 7.1* has specific regional and temporal expression patterns during early murine craniofacial embryogenesis especially tooth development in vivo and in vitro. Development 111:269–285

MacKenzie A, Ferguson MW, Sharpe PT (1992) Expression patterns of the homeobox gene, *Hox-8*, in the mouse embryo suggest a role in specifying tooth initiation and shape. Development 115:403–420

Maeda T, Abe M. Kurisu K, Jikko A, Furukawa S (2001) Molecular cloning and characterization of a novel gene, *CORS26*, encoding a putative secretory protein and its possible involvement in skeletal development. J Biol Chem 276:3628–3634

Meyer JM, Ruch JV, Kubler MD, Kupferle C, Lesot H (1995) Cultured incisors display major modifications in basal lamina deposition without further effect on odontoblast differentiation. Cell Tissue Res 279:135–147

Mina M (2001) Morphogenesis of the medial region of the developing mandible is regulated by multiple signaling pathways. Cells Tissues Organs 169:295–301

Osumi-Yamashita N, Ninomiya Y, Doi H, Eto K (1994) The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. Dev Biol 164:409–419

Peters H, Balling R (1999) Teeth – where and how to make them. Trends Genet 15:59–65

Russo LG, Maharajan P, Maharajan V (1998) Basic fibroblast growth factor (FGF-2) in mouse tooth morphogenesis. Growth Factors 15:125–133

Satokata I, Maas R (1994) *Msx1* deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet 6:348–356

Schlange T, Andree B, Arnold HH, Brand T (2000) BMP2 is required for early heart development during a distinct time period. Mech Dev 91:259–270

Schwartz SA, Snead ML (1982) Bromodeoxyuridine-DNA interactions associate with arrest of rat odontogenesis in vitro. Arch Oral Biol 27:9–12

Shiba H, Nakamura S, Shirakawa M, Nakanishi K, Okamoto H, Satakeda H, Noshiro M, Kamihagi K, Katayama M, Kato Y (1995) Effects of fibroblast growth factor on proliferation, the expression of osteonection (SPARC) and alkaline phosphatase, and calcification in cultures of human pulp cells. Dev Biol 170:457–466

Shum L, Sakakura Y, Bringas Jr P, Luo W, Snead ML, Mayo M, Crohin C, Millar S, Werb Z, Buckley S, Hall FL, Warburton D, Slavkin HC (1993) EGF abrogation-induced fusilliform dysmorphogenesis of Meckel's cartilage during embryonic mouse mandibular morphogenesis in vitro. Development 118:903–917

Smith CE, Warshawsky H (1975a) Cellular renewal in the enamel organ and the odontoblast layer of the rat incisor as followed by radioautography using ^{3}H-thymidine. Anat Rec 183:523–562

Smith CE, Warshawsky H (1975b) Movement of entire cell populations during renewal of the rat incisor as shown by radioautography after labeling with ^{3}H-thymidine. Am J Anat 145: 225–260

Smith CE, Warshawsky H (1976) Quantitative analysis of cell turnover in the enamel organ of the rat incisor. Anat Rec 187: 63–98

Srivastava D, Cserjesi P, Olson EN (1995) A subclass of bHLH proteins required for cardiac morphogenesis. Science 270: 1995–1998

Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN (1997) Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet 16:154–160

Tabata MJ, Kim K, Liu JG, Yamashita K, Matsumura T, Kato J, Iwamoto M, Wakisaka S, Matsumoto K, Nakamura T, Kumegawa M, Kurisu K (1996) Hepatocyte growth factor is involved in the morphogenesis of tooth germ in murine molars. Development 122:1243–1251

Thesleff I, Nieminen P (1996) Tooth morphogenesis and cell differentiation. Curr Opin Cell Biol 8:844–850

Thomas BL, Sharpe PT (1998) Patterning of the murine dentition by homeobox genes. Eur J Oral Sci 106 (Suppl 1):48–54

Thomas BL, Tucker AS, Qiu M, Ferguson CA, Hardcastle Z, Rubenstein JLR, Sharpe PT (1997) Role of *Dlx-1* and *Dlx-2* genes in patterning of the murine dentition. Development 124:4811–4818

Thomas T, Kurihara H, Yamagishi H, Kurihara Y, Yazaki Y, Olson EN, Srivastava D (1998) A signaling cascade involving endothelin-1, dHAND and Msx1 regulates development of neural-crest-derived branchial arch mesenchyme. Development 125:3005–3014

Tissier-Seta JP, Mucchielli ML, Mark M, Mattei MG, Gordis C, Brunet JF (1995) Barx1, a new mouse homeodomain transcription factor expressed in cranio-facial ectomesenchyme and the stomach. Mech Dev 51:3–15

Tucker AS, Matthews KL, Sharpe PT (1998) Transformation of tooth type induced by inhibition of BMP signaling. Science 282:1136–1138

Weiss K, Stock D, Zhao Z, Buchanan A, Ruddle F, Shashikant C (1998) Perspectives on genetic aspects of dental patterning. Eur J Oral Sci 106 (Suppl 1):55–63

Yamagishi H, Olson EN, Srivastava D (2000) The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. J Clin Invest 105:261–270

Zhao Z, Stock D, Buchanan A, Weiss K (2000) Expression of *Dlx* genes during the development of murine dentition. Dev Genes Evol 210:270–275