

## Role of *Islet1* in the patterning of murine dentition

Thimios A. Mitsiadis<sup>1,2,\*</sup>, Irene Angeli<sup>1,\*</sup>, Chela James<sup>1</sup>, Urban Lendahl<sup>3</sup> and Paul T. Sharpe<sup>1,†</sup>

<sup>1</sup>Department of Craniofacial Development, GKT Dental Institute, Kings College London, Floor 28 Guy's Tower, Guy's Hospital, London SE1 9RT, UK

<sup>2</sup>Laboratoire de Biologie Moléculaire et Cellulaire, UMR 5665 CNRS/ENS Lyon, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon Cedex 07, France

<sup>3</sup>Department of Cell and Molecular Biology, Karolinska Nobel Institutet, SE-171 77 Stockholm, Sweden

\*These authors contributed equally to this work

†Author for correspondence (e-mail: paul.sharpe@kcl.ac.uk)

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### SUMMARY

It is believed that mouse dentition is determined by a pre-patterning of the oral epithelium into molar (proximal) and incisor (distal) regions. The LIM homeodomain protein *Islet1* (ISL1) is involved in the regulation of differentiation of many cell types and organs. During odontogenesis, we find *Islet1* to be exclusively expressed in epithelial cells of the developing incisors but not during molar development. Early expression of *Islet1* in presumptive incisor epithelium is coincident with expression of *Bmp4*, which acts to induce *Msx1* expression in the underlying mesenchyme.

To define the role of ISL1 in the acquisition of incisor shape, we have analysed regulation of *Islet1* expression in mandibular explants. Local application of bone morphogenetic protein 4 (BMP4) in the epithelium of molar territories either by bead implantation or by electroporation stimulated *Islet1* expression. Inhibition of BMP signalling with *Noggin* resulted in a loss of *Islet1*

expression. Inhibition of *Islet1* in distal epithelium resulted in a loss of *Bmp4* expression and a corresponding loss of *Msx1* expression, indicating that a positive regulatory loop exists between ISL1 and BMP4 in distal epithelium. Ectopic expression of *Islet1* in proximal epithelium produces a loss of *Barx1* expression in the mesenchyme and resulted in inhibition of molar tooth development. Using epithelial/mesenchymal recombinations we show that at E10.5 *Islet1* expression is independent of the underlying mesenchyme whereas at E12.5 when tooth shape specification has passed to the mesenchyme, *Islet1* expression requires distal (presumptive incisor) mesenchyme. *Islet1* thus plays an important role in regulating distal gene expression during jaw and tooth development.

Key words: *Islet1*, LIM transcription factors, Incisor, Ameloblast, Tooth

### INTRODUCTION

Sequential and reciprocal interactions between cranial neural crest cells and oral epithelium are required for tooth development (Ruch, 1987; Lumsden, 1988). The molecular mechanisms involved in tooth formation have started to be increasingly well understood. Secreted signalling molecules, transcription factors and extracellular matrix molecules are expressed in different stages of mouse tooth development (reviewed by Thesleff and Sharpe, 1997). It has been well established that signalling molecules are capable of inducing specific gene expression in explants of dental tissues *in vitro* and affect tooth development *in vivo* (Peters et al., 1998). Although progress has been made in our understanding of the initiation and patterning of teeth, information on rodent tooth specification (i.e. molars and incisors) at the molecular level has remained limited (Thomas et al., 1997; Tucker et al., 1998a; Tucker et al., 1998b; Tucker et al., 1999; Ferguson et al., 1998; Ferguson et al., 2000). Mouse dentition pattern must be determined either by a pre-patterning of the epithelium into

molar and incisor regions or by a pre-patterning of cranial neural crest cells as incisor and molar populations. Recent results suggest that it is the oral epithelium that determines incisor and molar tooth fields. Based on the restricted expression domains of the well known signalling molecules fibroblast growth factor 8 (FGF8) and bone morphogenetic protein 4 (BMP4) in oral epithelium during early developmental stages, a model has been proposed whereby these two molecules control patterning. It has been shown that BMP4 is needed for incisor formation, while FGF8 is responsible for the molar tooth shape (Tucker et al., 1998b). Thereafter, these two molecules control the expression in the mesenchyme of homeobox genes that are important for jaw and tooth development (i.e. *Barx1*, *Dlx1* and *Dlx2*, *Msx1* and *Msx2*). Initiation of molar tooth buds occurs by epithelial invagination into mesenchyme that has a specific complement of expressed genes (*Dlx1*, *Dlx2*, *Barx1*). Incisor tooth germ mesenchyme has a different complement of genes (*Msx1*, *Msx2*). The particular complement of these genes programmes the development of the tooth germ to follow an incisor or molar

morphogenetic pathway (Thomas et al., 1997; Tucker et al., 1998a; Tucker et al., 1998b; Ferguson et al., 2000).

LIM homeodomain-containing proteins are considered as major transcriptional regulators that, in cooperation with other transcription factors, play critical roles in the control of pattern formation and cell type specification of various tissues (Dawid et al., 1995; Jurata et al., 1996; Thaler et al., 2002). The disruption of several LIM homeodomain genes has demonstrated their importance in embryonic development. Deletion of *Lim1* (*Lhx1*) in mice leads to inhibition of head formation and lethality around E10 (Shawlot and Behringer, 1995), while inactivation of *Lim3* (*Lhx3*) by targeted mutation leads to severe perturbations in pituitary development (Sheng et al., 1996). Mice lacking the *Islet1* (*Isl1*) gene die at E9.5 (Pfaff et al., 1996) indicating the importance of the LIM transcription factors in development. *Islet1* (*ISL1*) is the earliest marker for motor neuron differentiation (Karlsson et al., 1990; Ericson et al., 1995; Yamada et al., 1993) and inhibition of *Isl1* expression prevents motor neuron and interneuron specification (Pfaff et al., 1996). However, *Isl1* expression is not restricted to developing neuronal structures, and it has been demonstrated that *ISL1* also controls pituitary and pancreas organogenesis (Ahlgren et al., 1997; Sheng et al., 1997). Two LIM homeodomain encoding genes, *Lhx6* and *Lhx7*, are found to be preferentially expressed in the mesenchyme of the first branchial arch (presumptive dental mesenchyme) prior to initiation of tooth formation (Grigoriou et al., 1998), indicating that members of the LIM family of transcriptional regulators also control tooth formation and patterning.

In the present study we describe the expression patterns of the *Isl1* gene and *ISL1* protein in the developing mouse teeth, and we examine its implication in the cascade of molecular events governing odontogenesis. A role for *Isl1* in patterning of the dentition is proposed as an activator of BMP4 expression in incisor (distal) areas of the stomatodeal epithelium.

## MATERIALS AND METHODS

### Animals and tissue preparation

Swiss mice were used at embryonic and postnatal stages. Embryonic age was determined taking the day of appearance of the vaginal plug as day 0, and confirmed by morphological criteria. The heads from mice ranging in age from embryonic day 9 (E9) to the postnatal day 4 (P4) were dissected in Dulbecco's phosphate-buffered saline (PBS), pH 7.4. The tissues were fixed in 4% paraformaldehyde for 24 hours at 4°C and prepared for sectioning.

### Immunohistochemistry and in situ hybridisation on tissue sections

Affinity-purified rabbit antibody against the *ISL1* protein was a gift from Dr T. Edlund (Umea University, Sweden). Preparation and characterisation of this antibody has been described earlier (Karlsson et al., 1990).

Immunoperoxidase (ABC kit, Vector Laboratories, Burlingame, CA) staining was performed as previously described (Mitsiadis et al., 1992; Mitsiadis et al., 1995). Positive peroxidase staining produces red/brown colour visible with light microscopy. Omission of the primary antibody served as a negative control.

For in situ hybridisation, digoxigenin-labelled antisense rat *Isl1* riboprobe was used. In situ hybridisation on cryosections was carried out as earlier described (Wilkinson, 1995; Mitsiadis et al., 1995; Mitsiadis et al., 1998).

### Mandibular explants

Mouse mandibles were carefully dissected from the rest of the heads of E9-E11 embryos in Dulbecco's PBS and placed into a solution of Dulbecco's Modified Eagle Medium containing glutamax (DMEM; Gibco-BRL) containing 20 units/ml penicillin/streptomycin (Gibco-BRL). The first branchial arches were then placed on top of a 0.1 µm Millipore filter on a stainless steel wire mesh (0.25 mm diameter wire) (Goodfellows) in an organ culture dish (Marathon) containing medium consisting of DMEM, 20% foetal calf serum (FCS; Gibco-BRL) and 20 units/ml penicillin/streptomycin. The mandibles were cultured in a humidified atmosphere of 5% CO<sub>2</sub>, 40% O<sub>2</sub> at 37°C (Nuair incubator) for the appropriate length of time.

After the required period of culture, explants were made to stick to the filters by washing in ice cold methanol (BDH) for 2 minutes and then fixed in 4% PFA in PBS overnight at 4°C. They were then dehydrated through a graded series of ethanol and stored at -20°C until required.

The explants were used for whole-mount immunohistochemistry and whole-mount in situ hybridisation analyses.

### Recombinant proteins and treatment of beads

Recombinant BMP4 protein (1.12 mg/ml) was a kind gift from Dr V. Rosen (Genetics Institute, Cambridge, Massachusetts). The proteins were stored at -70°C in 0.5 mM arginine-HCl, 10 mM histidine (pH 5.6) until use.

Affi-gel agarose beads (75-150 µm diameter; Biorad) were used as carriers of BMP4 protein (Mitsiadis et al., 1995). Beads were washed once with PBS and pelleted. Recombinant proteins were diluted into PBS, pH 7.4, to concentrations 50-250 ng/µl/5 µl/50 beads (FGF4 and FGF8, 100-200; Sonic hedgehog (SHH), 50-150; BMP4, 50-250) and incubated for 30 minutes at room temperature. Beads were washed for 5-15 minutes in culture medium and then transferred with a mouth-controlled capillary pipette on top of E9-E11 explants. Control beads were treated identically with 0.1% BSA in PBS.

All bead experiments were accompanied by positive controls to check activity of the proteins used.

### Expression constructs

The cytomegalovirus (CMV) promoter construct pIRES2-EGFP (Clontech) was used. pIRES2-EGFP is a bicistronic expression vector, which has an internal ribosomal entry site (IRES), a nuclear localisation signal expressed downstream of the gene of interest and a green fluorescent protein (GFP), which allows visualisation of the targeting efficiency of the electroporation. This construct mediates gene expression in all cells that take up the DNA. Full-length coding fragments for BMP4 and Noggin were cloned into this vector, recombinant plasmids were purified using a standard plasmid purification kit (Qiagen) and sequenced. The *Islet1* construct used (pND21) was provided by Tanna Franz (Freiburg) and expression was driven by the EF1-alpha promoter. This construct was co-electroporated with pIRES2-EGFP.

The expression constructs were used at a concentration of 1 µg/µl in TE (Tris-EDTA) buffer or in PBS for all electroporations. Fast green 1/10,000 (Sigma Chemical Co., St. Louis, MO) was added to the DNA solution for visualisation within the mandible.

### Morpholinos

*Isl1* antisense morpholino oligonucleotides were obtained from Gene Tools (USA). The sequence was 5' TTGGTGGATCGCCCAT-GTCTCCCAT 3', tagged with 3' fluorescein and used at a concentration of 100 µM. A control antisense morpholino for the zebrafish *Sox9B* gene with a sequence of 5' GCAGAGAGAGAGTGTGAGT-GTGT 3' was used.

### Electroporation

The gene constructs were introduced to targeted area of the mouse

mandible using fine glass needles prepared from glass capillary tubes (1 mm in diameter) using a standard micropuller equipped with a heating element. Needles were filled with DNA solution in 1% carboxy methyl cellulose by capillary action. Needles were connected to a syringe pump through a fine silicone tube. Two electrodes (0.1 mm in diameter) (Goodfellows) set on a micromanipulator were placed parallel on the surface of the mandible, 2–4 mm apart. The tungsten microelectrode was inserted just beneath the epithelium and the platinum electrode connected to the anode was placed at the surface of the epithelium. The DNA solution was applied to the epithelium close to the electrodes and a few drops of DMEM were added to cover the electrodes. Square electrical pulses were applied immediately. Square wave current pulses (5–8) of 30–45 V and 50 msec duration were applied using a square wave electroporator (Intracapt TSS10, Intracel, UK). Pulses were generated every 1 second so that a pulse of 50 ms seconds was followed by a 950 ms second rest phase. With this method, the electric pulse results in a brief opening of plasma membrane channels allowing the entry of small molecules. Using this particular electrode set-up the electroporated DNA is restricted to the epithelium. Following electroporation of the GFP reporter constructs, one side of the mandible was GFP positive, whereas the other side was GFP-negative and thereby served as an internal control. All explants were cultured for 24 hours before further processing.

#### Whole mount immunohistochemistry and in situ hybridisation of mandibular explants

The explants were treated with 3% H<sub>2</sub>O<sub>2</sub>/PBS for 60 minutes before starting whole-mount immunohistochemistry and in situ hybridisation. Whole-mount immunohistochemistry using antibodies against ISL1 was performed as previously described (Mitsiadis et al., 1995). When the colour reaction was satisfactory, the explants were washed in tap water and then fixed in 4% PFA. Digoxigenin-labelled antisense rat *Isl1*, mouse *Bmp4*, *Barx1* and *Msx1* riboprobes were used. Whole-mount in situ hybridisation was carried out as previously described (Wilkinson, 1995; Mitsiadis et al., 1998).

#### Renal transfers

Following electroporation of *Isl1* into proximal epithelium and GFP control plasmid into the same region on the contralateral side of mandible explants, the explants were cultured for 24 hours and then dissected into two pieces: a piece that contained the molar region of the *Isl1*-positive side and another piece that contained the whole incisor region together with the molar region of the control side. These pieces were transferred separately into renal capsules of adult male mice and left to develop for 10–14 days. Teeth were isolated from the kidneys and counted.

#### Tissue recombinations

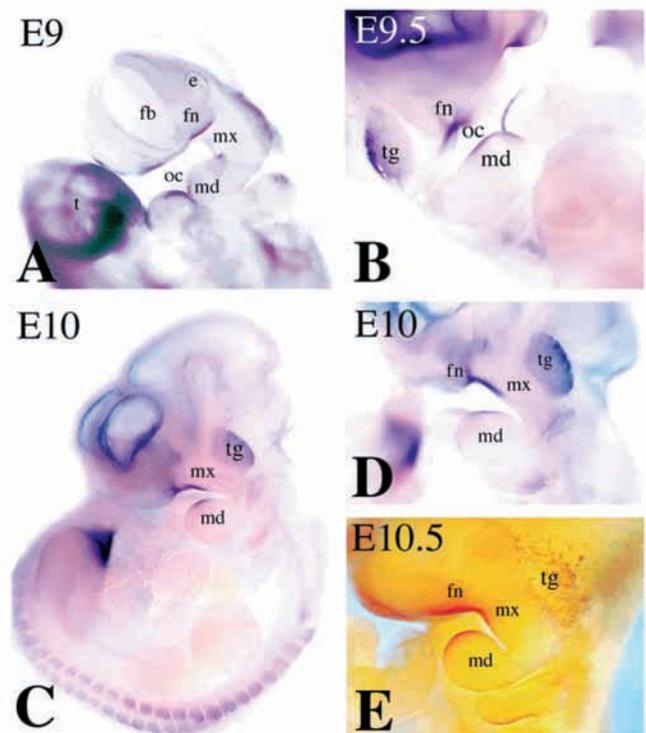
The regions where molar and incisor tooth germs will develop were carefully dissected in Dulbecco's PBS from the rest of the developing jaw of E10.5–E12.5 mouse embryos and incubated 5 minutes in 2.25% trypsin/0.75% pancreatin on ice. Epithelial and mesenchymal tissues were separated in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 15% foetal calf serum (FCS; Gibco). Isolated mesenchymal tissues were transferred with a mouth-controlled pipette onto pieces of Nuclepore filters (pore size, 0.1 µm) supported by metal grids (Trowell-type), while isolated epithelia were placed on top of the mesenchymal tissues. The recombinants were homochronic but heterotopic (epithelium of the molar region mesenchyme from the incisor region and vice versa), and were cultured for 24 hours in DMEM supplemented with 15% FCS in a humidified atmosphere of 5% CO<sub>2</sub> in air, at 37°C. After culture, explants were fixed in 4% PFA overnight at 4°C, washed in PBS and finally stored in methanol at –20°C until analysis by whole-mount in situ hybridisation.

## RESULTS

### *Isl1* gene and ISL1 protein expression in developing teeth

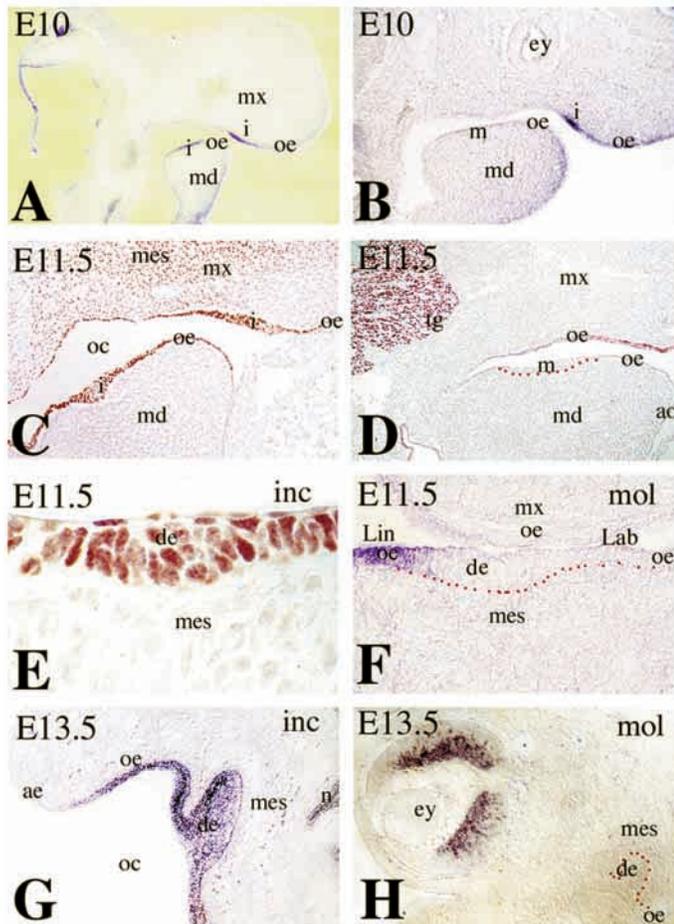
To determine the role of ISL1 in odontogenesis, we first analysed the expression pattern of the *Isl1* gene and the ISL1 protein distribution during mouse tooth development. Whole-mount in situ hybridisation and whole-mount immunohistochemical analyses showed that both the mRNA and the protein were present in the oral epithelium from E9. Expression was restricted at the distal parts of the mandibular and maxillary processes, where incisors will develop (Fig. 1A–E). The expression pattern of the ISL1 protein in the stomatodeal epithelium (Fig. 1E) was identical to that of the *Isl1* gene (compare Fig. 1D and E). No hybridisation signal was detected with sense probes at this or subsequent developmental stages (data not shown).

We then followed the expression of the *Isl1* gene and the ISL1 protein in tissue sections from embryos and pups (E10–P4). From E10 to E11.5, when the oral epithelium thickens and



**Fig. 1.** Expression of the *Isl1* gene and protein (ISL1) in the developing mandibular and maxillary processes of E9–E10.5 mouse embryos. Whole-mount in situ hybridisation using a digoxigenin-labelled probe (A–D) and whole-mount immunohistochemistry (E). (A,B) Frontal views of E9 and E9.5 mouse embryos showing strong *Isl1* mRNA expression (violet colour) in the distal parts of the oral epithelium and in a part of the nasal epithelium. (C) Lateral view of an E10 embryo showing *Isl1* expression in distal parts of the oral epithelium. (D) Higher magnification of C showing *Isl1* expression in oral epithelium and the trigeminal ganglion. (E) Lateral view of the head of an E10.5 mouse embryo demonstrating the distribution of the ISL1 protein in the distal parts of the oral epithelium and in the trigeminal ganglion. e, eye; fb, forebrain; fn, frontonasal process; md, mandibular process; mx, maxillary process; oc, oral cavity; t, tail; tg, trigeminal ganglion.

the incisor and molar placodes are visible, *Isl1* mRNA and ISL1 protein were found in the epithelium of the incisors (Fig.



**Fig. 2.** Patterns of the *Isl1* gene and protein expression in developing teeth of E10-E13.5 mouse embryos. Digoxigenin in situ hybridisation (A,B,F-H) and immunohistochemistry (C-E).

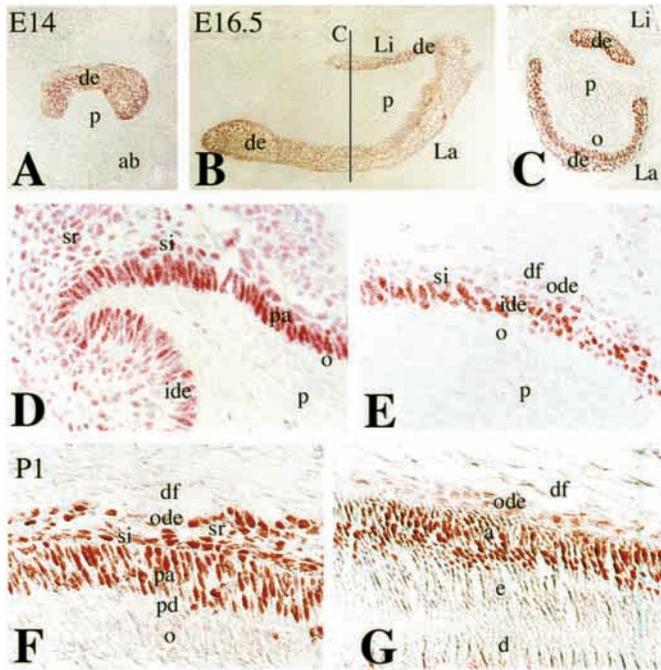
(A) Sagittal section through the head of an E10 mouse embryo shows *Isl1* expression in the oral epithelium where incisors will develop (placodes of incisors). (B) Section of an E10 mouse embryo demonstrating *Isl1* expression in the epithelium of the upper incisor and the absence of *Isl1* transcripts in epithelium of the lower molar. (C) Sagittal section of an E11.5 mouse embryo demonstrating ISL1 protein distribution in the distal part of the oral epithelium and the epithelium of the incisors. (D) Section of an E11.5 mouse embryo showing ISL1 protein expression in the oral epithelium of the maxilla and the trigeminal ganglion. Note the absence of ISL1 in the epithelium of the lower molar (red dotted line). (E) Frontal section. Higher magnification of an E11.5 incisor. Note the nuclear localisation of the protein. (F) Frontal section. Higher magnification of an E11.5 molar. *Isl1* transcripts are observed in a restricted part of the epithelium (lingual side) of the developing molar. (G) Frontal section through the head of an E13.5 mouse embryo showing *Isl1* expression in the oral epithelium and the epithelium of a developing upper incisor. The gene is absent in the aboral epithelium. (H) Frontal section through the head of an E13.5 mouse embryo demonstrating the absence of *Isl1* in the epithelium of a developing upper molar. ab, aboral epithelium; de, dental epithelium; ey, eye; i, incisor territory; inc, incisor; Lab, labial side; Lin, lingual side; m, molar territory; md, mandibular process; mes, mesenchyme; mol, molar; mx, maxillary process; frontonasal process; oc, oral cavity; oe, oral epithelium; tg, trigeminal ganglion.

2A-C,E), but not of the molars (Fig. 2B,D,F), suggesting that ISL1 is involved in tooth specification. While the initial stages of incisor development resemble those of molars, soon after the formation of the epithelial bud the developing incisor rotates and progressively acquires the form of a cylinder parallel to the proximodistal axis of the jaw. The dental epithelium at the lingual side of the incisor will give rise to two layers of cells (outer and inner dental epithelium), while the labial epithelium will form the inner and outer dental epithelia, the stellate reticulum and the stratum intermedium, also found in molars. Only the labial inner dental epithelium will give rise to ameloblasts. ISL1 was expressed during all the stages of embryonic incisor development in epithelial cells (Fig. 2G, Fig. 3A-E). By P1, the deposition of minerals in the dentin, the enamel and the bone matrices stabilise the shape of the incisors. ISL1 protein was strongly expressed in functional ameloblasts located at the median and anterior parts of the incisor (Fig. 3F-G). The protein was also detected in osteogenic areas of the mandibular and maxillary processes located in front of the incisal end of the erupting incisor (data not shown). ISL1 staining was absent in epithelial cells of the developing molars (Fig. 4A-C). However, during enamel formation at P4, ISL1 staining was detected in ameloblasts of the enamel-free cusp area (Fig. 4D), indicating that functional ameloblasts form a heterogeneous population.

#### BMP4 induces *Isl1* expression in oral epithelium

We investigated if signalling molecules involved in tooth initiation might regulate *Isl1* expression in oral epithelium. Previous studies have shown that SHH regulates *Isl1* expression in neural tissues (Echelard et al., 1993). Since *Shh* is also expressed in dental epithelial placodes (Bitgood and McMahon, 1995; Hardcastle et al., 1998), SHH-loaded beads were placed on top of dissected jaws at the sites where molars will develop in order to see if *Isl1* will be expressed at molar areas. Expression of *Isl1* in oral epithelium was not affected by SHH-releasing beads (Fig. 5A). Similarly, control beads incubated in BSA had no effect on *Isl1* expression (Fig. 5A). BMP4 signalling is involved in the shaping of incisors (Tucker et al., 1998b), suggesting that it might activate *Isl1* expression in incisor territories. To test this, beads loaded with different concentrations of BMP4 (50-250 µg/ml) were transplanted into future molar regions (proximal regions) of E9 to E10.5 mandibles and *Isl1* expression was detected by whole-mount in situ hybridisation. *Isl1* was expressed in molar regions after culture of the mandibular explants (Fig. 5B-F), suggesting that these regions acquired an incisiform potential after BMP4 activation. FGF8 is an antagonist of BMP4 action and is expressed in the proximal regions of the oral epithelium (molar regions) (Tucker et al., 1998b). Expression of *Isl1* in oral epithelium was not affected by FGF8-beads (Fig. 5B).

The bead implantation approach does, however, suffer from several disadvantages, in particular the non-physiological nature of beads soaked in very high concentrations of proteins and the inability to specifically target epithelium or mesenchyme. The technique of DNA electroporation was therefore used to deliver targeted gene expression to defined areas of mandibular arch epithelium (Angeli et al., 2002). Electroporation of DNA constructs into the epithelium was used to ectopically express BMP4 in more physiological concentrations and to inhibit BMP4 signalling by expression



**Fig. 3.** Patterns of ISL1 protein distribution in developing mouse incisors. Frontal (A,C) and longitudinal (B,D-I) sections. (A) ISL1 expression in the epithelium of an E14 upper incisor. (B) ISL1 distribution in the epithelium of an E16.5 lower incisor. The line represents the level of the frontal section in C. (C) ISL1 protein is restricted in the epithelium of the E16.5 incisor. (D,E) Higher magnifications showing in detail the expression pattern of ISL1 in the E16.5 incisor. Note the strong nuclear staining in pre-ameloblasts and inner dental epithelial cells. ISL1 is absent in dental papilla mesenchyme. (F) High magnification showing in detail the distribution of ISL1 in epithelial cells of the posterior part of a P1 incisor. Note the heavy nuclear labelling in pre-ameloblasts, stratum intermedium and outer dental epithelium. (G) High magnification showing the expression of ISL1 in epithelial cells of the anterior part of a P1 incisor. Note the strong nuclear staining in functional ameloblasts and the faint staining in cells of the outer dental epithelium. a, ameloblasts; ab, alveolar bone; d, dentin; de, dental epithelium; df, dental follicle; e, enamel; ide, inner dental epithelium; La, labial side; Li, lingual side; o, odontoblasts; ode, outer dental epithelium; p, dental papilla; pa, pre-ameloblasts; pd, predentine; si, stratum intermedium; sr, stellate reticulum.

of Noggin in the incisor areas. Electroporation of E10 mandibular explants with *Bmp4* DNA at the molar areas induced ectopic *Isl1* expression at these sites (Fig. 6A-E). Expression of Noggin in distal epithelium, where *Isl1* and *Bmp4* are co-expressed produced a localised loss of *Isl1* expression (Fig. 6H). Electroporation with a control construct had no effect on *Isl1* expression (Fig. 6G). *Bmp4* expressed in distal epithelium appears to induce/maintain *Isl1* expression.

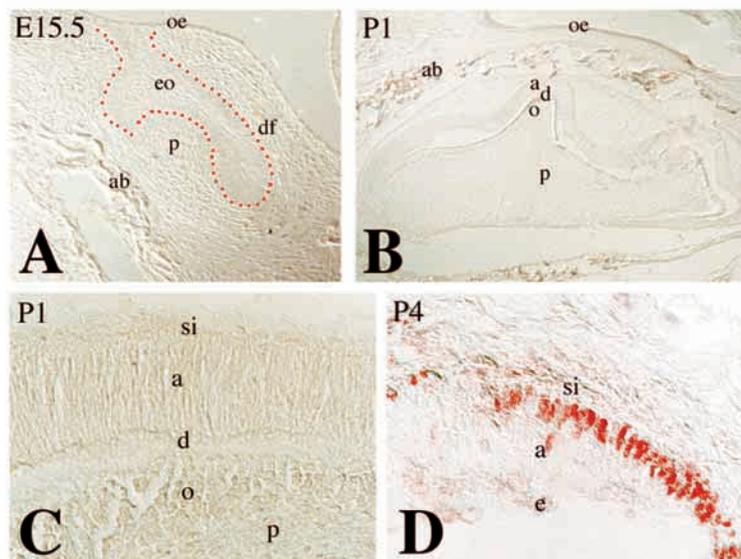
#### *Isl1* activates *Bmp4* expression in oral epithelium

In order to address the function of *Isl1*, we misexpressed it in proximal epithelium using electroporation and also inhibited its function in distal epithelium by electroporation of morpholino antisense oligonucleotides. When E10 mandibles were electroporated with an *Isl1* expression construct, ectopic *Bmp4* expression was seen in the epithelium at the site of

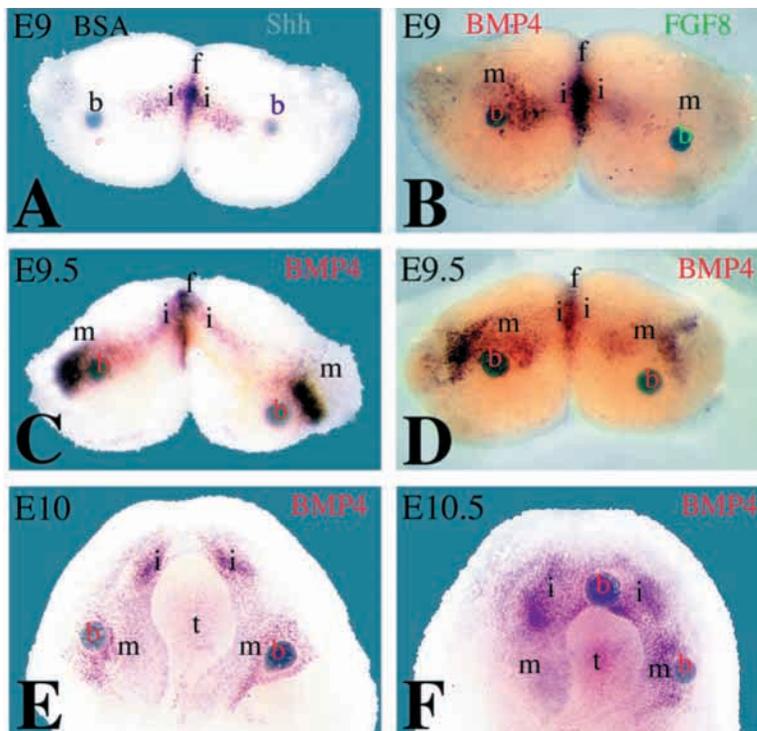
electroporation (Fig. 6F). Electroporation of oral epithelium with *Isl1* morpholinos resulted in the suppression of *Bmp4* expression, and a corresponding downregulation of *Msx1* expression in the underlying mesenchyme (Fig. 7E-L). As a control, Sox9B morpholinos (non-tagged) were co-electroporated with pIRES2-EGFP into the epithelium on the contralateral side of explants (Fig. 7A-D). These results clearly indicate that there is a positive feedback loop operating between ISL1 and *Bmp4* in distal epithelium. It is likely that ISL1 induces *Bmp4* transcription either as a direct or indirect target and BMP4 signalling acts downstream to maintain *Isl1* expression.

#### Ectopic *Isl1* expression represses *Barx1*

The localised expression and molecular interactions of *Isl1* in distal (presumptive incisor) epithelium suggested a role in dental patterning. In order to investigate the effects of *Isl1* expression on the development of molars, we ectopically expressed *Isl1* in proximal epithelium using electroporation. Electroporation of the *Isl1* expression construct into the proximal epithelium of E10 mandible explants followed by 24 hours culture resulted in a loss of *Barx1* expression in proximal mesenchyme (Fig. 8D). Since ISL1 can induce *Bmp4* expression the loss of *Barx1* expression is consistent with the previously reported *Barx1* repression by *Bmp4* (Tucker et al., 1998b). Expression of *Msx1*, which is normally induced by BMP4 in distal mesenchyme, was not induced in proximal mesenchyme



**Fig. 4.** ISL1 protein distribution in developing mouse molars. Frontal (A) and longitudinal (B-D) sections. (A,B) ISL1 protein is absent in dental epithelial cells of E15.5 (A) and P1 (B) molars. Red dotted lines in A indicate the cup shape of the enamel organ of a developing lower molar. (C) High magnification showing the absence of ISL1 in functional ameloblasts of a P1 molar. (D) High magnification demonstrating nuclear ISL1 staining in ameloblasts of the enamel-free cusp region of a P4 molar. a, ameloblasts; ab, alveolar bone; d, dentin; df, dental follicle; e, enamel; eo, enamel organ; o, odontoblasts; oe, oral epithelium; p, dental papilla; si, stratum intermedium.



**Fig. 5.** Effects of SHH, BMP4 and FGF8 signalling molecules on *Isll* gene expression in oral epithelium of E9-E10.5 mouse mandibular explants cultured in vitro.

(A,B) Oral views of E9 mouse mandibles implanted with SHH- (A, violet), BSA- (A, black), FGF8- (B, green) and BMP4 (B, red)-soaked beads showing ectopic *Isll* induction in oral epithelium only by BMP4. (C-F) Oral views of E9.5 (C,D), E10 (E) and E10.5 (F) mandibles cultured together with BMP4-beads. *Isll* expression is strongly induced in oral epithelium where molars will develop. b, bead; f, median area of the mandible; i, incisor territory; m, molar territory; t, tongue.

programme, mandible explants were dissected into incisor and molar regions following electroporation of *Isll* into proximal epithelium. The dissected explants were transferred to renal capsules and development of teeth assayed. From a total of 23 experiments, 25 molars and 3 incisors were recovered from control mandibles whereas only 6 molars and 1 poorly formed incisor were recovered following *Isll* expression. Since incisors develop poorly from manipulated mandibles, it is difficult to conclude that ectopic *Isll* is sufficient to transform molars into incisors. However, ectopic expression of *Isll* in presumptive molar epithelium and the resulting loss of *Barx1* expression in presumptive molar mesenchyme clearly results in repression of molar tooth development.

by ectopic *Isll* (Fig. 8H) (Chen et al., 1996). Induction and maintenance of ectopic *Msx1* expression thus requires more than a BMP4 signal.

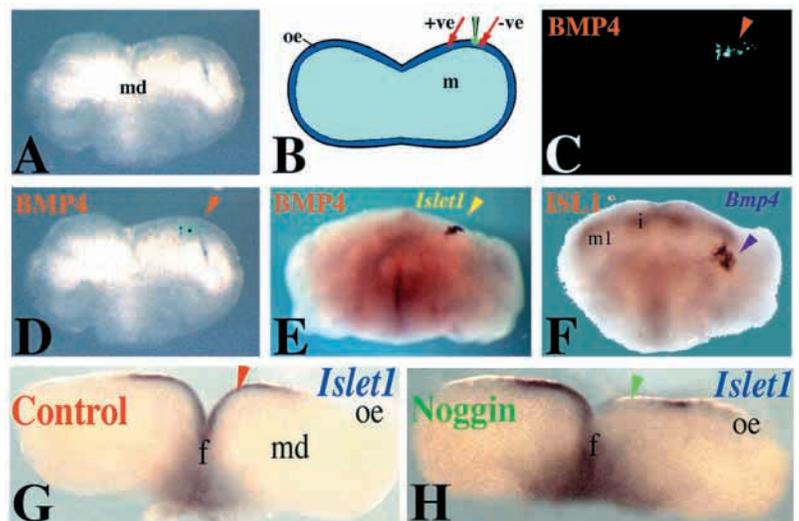
### Islet1 inhibits molar tooth development

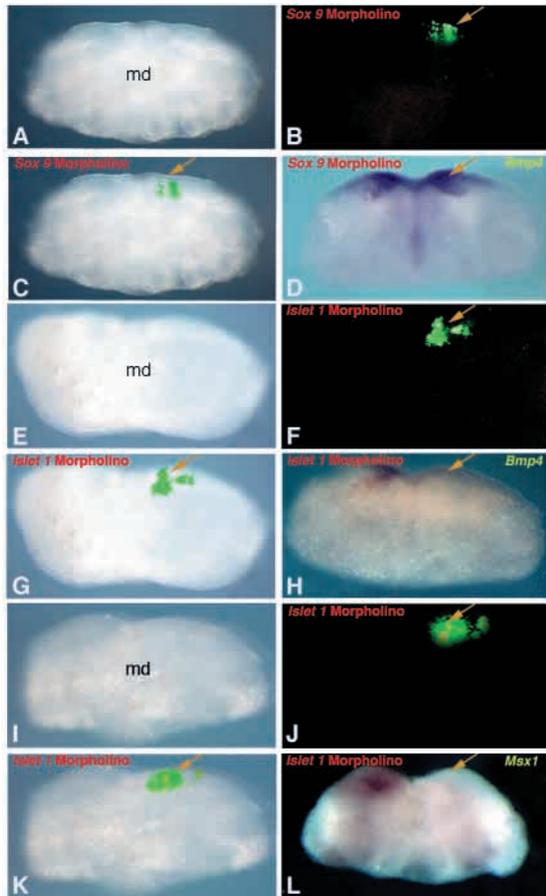
Ectopic expression of *Barx1* in distal mesenchyme either by inhibition of BMP4 signalling or via electroporation of expression constructs results in transformation of incisors into molars (Tucker et al., 1998b) (our unpublished data). In order to determine if expression of *Isll* in proximal epithelium and concomitant loss of *Barx1* could affect the molar development

### Mesenchymal requirements for *Isll* expression

In order to determine the influence of the underlying mesenchyme on *Isll* expression, epithelial/mesenchymal recombinations were performed. Recombination of distal, *Isll*-positive epithelium with proximal mesenchyme at E10.5 maintained *Isll* expression (Fig. 9A). In the reciprocal recombination of proximal *Isll*-negative epithelium with distal mesenchyme, no ectopic *Isll* expression was induced. (Fig. 9B). Similar recombinations were carried out at E12.5, a time when the spatial information specifying tooth shape has

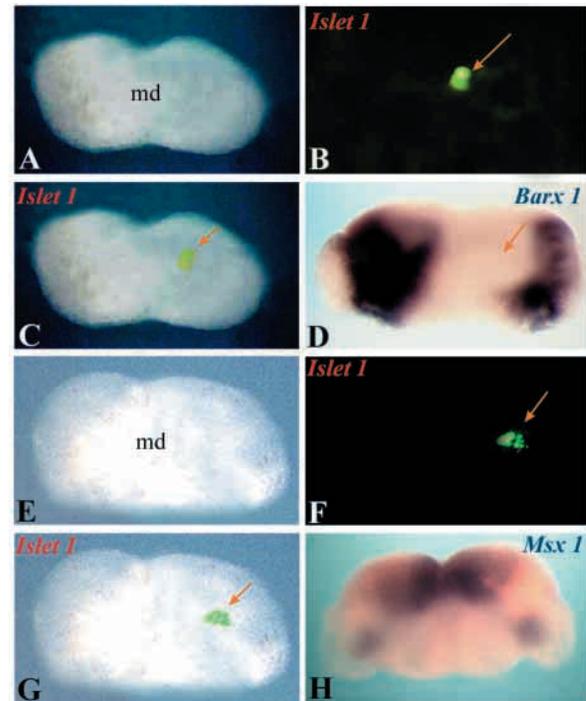
**Fig. 6.** Effects on *Isll* and *Bmp4* expression of electroporation of *Bmp4* and *Isll* expression constructs into the epithelium of E10 mandibular explants. (A) E10 mandibular explant prior to electroporation. (B) Schematic representation of the position of electrodes (red arrows) and glass needle containing the construct (green colour). (C) GFP expression of the *Bmp4*-pIRES2-EGFP construct (green colour, orange arrowhead). (D) Superimposition of A and C showing the localised epithelial expression of the *Bmp4*-pIRES2-EGFP construct (green colour, orange arrowhead). (E) Digoxigenin whole-mount in situ hybridisation showing *Isll* ectopic epithelial expression (yellow arrowhead) after electroporation with the *Bmp4*-pIRES2-EGFP construct. (F) Whole-mount in situ hybridisation showing *Bmp4* expression (violet arrowhead) after electroporation with the *Isll* expression construct. (G) *Isll* expression in a cultured E10 mandible after electroporation with the control pIRES2-EGFP expression construct. (H) Downregulation of *Isll* expression in the distal epithelium of an E10 mandible after electroporation with the *Noggin*-pIRES2-EGFP expression construct in the distal part, right side of the mandible. i, incisor territory; m, mesenchyme; f, median part of the mandible; md, mandibular explant; ml, molar territory; oe, oral epithelium; +ve, positive-voltage electrode/cathode; -ve, negative-voltage electrode/anode; BMP4, *Bmp4*-pIRES2-EGFP construct; ISL1, *Isll* expression construct; Noggin, *Noggin*-pIRES2-EGFP construct; Control, pIRES2-EGFP construct.





**Fig. 7.** Electroporation of *Islet1* morpholino expression construct into the epithelium of an E10 mandibular explant and effects on *Msx1* and *Bmp4* expression in the mesenchyme. (A,E,I) E10 mandibular explants prior to electroporation. (B) GFP expression following electroporation of Sox9B morpholino and pIRES2-EGFP (green colour, yellow arrow). (C) Superimposition of A and B showing the localised epithelial area of electroporated DNA. (D) Digoxigenin whole-mount in situ hybridisation, showing expression of *Bmp4* in epithelium (yellow arrow) following Sox9B electroporation. (F) Fluorescence image of explant following electroporation of *Islet1* morpholinos (yellow arrow). (G) Superimposition of E and F showing localised epithelial area of electroporated morpholinos. (H) Digoxigenin whole-mount in situ hybridisation showing expression of *Bmp4* following electroporation of *Islet1* morpholinos (yellow arrow). (J) Fluorescence image of explant following electroporation of *Islet1* morpholinos (yellow arrow). (K) Superimposition of I and J showing localised epithelial area of electroporated morpholinos. (L) Digoxigenin whole-mount in situ hybridisation showing expression of *Msx1* following electroporation of *Islet1* morpholinos (yellow arrow). md, mandibular explant.

transferred from the epithelium to the mesenchyme (Ferguson et al., 2000). Distal *Islet1*-positive epithelium, recombined with proximal (presumptive molar) mesenchyme resulted in a loss of *Islet1* expression (Fig. 9C). Proximal *Islet1*-negative epithelium recombined with distal (presumptive incisor) mesenchyme stimulated ectopic *Islet1* expression in the proximal epithelium (Fig. 9D). Thus, at E10.5, *Islet1* expression is intrinsic to distal epithelium and is not influenced by the underlying mesenchyme, whereas by E12.5, distal mesenchyme can



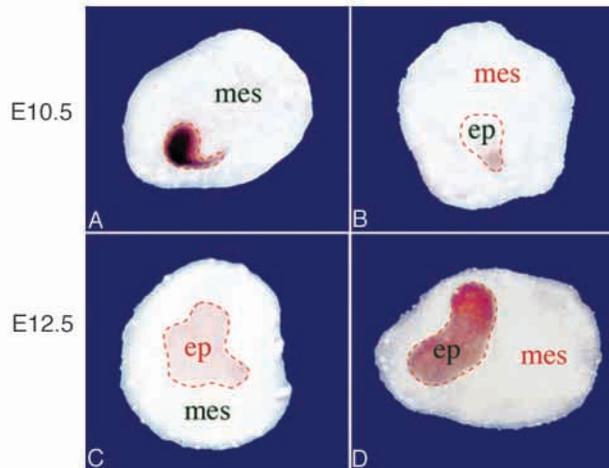
**Fig. 8.** Electroporation of *Islet1* expression construct into proximal (presumptive molar) epithelium of E10 mandibles. (A,E) Cultured E10 mandibles prior to electroporation. (B,F) Expression of GFP following electroporation of the *Islet1* expression construct in proximal epithelium (yellow arrows). (C,G) Superimposition of A,B and E,F to show the location of GFP to identify the region of ectopic *Islet1* expression. (D) Downregulation of *Barx1* expression in proximal epithelium following *Islet1* expression (yellow arrow). (H) *Msx1* whole-mount in situ hybridisation of a mandible electroporated with *Islet1* showing no change in expression. md, mandibular explant.

instruct overlying epithelium to express *Islet1*. Such instructive signals are not present in proximal mesenchyme.

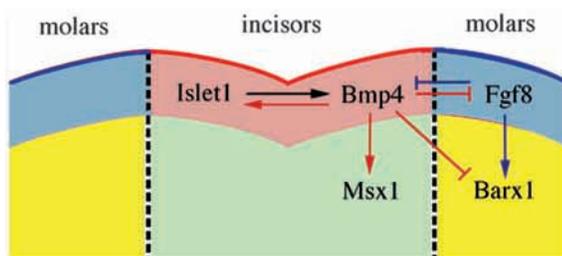
## DISCUSSION

The LIM homeodomain proteins constitute a distinct subclass of proteins characterised by a DNA binding homeodomain in addition to the two cysteine-rich LIM domains (Dawid et al., 1995). Vertebrate Lim genes can be grouped into small subfamilies (e.g. *Islet1* and *Islet2*, *Lim1* and *Lim2*, *Lhx6* and *Lhx7* subfamilies), which have almost identical homeodomains and a high degree of amino acid homology. Lim homeobox genes are likely to play critical roles in embryonic development and patterning of various tissues and organs. For example, the combinatorial expression of several Lim genes in the spinal cord delineates classes of motor neurons that are organised into discrete columns and innervate different groups of target muscles (Tsuchida et al., 1994). While the biological importance of the LIM transcription factors during embryogenesis is evident, comparatively little is known about their function in tooth development.

The present study shows that *Islet1* is expressed in distinct areas of the oral epithelium prior to initiation of tooth formation. During odontogenesis, *Islet1* is expressed in the



**Fig. 9.** Digoxygenin whole-mount in situ hybridisation showing expression of *Isll* following epithelial/mesenchymal recombinations. (A) Distal epithelium recombined with proximal mesenchyme at E10.5. (B) Proximal epithelium recombined with distal mesenchyme at E10.5. (C) Distal epithelium recombined with proximal mesenchyme at E12.5. (D) Proximal epithelium recombined with distal mesenchyme at E12.5.



**Fig. 10.** A model illustrating the molecular events directing murine dentition patterning. A regulatory loop exists between ISL1 and BMP4 in oral epithelium, where *Isll* stimulates *Bmp4* expression and *Bmp4* activates *Isll* expression. Thereafter, *Bmp4* activates *Msx1* expression in mesenchyme, whereas it acts as an antagonist for *Fgf8* expression from oral epithelium and downregulates *Barx1* expression in mesenchyme. Red and blue colours represent the incisor and molar territories respectively of the oral epithelium, green and yellow colours represent the incisor and molar territories respectively of the mesenchyme.

epithelium of the incisors, but not of molars. Early embryonic lethality of homozygous mutants prevents analysis of tooth formation in the absence of *Isll*. However, the fact that *Isll* expression in oral epithelium is missing from molar territories may suggest that its role is to pattern the mammalian dentition. ISL1 is specific for the epithelial compartment of the forming incisors throughout odontogenesis. The induction and maintenance of *Isll* expression in distal (presumptive incisor) epithelium is time dependent and reflects the changes in proximodistal potential of the cells. Thus at E10.5, when odontogenic potential and proximodistal specification is present in the epithelium, *Isll* expression in distal epithelium is intrinsic and not induced by the underlying mesenchyme. By E12.5, the proximodistal odontogenic potential originally present in the epithelium has been transferred to the

mesenchyme as a result of induction of FGF and BMP signalling from the epithelium regulating spatial expression of mesenchymal genes such as *Dlx*, *Msx* and *Barx1* (Tucker et al., 1998a; Tucker et al., 1998b). At E12.5, *Isll* expression in distal epithelium requires the presence of distal mesenchyme. Moreover, distal mesenchyme is able to induce ectopic *Isll* expression in proximal epithelium. The regulation of *Isll* in distal epithelium thus correlates with the changing potential to specify incisors. The ability of distal mesenchyme to induce ectopic *Isll* expression suggests the existence of signalling factors spatially localised in distal mesenchyme, although no obvious candidate molecules have been identified.

We suggest that *Isll* is involved in signalling between epithelium and mesenchyme that is necessary for normal progression of morphogenesis and cytodifferentiation events in incisors. Incisors of rodents are continuously growing teeth, characterised by distinct zones of cell proliferation, differentiation and maturation along their anterior-posterior axis. Another feature of incisors is that lingual dental epithelial cells do not differentiate into ameloblasts and thus cannot synthesise enamel matrix. *Isll* expression in epithelial cells of all incisor areas (proliferation and differentiation compartments) indicates a potential role of *Isll* in the progression of progenitor cells from the dividing and undifferentiated state to that of postmitotic ameloblasts secreting enamel matrix. The present results suggest that *Isll* expression comprises two phases, which are driven by different mechanisms: a first phase of position-dependent expression in the stomatodeal epithelium is followed by a second phase of expression in incisor epithelium probably maintained/initiated by mesenchyme-derived signals.

During late crown morphogenesis of molars, ameloblasts synthesise and secrete enamel matrix proteins. Functional ameloblasts localised at the 'enamel-free' cusp of the first molar express ISL1 protein. The 'enamel-free' area is quite different from other cusp regions where normal amelogenesis occurs. Ameloblasts of the 'enamel-free' cusp demonstrate a post-secretory appearance and seem to produce an extracellular matrix consisting of 'enamel-like' molecules. ISL1 expression in these ameloblasts may suggest that the molecular control involved in the 'enamel-free' cusp differs from those of other crown territories in molars.

Odontogenesis involves a series of epithelial-mesenchymal interactions, where BMP4 constitutes an essential early epithelial signal that has a crucial role in activating mesenchymally expressed genes (reviewed by Thesleff and Sharpe, 1997). The transient expression of BMP4 in distal epithelium of the facial primordia has been shown to be required for underlying expression of *Msx1* in ectomesenchyme. *Msx1* appears to have an important role in directing the development of incisor morphogenesis at this stage and forms part of a number of overlapping domains of expression of homeobox genes that provide the spatial information of dental patterning (Sharpe, 1995). The transcriptional regulation of *Bmp4* expression in distal epithelium has until now not been explored. We have shown that *Isll* is co-expressed with *Bmp4* in distal epithelium and is thus a candidate for a transcriptional regulator of *Bmp4* expression. Misexpression of *Isll* in proximal epithelium resulted in ectopic expression of *Bmp4*; inhibition of *Isll* in distal epithelium using morpholino antisense resulted in a loss

of *Bmp4* expression in the epithelium and, furthermore, suppressed *Msx1* expression in the underlying mesenchyme. Since BMP4 is known to regulate *Msx1* expression (Vainio et al., 1993), we assume that loss of *Msx1* is a direct result of loss of epithelial BMP4. Although not proven, it seems logical that BMP4 is a direct target of ISL1. Another possible candidate for regulating *Bmp4* expression is *Dlx2*, which is co-expressed with *Isl1* and *Bmp4* in distal epithelium. *Dlx2* does not, however, appear to be required for *Bmp4* expression and may have a role in regulating other signalling molecules (unpublished data).

The early distal expression of *Isl1* and its interactions with *Bmp4* suggest a specific role in incisor development. Ectopic expression of *Isl1* in molar epithelium resulted in the expected loss of *Barx1* expression, but surprisingly, *Msx1* was not induced. BMP4 is thus not sufficient to induce/maintain *Msx1* expression in proximal mesenchyme and either other factors are required or *Msx1* expression is actively inhibited. The outcome of loss of *Barx1* was inhibition of molar tooth development. Localised loss of *Barx1* expression in maxillary molar mesenchyme in *Dlx1/2* mutants also results in inhibition of molar development (Thomas et al., 1997). In both these cases, loss of *Barx1* expression is not accompanied by any gain of expression of distal genes such as *Msx1*. Significantly, redirection of cells from the incisor to molar pathway involves both gain of *Barx1* expression and loss of *Msx1* expression.

Since tooth development involves reciprocal signalling interactions between epithelium and mesenchyme, the possible role of signalling factors in regulation of *Isl1* expression was investigated. FGFs, BMPs and SHH are all possible candidates, having been identified as regulators of many of the key early interactions during tooth development. Moreover, Shh has been shown to participate in the regulation of *Isl1* expression in neural tube development (Ericson et al., 1995). Addition of FGF8 and Shh to mandibular primordia failed to induce *Isl1* expression. BMP4 protein supplied on beads or BMP4 expression induced by electroporation of an expression construct produced clear induction of *Isl1* expression. Since *Bmp4* and *Isl1* are co-expressed in distal epithelial cells at a time when there is no *Bmp4* expression in the underlying mesenchyme, it seems probable that the effect of BMP4 on *Isl1* expression occurs within the epithelium, although the possibility that there is an intermediate signal from BMP4 to *Isl1* via the mesenchyme cannot be excluded. *Isl1* and *Bmp4* thus appear to have a positive autoregulatory relationship (Fig. 10). We assume that this functions to maintain transient, high levels of BMP4 in distal epithelium to regulate *Msx1* expression in the underlying mesenchyme. Such positive autoregulation is a common feature among the oral epithelial signalling molecules since we have observed autoregulation of both *Shh* and *Fgf8* expression (unpublished).

Development of incisors and molars is controlled by parallel independent genetic pathways and may reflect the different responses to epithelial-derived signals of the cranial neural crest cells that populate the mandibular and maxillary processes (Ferguson et al., 2000). The *Isl1*-related homeobox genes *Lhx6* and *Lhx7* are expressed in the first branchial arch mesenchyme and are likely to play a critical role in its development and patterning (Grigoriou et al., 1998). The *Dlx1/Dlx2* double mutants have a tooth phenotype, in which only the development of maxillary molars is lost (Thomas

et al., 1997; Qiu et al., 1997). *Activin*  $\beta A$ , activin receptor *ActR*[*IIA*<sup>+/+</sup>;*IIB*<sup>-/-</sup>] and *Smad2*<sup>+/-</sup> mice also have a tooth phenotype in which the incisors and mandibular molars are missing but the maxillary molars develop normally (Ferguson et al., 1998; Ferguson et al., 2001). FGF8 has been shown to be upstream of *Lhx6* and *Lhx7*, *Dlx* and *activin*  $\beta A$  genes (Ferguson et al., 1998; Ferguson et al., 2000; Grigoriou et al., 1998; Thomas et al., 2000). The present data shows that ISL1 and BMP4 are part of the genetic programme that defines territories associated with incisor formation in the stomatodeal epithelium. These results identify a novel role for ISL1 in tooth patterning by regulation of BMP4 expression.

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