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Deletion of the Pitx1 genomic locus affects mandibular tooth morphogenesis and expression of the *Barx1* and *Tbx1* genes

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Abstract

Pitx1 is a bicoid-related homeodomain factor that exhibits preferential expression in the developing hindlimb, mandible, pituitary gland and teeth. *Pitx1* gene-deleted mice exhibit striking abnormalities in morphogenesis and growth of both hindlimb and mandible, suggesting a proliferative defect in these two structures. Here, we studied the expression and regulation of *Pitx1* in both mandible and developing teeth and analyzed tooth morphology, cell proliferation, apoptosis and expression of *Pitx2*, *Barx1* and *Tbx1* in dental tissues of *Pitx1*−/− mouse embryos. *Pitx1* expression is restricted to the epithelium of the growing tooth anlagen. Tissue recombination and bead implantation experiments demonstrated that bone morphogenetic protein-4 down-regulates *Pitx1* expression in both mandibular mesenchyme and dental epithelium. Deletion of the *Pitx1* locus results in micrognathia and abnormal morphology of the mandibular molars. Although *Pitx2* expression in teeth of *Pitx1*−/− embryos is not altered, expression of *Barx1* decreased in the mesenchyme of the mandibular molars. Furthermore, *Pitx1* deletion results in suppression of *Tbx1* expression in dental epithelium. Taken together, these results indicate that independent genetic pathways in mandibular and maxillary processes determine tooth development and morphology.

Keywords: Pitx1; Transcription factors; Tooth; Mandible; Mouse; *Barx1*

Introduction

Teeth are organs that develop as a result of sequential and reciprocal interactions between the oral ectoderm and neural crest-derived mesenchyme. Epithelial-derived ameloblasts synthesize the organic components of the enamel and mesenchyme-derived odontoblasts secrete the matrix of dentin (Ruch, 1987).

During recent years, considerable progress has been made in the molecular basis underlying epithelial–mesenchymal interactions during the different stages of mouse tooth development (for reviews, see Mitsiadis, 2001; Tucker and Sharpe, 2004). Functional analyses of transcription regulators (i.e. Msx1, Pax9, Pitx2, Dlx1 and Dlx2) have shown drastic effects on tooth development, including tooth abnormalities and/or agenesis in both mice and humans. Signaling molecules such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) are capable to induce specific gene expression in mandibular and tooth explants *in vitro* and affect tooth development *in vivo* (for reviews, see Mitsiadis, 2001; Tucker and Sharpe, 2004).

Although progress has been made in understanding the establishment of different tooth shapes in mice (i.e. incisors and molars), little is known about the molecular mechanisms that are involved in distinctions between teeth of the maxilla and mandible. Teeth of mandibular origin (lower teeth) are histologically and morphologically identical to teeth of maxillary origin (upper teeth), although their developmental pathways are different. The maxillary teeth are composed of elements derived from midbrain and forebrain neural crest, while the mandibular teeth receive neural crest cells derived from hindbrain (rhombomeres 1 and 2) and midbrain (Cobourne and Mitsiadis, 2006; Imai et al., 1996; Osumi-Yamashita et al., 1994; Trainor and Tam, 1995). Few transcription factors are differentially expressed in

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the maxillary and mandibular processes, and their early onset of expression indicates that they play a formative role in maxillary and mandibular tooth specification (for reviews see Mitsiadis, 2001; Tucker and Sharpe, 2004). *Dlx* genes in particular seem important for the morphogenesis of proximal jaw hard tissues and most specifically distinguish the upper from the lower jaw structures. In *Dlx1*/*Dlx2* double mutants, maxilla development is affected and maxillary molars, but not mandibular molars, failed to develop (Qiu et al., 1997). *Dlx5* and *Dlx6* are expressed in proximal mandibular mesenchyme in domains similar to *Dlx1* and *Dlx2*, but these two genes are not expressed in maxillary mesenchyme (Zhao et al., 2000). The fact that the *Dlx* genes are differentially expressed in the maxillary and mandibular processes indicates a basic genetic difference between upper and lower molar specification. *Pitx1* is another candidate gene for controlling mandibular/maxillary tooth identity. Pitx1 is a member of the novel *bicoid*-related family of homeoproteins that exert critical regulatory roles during development. It has been shown that *Pitx1* is expressed in the proximal mesenchyme of the developing mandible, hindlimb, oral epithelium, developing teeth and pituitary gland (Lamonerie et al., 1996; Szeto et al., 1996; Lanctôt et al., 1997; Shang et al., 1997). In *Pitx1* mice mutants the shape and growth of both hindlimb and mandible were severely affected (Lanctôt et al., 1999) while tooth development proceeds normally at least until E14.5 (Lanctôt et al., 1999; Szeto et al., 1999). The related gene products, Pitx2 and Pitx3, have similar transcription properties, but their expression patterns and developmental roles are different. *PITX2* is responsible for the Rieger syndrome in humans (Semina et al., 1996), an autosomal dominant disease characterized by anterior chamber ocular abnormalities, dental hypoplasia and/or agenesis and mild craniofacial dysmorphism. *Pitx2* is specifically expressed in dental epithelium (Mucchielli et al., 1997; Mitsiadis et al., 1998), and deletion of the *Pitx2* locus in mice results in loss of all dental structures (Lin et al., 1999; Lu et al., 1999). *Pitx3* is expressed in the eye lens, and mutations in the human *PITX3* gene lead to cataracts and anterior segment mesenchymal dysgenesis (Semina et al., 1998; Rieger et al., 2001).

In this paper, we investigate the expression and function of *Pitx1* in tooth development and we report evidence that *Pitx1* exerts critical roles in mandibular tooth morphogenesis. We also exploited the ability of epithelial–mesenchymal interactions to regulate *Pitx1* expression in mandibular and dental explants *in vitro*.

Materials and methods

Animals and tissue preparation

Swiss mouse embryos from embryonic day 9.5 (E9.5) to postnatal day one (P1) were used for in situ hybridization, tissue recombination and bead implantation experiments. E9.5, E10.5, E11.5, E12.5 and E17.5 wild-type, *Pitx1* +/− and *Pitx1*−/− mouse embryos were hybrid Sv129xBalb/c of the first three generations of crossing with Balb/c mice (Lanctôt et al., 1999). The age of the mouse embryos was determined according to the appearance of the vaginal plug (day 0) and confirmed by morphological criteria. Animals were killed by cervical dislocation and the embryos were surgically removed in Dulbecco's phosphate-buffered saline (PBS). Dissected heads were fixed overnight at 4 °C in 4% paraformaldehyde (PFA).

Probes and in situ hybridization

Digoxigenin and fluorescein-labeled (Boehringer Mannheim) antisense riboprobes for *Pitx1* (Lanctôt et al., 1997) and *Pitx2* (Mucchielli et al., 1997) and digoxigenin-labeled probes for *Barx1* (Mucchielli et al., 1997), *Tbx1* (Zoupa et al., 2006) and *Bmp4* (Mitsiadis et al., 2003) were used. Whole-mount *in situ* hybridization on explants and *in situ* hybridization on sections were performed as previously described (Mitsiadis et al., 2003).

Proteins and bead preparation

Recombinant BMP4 protein (Genetics Institute, USA) was used to load beads (100–200 mesh/100–200 μm diameter; Sigma). The protein was diluted with 0.1% bovine serum albumin (BSA) in PBS to concentrations of 200 μg/ml. As a control, we used beads loaded with 0.1% BSA in PBS.

Mandibular and dental explants, tissue recombination and bead implantation experiments

For tissue recombination and bead implantation experiments, E9.5–E10 mandibles and E13 and E14 lower first molars were used. Mouse mandibles were dissected in Dulbecco's PBS from the rest of the heads of E9.5–E10 embryos and placed into a solution of Dulbecco's modified Eagle medium (DMEM; Gibco). For tissue recombination experiments, the mandibles were carefully dissected in four different pieces: two pieces representing the proximal parts of the mandible (where molars will develop) and two pieces representing the distal parts of the mandible (where incisors will grow). The explants were incubated 5 min in 2.25% trypsin/0.75% pancreatin on ice. Epithelial and mesenchymal tissues were separated in DMEM supplemented with 15% fetal calf serum (FCS; Gibco). Isolated mesenchymal tissues were transferred with a mouth-controlled pipette on pieces of Nuclepore filters (pore size, 0.1 μm) supported by metal grids (Trowell-type), and thereafter isolated epithelia were placed in contact to the mesenchymal tissues. The recombinants were both homotopic (epithelium and mesenchyme from the same mandibular region) and heterotopic (epithelium from a different region than the mesenchyme) and cultured for 20 h in DMEM supplemented with 15% FCS and 20 U/ml penicillin/streptomycin in a humidified atmosphere of 5% CO2 in air, at 37 °C. For bead implantation experiments in mandible, BMP4 beads were transferred on top of E9.5–E10 entire mandibles or the proximal parts of the mandible, and the explants were cultured for 20 h. For experiments in dental tissues, E13–E14 molar tooth germs were carefully dissected from the rest of the mandible and incubated for 3 min in 2.25% trypsin and 0.75% pancreatin on ice. Dental epithelia were mechanically separated from mesenchyme in DMEM supplemented with 15% FCS. Isolated epithelia were placed on top of isolated mesenchymes. Beads were then transferred on top of dental epithelia and thereafter the cultured for 20 h. After culture, explants were fixed in 4% PFA overnight at 4 °C, washed in PBS and finally stored in MeOH at −20 °C until analysis by whole-mount in situ hybridization (for details, see Mitsiadis et al., 2003).

Analysis of apoptosis

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-TUNEL (in situ cell death detection kit) was used to investigate apoptotic DNA fragmentation. Briefly, after proteinase K pre-treatment (20 μg/ml at 37 °C for 30 min), 3% hydrogen peroxide was applied to the slides to avoid endogenous peroxidase reaction. Slides were then incubated with terminal deoxyribonucleotide transferase at 37 °C for 1 h. Anti-digoxigenin antibody conjugated with horseradish peroxidase was applied and 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) was used to visualize apoptotic DNA strand breaks (brown color). Sections were counterstained with hematoxylin. A positive control of TUNEL labeling was prepared using Nuclease (R&D Systems) treatment (5 μg/ml at 37 °C for 30 min). As a negative control, the terminal transferase was omitted from the labeling procedure (label solution only instead of TUNEL reaction mixture).