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Technical Article

A mouse mandibular culture model permits the study of neural crest cell migration and tooth development

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ABSTRACT A major issue in developmental biology is to determine how time and position-restricted instructions are signaled and received during morphogenesis of different phenotypes, of which tooth, Meckel's cartilage and tongue formation are classical examples. It is now evident that a hierarchy of growth factors and their downstream transcription factors regulate the timing, sequence and position of cells and tissues in forming different phenotypes during embryogenesis. Here we report the development of an early mandibular organ culture model. Explants of E8 and E9 first branchial arch were cultured and produced mandibular processes with cap stage tooth formation, Meckel's cartilage and tongue development. In tandem, vital dye (DiI) labeling studies confirmed that rhombomeres 1-4 give rise to craneal neural crest (CNC) cells which emigrate from the neural fold to the forming maxillary and mandibular arches. Furthermore, we have tested the feasibility of investigating the regulation of different phenotypes within the first branchial arch by a transcription factor using this early mandibular organ culture model. Lymphoid enhancing factor 1 (*Lef1*), a transcription factor, has been implicated to regulate tooth formation *in vivo*. We have analyzed the expression of *Lef1* and studied the biological effects of *Lef1* on E8 embryonic mouse first branchial arch explants in organ culture. Collectively, these results demonstrate that first branchial arch explant model is suitable for studies of rhombencephalic crest cell fate during mandibular morphogenesis and can be used as a model with direct access to investigate the molecular mechanism in regulating first branchial arch morphogenesis.

KEY WORDS: *mandibular morphogenesis and tooth formation, cranial neural crest cells migration, lymphoid enhancing factor 1 (Lef1)*

Introduction

A central issue in developmental biology is to understand the molecular controls for the initiation and development of complex morphological structures such as teeth, Meckel's cartilage and tongue. Less evident is the fact that each of these morphological structures are derived from a set of interactions associated with metameric organizations. Recent evidence indicates that along the antero-posterior axis of the early mouse embryo, the forebrain, midbrain and hindbrain develop from a metameric organization (Keynes and Lumsden, 1990; Graham *et al.*, 1994; Lumsden and Krumlauf, 1996). Moreover, several labeling studies have demonstrated that neuroectoderm cells of rhombomeres 1-4 (r1-4) in the forming posterior midbrain and hindbrain transform into cranial neural crest cells which migrate into the first branchial arch and thereafter become resident within the maxillary and mandibular processes (Osumi-Yamashita *et al.*, 1990; Serbedzija *et al.*, 1992; Bronner-Fraser, 1993; Selleck *et al.*, 1993). The migration of these rhombencephalic crest cells may be regulated by growth factors and downstream transcription factors before they become committed to a number of different phenotypes including progenitor tooth mesenchymal cells, osteoblasts and chondroblasts (Noden, 1983, 1991; Lumsden, 1988; Graham and Lumsden, 1993; Le Douarin *et al.*, 1993; Imai *et al.*, 1996).

The *in vitro* development of the mouse dentition provides an excellent model system to analyze the molecular mechanisms associated with the initiation, differentiation and morphogenesis of tooth forms. The initiation of tooth development is regulated by

Abbreviations used in this paper: CNC, cranial neural crest cells; *Lef1*, Lymphoid enhancing factor 1.

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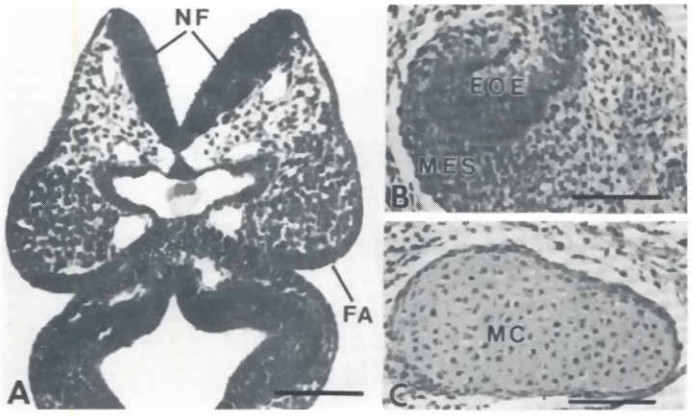

Fig. 1. E8 explant formed tooth organ, Meckel's cartilage and tongue. **(A)** *Transverse section of E8 first branchial arch (FA) and neural folds (NF).* **(B and C)** *E8 first arch and neural fold explants cultured for 10 days were permissive for the formation of tooth bud with enamel organ epithelium (EOE) and condensed odontogenic mesenchymal cells (MES), as well as Meckel's cartilage (MC). Bar, 100μm.*

sequential and inductive epithelial-mesenchymal interactions between cranial neural crest-derived odontogenic mesenchymal cells and an odontogenic placodal ectoderm (Lumsden, 1988; Chen *et al.*, 1996). Presumably, rhombomeres, 2 and 4 transform into cranial neural crest cells between E7.5 and E8 days of gestation in mouse embryos, and these cells migrate into the forming first branchial arch during E9 and become committed into different phenotypes (Osumi-Yamashita *et al.*, 1994; Sechrist *et al.*, 1994; Imai *et al.*, 1996). Subsequently, the first histological evidence for the odontogenic placodal thickening of oral epithelium is at E10-11. By E12-13 the odontogenic ectoderm invaginates into the adjacent mesenchyme and forms a tooth organ through the critical epithelial-mesenchymal interaction with cranial neural crest-derived odontogenic mesenchymal cells. One of the key regulatory molecule for this interaction is a transcription factor *Lef1* (lymphoid enhancing factor 1). It is a cell type-specific transcription factor expressed in lymphocytes of the adult mouse, and in the neural crest, mesencephalon, tooth bud, whisker follicles, and other sites during embryogenesis (Travis *et al.*, 1991; Waterman *et al.*, 1991; Oosterwegel *et al.*, 1993; van Genderen *et al.*, 1994; Zhou *et al.*, 1995). Targeted inactivation of the *Lef1* gene in the mouse germ line resulted in a pleiotropic phenotype in which the development of teeth, whiskers, hair follicles, and mammary glands was severely impaired (van Genderen *et al.*, 1994). Tooth development is initiated in *Lef1*$^{-/-}$ embryos, however, it is arrested at bud stage before the formation of a mesenchymal dental papilla.

We have developed an early mandibular organ culture model which permits direct access to study the migration of CNC cells. In particular, E8 and E9 explants formed cap stage tooth organ, Meckel's cartilage, osteoid-like tissue and tongue *in vitro*. Removal of the neural folds from the explant at less than six somite stage resulted in adontia (tooth agenesis) and premature chondrocytes formation which clearly indicated the critical role of these cranial neural crest cells in instructing the formation of tooth and Meckel's cartilage. In addition, the critical time period for the emigration of these neural crest cells is between 4 to 6 somites stage for the structures derived from the first branchial arch.

Using this model, we present DiI labeling evidence which indicated that cranial neural crest cells from the dorsal neural folds migrated into the forming first branchial arch. In tandem, we also used this model to investigate the molecular regulation of tooth formation. In particular, we described the expression patterns of mouse *Lef1* gene from E8 to E10 during mouse embryogenesis. Antisense oligonucleotide inhibition of *Lef1* during explant cultures retarded the process of tooth formation at bud stage, a phenotype identical to the *Lef1*$^{-/-}$ embryos. Collectively, these experimental evidences confirm that this early embryonic mouse mandibular organ culture model seems suitable for studies of cranial neural crest cell destination and of molecular mechanism in regulating craniofacial morphogenesis.

Experimental Protocols

Animals and tissue preparation

Timed-pregnant Swiss-Webster mice were purchased from Simonsen Laboratories and housed at our institution. The animals were maintained on a light-dark cycle with light from 0600 to 1800 h. The temperature was maintained at 70°F, and mice were fed a diet of Breeder Block (Wayne Pet Food, Chicago) and water *ad libitum*. Mouse embryos were dissected from uterine decidua. Reichert's membrane was removed from E8 and E9 embryos that were dissected free of the decidual mass. Embryonic age was determined according to the vaginal plug (day 0) and external staging was used to define embryonic development according to number of somite pairs and Theiler staging (1972). E8-E10 embryos were used for either whole-mount *in situ* hybridization, DiI labeling and microdissection to obtain the forming first branchial arch containing posterior midbrain and hindbrain segment used as explants in organ culture.

First branchial arch explant and whole-embryo organ culture

The first branchial arch (six explants per dish) along with its dorsal neural folds were dissected and cultured for periods up to

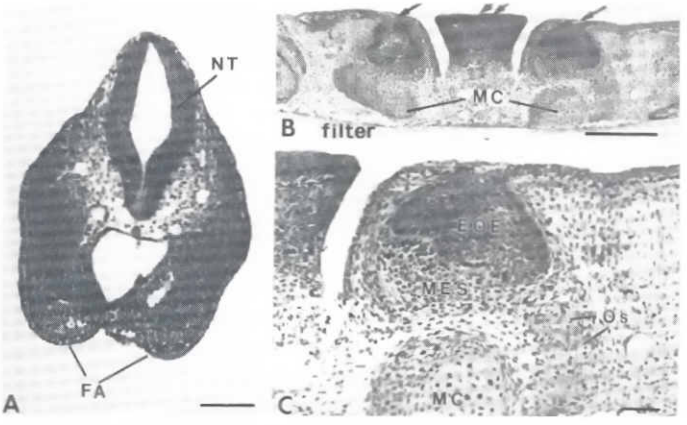

Fig. 2. E9 explant formed bilaterally symmetrical Meckel's cartilage, cap stage tooth organ and tongue. **(A)** *Transverse section of E9 first arch (FA) and neural tube (NT).* **(B and C)** *E9 first arch and neural tube explants cultured for 10 days were permissive for the formation of Meckel's cartilage (MC); tongue (); cap stage tooth organs () with its enamel organ epithelium (EOE) as well as dental mesenchymal cells (MES) and osteoid-like tissue (Os). Bar, 100μm.*

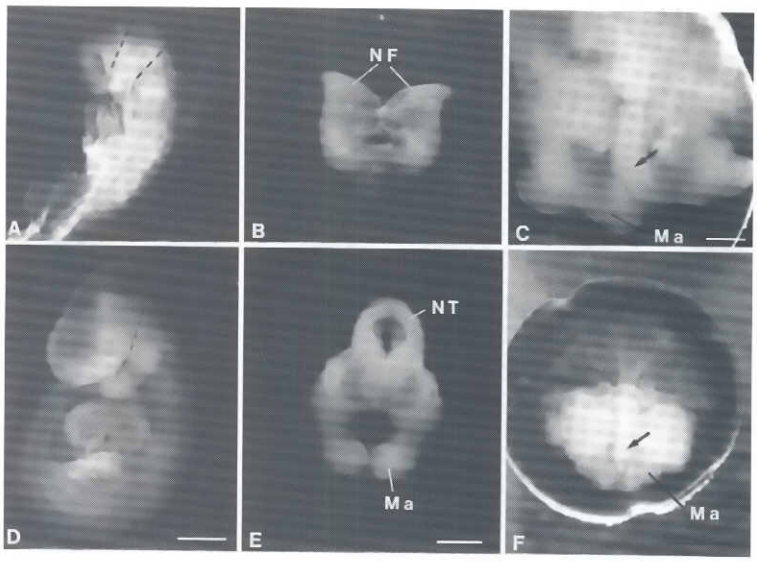

Fig. 3. Early embryonic mouse mandibular morphogenesis in a defined explant culture model. (A) *Lateral view of E8 (1-7 somite pairs, Theiler stage 12) embryo with the first branchial arch (....).* **(B)** *Dissected E8 explant with neural folds (NF).* ***(C)*** *Cultured E8 explants were permissive for the formation of mandible (Ma) and tongue () by 10 days in vitro.* **(D)** *Lateral view of E9 (13-20 somite pairs, Theiler stage 14) embryo with emerging mandibular prominence within the dotted lines.* **(E)** *Dissected E9 explants with neural tube (NT) and mandibular processes (Ma).* **(F)** *Cultured explant demonstrated the formation of mandible (Ma) and tongue (). Bar for panels A through E, 100μm. Bar for panel F, 1mm.*

10 days *in vitro* in a Trowell culture system. The explants were placed on type AA, 0.8 μm pore size Millipore filter discs 6 mm in diameter (Bedford, MA) and supported by stainless steel mesh triangles to insure that the oral epithelium would face upward, opposite to the surface of the filter. The explants were cultured in Grobstein Falcon dishes under optimal humidity conditions in an atmosphere containing 5% CO2 and 95% air. The medium consisted of 700 to 800 μl of BGJb with 10% fetal bovine serum (Gibco, Grand Island, New York) and NGF-2.5s (2.5ng/ml, Sigma) to enhance neuroectoderm viability and development. The medium was also supplemented with 0.1 mg ascorbic acid/ml and 50 units penicillin/streptomycin (Gibco). The medium was changed every other day and kept within a pH range of 7.0 to 7.4. There were three dishes per experimental group (total of eighteen explants). At the end of culture, all explants reached an identical developmental stage.

The same medium was used for whole embryo culture and changed every 2 h. To maintain the proper air exchange, the whole embryos were physically rotated every 20 to 30 min within the incubator and cultured up to 48 h.

DiI labeling

The methodology used for labeling has been previously published by our colleagues (Shuler *et al.*, 1992). We used this methodology to label pre- migratory cranial neural crest (CNC) cells in E8 embryos. Original DiI stock solution (10 mg/ml in absolute ethanol) was first diluted at 1:100 in physiological saline at 37°C for 5 min and followed by a brief centrifuge. DiI was further diluted with BGJb medium containing 10% FBS at 1:6. E8 embryos were incubated with DiI solution for two hours in a tissue culture incubator and then rinsed thoroughly with BGJb medium plus 10% FBS. The whole embryos were cultured and the forming first branchial arch along with its dorsal neural folds were evaluated for the migration of DiI labeled CNC cells into the first branchial arch.

Injection of DiI

The injection of DiI technique has been described previously (Shuler *et al.*, 1992; Chai *et al.*, 1997). DiI is a lipophilic carbocyanine dye which is capable of inserting into the cell membrane at the injection site and can be easily visualized under the dissecting microscope. Micropipettes were made using 50 μl Drummond Microcaps Disposable Pipettes (St. Louis, MI), pulled with a Narishige PB-7 micropipette puller (Greenvale, NY). The micropipettes were then filled with DiI. DiI was first dissolved at 10 mg/ml in absolute ethanol and then diluted 1:100 with saline at 37°C for 5 min. With a Narishige Joystick Manipulator MN-151, the pipette tip was placed at the desired position. By applying gentle air pressure, a controlled amount of dye was injected into a small group of cells. After injection embryos were microdissected and cultured with BGJb medium with 10% FBS for up to 24-48 h. The explants were fixed with freshly prepared 4% paraformaldehyde and 0.25% glutaraldehyde and washed with 1xPBS for 4 times, 30 min per wash. The examination of DiI labeled cell emigration was visualized with both conventional fluorescent or confocal laser scanning microscope.

Whole-mount in situ hybridization

Mouse *Lef1* cDNA probe was subcloned into vector (pGL1-4) containing both the T3 and T7 promoters for generation of sense (negative control) and antisense (experimental) riboprobes utilizing the T3 and T7 RNA promoters. Nonradioactive probes were prepared using RNA Labeling Kit (Boehringer-Mannheim, Germany), which included digoxigenin-labeled UTP. Whole mount in situ hybridization was performed following the method of Wilkinson and Nieto (1993).

Messenger RNA phenotyping

RT-PCR was performed according to Rappolee and colleagues (1989). Explants cultured for 9 days were isolated and placed in liquid nitrogen to ensure the stability of RNA. Six explants were pooled for each RNA extraction. The 5' and 3' amplimers used in the present study were derived from the *Lef1* sequence from position 2099 to 2530 of the cDNA. The amplimers used were YC-*LEF1*-1 (5' CAA GGT CAG CCT GTT TAT CCC ATC 3') and YC-*LEF1*-2 (5' CTG TCT CTC TTT CCG TGC TAG TTC 3'). The amplified product was 431bp and was sequenced to verify the

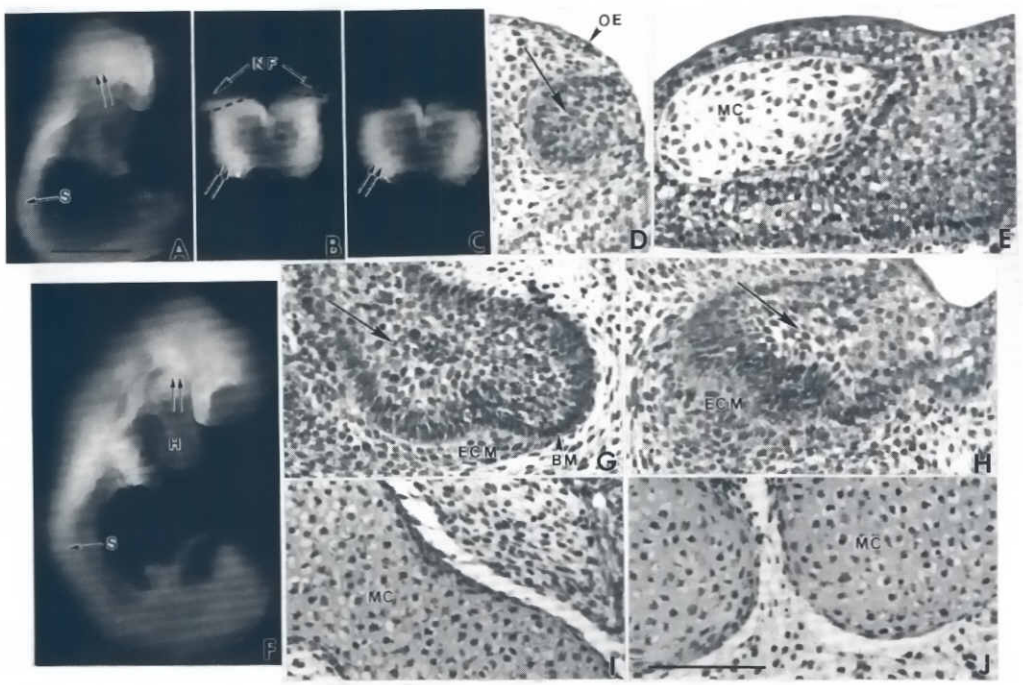

Fig. 4. Cranial neural crest cells at the neural folds of E8 embryos are associated with the formation of tooth and Meckel's cartilage. (A) *Lateral view of E8 embryo with less than six somites (S). The forming first branchial arch is labeled with double arrows.* **(B)** *Dissected E8 explant (double arrow) with intact neural folds (NF). [The dotted line indicated the location where the neural folds could be removed].* **(C)** *Dissected E8 explant with neural folds removed.* **(D)** *E8+10 days cultured explant (dissected from embryos with less than six somites and with intact neural folds) were permissive for the formation of tooth bud (arrow). OE, oral epithelium.* **(E)** *The removal of neural folds from E8 explants (obtained from embryos with less than six somites) resulted in adontia and premature chondrocytes within Meckel's cartilage (MC).* **(F)** *E8 mouse embryo with more than six somite pairs (S). Double arrow indicates the forming first branchial arch. H, heart.* **(G and I)** *E8+10 cultured explants with intact neural folds (from embryo with more than six somite pairs) produced early cap stage tooth organs (arrow) with condensation of extracellular matrix (ECM) and basement membrane (BM) as well as mature chondrocytes within Meckel's cartilage (MC).* **(H and J)** *E8+10 cultured explants with neural folds removed (from embryos with more than six somite pairs) also produced early cap stage tooth organ (arrow), extracellular matrix (ECM) and mature chondrocytes within Meckel's cartilage (MC). Bar for panels A, B, C, and F, 50μm. Bar for panels D, E, G, H, I, and J, 100μm.*

specificity. The β-actin amplification represented position 449-766 in the mouse β-actin cDNA using amplimers BF-11 (5' GTT TGA GAC CTT CAA CAC CCC 3') and BF-12 (5' GTG GCC ATC TCC TGC TCG AAG TC 3') which produced a product of 317bp. The marker used was (ϕ174 RF DNA/Hae III from Gibco-BRL. The PCR products were visualized on a 3% agarose gel by staining with ethidium bromide.

Antisense oligonucleotide inhibition

Lef1 oligodeoxynucleotides were designed based on the cDNA sequences of mouse *Lef1* nucleic acid sequences. The proposed oligodeoxynucleotides were cross-referenced with GenBank and IBI sequence analysis program (International Biotechnology, New Haven) to define the specificity of each oligodeoxynucleotides. They were synthesized and purified using reverse-phase HPLC (Bio-synthesis Inc., Lewisville, TX). Six explants per experimental group were used. They were *Lef1* antisense, 5' GGA AAG TTG GGG CAT 3' and corresponding control *Lef1* sense, 5' ATG CCC CAA CTT TCC 3' (Travis *et al.*, 1991). An additional control antisense and sense oligodeoxynucleotide for mouse amelogenin was used (Diekwisch *et al.*, 1993), since the mouse amelogenin is not expressed in mandibular incisor and molar tooth organs until E15 nor is it expressed during control explant cultures for 10 days. Antisense or control sense oligodeoxynucleotides were added to culture medium at the concentration of 30 μM and replaced with fresh medium changes every other day. Each experimental group (six mandibular explants) was processed for light microscopy.

Results and Discussion

Early first branchial arch explants produce mandibular morphogenesis with Meckel's cartilage, tooth and tongue formation in vitro

E8-E9 mouse embryonic explants were cultured in 10% FBS supplemented with 2.5 ng/ml NGF-2.5s for up to 10 days *in vitro*. As seen in Figure 1A, E8 explants from 1-7 somite pairs (Theiler stage 12) produced tooth buds (Fig. 1B), Meckel's cartilage (Fig. 1C), and tongue formation within 10 days *in vitro*. The formation of Meckel's cartilage was further confirmed by whole-mount Alcian blue staining (data not shown) which stains the chondroitin 4- and 6-sulfate components in cartilage (Chai *et al.*, 1994). There was a lack of bilateral symmetry associated with the final form of cultured E8 explants. However, all phenotypic features including bilateral symmetry were observed using E9 explants. E9 explants (Fig. 2A) from 13-20 somite pairs (Theiler stage 14) produced bilaterally symmetrical Meckel's cartilage (Fig. 2B), cap stage tooth organs (Fig. 2B and C) and tongue formation (Fig. 2B). Figure 3 shows the dissection scheme used to obtain the explants and the phenotypes produced by this *in vitro* model. At E8, the forming first branchial arch was dissected along with its dorsal neural folds (Fig. 3A). With a still wide open neural tube, the explants (Fig. 3B) were placed in the organ culture system. At the end of the culture, the first branchial arch produced the formation of tongue and mandible (Fig. 3C). Figure 3D shows the dissection of the first branchial arch from an E9 embryo. At this developmental stage, the fusion of the neural tube was completed (Fig. 3E). *In vitro* explant culture

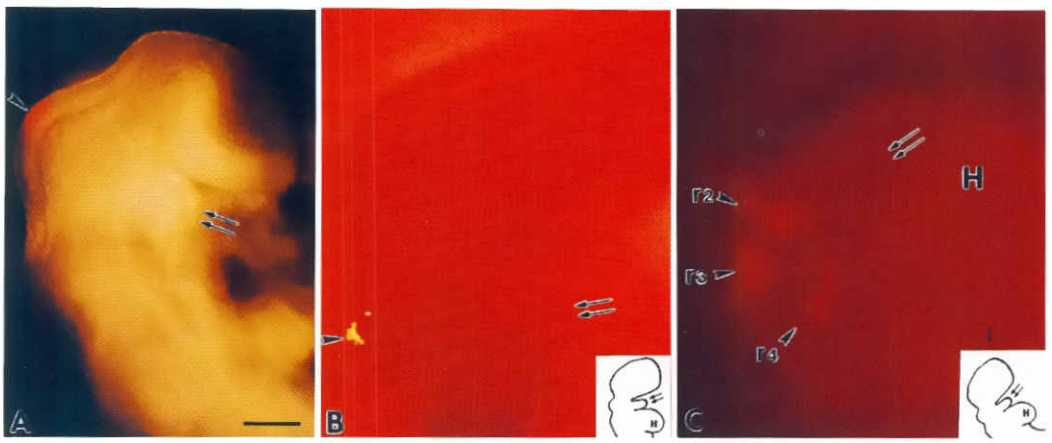

Fig. 5. Cranial neural crest cells emigrate from the neural folds into the forming first branchial arch. **(A)** *DiI focal injection (arrow) into the neural fold dorsal to the first branchial arch (double arrow).* **(B)** *Epifluorescence photography showed DiI-labeled CNC cells (arrow) in the neural fold dorsal to the first branchial arch (double arrow).* **(C)** *DiI-labeled CNC cells from rhombomeres 2 and 4 emigrated into the first branchial arch. (() DiI injection site; ((() the first branchial arch; (r2, r3, and r4) rhombomeres 2, 3, and 4. Inserts, outline the exact orientation and landmarks of the embryo. Bar, 100μm.*

produced the formation of tongue and a symmetrical mandibular form (Fig. 3F).

In order to determine the critical timing for emigration of cranial neural crest cells which engage in the formation of tooth and Meckel's cartilage during the morphogenesis of the first branchial arch, we removed the neural folds from explants at different developmental stages. Figure 4A shows the lateral view of early E8 embryo with less than six somites. The explants with intact neural folds (Fig. 4B) were cultured and formed bud stage tooth organs and Meckel's cartilage (Fig. 4D). If the neural folds were removed (Fig. 4C), there was no detectable tooth formation and there were premature chondrocytes within Meckel's cartilage (Fig. 4E). When mouse embryos of more than six somites were used to obtain explants (Fig. 4F), the removal of neural folds from explants did not affect tooth formation (Fig. 4H) and Meckel's cartilage development (Fig. 4J), [compare to control group using explants with intact neural folds in Figure 4G and Figure 4I, respectively]. These experiments were repeated three times with reproducible results.

The design of the early explant culture model was based on our previous E11 (42-44 somite stage) mandibular explant culture model in serumless, chemically-defined medium (Slavkin *et al.*, 1989; Chai *et al.*, 1994). Empirically, we determined that E8 and E9 explants form Meckel's cartilage, tooth buds and tongue. Furthermore, we discovered that a dissection scheme which included the dorsal neural folds and the forming first branchial arch, ensured cranial neural crest cell emigration and preserved the migration pathway. Predictably, we further discovered that removal of the neural folds from the explant at less than six somite stage resulted in adontia and premature chondrocyte formation. This reliable result suggested that cranial neural crest cells are required for instructing the initiation and formation of tooth and Meckel's cartilage development. In addition, the critical time period for cranial neural crest cell emigration into the first branchial arch is between the 4 to 6 somite stages of mouse embryogenesis in order to induce tooth and cartilage formation. These experiments tested and confirmed our hypothesis that cranial neural crest cells at the dorsal neural folds of the first branchial arch emigrated into the forming mandibular arch, and, through unique epithelial-mesenchymal interactions, formed Meckel's cartilage and tooth buds in this early embryonic mouse mandibular culture model.

Early embryo culture model is permissive for studies of cranial neural crest migration during first branchial arch morphogenesis

Previous studies indicated that cranial neural crest cells first emigrate into the first branchial arch from the midbrain and anterior hindbrain by four somite stage; this emigration process was completed by 11-14 somite stage (Nichols, 1981, 1986; Tan and Morriss-Kay, 1986; Serbedzija *et al.*, 1992). Furthermore, the timing of emigration of CNC cells from the neural fold and the eventual cessation of emigration paralleled each other at different axial levels of the developing embryonic brain; both were completed in approximately 9 to 12 h. Significantly, these previous studies demonstrated the contribution of midbrain and hindbrain crest cells to the first branchial arch during mouse embryogenesis. However, the only direct evidence that indicates crest cells to tooth formation during rat embryogenesis was recently shown by detecting DiI labeled crest cells from the posterior midbrain in the dental mesenchyme (Imai *et al.*, 1996).

We hypothesized that cranial neural crest cells at the dorsal neural fold of the first branchial arch emigrate into the forming maxillary and mandibular arches. Thereafter, through unique and instructive epithelial-mesenchymal interactions, these crest cells provide the cell lineages for Meckel's cartilage, tooth organ, and tongue development. To test our hypothesis, vital dye-DiI was used to label neuroectoderm cells at the dorsal neural fold of the first branchial arch which may transdifferentiate into cranial neural crest cells and emigrate from the neural folds into the forming mandibular arch (Fig. 5). Figure 5A shows the lateral view of E8 embryo with focal DiI injected into the neuroectoderm at the anterior portion of hindbrain, directly behind the first branchial arch (double arrow). Using fluorescent microscopy (Fig. 5B), a small and confined group of DiI labeled neuroectodermal cells were localized dorsal to the first branchial arch (double arrow). After culturing the whole embryo for 12 h, labeled presumptive cranial neural crest cells from dorsal neural fold emigrated into the first branchial arch (double arrow).

E8 whole embryos were incubated with DiI for 2 h, and subsequently cultured for 12 h. At E8, the neural grove is partically open with the formation of the first branchial arch in progress (Fig. 6A). After 12 h in culture, DiI-labeled cells from the dorsal neural folds were identified at distal positions in the first branchial arch (double

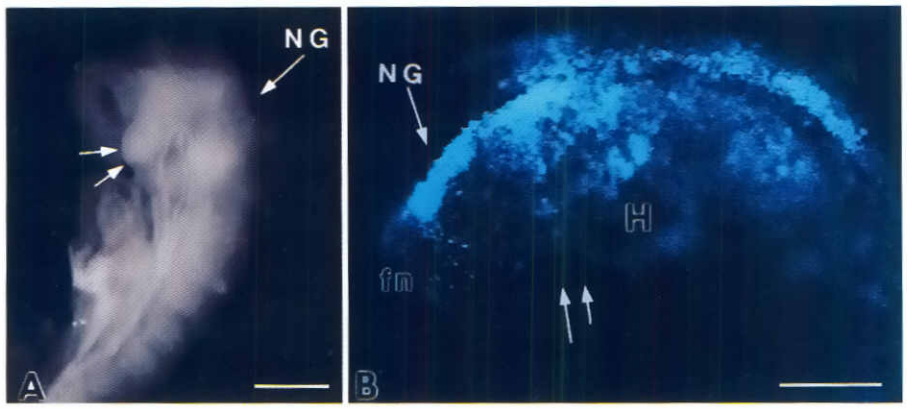

Fig. 6. Incubation of E8 whole embryos with DiI showed cranial neural crest cells emigrate into the first branchial arch. (A) *Lateral view of E8 embryo with first arch (I) and neural groove (NG).* **(B)** *E8 embryo labeled with DiI for 2 h and cultured for 12 h. DiI-labeled neural folds and CNC cells emigrating into the forming first arch (I) observed with confocal microscopy. Bar for panel A, 50μm, Bar, 20μm for panel B.*

arrow) using confocal microscopy (Fig. 6B) and epifluorescence photography.

This experimental approach permitted the DiI labeling of embryo by either whole embryo incubation or focal injection of DiI to label discrete groups of cells. Utilizing the whole embryo culture followed by early mandibular explant culture model described here, we can design future experiments to define the destination of a discrete crest cell population, migration pathway, duration of emigration, and their final phenotypes during mandibular morphogenesis.

Using early mandibular explant culture model to study the regulation of tooth formation by transcription factor *Lef1*

Two lines of evidence suggested that *Lef1* gene expression was associated with mandibular morphogenesis. First, RT-PCR analysis of microdissected specimens representing E8, E9, E10 and E11 showed *Lef1* gene expression in first branchial arch tissues (Fig. 7). Second, whole-mount *in situ* hybridization of E8 (1-7 somite pairs), E9 (13-30 somite pairs) and E10 (30-34 somite pairs) embryos demonstrated *Lef1* gene expression associated with frontonasal, maxillary and mandibular prominences, limb and heart morphogenesis (Fig. 8). At E8, *Lef1* was localized to the somites (Fig. 8A). At E9, *Lef1* was detected in the mandibular arch and in the presumptive maxillary prominence (Fig. 8B). The lateral view of E10 embryo demonstrated *Lef1* expression at the frontonasal, maxillary, mandibular prominences, second branchial arch, limb buds, and heart (Fig. 8C). These results confirm the pattern of *Lef1* gene expression previously described in neural crest, branchial arches and limb buds during E10.5 mouse embryogenesis (Oosterwegel *et al.*, 1993).

Based upon *Lef1* expression during early embryonic mouse mandibular development, we designed loss of function experiments using antisense inhibition directed against *Lef1*. Antisense *Lef1* oligonucleotide inhibition using E8 (10-12 somite pairs) first branchial arch explants with associated rhombencephalic neural crest produced bud stage tooth organs (Fig. 9C) which did not advance to cap stage. Sense-treated or non-treated controls produced early cap stage tooth organs (Fig. 9A and B). These experiments were repeated three times with reproducible results. Since amelogenin is not expressed in mandibular incisor or molar tooth organs until E15, an additional control using mouse amelogenin antisense oligodeoxynucleotide also produced early cap stage tooth organ by culturing E8 first branchial arch explants (data not shown). These results complement and are consistent with recent reports indicating that *Lef1* null mutation in transgenic mice arrested tooth morphogenesis before the formation of a mesenchymal dental papilla and also produced other developmental defects associated with epithelial-mesenchymal interactions (van Genderen *et al.*, 1994; Kratochwil *et al.*, 1996).

Using the early mandibular explant culture model, we demonstrate that tooth organ development was arrested at the bud stage when E8 explants were treated with *Lef1* antisense oligodeoxynucleotides; while sense and non-treated controls demonstrated formation of early cap stage tooth organ. The reasons for using E8 mandibular explants in the antisense experiment are two folds. First, E8 mandibular explant is the earliest starting material which

Fig. 7. *Lef1* expression during mandibular morphogenesis. *M: DNA marker (X 174/Hae III; Lanes 1-4, the expression of Lef1 in first branchial arch from E8, E9, E10 and E11 mouse embryos; Lanes 5-8, the expression of b-actin in first branchial arch.*

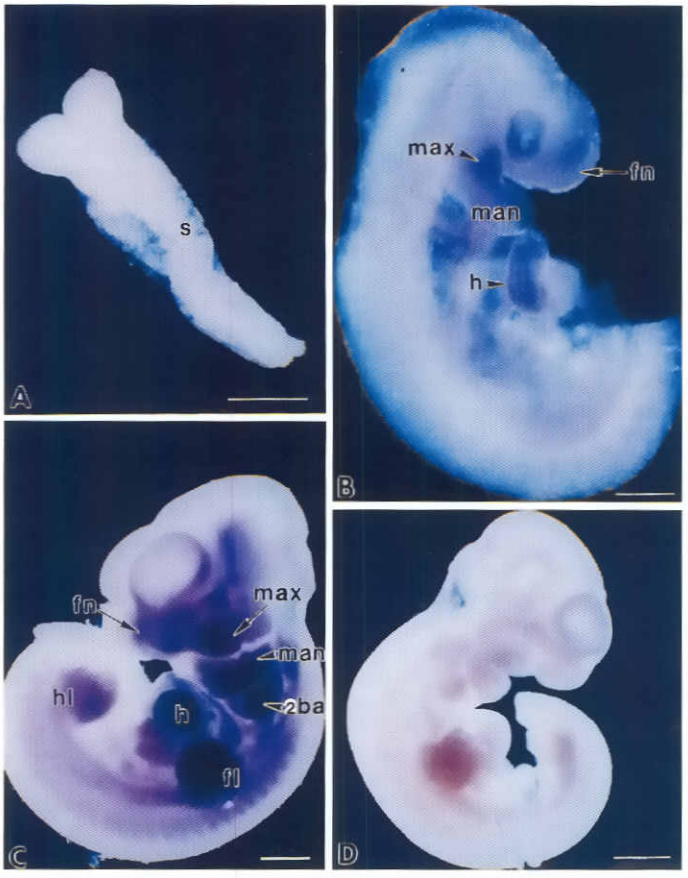

Fig. 8. *Lef1* patterns of expression during early embryogenesis. (A) *Dorsal view of E8 (1-7 somite pairs) mouse embryo with Lef1 expression in the somite region (s).* **(B)** *Lateral view of E9 (13-20 somite pairs) mouse embryo with Lef1 expression in the rostral region of mandibular prominence (man), the site of maxillary prominence formation (max), frontal nasal prominence (fn) and the heart (h).* **(C)** *Lateral view of E10 (30-34 somite pairs) mouse embryo with Lef1 expression in the mandibular prominence (man), maxillary prominence (max), second branchial arch (2ba), frontal nasal prominence (fn), heart (h), forelimb (fl) and hindlimb (hl).* **(D)** *Lateral view of E9 mouse embryo treated with Lef1 sense probe as a control. Bar, 100μm.*

can produce tooth organ in this *in vitro* model. Second, E8 is a critical time point for the migration of CNC cells which contribute to the formation of tooth and Meckel's cartilage. Interestingly, targeted inactivation of the *Lef1* gene resulted in an arrest of early tooth development at E13, after formation of the epithelial tooth bud and mesenchymal condensation, but before the formation of dental papilla (van Gendern *et al.*, 1994; Kratochwil *et al.*, 1996). The specific location of *Lef1* expression in the most basal cells of the epithelial tooth bud at E13 suggested a possible function in the formation of the enamel knot proposed to function as a signaling center for tooth morphogenesis (Jernvall *et al.*, 1994; Kratochwil *et al.*, 1996; Vaahtokari *et al.*, 1996). The conclusion from our study, and other previous studies, is that *Lef1* does not control the initiation of tooth formation which is marked by the formation of a dental lamina, but rather, it is involved in the regulation of epithelial-mesenchymal interactions during subsequent odontogenesis.

In summary, starting as early as E8, cranial neural crest cells emigrate from the dorsal neural folds into the forming first branchial arch. The critical time period for these crest cells to emigrate into the first branchial arch is between E8 and E9. These cells and their lineages appear to be associated with the formation of tooth and Meckel's cartilage. The early explant culture model described in this investigation is permissive for studies of CNC cell migration during first branchial arch and subsequent mandibular morphogenesis. Finally, this early mandibular explant culture model provides direct approach in investigating the role of transcription factor, such as *Lef1*, in regulating mandibular morphogenesis.

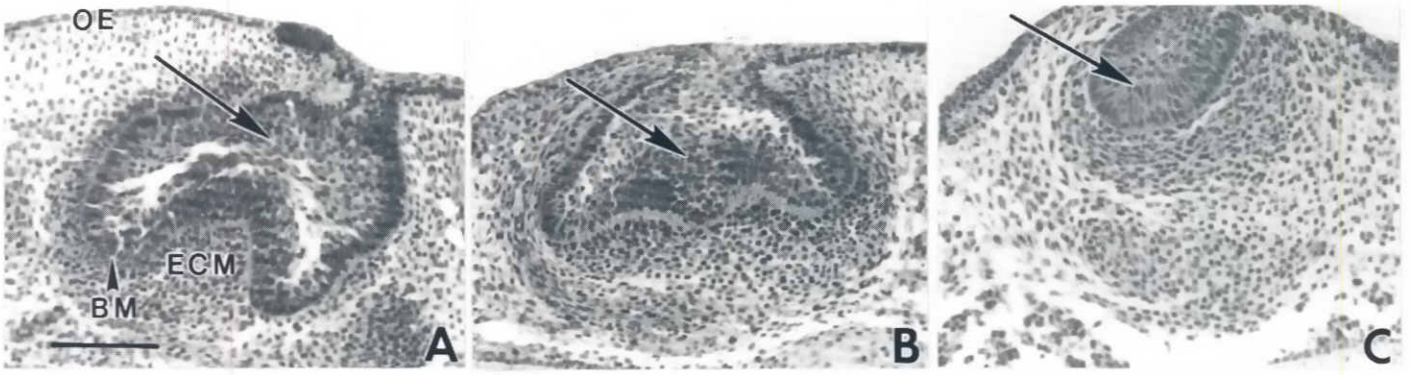

Fig. 9. *Lef1* abrogation resulted in arrested tooth formation. (A) *E8 + 10 days cultured explants were permissive for tooth formation with oral epithelium (OE), early cap stage tooth organ ((), basement membrane (BM), and ectomesenchyme surrounding the tooth organ (ECM).* **(B)** *Control Lef1 sense oligodeoxynucleotides treated E8 + 10 days cultured explant also permissive for early cap stage tooth formation (().* **(C)** *Lef1 antisense oligodeoxynucleotides treated E8 +10 days cultured explants showed bud stage tooth formation (() without the CNC-derived ectomesenchymal cells forming the future dental papilla. Ba, 100μm.*

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