**prx-1** functions cooperatively with another *paired*-related homeobox gene, **prx-2**, to maintain cell fates within the craniofacial mesenchyme

Mei-Fang Lu, Hui-Teng Cheng, Michael J. Kern, S. Steven Potter, Bao Tran, Thomas G. H. Diekwisch and James F. Martin

1 Alkek Institute of Biosciences and Technology, Center for Cancer Biology and Nutrition, Department of Medical Biochemistry and Genetics, Texas A&M University, Houston, TX 77030, USA
2 Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, 171 Ashley Avenue, SC 29425, USA
3 Division of Basic Science Research, Children’s Hospital Research Foundation, Cincinnati, 3333 Burnett Avenue, OH 45229, USA
4 Department of Biomedical Sciences, Baylor College of Dentistry, 3302 Gaston Avenue, Dallas, TX 75246, USA

*Author for correspondence (e-mail: Jmartin@ibt.tamu.edu)

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**SUMMARY**

The *paired*-related homeobox gene, **prx-1**, is expressed in the postmigratory cranial mesenchyme of all facial prominences and is required for the formation of proximal first arch derivatives. We introduced lacZ into the **prx-1** locus to study the developmental fate of cells destined to express **prx-1** in the **prx-1** mutant background. lacZ was normally expressed in **prx-1** mutant craniofacial mesenchyme up until 11.5 d.p.c. At later time points, lacZ expression was lost from structures that are defective in the **prx-1** mutant mice. A related gene, **prx-2**, demonstrated overlapping expression with **prx-1**. To test the idea that **prx-1** and **prx-2** perform redundant functions, we generated **prx-1**,**prx-2** compound mutant mice. Double mutant mice had novel phenotypes in which the rostral aspect of the mandible was defective, the mandibular incisor arrested as a single, bud-stage tooth germ and Meckel’s cartilage was absent. Expression of two markers for tooth development, **pax9** and **patched**, were downregulated. Using a transgene that marks a subset of **prx-1**-expressing cells in the craniofacial mesenchyme, we showed that cells within the hyoid arch take on the properties of the first branchial arch. These data suggest that **prx-1** and **prx-2** coordinate regulation of gene expression in cells that contribute to the distal aspects of the mandibular arch mesenchyme and that **prx-1** and **prx-2** play a role in the maintenance of cell fate within the craniofacial mesenchyme.

Key words: *Paired*-related homeobox, Craniofacial development, Genetic redundancy, Mouse, **prx-1**

**INTRODUCTION**

Cell lineage analysis performed primarily in the avian system has demonstrated that the craniofacial skeleton derives primarily from two embryologic sources: the cranial neural crest (CNC) and the cranial paraxial mesoderm with a minor contribution from the occipital somites. The majority of the skull is crest-derived, although there is disagreement about the origin of the bones of the calvarium (Coulby et al., 1993; Le Douarin et al., 1993; Noden, 1988). A migratory cell population that originates in the dorsal neural tube, the CNC, undergoes an epithelial-to-mesenchymal transition to form the mesenchyme of the craniofacial primordia. Among these is **prx-1** (previously called ***Mhox***) which are closely related members of the *paired*-related (**prx**) family of homeobox genes (Cserjesi et al., 1992; Kern et al., 1992). At 9.5 d.p.c., **prx-1** is expressed in the CNC-derived mesenchyme of the fronsal process, as well as the first and second branchial arches. At this stage, **prx-1** is also expressed within a group of cells ventral to the eye that will form the maxillary process of the first branchial arch. At later stages, **prx-1** expression is maintained within the mesenchyme of the maxillary and mandibular processes of the first branchial arch. Expression of **prx-1** is extinguished in mesenchymal cells as differentiation is initiated (Cserjesi et al., 1992; Kern et al., 1992; Kuratani et al., 1994).

Inactivation of **prx-1** in mice demonstrated that it played a
central role in development of skeletal elements derived from the proximal aspects of the first branchial arch (Martin et al., 1995). This suggested that there may be other, redundant genes functioning in the unaffected regions of the prx-1-expressing craniofacial mesenchyme. To follow the developmental progression of cells that are fated to express prx-1 in the prx-1 mutant background, we introduced the lacZ gene into the prx-1 locus. In prx-1neo; prx-1lacZ homozygous mutant embryos, we found that prx-1-expressing cells initially contributed normally but failed to be maintained in developing craniofacial skeletal elements derived from proximal first branchial arch mesenchyme. To test the possibility of genetic redundancy between the prx-1 and prx-2 in the craniofacial primordia, we generated prx-1neo; prx-2 double mutant mice and found novel defects of the distal aspects of the first branchial arch. Using a transgene that distinguishes between groups of prx-1-expressing cells in the craniofacial primordia, we provide evidence that subpopulations of cells within the larger field of prx-1 expression were reprogrammed to new fates. Our data show that prx-1, in cooperation with prx-2, function to stabilize and maintain cell fates within the craniofacial mesenchyme.

MATERIALS AND METHODS

Nomenclature

With the goal of simplifying vertebrate homeobox gene nomenclature, we now refer to Mhx and S8 as prx-1 and prx-2, respectively (Scott, 1992).

Generation of prx-1lacZ allele

The prx-1lacZ targeting vector was constructed by inserting the lacZ gene in frame into a unique StuI site at the 5′ end of the homeobox. A pSGneo resistance cassette was introduced 3′ of lacZ in the reverse transcriptional orientation. The 5′ arm was a 2.5 kb PvuII-StuI fragment and the 3′ arm was a 4.0 kb StuI-EcoRI fragment. The targeting vector was flanked on the 5′ side by a pMCI-thymidine kinase gene (Mansour et al., 1988). The targeting vector was linearized with PmeI.

The prx-1lacZ targeting vector was electroporated into ES cells (AK7) using a Bio-Rad gene pulser (500 rf, 240 V) and the ES cells were plated on SNL76/7 cells and cultured under positive and negative selection using G418 and FIAU (McMahon and Bradley, 1990). Surviving clones were analyzed by Southern analysis to identify targeted clones (Ramirez-Solis et al., 1992). The four targeted clones were expanded and genomic DNA extracted and analyzed using 5′ and 3′ probes to verify the integrity of the prx-1lacZ targeted locus. Two of these clones were injected into 3.5 d.p.c. blastocysts to generate chimeras. One clone transmitted the mutation through the germline.

Genotyping of mice

To identify mice carrying the prx-1 and prx-2 mutations, Southern blot was performed on genomic DNA obtained from tail biopsies of neonatal and 10-day-old mice and from the yolk sacs of mouse embryos. To isolate genomic DNA, tissue was incubated in lysis buffer (10 mM Tris pH 8.0, 25 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS, 0.2 mg/ml Proteinase K) at 55°C for 3 hours, followed by phenol-chloroform extraction and ethanol precipitation. Genomic DNA was digested with the indicated restriction enzyme and fractionated on a 0.7% agarose gel. Digested DNA was transferred to Zeta-Probe GT membranes and hybridized with prx-1- or prx-2-specific probes. To identify the prx-1lacZ mutant allele, genomic DNA was digested with EcoRI and EcoRV. Using the A probe for Southern blots, the wild-type allele migrated at 3.5 kb while the mutant allele was 2.5 kb. Details of the prx-1neo targeting strategy have been described (Martin et al., 1995). The prx-2 targeting strategy resulted in the deletion of the third exon which encodes the DNA binding domain and was shown to be a null allele. The details for generating and genotyping this allele will be published elsewhere (Kern et al., submitted). The prx-1neo; prx-2 double mutant phenotype was analyzed on a 129Sv × C57Bl/6 hybrid background. All phenotypes were 100% penetrant unless otherwise stated in the text.

Generation of transgenic mice

The prx-1 transgenic mice will be described in more detail elsewhere (J. F. M., unpublished data). Briefly, the construct used to generate the transgenic mice analyzed in this paper contains 2.7 kb of prx-1 5′ flanking region which extends into the 5′ UTR of prx-1 fused to the lacZ gene from the puc19 AUGlacZ plasmid, which contains its own initiator methionine (Cheng et al., 1993). Five transgenic lines have been generated with similar results to what is reported here.

Skeletal analysis

Skeletal preparations were performed essentially as described (Martin et al., 1995). The data presented here are based on the analysis of twelve prx-1neo/lacZ; prx-2−/− and nine prx-1neo/lacZ; prx-2+/− skeletons.

Histology

Mouse tissues were fixed in 4% paraformaldehyde overnight and then dehydrated through graded alcohols and embedded in paraffin. Paraffin blocks were sectioned at 7-10 μm and stained with hematoxylin and eosin.

Staining for β-gal

The expression of lacZ in developing tissues was detected essentially as described (Beddington et al., 1989).

In situ hybridization

Whole-mount and sectioned in situ hybridization was performed as described (Edmondson et al., 1994). Probes for patched (Neubuser et al., 1997), patched (Goodrich et al., 1996), prx-2 (Opstelten et al., 1991) and prx-1 (Cserjesi et al., 1992) have been described previously.

RESULTS

Introduction of lacZ into the prx-1 locus

In order to study the developmental progression of cells that are fated to express prx-1 in the prx-1 mutant background, we targeted lacZ to the prx-1 locus to generate the prx-1lacZ allele (Fig. 1). To do this, we made an in-frame fusion of lacZ to a unique StuI site in the 5′ end of the homeobox (Fig. 1A,B). The targeting vector, which contained 6.5 kb of homology, was linearized and electroporated into embryonic stem (ES) cells. After positive and negative selection, 96 colonies were analyzed by Southern analysis (Fig. 1C). Four (approximately 1 in 25) targeted events were identified and two clones were injected into C57Bl/6J blastocysts to generate chimeras. One of these clones transmitted the prx-1lacZ allele through the germline.

To confirm that the prx-1lacZ allele was genetically identical to the previously described prx-1neo allele (Martin et al., 1995), we crossed these two lines and analyzed the phenotypes of prx-1neo; prx-1lacZ mice. The phenotype of these mutant mice was a pheno type of the prx-1neo homozygous mutant mice (data not shown). From this, we conclude that the prx-1neo and prx-1lacZ are genetically comparable. We next performed an expression
expression of the prx-1lacZ allele at multiple developmental time points to confirm that the prx-1lacZ allele was expressed similarly to the endogenous gene.

**Expression analysis of the prx-1lacZ allele**

At 9.5 days post coitum (d.p.c.), lacZ expression was detected in the rostral aspect of the mandibular process, as well as within cells ventral to the eye that will contribute to the proximal components of the first branchial arch skeleton (Fig. 2A). Expression of lacZ was most intense at the distal half of the mandibular process. At this stage, we also found that lacZ was expressed in the mesoderm of the forelimb bud (Fig. 2B). At 10.0 d.p.c., expression of lacZ was maintained in the mandibular and maxillary process but was now found in more proximally located cells around the first branchial groove that will give rise to the external acoustic meatus and the external ear structures (Fig. 2C,D). lacZ was intensely expressed in the facial structures at 11.5 d.p.c. in a manner that was consistent with the earlier expression pattern. Intense staining was detected in the forming maxilla and mandible as well as in the frontonasal process (Fig. 2E). At this time point and at 12.5 d.p.c. (Fig. 2F), lacZ-expressing cells could be seen in cells that prefigure the dermal bones on the lateral aspect of the head such as the squamosal. From this analysis, we conclude that the prx-1lacZ allele expresses in an identical fashion to the endogenous gene (Cserjesi et al., 1992; Kern et al., 1992; Kuratani et al., 1994).

**lacZ expression is lost from a group of proximal first arch-derived cells**

We next analyzed the expression of lacZ in the prx-1neo: prx-1lacZ mutant background. lacZ expression was similar in wild-type and mutant embryos at 10.5 and 11.5 d.p.c. (Fig. 3A,B and not shown). However, at 12.5 d.p.c. a dramatic decrease in lacZ-expressing cells was detected in a region of the craniofacial primordia destined to contribute to the squamosal and other proximal first arch derivatives (Fig. 3C,D). This group of cells normally gives rise to the structures that were abnormal or missing in the prx-1neo homozygous mutant mice (Martin et al., 1995). Thus, cells fated to express prx-1 initially contributed normally to forming cranial structures in the prx-1 mutant; however, at later time points, a subpopulation of lacZ-expressing cells was lost or stopped expressing lacZ. Thus, prx-1 function is required for maintenance of prx-1-expressing cells in the proximal first arch mesenchyme. These defects were not observed in the distal components of the first arch suggesting that another gene was compensating for the loss of prx-1 function in that mesenchyme.

**prx-2 and prx-1 are coexpressed in the craniofacial primordia**

prx-2 is a closely related prx family member that has overlapping expression with prx-1 in the mandibular process of the first branchial arch, as well as in other regions of the embryo (Leussink et al., 1995). Additionally, prx-2−/− mice had normal skeletons suggesting that another gene may substitute for loss of prx-2 function in the skeletal primordia (M. J. K., unpublished data). To confirm that prx-1 and prx-2 were coexpressed in craniofacial primordia, we performed in situ analysis on serial sections of mouse embryos, as well as whole-mount in situ analysis of similarly staged mouse embryos. At 10.5 d.p.c., we found that prx-1 and prx-2 are coexpressed in cells within the mandibular, maxillary and frontonasal processes (Fig. 4A,B,E,F). prx-1 was expressed both more abundantly and more broadly at this stage. Although prx-1 and prx-2 were both expressed at high levels at the tip of the mandibular arch (Fig. 4A,B,E,F); prx-2 was also expressed at higher levels in the otic capsule. At 10.5 d.p.c., expression of prx-1 and prx-2 was detected in more distal regions of the nasofrontal primordium (Fig. 4A,B,E,F). Additionally, prx-2 was expressed in the maxillary process at 11.5 d.p.c. (Fig. 4A,B,E,F). At 12.5 d.p.c., expression of prx-2 was detected in the maxillary process but was now found in more proximally located cells around the first branchial groove that will give rise to the external acoustic meatus and the external ear structures. This expression pattern was similar in wild-type and mutant embryos at 10.5 and 11.5 d.p.c. (Fig. 3A,B and not shown). Additionally, a HindIII site has been shifted more 3′. (C) Southern blot analysis of gene targeted ES clones digested with EcoRI and HindIII on the left and EcoRI and EcoRV on the right. The genotypes of each ES clone is shown at top. With the introduction of lacZ into the prx-1 locus a new EcoRV site has been inserted. Additionally, a HindIII site has been shifted more 3′.
4A,E), prx-1 was also expressed at high levels in the proximal mandibular process where prx-2 expression was less intense (Fig. 4B,F). At 11.5 d.p.c., whole-mount in situ demonstrated that expression of both prx-1 and prx-2 was maintained at high levels in equivalent groups of cells in the mandibular and maxillary processes (Fig. 4C,G). At 12.5 d.p.c., expression of these two genes was downregulated in the mandibular process but expression in the maxilla and nasal processes was maintained (Fig. 4D,H). We identified some minor differences in the craniofacial expression patterns of prx-1 and prx-2. At 12.5 d.p.c., prx-2 was expressed slightly more medially in the frontonasal process than prx-1 (Fig. 4D,H).

We examined expression of prx-1 in the prx-2 mutant mouse to determine if there was a compensatory increase in prx-1 expression. No differences in expression of prx-1 in the prx-2 mutants as compared to wild-type mice were detected (data not shown). Similarly, expression of prx-2 in the prx-1 neo homozygous mutants was examined at 10.5 and 11.5 d.p.c. and no differences were detected between wild type and mutant (data not shown). Thus, prx-1 and prx-2 do not cross regulate each others’ expression.

**prx-1neo; prx-2 double mutant mice have severe defects of distal first branchial arch derivatives**

To generate double mutant mice, we crossed prx-1neo heterozygotes to prx-2 homozygous mutant mice which are viable and fertile (M. J. K., unpublished data). The majority of the analysis of double mutant mice was performed using the prx-1neo allele, although the phenotypes of prx-1neo; prx-2 double mutants were identical to the prx-1lacZ; prx-2 double mutants. Compound prx-1neo; prx-2 heterozygotes were viable and fertile, and had normal skeletons. Intercrosses between compound heterozygotes gave rise to doubly mutant neonatal mice in the expected Mendelian ratios demonstrating that the double mutants were able to progress through development (data not shown). We found that one copy of the wild-type prx-1 allele was sufficient for skeletal development, since prx-1neo heterozygotes on the prx-2 homozygous mutant background were normal.

The double mutant neonates never fed, had respiratory distress marked by gasping motions and cyanosis, and died...
within 24 hours of birth. All double mutants had a cleft secondary palate, which was likely to contribute to their inability to feed and breathe (Fig. 5A-D). *prx-1*neo; *prx-2* double mutant neonatal mice had more hypoplastic and posteriorly displaced auricles than the *prx-1*neo homozygous mutant mice, as well as open eyes secondary to failure of eyelid formation (data not shown). Double mutant mice also had severe defects in limb morphogenesis. A detailed analysis of the limb phenotype will be presented elsewhere (Lu et al., 1998).

Skeletal preparations of neonates demonstrated that the mandible was severely shortened and fused at its most rostral aspect (Fig. 5E-H). Wild-type neonatal mice and *prx-1*neo mutants had well-formed mandibles connected by a synphysis at its rostral aspect (Fig. 5E,F). The *prx-1*neo/-; *prx-2*+/− mice had a mandible that was fused rostrally and had only a single midline incisor tooth (Fig. 5G). *prx-1*neo/-; *prx-2*−/− mice had a severely shortened mandible that was also rostrally fused and failed to form an incisor tooth (Fig. 5H). Thus, the mandibular and incisor tooth phenotypes were sensitive to the dosage of the *prx-2* gene. We also detected defects in the skull base of double mutants although these phenotypes were less severe than the mandibular defects (Fig. 5A-D). In double mutant mice, the palatal and zygomatic process of the maxilla were deleted (Fig. 5D). In *prx-1*neo/-; *prx-2*+/− mice, the maxillary zygomatic process was intact and the palatal was reduced but present (Fig. 5B).

The primary cartilage of the mandibular process is Meckel’s cartilage. This structure was more severely affected in double mutant embryos as demonstrated by cartilage staining at 14.5 d.p.c. At this stage, Meckel’s cartilage is a well-defined, rod-shaped cartilage (Fig. 5I). In the *prx-1*neo/-; *prx-2*+/+ mice, Meckel’s cartilage had an abnormal sigmoidal morphology (Fig. 5J) whereas *prx-2* mutants had a normal Meckel’s cartilage. In the double mutant, Meckel’s cartilage was absent except for a remnant at the most rostral tip of the developing mandible (Fig. 5K).

**prx-1**neo; **prx-2** double mutants have abnormal mandibular incisor teeth

To further characterize the distal mandibular arch phenotype, we performed histological analysis of the developing teeth in double mutant embryos. In wild-type embryos at 12.5 d.p.c., the two incisor teeth had progressed from the bud stage to the early bell stage in which the base of the tooth bud had become invaginated by the underlying dental papilla (Fig. 6A). In the double mutants, mandibular incisor development arrested as a single tooth bud. Parasagittal and transverse sections through the double mutant incisor tooth bud demonstrated that, while mesenchyme had condensed around the dental epithelium, the morphogenetic events that lead to the bell-stage tooth failed to occur (Fig. 6B,C). Sections of embryos at 16.5 d.p.c. demonstrated the reduction in size of the tooth organ as well as epithelial hypertrophy and abnormal positioning of the tooth germ relative to the alveolar bone. In addition, there was a failure of odontoblasts and ameloblasts to differentiate (Fig. 6D,E).

We examined the expression of molecular markers that have been implicated in tooth formation. At 12.5 d.p.c., we examined the expression of *pax9* which has been demonstrated...
to be expressed in dental mesenchyme of all tooth germs and is required for normal tooth development (Neubuser et al., 1997). In double mutant mandibular incisors, expression of \textit{pax9}, was downregulated in comparison to wild type (Fig. 6F,G). In contrast, expression of \textit{pax9} in molars was unaffected in the double mutant embryos. We also examined expression of \textit{patched} which is expressed in developing teeth and is a component of the \textit{Shh} signaling pathway (Helms et al., 1997; Marigo et al., 1996). We found that, as for \textit{pax9}, expression of \textit{patched} was downregulated in the mandibular incisor tooth germ of double mutant embryos (Fig. 6H,I). These results demonstrate that \textit{prx-1} and \textit{prx-2} cooperatively function to maintain expression of \textit{pax9} and \textit{patched} in the forming mandibular incisor teeth.

To determine if the mesenchyme of the mandibular process had been correctly specified, we examined expression of \textit{sox9}, which marks the prechondrogenic mesenchyme of the branchial arches (Zhao et al., 1997; Thomas et al., 1997). At 11.5 d.p.c., \textit{sox9} is expressed, prior to chondrogenesis in the cells that will form Meckel’s cartilage in the mandibular process (Fig. 6J). In the double mutants at this stage, we found that \textit{sox9} was expressed normally (Fig. 6K). Thus, in the \textit{prx-1neo; prx-2} double mutant, the condensations are initiated normally, as determined by \textit{sox9} expression, but fail to be maintained, as determined by the failure of Meckel’s cartilage to form.

\textit{prx-1neo; prx-2} double mutant craniofacial mesenchyme ectopically expresses a \textit{prx-1} transgene

We sought to follow the developmental fate of cells destined to express \textit{prx-1} in the double mutant background. For this purpose, we used a \textit{prx-1} transgene that drives expression of \textit{lacZ} in a subset of craniofacial precursors that normally express the endogenous \textit{prx-1} gene (J. F. M., unpublished data). This transgene, called \textit{prx-1 2.7 lacZ}, first expresses \textit{lacZ} in the lateral aspect of the mandibular region at 14.5 d.p.c. Additionally, the incisor is absent (arrow). (J-K) Embryos at 14.5 d.p.c. stained for cartilage. The wild type (I) shows the well-formed Meckel’s cartilage (Mc) within the forming mandibular region. The morphology of Meckel’s cartilage is moderately abnormal in the \textit{prx-1neo/-} embryo (J), however, in the double mutant embryo (K) Meckel’s cartilage is absent (arrow) except for a remnant at the most rostral tip of mandible. The malleus found at the proximal end of the forming mandible is still present in double mutant embryos. AS, alisphenoid; E, eye; BS, basiophenoid; BO, basisphenoid; i, incisor tooth; M, maxilla; NC, nasal cartilage; OC, otic capsule; P, palatal; PT, pterygoid; s, synphysis; Ty, tympanic ring; Z, zygoma; ZP, zygomatic process.
developmental progression of these subpopulations of cells in the prx-1neo/-; prx-2-/- mice, we crossed this transgene into the double mutant background.

At 10.5 d.p.c., the prx-1 2.7 lacZ transgene does not express in the craniofacial primordia of wild-type embryos (Fig. 7A). Expression of lacZ was first detected in a small group of cells in the wild-type maxillary process at 11.5 d.p.c.. In 10.5 d.p.c. double mutant embryos, lacZ was detected in the maxillary process, the distal tip of the mandibular process, as well as in the hyoid arch (Fig. 7B). Therefore, in the double mutant embryos, expression of lacZ was expressed prematurely in the maxillary and mandibular processes and ectopically in the hyoid arch. The prx-1 2.7 lacZ transgene does not express in the hyoid arch in wild-type embryos. In the wild-type 12.5 d.p.c. embryo, expression of lacZ was seen in the lateral aspect of the maxilla, as well as in cells at the distal tip of the mandible (Fig. 7C). In double mutant 12.5 d.p.c. embryos, lacZ expression was detected in ectopic locations in cells derived from the hyoid arch, as well as in proximally located cells surrounding the developing external ear structures. lacZ was also more broadly expressed in the forming mandible and maxilla of double mutant embryos (Fig. 7D). Thus, in the double mutant embryos, mesenchymal cells within the hyoid arch and cells around the forming external ear have acquired the characteristics of cells normally found within the maxillary and mandibular processes.

**DISCUSSION**

We have introduced lacZ into the prx-1 locus and have found that prx-1 mutant cells initially contributed normally but failed to be maintained in the structures that are defective in prx-1neo mutant mice. We also showed that prx-1 and prx-2 perform redundant functions in the mandibular process of the first branchial arch mesenchyme. These genes are required for

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**Fig. 6.** Characterization of phenotypes within the mandibular process of prx-1-/-; prx-2-/- mutant mice. (A) Parasagittal section of 12.5 d.p.c. wild-type embryo through the bell-stage incisor tooth germ. At this point, a well-formed dental papilla (dp) has been surrounded by the dental epithelium (de). (B,C) In the double mutant, a parasagittal (B) and transverse (C) section at this time point demonstrates that the tooth germ has arrested at the bud stage. Although there is a condensed mesenchyme (cm) around the dental epithelium, the tooth germ has not progressed to the bell stage. (D,E) At 16.5 d.p.c., the incisor teeth (in) of the wild-type mouse (D) is a large structure that is embedded in alveolar bone (ab) while, in the double mutant (E), only a remnant of the normal incisor is evident and the position of this structure in relation to the alveolar bone is abnormal. Additionally, hypertrophy of the oral epithelium (oe) overlying the incisor tooth remnant is evident. (F,G) Whole-mount in situ at 12.5 d.p.c. using the pax9 antisense probe demonstrates that pax9 is expressed in the wild-type (F) dental mesenchyme of both the forming incisors and molars as denoted by the arrows. In the double mutant (G), expression of pax9 was downregulated in the mandibular incisor while expression was maintained in the molars (arrows). (H,I) Whole-mount in situ at 12.5 d.p.c. using the patched probe demonstrated that patched was expressed at high levels in the developing wild-type incisors (H) but was downregulated in the double mutant mandibular incisors (I). (J,K) Whole-mount in situ at 11.5 d.p.c. using the sox9 probe. Both the wild-type (J) and the double mutant (K) embryos demonstrate expression in the mandibular process at this stage (arrows). ab, alveolar bone; cm, condensing mesenchyme; de, dental epithelium; dp, dental papilla; in, incisor tooth; oe, oral epithelium.
formation of the mandibular incisor and the rostral mandible, as well as the majority of Meckel’s cartilage. Using a transgene that distinguishes between subpopulations of \( prx-1 \)-expressing cells in the craniofacial primordia, we showed that \( prx-1 \)-expressing cells are found at ectopic locations in the double mutant mice. Taken together, our data demonstrate that the \( prx-1 \) and \( prx-2 \) genes function to maintain and stabilize cell fates of craniofacial mesenchyme.

Cells fated to express \( prx-1 \) are lost from the structures that are defective in the \( prx-1 \) mutant mouse

The \( prx-1^{neo} \) mutant mouse had craniofacial defects that were confined to cells within the proximal aspect of the first branchial arch (Martin et al., 1995). We now show that cells that are fated to express \( prx-1 \) are allocated normally to the precursors of the defective structures. \( lacZ \)-positive cells were found at normal locations up until 11.5 d.p.c. However, at 12.5 d.p.c., we noted that \( lacZ \)-expressing cells were absent in structures derived from the maxillary process of the first branchial arch. From this, we conclude that \( prx-1 \) is required for maintenance of cell fates within this region of the first branchial arch mesenchyme. In the absence of \( prx-1 \) function, specific populations of cells are present but fail to express \( lacZ \).

Alternatively, these same cells may be lost either by failing to proliferate or by activating apoptotic programs. Lineage-tracing experiments using chimeric analysis are now underway to distinguish between these two possibilities.

\( prx-1 \) and \( prx-2 \) perform redundant functions in the mandibular process of the first branchial arch

The cranial abnormalities observed in the \( prx-1^{neo} \) mutant were confined to the proximal aspect of the first branchial arch (Martin et al., 1995). Thus, despite widespread expression in other regions of the forming craniofacial skeleton, no function for \( prx-1 \) was observed. We have found that \( prx-1^{neo}; prx-2 \)

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**Fig. 7.** Cells which are fated to express \( prx-1 \) are found in ectopic locations in \( prx-1^{neo}/-; prx-2/- \) embryos. (A,B) Expression of the \( prx-1 \ 2.7\ lacZ \) transgene that marks a subpopulation of \( prx-1 \)-expressing cells in the craniofacial primordia. At 11.0 d.p.c., the wild-type embryo does not yet express this transgene in the branchial arches, although expression is detected in the limb buds. \( lacZ \) expression in the posterior regions of the head is ectopic staining associated with this transgene construct in all lines analyzed. (B) \( lacZ \)-expressing cells are detected in the maxillary (mx) and mandibular (md) processes as well as the hyoid arch at 11.0 d.p.c. in the double mutant embryos (arrows). (C) At 12.5 d.p.c., expression of the \( prx-1 \ 2.7\ lacZ \) transgene is detected in cells at the lateral aspect of the forming maxilla and in a group of cells at the distal aspect of the forming mandible which are obscured in this lateral view. (D) In the double mutant embryo at this stage, \( lacZ \) expression is detected in the maxilla and mandible as well as in two ectopic locations, caudally in the hyoid arch derived cells and in cells that will contribute to the external ear structures (arrows). e, eye; md, mandibular process; mx, maxillary process.

**Fig. 8.** Diagram of developmental fate of cells that normally express \( prx-1 \) in wild-type and mutant backgrounds. (A-C) The entire \( prx-1 \)-expressing field at 11.0 d.p.c. within the craniofacial region of a wild-type mouse (A) (shaded yellow). (B) The subpopulation of \( prx-1 \)-expressing cells that normally express the \( prx-1 \ 2.7\ lacZ \) transgene in the wild-type mouse at 11.0 d.p.c. (shaded yellow). (C) The change in expression of the \( prx-1 \ 2.7\ lacZ \) transgene in the double mutant background. Note that aberrantly expressing cells (shaded gray) are found in the hyoid arch. The gray cells surrounding the yellow in the maxillary and mandibular processes signify cells that prematurely activated the transgene. (D,E) In the wild type (D), \( prx-1 \)-expressing cells are found in all facial prominences however, these cells are developmentally distinct (as denoted by the different colors). In the double mutant background (E), cell fates are reprogrammed such that cells in the hyoid arch (shaded green) now take on the properties of cells within the mandibular processes (shaded yellow). Fn, frontonasal process; hy, hyoid arch; md, mandibular process; mx, maxillary process.
double mutants had severe defects in structures derived from
the mandibular process of the first branchial arch, demonstrating that these two genes perform redundant functions in the distal mandibular arch precursors. In double mutants, the rostral aspect of the mandible was fused and the mandibular incisor arrested as a bud-stage tooth germ. Additionally, Meckel’s cartilage was severely deficient.

In order to gain insight into the molecular mechanisms underlying the incisor tooth defect, we studied the expression of two genes that are components of signaling pathways that have been implicated in tooth organogenesis. Expression of Pax9, an early marker for tooth development, was downregulated in the double mutant tooth germs. Function of pax9 is required for progression of tooth development past the bud stage (Neubuser et al., 1997). In addition, pax9 has been proposed to determine placement of tooth bud initiation by integrating FGF and BMP signaling pathways (Neubuser et al., 1997). prx-1;prx-2 double mutant incisors also arrested at the bud stage as a single incisor instead of the usual two. These data suggest that prx-1 and prx-2 function to maintain pax9 expression in the forming mandibular incisor. Downregulated expression of patched in the double mutant incisors also supports the notion that prx-1 and prx-2 are required for the maintenance of normal signaling pathways required for mandibular incisor tooth formation.

Although formation of Meckel’s cartilage in the double mutants was severely disrupted, we found that expression of sox9 in the mandibular process of double mutant embryos was intact prior to chondrogenesis at 11.5 d.p.c. Therefore, although the chondrogenic mesenchyme was correctly specified, it failed to be maintained and complete its developmental program.

**prx-1-expressing cells are found in ectopic locations in the prx-1neo; prx-2 double mutant mice**

We have made use of the prx-1 2.7 lacZ transgene that distinguishes between subpopulations of prx-1-expressing cells in the craniofacial primordia. In the double mutant embryos, we found ectopic expression of this transgene. Cells that did not express the transgene in wild-type embryos were reprogrammed to express in the double mutant. Aberrant expression of this transgene in the double mutant included cells that did not normally express, as well as cells that prematurely expressed the transgene. These data suggest that prx-1 and prx-2 function to maintain or stabilize cell fates in the post migratory cranial mesenchyme.

We believe these data provide insight into the developmental basis for the craniofacial phenotypes observed in both the prx-1 single mutant and the prx-1; prx-2 double mutant mice. Although prx-1-expressing cells are found in all facial primordia, these cells are not developmentally equivalent (Fig. 8A,D). Cells within the maxillary prominence respond to different positional cues than cells within the mandibular process. The prx-1 2.7 lacZ transgene marking experiment demonstrated that in double mutants, cells within the prx-1-expressing field have been reprogrammed (Fig. 8A-C). Thus, in double mutant embryos, hyoid arch mesenchyme expresses a transgene that, in the wild-type embryo, would only be expressed in the maxillary and mandibular processes of the first branchial arch. We propose that this reprogramming of the craniofacial primordia in single prx-1 mutant and prx-1; prx-2 double mutant embryos underlies the observed craniofacial phenotypes. The consequence of this change in cell fate is that subpopulations of cranial mesenchyme receive inappropriate positional information to which they cannot respond resulting in failure to maintain gene expression in forming craniofacial organs (Fig. 8E).

Our results have important implications for the developmental mechanisms underlying craniofacial development. Recent work has demonstrated that the CNC has some degree of intrinsic patterning capacity (Couly et al., 1998). Our data suggest that prx-1 and prx-2 are components of the genetic program that functions to maintain this patterning information during the postmigratory phases of craniofacial organogenesis.

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