Genetic dissection of *Pitx2* in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration

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Summary

***Pitx2*, a *paired*-related homeobox gene that encodes multiple isoforms, is the gene mutated in the haploinsufficient Rieger Syndrome type 1 that includes dental, ocular and abdominal wall anomalies as cardinal features. Previous analysis of the craniofacial phenotype of *Pitx2*-null mice revealed that *Pitx2* was both a positive regulator of *Fgf8* and a repressor of *Bmp4*-signaling, suggesting that *Pitx2* may function as a coordinator of craniofacial signaling pathways. We show that *Pitx2* isoforms have interchangeable functions in branchial arches and that *Pitx2* target pathways respond to small changes in total *Pitx2* dose. Analysis of *Pitx2* allelic combinations that encode varying levels of *Pitx2* showed that repression of Bmp signaling requires high *Pitx2* while maintenance of *Fgf8* signaling requires only low *Pitx2*. Fate-mapping studies with a *Pitx2 cre recombinase* knock in allele revealed that *Pitx2* daughter cells are migratory and move aberrantly in the craniofacial region of *Pitx2* mutant embryos. Our data reveal that *Pitx2* function depends on total *Pitx2* dose and rule out the possibility that the differential sensitivity of target pathways was a consequence of isoform target specificity. Moreover, our results uncover a new function of *Pitx2* in regulation of cell motility in craniofacial development.**

Key words: Homeobox, Craniofacial morphogenesis, Haploinsufficiency

Introduction

Pitx2 is a *paired*-related homeobox gene that was shown to be the gene mutated in Rieger Syndrome type I (RGS I) (Semina et al., 1996), an autosomal dominant, haploinsufficient disorder that includes tooth abnormalities as one of its primary features (Flomen et al., 1998). The craniofacial defects in individuals with RGS I, that have one half dose of *Pitx2*, include dental hypoplasia, anodontia vera, abnormally shaped teeth and a flattened midface (Amendt et al., 2000). Individuals with RGS I also have ocular anterior chamber disorders, which often result in glaucoma and umbilical abnormalities (Semina et al., 1996). *Pitx2* plays a central role in left right asymmetry (Capdevila et al., 2000; Harvey, 1998) and is a component of Wnt-β-catenin signaling in pituitary and cardiac outflow tract development (Kioussi et al., 2002). Experimental evidence supports the idea that the dominant genetics of RGS I results from haploinsufficiency; however, there is evidence for a dominant negative mechanism in a subset of patients (Saadi et al., 2003; Saadi et al., 2001).

Investigation of *Pitx2* function using loss-of-function approaches in mice has shown that *Pitx2* plays an important role in early stages of tooth development (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). *Pitx2*-null mutant embryos had arrested tooth development at placode or bud stage. Consistent with a haploinsufficient mechanism, tooth phenotypes were observed in *Pitx2* null $^{+/-}$ mice (Gage et al., 1999). Early epithelial-mesenchymal signaling was intact in *Pitx2*-null embryos as suggested by the presence of a condensed dental mesenchyme (Lin et al., 1999; Lu et al., 1999). Expression of markers such as *Shh* and mesenchymal *Bmp4* and *Msx1* also supported the idea that tooth initiation and specification occurred but tooth germ expansion failed in *Pitx2*-null embryos (Lin et al., 1999; Lu et al., 1999). In situ also showed that *Bmp4* expression was expanded, while *Fgf8* failed to be expressed or was downregulated in oral epithelium of *Pitx2*-null embryos (Lin et al., 1999; Lu et al., 1999). Taken together, these data suggest that the initial events in tooth development occurred in the absence of *Pitx2*, subsequent signaling events were deranged resulting in a premature extinction of *Fgf8* expression and failure of demarcation of *Bmp4* expression to dental epithelium. These experiments uncovered an early function for *Pitx2* in tooth morphogenesis but failed to address any later role for *Pitx2* in craniofacial development.

The *Pitx2* gene encodes three isoforms, *Pitx2a*, *Pitx2b* and *Pitx2c* in mice and a fourth *Pitx2* isoform, *Pitx2d*, has been identified in humans (Cox et al., 2002). The different isoforms are generated by both alternative splicing and alternative promoter usage (Shiratori et al., 2001) (Fig. 1A,B) and have both overlapping and distinct expression patterns. All *Pitx2*

isoforms have a common C terminus and distinct N termini (Fig. 1A). *Pitx2c* is the asymmetrically expressed isoform while *Pitx2a*, *Pitx2b* and *Pitx2c* isoforms are co-expressed in head mesoderm, oral ectoderm, eye, body wall and central nervous system (Kitamura et al., 1999; Liu et al., 2001; Schweickert et al., 2000; Smidt et al., 2000). *Pitx2c*, but not *Pitx2a* or *Pitx2b*, is expressed in hematopoietic stem cells (Degar et al., 2001). Co-expression of *Pitx2* isoforms is found in the three developmental fields that are most frequently affected in individuals with RGS I: eyes, teeth and anterior abdominal wall.

The observation that *Pitx2* regulated two fundamentally important signaling pathways in craniofacial morphogenesis raised the possibility that haploinsufficiency observed in humans and mice was a consequence of differential sensitivity of these important target pathways to total *Pitx2* dose. An alternative idea, suggested by multiple *Pitx2* isoforms with overlapping expression in developing teeth, was that *Pitx2* function in craniofacial development was a consequence of distinct isoform function. For example, it is conceivable that one *Pitx2* isoform functions to repress *Bmp4* while a separate isoform maintains *Fgf8* expression. In addition, *Pitx2* isoforms have been shown to form heterodimers in vitro suggesting that *Pitx2* isoform heterodimers may have distinct target genes (Cox et al., 2002). Overexpression of a *Pitx2* engrailed repressor (en^r) fusion protein in left lateral plate of chick embryos revealed that *Pitx2c* en^r but not a *Pitx2a* en^r fusion could interfere with endogenous *Pitx2c* function (Yu et al., 2001), consistent with the idea that *Pitx2* isoforms have distinct target genes. Experiments performed in *Xenopus* and zebrafish, as well as tissue culture studies, support the idea that *Pitx2* isoforms have distinct targets (Cox et al., 2002; Essner et al., 2000; Faucourt et al., 2001; Suh et al., 2002).

We investigated *Pitx2* isoform function in craniofacial morphogenesis by analyzing craniofacial phenotypes of isoform-specific deletions. We used *Pitx2* alleles that encode

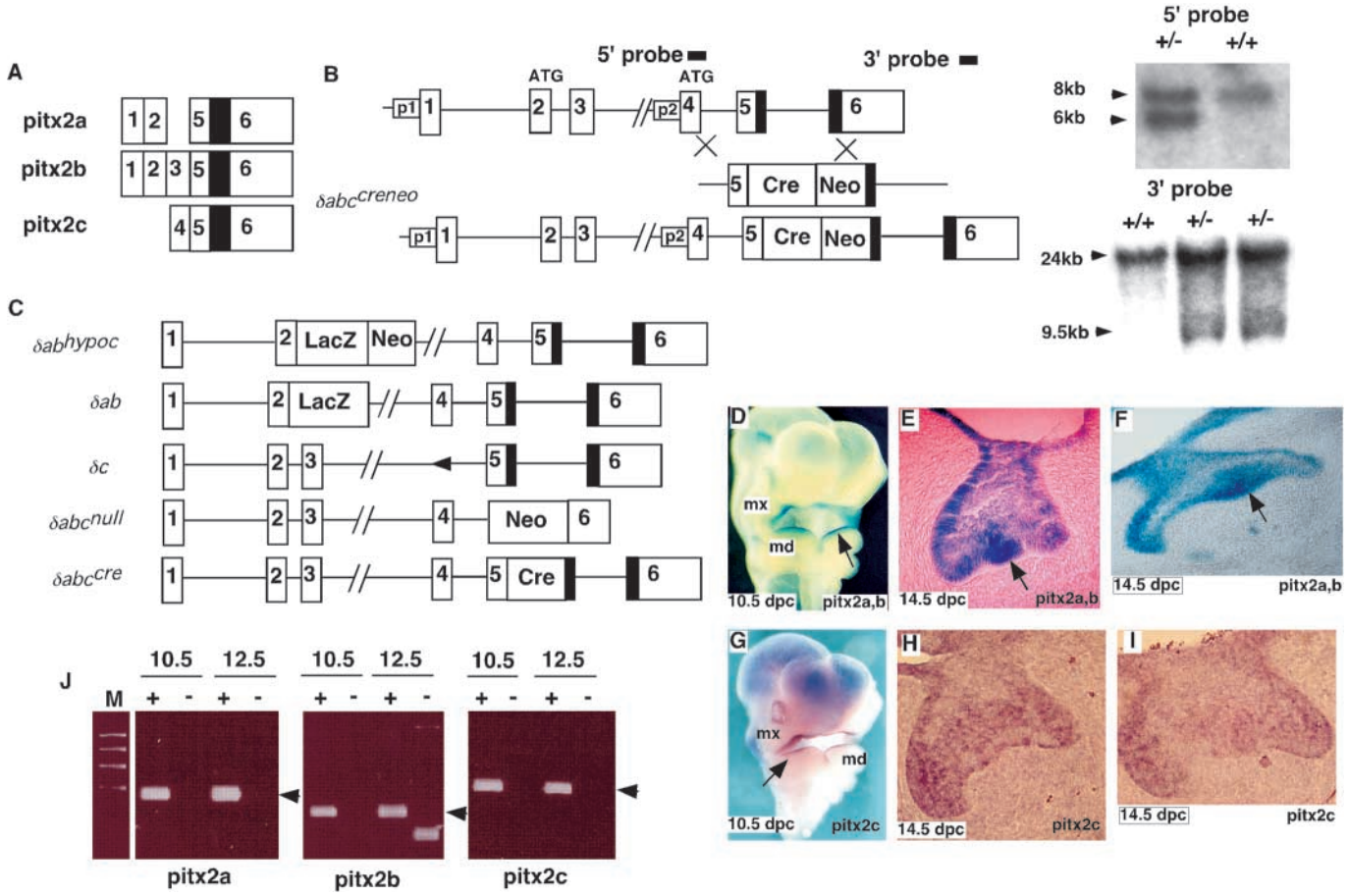

Fig. 1. *Pitx2* alleles and *Pitx2* isoform expression in developing teeth. (A) Summary of *Pitx2* isoforms. Numbered boxes represent exons and black boxes the homeodomain. (B) *Pitx2* δabc^{creneo} targeting strategy. At the top is a wild-type allele, the targeting vector is in the middle and at the bottom is the targeted allele. Numbered boxes represent exons and lines the intervening introns. Black shaded areas are homeobox. p1 and p2 are two alternate promoters located upstream of exon 1 and exon 4. On the right are Southern blots with 5′ and 3′ flanking probes. (C) Five *Pitx2* alleles: *Pitx2a* and *Pitx2b* isoform-specific deletions, δab^{hypoc} and δab alleles (Liu et al., 2001). The δabc^{null} allele is a homeobox deletion and a *Pitx2*-null allele (Lu et al., 1999) and δc allele is and isoform-specific deletion of *Pitx2c*. (D) *lacZ* staining of $\delta ab^{+/-}$ embryo showing expression in oral ectoderm (arrow). (E,F) Coronal (E) and parasagittal (F) sections through molar cap stage tooth showing *lacZ* expression in epithelial components and enamel knot (arrows). (G) Whole-mount in situ with *Pitx2c* probe showing expression in oral ectoderm (arrow). (H,I) Coronal (H) and parasagittal (I) section in situ with *Pitx2c* probe showing expression in epithelium of cap stage tooth. (J) RTPCR of *Pitx2* isoforms in oral and dental epithelium. Specific amplified bands for each isoform (arrow). + indicates inclusion of reverse transcriptase; – is control without reverse transcriptase. md, mandibular process; mx maxillary process.

differing levels of *Pitx2* to investigate the requirements for total *Pitx2* dose in craniofacial morphogenesis (Liu et al., 2001). Our results show that *Pitx2* isoforms have interchangeable function in craniofacial development and that signaling pathways that are regulated by *Pitx2* respond differently to changes in total *Pitx2* dose. The *Fgf8* maintenance pathway uses low *Pitx2* doses, while *Bmp4* repression requires high *Pitx2* doses. Our findings uncovered downstream functions for *Pitx2* in tooth development and fate mapping experiments with a *Pitx2 cre recombinase* knock-in allele revealed that *Pitx2* daughter cells are migratory. Movement of *Pitx2* daughters was aberrant in *Pitx2* mutants, suggesting that *Pitx2* regulates cell movement in craniofacial primordia.

Materials and methods

Whole-mount and section in situ hybridization

Whole mount and section in situ hybridization performed as described (Lu et al., 1999) with modifications for the use of digoxigenin labeled probes. *Bmp4*, *Barx1*, *Pax9*, *Fgf8*, *Pitx2c* and myogenin probes were described (Lu et al., 1999; Mitsiadis et al., 1998; Peters et al., 1998; Trumpp et al., 1999; Winnier et al., 1995; Liu et al., 2002).

lacZ staining and histology

Mouse embryos were fixed in Bouin's, dehydrated and embedded in paraffin wax. Sections were cut (7-10 μm) and stained with Hematoylin and Eosin. *lacZ* staining was as previously described (Lu et al., 1999).

Generation of the *Pitx2* alleles

The *Pitx2* δabc^{null}, δab^{hypoc}, δab and δc alleles have been described previously (Liu et al., 2001; Liu et al., 2002; Lu et al., 1999). For *Pitx2* δabc^{creneo} allele, a targeting vector was constructed that introduced *cre recombinase neofrt* into *Pvu*II and *Nru*1 sites in *Pitx2* fifth exon. Crosses to a *rosa26 eFlp* deletor strain resulted in neomycin removal (Farley et al., 2000). Crosses to *Pitx2* δabc^{null} allele confirmed that *Pitx2* δabc^{creneo} was a null allele and in situ hybridization experiments showed *cre* expression recapitulated endogenous *Pitx2*.

RT-PCR

Total mRNA was extracted using SV total RNA isolation system (Promega) and cDNA produced with M-MLV reverse transcriptase (Invitrogen). Four *Pitx2* primers detected *Pitx2* isoform expression: exon 2 (5′-attgtcgcaaactagtgtcgg-3′), exon 3 (5′-ccgtgaactcgaccttttga-3′), exon 4 (5′-tcctgggactcctccaaacat-3′) and exon 5 (5′-gtttctctgga-aagtggctcc-3′). A 104 bp *Pitx2b* fragment was amplified with exon 2 and exon 3 primers, 159 bp *Pitx2a* fragment with exon 2 and exon 5 primers and a 207 bp *Pitx2c* fragment with exon 4 and exon 5 primers.

Results

Pitx2 isoforms are co-expressed in oral and dental epithelium

The *Pitx2* δabc^{null} allele, a homeobox deletion, removes function of all isoforms, while the δab^{hypoc} and δab alleles delete the *Pitx2a* and *Pitx2b* specific exons and leave *Pitx2c* intact (Fig. 1A,C). The δab^{hypoc} allele, which retains PGKneomycin, encodes less *Pitx2c* function than the δab allele in which PGKneomycin was removed (Liu et al., 2001). We generated a deletion of the *Pitx2c* isoform (Liu et al., 2002), the δc allele, that was a replacement of the *Pitx2c*-specific exon 4 with a LoxP flanked PGKneomycin. In the final δc allele, PGKneomycin has been removed by crossing to the *CMVcre* deletor strain (Liu et al., 2002) (Fig. 1C). To study the developmental progression of *Pitx2* daughter cells (see below), we generated *Pitx2* δabc^{creneo}, a *Pitx2* cre recombinase knock-in allele (Fig. 1B; see Materials and methods). We introduced *cre* into *Pitx2* exon 5 that resulted in a *Pitx2* null allele and expressed *cre* in the same spatiotemporal pattern as endogenous *Pitx2* (see below). Excision of the PGKneomycin cassette by crossing to the rosa26 eFlp deletor strain resulted in the *Pitx2* δabc^{cre} allele.

We studied *Pitx2a* and *Pitx2b* isoform expression using the δab^{hypoc} and δab alleles that contain a *lacZ* knock-in into *Pitx2* exon 2 and deletes *Pitx2* exon 3 (Fig. 1C-F). As *lacZ* was introduced into exon 2, this analysis provides information about *Pitx2a* and *Pitx2b* specific expression but does not distinguish between these two isoforms because *Pitx2a* uses exon 2 and *Pitx2b* uses both exon 2 and exon3 (Fig. 1A). We used RT-PCR to distinguish between *Pitx2a* and *Pitx2b* expression (see below). We also performed in situ analysis using a *Pitx2c* probe. At 10.5 dpc, *lacZ* was expressed uniformly throughout the oral ectoderm, while at 14.5 dpc, *lacZ* expression was found in dental epithelium and primary enamel knot of cap stage tooth (Fig. 1D-F). Using a *Pitx2c* probe for in situ, we detected *Pitx2c* expression throughout the 10.5 dpc oral ectoderm (Fig. 1G). At 14.5 dpc, *Pitx2c* was expressed in dental epithelium similarly to *Pitx2a* and *Pitx2b* (Fig. 1H,I). To distinguish between *Pitx2a* and *Pitx2b* isoform expression in oral ectoderm, we performed RT-PCR with a primer set that distinguished between *Pitx2a*, *Pitx2b* and *Pitx2c*. We identified all three isoforms in the mandibular arch epithelium at 10.5 and 12.5 dpc (Fig. 1J). These data suggest that the *Pitx2a*, *Pitx2b* and the *Pitx2c* isoforms are co-expressed in oral ectoderm and, at later stages, within tooth epithelial structures.

Pitx2 isoforms have interchangeable functions in tooth development

Co-expression of *Pitx2* isoforms suggests a number of possibilities for the regulation of target pathways by *Pitx2*. It is possible that *Pitx2* isoforms would regulate distinct target genes in tooth formation or *Pitx2* isoforms may have redundant functions. Isoform co-expression also supports the idea that some *Pitx2* target genes have a requirement for *Pitx2* heterodimers (Cox et al., 2002). To address these ideas, we analyzed forming teeth of $\delta ab^{-/-}$ and $\delta c^{-/-}$ embryos.

As a control, we analyzed teeth of $\delta ab;\delta c$ mutant embryos. We reasoned that this allelic combination should encode near normal levels of all *Pitx2* isoforms, albeit from different chromosomes, and should be functionally similar to δabc^{null} heterozygous embryos. Analysis of coronal and sagittal sections through the teeth of $\delta ab;\ \delta c$ embryos at 14.5 and16.5 dpc revealed that tooth development was normal (Fig. 2A-D). From this, we conclude that the δab and δc alleles encode adequate levels of *Pitx2* isoforms to support normal tooth development.

To test the idea that *Pitx2* isoforms had distinct target genes and thus distinct functions in tooth development, we analyzed the teeth of $\delta ab^{-/-}$ embryos at two timepoints, 16.5 dpc and 18.5 dpc. We found that teeth of δab homozygous mutant embryos that lack *Pitx2a* and *Pitx2b* are normal suggesting that there is redundant function between the *Pitx2a*, *Pitx2b* and

Pitx2c isoforms in tooth development or that *Pitx2c* has the major role in tooth development (Liu et al., 2001) and Fig. 2G,H,J,K). Sections through *Pitx2c* mutant teeth at 16.5 and 18.5 dpc revealed normal molar tooth morphology suggesting that *Pitx2 a*, *Pitx2b* and *Pitx2c* isoforms have redundant function in tooth morphogenesis (Fig. 2G,I,J,L). These data argue against an absolute requirement for either *Pitx2* isoform-specific target genes or *Pitx2* isoform heterodimers in branchial arch morphogenesis and tooth development. These results suggest that common *Pitx2* target genes are differentially regulated by total *Pitx2* dose (Table 1).

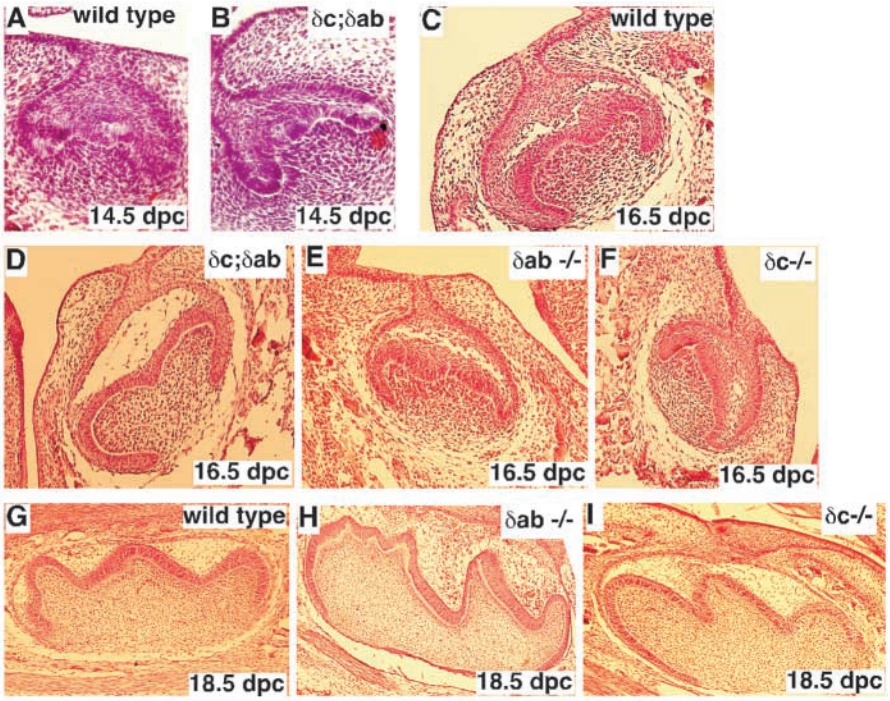

Fig. 2. Histological analysis of tooth morphology in *Pitx2* isoform deletions. (A-F) Coronal sections through molar teeth of 14.5 and 16.5 dpc embryos stained with Hematoxylin and Eosin. Genotypes and stage are labeled. (G-I) Parasagittal sections stained with Hematoxylin and Eosin through molar teeth (18.5 dpc).

Failure of *Fgf8* maintenance and defective rostral caudal mandibular arch polarity in *Pitx2* null mutants

Previous data suggested that *Fgf8* expression was absent in *Pitx2* δabc^{null} homozygous mutants (Lu et al., 1999) but was diminished only in embryos homozygous mutant for an independently generated *Pitx2*-null allele (Lin et al., 1999). One idea to explain this discrepancy is that *Fgf8* expression was induced but not maintained in *Pitx2*-null mutant embryos. To determine if *Pitx2* was required for the maintenance of *Fgf8* expression, we examined *Fgf8* expression in *Pitx2*-null mutants at earlier timepoints than previously reported. In 9.5 dpc δabc^{null} homozygous mutant embryos, low levels of *Fgf8* mRNA was expressed in the oral ectoderm (Fig. 3A,B). Sectioning revealed that the *Fgf8* expression domain was restricted to a small region of oral ectoderm at the proximal aspect of the mandibular process in *Pitx2* δabc^{null} homozygous mutants when compared with wild-type embryos (Fig. 3C,D). In the absence of *Pitx2*, the majority of the oral ectoderm loses the competency to express *Fgf8*, suggesting that *Pitx2* has a role in the demarcation of the *Fgf8* expression domain to the proximal aspect of the mandibular and maxillary processes. At later timepoints, *Fgf8* expression is lost in *Pitx2*-null mutants (Lu et al., 1999) (see below).

We examined expression of genes that are proposed *Fgf8* targets in mandibular mesenchyme. *Lhx6* expression was shown to be dependent on *Fgf8* function as *Lhx6* failed to be induced in mutants with an oral ectoderm specific inactivation of Fgf8 (Trumpp et al., 1999). In *Pitx2* δabc^{null} mutants, *Lhx6* expression was reduced (Fig. 3E,F). The residual *Lhx6* expression in the *Pitx2* δabc^{null} embryos was in the proximal mandible near the region where *Fgf8* was expressed in the *Pitx2* δabc^{null} mutant embryos (Fig. 3D). Expression of *Pitx1*, normally expressed in the oral ectoderm and proximal mandibular mesenchyme, has been shown to be induced by implantation of an *Fgf8* bead (St Amand et al., 2000). *Pitx1* expression was reduced in the proximal aspect of the *Pitx2* δabc^{null} mutant mandibular arch mesenchyme at 10.5 dpc (Fig. 3G,H). Expression of *Dlx2* in mandibular mesenchyme has also been shown to be upregulated by *Fgf8* bead implantation (Thomas et al., 2000). We found that the mesenchymal expression of *Dlx2* was reduced in *Pitx2* δabc^{null} mutants (Fig. 3I,J). As previous data suggested that induction of *Pitx1* and *Dlx2* expression was independent of *Fgf8*, our results suggest that *Fgf8* functions to maintain *pitx1* and *dlx2* expression in the mandibular mesenchyme (Trumpp et al., 1999). Expression of endothelin 1 (*Edn1*), also dependent on *Fgf8* function, was downregulated in the mandibular arch ectoderm of *Pitx2* δabc^{null} mutants (Fig. 3K,L). It is notable that expression of *Lhx6*, *Pitx1*, and *Dlx2* in the maxillary primordial of *Pitx2* δabc^{null} mutants was also reduced; however, further experiments are necessary to rule out the possibility that this

Table 1. Summary of phenotypes in *pitx2* mutant allelic combinations

Genotype	Molar phenotype	Fgf8 signaling	Bmp signaling
δabcnull; $^{+/-}$	Normal	Normal	Normal
δabcnull; $^{-/-}$	Arrested bud stage	Maintenance defect	Strongly expanded
δabcnull; δabhypoc	Defect prior to cap formation	Normal	Weakly expanded
δabcnull; δab	Molar orientation defect	Normal	Weakly expanded
δab; δc	Normal	n.d.	n.d.
δab; δab	Normal	Normal	Normal
δc; δc	Normal	Normal	Normal

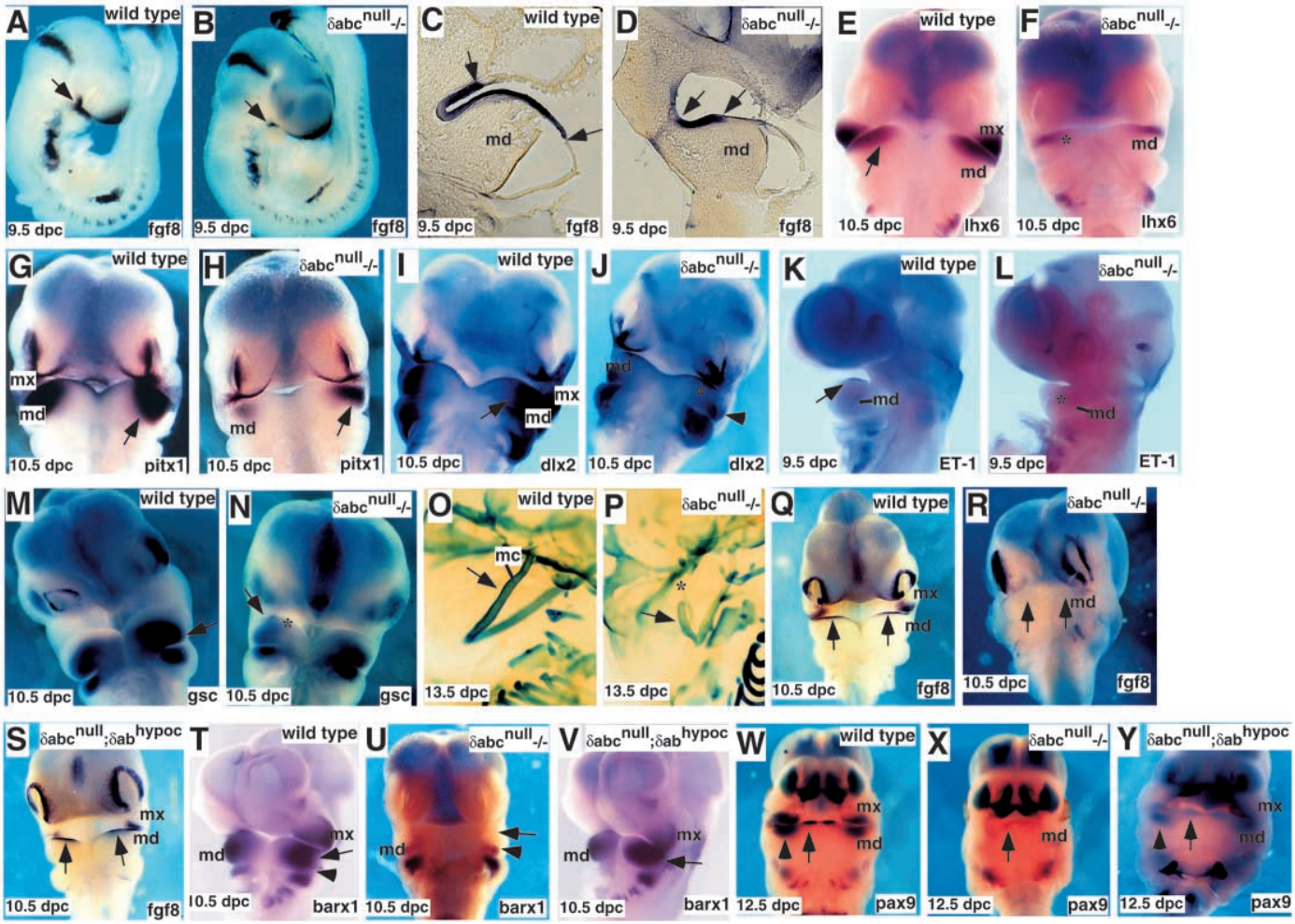

Fig. 3. *Fgf8* signaling pathways require low doses of *Pitx2*. (A,B) In situ analysis of 9.5 dpc wild-type (A) and *Pitx2* δabc^{null} embryo (B) with *Fgf8* probe. (C,D) Parasagittal cryosections of 9.5 dpc *Fgf8* whole-mount of wild-type (C) and *Pitx2* δabc^{null} mutant (D) embryos. (E-N) In situ of wild-type (E,G,I,K,M), *Pitx2* δabc^{null} homozygous mutant embryos (F,H,J,L,N). (O,P) Cartilage staining of 13.5 dpc wild-type (O), *Pitx2* δabc^{null} homozygous mutant (P) embryos. (Q-S) In situ of 10.5 dpc wild-type (Q), *Pitx2* δabc^{null} homozygous mutant (R) and δabc^{null}; δab^{hypoc} (S) embryos with *Fgf8*. Arrows indicate areas of oral ectoderm expression. (T-V) In situ of 10.5 dpc wild-type (T), *Pitx2* δabc^{null} homozygous mutant (U) and δabc^{null}; δab^{hypoc} (V) embryos with a *Barx1* probe. Arrows denote expression in proximal mandibular mesenchyme that is absent in *Pitx2* δabc^{null} mutant. Arrowhead indicates expression in caudal mandibular arch mesenchyme that is probably induced by *Fgf8* signaling from the caudal mandibular ectoderm. (W-Y) In situ of 12.0 dpc wild-type (W), *Pitx2* δabc^{null} homozygous mutant (X) and δabc^{null}; δab^{hypoc} (Y) embryos with *Pax9*. Arrows (mandibular incisor) and arrowheads (mandibular molar) indicate areas of expression in dental mesenchyme that are reduced in δabc^{null} homozygous mutant. md, mandibular process; mx maxillary process.

was secondary to reduction in the outgrowth of the forming maxilla (Fig. 3E-J).

We noted that *Dlx2* was still expressed in the caudal aspect of the *Pitx2* mutant mandibular mesenchyme (Fig. 3I,J). As *Pitx2* expression is restricted to the rostral mandibular arch ectoderm, continued expression of *Dlx2* in caudal mandibular mesenchyme suggested that *Fgf8* signaling from the caudal aspect of the mandibular ectoderm was intact in the *Pitx2* δabc^{null} mutant embryos and that patterning of the mandibular process was disrupted in the *Pitx2* δabc^{null} mutants. Goosecoid (*Gsc*), an *Fgf8* responsive homeobox gene, is normally expressed in the caudal mandibular arch mesenchyme. Caudal *Gsc* expression is normally maintained via a *Fgf8* repressive pathway that inhibits *Gsc* expression in the rostral mandibular process (Tucker et al., 1999). We reasoned that if maintenance of *Fgf8* signaling was disrupted in *Pitx2* δabc^{null} mutants, then *Gsc* expression should be expanded rostrally. We found that *Gsc* expression was weakly expanded in a subset of *Pitx2* δabc^{null} mutants embryos (Fig. 3M,N), while in the remainder of mutant embryos *Gsc* expression was caudally restricted (data not shown). The incomplete penetrance of expanded *Gsc* expression suggests that in the subpopulation of *Pitx2* mutant embryos with correct *Gsc* expression, the early *Fgf8* expression was sufficient to specify the correct *Gsc* expression domain.

Correct patterning of the mandibular mesenchyme is necessary for formation of Meckel's cartilage (Tucker et al., 1999). Based on the weak expansion of *Gsc* expression, we expected that *Pitx2*-null mutants would have a weak Meckel's cartilage phenotype. To assess this, we performed whole-mount cartilage staining on *Pitx2* δabc^{null} mutants and control wild-type littermate embryos. The *Pitx2* δabc^{null} mutants had a variable deficiency of Meckel's cartilage supporting the

notion that rostral caudal polarity of the mandibular process was weakly affected by loss of *Pitx2* function (Fig. 3O,P). Taken together, these data suggest that in the absence of *Pitx2*, *Fgf8* expression in oral ectoderm fails to be maintained. In the absence adequate *Fgf8* signaling, *Fgf8*-dependent signaling to underlying mesenchyme is reduced leading to defective mandibular arch rostral caudal polarity.

Differential sensitivity of *Pitx2* target pathways to changes in total *Pitx2* dose

To address the idea that *Pitx2* target pathways have distinct requirements for total *Pitx2* dose, we examined *Fgf8* and *Bmp*-signaling pathways in *Pitx2* allelic combinations that encode differing levels of *Pitx2* activity (Liu et al., 2001). We used the δabc^{null} allele, in conjunction with the δab and δab^{hypoc} alleles that encode reduced levels of *Pitx2c* in the absence of *Pitx2a* and *Pitx2b* to generate *Pitx2* allelic combinations with intermediate levels of *Pitx2* activity. Previously, we showed that the $\delta abc^{null+/-}$ embryos expressed ~58% of homozygous wild-type *Pitx2c* mRNA levels while the $\delta abc^{null};\delta ab$ and $\delta abc^{null};\ \delta ab^{hypoc}$ allelic combinations expressed ~50% and 38% of wild-type *Pitx2c* mRNA levels respectively (Liu et al., 2001).

At 10.5 dpc, *Fgf8* expression was not detectable in the *Pitx2* δabc^{null} homozygous mutant oral ectoderm, supporting the idea that *Pitx2* was required for maintenance of *Fgf8* expression in the oral ectoderm (Fig. 3Q,R) (Lin et al., 1999; Lu et al., 1999). In the rostral mandibular process of *Pitx2* δabc^{null} mutant embryos, *Barx1* and *Pax9*, mesenchymal targets of *Fgf8* signaling pathways (Neubuser et al., 1997; Tucker et al., 1998), were not expressed or had greatly diminished expression (Fig. 3T,U,W,X). Caudal mandibular arch expression of *Barx1* was maintained in *Pitx2* δabc^{null} mutant embryos as this expression is probably dependent on *Fgf8* and *Edn1* signaling from the caudal aspect of the mandibular process that does not express *Pitx2* (Fig. 3R,S). By contrast, the $\delta abc^{null};\delta ab$ and $\delta abc^{null};\delta ab^{hypoc}$ allelic combinations, that encode reduced levels of *Pitx2c* mRNA and lack *Pitx2a* and *Pitx2b* (Liu et al., 2001) (Fig. 1C), expressed *Fgf8* in the oral ectoderm of 10.5 dpc embryos (Fig. 3Q-S and data not sown). *Barx1* and *Pax9* were expressed in the $\delta abc^{null};\delta ab^{hypoc}$ embryos that encode low levels of *Pitx2* (Fig. 3T,W).

We investigated whether repression of Bmp signaling by *Pitx2* was also rescued in the $\delta abc^{null};\delta ab^{hypoc}$ allelic combination that encodes low levels of *Pitx2* function. To assess expansion of Bmp signaling, we examined *Bmp4* expression in oral ectoderm of 10.5 dpc *Pitx2* mutant embryos. In contrast to the *Fgf8* signaling pathway, Bmp repression required high levels of *Pitx2* function. In *Pitx2* $\delta abc^{null-/-}$ embryos *Bmp4* expression was expanded laterally in mandibular process ectoderm (Fig. 4A,B) (Lu et al., 1999). In wild-type embryos, *Bmp4* expression is found in the medial mandibular process and the distal aspect of the ectoderm of the maxillary process at 10.5 dpc (Fig. 4B,E). In *Pitx2* $\delta abc^{null};\delta ab^{hypoc}$ and $\delta abc^{null};\delta ab$ allelic combinations, *Bmp4* expression in the mandibular process was weakly expanded. Moreover, in the maxillary process ectoderm of *Pitx2*

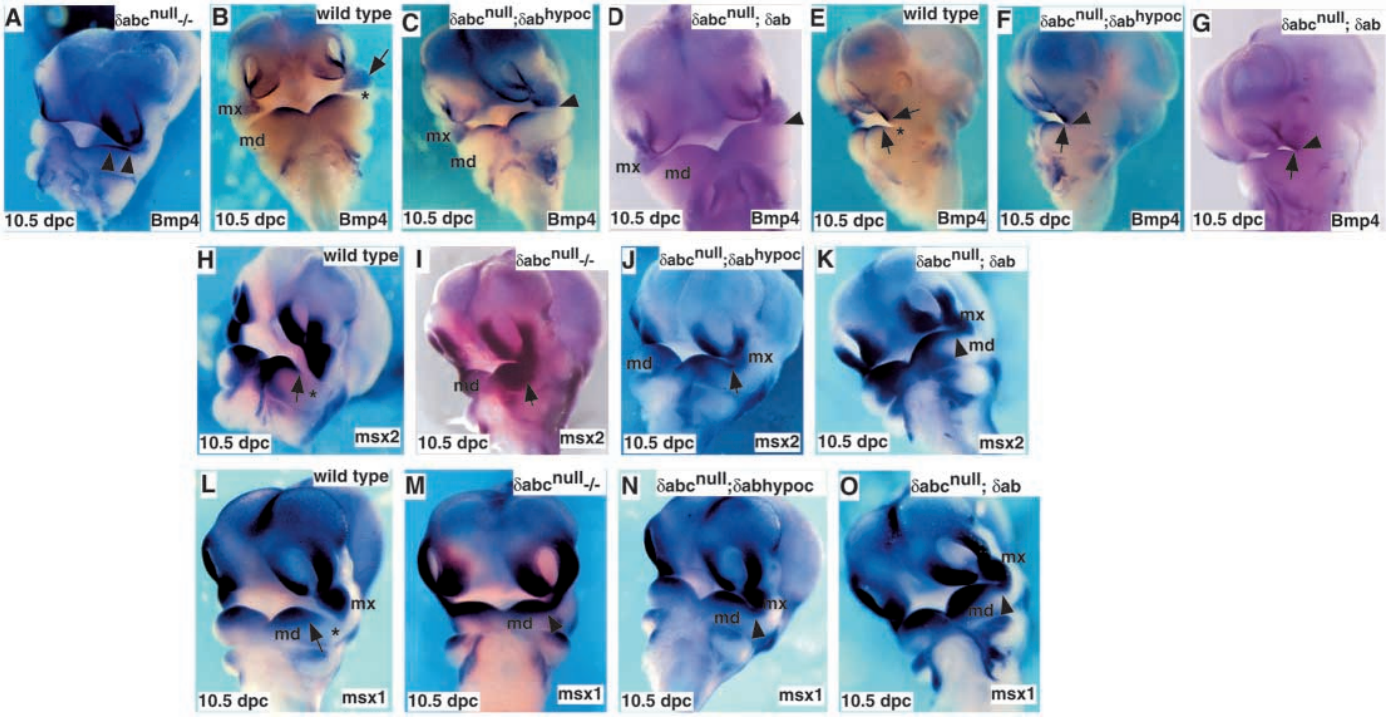

Fig. 4. Repression of *Bmp4*-signaling pathways requires high doses of *Pitx2*. (A-G) Whole-mount in situ analysis of 10.5 dpc *Pitx2* δabc^{null} homozygous mutant (A), *Pitx2* wild-type (B,E), δabc^{null}; δab^{hypoc} (C,F) δabc^{null}; δab (D,G) embryos with a *Bmp4* probe. (H-K) Whole-mount in situ analysis of 10.5 dpc *Pitx2* wild-type (H), δabc^{null} homozygous mutant (I), δabc^{null}; δab^{hypoc} (J) and δabc^{null}; δab (K) embryos with *Msx2*. (L-O) Whole-mount in situ of 10.5 dpc wild-type (L), *Pitx2* δabc^{null} homozygous mutant (M), δabc^{null}; δab^{hypoc} (N) and δabc^{null}; δab (O) embryos with *Msx1* showing normal (arrows) and expanded (arrowheads) areas of expression. *Areas in wild type with expanded expression in mutant. md, mandibular process; mx maxillary process.

δabc^{null}*;δab*^{hypoc} and *δabc*^{null}*;δab* mutants, *Bmp4* expression failed to be distally restricted and was detected all the way to the junction with the mandibular process (Fig. 4C-G).

We examined expression of *Msx1* and *Msx2* that are mesenchymal targets of *Bmp* signaling (Barlow and Francis-West, 1997; Vainio et al., 1993). In *Pitx2 δabc*$^{null-/-}$ embryos and *δabc*^{null}*;δab*^{hypoc} and *δabc*^{null}*;δab* allelic combinations, expression of *Msx2* (Fig. 4H-K) and *Msx1* (Fig. 4L-O) was expanded proximally in the mandibular and maxillary processes. These data also revealed that expression of *Msx1* and *Msx2* was more obviously expanded than the *Bmp4* ligand, particularly in the mandibular process in *Pitx2* mutant allelic combinations. We noted that expression of *Msx1* and *Msx2* was expanded in the branchial arch mesenchyme that probably contributes to the developing heart in some *Pitx2* mutant embryos (Fig. 4K,O). Taken together, these results suggest that maintenance of *Fgf8* expression and repression of *Bmp*-signaling pathways have distinct requirements for total *Pitx2* dose in the branchial arches (summarized in Table 1).

Pitx2 regulates tooth orientation and cap formation

We investigated the tooth morphology of the *δabc*^{null}*;δab*^{hypoc} and *δabc*^{null}*;δab* allelic combinations using histological analysis. Sections through 18.5 dpc wild-type, and *δabc*^{null}*;δab* mutant embryos revealed well-formed molars. We found that in the *δabc*^{null}*; δab* embryos, the orientation of the molar tooth was abnormal (Fig. 5A,C,E,G). In *δabc*^{null}*;δab*^{hypoc} 18.5 dpc mutant embryos, analysis of serial sections revealed that molar teeth were absent (Fig. 5B,F). As *lacZ* marks cells fated to express *Pitx2a* and *Pitx2b*, serving as a marker of dental epithelium, we performed *lacZ* staining on serial cryosections from heads of 14.5 dpc *Pitx2* allelic combinations. In *δab*$^{+/-}$ and *δabc*^{null}*; δab* embryos, well-formed cap stage molar teeth were clearly evident with *lacZ* staining (Fig. 5I,J). In *δabc*^{null}*; δab*^{hypoc} mutant embryos, the dental lamina invaginated but failed to form the dental cap (Fig. 5K). In *Pitx2 δabc*^{null} homozygous mutant embryos, tooth development arrested at the placode or bud stage. The molar phenotype in *δabc*^{null}*; δab*^{hypoc} embryos, with a more developed dental lamina, suggests that tooth development progressed further than in *δabc*^{null} mutant embryos. These data show that as the dose of *Pitx2* decreases there is evidence of increasingly severe defects in tooth morphogenesis.

From these results, we conclude that *Pitx2* has a late function in molar orientation and in morphogenesis of the cap stage tooth. The intermediate tooth phenotypes observed in the *δabc*^{null}*; δab*^{hypoc} and *δabc*^{null}*; δab* mutants most probably reflects a direct role for *Pitx2* in morphogenesis of dental epithelium. Although it is possible that expression of *Fgf8* in the *Pitx2 δabc*^{null}*; δab*^{hypoc} and *δabc*^{null}*; δab* oral ectoderm is inadequate to completely rescue molar tooth development, the expression of *Pax9* and *Barx1* in dental mesenchyme of these allelic combinations suggests that *Fgf8* signaling to mesenchyme is intact in these mutant embryos and argues that *Pitx2* directly regulates epithelial morphogenesis.

Pitx2 regulates cell movement from the oral ectoderm into oral cavity and facial ectoderm

Our previous data revealed that *Pitx2* functioned to regulate local cell movement in heart development (Liu et al., 2002). To determine if a similar mechanism was at work in craniofacial development, we used the *δabc*^{cre} knock in allele and the *Gtrosa 26* reporter mouse to follow the movement of *Pitx2* daughter cells within the first branchial arch. At 9.5-11.0 dpc, *cre* expression was detected in the oral ectoderm in both *δabc*$^{cre+/-}$ and *δabc*^{cre}; *δabc*^{null} embryos, although by 11.0 dpc *cre* expression was diminished in the *δabc*^{cre}; *δabc*^{null} embryos (Fig. 6A,B and not shown). *Cre* expression was restricted to oral ectoderm and was not found in facial ectoderm or epithelium lining the oral cavity (Fig. 6C-E). Fate mapping with the *GtRosa26* reporter showed that *Pitx2* daughters were detected in the oral ectoderm, periocular mesenchyme, guts, heart and body wall (Fig. 6F,G).

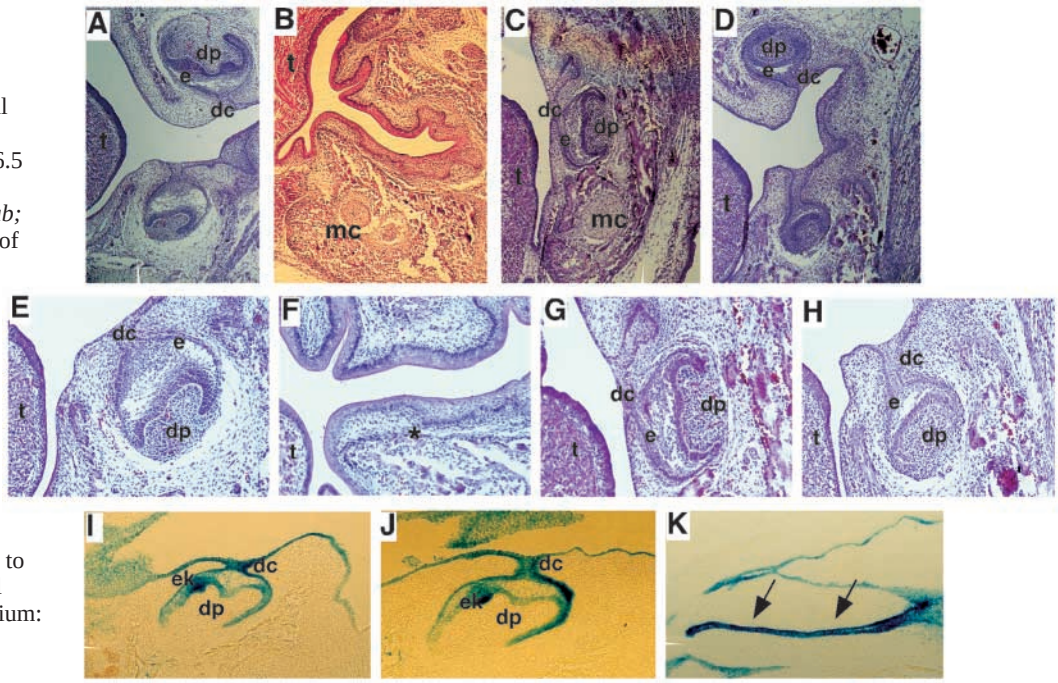

Fig. 5. Tooth phenotypes of *Pitx2* mutant allelic combinations. (A-D) Low-power view of coronal Hematoxylin and Eosin stained sections through molar teeth of 16.5 dpc wild type (A), *Pitx2 δabc*^{null}*; δab*^{hypoc} (B), *δabc*^{null}*; δab* (C), *δab; δab* (D). (E-H) High-power view of coronal Hematoxylin and Eosin stained sections through molar teeth of 16.5 dpc wild type (E), *Pitx2 δabc*^{null}*; δab*^{hypoc} (F), *δabc*^{null}*; δab* (G), *δab; δab* (H). (I-K) Parasagittal cryosections through 16.5 dpc embryos stained for *lacZ*. Wild-type (I) and *δabc*^{null}*; δab* (J) show cap stage teeth, whereas *δabc*^{null}*; δab*^{hypoc} (K) reveals a defect prior to cap formation (arrows). dc, dental cord; dp, dental papilla; e, epithelium: ek, enamel knot; mc, Meckel's cartilage; t, tongue;

In the craniofacial region, *Pitx2* daughters moved outwards from the oral ectoderm to the facial ectoderm in both wild-type and mutant embryos (Fig. 6H-K). As *cre* mRNA expression was restricted to oral ectoderm, these data reveal that *lacZ*-positive migrating cells were *Pitx2* daughters that had extinguished *Pitx2* expression. There were differences in the pattern of daughter migration in *Pitx2*-null mutant compared with wild-type embryos. In wild-type embryos, *Pitx2* daughters moved a short distance to cover the outer aspect of the mandibular and maxillary process. Some *Pitx2* daughters also contributed to the nasal process of wild-type embryos (Fig. 6H,J). In *Pitx2* mutant embryos, daughter cells moved aberrantly in a dorsal direction just inferior to the eye and failed to contribute to the mutant nasal process (Fig. 6I,K).

Pitx2 daughters extensively populated the floor and roof inside the forming mouth (Fig. 6L-O). In *Pitx2* mutants, fewer daughter cells populated the oral cavity roof as compared with wild type (Fig. 6N-Q). *Pitx2* daughters contributed to Rathke's pouch and dental epithelium, of both the wild type and mutant although in the *Pitx2* mutant tooth morphogenesis was arrested (Fig. 6N-S and not shown). These data reveal that *Pitx2* daughter cells exit the oral ectoderm and contribute to both facial ectoderm and the ectoderm lining the oral cavity and *Pitx2* function is necessary for correct deployment and expansion of daughter cells.

Discussion

In craniofacial development, the mechanisms that organize growth and morphogenesis of the branchial arches remain poorly understood. We investigated *Pitx2* isoform function in craniofacial morphogenesis using *Pitx2* exon-specific deletions. Analysis of *Pitx2* allelic combinations encoding different levels of *Pitx2* also uncovered the influence of variations in total *Pitx2* dose on *Fgf8* and *Bmp4* signaling (Table 1). Our data indicate that *Pitx2* isoforms have interchangeable function in craniofacial development and that *Pitx2* target pathways have distinct requirements for total *Pitx2* dose. Reduced *Pitx2* levels resulted in unbalanced interplay between *Fgf8* and *Bmp4* signaling pathways in craniofacial morphogenesis. We found that *Pitx2* daughter cells are migratory, eventually populating intraoral and facial ectoderm, and that *Pitx2* function is required for this movement. We

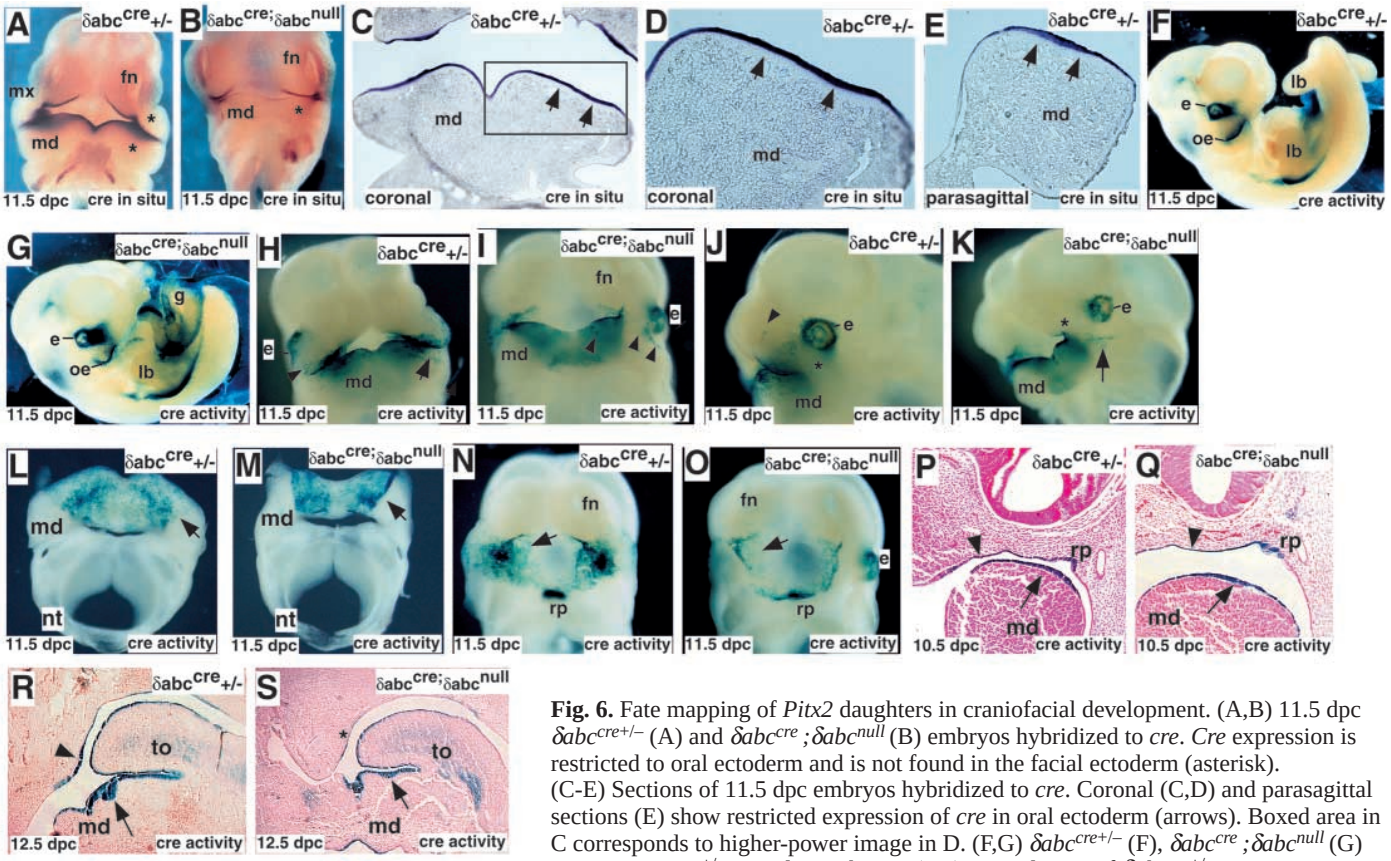

Fig. 6. Fate mapping of *Pitx2* daughters in craniofacial development. (A,B) 11.5 dpc $\delta abc^{cre+/-}$ (A) and δabc^{cre} ;δabc^{null} (B) embryos hybridized to *cre*. *Cre* expression is restricted to oral ectoderm and is not found in the facial ectoderm (asterisk). (C-E) Sections of 11.5 dpc embryos hybridized to *cre*. Coronal (C,D) and parasagittal sections (E) show restricted expression of *cre* in oral ectoderm (arrows). Boxed area in C corresponds to higher-power image in D. (F,G) $\delta abc^{cre+/-}$ (F), δabc^{cre} ;δabc^{null} (G) *rosa26 reporter*$^{+/-}$ 11.5 dpc embryos. (H,I) Ventral view of $\delta abc^{cre+/-}$ *rosa26 reporter* compound heterozygous 11.5 dpc embryos stained for *lacZ* (H) showing *lacZ*-positive *Pitx2* daughter cells (arrowhead). (J,K) Lateral view of $\delta abc^{cre+/-}$ (J) and δabc^{cre} ;δabc^{null} (K) *rosa26 reporter*$^{+/-}$ 11.5 dpc embryos stained for *lacZ*. Arrowhead (J) indicates *lacZ*-positive *Pitx2* daughter cells and arrow (K) denotes cells that move ectopically. (L-O) Oral view of floor of mouth (L,M) or roof of mouth (N,O) from $\delta abc^{cre+/-}$ (L,N) and δabc^{cre} ;δabc^{null} (M,O) *rosa26 reporter* $^{+/-}$ 11.5 dpc embryos showing migrating *Pitx2* daughter cells (arrows). (P-S) Parasagittal sections of 10.5 dpc (P,Q) and 12.5 dpc (R,S) $\delta abc^{cre+/-}$ (P,R) and δabc^{cre} ;δabc^{null} (Q,S) *rosa26 reporter*$^{+/-}$ embryos. Arrowheads indicate *lacZ*-positive *Pitx2* daughter cells contributing to oral cavity roof and asterisk indicates region that has diminished contribution in *Pitx2* mutant. Arrows indicate *Pitx2* daughters contributing to mandibular oral and dental epithelium. e, eye; g, gut; fn, frontonasal process; lb, limb bud; md, mandible; mx, maxilla; nc, nasal cartilage; nt, neural tube; oe, oral ectoderm; rp, Rathke's pouch; to, tongue.

provide evidence that *Pitx2* connects overall growth and morphogenesis of the first branchial arch through a mechanism involving differential sensitivity of target pathways to total *Pitx2* dose.

Pitx2 regulates mandibular morphogenesis by maintaining *Fgf8* and repressing *Bmp4* expression

Deletion of *Fgf8* in oral ectoderm revealed a role for *Fgf8* in survival and outgrowth of mandibular mesenchyme (Trumpp et al., 1999), while pharmacological suppression of Fgf signaling in explants suggested that Fgf functioned primarily by signaling to the underlying mesenchyme (Mandler and Neubuser, 2001). Bead implantation also suggested an early role for *Fgf8* in establishing the maxillo-mandibular region of the chick embryo (Shigetani et al., 2000). Importantly, antagonistic interactions between Fgf and Bmp signaling has been implicated in proximodistal mandibular arch patterning, placement of tooth organ formation and determination of the maxillo-mandibular region of the early embryo (Neubuser et al., 1997; Shigetani et al., 2000; Tucker et al., 1998).

Our data reveal that *Pitx2* maintains *Fgf8* expression in branchial arch ectoderm. Expression of prospective *Fgf8* target genes, such as *Barx1* and *Pitx1*, was severely reduced in *Pitx2* δabc^{null} homozygous mutant embryos. Consistent with a role of *Fgf8* signaling in mandibular rostral caudal polarity, expression of *Gsc* was expanded rostrally in the mandibular process of *Pitx2* δabc^{null} mutants. In addition, as *Pitx2* is normally expressed in rostral mandibular arch ectoderm that contributes to oral ectoderm, *Pitx2* δabc^{null} homozygous mutants lose *Barx1* expression in the rostral but not caudal mandibular arch. The *Pitx2* $\delta abc^{null}; \delta ab^{hypoc}$ and $\delta abc^{null}; \delta ab$ mutant embryos express *Fgf8* and *Fgf8* target genes, suggesting that maintenance of this pathway requires only low doses of *Pitx2*.

In contrast to *Fgf8*, high doses of *Pitx2* are required for repression of *Bmp* signaling. In the $\delta abc^{null}; \delta ab^{hypoc}$ and $\delta abc^{null}; \delta ab$ mutants, expression of *Bmp4* was expanded in maxillary ectoderm while *Msx1* and *Msx2* expression was expanded in mesenchyme of both maxillary and mandibular processes. Thus, expression of the Bmp target genes was more significantly expanded than expression of *Bmp4* ligand. This may reflect the induction of a signal relay cascade in the mandibular process. It is also interesting to note that *Dpp* has been shown to act as a classical morphogen in the wing imaginal disc of *Drosophila* (Entchev et al., 2000; Teleman and Cohen, 2000).

We found that in $\delta abc^{null}; \delta ab^{hypoc}$ and $\delta abc^{null}; \delta ab$ *Pitx2* mutants components of *Bmp4* and *Fgf8* signaling pathways, such as *Msx1* and *Barx1*, are co-expressed in mandibular mesenchyme. Previous work suggested an antagonistic interaction between these two signaling pathways (Neubuser et al., 1997; Tucker et al., 1998). It is likely that in the *Pitx2* mutant allelic combinations, Bmp signaling is only weakly expanded and this is insufficient to antagonize expression of *Barx1* in mandibular mesenchyme.

These data provide insight into the normal function of *Pitx2* in regulating gene expression. The *Fgf8* pathway and the *Bmp* suppression pathway have different requirements for total *Pitx2* dose. As *Pitx2*, *Fgf8* and *Bmp4* are co-expressed in many cells of the oral ectoderm, one can envision a mechanism where *Pitx2* would directly regulate *Fgf8* and *Bmp4* expression. In this model, one idea to explain the different requirements for *Pitx2* dose in regulating *Bmp4* and *Fgf8* would be that the regulatory regions of *Bmp4* and *Fgf8* contain different numbers of high-affinity *Pitx2*-binding sites, a mechanism suggested to underlie the haploinsufficiency of individuals with Holt-Oram syndrome that are heterozygous for *tbx5* (Bruneau et al., 2001). Thus, *Pitx2* target genes with more *Pitx2*-binding sites would require higher doses of *Pitx2* for correct levels of gene expression. However, this model is complicated by in vitro observations showing that *Pitx2* can cooperatively bind DNA (Dave et al., 2000; Wilson et al., 1993), suggesting that low levels of *Pitx2* can form higher order complexes on DNA. It is likely that there are other mechanisms, such as interaction with co-factors, to constrain or augment the ability of *Pitx2* to activate target genes. Further experiments are necessary to rule out the possibility that *Pitx2* indirectly regulates the *Fgf8* and *Bmp4* pathways.

Pitx2 in tooth morphogenesis and cell movement in craniofacial development

Pitx2-null embryos have arrest of tooth development at the placode or bud stage (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). In the *Pitx2* $\delta abc^{null}; \delta ab^{hypoc}$ and $\delta abc^{null}; \delta ab$ embryos, molar tooth morphogenesis was partially rescued in that an invaginated dental lamina formed without a cap or the orientation of the dental cap was abnormal. Our in situ studies showed that *Fgf8* was expressed in the oral ectoderm of $\delta abc^{null}; \delta ab^{hypoc}$ and $\delta abc^{null}; \delta ab$ embryos. Moreover, expression of *Pax9* was also detected in the prospective dental mesenchyme and *Barx1* was expressed in proximal mandibular mesenchyme of these embryos revealing that Fgf signaling to mandibular mesenchyme is intact in the *Pitx2* hypomorphic embryos. Although expanded *Bmp* signaling could account for tooth defects in the $\delta abc^{null}; \delta ab^{hypoc}$ and $\delta abc^{null}; \delta ab$ embryos, the abnormal tooth morphology was not suppressed by reducing *Bmp4* dose using a *Bmp4*-null allele (W.L. and J.F.M., unpublished). Based on these data, we favor the notion that *Pitx2* regulates tooth morphogenesis through a pathway that is distinct from *Fgf8* and *Bmp4* signaling, although further experiments are required to investigate these ideas.

Our fate-mapping studies show that *Pitx2* daughter cells move from oral ectoderm to populate facial and inner oral cavity ectoderm. *Pitx2*-expressing cells make a decision to extinguish *Pitx2* and become motile. It may be that *Pitx2* expression promotes cell compaction or inhibits cell motility. It is notable that one of the phenotypes of the *Pitx2*-null embryos was failure of compaction and differentiation of the periocular mesenchyme (Lu et al., 1999). *Fgf8* signaling was implicated in cell movement as *Fgf8*-null embryos had defects in cell migration through the primitive streak. Analysis of *Xenopus sprouty2*, an inhibitor of Fgf signaling, revealed that Fgf signaling in *Xenopus* regulated both mesoderm induction and convergent extension movements (Nutt et al., 2001). Thus, it is plausible that *Pitx2* regulates cell movement in the craniofacial primordia through an *Fgf8*-mediated pathway.

A direct connection of *Pitx2* to cytoskeleton and morphogenetic movement has been made by the observation that *Pitx2* controls *Rho GTPase* activity by regulating expression of the guanine nucleotide exchange factor, *Trio* (Wei and Adelstein, 2002). It has recently been proposed that *Pitx2* is a target of canonical Wnt β-catenin signaling pathway

in pituitary and cardiac development (Kioussi et al., 2002). This work uncovered a genetic interaction between *Pitx2* and dishevelled 2, a Wnt pathway branchpoint, in the heart. Other studies showed that *Rho* family GTPases are downstream components of non-canonical planar cell polarity (PCP) pathway (Habas et al., 2003; Strutt et al., 1997; Winter et al., 2001). Although further experiments are required, our data showing that *Pitx2* daughters are migratory supports the idea that *Pitx2* may be a component of a non-canonical *Wnt* pathway in craniofacial development.

Pitx2 and the phenotypic heterogeneity of Rieger syndrome I

The phenotypes in individuals with Rieger syndrome with *PITX2* mutations are heterogeneous. Our data reveal that slight changes in *Pitx2* dose can have a large influence on resulting phenotypes. This is illustrated most clearly by comparing the $\delta abc^{null}; \delta ab^{hypoc}$ and $\delta abc^{null}; \delta ab$ mutants that have only slight changes in *Pitx2* activity but dramatic differences in tooth morphogenesis (Liu et al., 2001). Many organ systems, such as heart and lungs, cannot distinguish between these small differences in Pitx2 activity (Liu et al., 2001).

The isoform deletions of *Pitx2* reveal functional redundancy between isoforms in tooth development. These data are consistent with the observation that all *Pitx2* mutations detected in individuals with Rieger syndrome are in regions common to all isoforms (Alward, 2000; Kozlowski and Walter, 2000; Priston et al., 2001; Saadi et al., 2001). Our data suggest that the *Pitx2* N terminus does not have a significant function in tooth morphogenesis because this region is not conserved between *Pitx2a*, *Pitx2b* and *Pitx2c*. This differs from pituitary and skeletal muscle where the N terminus has an influence on *Pitx2* function (Kioussi et al., 2002; Suh et al., 2002). It is also clear that *Pitx1* functions cooperatively with *Pitx2* in pituitary organogenesis and limb development (Marcil et al., 2003). As *Pitx1* is co-expressed with *Pitx2* in developing teeth, it will be interesting to investigate potential cooperative functions of *Pitx1* and *Pitx2* in oral and dental epithelium.

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