# *Shox2*-deficient mice exhibit a rare type of incomplete clefting of the secondary palate

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## Summary

**The short stature homeobox gene *SHOX* is associated with idiopathic short stature in humans, as seen in Turner syndrome and Leri-Weill dyschondrosteosis, while little is known about its close relative *SHOX2*. We report the restricted expression of *Shox2* in the anterior domain of the secondary palate in mice and humans. *Shox2*$^{-/-}$ mice develop an incomplete cleft that is confined to the anterior region of the palate, an extremely rare type of clefting in humans. The *Shox2*$^{-/-}$ palatal shelves initiate, grow and elevate normally, but the anterior region fails to contact and fuse at the midline, owing to altered cell proliferation and apoptosis, leading to incomplete clefting within the presumptive hard palate. Accompanied with these cellular alterations is an ectopic expression of *Fgf10* and *Fgfr2c* in the anterior palatal mesenchyme of the mutants. Tissue recombination and bead implantation experiments revealed that signals from the anterior palatal epithelium are responsible for the restricted mesenchymal *Shox2* expression. BMP activity is necessary but not sufficient for the induction of palatal *Shox2* expression. Our results demonstrate an intrinsic requirement for *Shox2* in palatogenesis, and support the idea that palatogenesis is differentially regulated along the anteroposterior axis. Furthermore, our results demonstrate that fusion of the posterior palate can occur independently of fusion in the anterior palate.**

Key words: Mouse, Cleft palate, *Shox2*, Epithelial-mesenchymal interaction

## Introduction

The mammalian palate forms by the union of the primary palate and the two secondary palatal shelves. In mice, the development of the secondary palate initiates at embryonic day 11.5 (E11.5). At E12.5 and E13.5, the palatal shelves grow vertically downward beside the tongue. Around E14.0 the tongue descends and a rapid elevation of the palatal shelves brings the two palatal processes into horizontal apposition above the tongue. Directed growth towards the midline leads to the contact and fusion of the medial edge epithelium (MEE) of the bilateral palatal shelves around E14.5 and the formation of a continuous palate, which separates the oropharynx from the nasopharynx (Johnston and Bronsky, 1995). Ossification subsequently occurs in the anterior two-thirds of the palate and forms the hard palate. The posterior third represents the bone-free region of the soft palate (Ferguson, 1988; Greene and Pratt, 1976). The whole process of palatal development is precisely regulated. Disruption at any stage of growth, elevation or fusion, genetically or environmentally, causes palate clefts, one of the most common birth defects in humans. Recent studies of targeted mutations in mice have revealed that an increased number of genes, including those encoding transcription factors and signaling molecules and their receptors, are implicated in normal palate development (Thyagarajan et al., 2003). Interestingly, among these genes, some exhibit restricted expression patterns in the developing palatal shelves along the anteroposterior (AP) axis, indicating an AP patterning in the palatal shelves (Zhang et al., 2002; Herr et al., 2003; Alappat et al., 2005; Cui et al., 2005). For example, a genetic pathway controlled by *Msx1* involving *Shh*, *Bmp2* and *Bmp4* is expressed and operates in the anterior region of developing palate to control cell proliferation in the palatal mesenchyme (Zhang et al., 2002). *Fgf10* also exhibits a restricted expression in the anterior palatal mesenchyme, regulating *Shh* expression, and cell proliferation and apoptosis in the palatal epithelium (Rice et al., 2004; Alappat et al., 2005). By contrast, *Tbx22* was reported to be expressed in the posterior palate (Herr et al., 2003).

The homeobox genes encode a class of transcription factors that control many developmental processes during embryonic development in organisms as diverse as humans and flies. Mutations in homeobox genes have been described in a number of human genetic diseases (Boncinelli, 1997), such as *PAX3* in Waardenburg syndrome (Baldwin et al., 1992), *PAX6* in aniridia (Glaser et al., 1992), *PITX2* in Rieger syndrome (Semina et al., 1996), and *MSX1* in familial tooth agenesis and

cleft palate (Vastardis et al., 1996; Van den Boogaard et al., 2000). The *SHOX* gene (short stature homeobox gene) was initially identified to be associated with idiopathic growth retardation and Turner syndrome and Leri-Weill dyschondrosteosis in humans (Ellison et al., 1997; Rao et al., 1997; Belin et al., 1998; Shears et al., 1998). *SHOX* has a closely related human homolog *SHOX2* (*SHOT* or *OG12X*) (Blaschke et al., 1998; Semina et al., 1998). They share 83% homology at the amino acid level and have an identical homeodomain. The expression of *SHOX2* has been detected in the limb bud, branchial arches, nasal processes, heart, central nervous system and genital tubercle of human embryos (Clement-Jones et al., 2000). However, *SHOX2* has not yet been linked to any known syndromes so far. A zooblot analysis of *SHOX* and *SHOX2* revealed an absence of both genes in all the invertebrates studied but their presence in all vertebrates studied suggests that these two genes have a central role in the development of the internal skeleton and its related structures (Clement-Jones et al., 2000). An *SHOX* ortholog does not exist in mice, but the true mouse *Shox2* ortholog has been identified (Rovescalli et al., 1996; Clement-Jones et al., 2000). The mouse Shox2 shares 99% identity at the amino acid level with its human counterpart (Blaschke et al., 1998; Semina et al., 1998). The expression pattern of *Shox2* in developing mouse embryo is very similar to that of human *SHOX2* (Blaschke et al., 1998; Semina et al., 1998; Clement-Jones et al., 2000). In humans, *SHOX* and *SHOX2* exhibit an overlapping while sometimes complementary expression pattern in a number of developing organs or tissues, including the first and second branchial arch and their derivatives, and the developing limb, suggesting a functional redundancy between them (Clement-Jones et al., 2000). The lack of an *SHOX* ortholog in mice implicates that mouse *Shox2* may play a broader function than human *SHOX2* in embryogenesis.

In this study, we identified a restricted expression pattern of *Shox2* in the anterior palatal shelves of both mouse and human embryos. We demonstrate that the initially restricted expression of *Shox2* in the mouse palatal mesenchyme is induced by signals derived from the anterior palatal epithelium, among which BMP activity is indispensable. We further generated *Shox2* mutant mice by gene targeting. Defective palatal growth and altered gene expression are found to be confined to the anterior palate where *Shox2* is expressed, leading to an incomplete cleft within the anterior region. Our results demonstrate a crucially intrinsic role for *Shox2* in mammalian palatogenesis.

## Materials and methods

### Generation and genotyping of *Shox2* knockout mice

A BAC clone covering the *Shox2* genomic region (from Invitrogen) was used to amplify homologous sequences by PCR. The targeting vector was constructed by placing a 2.5 kb 5′ homologous fragment and a 4.2-kb 3′ homologous arm into a modified *DTA-PGK-neo* vector, flanking the neomycin resistance gene. Correct targeting of the *Shox2* locus with this vector results in the *PGK-neo* cassette replacing exon 3, which encodes the homeodomain. The targeting vector was linearized and electroporated into JM1 embryonic stem (ES) cells. Clones were selected with G418 and targeting was screened by Southern blot analysis with both a 5′ probe and a 3′ probe outside the flanking homologous sequences. Targeted ES cells were injected into C57BL/6 blastocysts to produce chimeras. Chimeric mice that were derived from two independently targeted ES cell clones were mated with C57BL/6 mice to generate *Shox2* heterozygous animals, whose genotype was confirmed by Southern blotting of tail DNA. On the blots, digestion of genomic DNA with *Bam*HI and hybridized with the 3′ probe gave rise to a 5 kb wild-type band and a 6.7 kb mutant band. Homozygous mutant mice from both clones exhibit identical phenotypes. Mice and embryos, maintained on C57BL/6 background, were routinely genotyped by PCR analysis of tail DNA or yolk sacs of embryos. One set of primers (M1, 5′-AGAGGCTATTCGGCTATGA-3′; M2, 5′-AGCCATGATGGATACTTTCTCG-3′) amplified a 300 bp product from the mutant *Shox2* allele, the other set of primers (W1, 5′-GGTCCGACTTCGCCTCTGCTTGAT-3′; W2, 5′-CTTGCCGCGC-CCTTTAACCGAGAC-3′) produced a 520 bp product from the wild-type *Shox2* allele.

### RT-PCR

E13.5 mouse embryos were used to isolate mRNA using an RNAqueous-4PCR kit (Ambion). The first-strand cDNA synthesis was carried out using a SuperScript kit (Invitrogen). A set of primers (upstream, 5′-CTGCCCCATTGATGTGTTATT-3′; downstream, 5′-CCTCCTCCTCCAGCACCT-3′) that amplified a 368 bp sequence of exon 1 and exon 2, and a pair of primers (upstream, 5′-ACCAGCAA-GAACTCCAGCAT-3′; downstream, 5′-GCCACACTCCTTTG-TCCAGT-3′) that amplified a 371 bp sequence covering exon 4 to exon 6 of *Shox2* were used for RT-PCR detection of partial transcripts in the *Shox2* mutants. The primers (upstream, 5′-TTCCG-CAAGTTCACCTACC-3′; downstream, 5′-CGGGCCGGCCAT-GCTTTACG-3′) that amplify a 361 bp cDNA product of S15 RNA were included as the positive control for RT-PCT.

### Histology, in situ hybridization and scanning electron microscopy (SEM)

Mouse embryos were dissected in cold PBS and fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight. Surgically and medically terminated human embryos were collected, staged and fixed in 4% PFA, under the ethical permission of the Ethics Committee of Fujian Normal University. For histological analysis, samples were embedded in paraffin, sectioned at 8 μm and stained with Hematoxylin and Eosin. Samples used for section in situ hybridization were paraffin embedded and sectioned at 10 μm. At least two identical samples of mutant and wild type were used for in situ hybridization for each probe. Samples used for whole-mount in situ hybridization were bleached with 6% $H_2O_2$ after fixation, and dehydrated through a graded methanol series. Whole-mount and section in situ hybridization were performed as previously reported (Zhang et al., 1999). SEM was carried out as described before (Zhang et al., 2002).

### Tissue recombination and bead implantation

The secondary palatal shelves from E11.5 to E13.5 embryos were microdissected in PBS. To separate the palatal epithelium from the mesenchyme, isolated palatal shelves were incubated for 20 minutes in solution containing 0.5% trypsin and 2.5% pancreatin on ice, and then were transferred to α-MEM medium plus 20% fetal bovine serum on ice for an additional 10 minutes. Tissues were microsurgically separated. Tissue recombination and bead implantation were carried out according to procedures described previously (Chen et al., 1996; Zhang et al., 2002). Briefly, isolated palatal mesenchyme or intact palatal tissue (containing both the epithelium and mesenchyme) was placed on filter in the Trowell type organ cultures. For tissue recombination, donor palatal epithelia were placed on the top of mesenchymal tissues in organ culture. For bead implantations, protein-soaked beads (Affi-Gel blue agarose bead, 100-200 mesh, 75-150 μm diameter, from Bio-RAD, Hercules, CA) were implanted into the palatal tissues on the filter. Samples were harvested for whole-mount in situ hybridization analysis after 24 hours in culture in α-MEM media with 3500 mg/l glucose, 0.55 mM glycine, 0.056 mM ascorbic acid and 14% knockout serum replacement. The following

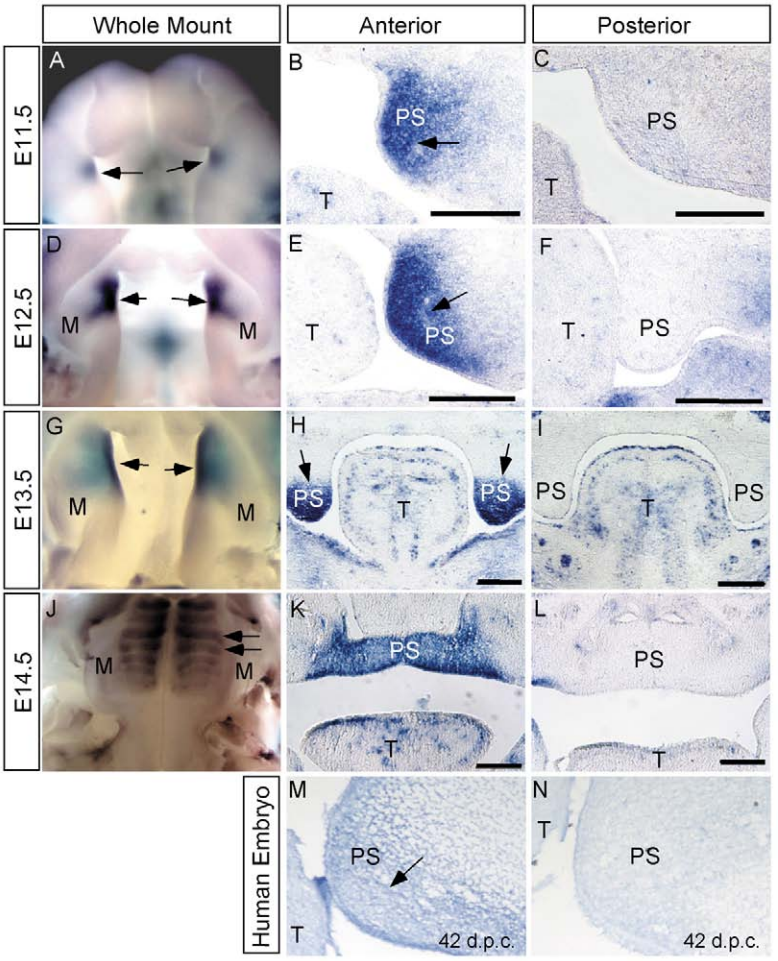


**Fig. 1.** Expression of *Shox2* in the developing palate. (A-C) *Shox2* expression (arrows) is detected by whole-mount (A) and section (B,C) in situ hybridization in the anterior (A,B) but not the posterior portion of palatal shelves at E11.5. The expression is restricted in the palatal mesenchyme (B). (D-F) At E12.5, *Shox2* expression (arrows) domain expands but is still restricted in the anterior palatal mesenchyme, as detected by whole-mount (D) and section (E) in situ hybridization. (G-I) *Shox2* expression (arrows) is still restricted in the anterior (G,H) but not in the posterior region (I) at E13.5. The expression at this stage expands to the palatal epithelium (H). (J-L) At E14.5, *Shox2* expression exhibits a pattern of alternative stripes in the anterior half of the palate (J). The expression is detected in both the palatal mesenchyme and epithelium (K). (M) *SHOX2* is also detected in the anterior palate of human embryo of 42 days post-conception (dpc), equivalent to mouse E11.5. (N) The posterior palate of human embryo of 42 dpc shows no *SHOX2* expression. All sections shown were made through a coronal plane. M, the first molar; T, tongue; PS, palatal shelf. Scale bar: 200 μm.

proteins (with the concentration at which they were used) were obtained from R&D System (Minneapolis, MN) and used for bead implantation: activin (200 ng/μl), Bmp2 (200 ng/μl), Bmp4 (200 ng/μl), Bmp5 (200 ng/μl), Bmp7 (200 ng/μl), Fgf2 (500 ng/μl), Fgf4 (500 ng/μl), Fgf8 (200 ng/μl), Fgf10 (500 ng/μl), noggin (200 ng/μl) and Shh (1 μg/μl). Anti-Shh antibodies (5E1), obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, were used at 400 ng/μl.

### Detection of cell proliferation and apoptosis

Cell proliferation was detected by an immunochemical assay using a biotinylated antibody against PCNA using a PCNA Staining Kit from Zymed Laboratories (South San Francisco, CA) or by BrdU labeling using a BrdU labeling and detection kit (Boehringer Mannheim) according to the manufacturer's instruction. PCNA-positive cells in arbitrarily defined area/region of the palatal epithelium and mesenchyme in wild-type and mutant embryos were counted. PCNA-positive cells in the laterally adjacent maxillary mesenchyme where *Shox2* is not expressed were also counted as controls. Student's *t*-test was used to analyze if significance of differences and *P* values were present in wild-type and mutant samples. For BrdU labeling, tissues in organ cultured were pulsed at a concentration of 10 μM for 45 minutes prior to being fixed in Carnoy fixative. Samples were processed for immunochemical staining as described previously (Zhao et al., 2000). The TUNEL assay was carried out to detect cell apoptosis in the palatal shelves in paraffin sections as described previously (Alappat et al., 2005). Three samples of mutant and wild-type control each were used for PCNA and TUNEL assays.

## Results

### *Shox2* expression is confined to the anterior domain of the developing palate

In an expression screen for genes that are differentially expressed along the anteroposterior (AP) axis in the developing palate, we identified *Shox2* as one such gene. *Shox2* expression appears in the anterior-most domain of the palatal tissue folds at E11.5 when palatogenesis begins (Fig. 1A), and expands with its posterior boundary to the middle level of the first molar at E12.5 (Fig. 1D) and E13.5 (Fig. 1G). *Shox2* expression is restricted to the palatal mesenchyme at E11.5 (Fig. 1B) and E12.5 (Fig. 1E), but is expressed in both the palatal mesenchyme and epithelium at E13.5 (Fig. 1H). At E14.5, *Shox2* expression is somewhat downregulated and exhibits a striped pattern in the mesenchyme and epithelium (Fig. 1J,K). *Shox2* expression was absent in the posterior palatal mesenchyme in all stages examined (Fig. 1C,F,I,L). The palatal *Shox2* expression subsequently disappears at E15.5 (data not shown). We further demonstrated that the expression of human *SHOX2*, which resembles that of its mouse counterpart in many sites in developing embryo (Clement-Jones et al., 2000), is also confined to the anterior region of the palate of the human embryo (Fig. 1M,N).

### *Shox2* homozygous mutants exhibit impaired palatal development

To investigate the role of *Shox2* in palatal development, we inactivated *Shox2* by deleting exon 3 which encodes the homeodomain of the Shox2 protein (Fig. 2A). The targeted locus in ES cells and germline transmission were confirmed by Southern blot analysis (Fig. 2B). Heterozygous and homozygous mice were maintained on the C57Bl/6 background and were genotyped by PCR (Fig. 2C). To confirm an absence of *Shox2* expression in *Shox2*-deficient mice, RT-PCT was performed with primers that amplify sequences spanning exon 1 to exon 2 and exon 4 to exon 6 of the *Shox2* gene, respectively. No partial *Shox2* transcripts were detected

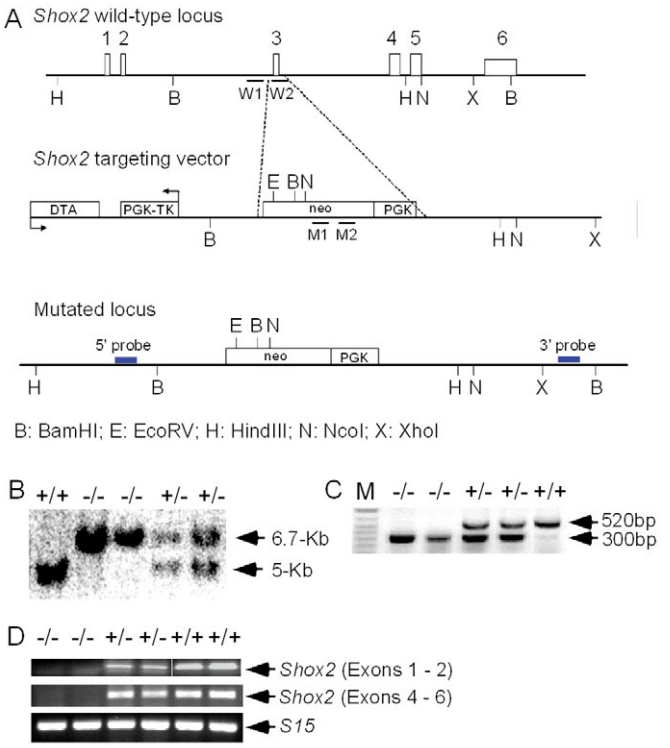


**Fig. 2.** Targeted disruption of the *Shox2* gene in mice. (A) The mouse *Shox2* genomic structure spans 8.3 kb, and contains 6 exons, as indicated by numbers. The targeting vector contains genomic fragments flanking the *PGK-neo* cassette which replaces exon 3 of *Shox2* when correct homologous recombination occurs in ES cells. (B) Southern blot analysis of genomic DNA (digested with *Bam*HI and probed with 3′-probe) derived from E11.5 embryos with different genotypes. (C) PCR analysis of DNA from DNA samples from yolk sac of embryos using W and M pairs of primers for wild-type and mutant alleles, respectively (see A). The W set of primers amplify the wild-type DNA fragment at the size of 520 bp, while the M set of primers amplify the mutant DNA fragment of 300 bp. (D) RT-PCR assays showing the absence of partial *Shox2* transcripts in *Shox2*-deficient embryos.

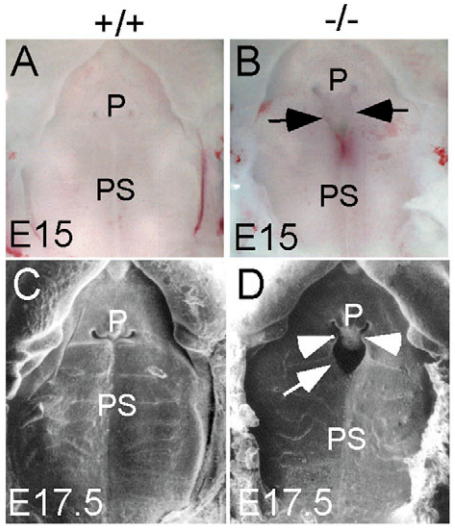


**Fig. 3.** *Shox2* mutants exhibit clefting in the anterior palate. (A,B) Oral view of E15.0 wild-type (A) and *Shox2*$^{-/-}$ (B) palate shows an anterior clefting in the mutant. Arrows indicate the cleft. (C,D) Scanning electron microscopic images of oral view of E17.5 wild-type (C) and *Shox2*$^{-/-}$ (D) embryonic palate show an incomplete clefting (arrows) in the mutant palate. Arrow indicates the cleft, while arrowheads indicate the regions where the secondary palate fails to fuse with the primary palate and nasal septum. P, primary palate; PS, palatal shelf.

in *Shox2* mutant embryos (Fig. 2D). Mice heterozygous for the *Shox2* mutation appeared normal and fertile. No *Shox2* homozygous mutant mice were identified at birth. Examination of staged embryos revealed that death of homozygous mutants occurred at mid-gestation stage. Of 187 homozygous mutant embryos recorded that present in a Mendelian ratio, 63% of mutant embryos died between E11.5 and E12.5, while the rest survived up to E17.5. Cardiac and vascular defects appeared to contribute to the embryonic lethality in *Shox2* homozygotes (R. Espinoza, L.Y. and Y.P.C., unpublished). Strikingly, the mutant embryos manifested an incomplete clefting in the anterior region of the palate (Fig. 3). At E15.0, when the secondary palate closes in the wild type, a clefting, from the anterior extremity of the secondary palate to the first molar level, could be seen in the mutant (Fig. 3). The AP length of the cleft appeared shortened at E17.5, probably owing to partial fusion of the palate at the posterior domain in the mutants (Fig. 3). Furthermore, it appears that the secondary palate failed to fuse with the primary palate and the nasal septum.

Histological analyses demonstrated that palatogenesis initiated normally in the *Shox2*$^{-/-}$ embryos at E11.5. At E12.5, the anterior palate appeared slightly shorter (data not shown). At E13.5, the mutant anterior palate showed a dramatically retarded growth and exhibited a broadened appearance owing to a shallow ventrolateral indentation of the shelf, when compared with the wild-type control (Fig. 4A,B). By contrast, the posterior palate developed normally (Fig. 4C,D). At E14.5, the wild-type palatal shelves have elevated above the dorsum of the tongue and have met each other at the midline along the AP axis (Fig. 4E,G). The mutant palatal shelves were elevated over the tongue, but had only made contact at the midline in the posterior region (Fig. 4H). The retarded anterior palatal shelves in the mutants, although reoriented to the horizontal position, failed to make contact at the midline, leaving an opening in the anterior palate (Fig. 4F). In *Shox2* mutants at E15.0, a cleft remained in the anterior palate where the hard palate (anterior two-thirds of the palate) is forming (Fig. 4I,J), while the posterior region of the palate, which made contact posterior to the mid-level of the first molar (Fig. 4K,L), began to fuse, as indicated by the disruption of the midline seam (inset in Fig. 4L). The anterior palatal defect was seen in all 33 mutants examined. The coincidence of the restricted *Shox2* expression pattern in the palate and the clefting phenotype seen in the mutant indicates that Shox2 is an intrinsic regulator of palatogenesis.

Cell proliferation and apoptosis assays were carried out to reveal the cellular mechanisms that are responsible for the retarded palatal growth in *Shox2*$^{-/-}$ embryos. Significant decreased level of cell proliferation, assayed by PCNA staining, was detected in the anterior regions, in both epithelium ($P$<0.0005) and mesenchyme ($P$<0.0005), of the

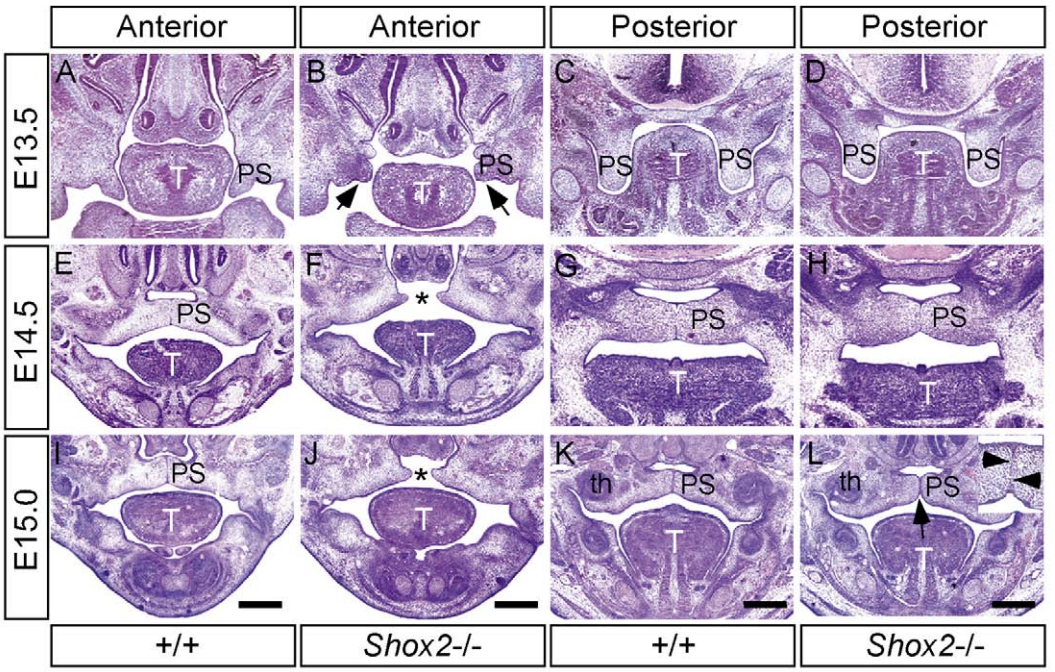


**Fig. 4.** *Shox2*$^{-/-}$ mice exhibit impaired palatal growth in the anterior domain. Coronal sections were made in all panels. Those anterior (A,B,E,F,I,J) and posterior (C,D,G,H) to the first molar are indicated. (K and L) Sections were made through mid-level of the first molar. (A-D) At E13.5, the palatal shelves are vertically oriented in the wild type (A,C). The anterior palatal shelves (arrows) in the *Shox2* mutants appear shorter and more rounded (B), but the posterior palatal shelves appear indistinguishable from the wild type (D). (E-H) At E14.5, the wild-type palatal shelves have elevated to the horizontal position above the tongue, closed and begun to fuse (E,G). The mutant palatal shelves have also elevated to the dorsum of the tongue, but have only made contact and fused in the posterior region (H). The anterior palatal shelves of the mutants appear too small to make contact at the midline, leaving an opening as indicated by a star (F). (I-L) At E15.0, palate fusion is still ongoing along the AP length in the wild-type embryo (I,K). A cleft (star) remains in the anterior palate of *Shox2* mutants (J). A section through the mid-level of the first molar of the mutant shows the anterior contact point (arrow) of the palatal shelves (L). Inset in L shows disruption of the midline seam (arrowheads) in the posterior palate of the mutant at E15.0. T, tongue; PS, palatal shelves; th, tooth. Scale bar: 300 μm.

*Shox2*$^{-/-}$ palate at E12.5 when the phenotype initially appears (Fig. 5A,B,E). However, comparable levels of cell proliferation in the posterior palate were found in the wild type and the mutants (data not shown). The altered cell proliferation in the palatal epithelium where *Shox2* is not normally expressed at this stage indicates a non-cell autonomous effect of the absence of *Shox2*. TUNEL assays have shown that cell death was unaltered in the *Shox2*$^{-/-}$ palatal shelves at E12.5 (data not shown), but an increased level of apoptosis was detected in the anterior palatal epithelium at E13.5 (Fig. 5C,D). As *Shox2* is also expressed in the palatal epithelium at E13.5 (Fig. 1H), *Shox2* might control cell apoptosis via a cell autonomous mechanism.

### Expression of genes implicated in palatogenesis in *Shox2* mutants

To establish a genetic hierarchy involved *Shox2* and genes that have been implicated in palatogenesis, we examined the expression of a number of candidate genes at E12.5 and E13.5. The expression of *Msx1*, *Bmp4* and *Fgf10* overlaps with that of *Shox2* in the anterior palatal mesenchyme. Mutations in either *Msx1* or *Fgf10* lead to complete cleft palate (Zhang et al., 2002; Rice et al., 2004; Alappat et al., 2005). Our results demonstrate a wild-type level of expression of *Msx1* and its downstream gene *Bmp4* in the *Shox2*$^{-/-}$ palate (Fig. 6A,B; data not shown). By contrast, *Fgf10* expression initially appeared normal in the mutant palate at E12.5 (data not shown), but showed an ectopic pattern in the anterior palatal mesenchyme immediately adjacent to the medial edge epithelium (MEE) at E13.5, while in the wild-type controls *Fgf10* expression has been displaced ventrolaterally away from the MEE at this stage (Fig. 6E,F) (Alappat et al., 2005). Fgf10 is known to signal through Fgf receptor 2b (Fgfr2b), which is expressed in the epithelium of developing palate (Fig. 6G) (Lee et al., 2001; Rice et al., 2004). Although *Fgfr2* knockout mice die at E10.5, specific deletion of *Fgfr2b* isoform, similar to *Fgf10* knockout, leads to a cleft palate phenotype (De Moerlooze et al., 2000; Rice et al., 2004). In situ hybridization using *Fgfr2* probe that covers both *Fgfr2b* and *Fgfr2c* transcripts, and *Fgfr2b*-specific probe revealed an ectopic *Fgfr2* expression in the anterior palatal mesenchyme of E13.5 *Shox2* mutant embryo (Fig. 6H). By contrast, *Fgfr2b* expression was not altered in the mutant palate, indicating that it is *Fgfr2c* that is ectopically activated in the palatal mesenchyme of *Shox2* mutants (data not shown). We have previously shown an altered expression of *Tgfb3* and *Jag2* in the palatal epithelium of *Fgf10* mice (Alappat et al., 2005). We further asked whether the ectopically expressed *Fgf10* in the palatal mesenchyme of *Shox2* mutant would alter the expression pattern of *Tgfb3* and *Jag2*. However, a comparable expression level of both genes was found in both wild-type and *Shox2* mutant embryos (data not shown). Together with the finding that *Shox2* maintains its expression pattern in both *Msx1* and *Fgf10* mutant palate (data not shown), we conclude that *Shox2* functions upstream to *Fgf10* and *Fgfr2c* but in parallel with *Msx1* in palatogenesis. Additionally, the expression of *Pax9*, *Lhx8* and *Osr2*, which are known to be critical for normal palatogenesis (Peters et al., 1998; Zhao et al., 1999; Lan et al., 2004), appeared normal in the *Shox2*$^{-/-}$ palate (Fig. 6C,D; data not shown), indicating that these genes are not downstream effectors of *Shox2*.

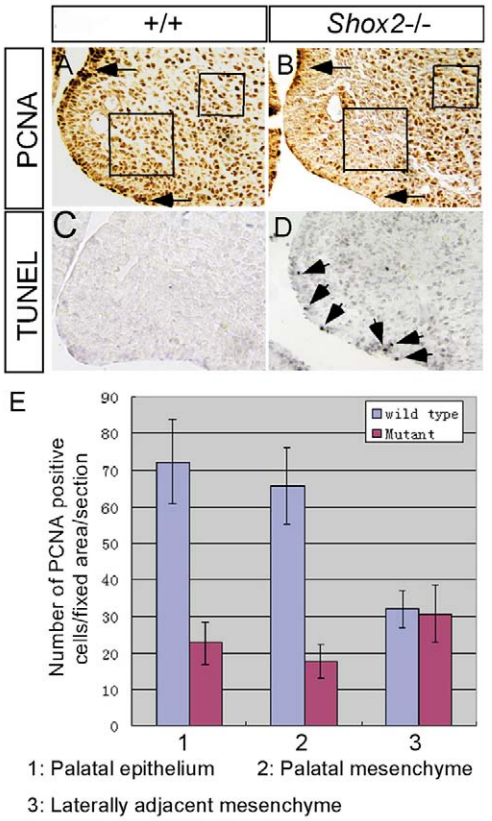


**Fig. 5.** Altered cellular processes in the *Shox2*$^{-/-}$ palatal shelves. (A,B) PCNA staining on coronal sections of E12.5 wild-type (A) and *Shox2*$^{-/-}$ (B) palate shows significantly decreased level of cell proliferation in the anterior region of the *Shox2*$^{-/-}$ palate. (C,D) The TUNEL assay on coronal sections of E13.5 wild-type (C) and *Shox2*$^{-/-}$ (D) palate exhibits an increased level of cell apoptosis (arrows) in the anterior palatal epithelium of the *Shox2* mutant. (E) Comparison of PCNA-positive cells in a fixed area of palate in wild-type and Shox2 mutant embryos. The number of PCNA-positive cells in the mutant type palatal apex epithelium (mean=22.7), as marked by arrows in A,B, is greatly reduced when compared with that in the wild type (mean=72.3) ($P$<0.0005). The number of PCNA-positive cells in the mutant palatal mesenchyme (mean=17.5), as counted within the large square in A,B, is reduced to about 30% when compared with that in the wild type (mean=65.6) ($P$<0.0005). However, the number of PCNA-positive cells in the laterally adjacent maxillary mesenchyme where *Shox2* is not expressed, as counted within the small square in A,B, is similar between the wild type and the mutant ($P$>0.5). Standard deviation values were indicated for the error bars.

To test whether the ectopic *Fgf10* expression in the anterior palatal mesenchyme of *Shox2* mutant accounts for the defective cell proliferation, we carried out in vitro bead implantation experiment using the anterior and posterior region of E13.5 wild-type palatal shelves. Beads soaked with BSA (1 mg/ml), Fgf10 (500 ng/μl) or Fgf2 (500 ng/μl) were implanted onto the tissue explants. Explants were cultured for 20 hours and were pulsed for BrdU for 45 minutes prior to fixation. A significant lower level of BrdU-labeled cells was observed in the anterior palatal tissues (16/20) implanted with Fgf10-soaked beads (Fig. 7B), when compared with controls implanted with BSA beads (Fig. 7A). Fgf10-soaked beads, however, did not alter cell proliferation in the posterior palatal tissue (11/11) (Fig. 7D,E), further demonstrating differential responses of palatal tissue along the AP axis to Fgf10 induction. By contrast, Fgf2, which binds to all four Fgf receptors, stimulate cell proliferation in both anterior (12/12) (Fig. 7C) and posterior palatal tissues (8/8) (Fig. 7F). Thus, ectopically applied Fgf10 appears to inhibit cell proliferation in the anterior palate. Different Fgfs apparently act differently on cell proliferation in the developing palate.

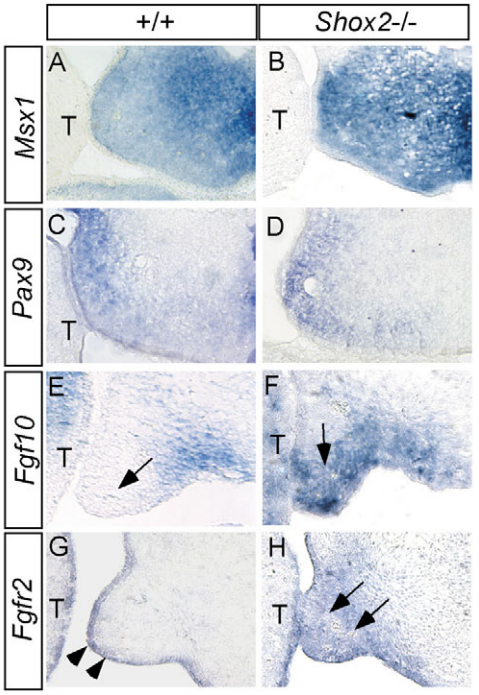


**Fig. 6.** Gene expression in the *Shox2*$^{-/-}$ palatal shelves. (A) *Msx1* expression in the anterior palatal mesenchyme of an E12.5 wild-type embryo. (B) *Msx1* exhibits a wild-type pattern of expression in the anterior palatal mesenchyme of an E12.5 *Shox2*$^{-/-}$ embryo. (C,D) *Pax9* expression is unaltered in the anterior palate of *Shox2* mutant (D) when compared with wild-type control (C). (E,F) Ectopic *Fgf10* expression is seen in the anterior palatal mesenchyme (arrows) of E13.5 mutant embryo (E) but not in the wild-type counterpart (F). (G,H) *Fgfr2*, which is restricted to the anterior palatal epithelium (arrowheads) in E13.5 wild-type embryo (G), is ectopically activated in the anterior palatal mesenchyme (arrows) of the age-matched *Shox2* mutants. b, bead.

## Regulation of *Shox2* expression in the palate

We further studied the regulation of *Shox2* expression in the palate. In vitro tissue culture and recombination experiments were performed to determine if *Shox2* induction/expression relies on the palatal epithelial signals. Palatal folds from E11.5 retained *Shox2* expression after 24 hours of culture in vitro (Fig. 8A), but lost *Shox2* expression if the palatal epithelium was removed (Fig. 8B). However, *Shox2* expression was restored in the mesenchyme when recombined with epithelia from the anterior palatal shelves (11/11; Fig. 8C). By contrast, *Shox2* expression was independent of the palatal epithelium in tissues isolated at E12.5 (Fig. 8D,E). Interestingly, when the epithelium from the anterior region of E12.5 palatal shelves was recombined with the posterior palatal mesenchyme, ectopic *Shox2* expression was activated (7/10; Fig.

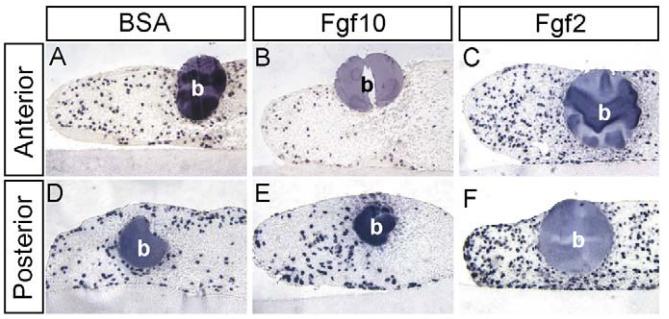


**Fig. 7.** Fgf proteins exert different effects on cell proliferation in the developing palate. (A-C) Fgf10 inhibits cell proliferation (B), while Fgf2 stimulates cell proliferation (C) in E13.5 anterior palatal tissue when compared with tissue treated with BSA (A). (D-F) Cell proliferation levels are similar in the posterior palatal tissue implanted with either BSA bead (D) or Fgf10 bead (E). However, cell proliferation level is significantly increased in the posterior palatal tissue implanted with Fgf2 bead (F). b, bead.

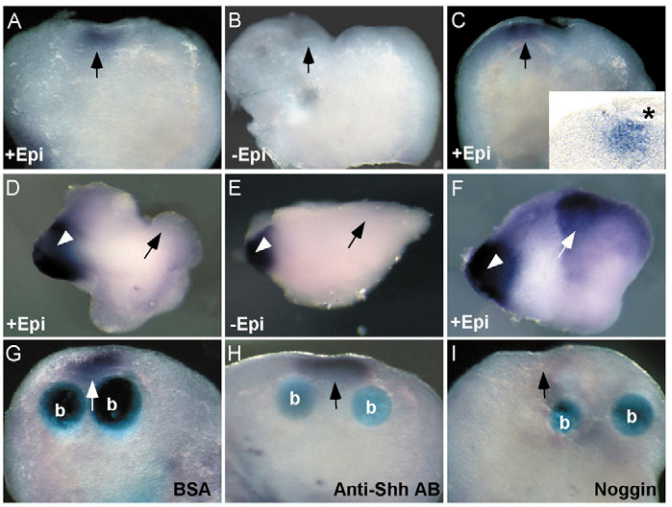


**Fig. 8.** Regulation of *Shox2* expression in the developing palatal mesenchyme. (A-C) *Shox2* expression (arrow) remains in the anterior palatal mesenchyme of an E11.5 palatal shelf after 24 hours in organ culture (A), but is not detected in the mesenchyme of an E11.5 de-epithelialized palatal shelf after 24 hours in organ culture (B). *Shox2* expression (arrow) is maintained/induced in E11.5 palatal mesenchyme recombined a piece of anterior palatal epithelium from a donor palatal shelf of the same stage (C). (C, inset) A section through a recombinant sample shows *Shox2* expression in the palatal mesenchyme immediately adjacent to the donor epithelium indicated by an asterisk. (D-F) At E12.5 *Shox2* is expressed in the anterior palatal mesenchyme (arrowheads) of the palatal shelves with (D) or without (E) palatal epithelium after 24 hours in organ culture. Ectopic *Shox2* expression (arrow) is induced in the mesenchyme of the posterior palatal shelf by an epithelium from the anterior region of the E12.5 palatal shelf after 24 hours in organ culture (F). Arrows in D-F indicate the posterior region of the cultured palatal shelves. (G-I) *Shox2* expression is not altered by application of either BSA (G) or anti-Shh antibodies (H) in the anterior mesenchyme of the E11.5 palatal shelves, but is downregulated by Noggin beads after 24 hours in organ culture (I). b, bead.

8F). The anterior palatal epithelium is thus responsible for *Shox2* expression in the developing palate.

In an effort to identify growth factors that are responsible for *Shox2* induction in the palate, we performed beads implantation experiments. Palatal mesenchyme from the E11.5 or E12.5 posterior palate was isolated and implanted with protein-soaked beads and cultured in vitro. The proteins tested (activin, Bmp2, Bmp4, Bmp5, Bmp7, Fgf4, Fgf8 and Shh) are either expressed in the developing palate or are known to induce gene expression in the dental mesenchyme, which has the same origin as the palatal mesenchyme (Chai et al., 2000; Ito et al., 2003). However, no single growth factor tested in this assay was sufficient to induce *Shox2* expression in the posterior palatal mesenchyme (data not shown): activin (0/10), Bmp2 (0/18), Bmp4 (0/14), Bmp5 (0/8), Bmp7 (0/15), Fgf4 (0/6), Fgf8 (0/18) and Shh (0/10). As Fgf and Bmp signaling pathways are often antagonistic to each other in many developing organs, we tested a combinatorial effect of Fgf and noggin on *Shox2* induction by using beads soaked with Fgf10 (200 ng/μl) and noggin (200 ng/μl). Ectopic *Shox2* induction was never seen in all 12 samples tested (data not shown). As *Shh* and *Bmp2* are expressed in the anterior palatal epithelium (Zhang et al., 2002), and the anterior palatal epithelium was shown to activate ectopic *Shox2* expression (Fig. 8F), we blocked their activities to determine if they are involved in *Shox2* induction. Noggin- or Shh antibody-soaked beads were implanted into explanted E11.5 palatal shelves close to the region where *Shox2* is normally expressed. Following 24 hours in organ culture, similar to the BSA controls, Shh antibody did not affect *Shox2* expression (0/8; Fig. 8G,H), but Noggin-soaked beads were able to inhibit *Shox2* expression (12/17; Fig. 8I), indicating that Bmp activity constitutes an indispensable component of the epithelial signals. Although not sufficient, Bmp activity is necessary for *Shox2* induction in the palatal mesenchyme.

## Discussion

### Palatal *Shox2* expression is regulated and regionally confined by epithelial signals

We have previously demonstrated a differential expression pattern of a number of genes along the AP axis of the developing palatal shelves (Zhang et al., 2002; Alappat et al., 2005), suggesting regional regulation of palatal growth and patterning along the AP axis. Here, we show that *Shox2* also exhibits a restricted expression pattern in the anterior palate. This restricted expression pattern implicates *Shox2* as a unique regulator of palatal growth and patterning.

Mammalian palatogenesis is governed by interactions between the palatal epithelium and mesenchyme (Slavkin, 1984; Ferguson and Honig, 1984). Our tissue recombination studies demonstrate the induction of *Shox2* expression in the palatal mesenchyme by the palatal epithelial tissue. Initial *Shox2* expression in the palatal mesenchyme relies on the presence of the palatal epithelium. Removal of the palatal epithelium at E11.5 led to loss of *Shox2* expression in the palatal mesenchyme. However, at E12.5, the epithelial signals are no longer necessary for the maintenance of the mesenchymal *Shox2* expression. Interestingly, at this stage, the anterior palatal epithelium retains its inductive capability, as the epithelium is able to induce ectopic *Shox2* expression in the posterior palatal mesenchyme. Only epithelial tissue from the anterior palatal shelves but not the posterior is capable of

doing so, indicating heterogeneity in the palatal epithelium along the AP length. The morphological heterogeneity of the palatal epithelium has been noticed, with the formation of pseudostratified, ciliated and columnar epithelia on the nasal aspect of palate, and stratified non-keratinized and squamous epitheliua on the oral side (Ferguson, 1988). However, our results unambiguously demonstrate the existence of epithelial heterogeneity in terms of gene induction capability along the palatal AP axis at the onset of palatogenesis. This epithelial heterogeneity along the palatal AP axis could be attributed to different origins of the epithelial tissues or distinct origins of cranial neural crest cells that the epithelial tissues exposed to. We have previously shown that the palatal mesenchyme becomes heterogeneous along the AP axis at E12.5, as evidenced by the fact that Bmp4 induces *Msx1* expression in the anterior palatal mesenchyme but not the posterior mesenchyme, while Fgf8 induces *Pax9* expression only in the posterior palatal mesenchyme (Zhang et al., 2002). This point is further strengthened by the fact that Fgf10 represses cell proliferation in the anterior but not the posterior palatal tissues (this study). The finding that the anterior palatal epithelium is able to induce *Shox2* expression in the E12.5 posterior palatal mesenchyme suggests that palatal mesenchyme at this stage retains a certain degree of plasticity in terms of competence to the induction by epithelial signals.

Diffusible growth factors play a pivotal role in mediating tissue interactions, often leading to activation of gene expression in the adjacent tissue (Thesleff et al., 1995; Chen and Maas, 1998). A number of growth factors are expressed in developing palate or are implicated in normal palate development, such as *Bmp2*, *Bmp4* and *Shh*, that are all expressed in the anterior palatal epithelium (Zhang et al., 2002), making them potential inducers of *Shox2*. However, none of the growth factors that were tested in this study was able to induce *Shox2* expression in the posterior palatal mesenchyme. By contrast, blocking of Bmp activity in the anterior palatal epithelium by the Bmp antagonist Noggin led to a downregulation of *Shox2* expression in the anterior palatal mesenchyme. These results demonstrate a necessary but not sufficient role for Bmp in the induction of *Shox2* expression. *Shox2* induction apparently requires participation of multiple factors.

### *Shox2* encodes an intrinsic regulator of the anterior palatal growth

Altered levels of cell proliferation and/or apoptosis in developing palate often cause abnormal palatal growth, leading to cleft palate formation (Zhang et al., 2002; Ito et al., 2003; Lan et al., 2004; Rice et al., 2004; Alappat et al., 2005). The anterior region of the *Shox2*$^{-/-}$ palatal shelves appears shorter and smaller, indicating an impaired palatal growth. The mutant anterior palatal shelves are obviously too small to make contact after elevation. This retarded growth in the anterior palate may also contribute to the lack of fusion of the secondary palate with the primary palate and the nasal septum. TUNEL analyses of the palatal shelves of *Shox2*$^{-/-}$ embryos at E12.5 reveal an unaltered level of cell apoptosis. However, a high level of apoptosis in the anterior palatal epithelium at E13.5 was detected when *Shox2* expression expands from the palatal mesenchyme to the epithelium, suggesting a likely cell-autonomous regulation of *Shox2* on apoptosis. Deficient cell proliferation in the anterior palatal mesenchyme was also identified. Thus, both altered apoptosis and cell proliferation, confined to the region where *Shox2* is expressed, contribute to the retarded palatal growth in the *Shox2* mutants. Gene expression analyses demonstrate that an unaltered expression of a number of genes known to be crucial for normal palatogenesis, including *Msx1* and its downstream gene *Bmp4*, *Pax9*, *Lhx8*, *Osr2*, *Jag2* and *Tgfb3*, in the *Shox2*$^{-/-}$ palatal shelves, suggesting that these genes do not reside downstream of *Shox2* in palatogenesis. However, *Fgf10* and its receptor *Fgfr2* were found to be ectopically expressed in the anterior palatal mesenchyme of the mutants. It has been demonstrated that Fgf10/Fgfr2 signaling is required for normal cell proliferation and survival in developing palate, and application of exogenous Fgf10 can stimulate cell proliferation in the palatal epithelium (Rice et al., 2004). In contrast to these observations, *Fgf10* and *Fgfr2c* were ectopically activated in the anterior palate of *Shox2* mutants, where defective cell proliferation and apoptosis were also found. In vitro application of Fgf10 protein to the anterior palate could actually inhibit cell proliferation. The opposite results observed in the current study and the studies by Rice et al. (Rice et al., 2004) could be attributed to the different concentrations of Fgf10 protein that was used, or different regions along the AP axis of the palatal shelves to which the protein was applied. It is true that a molecule may have opposite functions at different concentrations or in different developing organs. This can be exemplified by the fact that Bmp4 stimulates cell proliferation in palatal mesenchyme and mandibular mesenchyme but exerts an inhibitory effect on cell proliferation in developing lung (Bellusci et al., 1996; Barlow and Francis-West, 1997; Zhang et al., 2002). Furthermore, Bmp4 was found to activate at a lower concentration but to repress at a higher concentration the expression of *Shh* in the developing tooth germ (Zhang et al., 2000). Several signaling pathways operate and form a signaling network in the regulation of palatogenesis. Ectopic expression *Fgf10* and *Fgfr2c* in the palatal mesenchyme of *Shox2* mutants may disrupt a precisely tuned balance in the signaling network that regulate cell proliferation and survival in the anterior palate, leading to abnormal cell division and apoptosis. As Fgf10 does not signal through Fgfr2c, the ectopically expressed Fgfr2c might mediate signaling from other Fgfs that are expressed in the developing palate. In normal palatogenesis, *Shox2* appears to repress, although not necessarily directly, *Fgf10* and *Fgfr2c* expression in the anterior palatal mesenchyme.

### *Shox2*$^{-/-}$ mice exhibit an unusual type of cleft secondary palate

Genes that are harbored within the human genomic region 3q22-26 were thought to be responsible for blepharophimosis, Cornelia de Lange syndromes and 3q duplication syndrome (Ireland et al., 1991; Aqua et al., 1995; Fryns, 1995; Small et al., 1995; Allanson et al., 1997; Semina et al., 1998). The human *SHOX2* gene is located at this precise site of the human genome, and its mouse orthologue is mapped within the region syntenic to human 3q25-26 (Blaschke et al., 1998; Semina et al., 1998). Because of its chromosomal localization and the consistency of its expression pattern in developing human and mouse embryos with the congenital defects of these syndromes (Blaschke et al., 1998; Semina et al., 1998; Clement-Jones et

al., 2000), *SHOX2* was initially thought to be a potential candidate gene for these syndromes. However, recent studies have linked NIPBL to Cornelia de Lange syndrome and FOXL2 to blepharophimosis (Crisponi et al., 2001; Gillis et al., 2004; Krantz et al., 2004; Tonkin et al., 2004). Thus far, *SHOX2* has not yet been linked to any known syndrome in humans. The lack of an *SHOX* ortholog in mice implies that mouse *Shox2* may play a broader function than human *SHOX2* in embryogenesis. Indeed, *Shox2*$^{-/-}$ mice die prenatally, probably owing to cardiac failure, exhibiting a cleft palate phenotype (this study) and other defects including shortened limbs as is characteristic of the short stature syndromes (L.Y. and Y.P.C., unpublished).

Consistent with the confined *Shox2* expression in the anterior palatal shelves, *Shox2*-deficient mice show an incomplete anterior clefting. Thus, the cleft is limited within the future hard palate, while the future soft palate is unaffected. The unique anterior clefting phenotype in *Shox2*$^{-/-}$ mice is clearly different from those seen in mice carrying a genetically engineered or naturally occurring mutant gene reported previously. It is generally considered that the clefts of the hard palate invariably include soft-palate clefts (Sperber, 2001). In humans cleft hard palate with intact soft-palate is extremely unusual (Schupbach, 1983). This type of cleft was even not classified in the Veau classification system, the earliest widely accepted system that divided cleft anomalies of individuals into four subgroups (Shprintzen, 2002).

Human and rodents share great similarity in palate closure that begins at the earliest point of contact and proceeds in the anterior and posterior directions (Schupbach, 1983). As the initial closure point is located within the anterior third of the shelf, it is generally accepted that palatal closure occurs in an anterior-to-posterior sequence until fusion is complete (Schupbach, 1983; Kaufman and Bard, 1999; Sperber, 2001; Zhang et al., 2002). Clinically, the mildest form of cleft palate is bifid uvula or clefting of posterior soft palate. Increasingly, severe clefts always have posterior involvement and the clefting advances anteriorly in an opposite direction to that of normal palatal fusion (Sperber, 2001). Cleft hard palate with an intact soft-palate was seen in humans but was thought to be caused by a postfusion rupture mechanism (Fara, 1971; Mitts et al., 1981; Schupbach, 1983). The restricted *SHOX2* expression in the human palate makes it a potential candidate gene for this rare type of cleft. In a genetic study using *Msx1* mutant mice, *Msx1* was found to be expressed in the very anterior region of the secondary palatal shelves and controls cell proliferation in the anterior palatal mesenchyme via regulating *Bmp4* expression (Zhang et al., 2002). The *Msx1* mutant palatal shelves elevate to the dorsum of the tongue, but the anterior region of the palatal shelves appears too small to make contact at the midline, leading to complete cleft of secondary palate. Similar complete clefts resulted from a defect in the anterior palate growth were also observed in *Fgf10* mutants (Rice et al., 2004; Alappat et al., 2005). These studies support the anterior-to-posterior closure model and suggest a zipper-like mechanism for palatal closure (Zhang et al., 2002). However, in the present study, we showed that mice lacking *Shox2* have an incomplete clefting within the anterior palate, while the posterior palate, including the soft palate, closes and fuses normally. These results demonstrate that the posterior palate can fuse independently of anterior palate fusion, and call for a revision of the prevailing model on the palatal closure sequence. However, we certainly cannot rule out the possibility that in this particular *Shox2* mutant model increased *Fgf10* expression in the anterior palatal mesenchyme could signal to the posterior palate and lead to posterior fusion. Nevertheless, *Shox2*$^{-/-}$ mice represent a unique model for studying pathogenesis of cleft hard palate.

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