Odd-skipped related 2 (*Osr2*) encodes a key intrinsic regulator of secondary palate growth and morphogenesis

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Summary

Development of the mammalian secondary palate involves multiple steps of highly regulated morphogenetic processes that are frequently disturbed during human development, resulting in the common birth defect of cleft palate. Neither the molecular processes governing normal palatogenesis nor the causes of cleft palate is well understood. In an expression screen to identify new transcription factors regulating palate development, we previously isolated the odd-skipped related 2 (*Osr2*) gene, encoding a zinc-finger protein homologous to the *Drosophila odd-skipped* gene product, and showed that *Osr2* mRNA expression is specifically activated in the nascent palatal mesenchyme at the onset of palatal outgrowth. We report that a targeted null mutation in *Osr2* impairs palatal shelf growth and causes delay in palatal shelf elevation, resulting in cleft palate. Whereas palatal outgrowth initiates normally in the *Osr2* mutant embryos, a significant reduction in palatal mesenchyme proliferation occurs specifically in the medial halves of the downward growing palatal shelves at E13.5, which results in retarded, mediolaterally symmetric palatal shelves before palatal shelf elevation. The developmental timing of palatal growth retardation correlates exactly with the spatiotemporal pattern of *Osr1* gene expression during palate development. Furthermore, we show that the *Osr2* mutants exhibit altered gene expression patterns, including those of *Osr1*, *Pax9* and *Tgfb3*, during palate development. These data identify Osr2 as a key intrinsic regulator of palatal growth and patterning.

Key words: Cleft palate, Odd-skipped, *Osr2*, Palate development

Introduction

The mammalian secondary palate arises from the medial sides of the maxillary processes flanking the embryonic oral cavity as two bilateral outgrowths that initially grow vertically down the sides of the developing tongue. At a precise developmental stage, the bilateral palatal shelves elevate to a horizontal position above the dorsum of the tongue and fuse with each other at the midline to form the intact secondary palate that separates the nasal cavity from the oral cavity (Ferguson, 1988). Any disturbance of the growth, elevation or fusion of the palatal shelves could result in cleft palate, one of the most common birth defects in humans. Considerable efforts have gone into genetic and epidemiological studies of human orofacial clefting (Murray, 2002; Carinci et al., 2003). Several cleft-causing mutations have recently been identified, including mutations in the *IRF6*, *MSX1*, *PVRL1* and *TBX22* genes (van den Boogaard et al., 2000; Suzuki et al., 2000; Braybrook et al., 2001; Sozen et al., 2001; Kondo et al., 2002; Jezewski et al., 2003). In addition, studies of targeted mutations in mice have shown that mutations in a growing number of genes each results in cleft palate in nullizygous mutants (Thyagarajan et al., 2003). Most of these mutant mice also have gross craniofacial developmental defects (e.g. Gendron-Maguire et al., 1993; Rijli et al., 1993; Satokata and Maas, 1994; Martin et al., 1995; Sanford et al., 1997; Peters et al., 1998), indicating that cleft palate is a common secondary effect of other craniofacial abnormalities. Mutations in *Msx1* and *Tgfb3*, which are normally expressed during palate development, have been shown to cause cleft palate as a primary effect, because the gene products normally regulate palatal mesenchyme proliferation and palatal shelf fusion, respectively (Fitzpatrick et al., 1990; Pelton et al., 1990; Satokata and Maas, 1994; Kaartinen et al., 1995; Proetzel et al., 1995; Taya et al., 1999; Zhang et al., 2002).

Despite the large number of genes associated with cleft palate formation, strikingly little is known about the molecular processes governing normal palatal growth and patterning. For example, there is little understanding of what controls the initial phases of palatal outgrowth from the medial sides of the maxillary processes, although this occurs at the developmental time when many drugs are administered to experimentally induce cleft palate in animal models (Salomon and Pratt, 1979; Shah, 1984; Ferguson, 1988; Diehl and Erickson, 1997). In an attempt to identify genes regulating the initial phases of palate development, we previously carried out an expression screen in mouse embryos and showed that the odd-skipped related 2 (*Osr2*) gene is specifically activated in the nascent palatal mesenchyme at the onset of palatal outgrowth (Lan et al., 2001). The *Osr2* gene encodes a zinc-finger protein with extensive sequence similarity to the *Drosophila* Odd-skipped family of putative transcription factors (Lan et al., 2001). The

odd-skipped gene was initially identified as a pair-rule gene because mutations at this locus cause loss of the odd-numbered segments in the *Drosophila* embryo (Nusslein-Volhard and Wieschaus, 1980; Coulter et al., 1990). Gene expression and phenotypic analyses indicate that the *odd-skipped* gene product functions to prevent inappropriate expression of other segmentation genes (Coulter and Wieschaus, 1988; DiNardo and O'Farrell, 1987; Mullen and DiNardo, 1995; Saulier-Le Drean et al., 1998). During mouse embryogenesis, *Osr2* mRNA expression marks the medial maxillary regions where the initial palatal outgrowths occur and persists strongly in the downward growing palatal shelves. Upon palatal shelf elevation and fusion at the midline, *Osr2* expression in the palate is downregulated (Lan et al., 2001). The sequence homology to the Odd-skipped family of transcription factors and the dynamic expression pattern suggest that Osr2 may play an important role in palate development. Interestingly, recent annotation of the human genome database assigned the orthologous human *OSR2* gene to chromosome 8q23, in a chromosomal region strongly associated with non-syndromic orofacial clefting (Prescott et al., 2000). To analyze the function of *Osr2* in palate development, we have generated mutant mice carrying a targeted deletion of the *Osr2* coding region and found that *Osr2* mutants have specific defects in palatal shelf growth and morphogenesis.

Materials and methods

Targeted disruption of the mouse *Osr2* gene

A BAC clone containing the *Osr2* genomic region was isolated from the RPCI-22 129/SvEvTac mouse BAC library (BACPAC Resources, Children's Hospital of Oakland, Oakland, CA). A targeting vector was made with a 9.4 kb *Hin*dIII fragment containing all three coding exons of the *Osr2* gene subcloned from the BAC clone (Fig. 1A). The targeting vector contains a 2 kb 5′ homology arm containing the initial 15 codons of the *Osr2* open reading frame fused in-frame with a modified bacterial *lacZ* gene that encodes a nuclearly localized β-galactosidase, a loxP-flanked *PGK-neo* expression cassette, a 3.3 kb 3′ homology arm and a *PGK-DTA* expression cassette. Correct targeting of the *Osr2* locus with this vector results in the replacement of 2.6 kb of the *Osr2*-coding region with the *lacZ* gene and the neo expression cassette, which is expected to produce a β-galactosidase fusion protein containing the N-terminal 15 amino acid residues of the Osr2 protein.

The targeting vector was linearized and electroporated into CJ7 ES cells as previously described (Swiatek and Gridley, 1993). G418-resistant ES colonies were screened by Southern hybridization for homologous recombination. Two independently targeted ES cell clones were injected into blastocysts from C57BL/6J mice and the resultant chimeras bred with C57BL/6J females. F1 mice were genotyped by Southern hybridization analysis of tail DNA. Mice and embryos from subsequent generations were genotyped by PCR. PCR with primer 1 (5′-GAT ACG GGT AAG ACA GAA ACT G-3′) and primer 2 (5′-CTA CAA GGA TCT AGC ACA TGC TG-3′) amplified a product of 490 bp from the wild-type *Osr2* allele. PCR with primer 2 and primer 3 (5′-CTT CTT GAC GAG TTC TTC TGA GG-3′) amplified a mutant allele-specific product of 460 bp. Heterozygous F1 mice were backcrossed with C57BL/6J mice and N2 heterozygous mice were intercrossed for analysis of homozygous phenotype.

Detection of β-galactosidase and skeletal analysis

X-gal staining of whole-mount embryos and cryostat sections for β-galactosidase detection was performed as described previously (Hogan et al., 1994). Cryostat sections were counterstained with eosin after X-gal staining. Skeletal preparations were made from newborn mice as described previously (Martin et al., 1995).

Histology and in situ hybridization

For histology, embryos were fixed in Bouin's fixative, dehydrated through graded alcohols, embedded in paraffin wax, sectioned at 7 μm and stained with Hematoxylin and Eosin. For in situ hybridization, embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS. In situ hybridization of whole-mount embryos and tissue sections were performed as described previously (Lan et al., 2001).

Detection of cell proliferation and apoptosis

For detection of cell proliferation in the palatal shelves, pregnant *Osr2* heterozygous females were injected intraperitoneally on gestational day 12.5 or 13.5 with BrdU (Roche) labeling reagent (45 μg/g body weight). One hour after injection, embryos were dissected, fixed in Carnoy's fixative, dehydrated through graded alcohols, embedded in paraffin wax and sectioned in the coronal plane at 5 μm. Immunodetection of BrdU was performed using the BrdU labeling and detection kit (Roche) according to manufacturer's instructions and the sections were counterstained with Eosin. The total number of mesenchymal cell nuclei as well as the number of BrdU-labeled mesenchymal nuclei in a fixed area of 0.057 mm^2 beginning at the distal tip and encompassing more than two-thirds of the vertically-oriented palatal shelves were counted using an ocular scale grid. Sections were selected from the middle of the anteroposterior axis of the palatal shelves in comparable positions in the wild-type and mutant embryos and cell counts were recorded for each of the bilateral palatal shelves from five continuous sections of each embryo. The cell proliferation index was calculated as percentage of the cell nuclei with BrdU labeling. Student's *t*-test was used to analyze the significance of difference and a *P* value less than 0.01 was considered statistically significant.

Apoptotic cell death in the palatal shelves was assessed by TUNEL labeling of either paraffin wax or cryostat sections using the in situ cell death detection kit (Roche) following the manufacturer's instructions.

Results

Generation of *Osr2^{tm1Jian}* mutant mice

To examine the function of *Osr2* in palate development, we have generated mice carrying a targeted mutation replacing most of the *Osr2*-coding region with a modified bacterial *lacZ* gene through homologous recombination in ES cells (Fig. 1A). The targeted allele, *Osr2^{tm1Jian}*, is expected to encode a functional β-galactosidase fusion protein containing the N-terminal 15 amino acid residues of the Osr2 protein and to result in loss of *Osr2* function. Correctly targeted ES cell clones were used to generate chimeric animals and germline transmission of the targeted mutation was confirmed by Southern hybridization and PCR analysis of tail DNA samples of the F1 and F2 progeny of the chimeric founders (Fig. 1B,C). Embryos heterozygous for the *Osr2^{tm1Jian}* allele were isolated and stained with X-gal to reveal domains of β-galactosidase activity. As shown in Fig. 2, β-galactosidase staining was restricted to the mesonephros at E9.5 (Fig. 2A). By E10.5, β-galactosidase staining is also detected in the limb buds, in the rostrolateral regions of the mandibular processes, in the mesenchyme posterior to the optic placodes, and in the nascent palatal mesenchyme at the medial sides of the maxillary processes (Fig. 2B,C). At E13.5, β-galactosidase activity was detected throughout the downward growing palatal mesenchyme, with lateral sides of the palatal mesenchyme

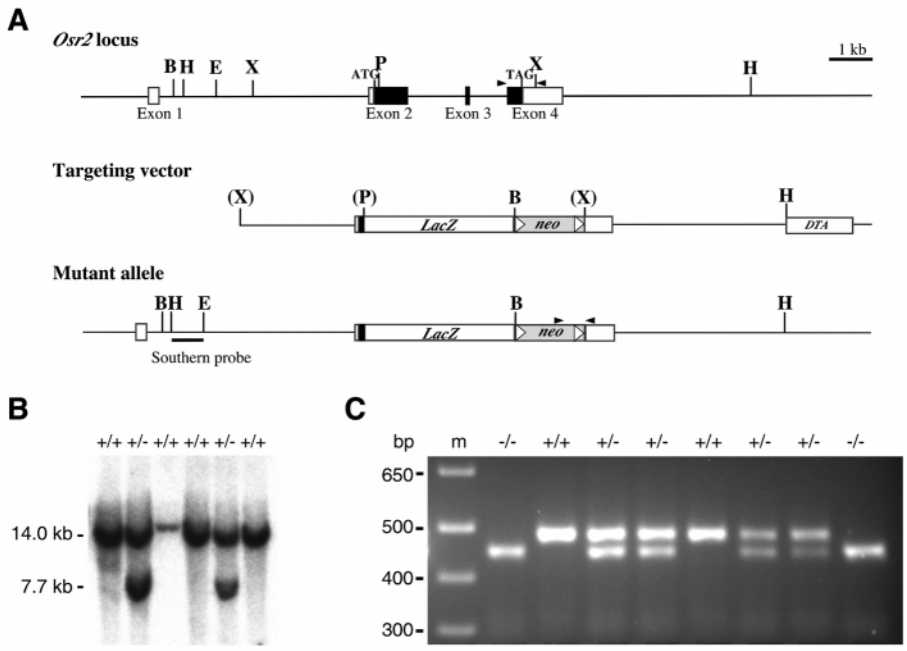

Fig. 1. Targeted disruption of the mouse *Osr2* gene. (A) The *Osr2* gene consists of four exons spanning ~8 kb of genomic DNA. Boxes indicate exons, with the protein-coding region marked in black. The positions of the translation start (ATG) and stop (TAG) codons are also indicated. Restriction sites are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I. The targeting vector used the 2.2 kb *Xba*I-*Pst*I fragment containing the intron 1/exon 2 junction as the 5′ arm and the 3.3 kb *Xba*I-*Hin*dIII fragment 3′ to the *Osr2*-coding region as the 3′ arm. A modified bacterial *lacZ* gene and a *neo* expression cassette were inserted in between the arms and a diphtheria toxin A (*DTA*) expression cassette was cloned 3′ to the 3′ arm for negative selection against random integration. Correct targeting results in the *lacZ* gene and the *neo* cassette replacing most of the *Osr2* coding region, from the sixteenth codon of the open reading frame to the *Xba*I site in the 3′ untranslated region. Arrowheads above the wild-type and mutant genomic schematics indicate the positions of PCR primers used for genotyping. (B) Southern hybridization analysis of tail DNA samples from a litter of F1 progeny of a chimeric male generated with a targeted ES clone. Tail DNA samples were digested with *Bam*HI, separated by electrophoresis through a 1% agarose gel, transferred onto a Zetaprobe nylon membrane (BioRad), and hybridized with random prime-labeled probes made from the 600 bp *Hin*dIII-*Eco*RI fragment isolated from the *Osr2* genomic region 5′ to the targeted region. The 14 kb *Bam*HI fragment corresponding to the wild-type allele was detected in all F1 progeny, while the 7.7 kb mutant allele-specific fragment was detected only in heterozygotes. (C) PCR analysis of tail DNA samples from a litter of newborn F2 progeny. The fragments amplified from wild-type and mutant alleles are 490 bp and 460 bp, respectively. Homozygous mutants were born at the expected Mendelian frequency (25%). m, DNA fragment size markers; +/+, wild type; +/–, heterozygote; –/–, homozygote.

showing higher β-galactosidase activity than the medial sides (Fig. 2D), which is similar to the *Osr2* mRNA expression pattern reported previously (Lan et al., 2001). At E14.75, the palatal shelves have elevated and β-galactosidase expression is downregulated in the palatal mesenchyme, while it is strongly expressed in the tooth bud mesenchyme, the olfactory mesenchyme, the periocular mesenchyme and the eyelids (Fig. 2E). These data indicate that expression of the β-galactosidase reporter fusion protein recapitulates the endogenous *Osr2* expression pattern reported previously (Lan et al., 2001), further confirming correct integration of the *lacZ* gene into the *Osr2* locus.

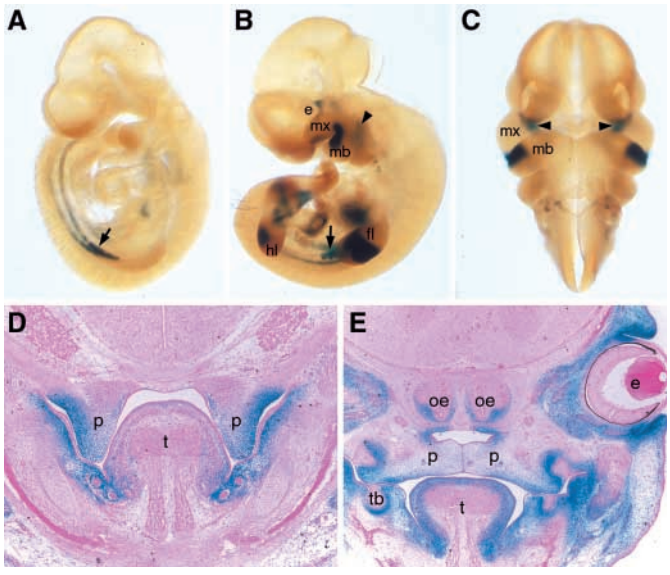

Fig. 2. Expression of β-galactosidase in *Osr2* heterozygous embryos. (A) At E9.5, β-galactosidase expression (blue) was detected specifically in the mesonephric vesicles (arrow). (B) At E10.5, β-galactosidase activity was detected in the mesonephros (arrow), in the limb buds, in the maxillary and mandibular processes, in the mesenchyme posterior to the eye and in the mesenchyme adjacent to the first branchial cleft (arrowhead). (C) Facial view of a stained E10.5 embryo showing β-galactosidase expression in the palatal primordia (arrowheads). (D,E) Frontal sections of E13.5 (D) and E14.75 (E) heterozygous embryos showing β-galactosidase expression in the palatal mesenchyme, olfactory mesenchyme, tooth bud mesenchyme, and the periocular mesenchyme and the eyelids. e, eye; fl, forelimb bud; hl, hindlimb bud; mb, mandibular process; mx, maxillary process; oe, olfactory epithelia; p, palatal shelf, t, tongue; tb, tooth bud.

Osr2$^{tm1Jian/tm1Jian}$ homozygous mutants die at birth with open eyelids and cleft palate

Mice heterozygous for the *Osr2*^{tm1Jian} allele are normal and fertile. Heterozygous animals were intercrossed and genotypes of their progeny were determined 2 weeks after birth. No mice homozygous for the mutation were found. Careful examination of staged embryos and newborn mice from heterozygous intercrosses revealed that the *Osr2*$^{tm1Jian/tm1Jian}$ homozygous mutants could complete embryogenesis but died within 24 hours after birth. All homozygous neonates exhibited open eyelids and bilateral cleft of the secondary palate (Fig. 3B,D),

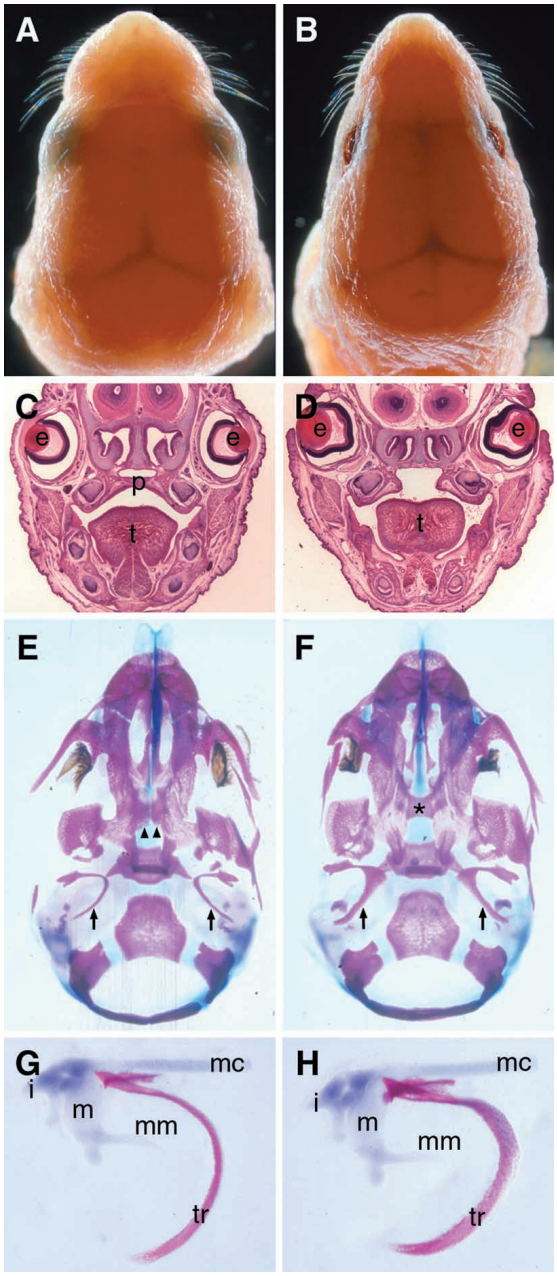

Fig. 3. *Osr2*$^{tm1Jian/tm1Jian}$ mutant mice are born with open eyelids and cleft palate. (A,B) Dorsal view of heterozygous (A) and homozygous (B) mutant newborn heads. (C,D) Frontal sections of heterozygous (C) and homozygous (D) newborn mutant heads. (E,F) Ventral view of stained skeletal preparations of heterozygous (E) and homozygous (F) mutant neonatal skulls. Arrowheads indicate palatal processes of the palatine bones that have fused to each other in the heterozygous mouse (E) but are absent in the homozygous mutant, exposing the presphenoid bone (marked with an asterisk) underneath (F). The tympanic rings are significantly thicker in the homozygous mutant than in the heterozygous mouse (arrows). (G,H) Comparison of tympanic rings with associated middle ear ossicles and the Meckel's cartilage dissected from wild-type (G) and homozygous mutant (H). Whereas the mutant tympanic ring is significantly thicker than that of the wild type, the associated Meckel's cartilage and middle ear ossicles are similar in size in wild-type and mutant newborns. e, eye; i, incus; m, malleus; mc, Meckel's cartilage; mm, manubrium of the malleus; p, palate; t, tongue; tr, tympanic ring.

which correlate with *Osr2* mRNA expression during normal eyelid and palate development (Lan et al., 2001). As *Osr2* also exhibits a dynamic expression pattern during kidney and limb development (Lan et al., 2001), we carefully examined development of these structures by histological and skeletal preparations, but did not find any abnormalities in them (data not shown). In addition to open eyelids and cleft palate, another abnormality observed in the homozygous mutants is that they have thickened tympanic rings (Fig. 3F), which correlates with *Osr2* expression in the mesenchymal cells that give rise to the tympanic rings proximal to the first branchial clefts (Fig. 2B). Other craniofacial structures, including the middle ear ossicles that develop from proximal first arch mesenchyme adjacent to the tympanic rings, are unaffected by the *Osr2*^{tm1Jian} mutation (Fig. 3G,H).

Osr2$^{tm1Jian/tm1Jian}$ mutants exhibit impaired palatal shelf growth

To investigate which palatal developmental processes require *Osr2* gene function, we carried out histological analyses of embryos throughout palate development from E12 to birth. As shown in Fig. 4, palatal outgrowth and initial downward palatal growth were normal in the homozygous mutants (Fig. 4A,B). Between E13.5 and E14.5, the vertically oriented palatal shelves undergo rapid growth and initiate reorientation to a horizontal position above the dorsum of the tongue in the wild-type and heterozygous embryos (Fig. 4C,E). By contrast, the *Osr2*$^{tm1Jian/tm1Jian}$ homozygous mutant littermates exhibit retarded palatal shelves that remain vertically oriented at this stage (Fig. 4D,F). Palatal shelf retardation is observed throughout the anteroposterior axis of the palatal shelves in all homozygous mutants examined at E14.5 (data not shown). By E15.5, the elevated palatal shelves have initiated fusion at the midline in wild-type and heterozygous embryos, whereas the homozygous mutant palatal shelves are elevated but failed to contact each other at the midline (Fig. 4G,H). As the palatal fusion process continues to generate intact secondary palates in wild-type and heterozygous embryos (Fig. 4I), the homozygous mutant palatal shelves are further separated from each other (Fig. 4J), resulting in bilateral cleft of the secondary palate at birth (Fig. 3D).

To investigate whether palatal shelf retardation in the *Osr2*$^{tm1Jian/tm1Jian}$ mutant embryos was due to impaired cell proliferation, we analyzed BrdU incorporation in E12.5 and E13.5 embryos. No difference in the cell proliferation index between wild-type and mutant palatal shelves was observed in E12.5 embryos (data not shown). However, whereas the wild-type and heterozygous embryos showed no significant differences in palatal cell proliferation at E13.5, the homozygous mutants exhibited a 26% reduction (P<0.01) in cell proliferation index in the palatal mesenchyme (Fig. 5C,D). The wild-type and heterozygous palatal shelves exhibit faster growth in the medial halves than the lateral halves (Fig. 5A,B,D), which is consistent with previous reports and results in the characteristic erectile shape of the palatal shelves before elevation (Ferguson, 1988). Remarkably, when the percentage of BrdU-labeled cells was recorded separately for the medial and lateral halves of the palatal shelves, there was a 37% reduction (P<0.01) in the cell proliferation index in the medial halves of homozygous mutant palatal shelves, while the cell proliferation index was not significantly different (P>0.05)

in the lateral halves of the palatal shelves in wild-type, heterozygous and homozygous mutant embryos (Fig. 5D). This preferential reduction in cell proliferation in the medial halves of the palatal shelves results in the appearance of retarded, mediolaterally symmetric palatal shelves in the homozygous mutants at E14.5 (Fig. 4D). We also examined whether abnormal cell death contributed to the palatal shelf retardation by in situ TUNEL assays, but did not find any differences in cell death between wild-type and homozygous mutant palatal shelves (data not shown).

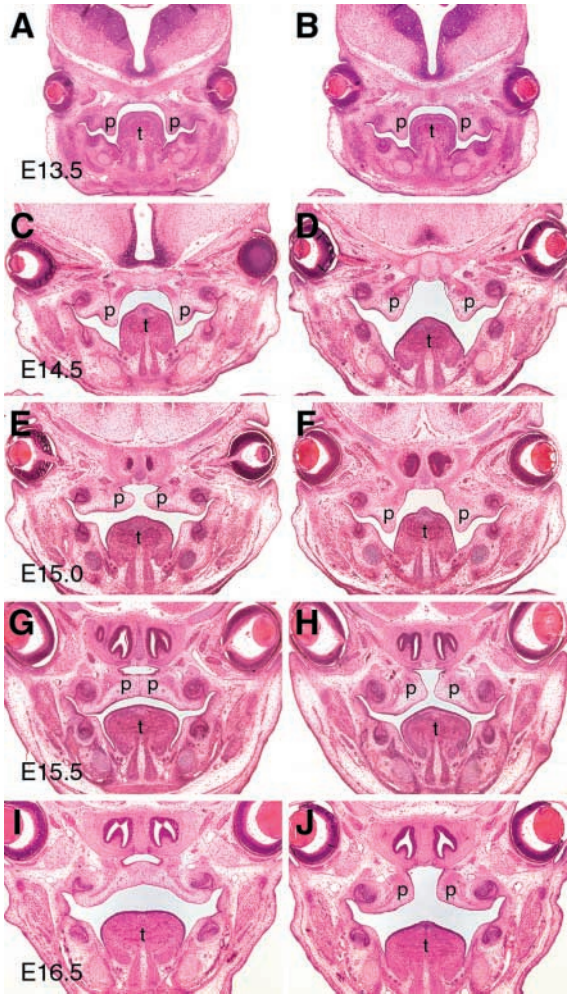

Fig. 4. Histological analysis of palate development in *Osr2*$^{tm1Jian/tm1Jian}$ mutants. (A,B) At E13.5, wild-type (A) and *Osr2*$^{tm1Jian/tm1Jian}$ homozygous mutant (B) embryos exhibited similar palatal shelf size and shape. (C,D) At E14.5, palatal shelves appeared retarded in the homozygous mutant (D) compared with the heterozygous littermate (C). (E,F) At E15.0, the heterozygous mutant (E) palatal shelves had elevated to the horizontal position above the tongue, while the homozygous mutant (F) palatal shelves were still vertically oriented. (G,H) At E15.5, the heterozygous mutant palatal shelves had made contact and initiated fusion at the midline, but the palatal shelves remained separated from each other in the homozygous mutant littermate (H). (I,J) At E16.5, the heterozygous mutant (I) palatal shelves had completed fusion, but the homozygous mutant (J) palatal shelves were retarded and separate from each other. p, palatal shelf, t, tongue.

The palatal mesenchyme proliferation defect in *Osr2*$^{tm1Jian/tm1Jian}$ mutants correlates with the spatiotemporal pattern of *Osr1* mRNA expression

As *Osr2* mRNA expression is activated at the onset of palatal outgrowth and persists throughout the downward growing palatal shelves during mouse embryogenesis (Lan et al., 2001), the normal initiation of palatal outgrowth and preferential reduction in cell proliferation in the medial halves of the palatal shelves in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants are surprising observations. One possible explanation for the mutant phenotype is that loss of *Osr2* function in the initial stage of palatal outgrowth is compensated by expression of a related gene product. The *Osr1* gene encodes a protein with 65% overall amino acid sequence identity and 98% sequence identity in the zinc-finger domain with the Osr2 protein (So and Danielian, 1999; Lan et al., 2001). No other *odd-skipped* related gene has been found in the mouse or human genomes. Thus, we examined *Osr1* mRNA expression and compared that with the expression pattern of *Osr2* mRNA during palate development. We also compared the expression patterns of *Osr1* and *Osr2* with that of *Pax9*, which encodes a paired-class homeodomain-containing transcription factor required for proper palatal patterning (Peters et al., 1998). From E12.5 to E13.5, *Osr2* mRNA is expressed abundantly throughout the palatal mesenchyme, with lateral regions expressing higher levels than the medial regions (Fig. 6A-C). *Osr1* mRNA is strongly expressed in the developing tongue and at the maxillary-mandibular junction but is only weakly expressed in the palatal mesenchyme at E12.5 and E13.0 (Fig. 6D,E). By E13.5, *Osr1* mRNA expression is significantly up-regulated in the lateral halves and is completely downregulated in the medial halves of the palatal shelves, forming a sharp boundary in the middle of the palatal shelves (Fig. 6F). *Pax9* mRNA is expressed in a pattern similar to that of *Osr2* mRNA in the palatal mesenchyme at E12.5 and E13.0 (Fig. 6G,H). However, at E13.5, *Pax9* mRNA expression is upregulated in the mediodistal regions and downregulated in the lateral regions of the palatal shelves (Fig. 6I). These data indicate that dynamic molecular changes occur between E13.0 and E13.5 during palate development. Interestingly, the defect in palate development in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants is first detectable at this stage and the specific reduction in cell proliferation in the mutant medial palatal mesenchyme correlates with the spatiotemporal downregulation of *Osr1* mRNA expression in those cells.

Defects in palatal shelf molecular patterning and elevation in *Osr2*$^{tm1Jian/tm1Jian}$ mutants

To investigate the role of *Osr2* in palate development further, we examined the expression patterns of several genes implicated in regulating palatal growth and/or patterning. *Msx1*, a homeobox gene required for anterior palate mesenchyme proliferation, is expressed weakly in the anterior but not posterior regions of the palatal mesenchyme and strongly in maxillary and mandibular mesenchyme at E14.5 (Zhang et al., 2002). No differences in *Msx1* expression were found between wild-type and *Osr2*$^{tm1Jian/tm1Jian}$ mutants, although the palatal shelves are retarded in the anterior region in the homozygous mutants (Fig. 7A,B). *Bmp4* has recently been implicated in regulating palate mesenchyme growth because a *Bmp4* transgene driven by the *Msx1* promoter was

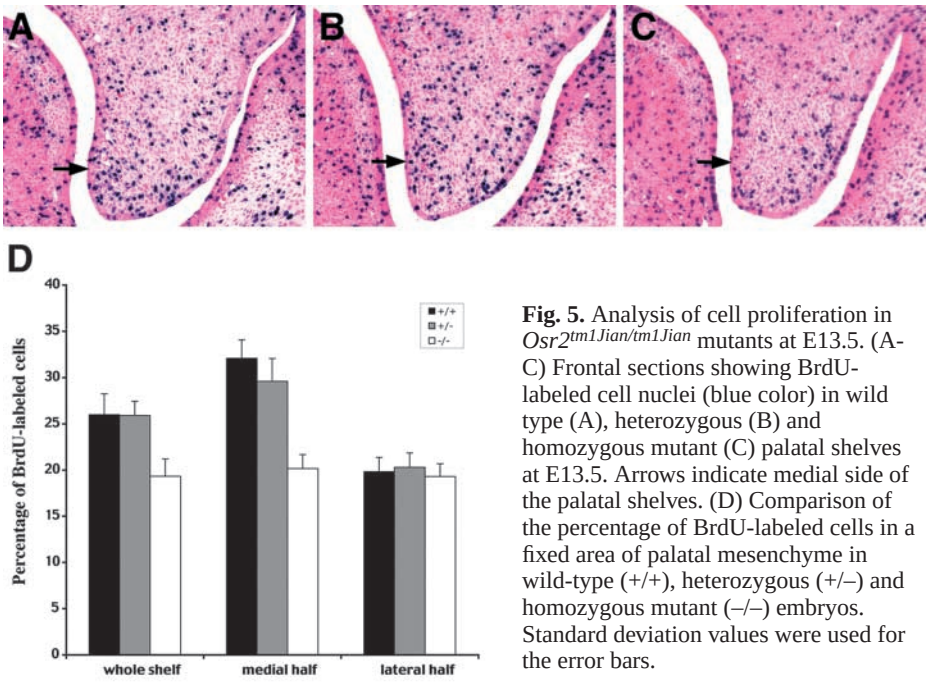

Fig. 5. Analysis of cell proliferation in *Osr2*$^{tm1Jian/tm1Jian}$ mutants at E13.5. (A-C) Frontal sections showing BrdU-labeled cell nuclei (blue color) in wild type (A), heterozygous (B) and homozygous mutant (C) palatal shelves at E13.5. Arrows indicate medial side of the palatal shelves. (D) Comparison of the percentage of BrdU-labeled cells in a fixed area of palatal mesenchyme in wild-type (+/+), heterozygous (+/–) and homozygous mutant (–/–) embryos. Standard deviation values were used for the error bars.

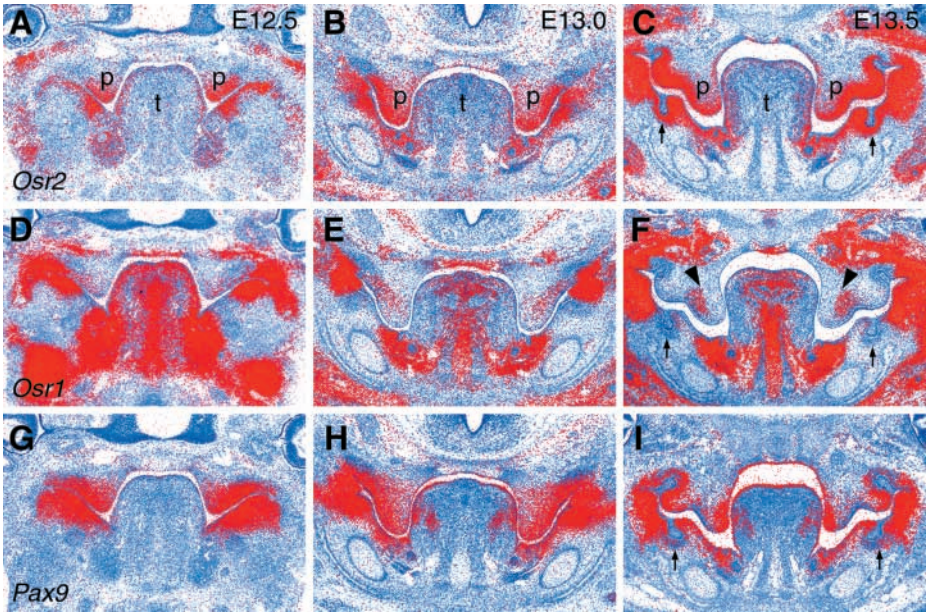

Fig. 6. Comparison of the patterns of *Osr2*, *Osr1* and *Pax9* mRNA expression during palate development. mRNA signals are shown in red in all panels. (A-C) *Osr2* mRNA is abundantly expressed throughout the developing palatal shelves from E12.5 to E13.5. *Osr2* mRNA is also highly expressed in the developing tooth bud mesenchyme at E13.5 (arrows in C). (D-F) *Osr1* mRNA expression is weak in the developing palatal shelves at E12.5 (D) and E13.0 (E) but it is highly abundant in the developing tongue and several regions of the mandible. By E13.5, the lateral halves of the palatal shelves express moderate levels of *Osr1* mRNA, while the medial halves of the palatal shelves completely lack *Osr1* mRNA expression (F). Arrowheads indicate the sharp boundaries between the lateral *Osr1*-expressing and the medial *Osr1*-nonexpressing palatal mesenchyme cells. In contrast to *Osr2*, *Osr1* is not expressed in the developing tooth bud mesenchyme (arrows in F). (G-I) *Pax9* mRNA exhibits a lateral to medial expression gradient in the downward growing palatal shelves at E12.5 (G) and E13.0 (H), with higher levels in the lateral regions. By E13.5, *Pax9* expression is upregulated in the medial regions of the palatal shelves and the *Pax9* mRNA gradient is reversed, with higher levels in the medial regions of the palatal shelves (I). *Pax9* mRNA is also expressed in the tooth bud mesenchyme at E13.5 (arrows in I). p, palatal shelf; t, tongue.

able to rescue palate growth defects in the *Msx1*$^{-/-}$ mutant mice (Zhang et al., 2002). However, *Bmp4* expression appears unaltered in the *Osr2*$^{tm1Jian/tm1Jian}$ mutant palatal shelves compared with the wild-type embryos (Fig. 7C,D). Expression of *Tbx22*, the mouse ortholog of the human X-linked cleft palate gene (Braybrook et al., 2002; Bush et al., 2002; Herr et al., 2003), is also unaltered in the mutant palatal shelves (Fig. 7E,F). *Pax9*, encoding a paired-class transcription factor required for proper palate patterning (Peters et al., 1998), exhibits a dynamic expression pattern during palate development (Fig. 6G-I). In the *Osr2*$^{tm1Jian/tm1Jian}$ mutants, *Pax9* expression is similar to that in wild-type embryos during early palatal outgrowth (data not shown). However, by E13.5 when the wild-type palatal shelves showed a strong mediolateral gradient of *Pax9* mRNA expression, the *Osr2*$^{tm1Jian/tm1Jian}$ mutants exhibited a uniform lower level of *Pax9* mRNA expression in the palatal mesenchyme (Fig. 8A,B). By E14.5, when strong *Pax9* expression is observed in the palatal shelves in wild-type littermates (Fig. 8C), significantly reduced levels of *Pax9* mRNA are found throughout the retarded palatal shelves in *Osr2*$^{tm1Jian/tm1Jian}$ mutants (Fig. 8D). The alteration in *Pax9* mRNA expression in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants is specific to the palatal mesenchyme, as *Pax9* mRNA levels in the tooth bud mesenchyme and in other craniofacial regions are similar in wild-type and mutant embryos (Fig. 8A-D). *Tgfb3*, which encodes a growth factor required for normal palatal shelf fusion (Kaartinen et al., 1995; Proetzel et al., 1995), is expressed in the medial and distal epithelia of the vertically oriented palatal shelves at E14.5 in wild-type embryos (Fig. 8E). In the homozygous *Osr2*$^{tm1Jian/tm1Jian}$ mutants, *Tgfb3* expression in the distal palatal epithelia is lost (Fig. 8F). Complementary to the *Tgfb3* expression pattern in the epithelia, *Osr1* is expressed in the mesenchyme of the proximolateral one-third of the palatal shelves in wild-type embryos at E14.5 (Fig. 8G). In the homozygous mutant palatal shelves, the *Osr1* expression domain extends to the distal tip of the palatal mesenchyme (Fig. 8H), most probably as a result

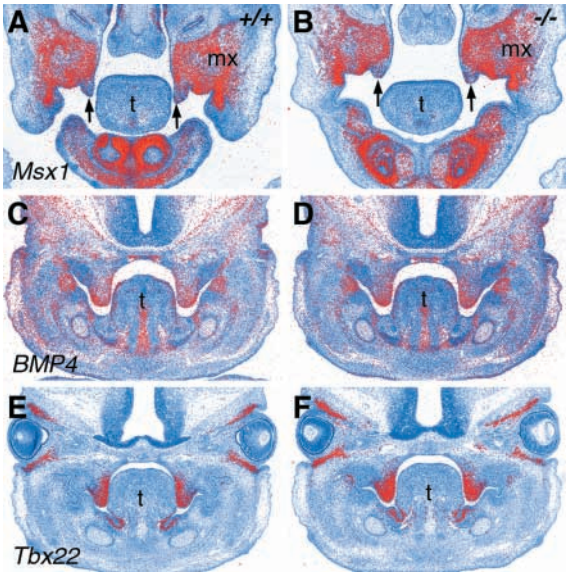

Fig. 7. Expression of *Msx1*, *Bmp4* and *Tbx22* in the developing palatal shelves is not altered in the *Osr2*$^{tm1Jian/tm1Jian}$ homozygous mutant embryos. (A,B) At E14.5, *Msx1* mRNA is abundantly expressed in medial maxillary processes and the tooth mesenchyme and weakly expressed in the anterior palatal shelves (arrows) in both wild-type (A) and the homozygous (B) mutant embryos. Note the palatal shelves are retarded in the homozygous mutant (arrows in B). (C,D) At E13.5, *Bmp4* mRNA is expressed at comparable levels in wild-type (C) and the homozygous mutant (D) palatal shelves. (E,F) At E13.5, *Tbx22* mRNA is abundantly expressed in the palatal mesenchyme and the base of the tongue in both wild-type (E) and the homozygous mutant (F) embryos. *Tbx22* is also expressed similarly in the periocular mesenchyme in wild-type and the mutant embryos. mx, maxillary process; t, tongue.

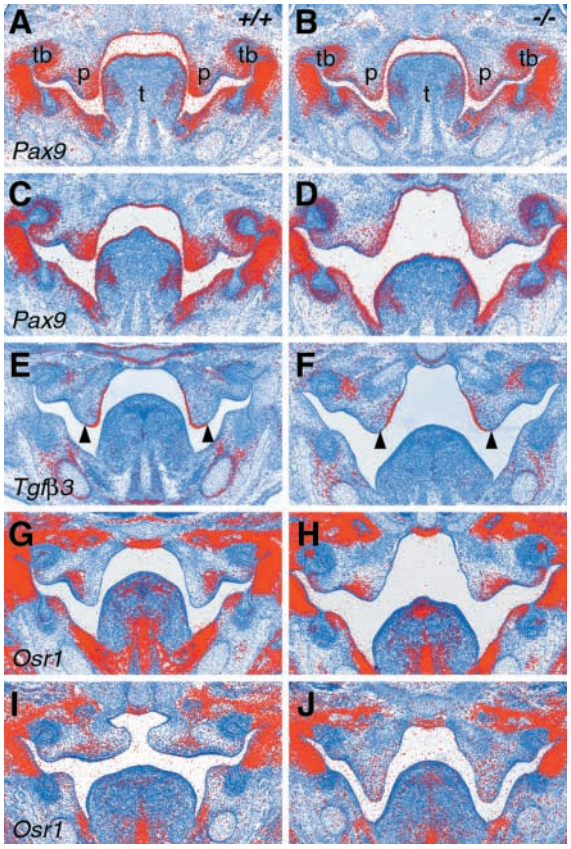

Fig. 8. Expression of *Pax9*, *Tgfb3* and *Osr1* is altered in the *Osr2*$^{tm1Jian/tm1Jian}$ mutant palatal shelves. (A,B) *Pax9* mRNA expression in wild-type (A) and mutant (B) embryos at E13.5. *Pax9* mRNA exhibits a mediolateral gradient in the wild-type palatal mesenchyme at this stage, with higher levels in the medial palatal mesenchyme (A). By contrast, uniform lower levels of *Pax9* mRNA expression are observed in the mutant palatal mesenchyme (B), although similar levels of *Pax9* mRNA are observed in the tooth bud mesenchyme in wild-type and mutant embryos. (C,D) At E14.5, *Pax9* mRNA in the palatal mesenchyme is significantly reduced in the mutant (D) compared with that in the wild-type littermate (C). *Pax9* mRNA levels in the tooth bud mesenchyme remains similar in wild-type and mutant embryos. (E,F) *Tgfb3* mRNA is abundantly expressed in the medial and distal regions of the palatal epithelium in wild-type embryos (E), but its expression domain is shifted medially in the mutant palatal epithelium (F) at E14.5. Arrowheads indicate the boundaries between *Tgfb3*-expressing and *Tgfb3*-nonexpressing cells in the distal palatal regions. (G,H) *Osr1* mRNA expression in wild-type (G) and the mutant (H) palatal shelves at E14.5. (I,J) *Osr1* mRNA expression in wild-type (I) and the mutant (J) palatal shelves at E15.0. p, palatal shelf; t, tongue; tb, tooth bud.

of the severe reduction in medial palatal mesenchyme proliferation. By E15.0, the palatal shelves have elevated to the horizontal position above the tongue and the *Osr1* expression domain is still restricted in the same regions of the palatal mesenchyme in the wild-type and heterozygous embryos (Fig. 8I). By contrast, the homozygous mutant littermates exhibit a low level of *Osr1* expression throughout the retarded, still vertically oriented palatal shelves (Fig. 8J). These data indicate, in addition to the role in palatal shelf growth, that Osr2 function is required for the normal mediolateral patterning and elevation of the palatal shelves.

Discussion

Osr2 is an intrinsic regulator of secondary palate development

Cleft palate is among the most common birth defects in humans and has been observed in a number of mice carrying mutations in genes encoding transcription factors (Gendron-Maguire et al., 1993; Rijli et al., 1993; Satokata and Maas, 1994; Martin et al., 1995; Qiu et al., 1997; De Felice et al., 1998; Peters et al., 1998; Zhao et al., 1999), growth factors and their receptors (Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997; Miettinen et al., 1999), other signaling molecules (Matzuk et al., 1995; Jiang et al., 1998), and extracellular matrix molecules (Pace et al., 1997; Lavrin et al., 2001). Whereas the cleft palate phenotypes at birth may appear similar, the underlying mechanisms in cleft pathogenesis are very different. Palate development occurs at the time of significant growth and morphogenesis of the entire craniofacial complex and is dependent on normal development of other craniofacial structures (Ferguson, 1988). The cleft palate phenotype observed in many mutant mice, such as those with mutations in *Dlx1*, *Dlx2*, *Hoxa2*, *Mhox* (*Prrx1* – Mouse Genome Informatics) and *δEF1* (*Zfhx1a* – Mouse Genome

Informatics) genes (Gendron-Maguire et al., 1993; Rijli et al., 1993; Martin et al., 1995; Qiu et al., 1997; Takagi et al., 1998), is accompanied by gross craniofacial and skeletal malformations and can be attributed, at least partly, to secondary effects of those malformations. Several mutations cause mechanical hindrance to palatal shelf elevation. For example, the palatal shelves fuse aberrantly to the lateral sides of the tongue in the *Jag2*$^{-/-}$ mutant mice and the tongue fails to descend during palatal shelf elevation in the *Foxf2*$^{-/-}$ mutant mice, causing cleft palate (Jiang et al., 1998; Wang et al., 2003). Another class of mutations specifically interferes with palatal shelf fusion. For example, *Titf2* and *Tgfb3* are normally expressed in palatal epithelium and mutations in these genes specifically disrupt palatal shelf fusion (Kaartinen et al., 1995; Proetzel et al., 1995; De Felice et al., 1998).

The cleft palate phenotype in the *Osr2*$^{tm1Jian/tm1Jian}$ mutant mice is clearly different from those of all previously reported mutant mice. All *Osr2*$^{tm1Jian/tm1Jian}$ homozygous mutants examined after E15.5 exhibited cleft palate. In addition to cleft palate, *Osr2*$^{tm1Jian/tm1Jian}$ mutants exhibited open eyelids and thickened tympanic rings, which correlated with *Osr2* mRNA expression during normal development of these structures. Open eyelids have been observed in several mouse mutant strains without cleft palate (Keeler, 1935; Bennett and Gresham, 1956; Jeriloff et al., 2000), indicating that it does not cause cleft palate. The thickened tympanic ring phenotype is unique to the *Osr2*$^{tm1Jian/tm1Jian}$ mutants, but it most probably reflects a developmental role for Osr2 that is independent from that in palate development as other craniofacial structures immediately adjacent to the tympanic rings are unaffected by the mutation. Moreover, *Osr2* mRNA is normally expressed during palatal outgrowth and *Osr2*$^{tm1Jian/tm1Jian}$ mutants exhibited specific impairment of palatal mesenchymal cell proliferation, which resulted in retarded, mediolaterally symmetric palatal shelves before palatal shelf elevation. Thus, whereas Osr2 is required for the normal development of several craniofacial structures, the cleft palate phenotype of the *Osr2*$^{tm1Jian/tm1Jian}$ mutants resulted from a primary defect in palatal shelf growth.

Impairment of palatal mesenchyme cell proliferation has been reported previously in the *Msx1*$^{-/-}$ mutant mice and recently in mice with neural crest-specific inactivation of the *Tgfbr2* gene (Zhang et al., 2002; Ito et al., 2003). *Msx1* is strongly expressed in the maxillary processes and tooth buds, but only very weakly expressed in the anterior region of the palatal shelves (Zhang et al., 2002). Reduction in cell proliferation was found in the anterior but not in the posterior regions of the palatal shelves in *Msx1*$^{-/-}$ mutants (Zhang et al., 2002). In mice with neural crest-specific inactivation of the *Tgfbr2* gene, palatal shelves grow normally up to E13.5 but palatal mesenchyme growth is reduced after palatal shelf elevation at E14.5 (Ito et al., 2003). These mutant mice also exhibited gross craniofacial skeletal growth defects (Ito et al., 2003), consistent with expression of *Tgfbr2* mRNA in migrating cranial neural crest cells (Wang et al., 1995). By contrast, *Osr2* mRNA expression is specifically activated in the nascent palatal mesenchyme at the onset of palatal outgrowth and persists strongly in the palatal mesenchyme during the downward palatal growth (Lan et al., 2001). Palatal growth retardation is observed throughout the anteroposterior axis of the palatal shelves before palatal shelf elevation in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants. Therefore, *Osr2* expression is a specific marker of the early palatal mesenchyme and is essential for the rapid downward growth of the palatal shelves before elevation.

In the *Msx1*$^{-/-}$ mutant mice, impairment of mesenchyme growth in the anterior palate was shown to be due to lack of *Bmp4* expression and transgenic expression of *Bmp4* under the control of the *Msx1* promoter was able to restore anterior palatal mesenchyme proliferation in the *Msx1*$^{-/-}$ mutant mice (Zhang et al., 2002). We found that *Msx1* and *Bmp4* are expressed normally in the developing palatal shelves in *Osr2*$^{tm1Jian/tm1Jian}$ mutant mice (Fig. 7). Furthermore, we observed no differences in *Shh* and *Bmp2* expression, which are believed to function downstream of the Msx1-BMP4 pathway in the palatal shelves (Zhang et al., 2002), between wild-type and *Osr2*$^{tm1Jian/tm1Jian}$ mutant palatal shelves (data not shown). The differences in phenotype and molecular marker expression patterns in *Msx1*$^{-/-}$ and *Osr2*$^{tm1Jian/tm1Jian}$ mutants indicate that Osr2 and Msx1 function in distinct molecular pathways to regulate palate development. The fact that impairment of palatal mesenchyme proliferation occurs earlier in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants than in mice lacking *Tgfbr2* in the neural crest derivatives suggests that Osr2 function in the palatal mesenchyme is also independent of Tgfβ signaling. The identification of Osr2 as an essential regulator of palatal mesenchyme growth warrants future investigation of how *Osr2* expression is activated in the nascent palatal mesenchyme and how it controls palatal mesenchyme proliferation. Interestingly, the human *OSR2* gene is located at chromosome 8q23, in a region with strong association to non-syndromic orofacial clefting (Prescott et al., 2000), suggesting that *OSR2* is a new candidate gene for human cleft palate formation.

The Osr genes and palatal patterning

Whereas the *Osr2* gene is activated at the onset of palatal outgrowth and is expressed throughout the downward growing palatal mesenchyme, the *Osr1* gene exhibits a unique, mediolaterally differentially regulated pattern of expression during palate development. During early palatal outgrowth, *Osr1* mRNA is expressed weakly throughout the palatal mesenchyme. By E13.5 of mouse development, *Osr1* mRNA expression is strongly upregulated in the lateral halves and completely downregulated in the medial halves of the palatal shelves (Fig. 6D-F). As the mediolateral pattern is laid down, *Pax9* mRNA expression is upregulated in the medial palatal mesenchyme (Fig. 6I). In the *Osr2*$^{tm1Jian/tm1Jian}$ mutants, early *Osr1* and *Pax9* expression patterns are unaltered during palatal outgrowth (data not shown). However, by E13.5, the upregulation of *Pax9* mRNA expression seen in the medial regions of the wild-type palatal shelves is not observed in the mutants (Fig. 8B), indicating a molecular patterning defect in the mutant palatal mesenchyme. At the same developmental stage, a significant reduction in BrdU incorporation is observed specifically in the medial palatal mesenchyme in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants (Fig. 5). Although mice deficient in the *Pax9* gene have cleft palate (Peters et al., 1998), it is not known whether Pax9 directly regulates palatal mesenchyme proliferation. Thus, it remains to be investigated whether the reduction in *Pax9* mRNA expression in the medial palatal mesenchyme is the cause of the region-specific reduction in palatal cell proliferation in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants.

Nevertheless, these data indicate that Osr2 plays an important role in the mediolateral patterning of the palatal mesenchyme. The differential regulation of *Osr1* mRNA expression in the medial and lateral halves of the palatal shelves suggests that Osr1 may also have a role in mediolateral palatal patterning.

In addition to alterations in gene expression in the palatal mesenchyme, we observed alteration of *Tgfb3* mRNA expression in the palatal epithelium at E14.5 in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants. Interestingly, the epithelial *Tgfb3* expression domain complements that of *Osr1* mRNA expression in the palatal mesenchyme in both wild-type and the *Osr2*$^{tm1Jian/tm1Jian}$ mutant embryos (Fig. 8E-H). Because the *Osr1* and *Tgfb3* mRNAs are expressed normally up to E13.5 in the *Osr2*$^{tm1Jian/tm1Jian}$ mutants (data not shown), the alterations in their expression in the palatal shelves at E14.5 are most probably secondary effects of the reduction in medial palatal mesenchyme proliferation. However, the corresponding shift in epithelial *Tgfb3* and mesenchymal *Osr1* expression domains suggest that *Osr1* expression in the palatal mesenchyme may regulate mesenchymal-epithelial interactions that in turn regulate *Tgfb3* expression in the palatal epithelium. Further characterization of the molecular pathways through which Osr2 and Osr1 regulate palate development will provide novel insights into the molecular mechanisms governing palate growth and patterning.

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