Development 139, 828 (2012) doi:10.1242/dev.079152

Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development

Jeffrey O. Bush and Rulang Jiang

There were errors published in *Development* **139**, 231-243.

On page 238, the citation to Jin and Ding (2006a) should be to Jin and Ding (2006b). The incorrect reference was listed for Pauws et al. (2009). The correct reference is given below.

The authors apologise to readers for these mistakes.

Pauws, E., Hoshino, A., Bentley, L., Prajapati, S., Keller, C., Hammond, P., Martinez-Barbera, J. P., Moore, G. E. and Stanier, P. (2009). *Tbx22*^{null} mice have a submucous cleft palate due to reduced palatal bone formation and also display ankyloglossia and choanal atresia phenotypes. *Hum. Mol. Genet.* **18**, 4171-4179.

Development 139, 231-243 (2012) doi:10.1242/dev.067082

Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development

Jeffrey O. Bush[1,*] and Rulang Jiang[2,*]

Summary

Mammalian palatogenesis is a highly regulated morphogenetic process during which the embryonic primary and secondary palatal shelves develop as outgrowths from the medial nasal and maxillary prominences, respectively, remodel and fuse to form the intact roof of the oral cavity. The complexity of control of palatogenesis is reflected by the common occurrence of cleft palate in humans. Although the embryology of the palate has long been studied, the past decade has brought substantial new knowledge of the genetic control of secondary palate development. Here, we review major advances in the understanding of the morphogenetic and molecular mechanisms controlling palatal shelf growth, elevation, adhesion and fusion, and palatal bone formation.

Key words: Cleft palate, Morphogenesis, Palate development, Secondary palate, Palatogenesis

Introduction

In mammals, the palate separates the oral from the nasal cavity and consists anteriorly of the bony hard palate and posteriorly of the muscular soft palate. The hard palate is crucial for normal feeding and speech, whereas the soft palate is movable and closes off the nasal airway during swallowing. Palatogenesis, which is the developmental process that generates the intact palate, is often disrupted by genetic and environmental perturbations, as reflected in the high frequency of cleft palate, a major congenital anomaly in humans that requires surgical intervention shortly after birth and has significant long-term health implications for affected individuals. Several decades of extensive epidemiological and human genetic studies have begun to reveal the genetic and environmental factors that underlie the etiology of cleft palate. In recent years, there has been an explosion of new knowledge about the genetic pathways coordinating palatogenesis. Much of this progress is due to the widespread application of sophisticated genetic manipulations in mice and detailed morphological and molecular analyses of mutant mouse models. Integrating these studies with experimental embryology approaches has revealed that palatogenesis is regulated by an extensive network of signaling molecules and transcription factors and involves abundant crosstalk between several distinct molecular pathways and cell types.

Here, we review recent advances in our understanding of the morphogenetic and molecular mechanisms of palatogenesis, focusing mainly on genetic studies in the mouse and briefly discussing contributions from other model systems. Many of the genes we discuss have been implicated in cleft palate in humans (Table 1) and several recent review articles provide excellent references for understanding the clinical and genetic aspects of cleft palate (Stanier and Moore, 2004; Gritli-Linde, 2007; Gritli-Linde, 2008; Dixon et al., 2011). We focus on how molecular pathways and cellular processes are integrated in the regulation of critical steps of palate morphogenesis. In addition, we highlight new technological approaches and genetic models that are being used for the study of palate development, and briefly discuss the implications that these studies have for an understanding of the fundamental principles of morphogenesis.

An overview of palatogenesis

In mammals, development of the face begins with the formation of five facial prominences surrounding the primitive mouth: the frontonasal prominence on the rostral side, a pair of maxillary prominences laterally and a pair of mandibular prominences caudally. These facial prominences are populated by cranial neural crest cells that originate at the dorsal edge of the developing rostral neural tube. As development proceeds, the frontonasal prominence is divided into the medial and lateral nasal processes by the formation of nasal pits. Subsequent morphogenetic fusion of the lateral and medial nasal processes forms the nostril, and fusion between the medial nasal processes and the maxillary prominences forms the upper lip (reviewed by Jiang et al., 2006). As the medial nasal processes fuse with the maxillary prominences, the presumptive primary and secondary palates first become morphologically visible as outgrowths from the oral side of the medial nasal and maxillary processes, respectively (Fig. 1A,B). The secondary palate arises as paired outgrowths, which in mammals initially grow vertically flanking the developing tongue (Fig. 1B,C,G,H,L,M,Q,R) and subsequently reorient to the horizontal position above the dorsum of the tongue in a process known as palatal shelf elevation (Fig. 1D,I,N,S). Following elevation, the paired palatal shelves grow towards the midline where they meet and fuse with each other (Fig. 1D-F,I-K,N-P,S-U). The fusion of palatal shelves involves the formation of a midline epithelial seam and its subsequent disintegration to allow mesenchymal confluence (Fig. 1O,P). In addition, the secondary palate fuses anteriorly with the primary palate and anterodorsally with the nasal septum, both of which are derived from the medial nasal processes, to form the intact roof of the oral cavity. In humans, palatogenesis is initiated in the sixth week and palatal fusion is complete by 12 weeks of gestation. In mice, palatal outgrowths are first detectable by embryonic day (E) 11.5 and palatal fusion is complete by E17.

Palatal shelf outgrowth

The palatal shelves are composed of mesenchyme derived mainly from the neural crest (Ito et al., 2003) surrounded by a thin layer of oral epithelium, and they exhibit distinct stereotyped shapes along the anterior-posterior (AP) axis. For example, at E13.5, the anterior part of the mouse palate exhibits a finger-like diagonal projection into the oral cavity (Fig. 1H), whereas the middle palatal region, which is flanked by the developing molar tooth germs, is

[1]Department of Cell and Tissue Biology and Program in Craniofacial and Mesenchymal Biology, University of California at San Francisco, San Francisco, CA 94143, USA. [2]Divisions of Developmental Biology and Plastic Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA.

*Authors for correspondence (Jeffrey.Bush@ucsf.edu; Rulang.Jiang@cchmc.org)

Table 1. Gene mutations identified in cleft palate in humans

Gene	Syndrome
Non-syndromic cleft palate	
BMP4	CLP
FGF8	CLP
FGFR2	CLP
FOXE1	CLP, CPO
IRF6	CLP, CPO
MSX1	CLP, CPO
PDGFC	CLP
SATB2	CPO
SUMO1	CLP
TBX22	CPO
Syndromic cleft palate	
BCOR	Oculofaciocardiodental
CHD7	CHARGE
COL2A1	Stickler type 1
COL11A1	Stickler type 2
COL11A2	Stickler type 3
DHCR7	Smith-Lemli-Opitz
DHCR24	Desmosterolosis
DHODH	Miller
ephrin B1 (*EFNB1*)	CFNS
FGFR1	Kallman
FGFR2	Crouzon, Apert
FLNA	Otopalatodigital types 1 and 2
FLNB	Larsen
FOXC2	Hereditary lymphedema-distichiasis
FOXE1	Bamforth-Lazarus
GLI3	Oro-facial-digital
IRF6	Van Woude, popliteal pterygium
KCNJ2	Andersen
MLL2	Kabuki
NIPBL	Cornelia de Lange
PQBP1	X-linked mental retardation
P63 (*TP63*)	EEC
SLC26A2	Diastrophic dysplasia
SOX9	Campomelic dysplasia, Pierre Robin
TBX1	DiGeorge
TBX22	CPX
TCOF1	Treacher Collins
TGFBR1	Loeys-Dietz
TGFBR2	Loeys-Dietz
TWIST1	Saethre-Chotzen

CLP, cleft lip with or without cleft palate; CPO, cleft palate only; CHARGE, Coloboma of the eye, Heart defects, Atresia of the nasal choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, and Ear abnormalities and deafness; CFNS, craniofrontonasal syndrome; EEC, ectrodactyly-ectodermal dysplasia-cleft syndrome; CPX, X-linked cleft palate and ankyloglossia. For more detailed information, see Dixon et al. (Dixon et al., 2011).

triangular in shape (Fig. 1M), and the posterior part of the palate, the presumptive soft palate, is vertical with a rounded distal end (Fig. 1R). Recent studies have revealed that the growth of the palatal shelves is controlled by reciprocal epithelial-mesenchymal interactions and involves distinct molecular mechanisms along the AP axis.

The roles of epithelial-mesenchymal interactions

Although the initiating signal has not yet been revealed, recent studies have identified key components of the signaling pathways that drive palatal shelf outgrowth. Sonic hedgehog (Shh), a secreted protein expressed throughout the early oral epithelium, is a key early signal that drives palatal shelf outgrowth (Rice et al., 2006). Studies of mice with epithelium-specific inactivation of *Shh* or mesenchyme-specific inactivation of smoothened (*Smo*), which encodes a transmembrane protein required for transducing Shh signaling, have demonstrated that Shh signals from the epithelium to the underlying mesenchyme to promote palatal cell proliferation and outgrowth (Rice et al., 2004; Lan and Jiang, 2009). Consistent with this, exogenous Shh application induces a mitogenic response in palatal explant cultures (Zhang et al., 2002; Rice et al., 2004). This mitogenic effect is mediated, in part, by the cell cycle regulators cyclin D1 and cyclin D2, the expression of which was reduced in the palatal mesenchyme of embryos with mesenchyme-specific inactivation of *Smo*. The expression of the forkhead box F1a and F2 (*Foxf1a* and *Foxf2*) and odd-skipped related 2 (*Osr2*) genes was also reduced in the palatal mesenchyme, indicating that these transcription factors might be downstream effectors of Shh signaling in this context (Lan and Jiang, 2009). Interestingly, Smo function was not required in the epithelium for palatal outgrowth (Rice et al., 2004), but its disruption in the palatal mesenchyme affected palatal epithelial cell proliferation, indicating that Shh signaling is necessary to activate a mesenchymal signal that regulates palatal epithelial cell proliferation (Lan and Jiang, 2009).

Fibroblast growth factor 10 (Fgf10) is a crucial mesenchymal signal that is required for palatal outgrowth. Mice homozygous for a null mutation in either *Fgf10* or the gene encoding its receptor, fibroblast growth factor receptor 2b (*Fgfr2b*), exhibited cleft palate with impaired palatal shelf outgrowth (Rice et al., 2004). Whereas *Fgf10* mRNA expression was restricted to the mesenchyme, *Fgfr2b* mRNA was most abundantly detected in the overlying epithelium. Fgfr2 function is required within the epithelium, as mice harboring an epithelial-specific deletion of *Fgfr2* also exhibited cleft palate (Hosokawa et al., 2009). Both epithelial and mesenchymal cell proliferation were reduced in the absence of either *Fgf10* or *Fgfr2b*, however, suggesting the presence of a factor that signals from the epithelium back to the underlying mesenchyme and that is dependent on Fgf10/Fgfr2b signaling. Indeed, *Shh* expression was dramatically reduced in the epithelium of $Fgf10^{-/-}$ and $Fgfr2b^{-/-}$ embryos, implying that the decreased palatal mesenchymal cell proliferation observed in these mutants might be a consequence of reduced *Shh* expression in the epithelium (Rice et al., 2004). The expression of *Fgf10* was also reduced in the palatal mesenchyme of embryos lacking mesenchymal *Smo*, indicating that Shh and Fgf10 function in a positive-feedback loop (Fig. 2A) that drives the outgrowth of the palatal shelves (Lan and Jiang, 2009).

Crosstalk between the Shh and bone morphogenetic protein (Bmp) signaling pathways has also been detected (Fig. 2A). The loss of *Smo* within the palatal mesenchyme led to upregulation of *Bmp4* and downregulation of *Bmp2* (Lan and Jiang, 2009). The positive regulation of Bmp2 by Shh signaling is consistent with the finding that exogenous Shh-containing beads induce *Bmp2* expression in palatal explant culture (Zhang et al., 2002). Exogenous Bmp2 can also positively regulate cell proliferation in the palatal mesenchyme (Zhang et al., 2002). Whereas complete inactivation of *Bmp4* resulted in early embryonic lethality, ablation of *Bmp4* function in the maxillary mesenchyme and throughout the oral epithelium (in *Nestin-Cre; Bmp4*$^{f/-}$ mice) caused a cleft lip phenotype, but no secondary palate defect was reported (Liu et al., 2005). Overexpression of the Bmp antagonist noggin specifically in the palatal mesenchyme led to retarded palatal growth and cleft palate in mice (Xiong et al., 2009), further supporting the involvement of Bmp signaling during palatal growth.

Recent studies indicate that Bmp signaling during palatogenesis occurs via the type I Bmp receptor Bmpr1a. Disruption of *Bmpr1a* in the maxillary mesenchyme and throughout the oral epithelium (in *Nestin-Cre; Bmpr1a*$^{f/-}$ mice) resulted in cleft lip and palate (Liu

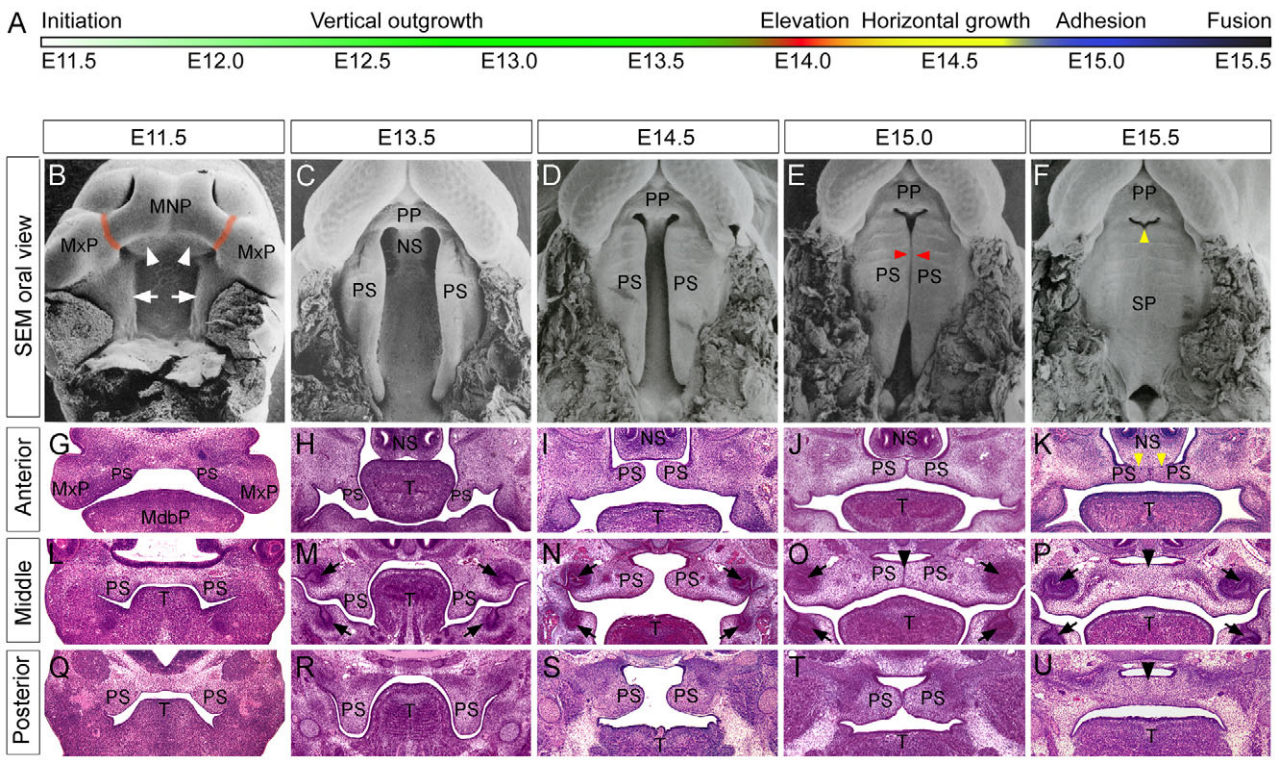

Fig. 1. Palatogenesis in the mouse. (**A**) Timecourse of palate development in mice. (**B-F**) Scanning electron micrographs showing oral views of the secondary palate at representative developmental stages [reprinted from Kaufman (Kaufman, 1992) with permission]. Orange lines mark sites of fusion between the medial nasal processes and maxillary processes, white arrowheads point to initial outgrowths of the primary palate, white arrows point to the initial outgrowth of the secondary palatal shelves, red arrowheads mark the initial site of palatal adhesion and fusion, and the yellow arrowhead points to the gap between the primary and secondary palates that will disappear following fusion between these tissues. (**G-U**) Representative histological frontal sections from anterior (G-K), middle (L-P), and posterior (Q-U) regions of the developing palate at each indicated stage. The middle palate region is flanked by the developing upper molar tooth germs (black arrows in M-P) and corresponds to the palatine region of the future hard palate. The posterior palate region corresponds to the future soft palate. At E11.5 (G,L,Q), the palatal shelf outgrowths arise from the oral surface of the maxillary processes. At E13.5 (H,M,R), the palatal shelves exhibit distinct shapes along the AP axis. By E14.5 (I,N,S), the palatal shelves have elevated to the horizontal position. At ~E15.0 (J,O,T), the palatal shelves make contact at the midline and initiate fusion by formation of the midline epithelial seam (MES) in the mid-anterior region (arrowhead in O). By E15.5 (K,P,U), palatal shelf fusion is evident in the middle and posterior regions, with complete removal of the MES (black arrowheads in P,U). Remnants of the MES can still be seen in the anterior region (K) at this stage and the palatal shelves also fuse superiorly with the nasal septum. Magnification is not equivalent between stages. MdbP, mandibular process; MNP, medial nasal process; MxP, maxillary process; NS, nasal septum; PP, primary palate; PS, palatal shelf; SP, secondary palate; T, tongue.

et al., 2005). The epithelial-specific inactivation of *Bmpr1a* (in *K14-Cre; Bmpr1a*$^{f/-}$ conditional mutant mice) did not result in a cleft palate phenotype, indicating that Bmpr1a function is likely to be required in the palatal mesenchyme (Andl et al., 2004). Conditional disruption of *Bmpr1a* in the neural crest and its derivatives (in *Wnt1-Cre; Bmpr1a*$^{f/-}$ embryos) caused dramatic retardation of the anterior region of the palatal shelves, whereas the posterior palate was less affected (Li et al., 2011). This phenotype was accompanied by other severe craniofacial abnormalities, including shortened mandible and hypoplastic maxillae, raising the question as to whether cleft palate in this situation resulted from a primary defect in palatogenesis or was secondary to earlier deficiencies in the maxillary prominences. Most recently, the disruption of *Bmpr1a* function specifically within the palatal mesenchyme was achieved using *Osr2-IresCre; Bmpr1a*$^{f/f}$ mice. These mutant embryos exhibited anteriorly restricted cleft palate and reduced cell proliferation in the anterior palatal mesenchyme (Baek et al., 2011). Interestingly, cell proliferation was also significantly reduced in the developing primary palate and expression of *Shh* was downregulated in both the primary palate and anterior secondary palate in the *Osr2-IresCre; Bmpr1a*$^{f/f}$ embryos, suggesting that interplay between Bmp and Shh signaling regulates both primary and secondary palate outgrowth (Baek et al., 2011). Furthermore, loss of noggin function resulted in cleft palate associated with aberrant apoptosis in the palatal epithelium and reduced cell proliferation of the anterior palatal mesenchyme (He et al., 2010b), indicating that Bmp signaling must be tightly regulated during palatogenesis.

In addition to classical signaling molecules, a recent study suggests that extracellular matrix (ECM) proteins participate in the regulation of palatal growth. Simultaneous disruption of the genes encoding two ADAMTS family metalloproteases, *Adamts9* and *Adamts20*, resulted in cleft palate with defects in early outgrowth, elevation and approximation of the palatal shelves (Enomoto et al., 2010). These secreted metalloproteases bind to the cell surface where they are actively involved in pericellular ECM proteolysis. A major substrate for these proteases is versican, a proteoglycan with space-filling properties. The cleavage of versican was indeed

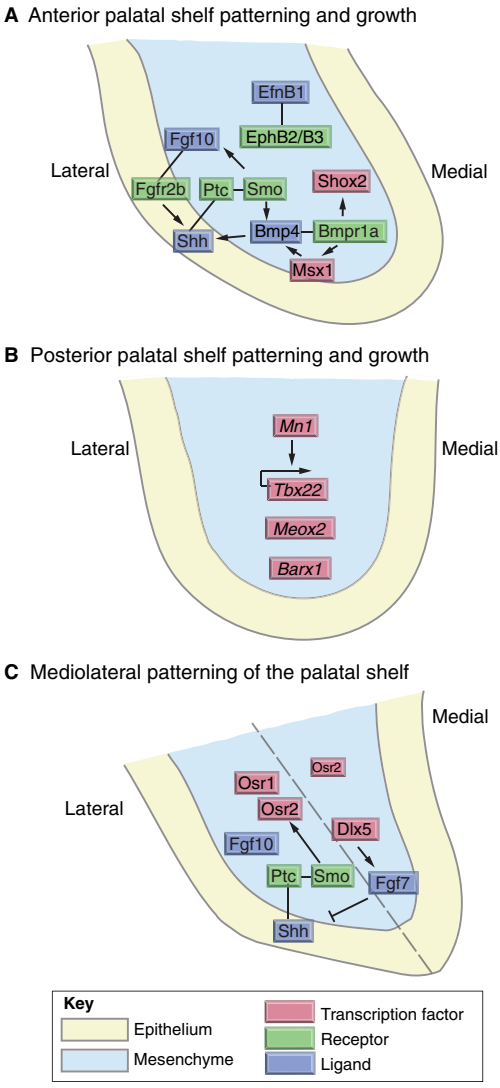

Fig. 2. Molecular control of palatal shelf growth and patterning. (**A**) Signaling interactions controlling anterior palatal growth. Sonic hedgehog (Shh) is expressed in the oral epithelium and binds to its receptor patched 1 (Ptc) in the underlying mesenchyme to permit smoothened (Smo) activation of palatal cell proliferation. Fibroblast growth factor 10 (Fgf10) is expressed in the palatal mesenchyme and binds to its receptor Fgfr2b in the palatal epithelium to regulate cell proliferation and survival. Fgf10 and Shh signaling maintain expression of each other to drive palatal outgrowth. Bone morphogenetic protein (Bmp) signaling through the Bmpr1a receptor in the mesenchyme regulates palate growth and expression of *Msx1* and *Shox2*. Bmp signaling is also involved in maintaining *Shh* expression in the palatal epithelium. Anterior palatal outgrowth is controlled additionally by ephrin B1 (EfnB1) signaling through its receptors EphB2 and EphB3. (**B**) Genes involved in development of the posterior palate. *Mn1*, *Tbx22*, *Meox2* and *Barx1*, which are expressed specifically in the posterior part of the palatal mesenchyme, regulate posterior palatal outgrowth, with *Tbx22* acting downstream of *Mn1*. (**C**) Pathways responsible for mediolateral patterning of the palatal shelves during vertical outgrowth. *Osr1* expression is restricted to the lateral mesenchyme whereas *Osr2* is expressed at high levels within the lateral part of the mesenchyme but also in the medial mesenchyme. The expression of both *Osr1* and *Osr2* is dependent on Shh signaling. An additional pathway involving Dlx5-Fgf7 signaling, which is able to repress Shh signaling, also controls outgrowth along the mediolateral axis. Although not marked in A and B, the mediolateral patterning pathways function throughout the AP axis of the developing palatal shelves; in C we have illustrated the AP axis (dashed line) in the middle palate. Illustrations represent the palatal shelves at specified positions along the AP axis at E13.5. Arrows represent inductive relationships, solid lines represent direct physical interaction, and blunt arrows indicate inhibition.

reduced in *Adamts9*$^{+/-}$; *Adamts20*$^{bt/bt}$ compound mutants. Interestingly, simultaneous disruption of versican and Adamts20 function also resulted in reduced palatal cell proliferation (Enomoto et al., 2010). It is possible that proteolysis of ECM molecules such as versican might produce bioactive fragments with growth-promoting activity. Alternatively, proteolysis of the ECM components might indirectly affect the distribution or activity of classical signaling molecules that regulate palatal growth. Further studies are necessary to test these possibilities.

Regionalized control of palatal outgrowth along the AP axis

Although the palatal shelves appear as continuous outgrowths that project from the maxillary processes into the oral cavity, gene expression studies have revealed remarkable molecular heterogeneity along the AP axis, and this heterogeneity is evident from the early stages of palatal outgrowth (Hilliard et al., 2005; Li and Ding, 2007; Welsh and O'Brien, 2009). Several transcription factor genes, including BarH-like homeobox 1 (*Barx1*), meningioma 1 (*Mn1*), Msh homeobox 1 (*Msx1*), mesenchyme homeobox 2 (*Meox2*), short stature homeobox 2 (*Shox2*) and T-box transcription factor 22 (*Tbx22*) are differentially expressed along the AP axis of the developing palatal shelves (Fig. 2A,B). The expression of *Msx1* and *Shox2*, for example, is restricted to the anterior part of the shelves, whereas *Meox2* and *Tbx22* mRNAs are restricted to the posterior region, with the AP gene expression boundary coinciding with a morphological landmark: the first formed palatal rugae (Zhang et al., 2002; Hilliard et al., 2005; Yu et al., 2005; Li and Ding, 2007; Pantalacci et al., 2008; Welsh and O'Brien, 2009). *Barx1* and *Mn1* mRNAs are also preferentially expressed in the posterior palate but their expression domains extend into the anterior half of the palatal shelves during palatal outgrowth (Liu et al., 2008; Welsh and O'Brien, 2009). Although mice lacking either *Msx1* or *Mn1* exhibited complete cleft palate, *Msx1*$^{-/-}$ mice exhibited specific cell proliferation defects in the anterior region, whereas *Mn1*$^{-/-}$ mice showed growth deficits in only the middle and posterior regions of the palatal shelves (Zhang et al., 2002; Liu et al., 2008). Mice lacking *Shox2* exhibited a cleft within the anterior palate, whereas the posterior palate fused normally, demonstrating a specific requirement for Shox2 in anterior palatal outgrowth (Yu et al., 2005). *Tbx22*^{null} mice displayed cleft palate, with the severity varying from a complete cleft palate phenotype due to decreased palatal shelf extension to submucous cleft palate in which palatal shelf elevation and fusion occurred normally (Pauws et al., 2009). *Tbx22* mRNA expression in the palate was significantly reduced in *Mn1*$^{-/-}$ mice, and Mn1

was able to activate *Tbx22* expression in cell culture assays, indicating that Tbx22 acts downstream of Mn1 in the regulation of posterior palatal outgrowth (Liu et al., 2008) (Fig. 2B).

The expression of *Msx1* and *Shox2* in the anterior palatal mesenchyme depends on Bmp signaling, as expression of both these genes was significantly reduced in the anterior palate of *Wnt1-Cre; Bmpr1a*$^{f/-}$ mice (Li et al., 2011). Remarkably, the addition of Bmp4-soaked beads in palatal explant cultures induced *Msx1* expression in the anterior, but not in the posterior, palatal mesenchyme (Hilliard et al., 2005). Exogenous Bmp4 was insufficient to induce *Shox2* mRNA expression in palatal mesenchyme explants, but the palatal epithelium from the anterior domain induced ectopic *Shox2* mRNA expression in the posterior palatal mesenchyme (Yu et al., 2005). These results further highlight intrinsic differences in both the epithelium and the mesenchyme along the AP axis.

Recently, Eph/ephrin family members have been demonstrated to control anterior palatal shelf outgrowth (Bush and Soriano, 2010). These signaling molecules have the capacity for bidirectional signaling, such that a forward signal can be transduced into the cell in which the Eph receptor tyrosine kinase is expressed, and a reverse signal can be transduced into the cell in which the ephrin (Efn) is expressed. The *Efnb1* gene exhibits a highly restricted expression pattern in the anterior palatal mesenchyme during all stages of palatogenesis, and *Efnb1*-null mice and *Efnb1*$^{+/-}$ heterozygous females exhibit cleft palate accompanied by decreased cell proliferation in the anterior palatal mesenchyme (Davy et al., 2004; Bush and Soriano, 2010). Analyses of mice carrying a series of targeted point mutations that specifically abrogate reverse signaling while leaving forward signaling by ephrin B1 intact revealed that reverse signaling is dispensable for palatogenesis (Bush and Soriano, 2009). In addition, null mutations in the EphB2 and EphB3 receptors, or specific disruption of forward signaling through these receptors, also resulted in reduced palatal shelf proliferation and cleft palate (Orioli et al., 1996; Risley et al., 2009). These results indicate that ephrin B1/EphB forward signaling controls anterior palatal shelf outgrowth (Bush and Soriano, 2009; Risley et al., 2009; Bush and Soriano, 2010). The *Efnb1* gene is X-linked and, owing to random X-inactivation, *Efnb1*$^{+/-}$ heterozygous embryos exhibit mosaic domains of *Efnb1* expression in the developing palatal shelves (Compagni et al., 2003; Davy et al., 2004; Bush and Soriano, 2010). Correspondingly, cell proliferation is affected in a correlated mosaic pattern that leads to more severe dysmorphogenesis of the palatal shelves in *Efnb1*$^{+/-}$ heterozygotes than in null mutant embryos (Bush and Soriano, 2010). These results indicate that cell proliferation must be tightly spatially regulated across the palatal shelf for normal outgrowth and for the establishment of proper palatal shape.

Most analyses of palatal outgrowth have focused on the downward growth of the palatal shelves; however, the maxillary processes also undergo significant rostrocaudal expansion from E12.5 to E14.5, and the palatal shelves correspondingly elongate along the AP axis. As the palatal shelves elongate, the domain of *Shox2* mRNA expression expands much more dramatically than that of *Meox2* (Li and Ding, 2007). Recently, it was demonstrated that the AP boundary of *Shox2* and *Meox2* expression coincides with the first formed palatal rugae (Pantalacci et al., 2008; Welsh and O'Brien, 2009). The rugae are metameric epithelial thickenings on the oral surface of the palate that are first apparent at E12.0 and develop by periodic reiterative interposition as the palatal shelves elongate along the AP axis (Pantalacci et al., 2008). Depending on the strain background, mice develop nine or ten rugae, which are maintained after birth. The rugae are thought to fulfill a number of mechanical functions, first in nursing and later in mastication (Pantalacci et al., 2008). Importantly, whereas the second and third rugae form sequentially and anteriorly to the first ruga, the fourth ruga forms between rugae 1 and 2, and, as the palatal shelves continue to elongate along the AP axis, subsequent rugae formation only occurs between the newly formed ruga and ruga 1 (Pantalacci et al., 2008; Welsh and O'Brien, 2009). These results indicate that there is a localized rugae growth zone immediately anterior to the AP boundary during the rostral elongation of the developing palatal shelves (Welsh and O'Brien, 2009).

Several laboratories have investigated cell proliferation in the developing palatal shelves using BrdU labeling, and these studies reported that cell proliferation rates are similar in the anterior and posterior regions of the palatal shelves at E13.5 (Zhang et al., 2002; Lan et al., 2004; Li and Ding, 2007). These studies were performed before the discovery of the localized rugae growth zone, however, and it is therefore possible that the palatal mesenchyme underlying the rugae growth zone might be proliferating at a faster rate than both the anterior and posterior regions. Additionally, a proliferation-independent mechanism might contribute to localized palatal elongation. When labeled cells were followed in explant cultures, anterior palatal mesenchyme migrated laterally while posterior palatal mesenchyme migrated anteriorly in a Wnt5a- and Fgf10-dependent manner (He et al., 2008). Furthermore, the finding that anterior palatal epithelium was able to induce *Shox2* mRNA expression in the posterior palatal mesenchyme in recombinant explant cultures (Yu et al., 2005) suggests that, once they have migrated into the anterior domain, posterior palatal mesenchymal cells might receive signals that activate *Shox2* mRNA expression. Taken together, these data suggest that directed migration of palatal mesenchyme from the posterior to the anterior palate with concurrent reprogramming of cell AP identity might be an important part of the mechanism that drives palatal shelf outgrowth and patterning along the AP axis.

Patterning along the mediolateral axis

The developing palatal shelves also exhibit morphological and molecular heterogeneity along the mediolateral axis during vertical outgrowth (Fig. 2C), with the lateral side corresponding to the oral side following palatal shelf elevation. Palatal rugae begin to form on the lateral side of the developing palatal shelves at E12, and the expression of *Shh* becomes restricted to the lateral palatal epithelium. *Osr1* and *Osr2*, which encode zinc-finger transcription factors, exhibit graded expression in the developing palatal mesenchyme along the mediolateral axis (Lan et al., 2004). At E13.5, *Osr1* mRNA expression is restricted to the lateral side, whereas *Osr2* exhibits graded expression that is strongest in the lateral mesenchyme and weaker in the medial mesenchyme (Fig. 2C). Targeted disruption of *Osr2* caused cleft palate associated with reduced cell proliferation in the medial side of the developing palatal shelves and with disrupted mediolateral patterning. The requirement of Osr2 for cell proliferation in the medial but not lateral side of the palatal shelf is likely to be due to partial functional redundancy of Osr2 with Osr1. Indeed, targeted replacement of the *Osr2* coding sequence with an *Osr1* cDNA rescued the cleft palate in *Osr2*-null mice (Gao et al., 2009).

A pathway involving the distal-less homeobox 5 (Dlx5) transcription factor is also implicated in mediolateral patterning and palatal expansion. *Dlx5* is co-expressed with *Fgf7* in the medial mesenchyme of the palatal shelf, and *Fgf7* expression was

dramatically reduced in this domain in *Dlx5*$^{-/-}$ mutant palatal shelves (Han et al., 2009). In these mutant embryos, *Shh* expression expanded to more medial palatal epithelium, possibly as a consequence of the loss of Fgf7, as exogenous Fgf7 could inhibit *Shh* expression in palatal explant cultures (Han et al., 2009). Although *Dlx5*$^{-/-}$ mutant palate shelves elevated and fused, the oral aspect of the palate was dramatically expanded and a malformed soft palate was observed. Interestingly, whereas *Msx1*$^{-/-}$ mice exhibited reduced *Shh* expression in the anterior palate, *Dlx5*$^{-/-}$; *Msx1*$^{-/-}$ compound mutant embryos exhibited *Shh* expression in the medial palatal epithelium, which was able to overcome the cell proliferation defects associated with *Msx1* loss-of-function (Han et al., 2009). Together, these results identify a new pathway involving Dlx5 and Fgf7 in the regulation of mediolateral patterning and palate growth (Fig. 2C). Since no obvious palatal abnormalities were detected in mice lacking Fgf7 (Guo et al., 1996), however, it is likely that another signaling molecule acts downstream of Dlx5, possibly in concert with Fgf7, to regulate *Shh* expression in the palatal epithelium.

Palatal shelf elevation/reorientation

At ~E14.0, the palatal shelves elevate to the horizontal position above the tongue. Because palatal shelf elevation occurs rapidly during midgestation, the exact molecular mechanisms have eluded characterization. Several hypotheses for palatal shelf elevation have been under consideration for decades, including: (1) rapid rotation of the shelves (Fig. 3A,B); and (2) growth-based regression of the distal portion of the shelves and outgrowth in the horizontal direction (Lazzaro, 1940). In 1956, Walker and Fraser noted that the process of palatal shelf elevation was too rapid to be explained by growth changes and proposed instead that the palatal shelves rapidly remodel themselves by a bulging of the medial wall and regression of the distal end (Fig. 3C,D) (Walker and Fraser, 1956). Subsequently, Coleman proposed that the mechanism of palatal shelf elevation is heterogeneous along the AP axis, whereby the anterior (rostral) part of the palatal shelves elevate by a swinging ‘flip-up’ process, while the posterior (caudal) and middle parts of the palatal shelves reorient through an oozing ‘flow’ remodeling mechanism (Fig. 3) (Coleman, 1965; Ferguson, 1988). The elevation of the palatal shelves often occurs asynchronously, with one palatal shelf elevating before the other (Lazzaro, 1940; Walker and Fraser, 1956). The physical forces driving this rapid change in position and shape are still somewhat unclear. Several hypotheses suggesting the involvement of the ECM have been proposed but none has been adequately proven (Brinkley and Bookstein, 1986; Ferguson, 1988). The palatal rugae were also hypothesized to play a role in palatal shelf elevation by serving as structural supports that stiffen the palatal shelves or by providing the force that drives elevation (Orban et al., 1972; Brinkley, 1980; Luke, 1984). Recent studies indicate that rugae are not required for elevation, however, as ablating canonical Wnt signaling in the palatal epithelium caused a complete loss of rugae formation but did not disrupt outgrowth and elevation of the palatal shelves (He et al., 2011).

A recent study investigated the palatal shelf elevation process by taking advantage of a mutant mouse strain with delayed palatal shelf elevation (Jin et al., 2010). Although they exhibit no apparent deficits in palatal cell proliferation or patterning, mouse embryos homozygous for a targeted disruption of the zinc-finger homeobox 1a (*Zfhx1a*, official nomenclature *Zeb1*) gene consistently exhibited delayed palatal shelf elevation by 24-48 hours (Jin et al., 2008). Thus, at E14.5, whereas the palatal shelves of control littermates are elevated, the palatal shelves of *Zfhx1a*$^{-/-}$ mutants remain vertical and express markers of the medial edge epithelium (MEE) along the medial side of the palatal epithelium. These results suggest that the MEE of post-elevation palatal shelves corresponds to the medial rather than the distal region of the pre-elevation palatal epithelium (Fig. 3C,D), supporting the hypothesis of Walker and Fraser that remodeling and horizontal outgrowth from the medial wall of the vertical palatal shelves give rise to the ‘elevated’ palatal shelves (Walker and Fraser, 1956). A recent histomorphological re-examination of this process (Yu and Ornitz, 2011) is in general

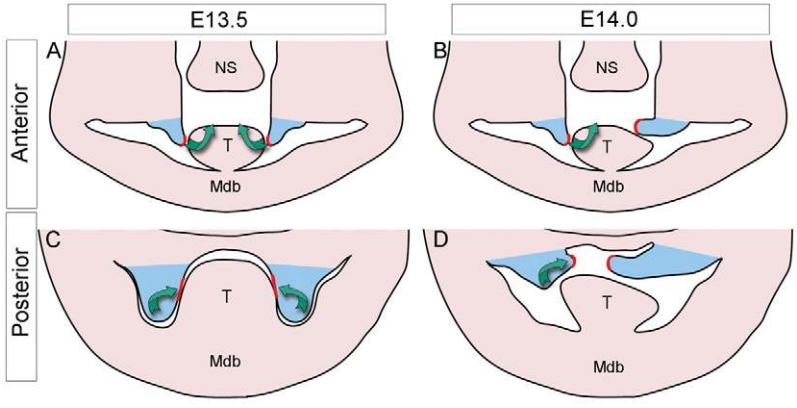

Fig. 3. Distinct mechanisms for palatal shelf elevation. Palatal shelf elevation at progressive stages, based on drawings from Walker and Fraser (Walker and Fraser, 1956). (**A**) At late E13, during the beginning of the palatal elevation stage, the anterior palatal shelves (blue) reorient by a ‘flip-up’ mechanism. (**B**) At ~E14.0, or mid-elevation, one palatal shelf has just completed elevation, while the other is still vertical. In this flip-up mechanism, the palatal shelves reorient such that the initially distally localized medial edge epithelium (MEE) (red) now becomes the medially located MES epithelium. (**C**) Initiation of horizontal outgrowth occurs from the medial wall of the vertically oriented palatal shelves in the mid-posterior region. (**D**) By E14.0, one palatal shelf has completed elevation, while the other is in the process of remodeling. In the posterior palate, the MEE (red) is initially localized to the medial surface of the vertical palatal shelves (Jin et al., 2010) and gives rise to the MES by the remodeling of the underlying mesenchyme. Green arrows depict distinct morphogenetic processes of palatal shelf elevation in the anterior and posterior regions; the anterior palatal shelves elevate in a flip-up process, whereas the posterior palatal shelves undergo remodeling and reorientation through horizontal outgrowth from the medial wall. Mdb, mandible; NS, nasal septum; T, tongue.

agreement with the report of Walker and Fraser but presented a refined timing for palatal shelf remodeling along the AP axis. Whereas Walker and Fraser described palatal shelf reorientation proceeding in a wave-like manner from posterior to anterior (Walker and Fraser, 1956), Yu and Ornitz showed histological evidence that horizontal outgrowth from the medial wall of the vertically oriented palatal shelves starts from the mid-posterior region, and palatal shelf reorientation occurs in a dynamic and regionalized manner along the AP axis (Yu and Ornitz, 2011).

Although the forces that drive palatal shelf elevation are still incompletely understood, recent progress has been made in identifying the genetic factors that control this event. In addition to defects in palatal shelf growth, *Osr2*$^{-/-}$ homozygous embryos exhibit a failure of palatal shelf elevation (Lan et al., 2004). It is possible that the reduction in medial palatal mesenchyme proliferation in these mutants impairs the horizontal outgrowth from the medial wall. Reduced medial mesenchymal cell proliferation could also affect contractile force in the medial aspect, leading to failure of shelf elevation.

A targeted point mutation in *Fgfr2* (*Fgfr2*$^{C342Y/C342Y}$) that leads to ligand-independent activation of the receptor resulted in increased palatal shelf mesenchyme proliferation in the lateral half of the shelf and delayed elevation, resulting in cleft palate (Snyder-Warwick et al., 2010). These embryos also exhibited reduced glycosaminoglycan (GAG) accumulation, providing further evidence for the correlation of GAG levels with palatal shelf elevation. It is notable that the mediolateral cell proliferation differential was perturbed in the same way as is observed in *Osr2*$^{-/-}$ embryos and with the same effect, suggesting that the regulation of cell proliferation along the mediolateral axis is intimately linked to the control of palatal shelf elevation. The ERK/MAPK signal transduction pathway is commonly utilized downstream of Fgf receptor signaling, and would be expected to be hyperactivated in *Fgfr2*$^{C342Y/C342Y}$ mutant palates. Disruption of sprouty 2 (*Spry2*), a negative regulator of the ERK/MAPK signal transduction pathway, resulted in failure of palatal shelf elevation, as well as in an increased cell proliferation rate in the anterior palatal shelves, suggesting that ERK/MAPK signaling plays an important role in palatal shelf elevation (Matsumura et al., 2011).

Palatal elevation defects were recently noted in mice deficient for the Wnt receptors frizzled 1 and frizzled 2 (Fz1 and Fz2, or Fzd1 and Fzd2) (Yu et al., 2010). Wnt ligands bind to Frizzled receptors to activate the canonical Wnt/β-catenin signaling pathway or the planar cell polarity (PCP) signaling pathway. Approximately 50% of *Fz2*$^{-/-}$ mice exhibited cleft palate, whereas all compound *Fz1*$^{-/-}$*; Fz2*$^{-/-}$ mutants exhibited cleft palate, suggesting functional redundancy between these receptors in palatogenesis (Yu et al., 2010). The PCP signaling pathway functions in diverse contexts to regulate multiple aspects of cell behavior, including cell movement and cell polarity, and PCP regulation of these cell behaviors might underlie palatal shelf elevation.

Glycogen synthase kinase 3β (Gsk3β) plays roles in multiple signal transduction pathways, most prominently by mediating degradation of β-catenin. Targeted disruption of *Gsk3b* resulted in completely penetrant cleft palate that could be rescued by chemically dependent re-expression of *Gsk3b* between E13.5 and E15.0 (Liu et al., 2007). Conditional disruption of *Gsk3b* in the palatal epithelium resulted in defective palatal shelf elevation accompanied by defects in epithelial cell proliferation and increased epithelial cell death (He et al., 2010a). Loss of *Gsk3b* did not affect Wnt/β-catenin reporter activity, and the epithelial-specific ablation of β-catenin did not result in defective palatal shelf elevation, suggesting that *Gsk3b* might work through a different pathway to regulate this morphogenetic process (He et al., 2010a; He et al., 2011).

Palatal shelf adhesion and fusion

Temporal and spatial control of shelf adhesion

Upon elevation to the horizontal position, the palatal shelves grow towards the midline, where they meet and fuse. Initial contact is made in the middle-anterior region of the shelves posterior to the second rugae, and fusion proceeds in the anterior and posterior directions. Adhesion between the palatal shelves at the midline is a critical initial step in palatal shelf fusion and requires proper regulation of MEE differentiation. This differentiation does not depend on contact between the palatal shelves, but instead is temporally programmed (Jin et al., 2008). Dysregulation of MEE differentiation and adhesion competence in time and space can result in cleft palate. Disruption of the Notch ligand jagged 2 (Jag2) caused cleft palate in *Jag2*$^{\Delta DSL/\Delta DSL}$ mice as a result of aberrant adhesion of the palatal shelves to the tongue (Jiang et al., 1998). Jag2 is expressed throughout the oral epithelium and is required for the maintenance of periderm cells (Casey et al., 2006), which are flattened epithelial surface cells that have been proposed to regulate fusion competence (Fitchett and Hay, 1989). Palate-tongue fusion, albeit less severe, also occurs in *Fgf10*$^{-/-}$ embryos, and *Jag2* expression was reduced in the mutant palatal epithelium, suggesting that Fgf10 signaling acts upstream of Jag2-Notch signaling to regulate palatal epithelial differentiation (Alappat et al., 2005).

Mice that are homozygous null for, or those harboring an R84C point mutation in, the interferon regulatory factor 6 (*Irf6*) transcription factor gene exhibited hyperproliferative epidermis that failed to differentiate, with multiple developmental consequences including inappropriate oral adhesions and cleft palate (Ingraham et al., 2006; Richardson et al., 2006). Irf6 regulates differentiation of the periderm in collaboration with Jag2, as compound *Irf6*$^{R84C/+}$*; Jag2*$^{\Delta DSL/+}$ mice exhibited palate-tongue fusion, oral adhesions, and cleft palate similar to that observed in mice homozygous for either individual allele (Richardson et al., 2009). The expression of each gene was unaffected in the reciprocal individual mutant, indicating that Irf6 does not directly regulate *Jag2* expression.

Mice deficient for the transcription factor p63 (Tp63 or Trp63) also exhibit cleft palate and a thin, undifferentiated epidermis (Mills et al., 1999; Yang et al., 1999), and *Irf6* expression is reduced in the palatal epithelium of *p63*$^{-/-}$ embryos (Thomason et al., 2010). The p63 protein binds to an enhancer upstream of the *Irf6* gene and can activate luciferase reporter expression driven by the *Irf6* enhancer, indicating that *Irf6* is likely to be a direct target of p63 (Thomason et al., 2010). Compound *p63*$^{+/-}$*; Irf6*$^{R84C/+}$ heterozygous mutant mice exhibited a failure of palatal shelf fusion associated with inappropriate maintenance of periderm cells. p63 has been shown to positively regulate *Jag2* and *Fgfr2* expression in other cell types (Sasaki et al., 2002; Candi et al., 2007). It is possible that a similar relationship between these genes is at play during palatal epithelial differentiation. Together, these data reveal a genetic network involving p63, Irf6, Fgf10-Fgfr2b and Jag2-Notch signaling in controlling palatal epithelial differentiation.

Palatal fusion: midline epithelial seam dissolution

The intervening epithelium between the abutted palatal shelves, termed the midline epithelial seam (MES), must be removed to provide mesenchymal continuity throughout the fused palate. Although recent studies have gone a great distance in clarifying the

cell biological mechanisms involved, some aspects of MES dissolution have remained contentious (Gritli-Linde, 2007). One of the foremost questions that remains is how, after apposition, is the MES abolished from the intervening shelves? Three non-exclusive hypotheses have been proposed. First, epithelial-to-mesenchymal transition (EMT) of the MES could allow the intervening epithelium to be incorporated into the mesenchyme of the intact palate. Second, death of the MES, by apoptotic or non-apoptotic mechanisms, could explain its removal from the midline. Third, the migration of MES cells in the oral and/or nasal direction could allow mesenchymal confluence. Decades of elegant cell biological and embryological studies that lend support to each of these hypotheses have been well-reviewed elsewhere (Dudas et al., 2007; Gritli-Linde, 2007). Here, we focus on recent studies utilizing mouse genetic approaches to address the question of MES disappearance.

In order to follow the fate of MES cells in vivo, genetic lineage tracing has been performed by coupling epithelially restricted Cre-expressing transgenic lines with the ROSA26R (R26R) reporter line (Vaziri Sani et al., 2005). When either *ShhGFPCre* or *K14-Cre* mice were crossed to R26R reporter mice, *lacZ* expression was irreversibly activated specifically within the epithelium. The subsequent examination of β-galactosidase staining during and following MES removal allowed the fate of MEE cells to be followed, to determine whether they contributed to the mesenchyme (i.e. if they underwent EMT). Using this approach, *lacZ*-expressing mesenchymal cells were not detected (Vaziri Sani et al., 2005), leading to the conclusion that EMT was not a significant contributor to the regression of the MES. These results were confirmed by a second group, who utilized *K14-Cre; R26R* lineage tracing (Xu et al., 2006). Different results, however, have been reported by a third group (Jin and Ding, 2006b), who showed that some mesenchymal β-galactosidase activity was present during and immediately after regression of the MES in *K14-Cre; R26R* embryos. The authors suggested that the discrepancy might have resulted from differences in Cre expression levels and/or patterns in the palatal epithelium in the different *K14-Cre* transgenic mouse lines used (Jin and Ding, 2006b).

Since K14-Cre is expressed throughout the palatal epithelium, the *K14-Cre; R26R* mouse approach cannot be used to determine whether migration of MES cells occurs. To address this issue, palatal shelves derived from wild-type embryos were recombined in culture with palatal shelves expressing *lacZ* in all cells (Jin and Ding, 2006b). *lacZ*-expressing epithelial cells migrated onto the wild-type palatal shelves, indicating that MES migration can occur during palatal fusion.

Several studies have shown that many MES cells are TUNEL positive and active caspase 3 positive during palatal fusion, indicating that apoptosis plays a role during MES dissolution (Cecconi et al., 1998; Cuervo and Covarrubias, 2004; Martinez-Alvarez et al., 2004; Vaziri Sani et al., 2005). To further investigate this, a recent genetic approach examined palatal fusion in the absence of *Apaf1*, a gene that encodes a crucial component of caspase 3-mediated apoptosis, and found that the *Apaf1*$^{-/-}$ embryos exhibited normal palate fusion and MES disintegration (Jin and Ding, 2006a). This result is in contrast to that of an earlier report indicating that palatal shelves made contact but did not fuse in *Apaf1*$^{-/-}$ embryos, although the secondary palate was not exhaustively examined in that study (Cecconi et al., 1998).

It has also been proposed that different mechanisms of MES dissolution might exist along the AP axis (Charoenchaikorn et al., 2009). Whereas the MES cells at the midline of the fusing secondary palatal shelves were intensely positive for TUNEL staining, only a few TUNEL-positive cells were detected in the epithelial seam between the fusing primary and secondary palate. Using the unpaired palatal shelf culture method, in which one palatal shelf is removed before midline contact to allow observation of the behavior of the MEE cells on the opposing shelf, it was demonstrated that MEE disappears without contact and adhesion from the middle and posterior regions of the palatal shelf but not from the region anterior to the second ruga (Charoenchaikorn et al., 2009). Thus, although programmed cell death plays a major role in MES dissolution, further research is necessary to elucidate the involvement of other cellular mechanisms, particularly the mechanism for fusion of the anterior secondary palate and between the primary and secondary palate.

Molecular mechanisms of palatal fusion

Transforming growth factor beta (Tgfβ) signaling has a prominent role in promoting fusion of the palatal shelves (Fig. 4). Palatal shelves in embryos lacking *Tgfb3* made contact at the midline but did not strongly adhere to each other and the MES was not removed, leading to complete cleft palate (Kaartinen et al., 1995; Proetzel et al., 1995). Furthermore, when placed in culture, *Tgfb3*$^{-/-}$ palatal shelves fail to abolish the MES, indicating that *Tgfb3* plays a crucial role in MES abolition (Kaartinen et al., 1997). Both *Tgfb3* and *Tgfb1* are expressed in the MEE prior to palatal shelf elevation, and their expression is maintained in the MES during palatal fusion, although the expression of *Tgfb1* is significantly lower than that of *Tgfb3* (Yang and Kaartinen, 2007). These ligands have partially overlapping functions, as a knock-in of the *Tgfb1* cDNA into the *Tgfb3* locus partially rescued palate fusion in the *Tgfb3*$^{-/-}$ mice (Yang and Kaartinen, 2007). Epithelial ablation of Tgfβ receptors *Alk5* (*Tgfbr1*) or *Tgfbr2* led to cleft palate phenotypes, wherein palatal adhesion occurred initially, but failure of the MES to dissolve caused the palatal shelves to pull apart (Dudas et al.,

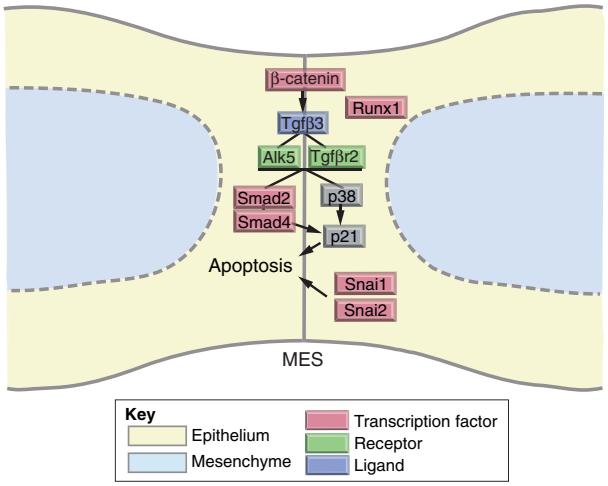

Fig. 4. Molecular control of palatal fusion. Tgfβ signaling plays a crucial role in palatal fusion, acting via the Alk5 and Tgfβr2 receptors to activate Smad2/Smad4 and the p38 MAPK pathways, which together regulate p21 expression in the MES. These, in parallel with the transcription factors Snai1 and Snai2, promote MES apoptosis and disintegration. Runx1, which is expressed in the MEE, is required for anterior palatal fusion. Arrows represent inductive relationships and solid lines indicate biochemical activation.

2006; Xu et al., 2006). In all of these cases, a dramatic reduction in apoptosis within the MES was observed, indicating that Tgfβ3 signaling regulates programmed cell death in the palatal epithelium (Dudas et al., 2006; Xu et al., 2006).

The signal transduction events downstream of *Tgfb3* in removal of the MES have also recently been investigated using mouse genetic studies (Fig. 4). The Tgfβ signaling pathway is initiated by ligand-induced heterotetramerization of a type I receptor dimer and a type II receptor dimer. Activated type I receptors phosphorylate R-Smads such as Smad2, which then partner with the obligate common mediator Smad4 to regulate transcription. Knockdown of Smad2 function in palatal explant cultures resulted in a failure of MES degeneration, and transgenic overexpression of Smad2 in the palatal epithelium partially rescued palate fusion in *Tgfb3*$^{-/-}$ mice (Cui et al., 2005; Shiomi et al., 2006). Epithelial-specific disruption of Smad4 in *K14-Cre; Smad4*$^{f/f}$ mice did not disrupt palatal shelf fusion, however, indicating that Smad-dependent signaling is not the only pathway at play (Shiomi et al., 2006; Xu et al., 2008). Tgfβ signaling can activate other intracellular signal transduction pathways, including p38 MAPK (Mapk14). Notably, p38 MAPK activation is elevated in the epithelium of the fusing palatal shelves (Xu et al., 2008). The treatment of *K14-Cre; Smad4*$^{f/f}$ palatal explants with a p38 MAPK inhibitor was able to block Tgfβ-dependent expression of the *p21* (*Cdkn1a*) cyclin-dependent kinase inhibitor gene in the MES, which correlated with reduced apoptosis and failed MES dissolution (Xu et al., 2008). Together, these results indicate that Smad- and p38 MAPK-dependent mechanisms are functionally redundant during palate fusion (Xu et al., 2008).

It has recently been reported that β-catenin can also regulate MES disappearance by regulating *Tgfb3* expression in the MEE. Epithelial-specific disruption of β-catenin (*Ctnnb1*) caused loss of *Tgfb3* expression in the MEE, reduction of apoptotic MES cells, and cleft palate due to failed palatal shelf fusion. Since β-catenin can function in either the canonical Wnt signaling pathway or as a component of adherens junctions, its mechanistic role in this context remains to be determined (He et al., 2011).

Several transcription factors have been implicated in palatal fusion (Fig. 4). Members of the Snail family of transcription factors are crucial regulators of palatal fusion, as *Snai1*$^{+/-}$*; Snai2*$^{+/-}$ compound mutants display failure of fusion concomitant with a reduction in MES apoptosis (Murray et al., 2007). *Tgfb3* expression was not affected in these mutants, however, indicating that these transcription factors regulate palatal fusion downstream of, or in parallel to, the Tgfβ3 pathway. By performing transgenic rescue of the hematopoietic defects in *Runx1*$^{-/-}$ embryos, a recent study was able to examine the role of this transcription factor in palate development (Charoenchaikorn et al., 2009). Although *Runx1* is expressed in the MEE throughout the AP axis from E13.5 through palate fusion, abrogation of its function resulted in an anterior-restricted failure of palatal shelf fusion, as well as in failed fusion with the primary palate. This anterior cleft correlates with a region of the MEE that has been observed to exhibit less TUNEL staining and to display distinct behavior as compared with the rest of the palatal shelf in unpaired palatal culture, suggesting that *Runx1* might play a part in this unique anterior MEE behavior (Charoenchaikorn et al., 2009). Interestingly, even after successful fusion and dissolution of the MES, the posteriorly restricted Meox2 transcription factor is required for the maintenance of palatal integrity, and *Meox2*$^{-/-}$ embryos exhibit a post-fusion split of the posterior palate (Jin and Ding, 2006a).

Palatal bone formation

The secondary palate-derived hard palate consists anteriorly of the palatal processes of the maxilla and posteriorly of the palatal processes of the palatine, whereas the primary palate consists of the small triangular piece of bone extending from anterior of the palatal processes of the maxilla to the alveolar bone of the incisors. Palatal bone formation occurs by intramembranous ossification, in which condensed neural crest-derived mesenchyme differentiates directly into osteoblasts. Recent studies have indicated that the palatal processes of the maxilla form from de novo ossification centers that are initially separate from the maxillary bone, whereas the palatal processes of the palatine form by the expansion of osteogenic fronts from the existing palatine towards the midline (Baek et al., 2011). Defects in palatal skeleton formation, after proper fusion of the palatal shelves, result in submucous cleft palate (SMCP). Despite the high frequency of SMCP in humans, only recently have mouse models of this congenital anomaly begun to provide insight into bone formation in the secondary palate. Inactivation of *Bmpr1a* in the palatal mesenchyme (*Osr2-IresCre; Bmpr1a*$^{f/f}$) caused SMCP due to lack of mesenchymal condensation and an absence of osteogenesis of the palatal process of the maxilla (Baek et al., 2011). A decrease in osteogenesis during formation of the palatal process of the palatine was also observed in these mutants.

The Tbx22 transcription factor is also required for palatal bone formation (Pauws et al., 2009). Some *Tbx22*^{null} embryos exhibited a complete cleft palate, whereas others displayed SMCP with reduced bone formation and delayed osteoblast differentiation. These results suggest two possibilities: (1) that Tbx22 is involved in two distinct aspects of palate development, i.e. palatal shelf growth and bone formation; and (2) that defects in bone formation in the *Tbx22*^{null} mice might be a consequence of mesenchymal insufficiency, even when palatal shelf fusion appears normal. Further investigation is needed to distinguish these possibilities.

Cleft palate as a consequence of defects in other craniofacial structures

Cleft palate in humans is clinically defined as either syndromic (occurring together with other developmental abnormalities) or non-syndromic (occurring in the absence of other developmental abnormalities). This designation, although highly valuable in clinical settings, has limited usefulness in understanding the developmental etiology of cleft palate. Instead, the crucial question is whether the cause of a cleft is intrinsic to the palatal shelves or secondary to deficiencies in other regions of the craniofacial complex. A cleft palate that is part of a syndrome can reflect either a secondary consequence of other developmental perturbations or an intrinsic defect within the palate. This distinction is best made by studying mouse models of a given syndrome because the embryological causes of cleft palate are most often indiscernible by the time patients are first examined.

Several examples of cleft palate as a secondary consequence of other craniofacial malformations exist, perhaps the most evoked of which is clinically referred to as the Pierre Robin Sequence, in which the lower jaw is either small (micrognathia) or set back from the upper jaw (retrognathia), resulting in a physical obstruction to palatal shelf elevation by the displaced tongue (Robin, 1994). Cleft palate as a secondary effect of mandibular or tongue developmental abnormalities has been reported in several mutant mouse strains (Table 2). It is notable, however, that in many such cases expression of the gene of interest was detected in the palatal shelves in addition to the mandible. Proof of cleft palate as a

Table 2. Cleft palate as a secondary consequence of mandibular defects

Mutation	Extrinsic cause	References
Hoxa2$^{-/-}$	Defective attachment of the hyoglossus muscle to the hyoid bone and consequently an inability to depress the lateral edges of the tongue	Gendron-Maguire et al., 1993; Barrow et al., 2000
Snai1$^{-/-}$; *Snai2*$^{-/-}$	Failure of palatal elevation due to physical obstruction by the tongue	Murray et al., 2007
csp1, *Prdm16*^{Gt683Lex}	Physical obstruction by the tongue as a consequence of mandibular defects	Bjork et al., 2010b
Wnt1Cre; Alk2$^{flox/flox}$	Mandibular hypoplasia leads to a small oral cavity preventing proper movements of tongue and palatal shelves	Dudas et al., 2004

secondary consequence of mandibular defects in each of these cases therefore awaits conditional ablation of gene function specifically in the developing mandible.

Insights from other animal models

Much of our understanding of the genetic control of palate development has been derived from mouse genetic studies. This is largely due to the striking similarity between palate development in humans and mice. In addition, the chick is a classical experimental embryology model system in which palate morphogenesis has been characterized (Ferguson, 1988). More recently, several studies have used the morpholino knockdown approach in zebrafish to assess gene function in palate development (Ghassibe-Sabbagh et al., 2011; Nakamura et al., 2011; Swartz et al., 2011). It is therefore important to understand the extent to which mechanisms for palatogenesis are conserved across these species.

Avians possess embryonic palatal shelves that are derived from the maxillae, but these palatal shelves begin their outgrowth in the horizontal direction and therefore do not undergo the morphogenesis of elevation (Ferguson, 1988). They also do not normally undergo fusion; instead, avian palatal shelves keratinize before making contact and remain constitutively cleft (Ferguson, 1988). Nevertheless, the expression of several genes involved in palatogenesis is conserved in the chick palatal shelves (Haenig et al., 2002; Fuchs et al., 2010; Sheehan-Rooney et al., 2010), and, as such, the chick is a useful model for studying the regulation of gene expression in the palate. *Tgfb3* is not expressed in MEE cells in the chick; when Tgfβ3 was exogenously added to chick palatal explant cultures, however, fusion of the palatal shelves was observed, indicating that *Tgfb3* expression might be a key difference between avian and mammalian palate development (Gato et al., 2002).

The zebrafish palate consists of a series of bones in the roof of the mouth that separates the oral cavity from the brain. The development of this structure does not involve palatal shelf formation, but instead entails condensation of cranial neural crest-derived mesenchyme above the oral ectoderm (Swartz et al., 2011). Thus, what is referred to as the palate in zebrafish is in fact homologous to part of the neurocranium, rather than the secondary palate, in mammals. Nevertheless, some promising molecular insights have been obtained from studies in zebrafish. An induced mutation in the *platelet derived growth factor receptor alpha* gene (*pdgfra*$^{b1059/b1059}$) in zebrafish resulted in hypoplasia of the roof of the mouth and abnormalities of the craniofacial skeleton, which resemble the cleft face phenotype exhibited by *Pdgfra*$^{-/-}$ mice (Soriano, 1997; Eberhart et al., 2008). *pdgfra* expression, as well as neural crest migration, are regulated by the microRNA miR140 in zebrafish (Eberhart et al., 2008). In mice, homozygous loss of the Pdgfc ligand, or mutations specifically abrogating the PI3K-binding ability of Pdgfrα, led to isolated cleft palate (Klinghoffer et al., 2002; Ding et al., 2004), indicating that the regulation of PDGF signaling is critical in mammalian palatogenesis. It is thus possible that the regulatory mechanisms of PDGF signaling in craniofacial development are conserved in zebrafish and mammals.

In mice, targeted disruption of the gene encoding the Sex-determining region Y-box 9 (*Sox9*) transcription factor led to multiple craniofacial defects including cleft palate, micrognathia and defects in chondrogenesis (Bi et al., 2001; Mori-Akiyama et al., 2003). A recent approach integrating mouse and zebrafish genetics with microarray analysis identified the gene encoding the WW domain containing E3 ubiquitin protein ligase 2 (*Wwp2*) as a direct downstream target of *Sox9* (Nakamura et al., 2011). Disruption of *Wwp2* function led to defects in chondrogenesis of the zebrafish palate that were similar to those resulting from *Sox9* loss of function. The authors concluded that this might be a molecular mechanism underlying cleft palate pathogenesis. However, the mammalian palatal bone forms by intramembranous ossification, which does not involve chondrogenesis. Therefore, although the regulation of chondrogenesis in the zebrafish craniofacial region by a Sox9-Wwp2 pathway is an important finding, the mechanism involving Sox9 in mammalian palatogenesis is likely to be different. Although the zebrafish is a valuable model for studying craniofacial development and neural crest biology, careful consideration of the differences is necessary when comparing defects in the zebrafish craniofacial skeleton to cleft palate pathogenesis in mammals.

Conclusions

In recent years, tremendous progress has been made in understanding the genetic control of palatogenesis. By analyzing gene expression changes in mutant embryos or in palate explant cultures treated with soluble factors, researchers have begun to define the signaling pathways and identify the pathway crosstalk crucial for controlling palate development. One challenge lies in obtaining insight into the direct mechanistic nature of these relationships. Although the genetic pathways governing palatogenesis are well established, the underlying biochemical mechanisms remain largely uncharacterized. Molecular and biochemical tools, such as chromatin immunoprecipitation, high-throughput gene expression profiling and mass spectrometry, can be combined with genetic, biochemical and cell culture studies to provide mechanistic insight into the direct relationships of molecules involved in palate development (Bush and Soriano, 2010; Thomason et al., 2010).

In addition to reverse genetic approaches, forward genetic screens utilizing N-ethyl-N-nitrosourea (ENU) mutagenesis in mice have identified new alleles that affect craniofacial development (Herron et al., 2002; Sandell et al., 2011). The ENU-induced recessive cleft secondary palate (*csp1*) allele was recently shown to be a loss-of-function mutation in the *Prdm16* gene (Bjork et al., 2010b). Knockdown of *Prdm16* using the PiggyBac transposon system (Ding et al., 2005) to deliver short hairpin RNA (shRNA)

resulted in a highly penetrant recapitulation of the cleft palate phenotype, suggesting that this technology can be used for rapid screening of gene function in mouse palate development (Bjork et al., 2010a).

The secondary palate presents an outstanding paradigm of general developmental mechanisms. The developing palatal shelves have a relatively simple structure in three dimensions and consist mainly of two basic cell types: epithelium and mesenchyme. Interrogation of reciprocal epithelial-mesenchymal interactions in the developing palatal shelves is relatively straightforward. Many of the genes involved in palatogenesis have crucial roles in other developmental and disease contexts, and studies of their roles and molecular pathways are therefore likely to be informative to their function in other settings, and vice versa. Furthermore, the abundance of signaling molecules and transcription factors involved in the development of the palate provides tremendous opportunity to understand signaling pathway function and crosstalk. As new technological tools are increasingly applied to the study of palatogenesis, our understanding of the fundamental principles governing morphogenesis will continue to advance rapidly.

Acknowledgements
We apologize to colleagues whose work we could not discuss owing to space limitations. We thank Kerstin Seidel for generously contributing E14.5 sections used in Fig. 1.

Funding
Palate development research in the Bush and Jiang laboratories was supported by the National Institutes of Health/National Institute of Dental and Craniofacial Research (NIH/NIDCR). Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

References

Alappat, S. R., Zhang, Z., Suzuki, K., Zhang, X., Liu, H., Jiang, R., Yamada, G. and Chen, Y. (2005). The cellular and molecular etiology of the cleft secondary palate in Fgf10 mutant mice. *Dev. Biol.* **277**, 102-113.

Andl, T., Ahn, K., Kairo, A., Chu, E. Y., Wine-Lee, L., Reddy, S. T., Croft, N. J., Cebra-Thomas, J. A., Metzger, D., Chambon, P. et al. (2004). Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. *Development* **131**, 2257-2268.

Baek, J. A., Lan, Y., Liu, H., Maltby, K. M., Mishina, Y. and Jiang, R. (2011). Bmpr1a signaling plays critical roles in palatal shelf growth and palatal bone formation. *Dev. Biol.* **350**, 520-531.

Barrow, J. R., Stadler, H. S. and Capecchi, M. R. (2000). Roles of Hoxa1 and Hoxa2 in patterning the early hindbrain of the mouse. *Development* **127**, 933-944.

Bi, W., Huang, W., Whitworth, D. J., Deng, J. M., Zhang, Z., Behringer, R. R. and de Crombrugghe, B. (2001). Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proc. Natl. Acad. Sci. USA* **98**, 6698-6703.

Bjork, B. C., Fujiwara, Y., Davis, S. W., Qiu, H., Saunders, T. L., Sandy, P., Orkin, S., Camper, S. A. and Beier, D. R. (2010a). A transient transgenic RNAi strategy for rapid characterization of gene function during embryonic development. *PLoS ONE* **5**, e14375.

Bjork, B. C., Turbe-Doan, A., Prysak, M., Herron, B. J. and Beier, D. R. (2010b). Prdm16 is required for normal palatogenesis in mice. *Hum. Mol. Genet.* **19**, 774-789.

Brinkley, L. L. (1980). In vitro studies of palatal shelf elevation. In *Current Research Trends in Prenatal Craniofacial Development* (ed. R. M. Pratt and R. L. Christiansen). Amsterdam: Elsevier/North-Holland.

Brinkley, L. L. and Bookstein, F. L. (1986). Cell distribution during mouse secondary palate closure. II. Mesenchymal cells. *J. Embryol. Exp. Morphol.* **96**, 111-130.

Bush, J. O. and Soriano, P. (2009). Ephrin-B1 regulates axon guidance by reverse signaling through a PDZ-dependent mechanism. *Genes Dev.* **23**, 1586-1599.

Bush, J. O. and Soriano, P. (2010). Ephrin-B1 forward signaling regulates craniofacial morphogenesis by controlling cell proliferation across Eph-ephrin boundaries. *Genes Dev.* **24**, 2068-2080.

Candi, E., Rufini, A., Terrinoni, A., Giamboi-Miraglia, A., Lena, A. M., Mantovani, R., Knight, R. and Melino, G. (2007). DeltaNp63 regulates thymic development through enhanced expression of FgfR2 and Jag2. *Proc. Natl. Acad. Sci. USA* **104**, 11999-12004.

Casey, L. M., Lan, Y., Cho, E. S., Maltby, K. M., Gridley, T. and Jiang, R. (2006). Jag2-Notch1 signaling regulates oral epithelial differentiation and palate development. *Dev. Dyn.* **235**, 1830-1844.

Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A. and Gruss, P. (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94**, 727-737.

Charoenchaikorn, K., Yokomizo, T., Rice, D. P., Honjo, T., Matsuzaki, K., Shintaku, Y., Imai, Y., Wakamatsu, A., Takahashi, S., Ito, Y. et al. (2009). Runx1 is involved in the fusion of the primary and the secondary palatal shelves. *Dev. Biol.* **326**, 392-402.

Coleman, R. D. (1965). Development of the rat palate. *Anat. Rec.* **151**, 107-117.

Compagni, A., Logan, M., Klein, R. and Adams, R. H. (2003). Control of skeletal patterning by ephrinB1-EphB interactions. *Dev. Cell* **5**, 217-230.

Cuervo, R. and Covarrubias, L. (2004). Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis. *Development* **131**, 15-24.

Cui, X. M., Shiomi, N., Chen, J., Saito, T., Yamamoto, T., Ito, Y., Bringas, P., Chai, Y. and Shuler, C. F. (2005). Overexpression of Smad2 in Tgf-beta3-null mutant mice rescues cleft palate. *Dev. Biol.* **278**, 193-202.

Davy, A., Aubin, J. and Soriano, P. (2004). Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev.* **18**, 572-583.

Ding, H., Wu, X., Bostrom, H., Kim, I., Wong, N., Tsoi, B., O'Rourke, M., Koh, G. Y., Soriano, P., Betsholtz, C. et al. (2004). A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. *Nat. Genet.* **36**, 1111-1116.

Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y. and Xu, T. (2005). Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell* **122**, 473-483.

Dixon, M. J., Marazita, M. L., Beaty, T. H. and Murray, J. C. (2011). Cleft lip and palate: understanding genetic and environmental influences. *Nat. Rev. Genet.* **12**, 167-178.

Dudas, M., Sridurongrit, S., Nagy, A., Okazaki, K. and Kaartinen, V. (2004). Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. *Mech. Dev.* **121**, 173-182.

Dudas, M., Kim, J., Li, W. Y., Nagy, A., Larsson, J., Karlsson, S., Chai, Y. and Kaartinen, V. (2006). Epithelial and ectomesenchymal role of the type I TGF-beta receptor ALK5 during facial morphogenesis and palatal fusion. *Dev. Biol.* **296**, 298-314.

Dudas, M., Li, W. Y., Kim, J., Yang, A. and Kaartinen, V. (2007). Palatal fusion – where do the midline cells go? A review on cleft palate, a major human birth defect. *Acta Histochem.* **109**, 1-14.

Eberhart, J. K., He, X., Swartz, M. E., Yan, Y. L., Song, H., Boling, T. C., Kunerth, A. K., Walker, M. B., Kimmel, C. B. and Postlethwait, J. H. (2008). MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nat. Genet.* **40**, 290-298.

Enomoto, H., Nelson, C. M., Somerville, R. P., Mielke, K., Dixon, L. J., Powell, K. and Apte, S. S. (2010). Cooperation of two ADAMTS metalloproteases in closure of the mouse palate identifies a requirement for versican proteolysis in regulating palatal mesenchyme proliferation. *Development* **137**, 4029-4038.

Ferguson, M. W. (1988). Palate development. *Development* **103 Suppl.**, 41-60.

Fitchett, J. E. and Hay, E. D. (1989). Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Dev. Biol.* **131**, 455-474.

Fuchs, A., Inthal, A., Herrmann, D., Cheng, S., Nakatomi, M., Peters, H. and Neubüser, A. (2010). Regulation of Tbx22 during facial and palatal development. *Dev. Dyn.* **239**, 2860-2874.

Gao, Y., Lan, Y., Ovitt, C. E. and Jiang, R. (2009). Functional equivalence of the zinc finger transcription factors Osr1 and Osr2 in mouse development. *Dev. Biol.* **328**, 200-209.

Gato, A., Martinez, M. L., Tudela, C., Alonso, I., Moro, J. A., Formoso, M. A., Ferguson, M. W. and Martinez-Alvarez, C. (2002). TGF-beta(3)-induced chondroitin sulphate proteoglycan mediates palatal shelf adhesion. *Dev. Biol.* **250**, 393-405.

Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993). Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

Ghassibe-Sabbagh, M., Desmyter, L., Langenberg, T., Claes, F., Boute, O., Bayet, B., Pellerin, P., Hermans, K., Backx, L., Mansilla, M. A. et al. (2011). FAF1, a gene that is disrupted in cleft palate and has conserved function in zebrafish. *Am. J. Hum. Genet.* **88**, 150-161.

Gritli-Linde, A. (2007). Molecular control of secondary palate development. *Dev. Biol.* **301**, 309-326.

Gritli-Linde, A. (2008). The etiopathogenesis of cleft lip and cleft palate: usefulness and caveats of mouse models. *Curr. Top. Dev. Biol.* **84**, 37-138.

Guo, L., Degenstein, L. and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* **10**, 165-175.

Haenig, B., Schmidt, C., Kraus, F., Pfordt, M. and Kispert, A. (2002). Cloning and expression analysis of the chick ortholog of TBX22, the gene mutated in X-linked cleft palate and ankyloglossia. *Mech. Dev.* **117**, 321-325.
Han, J., Mayo, J., Xu, X., Li, J., Bringas, P., Jr, Maas, R. L., Rubenstein, J. L. and Chai, Y. (2009). Indirect modulation of Shh signaling by Dlx5 affects the oral-nasal patterning of palate and rescues cleft palate in Msx1-null mice. *Development* **136**, 4225-4233.
He, F., Xiong, W., Yu, X., Espinoza-Lewis, R., Liu, C., Gu, S., Nishita, M., Suzuki, K., Yamada, G., Minami, Y. et al. (2008). Wnt5a regulates directional cell migration and cell proliferation via Ror2-mediated noncanonical pathway in mammalian palate development. *Development* **135**, 3871-3879.
He, F., Popkie, A. P., Xiong, W., Li, L., Wang, Y., Phiel, C. J. and Chen, Y. (2010a). Glycogen synthase kinase 3beta is required in the epithelium for palatal elevation in mice. *Dev. Dyn.* **239**, 3235-3246.
He, F., Xiong, W., Wang, Y., Matsui, M., Yu, X., Chai, Y., Klingensmith, J. and Chen, Y. (2010b). Modulation of BMP signaling by Noggin is required for the maintenance of palatal epithelial integrity during palatogenesis. *Dev. Biol.* **347**, 109-121.
He, F., Xiong, W., Wang, Y., Li, L., Liu, C., Yamagami, T., Taketo, M. M., Zhou, C. and Chen, Y. (2011). Epithelial Wnt/beta-catenin signaling regulates palatal shelf fusion through regulation of Tgfbeta3 expression. *Dev. Biol.* **350**, 511-519.
Herron, B. J., Lu, W., Rao, C., Liu, S., Peters, H., Bronson, R. T., Justice, M. J., McDonald, J. D. and Beier, D. R. (2002). Efficient generation and mapping of recessive developmental mutations using ENU mutagenesis. *Nat. Genet.* **30**, 185-189.
Hilliard, S. A., Yu, L., Gu, S., Zhang, Z. and Chen, Y. P. (2005). Regional regulation of palatal growth and patterning along the anterior-posterior axis in mice. *J. Anat.* **207**, 655-667.
Hosokawa, R., Deng, X., Takamori, K., Xu, X., Urata, M., Bringas, P., Jr and Chai, Y. (2009). Epithelial-specific requirement of FGFR2 signaling during tooth and palate development. *J. Exp. Zool. B Mol. Dev. Evol.* **312**, 343-350.
Ingraham, C. R., Kinoshita, A., Kondo, S., Yang, B., Sajan, S., Trout, K. J., Malik, M. I., Dunnwald, M., Goudy, S. L., Lovett, M. et al. (2006). Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). *Nat. Genet.* **38**, 1335-1340.
Ito, Y., Yeo, J. Y., Chytil, A., Han, J., Bringas, P., Jr, Nakajima, A., Shuler, C. F., Moses, H. L. and Chai, Y. (2003). Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. *Development* **130**, 5269-5280.
Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G. and Gridley, T. (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev.* **12**, 1046-1057.
Jiang, R., Bush, J. O. and Lidral, A. C. (2006). Development of the upper lip: morphogenetic and molecular mechanisms. *Dev. Dyn.* **235**, 1152-1166.
Jin, J. Z. and Ding, J. (2006a). Analysis of Meox-2 mutant mice reveals a novel postfusion-based cleft palate. *Dev. Dyn.* **235**, 539-546.
Jin, J. Z. and Ding, J. (2006b). Analysis of cell migration, transdifferentiation and apoptosis during mouse secondary palate fusion. *Development* **133**, 3341-3347.
Jin, J. Z., Li, Q., Higashi, Y., Darling, D. S. and Ding, J. (2008). Analysis of Zfhx1a mutant mice reveals palatal shelf contact-independent medial edge epithelial differentiation during palate fusion. *Cell Tissue Res.* **333**, 29-38.
Jin, J. Z., Tan, M., Warner, D. R., Darling, D. S., Higashi, Y., Gridley, T. and Ding, J. (2010). Mesenchymal cell remodeling during mouse secondary palate reorientation. *Dev. Dyn.* **239**, 2110-2117.
Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N. and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* **11**, 415-421.
Kaartinen, V., Cui, X. M., Heisterkamp, N., Groffen, J. and Shuler, C. F. (1997). Transforming growth factor-beta 3 regulates transdifferentiation of medial edge epithelium during palatal fusion and associated degradation of the basement membrane. *Dev. Dyn.* **209**, 255-260.
Kaufman, M. H. (1992). *The Atlas of Mouse Development*. London: Elsevier.
Klinghoffer, R. A., Hamilton, T. G., Hoch, R. and Soriano, P. (2002). An allelic series at the PDGFalphaR locus indicates unequal contributions of distinct signaling pathways during development. *Dev. Cell* **2**, 103-113.
Lan, Y. and Jiang, R. (2009). Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* **136**, 1387-1396.
Lan, Y., Ovitt, C. E., Cho, E. S., Maltby, K. M., Wang, Q. and Jiang, R. (2004). Odd-skipped related 2 (Osr2) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. *Development* **131**, 3207-3216.
Lazzaro, C. (1940). Sul meccanismo di chiusura del palato secondario. *Monit. Zool. Ital.* **51**, 249-273.
Li, L., Lin, M., Wang, Y., Cserjesi, P., Chen, Z. and Chen, Y. (2011). Bmprla is required in mesenchymal tissue and has limited redundant function with Bmprlb in tooth and palate development. *Dev. Biol.* **349**, 451-461.
Li, Q. and Ding, J. (2007). Gene expression analysis reveals that formation of the mouse anterior secondary palate involves recruitment of cells from the posterior side. *Int. J. Dev. Biol.* **51**, 167-172.
Liu, K. J., Arron, J. R., Stankunas, K., Crabtree, G. R. and Longaker, M. T. (2007). Chemical rescue of cleft palate and midline defects in conditional GSK-3beta mice. *Nature* **446**, 79-82.
Liu, W., Sun, X., Braut, A., Mishina, Y., Behringer, R. R., Mina, M. and Martin, J. F. (2005). Distinct functions for Bmp signaling in lip and palate fusion in mice. *Development* **132**, 1453-1461.
Liu, W., Lan, Y., Pauws, E., Meester-Smoor, M. A., Stanier, P., Zwarthoff, E. C. and Jiang, R. (2008). The Mn1 transcription factor acts upstream of Tbx22 and preferentially regulates posterior palate growth in mice. *Development* **135**, 3959-3968.
Luke, D. A. (1984). Epithelial proliferation and development of rugae in relation to palatal shelf elevation in the mouse. *J. Anat.* **138**, 251-258.
Martinez-Alvarez, C., Blanco, M. J., Perez, R., Rabadan, M. A., Aparicio, M., Resel, E., Martinez, T. and Nieto, M. A. (2004). Snail family members and cell survival in physiological and pathological cleft palates. *Dev. Biol.* **265**, 207-218.
Matsumura, K., Taketomi, T., Yoshizaki, K., Arai, S., Sanui, T., Yoshiga, D., Yoshimura, A. and Nakamura, S. (2011). Sprouty2 controls proliferation of palate mesenchymal cells via fibroblast growth factor signaling. *Biochem. Biophys. Res. Commun.* **404**, 1076-1082.
Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R. and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708-713.
Mori-Akiyama, Y., Akiyama, H., Rowitch, D. H. and de Crombrugghe, B. (2003). Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. *Proc. Natl. Acad. Sci. USA* **100**, 9360-9365.
Murray, S. A., Oram, K. F. and Gridley, T. (2007). Multiple functions of Snail family genes during palate development in mice. *Development* **134**, 1789-1797.
Nakamura, Y., Yamamoto, K., He, X., Otsuki, B., Kim, Y., Murao, H., Soeda, T., Tsumaki, N., Deng, J. M., Zhang, Z. et al. (2011). Wwp2 is essential for palatogenesis mediated by the interaction between Sox9 and mediator subunit 25. *Nat. Commun.* **2**, 251.
Orban, B. J., Sicher, H. and Bhaskar, S. N. (1972). *Orban's Oral Histology and Embryology*. Saint Louis: Mosby.
Orioli, D., Henkemeyer, M., Lemke, G., Klein, R. and Pawson, T. (1996). Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. *EMBO J.* **15**, 6035-6049.
Pantalacci, S., Prochazka, J., Martin, A., Rothova, M., Lambert, A., Bernard, L., Charles, C., Viriot, L., Peterkova, R. and Laudet, V. (2008). Patterning of palatal rugae through sequential addition reveals an anterior/posterior boundary in palatal development. *BMC Dev. Biol.* **8**, 116.
Pauws, E., Moore, G. E. and Stanier, P. (2009). A functional haplotype variant in the TBX22 promoter is associated with cleft palate and ankyloglossia. *J. Med. Genet.* **46**, 555-561.
Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W. and Doetschman, T. (1995). Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat. Genet.* **11**, 409-414.
Rice, R., Spencer-Dene, B., Connor, E. C., Gritli-Linde, A., McMahon, A. P., Dickson, C., Thesleff, I. and Rice, D. P. (2004). Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J. Clin. Invest.* **113**, 1692-1700.
Rice, R., Connor, E. and Rice, D. P. (2006). Expression patterns of Hedgehog signalling pathway members during mouse palate development. *Gene Expr. Patterns* **6**, 206-212.
Richardson, R. J., Dixon, J., Malhotra, S., Hardman, M. J., Knowles, L., Boot-Handford, R. P., Shore, P., Whitmarsh, A. and Dixon, M. J. (2006). Irf6 is a key determinant of the keratinocyte proliferation-differentiation switch. *Nat. Genet.* **38**, 1329-1334.
Richardson, R. J., Dixon, J., Jiang, R. and Dixon, M. J. (2009). Integration of IRF6 and Jagged2 signalling is essential for controlling palatal adhesion and fusion competence. *Hum. Mol. Genet.* **18**, 2632-2642.
Risley, M., Garrod, D., Henkemeyer, M. and McLean, W. (2009). EphB2 and EphB3 forward signalling are required for palate development. *Mech. Dev.* **126**, 230-239.
Robin, P. (1994). A fall of the base of the tongue considered as a new cause of nasopharyngeal respiratory impairment: Pierre Robin sequence, a translation. 1923. *Plast. Reconstr. Surg.* **93**, 1301-1303.
Sandell, L. L., Iulianella, A., Melton, K. R., Lynn, M., Walker, M., Inman, K. E., Bhatt, S., Leroux-Berger, M., Crawford, M., Jones, N. C. et al. (2011). A phenotype-driven ENU mutagenesis screen identifies novel alleles with functional roles in early mouse craniofacial development. *Genesis* **49**, 342-359.
Sasaki, Y., Ishida, S., Morimoto, I., Yamashita, T., Kojima, T., Kihara, C., Tanaka, T., Imai, K., Nakamura, Y. and Tokino, T. (2002). The p53 family member genes are involved in the Notch signal pathway. *J. Biol. Chem.* **277**, 719-724.
Sheehan-Rooney, K., Pálinkášová, B., Eberhart, J. K. and Dixon, M. J. (2010). A cross-species analysis of Satb2 expression suggests deep conservation across vertebrate lineages. *Dev. Dyn.* **239**, 3481-3491.

Shiomi, N., Cui, X. M., Yamamoto, T., Saito, T. and Shuler, C. F. (2006). Inhibition of SMAD2 expression prevents murine palatal fusion. *Dev. Dyn.* **235**, 1785-1793.

Snyder-Warwick, A. K., Perlyn, C. A., Pan, J., Yu, K., Zhang, L. and Ornitz, D. M. (2010). Analysis of a gain-of-function FGFR2 Crouzon mutation provides evidence of loss of function activity in the etiology of cleft palate. *Proc. Natl. Acad. Sci. USA* **107**, 2515-2520.

Soriano, P. (1997). The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. *Development* **124**, 2691-2700.

Stanier, P. and Moore, G. E. (2004). Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts. *Hum. Mol. Genet.* **13 Spec. No. 1**, R73-R81.

Swartz, M. E., Sheehan-Rooney, K., Dixon, M. J. and Eberhart, J. K. (2011). Examination of a palatogenic gene program in zebrafish. *Dev. Dyn.* **240**, 2204-2220.

Thomason, H. A., Zhou, H., Kouwenhoven, E. N., Dotto, G. P., Restivo, G., Nguyen, B. C., Little, H., Dixon, M. J., van Bokhoven, H. and Dixon, J. (2010). Cooperation between the transcription factors p63 and IRF6 is essential to prevent cleft palate in mice. *J. Clin. Invest.* **120**, 1561-1569.

Vaziri Sani, F., Hallberg, K., Harfe, B. D., McMahon, A. P., Linde, A. and Gritli-Linde, A. (2005). Fate-mapping of the epithelial seam during palatal fusion rules out epithelial-mesenchymal transformation. *Dev. Biol.* **285**, 490-495.

Walker, B. E. and Fraser, F. C. (1956). Closure of the secondary palate in three strains of mice. *J. Embryol. Exp. Morphol.* **4**, 176-189.

Welsh, I. C. and O'Brien, T. P. (2009). Signaling integration in the rugae growth zone directs sequential SHH signaling center formation during the rostral outgrowth of the palate. *Dev. Biol.* **336**, 53-67.

Xiong, W., He, F., Morikawa, Y., Yu, X., Zhang, Z., Lan, Y., Jiang, R., Cserjesi, P. and Chen, Y. (2009). Hand2 is required in the epithelium for palatogenesis in mice. *Dev. Biol.* **330**, 131-141.

Xu, X., Han, J., Ito, Y., Bringas, P., Jr, Urata, M. M. and Chai, Y. (2006). Cell autonomous requirement for Tgfbr2 in the disappearance of medial edge epithelium during palatal fusion. *Dev. Biol.* **297**, 238-248.

Xu, X., Han, J., Ito, Y., Bringas, P., Jr, Deng, C. and Chai, Y. (2008). Ectodermal Smad4 and p38 MAPK are functionally redundant in mediating TGF-beta/BMP signaling during tooth and palate development. *Dev. Cell* **15**, 322-329.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C. et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398**, 714-718.

Yang, L. T. and Kaartinen, V. (2007). Tgfb1 expressed in the Tgfb3 locus partially rescues the cleft palate phenotype of Tgfb3 null mutants. *Dev. Biol.* **312**, 384-395.

Yu, H., Smallwood, P. M., Wang, Y., Vidaltamayo, R., Reed, R. and Nathans, J. (2010). Frizzled 1 and frizzled 2 genes function in palate, ventricular septum and neural tube closure: general implications for tissue fusion processes. *Development* **137**, 3707-3717.

Yu, K. and Ornitz, D. M. (2011). Histomorphological study of palatal shelf elevation during murine secondary palate formation. *Dev. Dyn.* **240**, 1737-1744.

Yu, L., Gu, S., Alappat, S., Song, Y., Yan, M., Zhang, X., Zhang, G., Jiang, Y., Zhang, Z., Zhang, Y. et al. (2005). Shox2-deficient mice exhibit a rare type of incomplete clefting of the secondary palate. *Development* **132**, 4397-4406.

Zhang, Z., Song, Y., Zhao, X., Zhang, X., Fermin, C. and Chen, Y. (2002). Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* **129**, 4135-4146.