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Development of the Upper Lip: Morphogenetic and Molecular Mechanisms

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The vertebrate upper lip forms from initially freely projecting maxillary, medial nasal, and lateral nasal prominences at the rostral and lateral boundaries of the primitive oral cavity. These facial prominences arise during early embryogenesis from ventrally migrating neural crest cells in combination with the head ectoderm and mesoderm and undergo directed growth and expansion around the nasal pits to actively fuse with each other. Initial fusion is between lateral and medial nasal processes and is followed by fusion between maxillary and medial nasal processes. Fusion between these prominences involves active epithelial filopodial and adhering interactions as well as programmed cell death. Slight defects in growth and patterning of the facial mesenchyme or epithelial fusion result in cleft lip with or without cleft palate, the most common and disfiguring craniofacial birth defect. Recent studies of craniofacial development in animal models have identified components of several major signaling pathways, including Bmp, Fgf, Shh, and Wnt signaling, that are critical for proper midfacial morphogenesis and/or lip fusion. There is also accumulating evidence that these signaling pathways cross-regulate genetically as well as crosstalk intracellularly to control cell proliferation and tissue patterning. This review will summarize the current understanding of the basic morphogenetic processes and molecular mechanisms underlying upper lip development and discuss the complex interactions of the various signaling pathways and challenges for understanding cleft lip pathogenesis. *Developmental Dynamics 235:1152–1166, 2006.* © 2005 Wiley-Liss, Inc.

Key words: birth defect; cleft lip; cleft palate; craniofacial development; apoptosis; EMT; Bmp4; Fgf8; Shh; Wnt signaling

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INTRODUCTION

Cleft lip with or without cleft palate (CLP) has an occurrence of 1 in 500 to 2,500 live births worldwide, which represents the most common craniofacial birth defect in humans (Vanderas, 1987; Schutte and Murray, 1999; Gorlin et al., 2001). Clinically, cleft lip is a unilateral or bilateral gap between the philtrum and the lateral upper lip, often extending through the upper lip and jaw into the nostril and is sometimes accompanied by cleft of the secondary palate—the roof of the oral cavity. Another common form of orofacial clefting is cleft palate (CP), which appears as a gap in the secondary palate while the upper lip appears intact. Epidemiological and embryological studies suggest that CLP and CP have distinct etiology, although these two phenotypes sometimes appear in the same family (Fraser, 1970; Vanderas, 1987; Gorlin et al., 2001). Both CLP and CP have syndromic and nonsyndromic forms with the syndromic clefting often caused by single gene mutations, chromosomal abnormalities, or teratogenic exposure (Gorlin et al., 2001; Wyszynski, 2002). Approximately 70% of CLP cases are nonsyndromic for which the etiology and pathogenesis are complex and poorly understood.

To understand the etiology of CLP, it is necessary to understand the de-

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velopmental processes leading to the formation of the intact upper lip, at both the morphogenetic and molecular levels. However, elucidating the causes of CLP on even the morphological level has been hindered by a paucity of understanding of the fundamental processes of lip formation. Confusion exists in the literature with regard to the morphological processes leading to the formation of the intact upper lip. Whereas several studies describe that the upper lip forms from fusion between the maxillary and the medial nasal processes (e.g., Sun et al., 2000; Ashique et al., 2002; Sperber, 2002; Cox, 2004), others state that a cleft lip results when the epithelia of the opposing medial and lateral nasal processes fail to make contact (Trasler, 1968; Gaare and Langman, 1977a; Gong and Guo, 2003). The confusion may have arisen due in part to species differences (e.g., chick vs. mouse and human) in facial morphogenesis and in part to lack of synthesis of the fragmentary and often incomplete information gained from individual studies. Moreover, whereas it has been widely accepted that epithelial–mesenchymal transformation (EMT) of the epithelial seam is the major mechanism for both lip and palate fusion (Fitchett and Hay, 1989; Shuler et al., 1991, 1992; Griffith and Hay, 1992; Hay, 1995, 2005; Sun et al., 2000; Cox, 2004; Nawshad et al., 2004), recent studies have challenged this theory and demonstrated that the palatal epithelial seam gradually regresses by programmed cell death rather than by EMT (Cuervo and Covarrubias, 2004; Vaziri Sani et al., 2005). At the molecular level, recent studies in chick and mice have identified specific roles for several major signaling pathways, including Bmp, Fgf, and Shh signaling pathways in midfacial morphogenesis (Hu and Helms, 1999; Trumpp et al., 1999; Ashique et al., 2002; Trokovic et al., 2003; Jeong et al., 2004; Liu et al., 2005b). In addition, genetic studies in human and mice have also identified two *Wnt* genes involved in CLP pathogenesis (Juriloff et al., 2004, 2005; Niemann et al., 2004; Carroll et al., 2005). These data provide new insight into the molecular mechanisms underlying midfacial morphogenesis and CLP formation. This review will attempt to clarify the morphogenetic processes leading to formation of the intact upper lip and discuss the new advances in the understanding of the signaling pathways regulating upper lip development.

MORPHOGENESIS OF THE UPPER LIP

In 1985, Klaus Hinrichsen published a detailed scanning electron microscopy (SEM) study of a collection of various stage human embryos, focusing on the morphology and pattern of the developing face (Hinrichsen, 1985). Recently, Senders et al. (2003) presented high resolution SEM pictures of developing cynomolgus monkey embryonic faces. Comparing these with other histological and SEM studies of facial development in mouse and chick (Trasler, 1968; Gaare and Langman, 1977a,b; Yee and Abbott, 1978; Millicovsky and Johnston, 1981; Millicovsky et al., 1982; Trasler and Ohannessian, 1983; Cox, 2004) provides an accurate understanding of the morphological processes involved in facial development.

Development of the human face begins in the fourth week of embryogenesis (stage 10 according the Carnegie staging system for human embryos, O'Rahilly, 1972), with migrating neural crest cells that combine with the core mesoderm and the epithelial cover to establish the facial primordia. The neural crest-derived facial mesenchyme will give rise to the facial skeleton, whereas mesoderm-derived cells will form facial muscles (Noden, 1978, 1983, 1988; Couly et al., 1992, 1993). At stage 11 (approximately 24 days of gestation and corresponding to embryonic day [E] 9.0 of mouse embryogenesis), the primitive mouth, or stomodeum, is bound rostrally by the developing forebrain and caudally by the swelling mandibular arches (the first pharyngeal arch), whereas structures associated with the formation of the upper lip are not distinguishable yet at this stage (Yoon et al., 2000). By stage 12 (approximately 26 days of gestation, corresponding to E9.5 of mouse embryogenesis), the facial primordia consist of five separate prominences surrounding the stomodeum (Hinrichsen, 1985; Fig. 1A). At the rostral side of the stomodeum is a symmetrical, unpaired frontonasal prominence, which is fitted ventrolaterally to the forebrain and populated by mesenchymal cells derived from the fore- and mid-brain neural crest. The stomodeum is bound laterally by a pair of maxillary processes and caudally by the pair of mandibular processes, which are populated by neural crest cells originating from the first two rhombomeres of the hindbrain.

From stage 13 to stage 15 (fourth to fifth week) of human embryogenesis, the frontonasal prominence widens as the forebrain gives rise to the paired telencephalic vesicles (primordia of cerebral hemispheres), while the medial ends of the mandibular processes gradually merge in a caudal to rostral direction to form the mandible (lower lip and jaw; Hinrichsen, 1985; Yoon et al., 2000). At stage 14 (approximately 32 days of gestation and corresponding to E10.0 of mouse embryogenesis), thickening of surface ectoderm occurs bilaterally on the ventrolateral part of the frontonasal prominence, giving rise to the nasal placodes. The frontonasal process grows and bulges around the nasal placodes, resulting in the formation of nasal pits and the swelling horseshoe-shaped lateral and medial nasal processes (Hinrichsen, 1985; Sperber, 2002). In adaptation to the development of the telencephalic vesicles, the rostral end of the embryo forms a paired configuration with a median groove extending in between the paired medial nasal processes and into the stomodeum. The nasal pits are also in continuity with the stomodeum at this stage (Hinrichsen, 1985).

By stage 15 (approximately 35 days of gestation, corresponding to E10.5 of mouse embryogenesis), rapid growth of the mesenchyme in the maxillary processes have pushed the nasal pits medially, while the medial nasal processes have grown ventrolaterally, converting the nasal pits from round depressions into dorsally pointed slits (Fig. 1B). At this stage, the upper lip consists of the maxillary processes laterally and the medial nasal processes medially with the lateral nasal processes wedged in between the medial nasal and maxillary processes (Fig. 1C). Fusion between the medial and lateral nasal processes has initiated, while maxillary processes lie below the lateral nasal processes (Fig. 1C). By stage 16 (approximately 38 days of

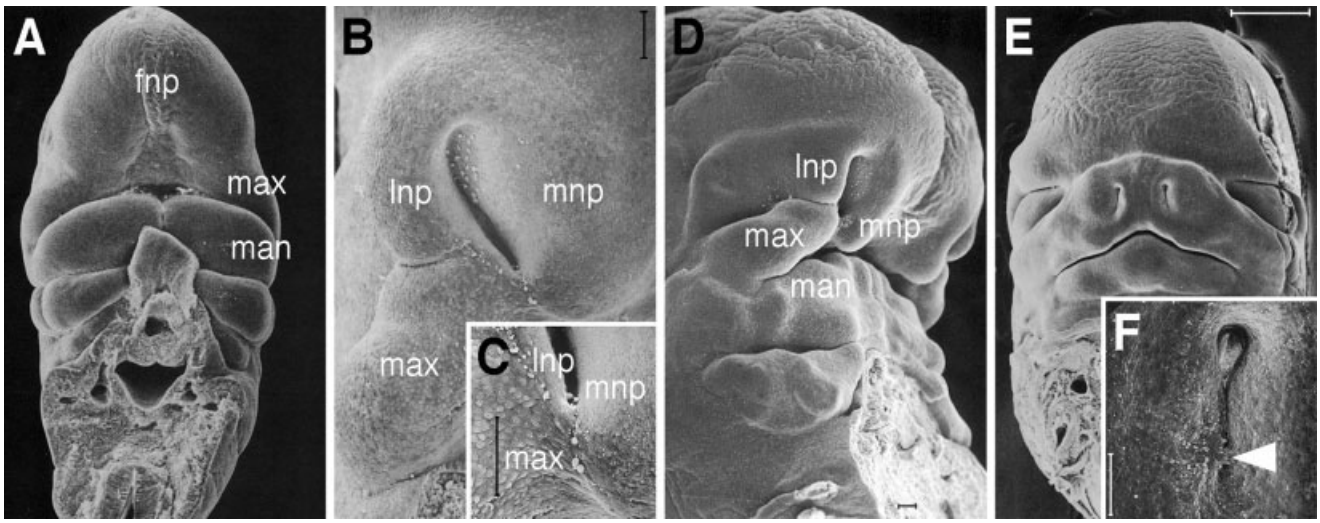

Fig. 1. Morphogenesis of the human upper lip. **A:** Scanning electron microscopy (SEM) facial view of a stage 13 human embryonic head. **B:** SEM micrograph of the right nasal pit of a late stage 15 human embryo. **C:** Enlarged detail of the lower nasal pit shown in B. The boundary between the maxillary and lateral nasal processes is clearly marked by the rounded cells at the surface. Rounded cells also appear at the contact site between the medial and lateral nasal processes. **D:** Lateral view of a stage 17 human embryonic head. The maxillary process is puffed laterally and wedges between the medial and lateral nasal processes. **E:** SEM micrograph of a stage 18 human embryonic head (facial view). **F:** Enlarged detail view of the left nostril of the embryo shown in E. Arrowhead points to distinct epithelial bridges in the lower part of the slit-shaped nostril, which continue to fuse and reduce the nostril. All panels are from Hinrichsen (1985; original figure numbers 4, 15, 17, 27, 46, and 52, copyright of Springer-Verlag Berlin Heidelberg 1985), with kind permission of Springer Science and Business Media. fnp, frontonasal prominence; lnp, lateral nasal process; man, mandibular process; max, maxillary process; mnp, medial nasal process. Scale bars = 100 μm in B–D, 1 mm in E, 10 μm in F.

gestation in human, corresponding to E11.0 of mouse embryogenesis), rapid growth of the maxillary and medial nasal processes have pushed the lateral nasal processes further rostrally in relative position and brought the distal ends of maxillary and medial nasal processes into direct contact (Fig. 1D). Lateral view of the human embryonic face at this stage gives the impression that the maxillary processes are wedged in between the medial and lateral nasal processes (Fig. 1D). High-resolution SEM micrographs of the cynomolgus monkey embryonic face at a similar stage also clearly demonstrated active fusion between the lateral nasal and medial nasal processes as well as between maxillary and medial nasal processes (Fig. 4 in Senders et al., 2003). Studies in mouse embryos showed that fusion between the nasal processes occurred initially at the posterior part of the nasal pits and proceeded in an anterior direction (Trasler, 1968; Gaare and Langman, 1977a), similar to what Hinrichsen described for human embryonic face development (Hinrichsen, 1985).

Facial morphogenesis in chick is slightly different from that in mammals, because the medial nasal process appears as a single entity sometimes referred as the frontal or frontonasal process, and the entire embryonic chick face appears in a square configuration before lip fusion (Yee and Abbott, 1978; Young et al., 2000; Cox, 2004). Despite the differences, close examination of SEM micrographs of the early fusion stage chick face showed that the initial contact and initiation of active cellular processes of fusion also begins between the lateral and medial nasal processes (Fig. 2 in Cox, 2004).

Trasler (1968) emphasized the importance of fusion between medial and lateral nasal processes and postulated that lateral cleft lip results when this fusion process does not occur. Ohbayashi and Eto (1986) carried out a microsurgical assay of relative contributions of the different facial processes in facial morphogenesis in rat embryos and found that surgical removal of either a lateral nasal or a maxillary process from one side of the face did not prevent fusion of the other process with the medial nasal process, whereas removal of the distal part of a medial nasal process resulted in cleft lip on the surgical side. These results indicate that contact and fusion between maxillary and medial nasal processes are not dependent on the prior fusion between the lateral and medial nasal processes. Once upper lip morphogenesis is complete (described below), the lateral nasal processes form the sides (alae) of the nose, whereas the intact upper lip is composed of tissues derived from the medial nasal and maxillary processes. Although the lateral nasal processes do not contribute to the final upper lip, the type of cleft lip in which the cleft extends into the nostril is clearly indicative of failure of fusion of the medial nasal processes with both maxillary and lateral nasal processes during upper lip development.

Whereas the union between the freely projected maxillary, lateral nasal, and medial nasal processes clearly involves active epithelial fusion, closure of the median groove between the paired medial nasal processes in mammals does not (Trasler, 1968; Millicovsky and Johnston, 1981; Millicovsky et al., 1982; Trasler and Ohannessian, 1983; Hinrichsen, 1985; Senders et al., 2003; Cox, 2004). As the epithelial fusion between maxillary, lateral nasal, and medial nasal processes continues from stage 16 to stage 18 (toward the beginning of the seventh week of gestation in human,

corresponding to E11.5 to E12.0 of mouse embryogenesis), the maxillary processes continue to grow rapidly and push the nasal pits and medial nasal processes mediofrontally (Hinrichsen, 1985). The groove between the medial nasal processes becomes gradually shallow and eventually smooth as a result of continued growth and confluence of medial nasal and maxillary mesenchyme (Fig. 1E). These morphogenetic processes also gradually convert the nasal pits to nose chambers and to nasal ducts as the fusion between the medial and lateral nasal processes is completed. The choanal membranes at the dorsal ends of the nose chambers, however, are not perforated until stage 18 to connect the nostrils to the posterior oral cavity. During the final stages of upper lip formation, the nostrils are transformed to small slits and their lower edge remodeled by the fusion between the medial nasal and maxillary processes (Hinrichsen, 1985; Fig. 1F).

By stage 19 (approximately 48 days of gestation in human, corresponding to E12.5 of mouse embryogenesis), after disintegration of the epithelial seams and mesenchymal confluence between medial nasal and maxillary processes, formation of the upper lip is complete, with the intermaxillary segment derived from the distal part of the medial nasal processes forming the central lip. The medialization of the nose chambers and the filling of the median groove by mesenchyme are followed by outgrowth of the intermaxillary segment into the oral cavity to form the anterior part of the palate (Hinrichsen, 1985). Some authors referred to this anterior, intermaxillary palate as the "primary palate," whereas others used "primary palate" to describe the tissues formed by fusion between the maxillary and medial nasal processes (Diewert and Wang, 1992; Wang et al., 1995; Sperber, 2002; Cobourne, 2004). The anterior palate derived from the intermaxillary process later fuses with the secondary palate derived from the maxillary processes.

Development of the secondary palate has been reviewed extensively (e.g., Ferguson, 1988; Murray and Shutte, 2004; Nawshad et al., 2004). Because fusion between the secondary palatal shelves, which arise bilaterally from the maxillary processes (Ferguson, 1988), and fusion between the primary and secondary palates occur much later in embryogenesis than the fusions between maxillary, lateral, and medial nasal processes during lip formation, failure of proper lip fusion often affects palatal contact secondarily. Therefore, cleft lip is often accompanied by cleft palate.

Normal lip fusion involves a series of remarkable cellular transformations as the freely projected medial nasal, lateral nasal, and maxillary processes are brought into proximity by proliferation of the neural crest-derived mesenchyme. In chick embryos, as the maxillary and medial nasal processes near each other and prepare for fusion, the periderm covering these processes undergo region-restricted apoptosis, resulting in their sloughing off (Sun et al., 2000). SEM analysis of human embryos at the beginning of lip fusion (stage 16) showed many rounded cells appearing to detach from the surface of the furrow between the maxillary and lateral nasal processes as well as at the caudal end of the nasal pits where the medial and lateral nasal processes are in direct contact (Hinrichsen, 1985; Fig. 1B,C). These rounded cells probably represent dead cells extruded during the fusion between the maxillary and lateral nasal processes and between the lateral and medial nasal processes. It has been hypothesized that death of periderm cells promote epithelial adherence by exposing basal layers of the opposed epithelia and permitting adherence junctions such as desmosomes to form between them (Sun et al., 2000). The death of periderm cells before contact of the prefusion epithelia of facial processes has also been observed in hamster, mouse, and rat embryos and has been proposed to play an important role in secondary palatal fusion (Lejour, 1970; Chaudhry and Shah, 1973; Hinrichsen and Stevens, 1974; Gaare and Langman, 1977b; Fitchett and Hay, 1989; Holtgrave et al., 2002).

As the free ends of the facial processes are brought into proximity, epithelial filopodia in highly localized primary fusion areas begin to span and establish bridges between these facial processes (Gaare and Langman, 1977b; Millicovsky and Johnston, 1981; Millicovsky et al., 1982; Hinrichsen, 1985; Senders et al., 2003; Cox, 2004). These filopodia anchor into the surface of the opposing prominences by penetrating between surface cells and are reinforced by the accumulation of larger cellular extensions and adhering junctions (Millicovsky and Johnston, 1981; Sun et al., 2000). Filopodial attachments are greatly reduced in A/WySn and CL/Fr mouse embryos, two strains with high frequency of spontaneous CLP (Millicovsky et al., 1982; Forbes et al., 1989). Similarly, filamentous projections have been observed in chick embryos between the fusing facial prominences and are notably missing from the *cleft primary palate* chick mutant embryos (Yee and Abbott, 1978, Cox, 2004). These observations, therefore, correlate the presence of filopodial processes spanning the prefusion primordia with an ability to fuse.

Comparisons of embryonic faces of cleft-predisposing and noncleft mouse strains indicated that facial geometry also plays an important role in lip development (Trasler, 1968; Millicovsky et al., 1982). It was demonstrated that embryos of both the A/J and CL/Fr strains, which have high frequency of spontaneous cleft lip, have more prominent and more medially convergent medial nasal processes than those of the C57BL/6 strain, which has a negligible spontaneous incidence of cleft lip (Millicovsky et al., 1982; Trasler and Ohannessian, 1983). It was postulated that the spontaneous cleft lip in the A/J and CL/Fr strains is a threshold character where a slight change in the divergence of the medial and lateral nasal processes leads to their partial or complete lack of fusion. Thus, the fusion process requires temporal coordination of surface changes in the prefusion epithelia and proper facial geometry for approximation of the facial prominences (Johnston and Millicovsky, 1985).

IS PROGRAMMED CELL DEATH, EMT, OR BOTH THE MECHANISM INVOLVED IN LIP FUSION?

Fusion of the medial and lateral nasal processes generates an intervening epithelial seam known as the nasal fin, which is subsequently broken

down and replaced by continuous mesenchyme between the processes (Trasler, 1968; Gaare and Langman, 1977b). Similarly, fusion between maxillary and medial nasal processes also generates an epithelial seam that is subsequently replaced by mesenchymal tissue (Wang et al., 1995; Sun et al., 2000). The fate of the epithelial seam cells during lip fusion primarily has been analyzed using transmission electron microscopy (TEM) and lipophilic dye cell labeling, whereas terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) assay was used to detect apoptotic cells. Gaare and Langman (1977b) investigated nasal fin regression during lip fusion in mouse embryos using TEM and reported that degenerating epithelial cells, characterized by an electron-dense nucleus and cytoplasm, were a prominent feature in the fusion of the nasal swellings. They showed that the number of degenerating cells in the contacting epithelial linings was considerably higher than in the nonfusing epithelia and surrounding mesenchyme and considered the fusing epithelia "cell-death zones." However, they also reported that most of the epithelial cells appeared healthy but did not mix with the mesenchyme at the stage of nasal fin regression and suggested that the surviving seam cells were probably incorporated into the neighboring epithelial linings rather than transformed into mesenchyme (Gaare and Langman, 1977b). Sun et al. (2000) examined lip fusion in chick embryos using TEM and TUNEL assays and also found that the epithelial seam cells were healthy looking and very few were TUNEL-positive. They then used 5,6-carboxy-2,7-dichlorofluoresscein diacetate succinimidyl ester, a lipophilic dye, to label the entire surface epithelia of chick embryos before lip fusion and found, after 24 hr, that there were labeled mesenchyme-like cells in the facial region after breakdown of the fusing epithelial seam between the medial nasal and maxillary processes. Thus, Sun et al. (2000) concluded that the epithelial seam cells transform into mesenchyme during lip fusion. However, questions remain about the fate of the epithelial seam cells. Could the few labeled cells be due to dye transfer into internal mesenchymal cells or to phagocytosis of dead labeled epithelial cells by macrophages? Even if the seam cells indeed transdifferentiate into mesenchyme, do they contribute to mesenchyme-derived structures later or do they die shortly after EMT?

With regard to the fate of the fusing epithelial seam, whether apoptosis or EMT, it is believed that similar mechanisms are involved in lip fusion and secondary palate fusion (Gaare and Langman, 1977b; Sun et al., 2000; Cox, 2004). In mammals, the secondary palate arises as bilateral palatal shelves that initially grow vertically and later elevate to the horizontal position above the tongue and fuse with each other at the midline to form the roof of the oral cavity (Ferguson, 1988; Murray and Schutte, 2004). In contrast to the few studies of the lip fusion process, the fate of the medial edge epithelial (MEE) cells of the secondary palatal shelves, which form the midline epithelial seam upon palatal shelf adhesion, has been studied extensively although considerable disagreement still exists. TEM and cell biological studies have provided clear evidence of apoptosis of at least a portion of the MEE cells (Glucksmann, 1965; Saunders, 1966; DeAngelis and Nalbandian, 1968; Smiley and Dixon, 1968; Shapiro and Sweney, 1969; Smiley and Koch, 1975; Mori et al., 1994; Taniguchi et al., 1995; Cuervo et al., 2002; Cuervo and Covarrubias, 2004). Others, however, reported that the midline epithelial seam cells looked healthy at the TEM level and found evidence of transdifferentiation of MEE cells into mesenchymal cells by using various cell labeling techniques (Fitchett and Hay, 1989; Shuler et al., 1991, 1992; Griffith and Hay, 1992; Sun et al., 1998; Martinez-Alvarez et al., 2000; Nawshad et al., 2004). Because large numbers of apoptotic cells in the fusing epithelial seam were only observed in palatal explant cultures (Martinez-Alvarez et al., 2000; Cuervo et al., 2002; Cuervo and Covarrubias, 2004), Nawshad et al. (2004) and Hay (2005) suggested that the observed dying cells in the seam were trapped dying periderm cells and argued in favor of EMT of palatal MEE cells. To answer definitively whether the MEE cells contribute to the palatal mesenchyme in vivo, Vaziri Sani et al. (2005) used the Cre/loxP-mediated genetic labeling approach to trace the MEE cells during mouse palate development. In their experiments, mice carrying the *shh-GFPCre* or *K14-Cre* transgene were crossed to mice carrying the *loxP-STOP-loxP-lacZ* cassette targeted into the *ROSA26* locus (*R26R*). The *Rosa26* gene promoter normally drives ubiquitous gene expression (Zambrowicz et al., 1997). However, the *loxP*-flanked transcription STOP cassette prevents the *lacZ* gene from being transcribed in the *R26R* mice (Soriano, 1999). Crossing the *shh-GFPCre* transgenic mice with the *R26R* mice results in the Cre recombinase specifically removing the STOP cassette from 5′ of the *lacZ* gene by excising sequences in between the loxP sites in the double transgenic mice, which activates β-galactosidase expression permanently from the *lacZ* gene at the *ROSA26* locus in all cells derived from *ShhGFPCre*-expressing cells. Because the *ShhGFPCre* fusion gene is expressed in the palatal epithelium but not in the palatal mesenchyme, any β-galactosidase–expressing palatal mesenchyme cell in the *ShhGFPCre;R26R* double transgenic mice would have to be derived from the palatal epithelium during palatal fusion. Similarly, the *K14-Cre* transgenic mice express Cre under the *keratin-14* promoter, which is activated in all epithelial cells after E11.75. Vaziri Sani et al. (2005) found well-labeled palatal epithelial cells, including palatal MEE cells before their developmental disappearance from the palatal midline, in both *ShhGFPCre;R26R* and *K14-Cre;R26R* embryos but never saw any evidence of palatal mesenchymal cells displaying specific β-galactosidase activity even after total disappearance of the β-galactosidase–positive midline epithelial seam. Furthermore, Vaziri Sani et al. (2005) reported that the regressing midline epithelial seam cells and epithelial islands formed during palatal fusion expressed activated Caspase-3, an early marker for apoptosis. These data indicate that MEE cells undergo programmed cell death rather than transdifferentiate into palatal mesenchyme during palatal fusion in vivo.

In light of the new evidence favoring

programmed cell death as the major mechanism for palatal fusion, we analyzed programmed cell death in mouse embryos during fusion of the medial and lateral nasal processes by using immunostaining for activated Caspase-3. As shown in Figure 2, we found that a lot of the epithelial seam cells between the fusing medial and lateral nasal processes express activated Caspase-3, indicating that many epithelial seam cells are fated to degenerate by apoptosis. These data suggest, like in secondary palatal fusion, that programmed cell death plays an important role in lip fusion.

It is conceivable that some epithelial cells of the fusing seam may remain viable and become incorporated into the facial epithelium as the facial mesenchyme rapidly expands. Epithelial seam cells in the secondary palate have been observed to migrate along the midline to contribute to the oral and nasal epithelia of the fused palate in some species (Carette and Ferguson, 1992). Further studies will be necessary to address whether any epithelial cells transdifferentiate and contribute to mesenchymal structures of the face or what was called EMT during lip fusion was just the cellular processes of shape changes, filopodial interactions, and intercalation of the epithelial seam cells before they degenerate.

GENES AND MOLECULAR PATHWAYS CRITICAL FOR UPPER LIP DEVELOPMENT

It is clear that growth and morphogenesis of the facial primordia have to be exquisitely coordinated to develop the intact face. Because most of the craniofacial mesenchyme is derived from neural crest cells, genes and molecular pathways regulating neural crest formation, migration, patterning, proliferation, and apoptosis, are all important for craniofacial development. Various aspects of cranial neural crest development and the roles of neural crest in craniofacial development have been reviewed recently by others (e.g., Wilkie and Morris-Kay, 2001; Chambers and McGonnell, 2002; Basch et al., 2004; Cox, 2004; Huang and Saint-Jeannet, 2004; Graham et al., 2004; Kulesa et al., 2004; Marazita and Mooney, 2004; Helms et al., 2005). We will focus on discussing the genes and molecular pathways critical for upper lip morphogenesis after the five facial prominences have formed.

Whereas rapid proliferation of the neural crest derived mesenchyme is the driving force of facial morphogenesis, fate mapping and tissue recombination experiments in chick showed that proliferation and directed expansion of the facial mesenchyme depend on signals from the facial epithelia (Wedden, 1987; Richman and Tickle, 1989; McGonnell et al., 1998). At the same time, signals from the mesenchyme also influence development of the facial ectoderm (reviewed in Francis-West et al., 1998; Jernvall and Thesleff, 2000). The reciprocal interactions involve many intercellular signaling pathways. We will discuss below the current understanding of the major molecular pathways critical for midfacial growth and upper lip morphogenesis.

The Bmp Pathway

Bmps (bone morphogenetic proteins) are a group of secreted signaling molecules of the transforming growth factor beta (Tgfβ) superfamily (Wozney et al., 1988). This family of ligands initiates signaling by binding and bringing together two types of receptor serine/threonine kinases on the cell surface (reviewed in Shi and Massague, 2003; Nohe et al., 2004). Upon ligand binding, the type II receptor phosphorylates and activates the type I receptor, which in turn phosphorylates a set of transcriptional coactivators called Smads and leads to their nuclear translocation and transcriptional activation of downstream target genes. The Bmp signaling pathway has been shown to regulate diverse developmental processes, including cell proliferation, differentiation, apoptosis, and tissue morphogenesis (reviewed in Wan and Cao, 2005). Francis-West et al. (1994) first showed that *Bmp2* and *Bmp4* mRNAs were expressed in dynamic, spatiotemporally regulated patterns in the developing chick facial primordia, with *Bmp4* having highly restricted expression in the distal epithelia of the medial nasal, lateral nasal, maxillary and mandibular processes. Ectopic application of Bmp2 or Bmp4 protein induced overgrowth and changed the patterning of the chick facial primordia (Barlow and Francis-West, 1997). On the other hand, inhibiting Bmp signaling by application of Noggin, a specific Bmp antagonist, in the chick facial primordia caused reduced mesenchymal proliferation and outgrowth (Ashique et al., 2002; Wu et al., 2004). Moreover, recent expression and functional assays in fish and birds also suggested that Bmp signaling plays an important role in the evolution of facial shape and size (reviewed in Helms et al., 2005, and references therein).

Interestingly, expression patterns of *Bmp2* and *Bmp4* in the facial ectoderm correlated with the largely overlapping mesenchymal expression domains of the homeobox genes *Msx1* and *Msx2* in the developing facial primordia. Moreover, ectopic Bmp2 or Bmp4 activated *Msx1* and *Msx2* gene expression in the facial mesenchyme (Barlow and Francis-West, 1997), suggesting the Msx1 and Msx2 are downstream transcription factors of the Bmp pathway. *Bmp4* is also expressed in the distal ectoderm of the facial primordia surrounding the stomodeum before and during lip fusion in mouse embryos (Gong and Guo, 2003; Fig. 3A), whereas *Msx1* and *Msx2* are expressed in the adjacent facial mesenchyme (Fig. 3B,C). Heterozygous loss of function of the *MSX1* gene has been associated with CLP and tooth agenesis in humans (van den Boogaard et al., 2000). Furthermore, missense mutations and variants in the *MSX1* gene have been associated with nonsyndromic CLP (Lidral et al., 1998; Jezewski et al., 2003). Although mice deficient in *Msx1* have cleft palate but not CLP (Satokata and Maas, 1994), mice lacking both *Msx1* and *Msx2* gene function exhibit bilateral CLP (Y. Chai, personal communication). Msx1 and Msx2 likely play critical roles in facial mesenchymal proliferation, as $Msx1^{-/-}$ mutant mice have shortened maxilla and mandibles as well as defects in palatal mesenchyme proliferation (Satokata and Maas, 1994; Zhang et al., 2002). Introduction of a *Bmp4* transgene under the control of *Msx1* promoter rescued the palatal growth defect in $Msx1^{-/-}$ mutant mice (Zhang et al., 2002). These data

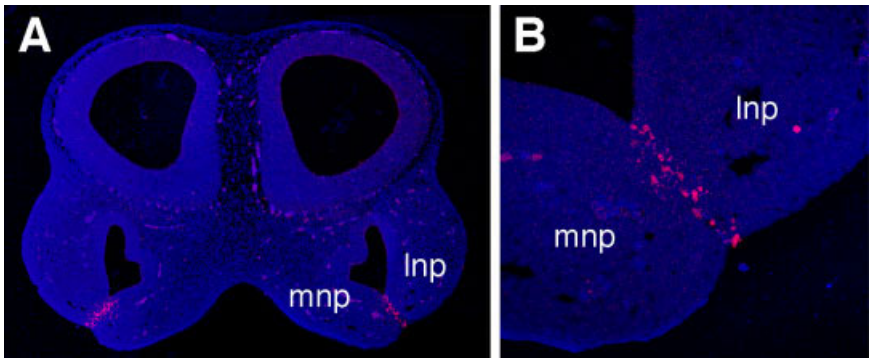

Fig. 2. Apoptosis plays an important role in breakdown of the epithelial seam during lip fusion. **A:** Frontal section of an embryonic day (E) 11.0 mouse embryo through the telencephalon and the fusing medial and lateral nasal processes. Red signal marks specific anti-active Caspase-3 antibody staining. **B:** High-magnification view of the fusing epithelial seam between the medial and lateral nasal processes shown in A. Many of the fusing epithelial cells express active Caspase-3, while very few nasal mesenchyme cells and epithelial cells in other regions express active Caspase-3, indicating specific programmed cell death of the fusing epithelial cells. lnp, lateral nasal process; mnp, medial nasal process.

indicate that Bmp4 and Msx1/Msx2 function in a common molecular pathway essential for facial growth and upper lip morphogenesis.

Recently, Liu et al. (2005b) reported that tissue-specific inactivation of either *Bmp4* or a Bmp type I receptor (*Bmpr1a*) gene in the facial primordia caused cleft lip. Interestingly, inactivation of *Bmpr1a* caused elevated apoptosis in both the prefusion epithelium and the distal medial nasal mesenchyme (Liu et al., 2005b), whereas inhibition of BMP signaling in the chick facial primordia with Noggin increased epithelial survival (Ashique et al., 2002). Another interesting finding by Liu et al. (2005b) was that many of the mouse embryos with fa-

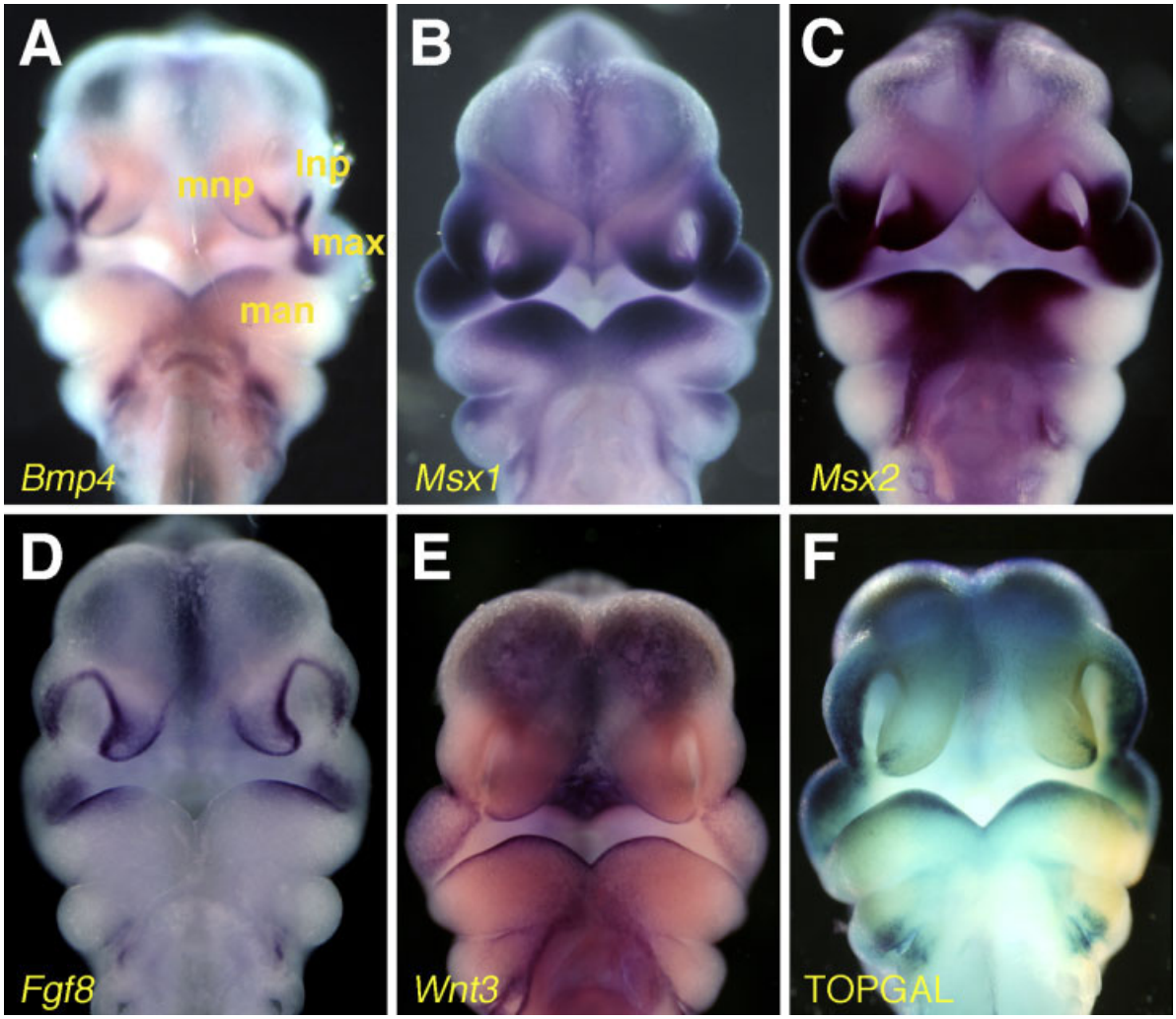

Fig. 3. Selected gene expression patterns in the developing facial primordia of embryonic day (E) 10.5 mouse embryos. **A:** Whole-mount in situ hybridization showing specific expression of *Bmp4* mRNA (blue/purple staining) in the distal ectoderm of the lateral nasal, medial nasal, maxillary, and mandibular processes. **B,C:** *Msx1* (B) and *Msx2* (C) mRNAs are expressed in overlapping patterns in the distal lateral nasal, medial nasal, maxillary, and mandibular mesenchyme. **D:** *Fgf8* mRNA is expressed dynamically in the ectoderm around the nasal pits as well as in the proximal maxillary and mandibular ectoderm. **E:** *Wnt3* mRNA is expressed in the maxillary and rostral mandibular ectoderm as well as in the distal medial nasal ectoderm. **F:** X-gal staining of an E10.5 hemizygous *TOPGAL* transgenic mouse embryo showing β-galactosidase activity in the distal ectoderm of the lateral nasal, medial nasal, maxillary, and mandibular processes. lnp, lateral nasal process; man, mandibular process; max, maxillary process; mnp, medial nasal process.

cial epithelial inactivation of *Bmp4* had delayed lip fusion, but the initial cleft lip was repaired by E14.5 in most mutants, perhaps due to functional complementation by or cross-regulation of other *Bmp* family genes. In addition, Ashique et al. (2002) showed that either inhibition or enhancement of BMP signaling in the facial primordia caused defective lip fusion. These data indicate that Bmp signaling is tightly regulated during upper lip development. Whereas defects in maxillary mesenchyme proliferation in the *Bmpr1a* conditional mutants is consistent with a role for Bmp signaling in promoting facial primordial outgrowth (Liu et al., 2005b), the role of Bmp signaling in facial ectoderm survival and in the lip fusion process needs to be further investigated.

The Fgf Pathway

Fgfs (fibroblast growth factors) and their cell surface receptors (Fgfr) make up a large and complex family of signaling molecules that play important roles in a variety of processes of embryogenesis and tissue homeostasis (for recent reviews, see Itoh and Ornitz, 2004; Chen and Deng, 2005; Dailey et al., 2005; Eswarakumar et al., 2005). There are 22 *Fgf* genes in humans and mice, several of which are expressed in partially overlapping and dynamic patterns in the developing mouse facial primordia (Francis-West et al., 1998; Colvin et al., 1999; Bachler and Neubuser, 2001). In particular, *Fgf8* is expressed broadly in the frontonasal and mandibular epithelia before outgrowth of the nasal processes and its expression becomes highly localized to around the nasal pits as well as in the maxillary and mandibular epithelia during active facial primordial outgrowth (Bachler and Neubuser, 2001; Fig. 3D). Studies using mandibular and nasal explant cultures showed that Fgf8 protein can substitute for the facial ectoderm to stimulate mesenchymal proliferation and maintain mesenchymal gene expression (Neubuser et al., 1997; Firnberg and Neubuser, 2002), suggesting that Fgf signaling regulates facial primordial outgrowth. Direct genetic analysis of the roles of *Fgf* genes in facial morphogenesis, however, has been complicated by early embryonic lethality and functional redundancy (reviewed in Dailey et al., 2005). Nevertheless, analysis of mouse mutants carrying hypomorphic alleles of *Fgf8* demonstrated that it is required for survival of the neural crest derived facial mesenchyme (Abbu-Issa et al., 2002; Frank et al., 2002). Moreover, tissue-specific inactivation of *Fgf8* in the mandibular epithelium showed that it is required for mandibular mesenchymal survival as well as proximodistal patterning (Trumpp et al., 1999), whereas specific inactivation of *Fgf8* in the forebrain and facial ectoderm led to severe facial defects, including midfacial cleft (Firnberg and Neubuser, 2002). In addition, despite broad overlapping expression of *Fgfr1* and *Fgfr2* in the developing facial primordia, analysis of various mutations in these genes in mice have demonstrated essential roles of Fgf signaling in neural crest migration, survival, proliferation, and patterning of both the facial epithelia and mesenchyme (Trokovic et al., 2003; Rice et al., 2004). These, together with the recent findings that nonsense mutations and deletions in the *FGFR1* gene in humans cause Kallmann syndrome, an autosomal dominant disorder characterized by infertility and anosomia but in which 5% of patients have CLP (Dode et al., 2003; Kim et al., 2005), indicate that Fgf signaling plays essential roles in midfacial growth and upper lip development.

The Shh Pathway

Shh is a member of the Hedgehog family of secreted proteins and possesses remarkable morphogenetic patterning activity (reviewed in Ingham and McMahon, 2001). It is involved in numerous key developmental events during embryogenesis, including left–right axis establishment, dorsoventral patterning of the neural tube, endoderm development, limb and craniofacial development, brain and pituitary development, among others (reviewed in Ingham and McMahon, 2001; McMahon et al., 2003; Roessler and Muenke, 2003, and references therein). The Shh signaling pathway is also involved in many human diseases, particularly holoprosencephaly and cancer (reviewed in Mullor et al., 2002; Roessler and Muenke, 2003). Shh signals to cells by binding to its cell surface receptor Patched1 (Ptch1) to relieve its inhibition of Smoothened (Smo), a seven-transmembrane protein obligatory for the activation of downstream targets of the Shh pathway. Through a series of steps that are currently not entirely understood, Smo activation leads to conversion of members of the Gli family of transcription factors from repressors to transcriptional activators and to activation of downstream gene expression. One of the downstream target genes of Shh signaling is *Ptch1*, thus establishing a feedback regulatory loop (reviewed in Ingham and McMahon, 2001; McMahon et al., 2003).

During facial outgrowth, *Shh* is expressed in the ectoderm of the facial primordia (Echelard et al., 1993; Hu and Helms, 1999; Jeong et al., 2004). Whereas a targeted null mutation in *Shh* caused severe cranial deficiencies that initially precluded direct assessment of the role of *Shh* in facial morphogenesis (Chiang et al., 1996), inhibition of Shh signaling in the outgrowing chick frontonasal process with a function blocking antibody inhibited facial outgrowth and caused cleft lip (Hu and Helms, 1999). Ahlgren and Bronner-Fraser (1999) showed that inhibition of Shh in the cranial mesenchyme also caused neural crest mesenchymal cell death. Moreover, Ahlgren et al. (2002) demonstrated that application of Shh protein rescued cranial mesenchymal death in chick embryos induced by ethanol treatment. These data indicate that Shh signaling is required for facial mesenchyme survival. In addition, Hu and Helms (1999) demonstrated that Shh might also regulate facial mesenchyme proliferation as ectopic application of Shh protein to the frontonasal process caused mediolateral expansion of that tissue. Tissue specific inactivation of *Smo* in the cranial neural crest further confirms that Shh signaling is required for both survival and proliferation of the facial mesenchyme (Jeong et al., 2004). Cranial neural crest cells lacking *Smo* migrated and formed facial primordia normally in mouse embryos but exhibited high levels of apoptosis from E9.5 to E10.5 and reduced cell proliferation at E11.5, indicating that *Shh* expression in the facial ectoderm specifically

supports cell survival during early stages and promotes proliferation at later stages to control the size of the facial primordia (Jeong et al., 2004). Interestingly, whereas overactivation of Shh signaling by loss of the inhibitor *Gli3* or constitutive activation of *Smo* in the neural crest causes slight overgrowth of the facial primordia, some patients with mutations in *PTCH1* have bilateral CLP (Hahn et al., 1996; Aoto et al., 2002; Jeong et al., 2004), suggesting that Shh signaling is regulated at multiple levels during facial morphogenesis.

The Wnt Pathway

The Wnt family of secreted glycoproteins bind cell surface receptors of the Frizzled (Fzd) family and signal through several different intracellular signal transduction pathways to regulate diverse developmental processes, including cell proliferation, cell fate determination and differentiation, and cell survival (reviewed in Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Eastman and Grosschedl, 1999; Huelsken and Birchmeier, 2001). The best characterized Wnt signaling pathway, termed the canonical Wnt pathway, signals through β-catenin, a dual functional protein involved in cell adhesion and signaling (reviewed in Bienz, 2005). In cells without Wnt signaling, cytoplasmic β-catenin is rapidly degraded through the ubiquitin–proteosome pathway. In cells responding to canonical Wnt signaling, β-catenin is stabilized and enters the nucleus to activate the Tcf/Lef family transcription factors and regulate transcription of downstream genes. Although several *Wnt* genes as well as *Tcf1* and *Lef1* are known to be expressed in the developing facial primordia (Gavin et al., 1990; Oosterwegel et al., 1993; Parr et al., 1993; Christiansen et al., 1995; Wang and Shackleford, 1996), a direct role for Wnt signaling in facial morphogenesis was not known until recently. In search for genes conferring susceptibility to spontaneous CLP in the A strains of mice, Juriloff and colleagues genetically mapped an essential causal recessive mutation, *clf1*, to a small region of mouse chromosome 11 containing the closely linked *Wnt3* and *Wnt9b* genes (Juriloff and Mah, 1995; Juriloff et al., 1996, 2001). Recently, Niemann et al. (2004) reported associations of a nonsense mutation in the *WNT3* gene with tetra-amelia, a rare recessive genetic disorder in humans characterized by complete absence of all four limbs and other anomalies, including CLP. Carroll et al. (2005) reported that a targeted mutation in the *Wnt9b* gene in mice caused severe kidney developmental defects and an incomplete penetrance of CLP. Although the *clf1* locus did not contain any coding mutation in the *Wnt3* and *Wnt9b* genes, direct sequence analysis showed that *clf1* is associated with a retrotransposon insertion at 6.6 kb downstream of the *Wnt9b* gene (Juriloff et al., 2004, 2005). These data indicate that both Wnt3 and Wnt9b play important roles in midfacial morphogenesis.

To understand what roles *Wnt3* and *Wnt9b* may have during facial development, we analyzed their expression patterns during mouse embryogenesis. We found that both *Wnt3* and *Wnt9b* mRNAs are expressed in the ectoderm of the developing facial primordia (Ryan et al., manuscript submitted for publication; Fig. 3E). Furthermore, we found that canonical Wnt signaling is specifically activated in the prefusion epithelia and in the underlying mesenchyme in the medial nasal, lateral nasal, and maxillary processes, as demonstrated by expression of the specifically responsive *TOPGAL* transgene (DasGupta and Fuchs, 1999; Merrill et al., 2004; Ryan et al., manuscript submitted, Fig. 3F). These data, together with the CLP phenotype in *WNT3*$^{-/-}$ humans and *Wnt9b*$^{-/-}$ mutant mice, suggest that the canonical Wnt signaling pathway directly regulates facial mesenchymal growth and lip fusion. Of interest, the domains of active canonical Wnt signaling in the developing facial primordia overlap significantly with the domains of *Bmp4* gene expression (Gong and Guo, 2003; Liu et al., 2005b; compare Fig. 3A with F). Previously, it has been demonstrated that Wnt/β-catenin signaling acts upstream of *Bmp4* expression during limb and lung development and that in cell transfection assays Wnt/β-catenin signaling can activate the mouse *Bmp4* promoter directly through evolutionarily conserved Tcf/Lef binding sites (Barrow et al., 2003; Soshnikova et al., 2003; Shu et al., 2005). Thus, it is possible that Wnt signaling acts upstream or interacts with the Bmp4 pathway to regulate midfacial morphogenesis.

Other Genes and Pathways

Many other genes have been implicated in upper lip development. Over 300 Mendelian syndromes in humans include CLP as part of the phenotype (Gorlin et al., 2001). Genes for several of these have been identified, including *PVRL1* in CLP-ectodermal dysplasia syndrome (CLPED1), *P63* in dominant ectrodactyly with ectodermal dysplasia and CLP (EEC) and related syndromes, and *IRF6* in Van der Woude syndrome (recently reviewed in Cobourne, 2004; Cox, 2004; Marazita and Mooney, 2004). In addition, mutations in *E-cadherin* (*CDH1*) were recently found in two families with hereditary diffuse gastric cancer associated with CLP (Frebourg et al., 2005) and mutations in *EFNB1* in craniofrontonasal syndrome (CFNS; Twigg et al., 2004; Wieland et al., 2004). Interestingly, *PVRL1*, *P63*, *IRF6*, and *CDH1* are all predominantly expressed in epithelial tissues, indicating that proper epithelial differentiation, organization, or patterning play important roles in lip development.

Whereas CLP is common in humans, CLP is rare in mice, although many mutant mouse strains exhibit CP. In addition to the A strains of mice described above, mice homozygous for either of two spontaneous mutations, *Dancer* and *Twirler*, exhibit high penetrance of CLP (Lyon, 1958; Deol and Lane, 1966, Gong et al., 2000). Whereas the *Twirler* gene remains to be identified, Bush et al. (2004) recently positionally cloned the *Dancer* mutation and showed that the CLP phenotype in the *Dancer* homozygous mutants results from widespread misexpression of the *Tbx10* gene due to insertion of a heterologous promoter. How *Tbx10* misexpression disrupts the normal molecular and cellular programs of facial morphogenesis remains to be determined.

Components of several other signaling pathways, including Tgfα/Egf, Pdgf, and retinoic acid pathways are

expressed during craniofacial development and gene knockout studies in mice have confirmed the involvement of these pathways in upper lip morphogenesis (reviewed in Francis-West et al., 1998, 2003). Mice lacking *Egfr* exhibit a low penetrance of CLP (Miettinen et al., 1999), whereas the *TGF*α locus has been associated with nonsyndromic CLP in some human populations (reviewed in Schutte and Murray, 1999; Cobourne, 2004). Mice carrying a null mutation in *Pdgfr*α and mice homozygous for mutations in both the *Pdgfr*α and *Pdgfc* genes have a median cleft (Soriano, 1997; Ding et al., 2004). Pdgfr function is apparently autonomous to the neural crest, because conditional disruption of *Pdgf*α in neural-crest cells results in a similar facial cleft (Tallquist et al., 2003). Mice harboring mutations in both the retinoic acid receptor genes *RAR*α and *RAR*β also display a severe median cleft and defects in other neural crest-derived structures (Lohnes et al., 1994; Johnston and Bronsky, 1995).

Many transcription factors of different classes are expressed in spatiotemporally regulated patterns in the developing facial primordia (reviewed in Francis-West et al., 1998, 2003). A subset of the Aristaless-like family of homeobox transcription factors apparently plays an important role in regulating morphogenesis of the frontonasal processes (Meijlink, 1999; Qu et al., 1999; Beverdam et al., 2001). Although single mutations in any of the *Alx3*/*Alx4*/*Cart1* genes do not display orofacial clefting, $Alx3^{-/-}Alx4^{-/-}$ or $Alx4^{-/-}Cart1^{-/-}$ double mutants display median cleft lip and cleft palate, indicating a degree of redundancy in this subfamily of transcription factors (Qu et al., 1999; Beverdam et al., 2001). In the case of $Alx3^{-/-}Alx4^{-/-}$ double mutants, the median cleft phenotype has been attributed to defects in survival of the frontonasal mesenchyme and failure of the medial nasal processes to merge properly (Beverdam et al., 2001). The *AP2*α gene also plays an important role in midfacial morphogenesis, because mice chimeric for a null mutation in *AP2*α exhibited CLP (Nottoli et al., 1998). Further compound mutant and conditional gene inactivation studies will help elucidate how interactions of different transcription factors integrate various signals from the facial ectoderm to regulate facial primordial outgrowth and upper lip morphogenesis.

SUMMARY AND PERSPECTIVES

In summary, upper lip development involves a series of highly coordinated, genetically programmed morphogenetic events that include directed growth and expansion of the facial prominences, programmed cell death, active fusion, and subsequent breakdown of the epithelial seam between the initially freely projected maxillary, medial nasal, and lateral nasal processes. Even subtle abnormalities in any one of these events may lead to a CLP phenotype. These developmental weak points along with the significant number of genes and signaling pathways involved in the morphogenetic processes provide an explanation for the frequent occurrence and genetic heterogeneity of CLP in humans.

The complete sequencing of the human genome brought development of increasingly high throughput genotyping capabilities, which has led to rapid identification of genes involved in Mendelian syndromes as well as candidate genes for complex genetic diseases such as CLP (reviewed in Lidral and Murray, 2004). At the same time, more and more sophisticated approaches are being developed to efficiently analyze gene function in specific developmental and cellular processes in animal model systems, which have significantly advanced our understanding of genes and molecular pathways involved in craniofacial development. Whereas continued gene identification will certainly improve our understanding of the molecular mechanisms of craniofacial development and malformations, the major challenges are (1) to understand the complex interactions between and integration of various signaling pathways, (2) to understand gene–environment interactions and epigenetic control of craniofacial development, and (3) to understand the relationship between genetic variation and susceptibility to craniofacial malformations.

There is clear genetic evidence that the major signaling pathways, including Bmp, Fgf, Shh, and Wnt pathways, interact synergistically or antagonistically during many developmental processes. The best characterized developmental system where these signaling interactions occur extensively is the developing limb (reviewed in Niswander, 2002). Limb bud formation is initiated by Wnt molecules (Wnt2b and Wnt8) expressed in the lateral plate mesoderm, which signal through β-catenin to restrict *Fgf10* expression to the presumptive limb mesoderm (Kawakami et al., 2001). Fgf10 then induces expression of another Wnt gene (*Wnt3a* in chick and *Wnt3* in mice) in the limb ectoderm, which in turn signals through β-catenin and acts in conjunction with Bmp signaling to induce and restrict *Fgf8* expression in the apical ectodermal ridge (AER; Kawakami et al., 2001; Barrow et al., 2003; Soshnikova et al., 2003). The Wnt3/β-catenin signaling in the limb ectoderm appears to be regulated by Bmp signaling by an unidentified ligand but through the Bmpr1a receptor (Soshnikova et al., 2003). Wnt3/β-catenin signaling also directly regulates *Bmp4* expression in the limb ectoderm, generating a positive feedback loop to pattern the proximal–distal axis of the limb (Barrow et al., 2003; Soshnikova et al., 2003). Moreover, during limb outgrowth, Fgf signaling from the AER interacts with Wnt7a signaling from the dorsal ectoderm to induce *Shh* expression in the posterior–distal limb mesenchyme (reviewed in Niswander, 2002). Shh induces expression of Gremlin, an antagonist of Bmp signaling, which in turn regulates *Fgf4* expression in the posterior AER, and Fgf signaling from the AER maintains Shh expression in the posterior–distal mesenchyme, forming a signaling loop (reviewed in Niswander, 2002). Some of these signaling interactions have been found in other developmental processes, including craniofacial development (Neubuser et al., 1997; St. Amand et al., 2000; Liu et al., 2005a; Shu et al., 2005). For example, *Fgf8* and *Bmp4* are expressed in complementary proximal–distal patterns in the rostral mandibular ectoderm and Bmp4 signaling appears to regulate *Fgf8* expression in a dose-dependent manner (Liu et al., 2005a). Bmp4 and Fgf10 have been shown to regulate expression of *Shh* in the palatal ectoderm, which, in turn, regulates *Bmp2*

expression in the palatal mesenchyme (Zhang et al., 2002; Rice et al., 2004; reviewed in Murray and Schutte, 2004). As discussed above, the canonical Wnt signaling activity overlaps with *Bmp4* expression in the distal ectoderm of the facial primordia during facial outgrowth and lip fusion. *Fgf8* is expressed dynamically in the facial ectoderm and exhibits both overlapping and complementary domains with *Bmp4* during facial outgrowth. In addition to cross-regulation at the transcriptional level, these signaling pathways also converge and crosstalk through interactions of the intracellular signaling components. Bmp4 and Fgf8 have been shown to interact antagonistically to regulate expression of downstream transcription factors involved in proximal–distal patterning of the mandible and teeth (Neubuser et al, 1997; St. Amand et al., 2000). The Smad proteins in the Tgfβ/Bmp signaling pathway have been found to directly interact with Tcf/Lef proteins, transcription factors of the Wnt/β-catenin pathway (Nishita et al., 2000). Fgf signaling has been shown to induce phosphorylation of GSK3β and influence the stability and nuclear entry of β-catenin in a cell-type dependent manner (Torres et al., 1999; Israsena et al., 2004). That the same major signaling pathways are involved in regulating cell proliferation and survival in various developmental contexts to pattern different tissues and organs highlights the complexity and importance of understanding the interactions and integration of these signaling pathways at the molecular and cellular levels.

In both humans and mice, it is known that environmental and epigenetic factors affect CLP susceptibility (reviewed in Murray, 2002; Finnell et al., 2002). Folate supplementation has been shown to decrease the prevalence of CLP in the A/WySn mouse strain (Angela Paros, 1999), and some studies have shown a protective effect in humans as well (reviewed in Prescott and Malcolm, 2002). Presumably these environmental factors act on both the maternal and embryonic genotype; however, the molecular mechanisms have not been discerned. Furthermore, genetic variation at some loci likely sensitizes the embryo to other genetic and environmental insults. For example, modifications of *Bmp4* expression or activity have been implicated in the evolution of facial shape in fish and birds (reviewed in Helms et al., 2005). Bmp4 is an essential regulator of facial primordial outgrowth and lip fusion, as discussed above. Differences in facial shape, such as slight changes in the shape of the medial and lateral nasal processes during facial development, has been proposed to be a threshold factor underlying CLP in the A/WySn and CL/Fr strains of mice (Millicovsky et al., 1982; Trasler and Ohannessian, 1983) and may account for the different frequencies of CLP in different human populations (Fraser and Pashayan, 1970). Considering the complexity involving the interactions and integration of signaling pathways and complex cellular processes involved in facial morphogenesis, genetic variation causing subtle changes of activity in one molecular pathway may tip the balance and result in higher susceptibility to developmental malformations such as CLP. Thus, facial morphogenesis is truly a quantitative genetic trait and an excellent model for understanding the molecular mechanisms of organogenesis and complex diseases.

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