# EPH AND EPHRINS IN PALATE DEVELOPMENT

A Dissertation

by

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# ABSTRACT

Cleft palate (CP) is one of the most common birth defects. It may not be life threatening but many functions, such as feeding, digestion, speech, middle-ear ventilation, hearing, respiration, and facial and dental development, can be disturbed because of the structures involved. These problems can also cause emotional, psychosocial, and educational difficulties. It imposes a tremendous health burden and often leaves lasting disfigurement. In humans and mice, the secondary palate forms from outgrowths of neural crest-derived mesenchyme covered with a double layer of epithelial cells. The shelves elevate over the tongue and grow toward each other. The medial edge epithelium (MEE) adheres to form the medial epithelial seam (MES). MES cells then undergo epithelial to mesenchymal transition (EMT), cell apoptosis or migrate to the oral and nasal surfaces to form a mesenchymal cell confluence.

This fusion process requires transforming growth factor β3 (TGFβ3), and blocking the expression of this protein or its downstream signaling cascade results in CP.

Eph receptors tyrosine kinases and their ephrin ligands are responsible for multiple developmental events such as adhesion and migration. Binding of ephrins to Ephs on opposing cells causes tyrosine kinase activation in the Eph- bearing cells (forward signaling), while binding of Ephs can activate intracellular signaling inside ephrin-bearing cells (reverse signaling). Activation of ephrin reverse signaling in chicken palates induces fusion, and it requires phosphatidylinositol-3 kinase (PI3K). Blockage of reverse signaling inhibited TGFβ3 induced fusion in the chicken and natural fusion in

the mouse palate. Thus, ephrin reverse signaling is necessary to induce palate fusion independent of TGFβ3.

EMT is orchestrated by a complex network of signaling molecules and it is a critical step for palatal fusion. TGFβ family is a multifunctional cytokine that oversees and directs all aspects of cell development, differentiation and survival of essentiall cell types and tissues. Also, it is a suppressor of cell growth and proliferation particularly in tumor cells of epithelial and mesenchymal origins.

Ephrin signaling promotes elevation of TGFβ signaling. These findings lead to the central hypothesis that the TGFβ and Eph/ephrin pathways cooperate in EMT in palatal fusion. Thus, the goal of this research project is to use the palate model system to generate cellular responses and changes to study the basic mechanisms that control EMT during palatogenesis.

Therefore the aims of this work are as follows: a) Determine if Eph and ephrins play a role in palatal fusion and b) Establish if ephrin reverse signaling is necessary and sufficient to induce EMT in palatal fusion independent of TGFβ.

# DEDICATION

To God for providing me with the inspiration, perseverance and strength to pursue my dreams. To my mother, husband, son, family and friends for their love, understanding and support during this long journey.

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NOMENCLATURE

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|---|---|
| A-P | Anterior to Posterior |
| ADAMTS | Disintegrin And Metalloproteinase with Thrombospondin Motifs |
| ANOVA | The Analysis Of Variance |
| ASCs | Adipose Stem Cells |
| ATCC | Cell lines |
| BMP | Bone morphogenetic proteins |
| Cbf | Core Binding proteins |
| CD1 | Cluster of differentiation (cell surface protein) |
| CHO-K1 | Cell line |
| CL | Cleft Lip |
| CLP | Cleft Lip and Palate |
| CNC | Neural Crest Derived |
| CP | Cleft Palate |
| DNA | Deoxyribonucleic acid |
| DO | Distraction Osteogenesis |
| ECM | Extracellular Matrix |
| EGFR | Epidermal Growth Factor Receptor |
| EMT | Epithelial Mesenchymal Transition |
| Eph | Erythropoietin-producing human hepatocellular carcinoma cell |
| ERK | Extracellular signal-regulated kinases |

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|---|---|
| Fc | Crystalized fraction |
| Fgf | Fibroblast Growth Factor |
| FgfR | Fibroblast Growth Factor Receptor |
| FOXE1 | Forkhead Box Protein E1 |
| GABA | Gamma Aminobutyric acid |
| GPI | Glycosylphosphatidyl-inositol |
| GRIP1 | Glutamate receptor interacting protein |
| GSK | Glycogen synthase kinase |
| GSTT | Glutathione S-transferase theta |
| H&E | Hematoxylin and Eosin |
| HA | Hydroxyapatite |
| hh | Hedge hog |
| Ig G | Immunoglobulin G |
| Irf | Interferon Regulatory Factor |
| Lhx | LIM homeobox |
| MAPK | Mitogen-activated protein kinases |
| MEE | Middle Edge Epithelium |
| MES | Medial epithelial Seam |
| MFS | Mean Fusion Score |
| Mmps | Matrix metaloproteinases |
| MP | Mid Palatal |
| mRNA | Messenger RNA |

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|---|---|
| MSCs | Mesenchymal Stem Cells |
| Msx1 | Msh homeobox 1 |
| NFAT | Nuclear Factor of Activated T-cells |
| NS | Nonsyndromic |
| NOS | Nitric oxide synthase |
| OO | Orbicularis Oris |
| OR | Odds Ratio |
| Osr | Protein odd-skipped-related |
| Pax | Paired box |
| PDGF | Platelet-derived growth factor |
| PI3K | Phosphatidylinositol-3 Kinase |
| PLA | Poly lactic acid |
| PLGA | Poly lactic glycolic acid |
| RTKs | Receptor Tyrosine Kinases |
| SEM | Standard error of the Mean |
| SH2 | Src Homology 2 (Binding Domain) |
| Shh | Sonic hedge hog |
| Shox2 | The short stature homeobox |
| siRNA | Small interfering RNA |
| SPSS | Statistical Package for the Social Sciences |
| SUMO | Small ubiquitin-like modifier |
| SVF | Stromo-Vascular Fraction |

| | |
|---|---|
| Tbx22 | T-box transcription factor |
| TGFβ3 | Transforming Growth Factor β3 |
| Tim | Tissue inhibitor of metalloprotein |
| TP | Trans Palatal |
| VAX1 | Ventral anterior homeobox 1 |

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# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW*

Orofacial clefts are the most prevalent craniofacial birth defects and the second most common birth anomaly (1). Among of all the possible craniofacial defects observed in newborns; perhaps the most well-known defect is the Cleft palate (CP) (2). Occurring with a frequency of approximately 1 in every 700 births per year in the US, the incidence of CP is equal to 475 cleft palates per month or 15 clefts per day (3,4). In other words, 1% of infants born worldwide (1 million) each year exhibit some form of facial dysmorphology, but the most dramatic observation is that in this country alone, a baby is born with a facial cleft every hour, of every day of the year (5).

Clefts of the palate (CP) and the lip (CLP) require a complex multidisciplinary treatment and having lifelong implications for affected individuals. In the United States of America, $100,000 is the amount estimated to be to rehabilitate a child born with an oral cleft.

The approach of the patient with cleft lip (CL) and palate requires a team that should be ideally composed by craniofacial surgeons, otolaryngologists, geneticists, anesthesiologists, speech-language pathologists, nutritionists, orthodontists,

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prosthodontists, and psychologists, and to be capable of treating even rare facial clefts with excellence, neurosurgeons, and ophthalmologists (6). In this manner, it is possible to provide long-term follow up through the entire child's development and achieve all of the following treatment goals: facial aesthetic, integrity of the primary and secondary palate, normal speech and hearing, class I occlusion with normal masticatory function, good dental and periodontal health, and normal psychosocial development (6,7).

**Palate Development**

The palatal structures are composed of the cranial neural crest (CNC)-derived mesenchyme and pharyngeal ectoderm (8-11). The epithelia that cover the palatal shelves are regionally divided into oral, nasal and medial edge epithelia (MEE). The nasal and oral epithelia differentiate into pseudostratified and squamous epithelia, whereas the MEE is removed from the fusion line (12) (Fig. 1-1).

The secondary palate originates as an outgrowth of the maxillary prominences at approximately embryonic day 11.5 in the mouse (E11.5-m) (Fig. 1-1) and post coital 6 weeks in humans (p.c.6wk-h). The palate shelves initially grow vertically along the sides of the tongue (E13.5-m; p.c.7wk-h) and then rise above the tongue as the latter drops in the oral cavity due to the forward and downward growth of the mandible (E14.0-m; p.c.8wks-h). With continued growth, the shelves appose at the midline (E14.5-m; p.c.10wks-h) and eventually fuse (E15.5-m; p.c.13wk-h) (13). Numerous genes that are similar in mice (14) and humans (13,15,16) are expressed (Table 1and 2) during palatal

development (12). Some of the cleft palate are also associate with other birth defects syndromes (Table 1) while others occur independently (Table 2).

During fusion, the epithelium covering the tip of the opposing palatal shelves adheres, intercalates and thins into a single-layer midline epithelial seam (MES) (10). The disintegration of this seam results in the confluence of the palatal mesenchyme. Tremendous interest has arisen in the cellular mechanisms underlying MES degradation. Epithelial-mesenchymal transition (EMT) is one of the proposed models that regulate the medial edge epithelial (MEE) cell fate (10,17-24). However, other mechanisms have been proposed, such as apoptosis (25-28), in which all MEE cells are theorized to die during fusion (12). Alternatively, it is hypothesized by some researchers that MES cells disappear by migrating from the midline towards the nasal and oral epithelia (29,30). Other investigators postulate that all events, including apoptosis, migration and EMT, may occur (10,27,31). Interestingly, the fusion of the external surface of the bilateral maxillary processes with the nasofrontal prominence in the chick is similar to palatal fusion (Fig. 1-2) (32). The outer periderm layer dies through apoptosis, and the lateral edge epithelium of the intermaxillary segment of the nasofrontal process fuses with the medial edge epithelium of the external maxillary process to form a seam that transitions to a confluent mesenchyme (Fig. 1-2) (32,33).

**Molecular Genetics Behind Cleft Palate**

Much of the general 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 (12,34-37). The understanding of the molecular causes of CP is complicated due to the amount of factors that can, when mutated, result in various forms of clefting (38).

***TGFβ3***

TGFβ3 is a member of the TGFβ superfamily expressed in the medial edge epithelial (MEE) cells before fusion of the palatal shelves. TGFβ3 is required for palatal shelf fusion (34,38,39), as evidenced by homozygous TGFβ3 null mice newborns, which have a cleft secondary palate. The function for TGFβ3 in palatogenesis relates to regulation of the breakdown of epithelial cells that lie between the palatal shelves (40). In the TGFβ3-null mice, the palatal shelves appear to approximate and adhere, but the epithelial seam remains preventing fusion (38). 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 TGFβ3–/– mice (12,40). TGFβ signaling can activate other intracellular signal transduction pathways, including p38 MAPK. The activation of p38 MAPK is elevated in the epithelium of the fusing palatal shelves (41). The treatment of K14-Cre; Smad4f/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. These results indicate that Smad- and p38 MAPK-dependent mechanisms are functionally redundant during palate fusion (12,41).

Previous studies had shown that TGFβR3 is expressed in the epithelium and was specifically localized to the MEE during palatal shelf fusion in mice (42). Knockdown of TGFβr3 with siRNA in a palatal shelf culture model inhibited in vitro palatal shelf fusion due to persistence of the palatal epithelium (43,44). Recent findings demonstrated a partial rescue of the CP phenotype in Wnt1-Cre; TGFβr2 $^{F/F}$;TGFβr3$^{+/-}$ mice suggesting that TGFβR3 played a pivotal role in maintaining homeostasis of TGFβ signaling during palatal vascular and bone development (44,45).

***Ephs and Ephrins***

Eph/ephrin family members have been demonstrated to control anterior palatal shelf outgrowth (46). 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 CP accompanied by decreased cell proliferation in the anterior palatal mesenchyme (47). 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 (47). This study also showed that ephrin reverse signaling using EphB2 is required and sufficient for chicken palate (47).

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 CP (48,49).

***PDGF Signaling***

Platelet-derived growth factor (PDGF) and its receptors (PDGFRα and -β) have specific roles in promoting tissue-tissue interactions to control cell migration, proliferation and survival during embryonic development (50). Deletion of Pdgfrα in the neural crest leads to defects in palatal fusion, nasal septation and abnormal development of several facial bones and cartilage structures in mouse models. Pdgfc-null neonates have a complete cleft of the secondary palate, accompanied by failure of the palatal bones to extend across the roof of the oronasal cavity (51).

***Wingless Type (Wnt) Protein Signaling***

Wnt pathway activity is specifically localized to facial epithelia and underlying mesenchyme in the lateral nasal, maxillary and mandibular prominences. In neural crest mesenchyme, Wnts promote proliferation; thus, promoting the growth of the maxillary prominences that come together to form the palate (52). In the facial epithelium, expression of multiple Wnts is essential for the fusion of facial prominences (53). Some abnormalies are linked to disruptions in various Wnt genes (54). Some perturbations of the pathway produce mild to severe facial clefting in various animal models as well as in humans (55). Mutation of Wnt9b in mice leads to CLP (54). Abnormal expression of the

lipoprotein receptor-related protein 6, Lrp6, a Wnt pathway coreceptor, also results in CLP.

***Irf 6 (Interferon Regulatory Factor 6)***

Irf6 is a member of a large family of transcription factors that bind to specific DNA sequences and regulate gene expression. In mice, disruption of this gene results in clefting (38). In humans, mutations in IRF6 have been shown to cause Van Der Woude syndrome and popliteal ptyergium syndrome, two disorders that are characterized by the presence of CP. Variations in IRF6 increased the risk for isolated CLP (56). *Irf6* Irf6 mutant mice exhibit a hyper-proliferative oral epithelium that fails to undergo terminal differentiation, causing epithelial adhesions that occlude the oral cavity and result in CP (57). Taken together, these data suggest Irf6 mutations may result in defective elevation of palatine shelves, secondary to inappropriate adhesions with oral epithelium (38).

***VAX1***

VAX1 gene is a member of the Emx/Not gene family and encodes a transcriptional regulator with a DNA-binding homeobox domain. Single nucleotide polymorphisms in the VAX1 genes were overrepresented in patients with CLP, suggesting that variants in VAX1 itself may contribute to development of clefting. Mouse knockouts for Vax1 show CP, and this gene was expressed widely in developing craniofacial structures (58). Therefore, variants in VAX1 are strong candidates in the etiopathogenesis of CLP.

***ADAMTS Family Metalloproteases***

Recent studies suggest 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 CP with defects in early outgrowth, elevation and approximation of the palatal shelves (59). 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 reduced in Adamts9$^{+/-}$; Adamts20 $^{bt/bt}$ compound mutants. Interestingly, simultaneous disruption of versican and Adamts20 function also resulted in reduced palatal cell proliferation (59). It is possible that proteolysis of ECM molecules such as versican might produce bioactive fragments with growth-promoting activity.

***Fibroblast Growth Factor 10 (Fgf10)***

Fgf10 it 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 CP with impaired palatal shelf outgrowth (60). Expression of Fgf10 mRNA was restricted to the mesenchyme, but Fgfr2b mRNA was detected in the overlying epithelium. Fgfr2 function is required within the epithelium, mice having a epithelial-specific deletion of Fgfr2 also exhibited CP (61). Both epithelial and mesenchymal cell proliferation were reduced in the absence of either Fgf10 or Fgfr2b. Thus, suggesting the presence of a factor that signals from the epithelium back to the underlying mesenchyme dependent on Fgf10/Fgfr2b signaling.

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 that drives the outgrowth of the palatal shelves.

***FOXE1 (Forkhead Box Protein E1)***

FOXE1 is a forkhead containing transcription factor that is involved in embryonic pattern formation. The FOXE1 gene is expressed at the point of fusion between maxillary and nasal processes during palatogenesis. Positional cloning and candidate gene sequencing show a correlation between mutations in FOXE1 and the occurrence of CLP (62). FOXE1 is expressed in the secondary palate epithelium of both mice and human embryos (63). Mice with a null mutation in FOXE1 have CP (64).

**Molecular Signaling Events in Embryonic Palatal Development**

As stated above, CP with or without CL is a complex trait triggered by a combination of numerous genes and environmental factors (65,66). The palatal shelf development defects will be divided in five categories: failure of palatal shelf formation, fusion of the palatal shelf with the tongue or mandible, failure of palatal elevation, failure of palatal shelves to meet after elevation, persistence of middle edge epithelium.

***Failure of Palatal Shelf Formation***

The failure of palatal shelf formation is a rare severe defect. Recent studies have identified several molecular networks operating between the palatal shelf epithelium and mesenchyme during different steps of palatogenenesis. These networks include signaling molecules and growth factors such as sonic hedge hog (Shh), members of the

transforming growth factor β (TGFβ) super family, including bone morphogenetic proteins (Bmps) and TGFβs, fibroblast growth factors and their receptors (FgfR), effectors and targets (13,67). Studies addressing the role of Fgf signaling during early palatal development by analyzing Fgf10 and FgfR2b mutants found altered cell proliferation within both mesenchyme and epithelium in the palatal shelves and increased apoptosis within the epithelium. Fgf10 and FgfR2b mutations affected the initial development of palatal shelves, and the mouse pups had complete CP (68). By signaling via its receptor, FgfR2b, in the palatal shelf epithelium, the mesenchymal derived Fgf10 supports epithelial proliferation and survival and also induces the expression of Shh within the epithelium. Shh, in turn, signals to the mesenchyme and stimulates cell proliferation. In general, the signaling activities are subject to tight spatiotemporal control, and, in many instances, too much or too control little is detrimental to the developing organ (12).

This situation is well illustrated in anomalies caused by deregulated hedgehog (hh) and Fgf signaling (69,70). While Fgf10/FgfR2b activity plays a crucial role during palatogenenesis, it appears to be subject to the tight spatiotemporal regulation shown in mice lacking *Shox2*. *Shox2* mutant mice develop a very rare type of CP that may also be found in humans (36): the soft palate is intact whereas, the hard palate has a cleft. Abnormal proliferation and apoptosis were theorized to be the cause of the cleft. Surprisingly, a number of protagonists implicated in palatogenesis, including *Msx1, Bmp4, Pax9, Lhx8, Osr2*, *TGFβ3* and *Jag 2*, were expressed normally (36). In contrast, Fgf10 and Fgfr2b were expressed at ectopic sites within the mesenchyme of the Shox2

mutant mice (71). These studies emphasize the importance of the precise timing and determination of sites of signaling activities necessary for normal development. The mutation of activin-βA causes a severe facial primordial development defect, which may be responsible for the retardation of palatal shelf development and complete CP. In addition, other genes, including *Msx1, Lhx8, Shox2* and *Osr2*, assume important roles in the palatal shelf growth. The targeted mutation of these genes in mice generates CP, indicating the intrinsic requirement of these factors during palatogenesis (71).

***Fusion of the Palatal Shelf with the Tongue or Mandible***

Under normal conditions, palatal shelves do not fuse with other oral structures. However, in mice that do not express Fgf10, the palatal shelf epithelium fuses with the tongue and mandible (68). The loss of function mutations of Fgf10 results in anterior palatal shelf fusion with the tongue, whereas the middle and posterior palatal shelf regions adhere to the mandible, thus preventing the elevation of the palatal shelf (72). There is a severe reduction of the expression of *Jagged 2*, thereby encoding a ligand for the Notch family receptors and ectopic TGFβ3 production in the nasal epithelia of these mice. The analysis of *Jag2* mutant embryos indicates that Jag2-Notch signaling prevents inappropriate palatal shelf adhesion to other oral epithelia through the control of oral epithelial differentiation. Mutations in TBX22 have been reported in families with X-linked CP and ankiloglossia (73-75). *Tbx22* is expressed in the developing palate and tongue in mice, suggesting an important role in regulating tongue and palate development.

***Failure of Palatal Elevation***

Palatal shelf elevation is a rapid movement triggered by both intrinsic forces within the palatal shelves proper and by influences from other craniofacial and oral structures, including the movement of the tongue, and growth of the cranium and mandible (76,77). The role of the extracellular matrix in palatal shelf elevation has been supported by some studies and is presently accepted as an important determinant of palatal shelf elevation (78,79). Those studies (77) suggested that a progressive accumulation of glycosaminoglycans, primarily hyaluronan in the palatal shelves, plays a role in their elevation (78,79). Hyaluran is a highly charged glycosaminoglycan that retains high amounts of water, forming hydrated gels leading to the expansion of the extracellular matrix. Other constituents of the palatal shelves including collagen fibers, vascularization, and the epithelial covering; the polarized alignment of the mesenchyme cells may also contribute to the intrinsic elevation force of the palatal shelves. Mutations of *Pax9, Pitx1* or *Osr2* can lead to failed palatal shelf elevation and CP defect (80-83). The cellular defect is associated with the CNC-derived palatal mesenchyme, suggesting the important functions of these transcription factors in regulating the fate of the CNC cells during palatogenesis. Early studies attributed a role to the neurotransmitters during palatal shelf elevation (77). At present, it is widely accepted that neurotransmitter γ-aminobutiric acid (GABA) regulates not only neuronal activities but also cell migration, survival, proliferation and differentiation of neuronal and non-neuronal cells (84-86). Teratological studies in rodents showed that GABA or GABA agonists generate CP by inhibiting palatal shelf elevation, whereas GABA antagonists stimulate the process (87).

The implication of GABA in palate development was demonstrated by genetic studies of mice lacking the β3 subunit of the GABA receptor that developed CP without other craniofacial malformations (88).

***Failure of Palatal Shelves to Meet After Elevation***

Fusion of the opposing palatal shelves is an important step, taking place through a sequence of events that includes the removal of the flat peridermal cells, contact and adhesion of the opposing MEE, which creates the MES. The MES disintegrates and the mesenchymal confluence is achieved at the midline (9,10,22). Failure of shelf fusion is the most common type of CP defect documented in animal studies. Mutations in *Msx1* and *Lhx8* and conditional inactivation of *TGFbr2* in CNC cells or *Shh* in the epithelium all result in retarded palatal shelf development (12,68).

***Persistence of Middle Edge Epithelium***

Adhesion of the opposing MEE is an important event in both human and mouse embryos (8,15,22,32,89). E-cadherin is expressed in the epithelia covering the frontonasal and medial nasal processes as well as during the different stages of palate development, including the epithelial islands, remnants of the MES (90-92). Mutations of *CDH1*/E cadherin, which deletes the extracellular cadherin repeat domains required for cell-cell adhesion, have recently been associated with CLP in families with hereditary diffuse cancer (93). E-cadherins are known to form dimers, indicating that the mutant proteins may have trans-dominant negative effects over the normal proteins (93).

Extensive efforts have been made to elucidate the role of TGFβ3 during palatal fusion (94-97). Adhesion of the MEE upon palatal shelf contact is a necessary step for

fusion. TGFβ3 is expressed in the MEE before and during fusion, and mediates MEE adhesion of the opposing palatal shelves through filopodia. E-cadherin is required for fusion, whereas filopodia seem to be crucial for proper alignment and guidance of cell sheets that are fated to fuse, but not for fusion itself (98). TGFβ3 is implicated in controlling the remodeling of the extracellular matrix through regulation of the expression of the matrix metaloproteinases (Mmps) Mmp13, Mmp2 and the tissue inhibitor of metalloproteinase-2 (Timp) (99). TGFβ3 signaling functions in the MEE by mediating the epithelial-masenchymal interactions leading to tissue changes that regulate palatal fusion. For example, EMT of the MES has been proposed as the major mechanism underlying the disappearance of the MES to generate mesenchyme continuity, thus preventing palatal clefts (22). The establishment of the concept of EMT as the prevailing mechanism of MES disappearance leading to studies attributing roles to different molecules, including TGFβ3, Lef1, Smad, RhoA, phosphatidylinositol 3-kinase (PI-3 kinase), Mmps, Twist and Snail (9,21,100). In *TGFβ3* or *Egfr* mutant mice, there is an alteration of the fate of MEE cells (101,102). In TGFβ3 null mutant mice, MEE cells fail to undergo apoptosis and remain along the midline, preventing normal fusion.

**SUMO Modification of Signaling Pathways in Palatogenesis**

The molecular understanding of NS (nonsyndromic) CLP is further complicated when one considers that large differences in penetrance often occur when the same mutations are placed on different strains, indicating a potential role for both genetic and or environmental modifiers in the pathogenesis of CLP. Several lines of evidence point

to the involvement of the small ubiquitin-like modifier (SUMO) posttranslational modification machinery (12,103). SUMO proteins are a family of small proteins that are covalently attached to and detached from other proteins in cells to modify their function. SUMOylation is a post-translational modification involved in various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle. A surprisingly specific role in orofacial development has been revealed for protein modification by the SUMO, which hints at a possible interaction with environmental factors. Small ubiquitin-related modifiers belong to the ubiquitin-related protein family, and SUMO proteins are ubiquitously expressed throughout the eukaryotic kindom (12,104). SUMO1 shows strong expression in the edge epithelial of the secondary palate (105). A translocation breakpoint interrupting SUMO1 was found in a patient with CLP (12,105). The causative nature of the translocation defect has been confirmed in SUMO1-deficient mice having a distinct CP phenotype (12,105). Furthermore, it was recently shown that mutations in TBX22 have a profound effect on its ability to be sumoylated, which is at least partially responsible for its loss of function (12,106). Other SUMO targets include Smad4, Msx1, p63, Pax9, Eya1 and FGF signaling (12,103). It seems likely that some of these factors may manifest through the disturbance of the SUMO pathway. Destabilizing the normal balance of expression and activity for genes such as TBX22, Msx1, SATB2, and p63 during early pregnancy is likely to provide a high-risk environment for the occurrence of CLP (12). Elucidating the relationship among environmental factors, the SUMO pathway, and the networks of craniofacial genes influenced by this

posttranscriptional modification may be crucial to the understanding of the idiopathic forms of orofacial clefts (12).

**A-P Gradient of Molecular Signaling in Palatal Development**

Multiple genes are critical for the development of the anterior region of the palate. *Msx1, Bmp4, Bmp2, Fgf10*, and *Shox2* have restricted expression patterns in the anterior region of the palate (12,68,107). In addition to the differential gene expression patterns along the A-P axis of the developing palate, there is also mesenchymal heterogeneity between the medial and lateral regions of the palatal shelf. The odd-skipped related genes *Osr1* and *Osr2* are expressed in a medial-lateral gradient in the palatal shelf. The mutation of the *Osr2* gene results in the compromised development of the medial aspect of the palatal shelf and retards palatal shelf elevation (12,83,108). The expression of *Fgfr2* is focused on the medial aspect of the developing palatal shelf, suggesting a possible functional significance in regulating its development and elevation.

An important discovery has been the confirmation of genetic heterogeneity along the anterior-posterior and medial-lateral axes of the developing palate (36). This heterogeneity may provide a differential regulatory mechanism for the fusion of the anterior vs. posterior region of the palate. MEE cells undergo apoptosis at different times during palatal fusion. It has been shown that the apoptosis of MEE cells is triggered by palatal shelf contact in the anterior region, whereas it is initiated before any contact between the opposing shelves in the posterior region (26). This difference may be the result of dissimilar molecular signals in the palatal mesenchyme along the

anteroposterior axis that instruct different fates to the palatal epithelium (12,109). Recent studies have demonstrated that constant and reciprocal interactions between the palatal epithelium and the CNC-derived mesenchyme are responsible for setting up this genetic heterogeneity along the AP axis and are crucial for normal palatal development and fusion (13,68,110). The specific gene expression patterns in the posterior region of the palatal mesenchyme are less understood. *Fgfr2* is expressed in the epithelium, and the CNC-derived mesenchyme is found in the middle and posterior palate. FGF8 signaling selectively induces the expression of *Pax9* in the posterior region of the palatal mesenchyme. The loss of *Pax9* results in a palatal shelf development defect and a CP (36,81).

**Types of Cleft Palate**

CP or CLP may involve both the primary or secondary palate (frequently both complete clefts). Those involving the primary palate are associated with clefts of the lip. Palatal clefts may also be unilateral or bilateral. Isolated clefts of the secondary palate (incomplete clefts) occur in the absence of defects in either the lip or the alveolar process. Because palatal fusion occurs in an anterior to posterior direction, clefts of the secondary palate may involve only the soft palate or both the soft and hard palates together (111).

Clinically, clefting in the secondary palate extends anteriorly from the uvula to varying degrees, often involving the hard palate (112). In complete forms, the cleft can affect the entire secondary palate, reaching the incisive foramen, leaving the

nasopharynx in direct communication with the oral cavity. Thus, the vomer can be seen as a midline structure extending from the base of the skull. While complete and incomplete clefts of the palate may be readily apparent on physical exam, other forms may also exist with regard to feeding, speech development and ear infections (113).

**Epidemiology**

CLP affects with variability across geographic origin, racial and ethnic groups, as well as environmental exposures and socio economic status. In general, Asian and Amerindian populations have the highest reported birth prevalence rates, often as high as 1/500, European-derived populations have intermediate prevalence rates at about 1/1000, and African-derived populations have the lowest prevalence rates at about 1/2500 (114,115). These observations suggest the relative contribution of individual susceptibility genes may vary across different populations. The frequency of CLP also differs by sex and laterality: there is a 2:1 male to female ratio for clefts involving the lip and approximately a 1:2 male to female ratio for clefts of the palate only; and there is a 2:1 ratio of left to right sided clefts among unilateral CL cases (114).

**Environmental Factors and Gene Interactions**

The etiology of CL with or without palate (CLP) is theorized to be a combination of factors associated with genes and environment (65,116). The advent of gene targeting technology and basic conventional techniques using animal models has led to the identification of genes associated with known and unknown etiologic factors. The

characterization of the genomic sequences will greatly impact the regulation of gene networks and pinpoint any variations in the different stages of craniofacial morphogenesis. There is emphasis placed on the different genes associated with the classifications of CLP into syndromic (Table 1) and nonsyndromic genes (Table 2). Each classification plays a significant role in the understanding of the molecular and genetic mechanisms affecting these types of craniofacial defects (66,67,117). In addition to known genes, there is strong evidence that several environmental factors (e.g., alcohol consumption, tobacco, and anti-convulsants) increase the risk of CLP (118,119). In contrast, several studies have shown that folic acid may have a protective effect on CLP and neural tube defects (120-124). Data from the National Birth Defect Prevention Network have indicated a decrease in neural tube defects from 5/10,000 to less than 2/10,000 after the fortification of the food supply with folic acid, indicating that this vitamin and the proteins that facilitate the uptake and metabolism of folic acid may be candidate genes in craniofacial development (122,125-128).

Maternal smoking has been associated repeatedly with increased risk of CLP analysis supports an overall odds ratio (OR) for having CLP of ~1.3 among offspring of mothers who smoke (16). Increased risk from exposure to maternal smoking during the peri-conceptual period raises the possibility that genes in certain metabolic pathways can play a role in the development of CLP. Markers in GSTT1 (glutathione S-transferase theta) or NOS3 (nitric oxide synthase 3) genes appear to influence risk of CLP in the presence of maternal smoking (129). The GSTT1 markers are gene deletion variants, which suggest deficiencies in detoxification pathways may underlie some of this

susceptibility. Smoking has also been associated with a joint risk with variants in the IRF6 gene and the same study reported interactions between multivitamins and IRF6 variants. Some specific teratogen, for example valproic acid have evidence of association with CP. Exposure to maternal alcohol consumption has also been suggested as a risk factor, but the evidence has been more inconsistent. Studies also suggest that drinking patterns (high doses of alcohol in short periods of time) increase risk (130). This is supported by variation in the ADH1C alcohol dehydrogenase gene. Nutritional factors, such as folate deficiency, have also been suggested to influence risk of CLP, based on both observational studies and trials using folate supplementation to prevent recurrences of CLP in families. However, the studies of vitamin supplementation with folate remain controversial, and recent studies of levels of folate receptor antibodies did not find an association with CLP. Furthermore, food fortification programs using folic acid have shown detectable decreases in the rates of clefting in some but not all studies. In the future, other nutrient and micronutrient studies will need to be studies in otder to find evidence of effects. For example, there is data to support roles for zinc deficiency in risk of oral clefts in populations in which zinc status is highly compromised, for cholesterol deficiency in facial clefting, as well for as multivitamins in general in cleft prevention.

Recent studies found that mothers who ate liver periconceptionally were at a decreased risk of having a child with a CLP compared to mothers who did not (131). A 3-oz portion of pork, beef, or veal liver contains more than 600% of the recommended

dietary allowance for vitamins A and B12 and more than 50% for folate, zinc, and vitamin B6 (US Department of Agriculture, 2013).

Besides nutrients and toxins other environmental exposures have been, and should continue to be, assessed for possible roles in clefting. These exposures include hyperthermia, stress, maternal obesity, occupational exposures, ionizing radiation and infection (114,132).

**Morbidity**

Researchers hoping to study the genetics of CLP were haunted by reviews stating that "babies born with clefts can be treated in their first year of life and understanding the genetics contribution to clefts will not change the outcome of these cases" (133). Recent work has suggested that this was an underestimation of the consequences of being born with facial clefts. Individuals born with clefts have a shorter lifespan, with increased risk for all major causes of death, when compared with individuals born without clefts. Contributing to these higher mortality rates are probably psychiatric disorders and cancer (134). Facial clefts increase the risk of hospitalization for psychiatric diseases in adults. Also, an increased occurrence of breast and brain cancer among adult females born with oral clefts, and an increased occurrence of primary lung cancer among adult males born with oral clefts have been reported (131).

Psychiatric disorders can be interpreted under the assumption that the development of the brain and that of the face are intimately related in both normal and pathologic conditions, and suggest that abnormal brain development might accompany

an abnormality in facial development. Animal models have shown that forebrain development and facial development are linked. Molecular signaling in the forebrain regulates the establishment of a signaling center in the face, and thus controls its subsequent morphogenesis (135). The molecular dialogue that exists between these tissues is essential for patterned outgrowth of the middle and upper face. It appears that defects in signaling within the forebrain can lead to a wide variety of craniofacial malformations, including CLP (135).

**Ossification of the Palate**

Palatal fusion signals the start of the ossification process in the anterior two-thirds of the palate to form the hard palatal tissues. This process entails the successful fusion of the three embryonic structures, namely, the lateral edges of the primary palate with the two anterior edges of the secondary palate. This process requires the synchronization of shelf movements together with the growth and withdrawal of the tongue and the growth of the mandible and head (136). Any form of disruption during the formative stages results in a pathological cleft.

A wide range of studies on craniofacial skeletal maturation has shown that the fusion of the palatal shelves along their length to form the mid-palatal (MP) suture occurs during the ossification of the maxillae and palatine bones before the mandibular condyle develops (36,137,138). Ossification is observed where mesenchymal cells condense, the surrounding tissue vascularizes and the cells differentiate into osteoblasts. There is a number of growth and differentiation factors involved in this process, such as

Bmps, core binding proteins (Cbf), Fgfs, and hh proteins that interact with various signaling pathways to regulate the patterning of the undifferentiated mesenchyme. Bmp-6 and the transcription factor Gli1 are also expressed during intramembranous bone formation (139-141). As in the craniofacial sutures, the mid-palatal (MP) and trans-palatal (TP) suture osteoblasts express TGFβ 1,2 and 3, while the suture cells express primarily TGFβ3 (142,143).

It has been established that cranial sutures are the growth sites for the neurocranium and that the dura mater provides the signaling molecules to regulate suture patency (144). The MP and TP sutures have different morphology, so they are not in contact with the dura mater. There is an hypothesis, that these facial sutures are growth centers (142,143) and that the nasal capsular cartilage produces signaling molecules to regulate the fusion of the MP and TP sutures (Fig. 1-3) (143). The nasal cartilage maintained the TP sutures as growth sites in experiments on rat palatal organ cultures (E20) with or without nasal cartilage. Thus, it was theorized that the nasal cartilage may regulate mid-facial growth (12,143).

Animal models have been developed to understand the etiology and pathogenesis of orofacial clefts and the mechanisms of normal palatal ossification. The application of cyclic forces is an effective mechanical stimulus for the regulation of osteogenesis and osteoclastogenesis in the sutural growth of neonatal rats (145). The process of tissue response and regeneration in the palato-maxillary suture under tensile forces was examined histologically and fluorescently. A cyst-like zone appeared in the conjuncture of the bony front and the sutural connective tissue at the early stage of sutural expansion

with increased proliferating osteoblasts and fibroblasts. New bone was deposited along the nasal septum and the front of the cyst until the new bone front formed and the suture restored its original morphology (146).

The approach of utilizing MP suture expansion in mice has provided new insights into mechanical stress modulation as an important factor for the skeletal remodeling of bones and cartilage. The expansive force across the MP suture promotes both bone resorption through the activation of osteoclasts and bone formation through the increased proliferation and differentiation of the periosteal cells (147). Similarly, the use of orthodontic wire expansion in growing rats showed that secondary cartilage could undergo chondrogenic and osteogenic differentiation in the maxillary arch. Interestingly, these induced changes were attributed to the alteration of the differentiation pathway of progenitor cells from chondroblastic to osteoblastic, in which many sutures temporarily form secondary cartilage during early development. Histological observations at days 7, 10, and 14 indicated that intramembranous bone formation, which is partially recognized as mature bone (148), occurred at the boundary between the precartilaginous and cartilaginous cell layers where the calcified matrix was positive for osteocalcin antibody. The cellular events taking place at the MP suture cartilage in rat models as a result of expansion force have been observed as endochondral bone formation at the boundary between the maxillary bone and cartilage. Whereas, intramembranous osteogenesis has appeared at the internal side of the cartilaginous layer (149). To stimulate new bone formation in defective tissues, rat organ cultures with distracted palatal sutures were treated with Bmp-7 and Nell-1 for 8 days *in vitro*. The presence of Nell-1 increased

chondrocyte hypertrophy and endochondral bone formation while Bmp-7 enhanced both chondrocyte proliferation and differentiation in the distracted palates of 4-week-old male rats. This study indicates that Nell-1 was involved in the rapid osteoblast differentiation in palate sutures (150). In another study, the application of TGF-β1 during the early stages of rat MP expansion induced rapid bone formation at the suture site (151).

Many research investigations have also explored the importance of distraction osteogenesis (DO) since its introduction by Ilizarov in the 1950s. This treatment is a special form of bone healing that has applied both basic and clinical research models in which well-controlled distraction stresses and subsequent tensile strains within callus tissues produce new bone formation at an unusual rate (152). The application of DO to craniofacial disorders is being actively investigated for purposes such as midfacial advancement, hard palate suture expansion, elongation, and alveolar cleft closure (153-157). However, a review of studies revealed that no single investigation concerning the use of DO has been done to close a hard palate cleft in humans due to the thin layer of hard palatine bone. One possible method to supplement existing treatment modes is to induce new bone formation and soft tissue migration over the cleft before definitive surgery. The technique of DO in which bone is lengthened gradually under tension after an osteotomy was utilized in dogs. Through histological analysis of the specimens, the results of this trial confirmed that distraction promoted bone healing at the cellular level. The appearance of osteoclasts and resorption lacunae signaled the initiation of the remodeling process. With time, an increased area of bone surface was covered with osteoid. There was evidence of numerous bone-forming osteoblasts interfacing with the

surface of nonmineralized bone matrix. The bone surface was extensively double-labeled with bone fluorochromes, indicating a high turnover of bone during the healing process. By 10-12 weeks, the osteoid formed was almost completely mineralized, as demonstrated by the bone occupying the surgically created cleft (157).

**Oral and Palatal Musculature and Related Deformities**

Overt CLP encompasses a broad spectrum of defects, ranging from so-called microform clefts; thus, unilateral or bilateral clefts of the lip and palate are completed. The orbicularis oris (OO) muscle consists of numerous differently oriented strata of muscular fibers that surround the orifice of the mouth. At approximately 7 weeks post-conception (p.c.) in humans, the two maxillary prominences fuse with the medial nasal prominence; however, lip fusion is not complete until the epithelial seam disappears through EMT and/or apoptosis (136) (Fig. 1-2A-C). By 8 weeks p.c., a dense, continuous band of mesenchymal cells corresponding to the future OO muscle can be seen, with discernable OO muscle fibers present by 12 weeks. The complete OO muscle architecture forms by 16 weeks. Any delay in fusion may result in subepithelial OO defects, such as the altered migration of the mesenchymal cells. Subepithelial (non-visible) defects of the orbicularis oris muscle represent the mildest form of CL, and such defects are part of the phenotypic spectrum of CLP. This defect usually is visualized as a ridge of tissue, resembling a scar on the upper lip along the philtrum (158).

Histological studies have demonstrated that such defects spread to the muscle fibers of the superior OO muscle. A method using high-resolution ultrasonography was

developed to visualize the OO muscle non-invasively (159). Significant differences in the defects of the OO are found between in the first-degree relatives of CLP individuals and controls. The OO muscle defect detected by ultrasound is consistent with the histological examination of cadavers (159). Interestingly, the Bmp4 knockout mouse model shows bilateral CL at E14.5, although this condition occurs at a rate of 22% after birth (160), suggesting the initial CL is rescued or healed *in utero*, leaving only the subepithelial OO defect. Potential mutations in BMP4 were found in 2 individuals with OO defects and none in the controls (161). The strong evidence that OO discontinuities are indeed part of the phenotypic spectrum of CLP provides an important clue for the clinical recurrence risk estimation for families with members affected with CLP.

The mildest form of CP is termed a "submucosal cleft palate", which is described as a bifid uvula, palatal muscle diastasis, and a notch in the posterior surface of the hard palate (162). Defects in the nasopharyngeal anatomy and/or physiology may lead to velopharyngeal incompetence (VPI). Although most VPI is caused by CP, the population prevalence of VPI due to other causes is estimated to be approximately 2.5% (163). In such cases, VPI may be caused by submucosal muscular defects of the levator palatine or musculus uvulae. Most of the soft palate muscles are derived from myotome cells, which first invade pharyngeal arch 4 and then migrate to the palate, carrying their innervations from the vagus nerve. One muscle (tensor veli palatini) is derived from myotome cells that first invade arch 1 and are innervated by the trigeminal nerve (164). In the mouse, the tensor veli palatine, levator veli palatine, medial pterygoid, and lateral pterygoid muscles are identified as myogenic fields as early as gestational day 15. The

palatoglossus, palatopharyngeus, and musculus uvula. However, are not clearly visible (165). In principle, the presence of these anatomical features in unaffected individuals may signify an elevated risk for producing clefts in offspring (166).

**Palate Repair**

Today, surgical repair of CL is performed around 2–3 months of age, with CP closure performed at 6–12 months (167). The earliest written account of a cleft surgery is from ancient China; the annals of the Chin dynasty from 390 AD recount the repair of CL in an 18-year-old man. Some of the features of the surgery (cutting and stitching the cleft edges) are essentially the same today, although various refinements now give more functional and aesthetic results. Surgical treatment of CP was first described in 1817 and was followed by many years of refinement of surgical techniques (167).

The first report of a CP repair is attributed to LeMonnier, who incised the cleft edges and placed sutures leading to suppuration and then healing across the defect (38,111). Von Langenbeck in 1931 introduced the use of mucoperiosteal flaps to close clefts involving the hard palate (38). The most widely used techniques include Von Langenbeck's, the Vaeu-Wardill- Kilner and the two-flap repair described by Bardach (38). While many modifications of each exist, the main principles across all CP repairs include tension-free closure of the oral and nasal layers, dissection of muscles from the posterior edge of the hard palate and construction of a horizontally oriented palatal sling to restore normal velar function (38).

Repair of the CP begins with an incision along the cleft margin at the junction between oral and nasal mucosa. The incision is carried anteriorly along the gingiva, allowing elevation of mucoperiosteal flaps off the hard palate (38). The tendon of the tensor veli palatini can also be divided medial to the hamulus to facilitate medialization of the levator muscle. The nasal mucoperiosteum is then widely mobilized from the undersurface of the hard palate. Posteriorly, an intravelar veloplasty is typically performed, with separation of the oral, muscle and nasal linings and release of the muscles from their abnormal attachment to the posterior edge of the bony palate (38). Closure of the defect is performed in three layers (nasal mucosa, velar muscle and oral mucosa), with horizontal reorientation of the levator veli palatini establishing proper orientation of the sling. In the region of the hard palate, a two-layer repair is performed, with the nasal layer sometimes requiring a vomerine flap (38,111).

In patients with either clefting of the soft palate or a submucous cleft, a Furlow palatoplasty can also be performed. Double opposing z-plasties are fashioned on the velum, with release of the levator muscle from the posterior edge of the hard palate. Transposition of the flaps yields retropositioning of the muscle to a more medial-lateral position (38,168). With this technique, simultaneous palatal lengthening and reconstruction of the levator sling is established along with additional narrowing of the nasophayngeal aperture (38,169). Velopharyngeal competence allowing development of normal speech is one of the most critical outcomes in cleft surgery, and the Furlow technique has been associated with some of the lowest rates of persistent velopharyngeal insufficiency following primary repair (38,168,170).

While techniques for CP repair have become well established, postoperative development of oronasal fistulas still remains a significant problem. Reports have noted an incidence ranging from 11% to 23%, with the most likely site being the junction of the hard and soft palates (38,171). Several retrospective studies have identified the extent of cleft to be a significant factor, as patients with bilateral clefts were found to have a 2- to 3-fold higher incidence of postoperative fistula development compared with unilateral clefts (172). Operator experience has also been shown to play a role (172). Recent studies have evaluated the utility of the buccal fat pad as adjunctive tissue for use in both primary palatal cleft repair and treatment of postoperative fistulas. Used in a pedicled fashion with overlying mucosa, the buccal fat pad has been shown to successfully treated wide oroantral and oronasal clefts (38,173). More recently, buccal fat has also been employed to cover laterally exposed bone adjacent to gingival mucosa following medialization of the mucoperiosteal flaps (174,175). As this was found to re-epithelialize within 2 weeks, use of the buccal fat pad may result in an eventual reduction of palatal scarring, which may limit subsequent growth restriction of the maxilla (38).

Surgical repair of CP is the clinical standard of care. However, recent investigations showed the role of GSK-3 in the process. CP resulting from loss of GSK-3β could be rescued by protein stabilization during a specific window in embryogenesis in the mouse model. A mutant mouse carrying alleles for GSK-3 was injected with rapamycin to inducibly stabilize GSK-3β during various 2-d windows in embryogenesis within the timeline of palate development. This transgenic mouse was engineered to

carry alleles for GSK-3β such that without drug addition the unstable FRB* tag would necessarily cause protein degradation, and the mouse would exhibit a null mutation phenotype. Subsequent histologic analysis revealed that without rapamycin, no GSK-β3F/F embryos were able to rescue the CP; however, with rapamycin injection of the pregnant dam between E13.5 and E15.0, the majority of conditional GSKβ3F/F mutant animals could be partially or completely rescued from their CP in utero. Rescue was not seen in other injection windows during palatogenesis, suggesting a critical role for GSK-3β function in normal palatogenesis between E13.5 and E15.0 in the mouse model (176,177).

GSK-3β has been implicated as a key regulator of a wide variety of developmentally important molecular pathways including Wnt, nuclear factor of activated T-cells (NFAT), Hedgehog, and insulin signaling. These signaling pathways are essential components of many biologic responses and associated diseases, including embryonic development and cell fate determination, diabetes, neurodevelopment and neurodegeneration, psychiatric disorders, cell cycle regulation and cancer, hematopoiesis, and immunity. GSK-3β has not previously been implicated in the development of the mammalian palate. However, because it is positioned at the "node" of so many significant developmental pathways, analysis of GSK-3β function during palatogenesis will likely provide important insight into this common birth defect. Because of the "promiscuous" nature of GSK-3β, it has become a potentially important therapeutic target. Many potent and selective inhibitors of GSK-3 function are being developed by the pharmaceutical industry (178).

Although GSK-3β mutations have not been documented to be a cause of human orofacial clefting, recent findings suggest it is clinically relevant because of the potential to devise methods for improved treatments, including in utero rescue, for human orofacial clefting. Investigations of GSK-3β role in palatogenesis promise future clinical applicability, because it has the potential to reveal signaling pathways underlying cleft formation and lay the groundwork for potentially improved treatments using small molecules.

**Palatal Tissue Engineering**

The management of CP in patients has improved significantly over 20 years. The use of autogenous grafted material is now the standard of care, but tissue engineering is an attractive alternative that could greatly reduce the morbidity of surgery and potentially enhance the healing process (38). Regarding mucosal repair, cultured epithelial grafts, dermal substitutes and a combination of the two, called mucosa equivalents, are commonly used to provide extra tissue and aide in wound healing after CP repair (38). Cultured epithelial grafts can provide coverage for large areas while being derived from only a small biopsy, but they are prone to infection and fail to reduce scarring or contraction in full-thickness wounds due to absence of a dermal component (179). Such grafts can be either allogenic or autologous. Allogenic grafts have the advantage of being readily available but have a low take rate and are generally only used for temporary coverage, while autologous grafts take require extra time to culture but have a higher take rate (38). Dermal substitutes made from polymers, purified collagen

or de-epidermized dermis (DED or AlloDerm) provide additional physical support that is often lacking in epithelial grafts. However, some require a secondary procedure to apply a split thickness skin graft or cultured epithelia. In recent years, repair of palatal fistulas have begun to employ acellularized dermal matrix (AlloDerm) with promising results. Using AlloDerm as an interpositional layer between nasal and oral mucoperiosteum has significantly reduced fistula recurrence rates (38,180,181). Recent studies have also demonstrated the utility of AlloDerm as an adjunctive measure in the primary repair of wide clefts (38).

Cultured mucosa equivalents provide an epithelial and dermal substitute in a one-step process, which seem to be the optimal replacement for mucosa, since this provides material for repair with properties closest to the original tissue. In CP repair, one of the major challenges lies in reconstructing the bony hard palatal and alveolar defects. Surgical repair with autogenous bone grafts is the current standard of care. Bone is most commonly harvested from the iliac crest but can be taken from the rib, tibia, calvarium or mandibular symphysis (38). This often requires multiple operations and extensive healing time and is associated with high donor site morbidity, including postoperative pain, altered sensation, scarring and infection. In addition, bone graft harvest ultimately yields a very limited quantity of bone for reconstruction (38,175).

This bone often does not fully integrate into the host site and can undergo some resorption. Bony repair needs to be very strong to support tooth eruption and to withstand physical stress from muscles of mastication. There are also allogeneic and synthetic materials available for grafting, and while these solve the problem of donor site

morbidity, there is still the risk of infection, elicitation of an immune response and problems with structural integrity and contour (38). The use of tissue engineering could avoid many disadvantages of autogenous grafting, such as donor site morbidity, and could potentially decrease the number of surgeries needed while providing improved outcomes.

## Craniofacial Engineering Using Stem Cells

### *Mesenchymal Stem Cells (MSCs)*

The identification of pluripotent MSCs in the bone marrow stroma over 25 years ago has led to a variety of research avenues. Capable of differentiating to multiple mesodermal lineages, including bone and cartilage, MSCs have become a standard in the field of adult stem cell biology and in regenerative medicine (182,183). It is only natural that these stem cells would be used in the repair of significant bony defects caused by trauma, surgery, or disease. Consistent with this, multiple studies have reported the formation of bone tissue both in vitro and in vivo upon the combination of MSCs and 3D scaffold supports. In vitro, a wide spectrum of scaffolds are being combined with MSCs, including chitosan or gelatin, electrospun collagen nanofibers, honeycomb collagen scaffolds, and titanium meshes (184). In animals, the scaffolds and model systems used have varied from Hydroxiapatita (HA) ceramics or hydroxyapatite and tricalcium phosphates (HA/TCP) constructs for the healing of small bone defects in rodents or larger defects in dogs, rabbits, or sheep to complicated biosynthetic composites to silk-based biomaterials in the healing of segmental femoral defects in nude mice(138). Each

of these studies report encouraging results and espouse the use of bone marrow MSCs in the repair of bony defects (2,185).

***Adipose-derived Stem Cells (ASC)***

Historically, the adipose compartment has been considered primarily a metabolic reservoir of storing, and releasing high-energy substrates. Today, the adipose compartment may be a site for an abundant population of stem cells, ASC or adipose-derived stem cells (2). Like the bone marrow, adipose tissue contains an extensive cellular stroma comprised of fibroblastic-like cells termed by Rodbell in 1964 as the stromo-vascular fraction or SVF. Further work by Hauner expanded this knowledge and postulated that the preadipocytes within the SVF represented a progenitor population, though apparently limited to the adipocytic lineage (186). However, in 2001, Zuk et al. showed that the SVF fraction isolated from human lipoaspirates in fact contained cells with multilineage potential and termed these cells pressed lipoaspirate cells (2). Now renamed ASCs, these cells undergo adipogenesis, osteogenesis, chondrogenesis, and myogenesis in vitro, suggesting that the SVF fraction of adipose tissue may, in fact, be comprised not just of lineage limited preadipocytes but of multipotent stem cells. ASCs have also become a hot topic in the world of tissue engineering. Numerous studies have begun to explore the osteogenic potential of ASCs in vivo through their combination with a wide variety of scaffolding materials. Groups led by Lee and Hicok were the first to show that implantation of human ASCs loaded onto polyglycolic scaffolds could result in the formation of an osteoid material (2,138,187,188).

To improve their ability to form bone, many of these studies treat ASCs with the osteogenic growth factor BMP2. Both Peterson and Dragoo were the first to describe the engineering of well-formed bone by ASCs in rodents with the help of bone morphogenic protein 2 (BMP2) and several MSC studies have shown that this osteogenic factor can be used in concert with these stem cells also. Many of these studies claim that increased bone formation can be attributed to the presence of BMP2- treated ASCs. However, work by Leboy has suggested that BMP2 may not promote osteogenic differentiation of human MSCs. Similarly, in patients receiving recombinant BMP2 treatment, the regenerative response is several times lower than that previously measured in animal studies, suggesting that the response of human cells to BMP2 may not be directly comparable to that observed by animal cells. Although several studies have begun to combine BMP2 and ASCs, surprisingly, to date, no in-depth in vitro studies have been performed to confirm if BMP2 can actually promote ASC osteogenesis (2).

***Scaffolds***

Efficient use of 3D scaffold systems in bone repair is dependent upon their bond-bonding or bioactive ability. Alhough scaffolds such as Poly lactic glycolic acid (PLGA) or Poly lactic acid PLA composites provide the reconstructive surgeon with a biodegradable platform for stem cell adhesion and differentiation, their bioactivity can be limited. However, studies have suggested that their bioactivity can be strengthened through the formation of a layer of hydroxyapatite (HA) at the bone-implant interface (2). Several HA materials for use in bone differentiation have been developed within the last 20 years and are thought to possess superior in vivo bioactivity. However, much

excitement has been generated regarding the osteoinductive capacities of biomimetic apatite coatings. Typically created through the immersion of 3D scaffolds in ionic solutions with compositions similar to blood plasma called Simulated Body Fluids biomimetic apatites are composed of plate-like crystals of calcium phosphate capable of coating the entire 3D scaffold architecture (189,190). An improvement on biomimetic apatites presented by Wu and colleagues through their development of accelerated biomimetic approaches, which have dramatically shorten the time required for coating from approximately 2 weeks to 2 days (191). Such convenience may make the accelerated biomimetic apatite more attractive for in vivo applications such as bone healing. In support of this, accelerated apatite coatings have been shown by Wu and his group to promote bone in-growth and differentiation of preosteoblasts and bone marrow stem cells and to enhance direct bone to bone contact. Recently, accelerated apatites have also been shown to promote the osteogenic capacity of ASCs. In a paper by Cowan et al., murine ASCs seeded onto accelerated apatite coated PLGA scaffolds healed critical-sized cranial defects without the need for exogenous stimulation such as BMP2 treatment (192). Although the ASCs used were murine and no further studies using human ASCs have been reported, these results remain exciting because they show the reconstructive surgeon that methods other than conventional growth factor stimulation may be used to induce stem cells to make and heal bone (2).

## Conclusion

CLP is caused by many factors, including both genes and environment. Gene targeting technology and basic conventional techniques using animal models led to the identification of genes associated with known and unknown etiologic factors. However, it should be noted that there are other cases where the human gene deficiency was identified first and replicated in an animal model. It is also clear from this extensive list of possible contributing genes that the molecular and cellular interactions associated with CLP are not all understood. Tissue engineering approaches also remain an exciting and potentially profitable direction of investigation as the field of regenerative medicine advances.

CHAPTER II

# EPHRIN REGULATION OF PALATE DEVELOPMENT*

## Synopsis

Studies of palate development are motivated by the incidence of cleft palate. Although, mechanistic studies of palate growth and fusion have focused on growth factors such as TGFβ3, recent studies have revealed that the ephrin family of membrane bound ligands and their receptors, the Ephs, play central roles in palatal morphogenesis, growth, and fusion. In this chapter, the recent findings on the functions of ephrins in palatal development will be discussed.

## Introduction

### *Ephrin Involvement in Palatal Growth and Fusion*

The Eph family is the largest family of mammalian receptor tyrosine kinases. Ephs and their membrane-bound ephrin ligands are responsible for multiple adhesion, migration, and boundary forming events throughout development, particularly midline fusion events such as urorectal closure (193-195). Binding of ephrins to Ephs on opposing cells causes kinase activation in the Eph-bearing cells (forward signaling),

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while binding of Ephs can activate intracellular signaling inside ephrin-bearing cells (reverse signaling). Ephrin-Bs are transmembrane proteins that have conserved intracellular signaling domains while ephrin-As are glycosylphosphatidyl-inositol (GPI)-linked and use co-receptors to signal. Ephrin-As preferentially bind to the EphA subclass of receptors, while ephrin-Bs bind to EphBs, although there is physiologically relevant binding across classes, most notably between EphA4 and all three B ephrins.

Over the years, a number of genetically modified Eph and ephrin alleles have been created in mice to both track expression of these molecules and to examine the roles of forward and reverse signaling in developmental processes. In addition to traditional gene knockouts, several LacZ knock-in alleles have been generated. In these, either the entire protein or just the cytoplasmic domain of the Eph or ephrin-B in question is replaced with a bacterial beta-galactosidase moiety that can be visualized in tissue by incubation with X-gal to produce a blue precipitate. The chimeric alleles are especially useful because they lack intracellular signaling ability while retaining activity as ligands from their extracellular domains. Thus, they can be used to separate forward and reverse ephrin signaling pathways.

The first evidence that ephrins play a role in palate development came with the linkage ephrin-B1 mutations to craniofrontonasal syndrome in humans, of which CP is a prominent feature (196,197). At the same time, Davy, et al. reported that deletion of ephrin-B1 in cranial neural crest cells in mice caused craniofacial deformities, including CP (198). The fact that these defects resulted from cell-autonomous ephrin-B1 deletion suggested that ephrin-B1 reverse signaling is important for palate formation. Five years

later, Risley et al. reported that forward signaling through the combination of EphB2 and EphB3 is necessary for growth of palatal mesenchyme (49). These authors used EphB2 $^{LacZ/LacZ}$; EphB3$^{-/-}$ compound mutant mice to create forward signaling double knockout mice (EphB3 signaling is removed while EphB2 forward signaling is removed and reverse signaling is still intact). These mice had CP from stunted palatal shelf growth, while EphB2 and EphB3 single mutants alone did not. Shortly after the Risley study, Bush et al. found that forward signaling from ephrin-B1 in palate mesenchyme was required for mesenchymal proliferation through a mechanism requiring MAPK/ERK activation. Without ephrin-B1, mice displayed CP because the shelves failed to grow to midline (46). These data together suggest that Ephs B2 and B3 function as the receptors for ephrin-B1 in palate mesenchyme. The EphB2 kinase was recently shown to increase proliferation in intestinal cypts through stimulation of Cyclin-D1 levels downstream of Abl activation (199).

When palatal shelves from EphB2 $^{LacZ/LacZ}$; EphB3 $^{-/-}$ compound mutants were placed in contact with each other in culture, they adhered to form an MES and fused normally (49). This demonstrated that EphB2 and EphB3 forward signaling are not required for fusion, and that reverse signaling from EphB3 alone is not critical for fusion, although the extracellular domain of EphB2 was still able to act as a ligand for reverse signaling in these mice.

In embryonic palate, the expression of Ephs and ephrins was examined using LacZ indicator mouse lines. A summary of these expression patterns in fusing palate combined with those for Ephs and ephrins in the published literature is presented in

(Figure 2-1). Ephrin-B2 and EphB2 were found to express specifically in the MES immediately prior to and during its degradation. This suggested that ephrin signaling contributes to palatal EMT and fusion.

A group of researchers had reported a study in which 26% of mice homozygous for the ephrin-B2$^{LacZ}$ allele had CP (200). In these experiments only a minority of these embryos had CP, suggests that other Eph and ephrin family members contribute to reverse signaling at the midline and remain unaccounted for. This is not surprising, as Eph/ephrin mediated developmental processes are frequently under redundant control by multiple family members, including in palate, as noted above. Interestingly, the study by Dravis et al. also showed ephrin-B2 expression in the mesenchyme before its re-localization to the MEE at the time of fusion, suggesting that ephrin-B2 plays a role in palatal shelf growth alongside ephrin-B.

They also showed EphB3 expression in the MEE at fusion, implicating it in the fusion process. These data emphasize the likely involvement of multiple Eph and ephrin family members in both phases of palatal development.

If ephrin-B1 is expressed in the palatal mesenchyme, and ephrin-B2 in the epithelium, how might these two molecules combine to mediate MES degradation and fusion? One possible answer may be found in recent studies on the role of ephrin signaling in cancer cell migration. (201). Astin et al., demostrated that prostate cancer cells are prodded along in their migration through fibroblasts by the activation of EphB3/EphB4 forward signaling in response to ephrin-B2 ligand from the surrounding fibroblasts (201). This forward signal activates Cdc42 within the cancer cells to

eliminate contact inhibition and increase their invasiveness. It may be that a similar mechanism is at work in palatal MEE cell migration through the ephrin-B1-expressing mesenchyme. Whereas reverse signaling in MEE cells initiated by contact with Ephs (acting as ligands in reverse signaling) on the opposing shelf MEE begins the process of EMT, mesenchymal ephrin-B1 (acting as ligand) activates forward signaling in the former epithelial cells to continue their migration and complete MES degradation. Ephrin-B1 may also provide a signal to the migrating former MEE cells that causes their eventual apoptosis, as B ephrin forward signaling is known to cause apoptosis in other systems (202).

***Ephrin Signaling in Palatal EMT and Fusion***

Ephrin reverse signaling was found in the chicken palate and it was sufficient to cause palatal fusion without the presence of TGFβ3, and that TGFβ3 cannot cause fusion without the ephrin signal. Yet there is clearly a question of signaling level. The fact that chicken palates cultured without TGFβ3 will not fuse unless exogenous EphB2/Fc is added, and that TGFβ3 knockout mouse palates do not normally fuse, indicates that the level of ephrin reverse signal naturally present in palatal tissue is not enough to overcome a lack of TGFβ signaling. The TGFβ3 and ephrin pathways must interact in one of two ways. The first possibility is that TGFβ3 activates expression of ephrins and/or Ephs in palate tissue to reach a threshold level required to activate fusion. In this model, ephrins are genetically and mechanistically downstream of TGFβ3. The second is that the two act in parallel, but intersect such that the TGFβ3-activated signals add to those elicited by ephrin activation to reach the level necessary to cause MES

degradation. The ephrin signal must still be preeminent; however, as elevated ephrin stimulation obviated the need for TGFβ3 in the palate fusion assay, while addition of exogenous TGFβ3 did not compensate for a lack of ephrin signal. The activity of phosphatidylinositol 3-kinase (PI3K) is required for TGFβ3 stimulation of fusion (203) and a recent study discovered that the same is true for ephrin reverse signaling as palates stimulated in culture with EphB2/Fc did not fuse in the presence of the PI3K inhibitor LY294002.

The data on ephrin-B2 expression supports the EMT model of palatal fusion in that the cells of the ephrin- B2-positive MES was observed in the act of dispersing into palatal mesenchyme during fusion. Epithelial cells have a polarized, inflexible morphology maintained by specific networks of intermediate filaments, cell-cell junctions, and adhesions to the extracellular matrix. The transition to a more fibroblastic, motile phenotype such as is observed in the palatal MES, requires the dismantling of these networks in favor of a more fluid cytoskeletal arrangement and more plastic cell-cell contacts. Cytokeratin intermediate filaments disappear in favor of vimentin; laminin-1 content in the extracellular matrix decreases as fibronectin increases, and E-cadherin based adherins junctions are replaced by N-cadherin based cell-cell contacts (204,205). These changes in expression are governed by a set of transcription factors such as Twist1 and Snail, both of which are regulators of EMT during gastrulation and palate development (14,204,206). Thus, EMT involves a reorganization of the cytoskeleton and a major shift in gene expression.

So, how do ephrins contribute to these events? Part of the answer is found in the EMT that is required for metastasis of epithelia-derived tumors (207). In certain settings, repulsion between Ephs and ephrins serves to keep potentially cancerous cells within their niche, such as in the colon, where ephrins keep intestinal crypt stem cells from migrating to the luminal ends of villi to form tumors (199,208). In instances such as these, Ephs appear to function as tumor suppressors. In many other cases; however, ephrins are upregulated in cancers, and their expression is associated with increased EMT and metastasis of malignancies. As mentioned above, the study by Astin et al. demonstrated that ephrin reverse signaling enables the loss of contact inhibition seen in prostate cancer cells and promotes their migration past normal fibroblasts (201). A novel finding of PI3K involvement in ephrin reverse signaling provides a connection to this migration mechanism. PI3K signals to Akt, which activates the mTor complex, leading to migration of cancer cells. This pathway is frequently activated in malignancies, and inhibition of the mTor complex proteins Raptor and Rictor retards cancer cell invasiveness and suppresses the EMT required for metastasis (209). This mechanism may control the EMT and migration of epithelial cells during palatal fusion (Figure 2-2).

The PI3K/Akt/mTor system also connects to transcriptional activation associated with cancer EMT. The mTor kinase phosphorylates the signal transducer and activator of transcription 3 (Stat3) on Ser727, and thereby activates a transcriptional program of growth and invasiveness (210,211). Stat3 activation is frequently associated with carcinoma invasiveness and poor prognosis (212). Active Stat3 upregulates Twist1 and Snail, which in turn suppress E-cadherin expression (206,213). Svoboda et al.

demonstrated that Twist1 regulates palatal fusion (214). Thus, PI3K potentially connects ephrin-B reverse signaling to an EMT-associated gene expression program in palate.

Phosporylated ephrin-B1 was also reported to bind directly to Stat3 in embryos and tumor cells, suggesting that direct recruitment of this transcription factor to the cytoplasmic domain of ephrin-Bs contributes to its activation (215). In addition to being a transcriptional activator, the ephrin-B1 cytodomain has been shown to bind the transcriptional repressor Groucho/TLE (216). Though the significance of this binding to EMT is unknown, Groucho has been reported to repress transcription downstream of TGFβ signaling, thus providing another potential cross-interaction with the TGFβ3 system in palate (217).

**Conclusion**

The study of ephrins in palate development is still in its beginnings. It is now known that ephrin forward signaling is necessary for early palatal shelf growth, and that ephrin reverse signaling is required for fusion of those shelves. But some important questions still remain: (1) which Ephs and ephrins control fusion? (2) what are the specific downstream effectors of Ephs and ephrins in palatal mesenchyme and epithelium? (3) how do TGFβ3 and ephrin signaling pathways intersect? and (4) what elements of the transcriptional program in palatal EMT are controlled by ephrin signaling?

The large collection of molecular and genetic tools available for studying ephrins in development makes it certain that efforts to answer these questions will accelerate in the coming years, and this will benefit both the fields of craniofacial biology and cancer.

# CHAPTER III

# EPRHIN REVERSE SIGNALING CONTROLS PALATE FUSION PI3 KINASE DEPENDENT MECHANISM*

## Synopsis

Secondary palate fusion requires adhesion and epithelial to mesenchymal transition (EMT) of the epithelial layers on opposing palate shelves. This EMT requires transforming growth factor β3 (TGFβ3), and its failure results in cleft palate. Ephrins, and their receptors the Ephs, are responsible for migration, adhesion, and midline closure events throughout development, and both can act as signal transducing receptors in these processes (termed "reverse" and "forward" signaling, respectively). Activation of ephrin reverse signaling in chicken palates was found to induce fusion in the absence of TGFβ3, and that PI3K inhibition abrogated this effect. Further, blockage of reverse signaling inhibited TGFβ3-induced fusion in the chicken and natural fusion in the mouse. Thus, ephrin reverse signaling is necessary and sufficient to induce palate fusion independent of TGFβ3 (218). The data in this chapter describe a novel role for ephrins in palate morphogenesis, and a previously unknown mechanism of ephrin signaling.

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## Introduction

The molecular mechanisms that control mammalian palate development are poorly understood. In the mouse, the mesenchymal extrusions of tissue that will ultimately form the hard palate elevate over the tongue starting at E11.5. By E14.5, they have grown to meet at the midline, at which time the layers of epithelium on the opposing shelves adhere (219). Current evidence indicates that these cells then undergo epithelial to mesenchymal transition (EMT) and apoptosis to achieve a fused palate of confluent mesenchyme, and both these aspects of the fusion process require the action of transforming growth factor beta 3 (TGFβ3) (220,221). Kang and Svoboda showed that PI3 kinase signaling is required for fusion (222). And recently, Xu et al. demonstrated that both Smad4 and p38 MAP kinase act downstream of TGFβ to cause palate fusion, although either alone appeared dispensable (41,218).

Early migration of embryonic neural crest cells that form the mouse palate require the action ephrin-B1, and its cognate Eph receptors (218,223). The Ephs are the largest family of receptor tyrosine kinases (RTKs), and are subdivided into A and B groups based on their preferential binding to the glycosylphosphatidyl inositol-linked A ephrin or the transmembrane B ephrin ligands, although binding can be promiscuous across classes (218,224). Ephrins are unique among RTK ligands in that they can also act as receptors, with the Eph acting as ligand, a process called “reverse signaling” (194,218). In humans, ephrin-B1 mutations are associated with syndromes that include CP (225-227), highlighting a likely conserved role for ephrin signaling in palate development. Recent work by Risley, et al. reported a role for EphB forward signaling in

the proliferation of palate shelf mesenchyme prior to midline apposition (49,218). This study was closely followed by a report from Bush and Soriano (2010) showing that ephrin- B1 forward signaling controls NCC-derived mesenchyme proliferation through the mitogen-activated protein kinase (MAPK) pathway (218). These findings are consistent with known roles for EphB forward signaling in progenitor proliferation in other systems, such as the hippocampus and the intestine (208,228). Both the Risley et al. (2009) and the Bush and Soriano (2010) studies showed evidence of Eph and ephrin in palate at a stage when the shelves begin to undergo EMT and fuse (218).

Risley et al. (2009) reported Ephs B2 and B3 and all three B ephrins in mouse palate epithelium and mesenchyme at E14.5, and Bush and Soriano (2010) showed ephrin-B1 at the same stage, only in the mesenchyme. Ephs and ephrins direct midline adhesion and fusion events in other developmental processes, such as urethral closure and urorectal septation (195,218). Thus, the question is whether they play a similar role in palate fusion. Here, in this chapter it is documented that the expression of B Ephs and ephrins in pre-fusion palate epithelium and the requirement for ephrin reverse signaling in palate fusion. The findings describe a novel role for ephrins in craniofacial development and point to a unknown mechanism of ephrin reverse signaling (218).

## Experimental Procedure

### *Chemicals*

TGFβ3 was obtained from Invitrogen (Carlsbad, CA). LY294002 PI3K inhibitor was from Cell Signaling Technology (Danvers, MA). EphB2 ectodomain was from

R&D Systems (Minneapolis, MN). IgG Fc protein was from Calbiochem (EMD Chemicals, Gibbstown, NJ) (229). Ephrin-B2 ectodomain fused to human Fc was produced by cloning a PCR product encompassing the extracellular portion of the coding sequence for murine ephrin-B2 into pFUSE-hIgG1e3-Fc2 (InvivoGen, San Diego, CA). The resulting plasmid was transfected into CHO-K1 cells (ATCC). Fusion protein was collected from conditioned supernatant by protein A chromatography and analyzed by Western blot with anti-Fc antibody (Jackson Immunoresearch). Detailed cloning and purification protocols available upon request. Fc clustering was accomplished with the same anti-Fc antibody (218).

***Embryonic Palate Culture***

Chicken palate culture was performed as previously described (203). Briefly, palate shelves were dissected from eight day old chicken embryos and placed nasal side down on nucleopore polycarbonate membranes and cultured with in BGJb medium (InvivoGen, San Diego, CA) for 72 h in 37oC with 5% CO2. Medium was replaced every 24 h with fresh treatments. TGFβ3 was used at 50 ng/ml. EphB2, ephrin-B2, and control IgG Fc proteins were used at 5 ng/ml. EphA4/Fc was used at 20 ng/ml (218). IgG Fc was added at 20 ng/ml when used as control for EphA4/Fc. LY294002 was used at 10 μM. To cluster Fc proteins, protein was mixed with anti-Fc in a 4 to 1 w/w ratio as a 50x or 100x stock and incubated at 22C for 1 h or overnight at 4C (218,230).

***Histological Analysis***

Cultured palates were fixed in 4% formaldehyde/phosphate buffered saline for 2 days. They were then stabilized in low melting point agarose and processed for paraffin

embedding. Serial 6 mm sections were collected in the coronal orientation from anterior to posterior. Sections were stained with hematoxylin and eosin (H&E) and photographed on a light microscope with a digital camera (229). Every twentieth section was scored for fusion by at least two independent, blinded observers using the previously described scale (Kang and Svoboda, 2002) (203): 1=non-fusion with no adhesion, 2=non-fusion with some apparent adhesion, 3=partial adhesion with some disintegration of MEE layers, 4=complete fusion with some traces of MEE cells or seam remaining, 5=complete fusion with no evidence of MEE cells or seam visible (229).

## Results

### *Eph and Ephrin Expression in Fusing Palate Epithelium*

The expression of beta-galactosidase (βgal) was examined in mouse embryos that were genetically engineered to express a chimeric ephrin-B2 allele in which the intracellular domain was replaced by a βgal moiety (218,231). At E14.5, ephrin-B2/βgal specifically was found in the palate epithelial cells, many of which had begun to migrate inward to mix with mesenchymal cells in the interior of the shelf (Figure 3-1A). This migration parallels the previously documented movement of fluorescently labeled epithelial cells during the EMT process that leads to fusion (192,218,232) and validates ephrin-B2 as a marker for palate epithelium. It was also noted βgal expression in the same layer in the EphB2/LacZ mouse, in which the lacZ gene replaces the EphB2 allele (218,233) (Figure 3-1B). These data suggested that Eph/ephrin forward and/or reverse signaling in the epithelial layers may play a role in palate adhesion and fusion (229).

***Activation of ephrin reverse signaling causes palate fusion***

To examine the role of ephrins in palate fusion, It was employed a well established *ex vivo* chicken palate culture system. The chicken palate does not naturally fuse, unlike the mouse palate. However, the palate shelves will fuse if placed in contact and given exogenous TGFβ3 (234). In this respect, the functional difference between mouse and chicken is that the mouse palate makes its own TGFβ3 (218,235); anti-TGF antibodies will block mouse palate fusion (40,218). Thus, the chicken system allows to examine ephrin signaling apart from TGFβ3 simply by adding or withholding this factor. To stimulate Eph forward signaling, purified recombinant ephrin-B2 extracellular domain fused to human Fc was used. In order for this protein to be biologically active, it must be artificially clustered by the addition of anti-Fc antibody to stimulate the receptor aggregation that is required to initiate intracellular signaling (218,230). The protein was applied, with or without TGFβ3, to palate shelves placed in contact for 72 h. The tissues were fixed and evaluated them histologically for fusion using the previously published one to five scale for mean fusion score (MFS) (203). Palates treated with IgG Fc as a negative control did not fuse (MFS=2.2±0.20), while those treated with TGFβ3+IgG Fc fused as expected (MFS=3.6±0.20; Figure 3-2). In comparison, palates treated with clustered ephrin-B2/Fc exhibited mildly increased partial fusion (MFS=2.6±0.23)(218).

More dramatic results were observed when the palates were treated with clustered EphB2/Fc recombinant protein to activate reverse signaling through endogenous ephrin-Bs. Clustered EphB2/Fc gave an MFS of 3.4±0.24, essentially equivalent to TGFβ3 treatment. When applied without clustering, Eph and ephrin Fc

proteins serve as effective blocking reagents because they bind to their target ligands and receptors without activating biological signaling, thus acting as competitive inhibitors (218,236). This strategy was employed by adding unclustered EphA4/Fc to palate cultures. EphA4 binds to all A and B ephrins and so serves as a good pan-ephrin blocker (237). This treatment effectively blocked palate shelf fusion, even in the presence of TGFβ3 (MFS=2.1±0.13; Figure 3-2). Thus, these data indicate that ephrin reverse signaling is required for palate fusion and is genetically downstream of TGFβ3 (218).

If this role of ephrins in fusion is conserved in mammals, then it would expect that inhibiting ephrin action in mouse palate would prevent the naturally occurring fusion normally observed in culture (218). To test this prediction, unclustered EphA4/Fc to E14.5 mouse palate culture was applied, and observed the same effect as in the chicken: an inhibition of palate fusion (MFS=2.55±0.12 vs 4.26±0.08) (Figure 3-3. Interestingly, it was observed in the mouse that fused areas were interspersed with non-fused areas along the length of touching epithelium. Nevertheless, the data clearly demonstrate that the requirement for ephrin signaling is conserved between chicken and mouse (218).

***Ephrin-Dependent Fusion is PI3 Kinase Dependent***

The palatal culture evidence indicated that ephrin signaling in palate fusion acts downstream of the receptor for TGFβ3. As noted above, previous work showed that TGFβ3-induction of fusion requires active PI3K, as PI3K inhibition abrogates or delays fusion (218). This would appear to place a required TGFβ3 effector downstream of ephrin signaling. Therefore it was tested whether ephrin reverse signaling also acts

through PI3K. Chicken palates cultured in the presence of the PI3K inhibitor LY and either TGFβ3 or clustered Eph-B2 failed to fuse over the test period (Figure 3-4). Thus, it can be concluded that ephrin reverse signaling acts through the PI3K pathway to cause fusion (218).

**Discussion**

Collectively, this study shows that ephrin reverse signaling is both required and sufficient for chicken palate fusion, and that PI3K is part of this signaling mechanism. As the stimulating and blocking reagents used here are not specific to a single Eph or ephrin member, we are not able to identify the individual ephrin that mediates fusion, or even whether t is a single or multiple ephrins (218). We do not know from our own experiments the expression of the remaining B ephrin, ephrin-B1, in the fusing palate. Our expression data for EB2 and EphB2 conflict with those of Risley et al., which reported all three B ephrins and EphB2 in both epithelium and mesenchyme at e14.5 (49). Although ephrin-Bs are more likely candidates for receptors of the EphB2 used in experiments (193,194,218). EphB2 is activated by at least one ephrin-A ligand (238). The initial expression evidence does suggest ephrin-B2 as the most likely candidate due to its presence in the midline epithelium at the time of palate contact. As mentioned, the observed migration of ephrin-B2/LacZ expressing cells into the mesenchyme during fusion parallels that seen in MEE cells labeled with vital dyes (239), and defines ephrin-B2 as a marker of these cells (218).

Another important question is how the signaling pathways of TGFβRs and ephrins intersect within cells of the fusing palate. Up to now, study of signal transduction in palate fusion has focused on events downstream of TGFβ3, as this was thought the chief requisite growth factor for EMT and fusion (218,221,240). The chicken palate findings show that ephrin signaling is downstream, or at least independent of, TGFβR signaling in that it was possible to dispense with the TGFβ3 treatment. It is now know that the PI3K mechanism is a point of intersection, but the up-and downstream details of the interaction are unknown (Figure 3-5) The action of the transcription factors Twist1 and Snail were shown by Yu et al. to mediate at least a substantial part of TGFβ3's fusion-promoting activity, as RNAi against these genes' mRNAs partially inhibited fusion (218,241). It will remain to be seen whether ephrin reverse signals impact these factors as well.

The EphA4/Fc blocking experiments demonstrated that the ephrin requirement is conserved between mouse and chick (218). However, previous data suggests that ephrins may not be sufficient in the mouse. Blocking antibodies against TGFβ3 inhibit mouse palate fusion, and genetic ablation of TGFβ3 in mice yields cleft secondary palate (34,40,220,235). This suggests that endogenous ephrin signals are not sufficient to compensate for loss of TGFβ3 (218). Thus, there may be a difference in the hierarchical roles of TGFβ3 versus ephrins between the two species. Alternatively, ephrin expression in the palate epithelium may be under the control of TGFβ3 such that loss of the TGFβ3 signal eliminates the endogenous ephrin signal as well (218).

The findings in this study have broader implications for ephrin biology beyond the example of palate fusion. Ephs are known to mediate EMT in other processes, such as cancer metastasis, and although PI3K signaling was implicated in Eph/ephrin systems, it was in the forward direction, downstream of the Eph RTKs (199,218). There is a report of PI3K being required for *in vitro* ephrin induced proliferation in an endothelial cell line via reverse signaling (242), but this work is the first evidence of reverse signaling mediating a biologically critical developmental EMT event via PI3K. Although PI3K is associated with cell motility, and ephrin reverse signaling is associated with cell migrations, this pathway has not been connected with the known signaling elements of the ephrin-B cytoplasmic domain (218).

The cytoplasmic domains of B class ephrins contain conserved tyrosines that are phosphorylated upon stimulation to form SH2-binding domains that bind the adaptor Grb4 (218,243), although it is possible they may bind other SH2 domain proteins in different contexts. Grb4 complexes with other factors such as the GTPase activating protein 1 (GIT1) and Glutamate receptor interacting protein 1 (GRIP1) in the case of synapse formation (218,244,245). B ephrins also have a C-terminal PDZ-binding domain, which is required for a number of biological functions (47,246). Binding of SH2 proteins in the PI3K pathway to ephrin-B2 is certainly plausible, but not yet demonstrated. A-class ephrins, on the other hand, do not have cytoplasmic domains. Still, they can participate in reverse signaling via a co-receptor (218,247,248). Of course, it is also possible that the reverse signal does not directly activate PI3K, but promotes is

action indirectly (218). This finding therefore encourages increased investigation along a new direction of ephrin signaling.

The ongoing studies will focus on identification and regulation of the specific Eph and ephrin molecules involved in palate fusion, the signal transduction pathways downstream of those ephrins, and their intersection with TGFβR-mediated pathways (38,218).

# CHAPTER IV

# EPHRIN REVERSE SIGNALING MEDIATES PALATAL FUSION AND EPITHELIAL-TO-MESENCHYMAL TRANSITION INDEPENDENTLY OF TGFβ3

## Synopsis

The mammalian secondary palate forms from shelves of epithelia-covered mesenchyme that meet at midline and fuse. Failure of the midline epithelial seam (MES) to degrade blocks fusion and causes cleft palate. It was previously thought that transforming growth factor β3 (TGFβ3) is required to initiate fusion. Members of the Eph tyrosine kinase receptor family and their membrane-bound ligands, the ephrins, are expressed on the MES. It was discovered that activation of ephrin reverse signaling (where the ephrin acts as a receptor and transduces signals from its cytodomain) was sufficient to cause fusion in cultured mouse palates and epithelial-to-mesenchymal transition (EMT) in palatal epithelial cells, even in the absence of TGFβ3 signaling. In this chapter, cultured mouse palates in the presence of either a blocking antibody against TGFβ3 or an inhibitor of the TGFβrI serine/threonine receptor kinase were shown. Fusion was abolished by both treatments, but was significantly rescued by the addition of EphB2/Fc recombinant protein to activate ephrin reverse signaling. Cultured palate epithelial cells traded their expression of epithelial cell markers for that of mesenchymal cells after treatment with EphB2/Fc and became motile. They concurrently increased their expression of the EMT-associated transcription factors Snail, Sip1, and Twist1. The

data confirm that ephrins direct palatal fusion in mammals and activate a gene expression program not previously associated with reverse signaling.

**Introduction**

The secondary palate in humans and mice forms from shelves of mesenchyme covered by epithelium. These shelves grow out bilaterally from the internal surfaces of the maxillary processes, elongate on each side of the tongue and become horizontal above the tongue as it descends (249,250). As soon as the opposing shelves reach each other, the lateral surfaces of the medial edge epithelia (MEE) cells form the medial epithelial seam (MES) (249,251). Complete disintegration of the MES is essential to form a confluent structure, and failure of palatal fusion causes CP, one of the most common birth defects (252). Thus, understanding the mechanism of fusion is an important goal of craniofacial biology.

Palatal fusion has been thought to require Transforming Growth Factor β-3 (TGFβ3) because TGFβ3 knockout mice, as well as naturally TGFβ3-null avian systems, display CP, and treatment of either with exogenous TGFβ3 rescues palatal fusion (234,253,254). Genetic and phamacological studies have shown that the TGFβ3 signal, acting through serine/threonine kinase TGFβ receptors (TGFβr) on MEE cells, activates Smad, p38 mitogen-activated protein kinase (MAPK) and phosphotidyl inositol 3 kinase (PI3K) pathways in palate epithelium (255,256). Fusion requires PI3K and either (but not necessarily both) the Smad or p38 pathways (255). However, the mechanism of MES degradation is still in question. Numerous studies suggest that the epithelial cells

undergo epithelial-to-mesenchymal transition (EMT), apoptosis, or both (thoroughly reviewed in (250). Recent work on cultured primary MEE cells indicates that TGFβ3 causes these cells to shift gene expression patterns away from epithelial markers to fibroblastic ones, while assuming a migratory phenotype (the definition of EMT). They then initiate caspase-dependent apoptosis. This entire process occurs in culture over the same 72 h time frame as does fusion in the mouse embryo, consistent with a mechanism that is reflective of the actual process *in vivo* (257).

It was recently reported a role for ephrin signaling in palatal fusion. The Ephs are the largest family of receptor tyrosine kinases. They are classified as A or B based on sequence homology and on their binding preference for the transmembrane B ephrin or the glysosyl phosphotidyl inositol linked A ephrin ligands (258). Eph-ephrin systems control a number of contact-dependent processes in development, including cell migration, boundary formation, and proliferation (198,259,260). Ephs function as traditional receptor tyrosine kinases when bound by their ephrin ligands, but they can also act as ligands that activate signaling downstream of the ephrin, which assumes the role of receptor in what is called "reverse signaling" (261). EphB and ephrin-B expression was reported in the MEE during fusion, and that ephrin-B reverse signaling was found to be required for palate fusion in mice and is sufficient to cause fusion in chicken palates without TGFβ3 (218). This finding was supported by a report of CP in ephrin-B2 reverse signaling-deficient mutant mice (200). Interestingly, it was discovered that the ephrin reverse signal passes through PI3K, a signaling pathway not previously associated with reverse signaling (218).

It was found that activation of reverse signaling in mouse palates is sufficient to cause fusion independently of TGFβr, and that the ephrin signal activates an EMT-like program in palatal epithelial cells. This data describe a role for ephrins in craniofacial development, and help to clarify their role in palatal fusion.

## Experimental Procedure

### *Chemicals and Reagents*

Anti-TGFβ3 was obtained from R&D Systems (Minneapolis, MN). The TGFβrI Kinase Inhibitor VI (SB431542) was from Calbiochem (EMD Millipore Cat#616465) (Billerica, MA). EphB2 ectodomain Fc fusion protein was from R&D Systems (Cat #467-B2) (Minneapolis, MN). IgG Fc protein was from Calbiochem (EMD Millipore Cat #401104) (Billerica, MA). Exogenous recombinant TGFβ3 (R&D systems, CA). For Immunofluorescence, primary antibodies used (and their source) included the following: E-Cadherin, Desmoplakin and Plakoglobin (kindly provided by Dr. James Wahl, University of Nebraska Medical Center), Vimentin (Sigma-Aldrich, MO), Fibronectin (Abcam, MA), ZO-1 (Invitrogen, CA), All antibodies and inhibitors were used at the concentration and time point recommended by the respective manufacturer/provider (262).

### *Embryonic Palate Culture*

Mouse palate culture was performed as previously described (204,218,256). In brief: Palatal shelves were dissected from E13.5 CD1 mouse embryos and placed nasal side down on polycarbonate membranes (Nucleopore Corp.) with their medial edges in

contact. The tissues were cultured with BGJb medium (Gibco) for 72 hrs (214). Medium was replaced every 24 hr with fresh treatments. Anti-TGFβ3 was used at concentration of 10μM. TGFβrI Kinase Inhibitor VI (SB431542) was used at a concentration of 25 μM. Based on the initial dose-response experiments (not shown), this was the concentration of kinase inhibitor that abolished MES degradation in cultured palates while showing no signs of altered cell morphology. EphB2/Fc and control IgG Fc proteins were used at 5 μg /ml, as in previously published studies. Fc proteins were clustered by mixing with anti-human Fc in a 4 to 1 w/w ratio and incubated at 22ºC for 1 hr or overnight at 4ºC. This treatment allows the soluble Fc proteins to mimic the clustering that occurs on cell membranes and is required to initiate biologically relevant signaling.

***Histological Analysis***

Cultured palates were fixed in 4% formaldehyde/phosphate buffered saline, stabilized in low melting point agarose, and processed for paraffin embedding. Serial 6-μm sections were collected in the coronal orientation from anterior to posterior (30,218,232). Sections were stained with hematoxylin and eosin (H&E) and scored for fusion by at least two independent blinded observers using the previously described scale as follows (256): A score of 5 denotes complete fusion with no epithelia persisting in the midline. A score of 4 means epithelial triangle or islands remained, but they are less than 1/3 the total width of the palatal shelf interface(214). A score of 3 signifies mesenchymal confluence was achieved in places, but over 1/3 or less of the palatal shelf interface, with large epithelial islands or triangles remaining. A score of 2 means that a

continuous epithelial seam persisted in the midline. Palatal shelves that were not touching each other in the midline received a score of 1.

***Statistical Analysis***

**Palate Culture Experiments:** All palate fusion experiments were performed at least three times for a total n=12 to 18 for each treatment group. Fusion scores reported are the mean ± standard error of the mean (SEM) of the pooled scores across all experiments. Statistical analyses were made using SPSS software. Mean Fusion Scores were analyzed using Kruskal–Wallis test with the Mann-Whitney U test used to analyze specific sample pairs for significant differences. Differences in fusion score between groups with $p<0.01$ were considered to be statistically significant. The statistical power of the samples in experiments was evaluated by G*POWER software (Version 3.1). The power with respect to the seriousness of types I and type II errors rate was calculated with the settings type I error, $\alpha = 0.01$ and type II error, $\beta=0.05$. It was expected that the power analysis under these settings and with a sample size large enough would yield a statistically significant effect.

**Palate Culture Experiments:** Data from at least three replicates for each parameter were evaluated and analyzed for significance by SPSS 14.0. The treatment groups included TGFβ3, EphB2/Fc and the control groups (IgG Fc). The observation times were collapsed due to the convenience of the study, and one-way ANOVA was conducted. The significance level was set as 0.05. AP-value of $\leq 0.05$ was considered significant. The one-way ANOVA indicated that the values differ significantly across the treatment groups. Bonferroni post-hoc comparisons of the treatment groups indicated

that the negative control control group significantly differ from each other ($P \leq 0.005$). The comparison of each treatment group (time and dose) showed EphB2/Fc treatments groups also differed significantly from the negative control groups ($P \leq 0.005$).

***Culture of Isolated Primary MEE Cells***

The following four experiments were designed and discussed at Baylor College of Dentistry, in the Department of Biomedical Sciences. However, these were done as collaboration with Jingpeng Liu at the University of Nebraska Medical Center. Embryonic MEE cell culture was performed as previously described (249,257,263,264). The single cell thick periderm covering on each shelf was removed by incubating the shelves with Proteinase K for 1hr at 37$^{o}$C. The shelves were then cultured at 37$^{o}$C for 12 hrs to allow brief adherence to the corresponding opposite shelf (adhered). Adhered shelves in organ culture were then cut close to the seam to ensure limited or no mesenchymal tissues attached to isolated seam (265). The shelves were then separated and treated with Dispase II for 30 min to allow the primary MES cells to separate from the underlying basement membrane so that epithelial cells could be collected without any mesenchymal contamination. Cells were then cultured in flasks and harvested at the exponential growth stage (~80% confluence) before any exogenous treatment began (265).

***Scratch-Wound Assay***

The Scratch Wound Assay was conducted as previously described (263). MES cells were grown to 80% confluency in 6-well culture plates, and a uniform straight line scratch was made with a sterile pipette tip (249). Scratches in EphB2/Fc (2, 5 and 10

μg/mL) treated and IgG Fc (control) wells were examined for 48h. The migration of cells (or gap filling) was monitored every 12h with phase contrast microscopy where cells were morphologically assessed for the migratory phenotype (249).

***Cell Motility Assay***

The Cell Motility Assay was conducted reported (263). 8 μm pore size Transwell migration chambers of a 6-well plate (BD BioCoat, MA) were used for migration analyses. $5\times10^5$ MES cells were seeded in the presence of 5mg/mL EphB2/Fc in 8μm pore size Transwell migration upper chambers of a 6-well plate. Treated and control (Ig Fc) MES cells were allowed to migrate through the filter toward media containing serum (10%) for 24 to 48 hours at 37 °C. Cells that did not migrate through the filter were removed with a cotton swab from inside the upper chamber. Each filter was fixed in 4% Paraformaldehyde for 10 minutes, washed three times, each time for five minutes with 1x PBS, placed in Hematoxylin stain (Dako, Mayer's hematoxylin) for 20 minutes, rinsed with water, and placed in bluing reagent (alkaline solution such as a weak ammonia solution, 0.08% in water) until the stain turned blue. Subsequently, the filters were washed again using deionized water. Migrating MES cells on the lower side of the filter were randomly counted at 10 areas per field by phase-contrast microscopy. The mean of the 10 areas was determined and is represented in the bar graph in Fig. 4B

***Immunohistochemistry, Immunofluorescence, and Immunobloting***

The MES cells and embryonic palates from 14.0 to 16.5 dpc underwent Immunohistochemistry, Immunofluorescence and Immunoblotting techniques as described previously (249,257,263,264). For protein expression of MES cells by western

blot, the cells were grown to confluency in 10% FBS and serum starved in 1% FBS for 24 h, followed by treatment with TGFβ3 (2 and 5 ng/ mL) and EphB2 ( 2 and 5μg/mL) in 1.0% FBS DMEM for 0–24 h for total protein extraction. For total proteins, the nuclear extraction kit from Chemicon total protein Extraction Kit (Millipore) was used as done previously (257,264). The concentration of the total proteins was obtained with the Genesys 10 UV scanner (Thermoscientific) at 595 nm. The 25μg of protein extract was electrophoresed on a 10% denaturing gel and transferred onto a nitrocellulose membrane. The membranes were blocked with gelatin, washed with PBS-Tween, incubated with the EphB2 (Source) and TGFβ3 (R&D Systems) antibodies and reacted with anti-goat (1:1000) and anti-rabbit (1:2000) secondary antibodies (Cell Signaling). The bands were then visualized by using an odyssey scanner (Li-Cor). Intensity of the band was measured using the Carestream Molecular Imaging Software version 5.3.1 (Rochester). To perform a t-test analysis of mean intensity measurements, an ROI analysis was done from the data to Microsoft Excel software from the exported ".txt" files. Data points for all samples are paired by spatial arrangement on gel and compared pairwise to minimize the impact of subtle background artifacts on image analysis. For protein expression on embryonic palates, 8μm sections of 14.5 dpc palates from WT and TGFβ3 knockout mice underwent Immunohistochemitsry as well as MES cells underwent Immunofluorescence as described previously (257,263,266). Immunofluorescence secondary antibodies were obtained from: Rhodamine, 1:100 (Invitrogen) and FITC, 1:200 (Jackson ImmunoResearch).

***Gene Expression***

As described previously, (267,268) RNA from MEE cells treated with clustered EphB2/Fc (1, 2, or 5 μg/ml) for 48 h, was harvested using the RNeasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions. RNA integrity was assessed using formaldehyde gels in1XTAE buffer, and RNA purity and concentration were determined by the 260/280 ratio on a Nanodrop 2000C (Thermoscientific, MA). The Ct values were exported into a Microsoft Excel Spreadsheet and analysed according to the ΔCt system. The –ΔΔCt (Snail, Sip1, Twist and E-Cadherin/vs IgG Fc control) values were plotted to show the genes that are up or downregulated in fold/s increase.

The sequences of primers were obtained from the Invitrogen online PCR primer design site, and were synthesized at the Molecular Biology Core Facility, UNMC.

| | |
|---|---|
| Mouse Snail | 5'- TGAGGTACAACAGACTATGCAATAGTTC -3' |
| | 5'- CCTGCTGAGGCATGGTTACA -3' |
| Mouse Twist | 5'- TCCGCGTCCCACTAGCA -3' |
| | 5'- TTCTCTGGAAACAATGACATCTAGGT -3' |
| Mouse Sip1 | 5'- TTGTGCCCATCACGAAAAAG -3' |
| | 5'- GTGCACAGTTTGACAATTTAATTGAA -3' |
| Mouse E-cadherin, | 5′-AAGTGACCGATGATGATGCC-3′ |
| | 5′-CTTCTCTGTCCATCTCAGCG-3′. |

Gene expression was determined by normalization with the control gene, GAPDH. Each RT-PCR experiment was performed in triplicate.

## Results

### *Ephrin Reverse Signaling Mediates Palatal Fusion Independently of TGFβ3.*

It was previously reported that exogenous ephrin activation causes fusion in chicken palates without the need for TGFβ3 (218). The questions then arises on whether the same is true of mouse palates if the TGFβ signal is romoved. This question can be answered in two ways using the mouse palate culture system. These experiments were preformed by placing embryonic mouse palatal shelves in contact on a support, and observing MES degradation and fusion over 72 h. After histological processing, each palate was scored for fusion on a one to five scale to generate a mean fusion score (MFS) for anterior, middle, and posterior regions, as detailed above. First, a set of embryonic mouse palates was cultured in the presence of a blocking antibody against TGFβ3 with or without clustered EphB2/Fc protein to activate ephrin-B reverse signaling (preclustering with anti-Fc is necessary to induce signaling). Secondly, another set with a chemical inhibitor of the TGFβr kinase (SB 431542) was cultured, again with or without EphB2/Fc. Control palates in the anti-TGFβ3 experiment fused normally over the three-day time window of these experiments, although fusion was on average incomplete in the posterior region (MFS±SEM= 4.5±0.08 for anterior, 4.6±0.09 for middle and 3.0±0.24 for posterior regions; Fig.4-1). Antibody treatment abolished MES degradation and palatal fusion (MFS=1.4±0.08 anterior, 2.0±0.10 middle, and 1.3±0.23 posterior; Fig. 4-1A) such that the epithelial layers in the MES remained almost entirely intact (Fig. 1B). Kinase inhibitor treatments had the same effect of abrogating fusion (MFS= 1.2±0.13 anterior, 1.7±0.20 middle, and 1.1±0.11 posterior with SB 431542 vs.

MFS= 3.5±0.17 anterior, 4.7±0.22 middle and 3.4±0.10 posterior for untreated controls; Fig. 1B and D). In both cases, addition of recombinant EphB2/Fc restored wide spread, though not complete, seam degradation and largely rescued fusion (MFS= 2.6±0.17 anterior, 3.9±0.11 middle, 2.6±0.08 posterior for anti-TGFβ3+EphB2, and MFS=1.9±0.08 anterior, 3.8±0.11 middle, 3.0±0.20 posterior for SB 431542+EphB2; Fig. 4-1A and C). Thus, although fusion was not restored to control levels, these data demonstrate that exogenous activation of ephrin reverse signaling is capable of causing MES degradation and palatal fusion in the absence of a TGFβ signal.

Results indicated that ephrins are downstream of TGFβ3 in palatal fusion, and so the possibility that TGFβ3 may simply activate Eph expression in the MEE to cause the fusion signal was investigated. Because it is known that EphB2, at least, is capable of acting as a ligand to induce fusion, its expression in the palatal MEE in the absence of TGFβ3 was examined. As shown in Figure 4-2A, EphB2 protein expression levels were found in the palates of TGFβ3 knock out mice were comparable to those in wild type mice. Further, primary palatal MEE cells was cultured in the presence of either TGFβ3 or clustered EphB2/Fc, and found that TGFβ3 did not appreciably increase EphB2 levels on Western blot, nor did EphB2 increase TGFβ3 protein (Fig. 2B). Therefore, it can be concluded that it is likely not the role of TGFβ3 in fusion to simply induce Eph expression and thereby initiate fusion.

It was previously found that MEE cells in fusing palates show signs of epithelial to mesenchymal transition (EMT), and that TGFβ3 added to MEE cells in culture causes EMT-like phenotypic changes, cell migration, and gene expression (218,257). The

behavior of these cells after activation of the ephrin reverse signal was examined to investigate the mechanism of ephrin function in fusion. MEE cells grown to confluence exhibit the hallmarks of epithelial cells: tightly packed, cuboidal cells joined in a sheet by desmosomes and tight junctions. E-cadherin, desmoplakin, and plakoglobin are among the proteins that are conspicuously and highly expressed in these epithelia-specific junctions. After 24 h of exposure to clustered EphB2/Fc, the expression of these proteins was markedly diminished, and by 48 h, largely disappeared (Fig. 3A). At the same time, fibronectin and vimentin, both fibroblast markers, rapidly increased on the cell surface (Fig. 3B). EphB2/Fc-treated cells also lost their tight packing over this time and assumed a looser, mesenchymal shape (Fig. 3A and B).

At the same time as these cells were changing their cell junction compositions and morphology, they became more motile. A scratch-wound assay on monolayers of MEE cells was performed to be treated with EphB2/Fc or control Fc. Fig. 4A shows that substantial numbers of EphB2/Fc treated cells moved into the cleared scratch area over a 48 h period, whereas control cells moved very little. By 48 h of treatment, it was observed a six-fold increase in motile cell number in EphB2/Fc cultures over that observed in controls (Fig. 4B). It can be concluded that activation of ephrin reverse signaling in MEE cells causes them to assume a mesenchymal phenotype.

The EMT that was observed requires a shift in gene expression, and so the levels of some key transcription factors were examined to be associated with gene expression profile changes in EMT. Both the basic helix-loop-helix (bHLH) transcription factor Snail and the Smad-interacting protein 1 (Sip1) are upregulated during developmental

EMT and have been shown to repress E-cadherin expression (249). It was recently shown that the EMT-associated bHLH factor Twist1 is also upregulated during palatal fusion and plays a role in MES degradation (204,269,270). Therefore it can be quantified the mRNA levels of these three genes in MEE cells after 48 h of EphB2/Fc treatment using real-time PCR. Snail mRNA doubled at the 5 μg/ml dose of EphB2/Fc used for palate and MEE culture experiments, and Sip1 increased more than 5-fold versus control. Although Twist1 mRNA increased only 30%, the change was significant and reproducible (Fig. 5). At the same time, E-cadherin mRNA was reduced 60% compared to control. This result is consistent with a role for ephrin reverse signaling in activation of the EMT gene expression program in MEE cells.

**Discussion**

It was previously showed that ephrin reverse signaling is required for mouse palatal fusion (218). Here it can be shown that it is sufficient to cause fusion in the absence of TGFβ3, a growth factor that was previously considered indispensible for fusion. Further, it can be proved that the ephrin signal causes EMT in palatal epithelial cells. These findings are significant for three reasons. First, the fact that ephrins cause EMT in palatal epithelial cells adds significance to the argument that palatal fusion proceeds through an EMT mechanism. Second, the discovery that ephrin signaling during fusion is separate from, and can supersede, TGFβ3 shifts the focus of intracellular signaling away from purely those pathway intermediates affiliated with the TGFβr serine/threonine kinase receptor. Third, the association of ephrin reverse signaling with

EMT reveals a previously unknown role for ephrins in activation of a gene expression program.

There are two prevailing theories of the mechanism of MES degradation in palatal fusion. One argues that the MEE cells proceed through EMT to achieve mesenchymal confluence in the palate (232,234,270-273). The other says that these cells are removed by apoptosis to allow the mesenchyme to join (274-276). Both of these views have been supported with strong evidence. Recent data suggest that these theories are not mutually exclusive. Ahmed, et al. reported that MEE cells in culture exposed to TGFβ3 undergo EMT, with appropriate changes in morphology and gene expression, followed by apoptosis (257). Their studies are consistent with genetic evidence from mouse studies of palatogenesis. Jin and Ding showed that *Apaf1* knockout mice, while deficient in apoptosis, developed fused palates, indicating that fusion does not rely on apoptosis alone. However, histological examination revealed that the triangles of epithelial cells normally found at the oral and nasal edges of fusing palates persisted in *Apaf1* knockouts, whereas they eventually disappear in wild type animals (277). These same triangles were observed by Ahmed and coworkers in cultured palates treated with a caspase inhibitor. Thus, it would seem that both EMT and apoptosis combine to remove the MEE cells and complete palatal fusion. This finding that EphB2 treatment both induces fusion and initiates EMT in MEE cells independently of TGFβ3 reinforces the view that EMT is a part of the fusion mechanism. Ephrin-B signaling has been shown to induce apoptosis in other systems (278,279), and it will be interesting to discover in the ongoing studies if it does so in the palate. EphB2 treatments did not completely rescue

fusion in TGFβ-blocked palates, and this observation could be explained by an insufficiency of ephrin reverse signaling to activate a specific part of the fusion program, such as an apoptotic activity that removes remaining MEE cells. Alternatively, it could be that there is a TGFβr-specific signal (e.g: one that is Smad-associated) that, while not formally required for fusion, combines with the ephrin signal to complete fusion in the observed time window.

The B ephrin cytodomain contains docking sites for a number of signaling proteins. Conserved tyrosines can be phosphorylated and function as SH2 domain binding sites (233,280). The SH2/SH3 adaptor protein Grb4/Nckß was shown to bind to activated ephrin-B1 and signal the disassembly of actin cytoskeletal elements (243). The C-terminal end also carries a PDZ domain binding motif (281). Any of these signaling motifs may participate in signaling fusion in the palate. However, the Henkemeyer group demonstrated that mutation in mice of all known conserved ephrin-B2 tyrosines and the PDZ binding domain does not produce CP, even though homozygous deletion of the entire cytodomain in ephrin-B2/LacZ mice does (200). This means that ephrin-B2 contains an as yet unidentified signaling domain that is crucial for palatal seam degradation. Previously published work shows that PI3K signaling is required for ephrin-mediated fusion (218). This pathway has not previously been associated with reverse signaling and represents uncharted territory in the ephrin field. Efforts are focused on identification of the ephrin-B domain responsible for the PI3K signal and its binding proteins.

PI3K phosphorylates Akt, which in turn activates mTor complexes to induce cell migration (282). Activation of mTor is associated with carcinoma EMT and metastasis, and so the connection of ephrin-Bs to PI3K provides an explanation for why Eph/ephrin signal activation is so often associated with tumor metastases. The PI3K/Akt/mTor axis also connects to the EMT transcriptional program. The mTor kinase phosphorylates the transcriptional activator Stat3 (210,211), which in turn activates expression of Twist1 and Snail as part of the EMT transcriptional program (213,283), and both Twist1 and Snail are important for palatal fusion (204,284). Although ephrin-B reverse signaling was previously shown to associate with both Stat3 and the Groucho repressor of Stat3 (215,216), very little is known about the potential for reverse signaling to access a gene expression program. The connection of ephrin-B signals to the PI3K pathway in previous work showed that a connection to transcriptional activation in EMT is plausible. The data presented here indicate that such a connection exists and is functional during the developmental process of palatal fusion. It also implies that the same connection functions in cases of metastatic EMT, and suggests that ephrin-mediated pathways may be valid targets for cancer therapies. The ongoing efforts will identify the cytoplasmic and nuclear intermediates that connect ephrins to EMT.

# CHAPTER IV

# DISCUSSION

Palatogenesis is a very important event during craniofacial development. The stages of palatal development traditionally have been defined by the position of the palatal shelves in the oral cavity and the level of union at the midline (77). The palatal shelves develop from the maxillary prominence of the first branchial arch and initially grow vertically along the lateral sides of the developing tongue. At the precise stage of embryonic development, the palatal shelves are remodeled to become reoriented to a horizontal position above the tongue, and the medial edges of epithelial cells of the two palatal shelves meet at the midline. The medial edge epithelia (MEE) of the two opposite palatal shelves that arise from the maxillary processes join to form a two- layered medial epithelial seam (MES) (77). Then, the epithelial seam disappears and the palate mesenchyme becomes confluent. These steps are tightly regulated; failure of palatal shelf growth, elevation, adhesion, mesenchymal differentiation and fusion can cause CP (67).

Formation of the medial epithelial seam (MES) by palatal shelf fusion is a crucial step of palate development. Complete disintegration of the MES is the final essential phase of palatal confluency with the surrounding mesenchymal cells (285).

The cellular mechanism underlying seam degeneration and the fate of MES cells has long been the focus of the field for years. Three major models have been proposed for seam degeneration: EMT, migration and apoptosis (22,286).

EMT consists of the entire series of events involved in the transition of epithelial to mesenchymal cell characteristics (287,288). During EMT, epithelial genes are repressed, which allows properties of mesenchymal cells to be activated (288). Epithelial and mesenchymal cells have their own unique characteristics and morphology. Epithelial cells can be arranged in a single layer, or they can form layers of cells that are in close contact with neighboring cells connected by tight junctions, adherent junctions, and desmosomes (289). E-Cadherin is the most common adherent protein, while occludin, claudins, and zonula occludens (ZO1) are important components of tight junctions (290). Desmosomes provide additional strength for intercellular adhesion and are structurally similar to adherent junctions. Desmoplakin, plakophilin, and plakoglobin are the common desmosomal proteins. All these junctional proteins help epithelial cells to remain in a regimented structure (291) and during EMT, these proteins are lost to promote significant changes in epithelial cell structure and behavior by changing their functional and phenotypic characteristics and acquiring mesenchymal characteristics.

For almost two decades, it has been understood that MEE degradation and palatal fusion requires TGFβ3, a potent inducer of EMT. In mammalian palates, which normally fuse on their own, this factor is produced by the palatal tissue itself (34,235), and genetic removal of TGFβ3 results in CP. Chicken palates, which do not normally fuse, can be induced to fuse by adding exogenous TGFβ3 (234).

The main purpose of this research project was to investigate a new set of proteins involved in palatal fusion, the Eph and ephrins. The first evidence that ephrins play a role in palate development came with the linkage of ephrin-B1 mutations to

craniofrontonasal syndrome in humans, of which CP is a prominent feature (226). At the same time, it was reported that deletion of ephrin-B1 in cranial neural crest cells in mice caused craniofacial deformities, including CP (223). The fact that these defects resulted from cell-autonomous ephrin-B1 deletion suggested that ephrin-B1 reverse signaling is important for palate formation.

The expression of Ephs and ephrins in embryonic palate was analyzed, using LacZ indicator mouse lines (Figure 3-1). It was found that ephrin-B2 and EphB2 were expressed specifically in the MES immediately prior to and during its degradation (229). Also, EphB2 was expressed in the palatal MEE in the absence of TGFβ3. EphB2 protein expression levels in the palates of TGFβ3 knock out mice were comparable to those in wild type mice (Figure 4-3). This suggested that ephrin signaling contributes to palatal EMT and fusion.

Also, it was found that fusion of the chicken palates occurred by adding EphB2/Fc, even without adding the TGFβ3 that is normally required for fusion. This confirmed that EphB2 can indeed act to induce fusion. Furthermore, it was demonstrated that ephrin-B reverse signaling is necessary for palate fusion when the unclustered EphA4/Fc protein was added. EphA4/Fc promiscuously binds all B-ephrins without activating signaling acting as competitive inhibitor, blocking fusion even in the presence of TGFβ3.

TGFβ3 requires the activity of phosphatidylinositol 3-kinase (PI3K) for fusion (203), and the same is true for ephrin reverse signaling, as palates stimulated in culture with EphB2/Fc did not fuse in the presence of the PI3K inhibitor LY294002. The

chicken palate findings show that ephrin signaling is downstream, or at least independent of TGFβR signaling. The EphA4/Fc-blocking experiments demonstrated that the ephrin requirement is conserved between mouse and chick. However, blocking antibodies against TGFβ3 inhibit mouse palate fusion, and genetic ablation of TGFβ3 in mice yields cleft secondary palate (34,40,235,265). Thus, endogenous ephrin signals are not sufficient to compensate for loss of TGFβ3.

In the final set of experiments, two chemical inhibitors were used: anti-TGFβ3 and the TGFβr kinase inhibitor (SB 431542) with or without EphB2/Fc. SB 431542 is a strong and selective inhibitor of TGF-β superfamily type I a receptor-like kinase (ALK) receptors. Inhibition of TGF-β signaling is known to induce the repression of epithelial fate. The results were consistent in both experiments, control palates in these experiments fused normally over the three-day time window, although fusion was incomplete in the posterior region. The groups treated with the anti-TGFβ3 antibodies and SB 431542 abolished MES degradation and palatal fusion such that the epithelial layers in the MES remained almost entirely intact. In both treatment groups, addition of recombinant EphB2/Fc restored widespread, but not complete, seam degradation and largely rescued fusion (Figure 4-1 and Figure 4-2). These data demonstrated that exogenous activation of ephrin reverse signaling was capable of causing MES degradation and palatal fusion in the absence of a TGFβ signal. The EphB2 treatments did not completely rescue fusion in TGFβ-blocked palates, and this observation could be explained by an insufficiency of ephrin reverse signaling to activate a specific part of the fusion program, such as an apoptotic activity that removes remaining MEE cells.

The MEE cells were isolated and their comportment was investigated after activation of the ephrin reverse signal. MEE cells grown to confluence exhibit the characteristics of epithelial cells. Packed, cuboidal cells joined in a sheet by desmosomes and tight junctions. E-cadherin, desmoplakin, and plakoglobin are among the proteins that are highly expressed in these epithelia-specific junctions. After 48 hrs of exposure to clustered EphB2/Fc, the expression of these proteins disappeared. At the same time, fibronectin and vimentin, both fibroblast markers, rapidly increased. EphB2/Fc-treated cells assumed a looser, mesenchymal shape and became motile.

In a scratch-wound assay on monolayers of the same MEE cells treated with EphB2/Fc showed an increased number of cells moved into the cleared scratch area over a 48 h period; whereas, control cells moved in fewer number over the same period of time. By 48 h of treatment, a six-fold increase was observed in motile cell number in EphB2/Fc cultures.

The results for gene expression were consistent with the changes expected for ephrin reverse signaling in activation of the EMT program. The transcription factors Snail and Sip1 are upregulated during developmental EMT and repress E-cadherin expression (249). Therefore, the mRNA levels were quantified using real-time PCR of in MEE cells after 48 h of EphB2/Fc treatment. Snail and Sip 1 increased, E-cadherin mRNA decreased when compared with the controls. Twist1 mRNA increased slightly; however, the change was significant. The results were consistent with a role for ephrin reverse signaling in activation of the EMT gene expression program.

The finding of PI3K involvement in ephrin reverse signaling provides a connection to this migration mechanism. PI3K signals to Akt, which activates the mTor complex, leading to migration of cancer cells. This pathway is frequently activated in malignancies, and inhibition of the mTor complex proteins Raptor and Rictor retards cancer cell invasiveness and suppresses the EMT required for metastasis (282). This mechanism may control the EMT and migration of epithelial cells during palatal fusion (Figure 2-2).

The discovery that EphB2 treatment both induces fusion and initiates EMT in MEE cells independently of TGFβ3 reinforces the view that EMT is a part of the fusion mechanism. Thus, Ephrin-B signaling could induce apoptosis in other systems (279). It also implies that the same connection functions in cases of metastatic EMT, and suggests that ephrin-mediated pathways may be valid targets for cancer therapies.

Understanding normal palate development as well as aberrant pathways involved in abnormal palate development is crucial to allow us to better develop therapeutic modalities to treat patients. Identifying the major pathways involved and manipulating those pathways prior to birth would represent a monumental step to prevent the primary and many secondary complications caused by CLP.

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APPENDIX A FIGURES

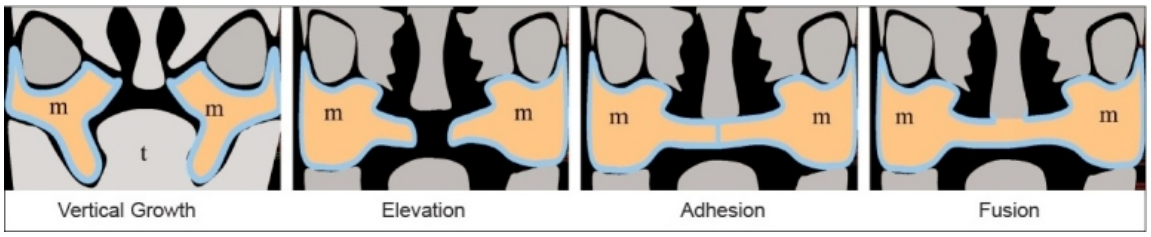


**Figure 1-1. Schematic drawing showing coronal view of a normal palate shelf and key stages of mouse palatal development.** At E12-E13 days in the mouse gestation, the palatal shelves grow downward along the tongue (t). At E13-E13.5 days, the palatal shelves become elevated above the tongue. At E14.5, the palatal shelves adhere to each other in the midline. After E15.5 days, the MES completely degrades, and the palate fuses.

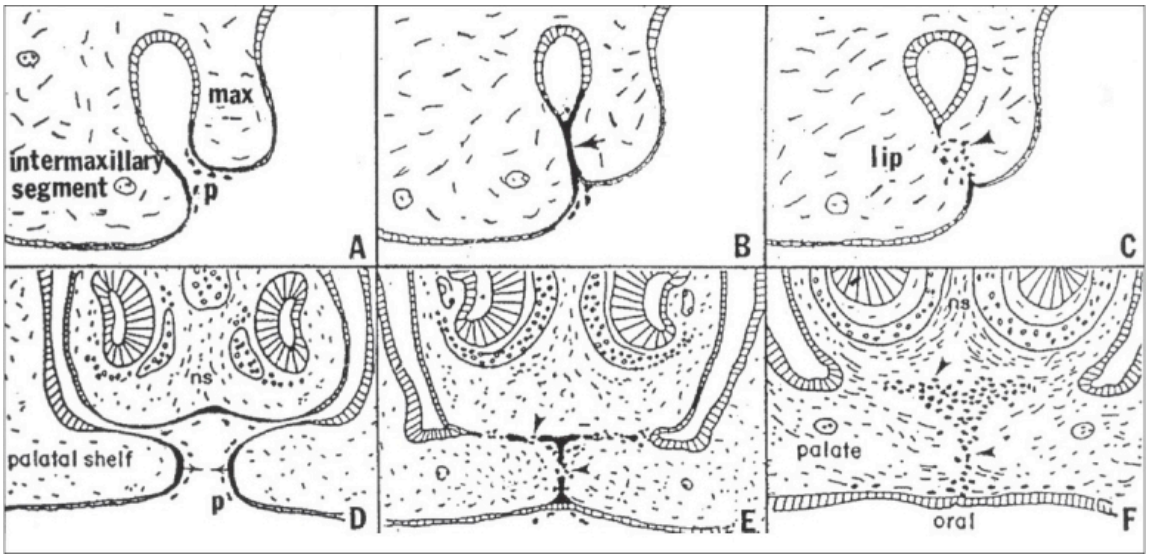


**Figure 1-2. Comparison of the morphogenesis of the upper lip with that of the palate** (D-F). After the bilateral maxillary processes (max) fuse externally with the inter-maxillary segment, the resulting epithelial seam (arrow, B) gives rise to mesenchyme (arrowhead, C) to produce a confluent lip. At a later time, the palatal shelves arising internally from the maxillary processes fuse with each other (arrows, D) and with the nasal septum (ns) above them, creating an epithelial seam that transforms to mesenchyme (arrowheads, E) to produce the confluent palate (arrowheads, F). p, sloughed periderm cells (32).

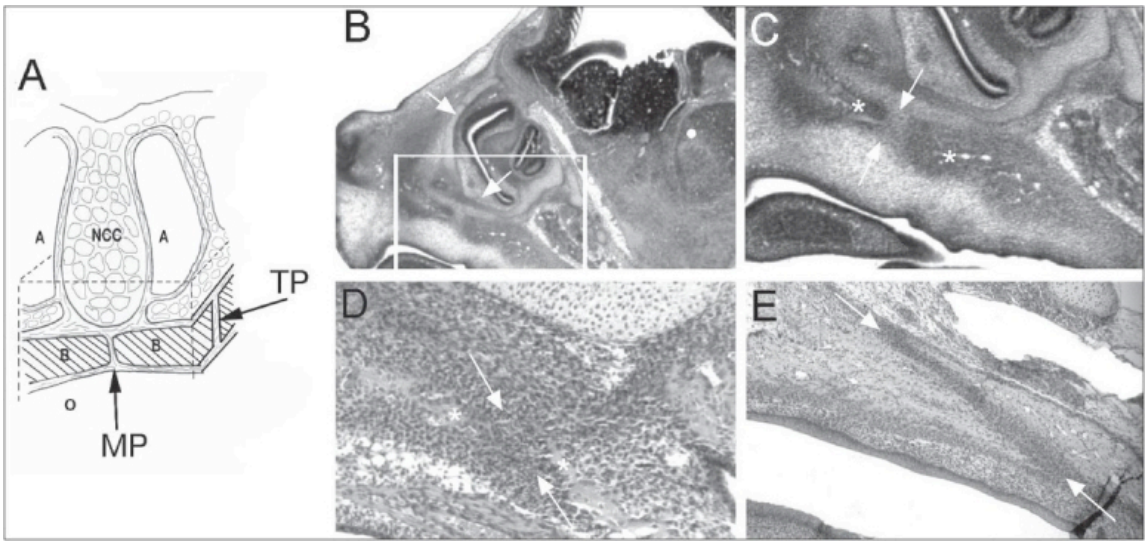


**Figure 1-3. Diagram showing the relationship between the NC cartilages and the transpalatal suture. A)** Dotted lines indicate cut lines for removing the palate from the embryo and the NC cartilage from above the sutures. (B-E) Micrographs of parasagittal sections of foetal rat heads show the pre-natal development of TP sutures. (B) At E16, NC cartilages (arrows) can be seen directly above the presumptive TP suture region (in box). (C) High-power micrograph of the region in the box, showing the advancing palatal plate of the maxilla and horizontal plate of the palatal bone (asterisks) on either side of the presumptive TP suture (between arrows). (D) At E18, the advancing bone fronts (asterisks) begin to overlap one another, creating a highly cellular suture blastema (between arrows). (E) By E20, an elongated TP suture (between arrows) continues to form as the bone fronts proceed to overlap one another. A, airway; B, shelves of maxillary bones; MP, midpalatal suture; NCC, nasal capsular cartilage; O, oral cavity; TP, transpalatal suture (143).

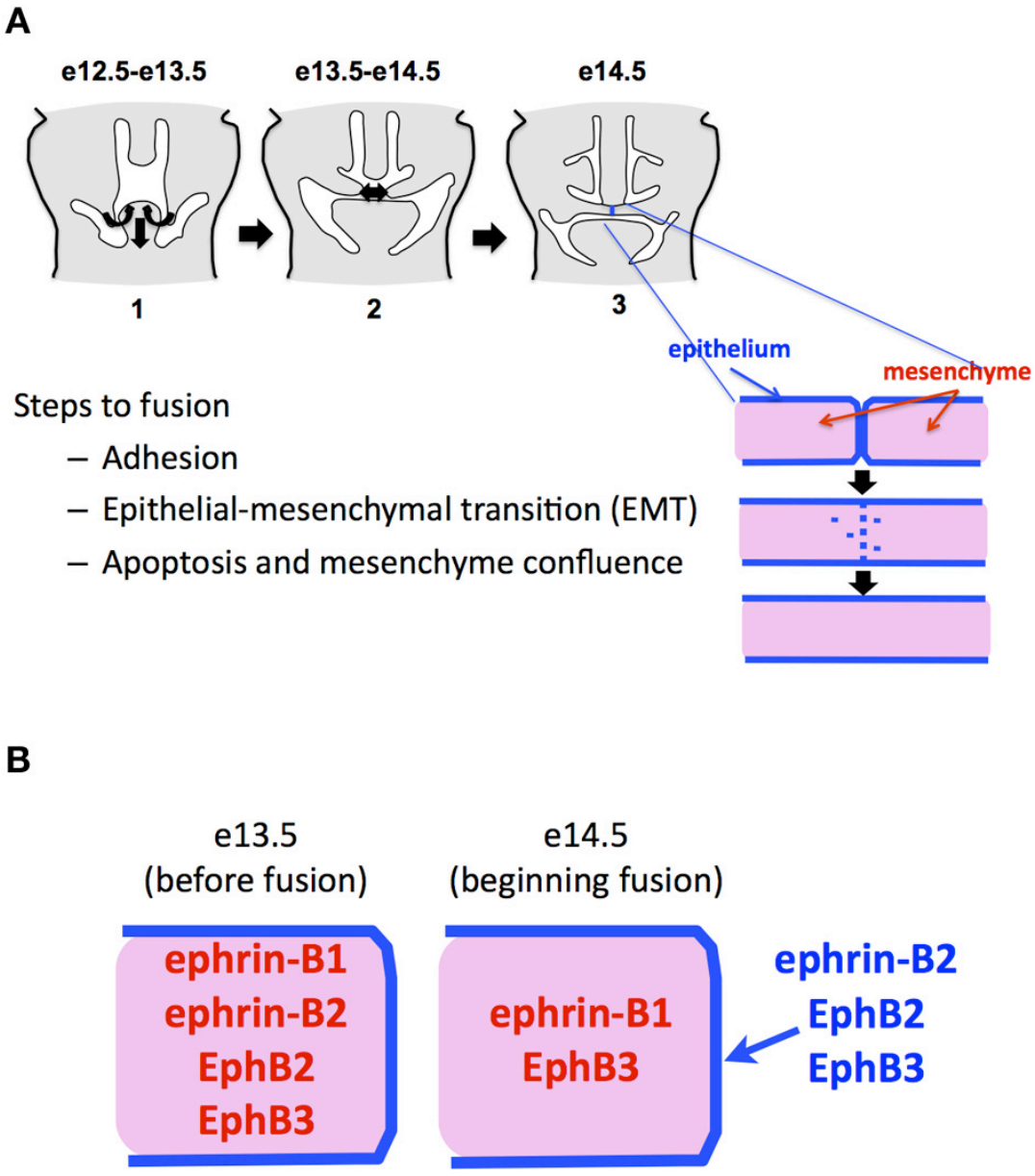


**Figure 2-1. Ephs and ephrins in fusing palate.** (A) Steps in mammalian palatal fusion. Palatal shelves of mesenchyme ensheathed in a two-cell thick epithelial layer elevate over the tongue and grow to midline. This happens beginning at about embryonic day 12.5 in the mouse. At e14.5, the epithelial cells adhere, migrate into the mesenchyme and/or die, leaving a confluent mesenchymal shelf. (B) Summary of published patterns of Eph and ephrin expression in the palate just before and during fusion.

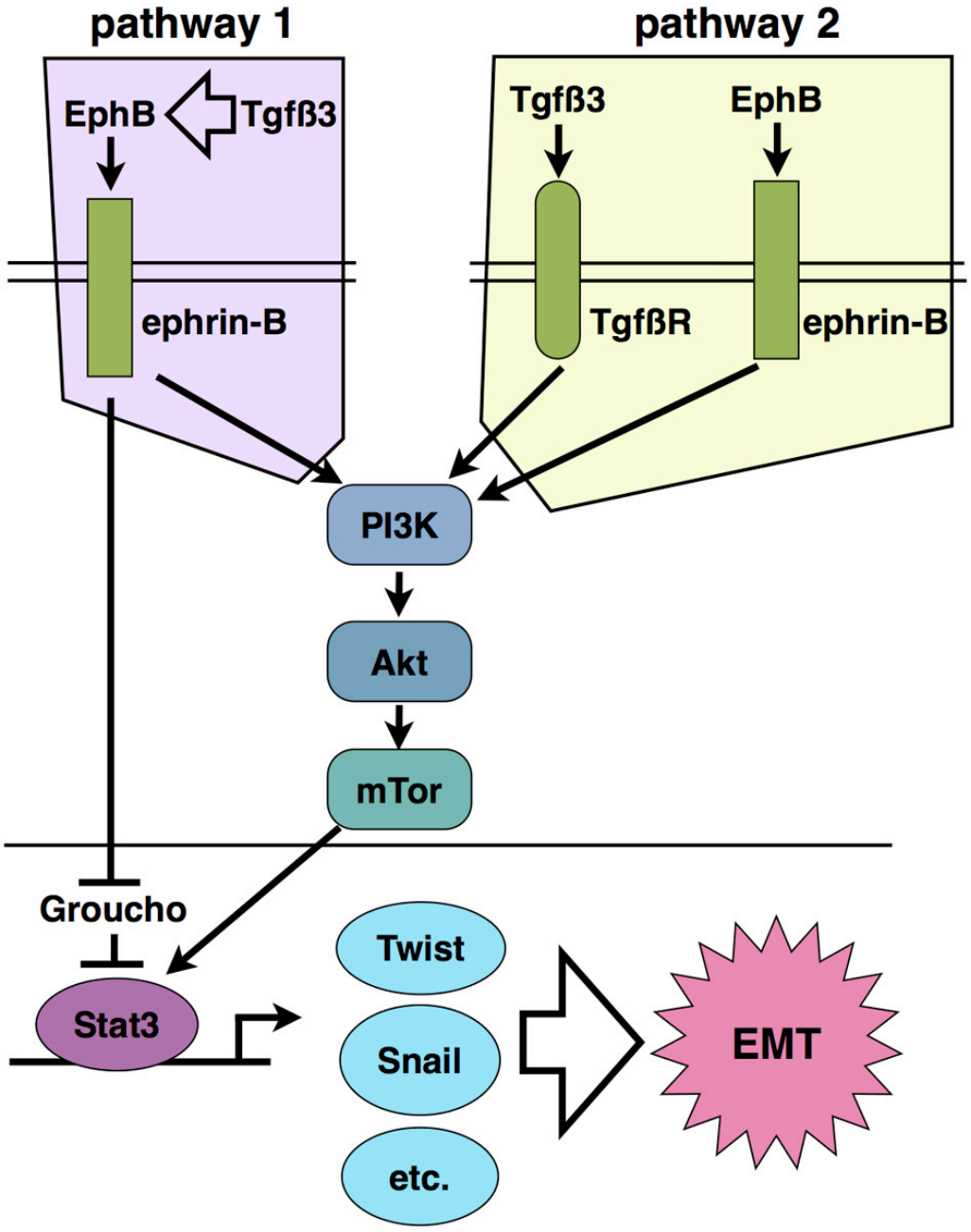


**Figure 2-2. Two proposed models of ephrin-B reverse signaling in palatal EMT based on current evidence.** Pathway 1 places ephrin signaling downstream of TGFβ3 signaling such that TGFβ3 stimulates expression of EphBs and/or ephrin-Bs, leading to activation of PI3K signaling. In pathway 2, TGFβ3 and ephrin-B signaling act in parallel to stimulate PI3K together.

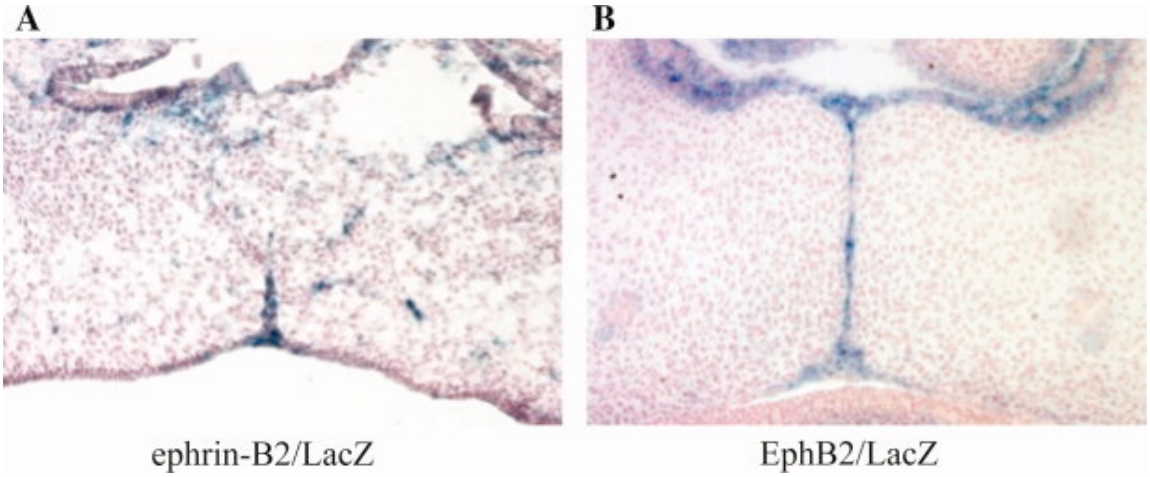


**Figure 3-1. EphB2 and ephrin-B2 expression in fusing palate epithelium.** Day 14.5 embryos from mice harboring: **(A)** the EB2/LacZ chimeric allele or **(B)** the LacZ knock-in to the EphB2 locus were sectioned coronally and stained with X-gal. Counter stain is Nuclear Fast Red. βgal expression was found in the palate epithelium, suggesting a role in adhesion and/or fusion. Note the breakup and dispersion of EB2/βgal during EMT.

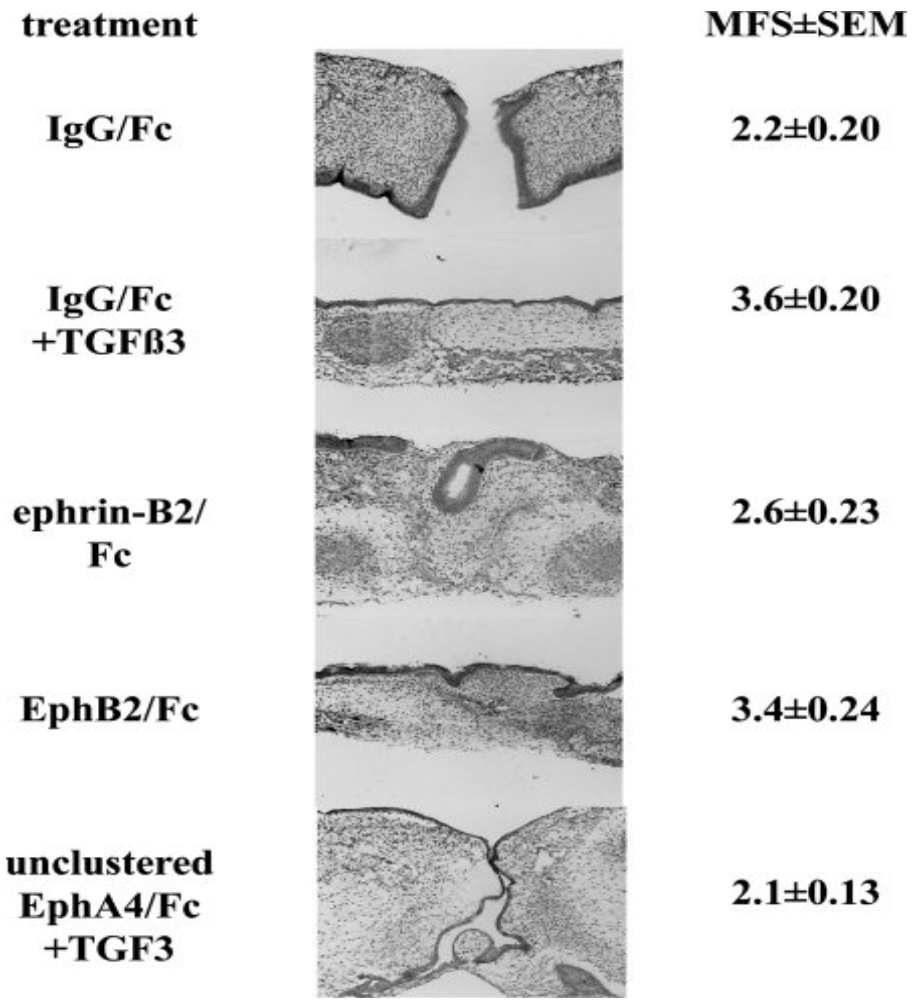


**Figure 3-2. Eph and ephrin effects on palate fusion.** Palatal shelves were dissected from eight day old chicken embryos and cultured in contact on a support for 72 h in the presence of specific treatments, as indicated. Tissues were fixed, paraffin processed, and sectioned. Shown are representative H&E stained sections from each treatment. Note the darkened epithelial layer that disappears as fusion proceeds. H&E stained sections from anterior to posterior were scored on for fusion on a scale of 1 to 5 at anterior, middle, and posterior points and these scores averaged to yield the mean fusion score (MFS) shown.

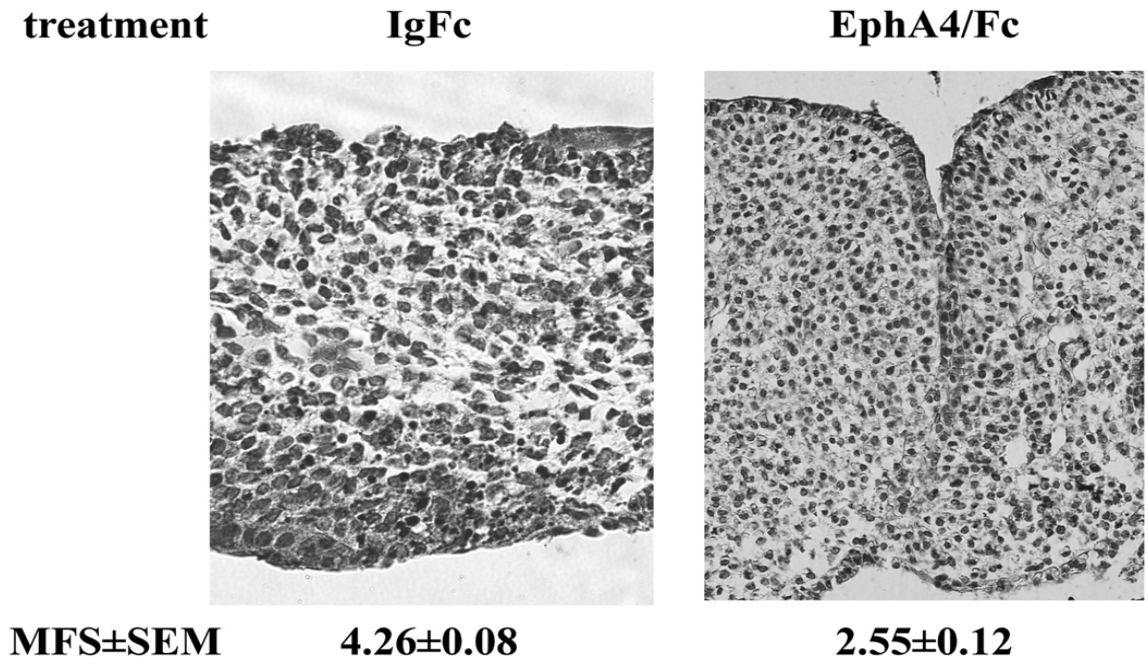


**Figure 3-3. Ephrin dependence of mouse palate fusion.** Embryonic day 14.5 mouse palates were cultured in the presence of unclustered EphA4/Fc soluble recombinant protein or IgG Fc control protein as described in the text. Tissues were fixed, paraffin processed, and sectioned. Shown are representative H&E stained sections from each treatment. H&E stained sections from anterior to posterior were scored on for fusion on a scale of 1 to 5 and these scores averaged to yield the mean fusion score (MFS) shown. Values are ±SEM for n=14 palates over four independent experiments. Magnification is 200×.

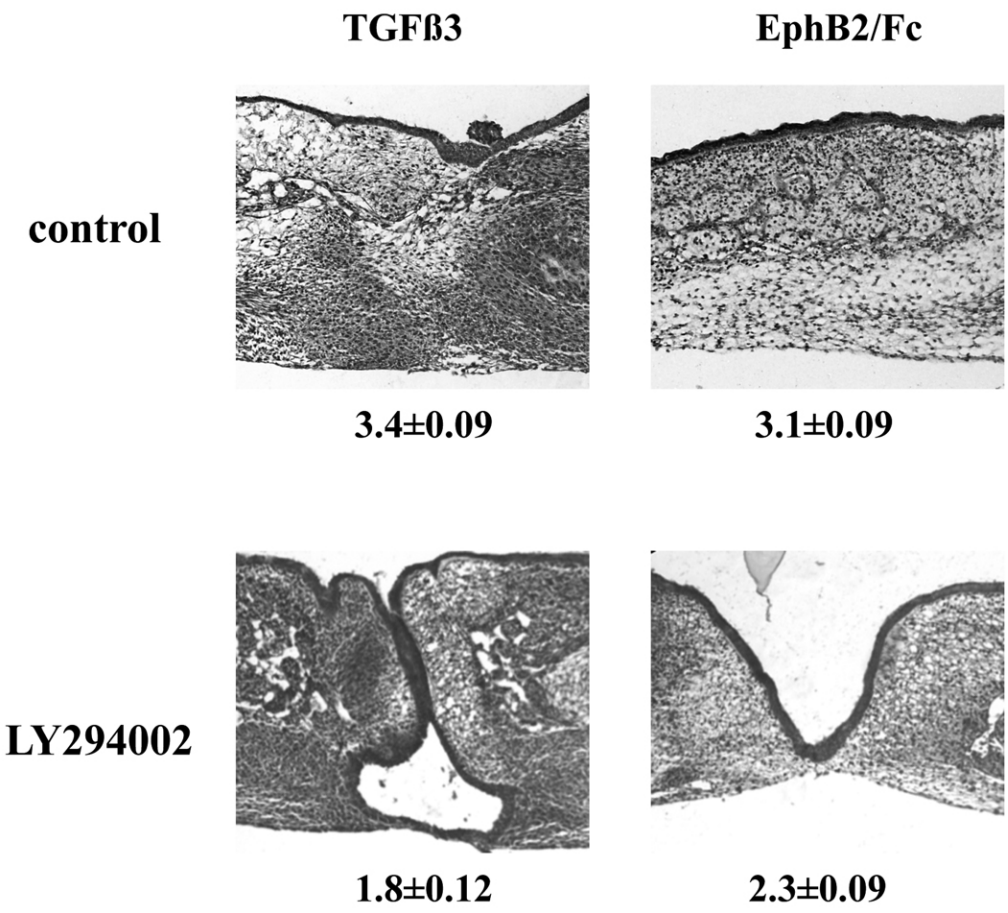


**Figure 3-4. Effect of PI3K inhibition on Eph-induced palate fusion.** Chicken palates were grown with the treatments indicated under the conditions described in the text. Samples grown in TGFβ3 or EphB2 alone fused almost completely. Addition of the PI3K inhibitor LY294002 abrogated fusion with either TGFβ3 or clustered EphB2/Fc. Shown are H&E stained examples of each group with n=16 to 19 for each group from 3 independent experiments. Mean fusion score (MFS) for each is shown ± SEM. Magnification is 100×.

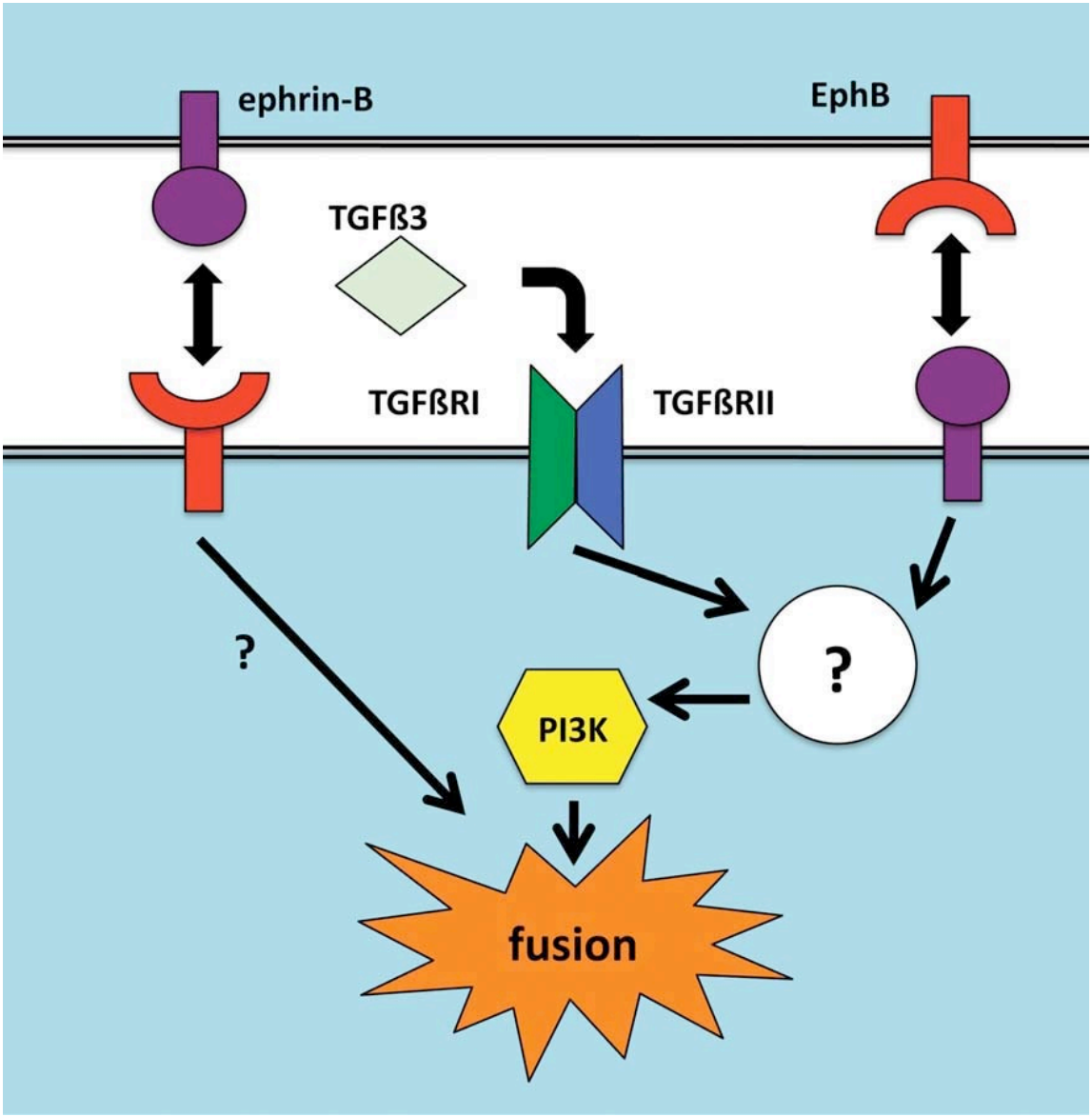


**Figure 3-5. Model of ephrin and TGFβ3 signal transduction in palate fusion.** Ephrin and TGFβR signals intersect at a point upstream of PI3K, which is required for fusion. Other possible pathways from eprhin-Bs that do not go through PI3K are not diagrammed. Known possible effectors or ephrin-Bs in reverse signaling are described in the text. Signals from Eph RTKs that induce partial fusion are unknown.

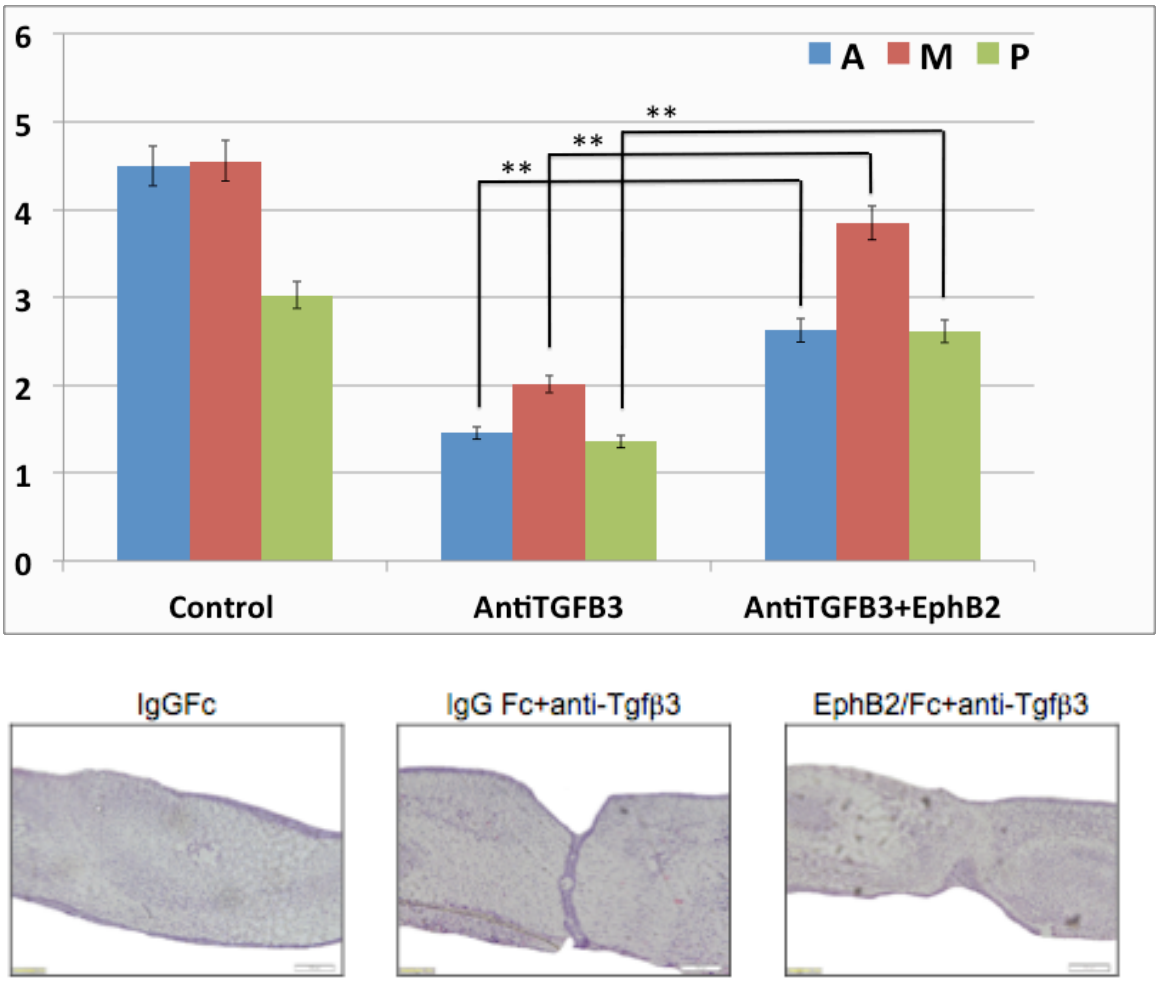


**Figure 4-1. Ephrin reverse signaling induces palatal fusion without TGFβ3**. Mouse E13.5 palatal shelves were dissected and grown with their medial edges in contact for 72h in the presence of treatments as indicated. All samples received either EphB2/Fc or IgG Fc protein at 5 µg/ml. Tissues were then fixed, paraffin-embedded and sectioned in the coronal orientation from anterior to posterior for histological analysis. Anterior, medial, and posterior regions were scored for fusion based on a one to five scale. Values shown are mean±SEM with n=12 to 18 palates for each group pooled from three independent experiments. (A) Control palates were treated with IgG Fc control protein and fused normally, with a slight decrease in posterior score indicative of the incomplete fusion commonly observed in some embryos during the 72h experimental period (MFS= 4.5 anterior, 4.6 middle, 3.0 posterior). Palates treated with 10 µM anti-TGFβ3 failed to fuse (MFS= 1.4 anterior, 2 middle, 1.3 posterior) and displayed intact MES. Palates treated with anti-TGFβ3 antibody+EphB2/Fc fused substantially better, especially in the middle region, displaying significant MES degradation (MFS= 2.6 anterior, 3.9 middle, 2.6 posterior). (B) Example H&E stained sections from each experimental group in A.

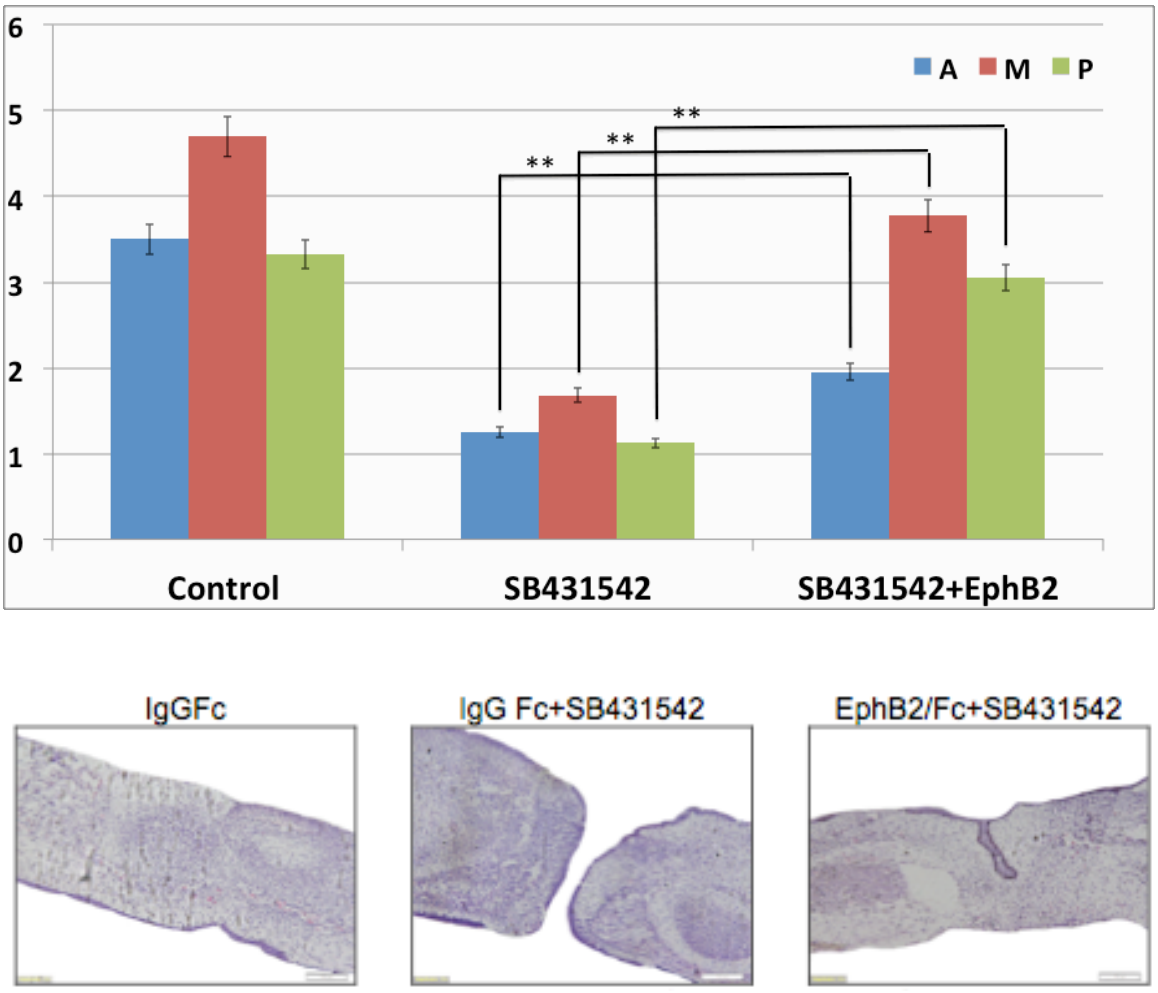


**Figure 4-2. Ephrin reverse signaling induces palatal fusion without TGFβr.** (A) Experimental conditions were the same as in A, except that the SB431542 inhibitor of the TGFβr kinase was used at 25 μM instead of anti-TGFβ3. IgG Fc control palates fused normally (MFS= 3.5 anterior, 4.7 middle, 3.4 posterior), and SB431542 abolished fusion (MFS= 1.3 anterior, 1.7 middle, 1.1 posterior). EphB2/Fc largely rescued fusion in the presence of kinase inhibitor (MFS= 2.0 anterior, 3.8 middle, 3.1 posterior). (B) Example H&E stained sections from each experimental group in C. Differences between antibody or inhibitor treated groups and their corresponding EphB2/Fc treated groups were statistically significant as determined by Mann Whitney U Test (**p<0.001).

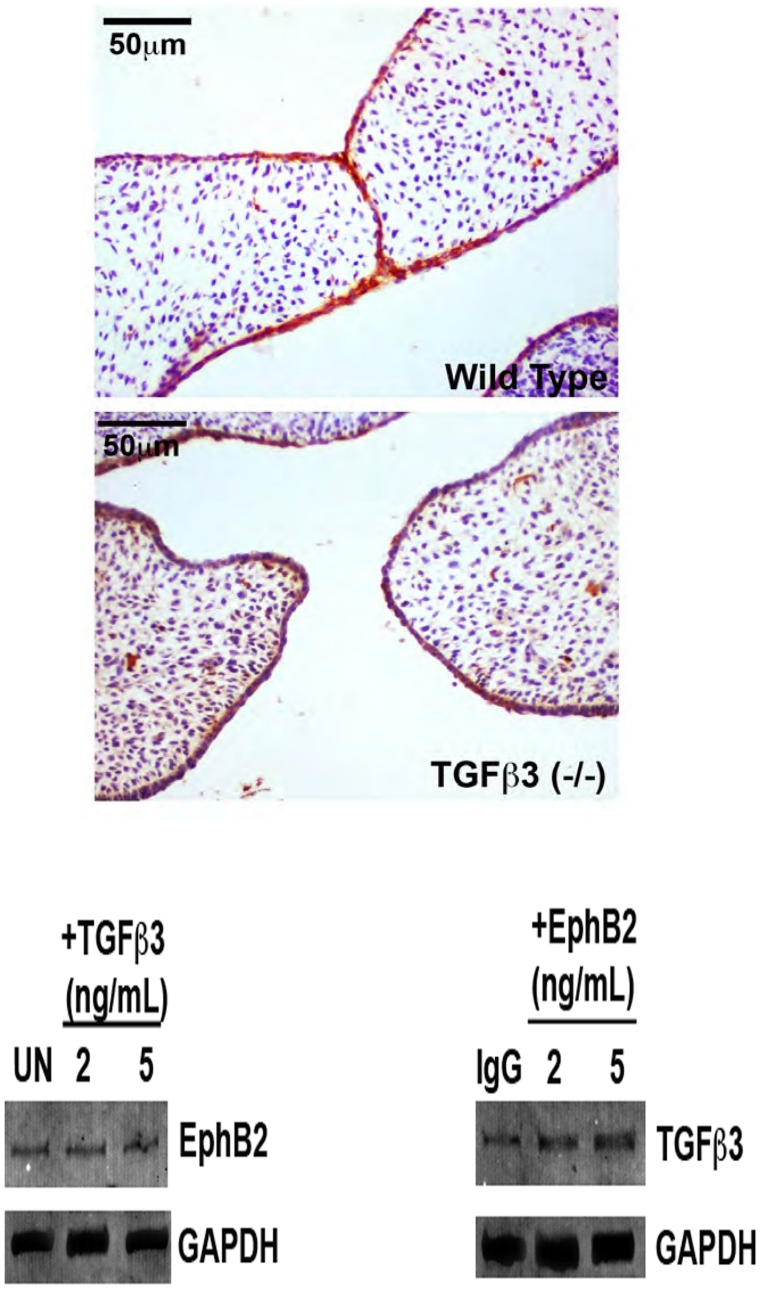


**Figure 4-3. TGFβ3 is not required for EphB2 expression**. (A) Sections of palates from wild type and TGFβ3 knockout mice were stained with antibody against EphB2. Staining (reddish-brown, DAB) is apparent in the MEE with both genotypes. (B) Mouse palatal MEE cells were grown in the presence of the indicated doses of either 10 μM TGFβ3 or 5 μg/ml EphB2/Fc for 48 h before being harvested for Western analysis with anti-TGFβ3 or anti-EphB2. UN=untreated; IgG=IgG Fc treated control. TGFβ3 treatment did not increase EphB2 levels while EphB2 treatment increased TGFβ3 levels modestly. Thus, the ability of TGFβ3 to cause palatal fusion cannot be explained by simple stimulation of EphB expression.

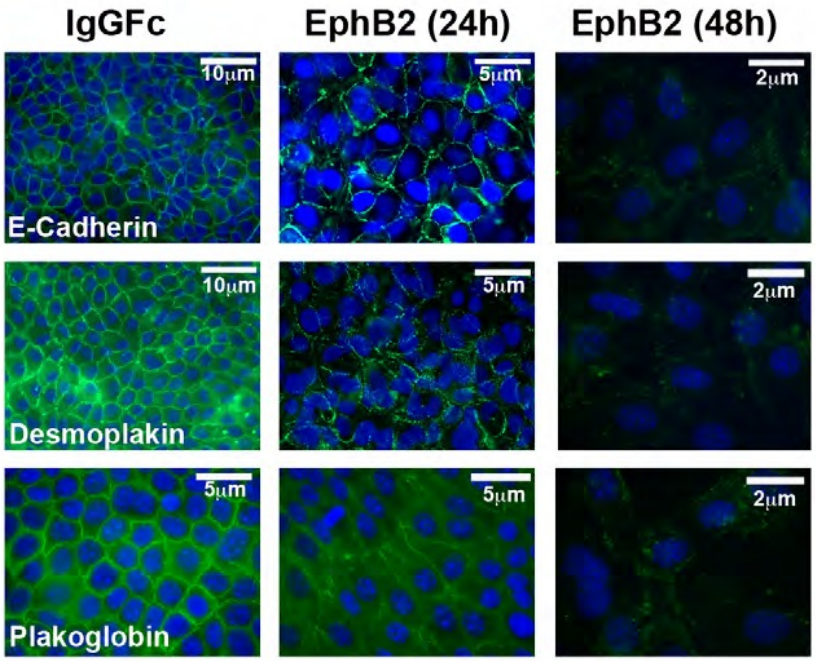


**Figure 4-4. Ephrin reverse signaling causes EMT in mouse palatal MEE cells.** Embryonic mouse MEE cells were cultured for 48 h in either IgG Fc or EphB2/Fc protein at 5 ng/ml, then fixed and processed for immunofluorescent detection of epithelial or mesenchymal markers. (A) Expression of the epithelia-specific cell junction markers E-cadherin, demosplakin, and plakoglobin (green) virtually disappeared after 48 h of EphB2/Fc treatment. (B) Expression of the mesenchymal markers fibronectin (green) and vimentin (red) increased dramatically after 48 h of EphB2/Fc exposure while expression of epithelia-associated proteins E-cadherin (red) and Z01 (green) essentially disappeared.

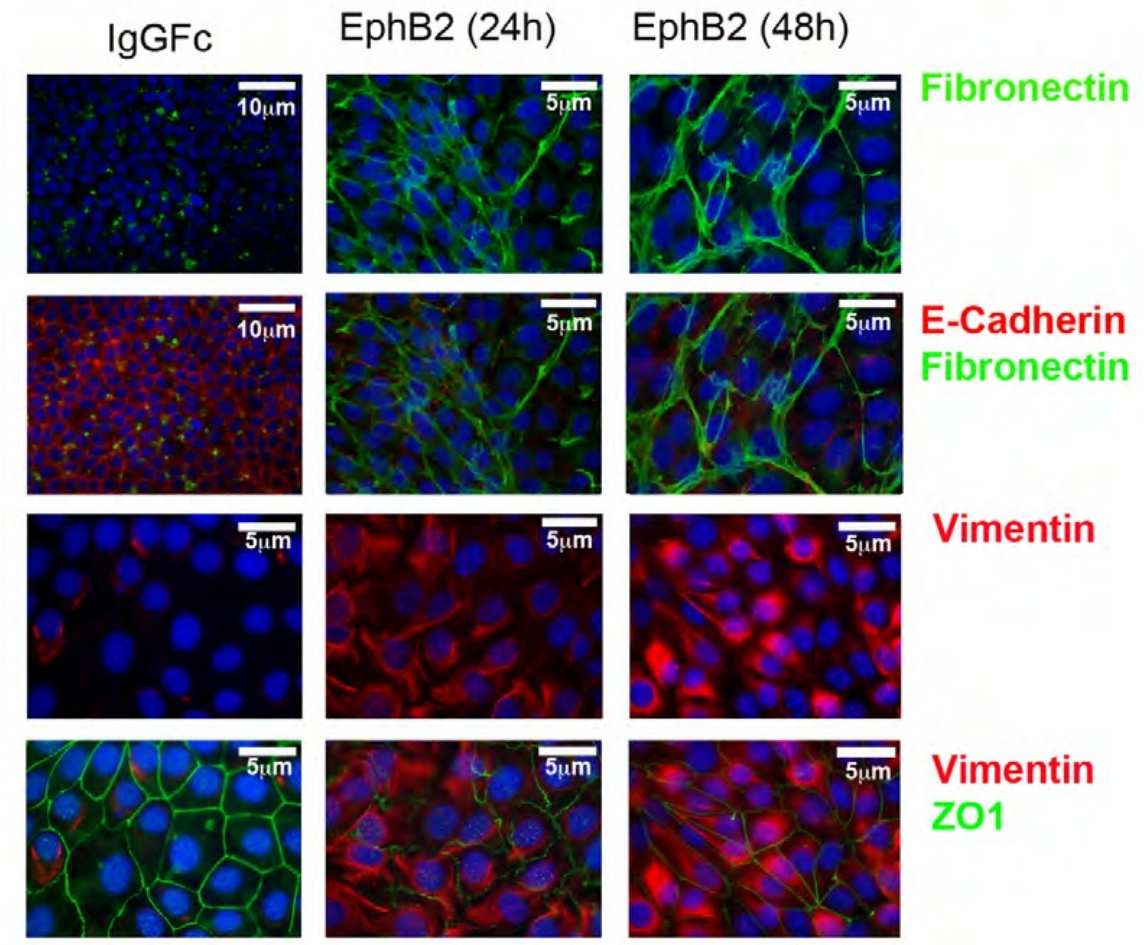


**Figure 4-5. Expression of the mesenchymal markers** fibronectin (green) and vimentin (red) increased dramatically after 48 h of EphB2/Fc exposure while expression of epithelia-associated proteins E-cadherin (red) and Z01 (green) essentially disappeared.

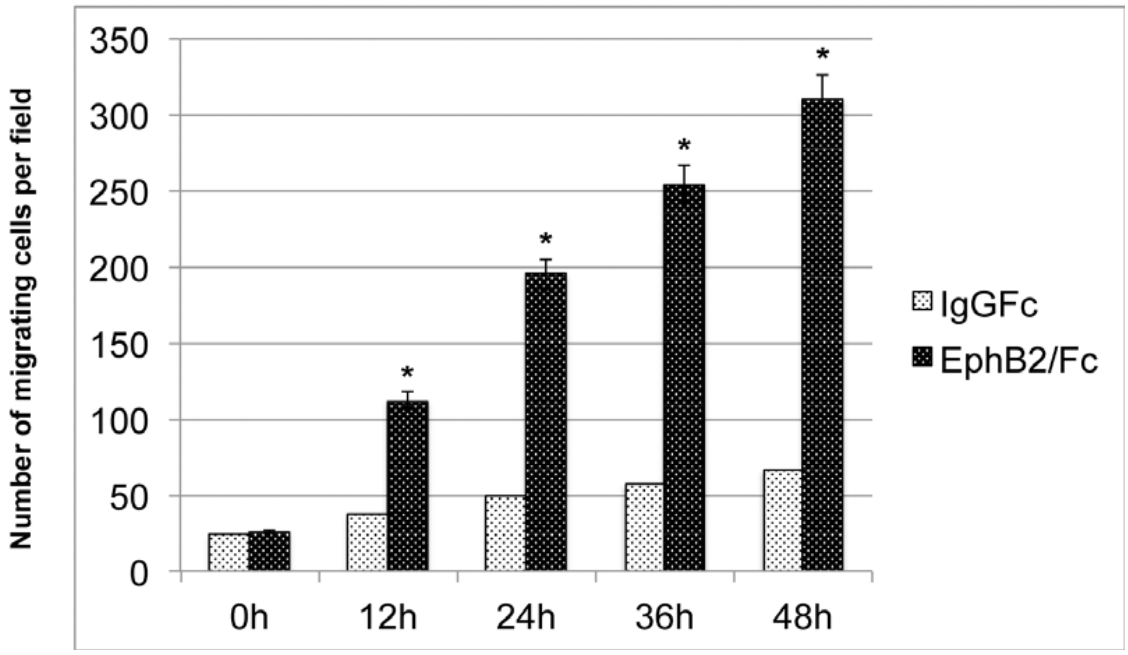


**Figure 4-6. Ephrin reverse signaling induces migration of mouse palatal MEE cells.** (A) Embryonic MEE cells were grown to confluence and then scratched with a needle to create a cleared area with uniform borders. The cells were treated with IgG Fc or EphB2/Fc for 48 h. (B) The number of cells that migrated across the borders into the cleared area was counted at 24 and 48 h. The change in the number of cells was determined by comparison to control (IgG Fc) and plotted as actual numbers of migrating cells (mean±SD.; n=3; *P<0.005 compared with controls AP-value of ≤0.05 was considered significant. The one-way ANOVA indicated that the values differ significantly across the treatment groups. All EphB2 treatment (time dependent) differed significantly (*P≤0.005) from the control groups (IgG Fc).

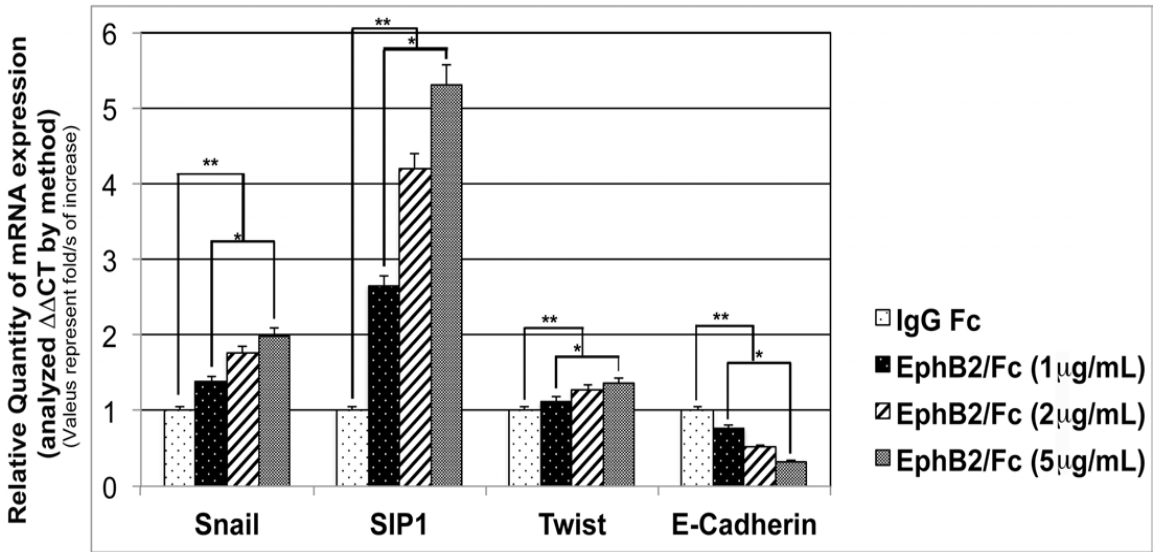


**Figure 5. Ephrin reverse signaling induces EMT-associated gene expression in palatal MEE cells.** RNA was harvested from mouse palatal MEE cells cultured for 48 h with 1, 2, or 5 μg/ml EphB2/Fc as indicated. Quantitative real time PCR analysis showed that messages for Snail, Sip1, and Twist1 were all significantly increased by EphB2/Fc treatment, demonstrating that ephrin reverse signaling activated expression of EMT-associated transcription factors. The change in mRNA levels was determined by comparison to control (IgG Fc) and plotted as fold change/s (mean±SD.; n=3; *P<0.005 compared with controls; AP-value of ≤0.05 was considered significant. The one-way ANOVA indicated that the values differ significantly across the treatment groups. All EphB2 treatment (dose dependent) differed significantly from the control groups (IgG Fc) **P<0.0005.

APPENDIX B TABLES

**Table 1.** Syndromic Genes Associated with Cleft and Palate

| SYNDROME | CLINICAL FEATURES | GENES | REFERENCE |
|---|---|---|---|
| Apert Syndrome (AS) | AD; high arched palate, bifid uvula, and cleft palate. | FGFR2 | (116,292-295), (296) |
| Bamforth-Lazarus Syndrome (BLS) | AR; hypothyroidism, athyroidal, CPO, choanal atresia, spiky hair. | FOXE1 | (116,297, 298) |
| Branchio-oculo facial syndrome (BOFS) | AD; pseudocleft of the upper lip resembling a poorly repaired cleft lip. | TFAP2A | (116,299) |
| Down syndrome (DS) | Macroglossia, microstomia, atlantoaxial subluxation | duplication of portion of chromosome 21 | (300) |
| Ectrodactyly-ectodermal dysplasia-cleft syndrome (EEC) | AD; triad of ectrodactyly, ectodermal dysplasia, and facial clefting. | P63 | (116,301, 302) |
| Fetal alcohol syndrome(FAS) | Disorder characterized by a pattern of minor facial anomalies, prenatal and postnatal growth retardation. | alcohol dehydrogenase 1B (ADH1B) | (303-305),(306) |
| Goldenhar syndrome (GS) | Oculo auricular vertebral dysplasia; AD; incomplete development of the ear, nose, soft palate, lip, mandible . | Pericentric inversion of chromosome 9 | (307,308) |
| Hereditary lymphedema-distichiasis syndrome (HLD) | AD; lymphedema of the limbs, double rows of eyelashes, cardiac defects, and cleft palate. | FOXC mutations | (309) |
| Kallmann Syndrome (KS) | AR disorder; Hypogonadotropic hypogonadism and anosmia | FGFR1 mutations | (116,310, 311) |
| Margarita Island ectodermal dysplasia (ED4) | AR; unusual facies, dental anomalies, syndactyly, and cleft lip/cleft palate. | PVRL1 (nectin-1) mutation | (116,312) |
| Pierre Robin Sequence (PRS) | AD; triad of micrognathia, glossoptosis, and cleft palate. | Loci 2q24.1-33.3, 4q32qter,11q23.1,and17q24.325.1. | (313,314) |
| Smith–Lemli-Opitz Syndrome (SLMOS) | AR; defects in cholesterol biosynthesis, growth retardation, dysmorphic facial features including CLP/ CPO, postaxial polydactyly | DHCR | (116,315,316) |
| Stickler Syndrome (SS) | AD; midface hypoplasia, micrognathia, Pierre Robin sequence, retinal detachment and early cataracts deafness, hypermobility of joints. | Col11A1, Col11A2, Col2A1 | (317,318) |
| Treacher Collins (TC) | AD ; craniofacial deformities such as downward slanting eyes, micrognathia, conductive hearing loss, underdeveloped zygoma. | Mutation in TCOF1 gene at chromosome 5q32-q33.1 | (319);(320) |

| SYNDROME | CLINICAL FEATURES | GENES | REFERENCE |
|---|---|---|---|
| van der Woude syndrome (VDWS) | AD; cleft lip palate, distinctive pits of the lower lips, or both. | IRF 6 (interferon regulatory factor 6) mutations | (116,321) |
| Velocardiofacial Syndrome (VCFS) | AD ; cleft palate, heart defects, abnormal facial structure, and learning problems. | Chromosome 22q11 microdeletion | (322,323) |
| Unnamed syndrome | CLP and hereditary diffuse gastric cancer | CDH1 | (93) |
| Unnamed syndrome | Chromodomain helicase DNA-binding proteins; CLP in Charge syndrome | CHD7 | (324,325) |
| Unnamed syndrome | Bilateral CLP, colobomas of the optic nerve and retina, agenesis of the corpos callosum. Dysphagia, reduced esophageal peristalsis | PAX 9 | (116,326) |
| Unnamed syndrome | X-linked mental retardation and CLP | PH8 | 6, (327) |
| Unnamed syndrome | Holoprosencephaly 7, a spectrum of forebrain and midline anomalies and midline CL | PTCH(315,328,329) | (116) |
| Unnamed syndrome | CPO, craniofacial anomalies, osteoporosis, and cognitive defects | SATB2 | (116,330) |
| Unnamed syndrome | Holoprosencephaly, a spectrum of anomalies ranging from severe (cyclopia) to subtle midline assymetries. CLP part of the spectrum | SHH | (116,315) |
| Unnamed syndrome | Anomalies with most features of DiGeorge/velocardiofacial syndromes: CPO, thymus and parathyroid gland hypoplasia, vertebra, facial and cardiac outflow anomalies. | TBX1 | 6, (331) |
| Unnamed syndrome | X-linked CPO and ankyloglossia | TBX22 | 6, (73,74) |
| Unnamed syndrome | Cardiovascular, craniofacial, skeletal, and cognitive alterations,bifid uvula and or/CPO | TGF Beta receptor | 6, (332) |

**Table 2.** Non Syndromic Genes: Interaction Effects of Genes and Environmental Risk Factors on Oral Clefts.

| GENE | FUNCTIONAL ROLE | RISK FACTOR | REFERENCE |
|---|---|---|---|
| Cytochrome P450 Proteins (CYP) CYPIA1, CYPIA2, CYPIB1 CYP2E1 | Highly polymorphic, having multiple functional alleles; Role in detoxification; metabolism of endogenous morphogens in the developing fetus. | Negative gene; Smoking inter-action effect | (333-335) |
| Epoxide Hydrolase (EPHX) | Class of proteins that catalyze the hydration of chemically reactive epoxides into their corresponding dihydrodiol products. | | |
| EPHX | Plays an important role in both the bioactivation and detoxification of exogenous chemicals such as PAHs, which are present in cigarette smoke. | Negative gene; Smoking inter-action effect | (333,336) |
| EPHX1 Y113H | Variant of EPHX 1 found in the fetus and maternal smoking. | Positive gene; Smoking inter-action effect | (16,337) |
| Glutathione Transferase Gene Family (GST) | Families of dimeric phase II enzymes that catalyze the conjugation of reduced glutathione with electrophilic groups of a wide variety of environmental agents. | | |
| GSTM1 | Major gene detoxifying PAHs and widely studied in many disorders and cancers. | Negative gene; Smoking inter-action effect | (338,339) |
| GSTT1 | Expressed in a variety of tissues/organs such as erythrocytes, lung, kidney, brain, skeletal muscles, heart, and small intestine; elevated expression profile at the craniofacial regions during embryonic development. | Positive gene; Smoking inter-action effect | (340) (16,335,337,340) (16,337) |
| GSTP1 | Major gene detoxifying PAHS; involvement in variety of disorders and cancers. Major enzyme involved in the inactivation of cigarette smoker's metabolites; most important isoform at the embryonic and early fetal developmental stages. | Positive gene; Smoking inter-action effect | (341) (16,337) |
| GST A4 / GSTM3 | Two other types of GST gene family members. | Positive gene; Smoking inter-action effect | (16,337) |
| Hypoxia-Induced Factor-1 (HIF1A) | Mechanism that maternal smoking may affect embryonic development due to the production of carbon monoxide, which interferes with oxygen transfer to the placenta, or nicotine, which constricts the uterine wall resulting in | Positive gene; Smoking inter-action effects | (16,337) |

| | | | |
|---|---|---|---|
| | hypoxia. | | |
| **GENE** | **FUNCTIONAL ROLE** | **RISK FACTOR** | **REFERENCE** |
| Arylamine N-Acetyltransferase gene Family | N-conjugation of arylamine by the action of N-acetyltransferases (NATs), UDP glucoronosyltransferases (UGTs), or sulfotransfereases (SULTS) produces nontoxic compounds. | | |
| N-acetyltransferases1 (NAT 1) | Expressed in many tissues such as erythrocytes, bladder, lymphocytes, neural tissues, liver and intestines. | Negative gene; Smoking inter-action effects | (127,342,343) |
| N-acetyltransferases pseudogene, (NATP1) | Pseudogene identified, which is located at chromosome 8p23.1-8p21.3. | | (127,342,343) |
| N-acetyltransferases2 (NAT 2) | Expressed in the liver and epithelial cells of the intestine. | Positive gene; Smoking inter-action effects | (16,335,337) |
| Methylenetetrahydrofolate reductase (MTHFR)<br><br>MTHFRC677T | Metabolism of folate by reducing methylenetrahydrofolate, primary donor for methionin synthesis.<br><br>Variant of methylenetetrahydrofolate reductase. | Positive gene; Vitamin inter-action effect<br><br>Negative gene; Smoking inter-action effect | (344-348),(349,350) |
| | | | |
| **OTHER METABOLIC GENES** | | | |
| NAD(P)H quinine oxidoreductase (NQO1) | Flavoenzyme that catalyzes two electron reduction of quinine compounds to hydroquinone and is inducible by oxidative stress, dioxin, and PAHS found in cigarette smoke | Negative gene; Smoking inter-action effect | (16,337) |
| SULT1A1 | Catalyzes transfer of the sulfonate group from the active sulfate to a substrate to form the respective sulfate or sulfamate ester. | Negative gene; Smoking inter-action effects | (16,337) |
| UDP glycosyltransferases (UGTs) UGT1A7 variant | Catalyzes conjugation reactions where hydrophobic chemicals are transformed into water-soluble compounds. Potential maternal effects on embryonic development. | Positive gene; Smoking inter-action effects | (337,351,352) |
| | | | |
| **DEVELOPMENTAL GENES FOR ORAL CLEFTS** | | | |
| Transforming Growth Factor A (TGF a) | Transmembrane protein expressed at the medial edge of the epithelium (MEE) of fusing palatal shelves. Its receptor epidermal growth factor (EGFR) is expressed in the degenerating MEE. | Positive gene; Interaction effects (smoking, alcohol drinking, vitamins) | (353-355) |

| | | | |
|---|---|---|---|
| Transforming growth Factor b-3 (TGF b3) | Regulator of many biological processes such as proliferation, differentiation, epithelial mesenchymal transformation and apoptosis. | Positive gene; Interaction effects (smoking, alcohol drinking) | (102,354,356) |
| **GENE** | **FUNCTIONAL ROLE** | **RISK FACTOR** | **REFERENCE** |
| Muscle Segment Homeobox1 (MSX1) | Transcriptional repressor important in craniofacial, limb, and nervous system development. | Positive gene; Interaction effects (smoking and alcohol drinking) | (354,357,358) |
| MSX2 | Similar to MSX1; rare cause of isolated cleft lip with or without cleft palate. | | (357,358) |
| Acyl-CoA desaturase ACOD4 | Pericentric inversion disrupts a gene (ACOD4) on chromosome 4q21 that codes for a novel acyl-CoA desaturase enzyme that occurs in a single two-generation family with CL. | | (359) |
| Retinoic acid receptor ( RAR) | Odds ratios for transmission of alleles at THRA1 were significant when ethnic group was included. | Negative gene; Smoking inter-action effects | (354) |
| CHD7 | Chromodomain helicase DNA-binding proteins. | | (360) |
| ESR1 | Ligand-activated TF estrogen receptor. | | (361) |
| FGF/ FGFR families FGF8 FGF3 FGF10 FGF18 FGFR1 FGFR2 FGFR3 | Expressed during craniofacial development and can rarely harbor mutations that result in human clefting syndromes. | | (362) (362) |
| SPRY1/SPRY2 | Loss of function mutations in FGFR1 cause a syndromic form of clefting. | | (363) |
| TBX10 | Ectopically expressed in dancer cleft lip and palate mutant mice. | | (363) |
| GABRB3 | b3 subunit of GABA receptor CLP. | | (85),(364),(116) |
| GLI2 | Mutations in GLi2 cause holoprosencephaly-like features with cleft lip and palate. | | (363) |
| ISGF3G | Similar to IRF6. | | (363) |
| | | | |
| **OTHER CANDIDATE GENES** | | | |
| SKI, FOXE1, JAG2, LHX8 | Rare causes of isolated cleft lip with or without cleft palate | | (363) |