

Epithelial-Mesenchymal Transformation during Craniofacial Development

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ABSTRACT

Epithelial to mesenchymal phenotype transition is a common phenomenon during embryonic development, wound healing, and tumor metastasis. This transition involves cellular changes in cytoskeleton architecture and protein expression. Specifically, this highly regulated biological event plays several important roles during craniofacial development. This review focuses on the regulation of epithelial-mesenchymal transformation (EMT) during neural crest cell migration, and fusion of the secondary palate and the upper lip. Abbreviations used in this paper: BMP, bone morphogenic protein; CCFSE, 5 (and 6) carboxy 2,7' dichlorofluorescein diacetate succinimidyl ester; CNC, cranial neural crest; DiI, 1,1-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate; EMT, epithelial-mesenchymal transformation; FGF, fibroblast growth factor; ILK, integrin-linked kinase; LEF1, Lymphoid enhancer factor-1; MEE, medial edge epithelia; MFS, mean fusion score; MMP, matrix metalloproteinase; PDK, 3-phosphoinositide-dependent protein kinase; Pax, paired box-1 to -9; PI-3 kinase, phosphatidylinositol-3 kinase; Ptc, patched; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Shh, Sonic hedgehog; Tbx, T-box family; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase.

KEY WORDS: palate, epithelial-mesenchymal transformation (EMT), TGF β , PI-3 kinase, Akt, Smad.

INTRODUCTION

Cell phenotype transformations, from epithelial to mesenchymal (epithelial-mesenchymal transformation, EMT), have been well-documented in embryonic development, wound healing, and tumor metastasis. Epithelia serve as the boundary between the external environment and the internal structures, while mesenchymal cells are found in the connective tissue compartment. The epithelial barrier function is partly supported by firm cell-cell junctions, such as tight junctions and desmosomes. In addition, epithelial cells normally have apical-basal polarity and attach to basal lamina by hemidesmosomes. In contrast, mesenchymal cells are more mobile and surrounded by extracellular matrix. They have anterior-posterior polarity, and they form only transient contacts with their neighboring cells. A phenotype transformation from epithelial to mesenchymal requires a regulated gene expression sequence.

EMT and the opposite, mesenchymal-epithelial transformation occur during normal developmental processes. One example is EMT during one of the earliest developmental events—gastrulation—that involves the invagination of epiblast-derived cells to form mesoderm (Sanders and Prasad, 1989). Also, during neurulation, as the cranial neural folds elevate, cranial neural crest (CNC) cells migrate away from an embryonic neuroepithelial layer by changing their cell-cell adhesion and shape. The cells then migrate to specific destinations after basal lamina degradation (Weston, 1982; Duband *et al.*, 1995). In addition, several other developmental processes—such as sclerotome formation (Solursh *et al.*, 1979), and cardiac cushion mesenchyme development (Runyan and Markwald, 1983; Potts and Runyan, 1989; Boyer *et al.*, 1999b)—require EMT. In contrast, mesenchymal-epithelial transformations occur in the formation of somites, kidneys, and caudal or secondary neural tubes (Griffith *et al.*, 1992).

The EMT phenotype change occurs through a regulated sequence of events that can be divided into stages. EMT can occur from simple, stratified, or fused epithelia. The cranial neural crest (CNC) transforms from a single layer of neuroepithelium (Fig. 1), whereas palate and lip fusion occurs when 2 epithelial sheets fuse and then transform through EMT (Fig. 1). Both tissues have a loss of cell-cell attachment, breakdown of basal lamina, and increased mobility. However, the palate is complicated by the process of 2 epithelial surfaces first bonding to each other (Fig. 1) after the surface cells have sloughed and before proceeding through EMT. During the mid-1990s, several reviews were published on EMT in development and pathogenesis (Duband *et al.*, 1995; Hay, 1995; Hay and Zuk, 1995; Shuler, 1995; Guarino *et al.*, 1999; Boyer *et al.*, 2000).

Describing CNC and palatal EMT will be the focus of this review. In addition, the current information on gene expression changes and signal transduction pathways involved in these events will be discussed. First, we will introduce the various experimental models that have been used to study EMT in craniofacial and other tissues. This will be followed by a review of the literature on CNC, and palate- and lip-fusion EMT. The signaling molecules and signal

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transduction pathways will be discussed, focusing mainly on the Tgfβ family of proteins. Finally, the clinical applications and implications of future research will be explored.

EXPERIMENTAL MODELS

Several organ culture models and three-dimensional culture methods have been used for the study of EMT, including cardiac cushion, palate, and lip organ culture models. The gelling collagen tissue culture model has also been a useful tool for studying the gain and loss of function experiments with transient gene expression *in vitro*. Finally, the contributions of transgenic animals to our understanding of EMT mechanisms and human disorders will be discussed.

ORGAN CULTURE MODELS

The embryonic cardiac cushion has been one of the models used for the study of EMT mechanisms. In embryonic hearts, endothelial cells transform and migrate into mesenchyme to form the future valves and septa. When this tissue is placed in a three-dimensional culture system, the endocardial cells migrate into the collagen gel (Runyan and Markwald, 1983). Antibodies or/and antisense oligonucleotides to transforming growth factor β2 (Tgfβ2), β3 (Tgfβ3), Tgfβ receptor types II (TβrII) and III (TβrIII), differentially inhibited EMT during avian or mouse embryonic cardiac cushion formation (Runyan *et al.*, 1992; Boyer *et al.*, 1999a; Boyer and Runyan, 2001; Camenisch *et al.*, 2002). Antibodies specific for Tgfβ2 blocked cell-cell separation, while antibodies to Tgfβ3 blocked mesenchymal invasion but not separation. These different experimental outcomes support the hypothesis that EMT is a multistep process. More recently, this model was used to show that Mox-1 played a necessary role to sustain EMT (personal communication, R. Runyan). Numerous growth factors and signaling pathways have been reported to control proliferation and EMT, and many parallel the pathways reported for the CNC and palate.

Several craniofacial research groups studied the regulation of EMT using organ cultures of embryonic rodent or chicken palates and lips. In most of the experiments, the palates were harvested at palate elevation stages [mice, E13-14 (Figs. 2A, 2B); rats, E16; and chickens, E8] (Shuler and Schwartz, 1986; Griffith and Hay, 1992; Shuler *et al.*, 1992; Sun *et al.*, 1998b,

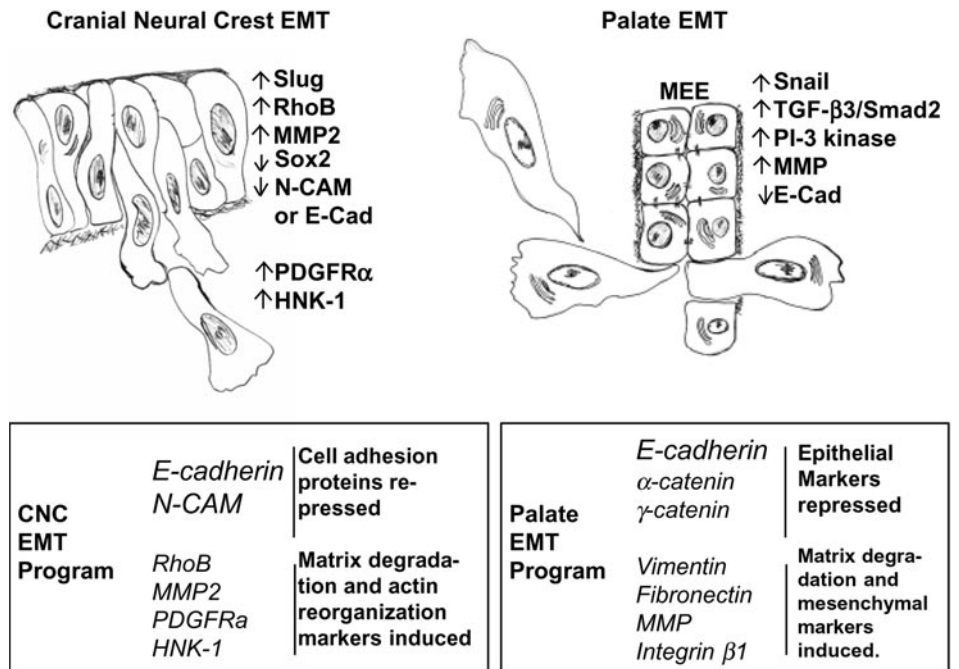
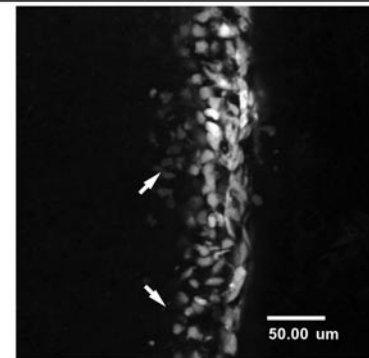


Figure 1. Comparison of cranial neural crest and palate EMT. Cranial neural crest (CNC) is derived from a single layer of epithelial cells located at the transition zone between neuroepithelium and surface ectoderm (Weston *et al.*, 2004). The cells have increased Slug, and decreased Sox2 expression. The cells lose cell-cell adhesion molecules [(N-cam or E-Cadherin [E-Cad])] and increase actin (RhoB) and extracellular matrix remodeling (Mmp2) proteins and growth factor receptors (Pdgfrα). After the crest cells have moved away from the neuroepithelium, they increase expression of the HNK-1 epitope (Del Barrio and Nieto, 2004). Palate EMT requires several more steps than CNC, since the periderm cells are sloughed through apoptosis, two epithelial sheets fuse at the apical membranes, and some cells may move to the oral or nasal epithelium. The cells increase Snail, Tgfβ3 signal transduction through Smad2, PI-3 kinase, and MMP, while decreasing E-cadherin. In both tissues, cell adhesion proteins are repressed, and matrix degradation and mesenchymal or tissue-specific proteins are increased. A confocal image of a single optical plane through palatal MEE cells that were labeled with CCFSE and then cultured for 72 hrs demonstrates the extensive remodeling at the seam as cells undergo EMT.



2000; Kang and Svoboda, 2002, 2003). The palatal shelves were removed from the maxilla and placed on a substrate, either in pairs or as single palatal shelves, and then maintained in static organ culture at the air-medium interface in media for 2-72 hrs (Fig. 2C) (Kang and Svoboda, 2002, 2003). *In vitro*, palates fused and EMT progressed, with complete mesenchymal confluence by 72 hrs. Chicken palates required additional Tgfβ3 (Sun *et al.*, 1998b), whereas rodent palates and chicken lips (Sun *et al.*, 2000) did not need additional growth factors. The transformation and migration of epithelia were investigated by a variety of techniques. For example, tissues were incubated with lipophilic markers such as 5 (and 6) carboxy 2,7' dichlorofluorescein diacetate succinimidyl ester (CCFSE; Figs. 1, 3) (Griffith and Hay, 1992; Kang and Svoboda, 2002, 2003), or with 1,1-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) (Shuler *et al.*, 1992) to label the epithelia prior to culture. Labeled cells were

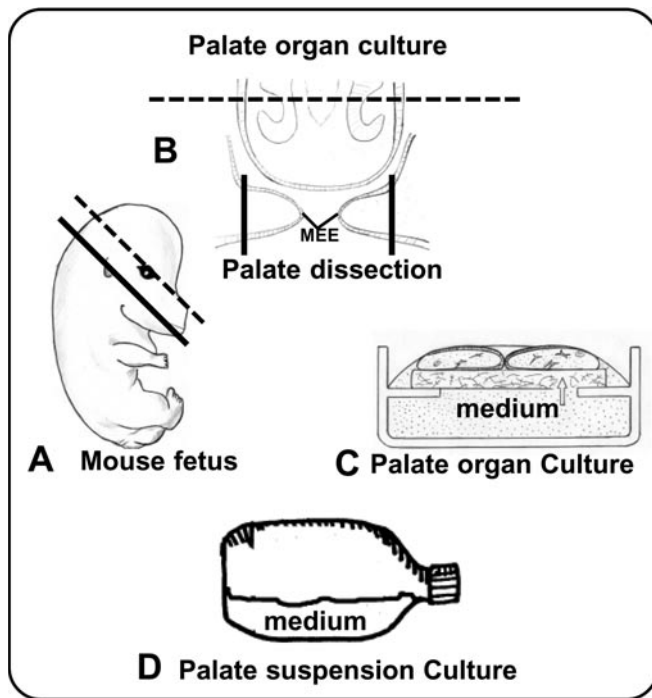


Figure 2. Experimental models for the study of EMT. (A) Embryonic mouse palatal shelves were dissected from the 12.5-gestational-day mouse embryos. (B) The palatal shelves are in the horizontal position, but are not touching or fused at this stage. (C) The dissected palatal shelves were placed in organ culture on a filter at the air-media interface for 20 to 72 hrs in the static organ culture model (Kang and Svoboda, 2002). (D) The palatal region can also be cultured in suspension cultures. The palatal region was dissected by a horizontal incision made through the oral opening (A - solid line), and the upper part of the head was resected by a second incision made parallel to the first at the level of the eyes (dashed lines in A and dashed line in B). The tongue and brain tissue were removed from the explants. The palatal explants were placed in 50-mL penicillin bottles ($n = 3$ to 6 explants/bottle) on a roller bottle culture system (Shiota *et al.*, 1990; Chou *et al.*, 2004). In this system, the palatal shelves fuse in 24 hrs.

then visualized by fluorescent or TEM microscopy after different culture periods. The design of this type of experiment was not complicated; however, major disadvantages included lack of label incorporation into the basal medial edge epithelium (MEE) cells and loss of labeling through time. Another type of cell lineage study used epithelia infected with virus *in vitro* or *in vivo*, and the labeling was then detected after static organ culture (Martinez-Alvarez *et al.*, 2000a). Unfortunately, the palatal cells have been difficult to infect with viruses consistently.

Using a different approach that cultured mouse maxilla with some associated tissues in suspension (Figs. 2A, 2D), Shiota's group established that the anterior, middle, and posterior palates may close by different mechanisms (Chou *et al.*, 2004). This group used carbon particles to mark palatal locations prior to cultivation for up to 48 hrs. They found that the midpalate closes by rotation and medial elongation, whereas the anterior and posterior regions appeared to have more remodeling (Chou *et al.*, 2004).

In new experiments comparing the suspension culture (Fig.

2D) with the traditional static cultures (Fig. 2C) described above, Takigawa and Shiota found that MEE cells can disappear throughout the medial edge, even without contact and adhesion to the opposing MEE in suspension culture (Takigawa and Shiota, 2004). Apoptosis, EMT, and epithelial cell migration all occurred at various stages of MEE cell disappearance, including the transient formation and disappearance of epithelial triangles and islets in the suspension cultures. In contrast, MEE cells showed poor differentiation in static culture in a CO_2 incubator. Furthermore, mouse and human amniotic fluids were found to prevent MEE cell differentiation in the cultured single palatal shelf, although paired palatal shelves fused successfully even in the presence of amniotic fluid. These experiments demonstrate that the palatal shelves behave differently when separated from the maxilla, and that maintaining them in their three-dimensional space may be critical to our understanding of the mechanisms of palatogenesis. Clearly, more experiments examining individual cell fates need to be conducted before we can fully understand palatal fusion.

More recently, a transgenic mouse has been developed that expresses β -galactosidase (β -gal) in the CNC cells (Chai *et al.*, 2000) (see complete description in the 'transgenic mouse' section). Palates from these embryos were incubated in DiI and then cultured according to the static organ culture method. In the resulting tissues, all CNC were labeled blue, and surface epithelium was labeled red (DiI). Non-labeled cells were neither CNC nor epithelial in origin. These mice will be very valuable in future studies using other markers, and as controls for selective gene knockouts or other alterations (Ito *et al.*, 2003).

Several studies using *in vitro* models have provided information on the necessary molecules that facilitate EMT. One *in vitro* model used the transformation of adult and embryonic epithelial cells (notochord, lens, cornea, limb ectoderm, and thyroid) into fibroblast or mesenchymal cells, when they were cultured in three-dimensional (3D) gelling type I collagen (Greenburg and Hay, 1982, 1986, 1988). The lens epithelial cells changed phenotype by decreasing cell-cell adhesion, increasing cell-matrix adhesion, and switching from epithelial to fibroblast intermediate filaments. The lens epithelial apical cell surface extended filopodia into the surrounding collagenous matrix. These cells began producing type I collagen and stopped producing crystallins, type IV collagen, and laminin. The apical surface started expressing $\beta 1$ integrin, and the mesenchymal cells up-regulated $\alpha 5 \beta 1$ integrin and started producing fibronectin (Zuk and Hay, 1994). It was found that blocking the cell-matrix interaction with antibodies to $\beta 1$ integrin partially inhibited transformation of lens epithelial cells to fibroblasts in collagen gels (Zuk and Hay, 1994). In contrast, thyroid follicle cells extended filopodia from the basal cell surface and decreased thyroglobulin and cytokeratin (cell-type-specific proteins) in 3D type I collagen gel cultures. The transformed cells also increased expression of vimentin, an intermediate filament family found in fibroblastic cell types (Greenburg and Hay, 1988), demonstrating that the cells lost epithelial-specific proteins. The ECM composition also played an important role, since culturing the thyroid follicle cells in a 3D gelling basal lamina extract (Matrigel) prevented the EMT phenotype change (Greenburg and Hay, 1988).

During EMT, epithelial cells lose polarity and differentiated cell-cell contacts, to become mobile mesenchymal cells. In contrast, when cells transform from mesenchyme to epithelial phenotype, they need to increase differentiated cell-cell contacts. The loss of cell-cell junctions was observed in normal MDCK cells incubated in neutralizing antibodies directed toward *E-cadherin* (Behrens *et al.*, 1989). The gene for *E-cadherin* has been shown to cause fibroblastic cell lines to become epithelioid in culture. To determine if the *E-cadherin* gene could cause a definitive embryonic mesenchyme to transdifferentiate into an epithelial phenotype, investigators co-transfected primary corneal fibroblasts by impact-loading with plasmids containing *E-cadherin* (Vanderburg and Hay, 1996). The fibroblasts expressing *E-cadherin* aggregated, localized *E-cadherin* to lateral surfaces, and formed stratified epithelia that developed zonulae occludentes and adherents. These epithelial cells expressed connexin 43, cytokeratin, and desmoplakin, and developed desmosomes. However, the cells continued to contain vimentin intermediate filaments, and the basement membranes did not develop, even though the cells synthesized laminin and type IV collagen. These types of cell culture experiments established that cell phenotype (epithelial *vs.* mesenchymal) was determined by both the extracellular environment and the gene expression program of the cells. Furthermore, the expression of cell-cell junction proteins such as E-cadherin was instrumental in determining the cell type (Hay, 1995; Hay and Zuk, 1995).

TRANSGENIC ANIMALS

Palatal clefting has been a popular model for the study of EMT during craniofacial development. Twenty or more genes have been related to facial clefting defects (Slavkin, 1995; Thyagarajan *et al.*, 2003). These include genes for growth factors, extracellular matrix, signaling molecules, and homeobox genes. A recent review has a detailed list of the genes responsible for craniofacial defects, including palatal clefts (Thyagarajan *et al.*, 2003). Two recent studies have identified genes associated with cleft palate in humans: The transcription factor, *interferon regulatory factor 6 (IRF6)*, is responsible for the autosomal-dominant disorder, Van der Woude syndrome (VWS) (Murray and Schutte, 2004), and nonsense mutations and deletions in the *fibroblast growth factor receptor 1 (FGFR1)* gene have been identified with Kallmann syndrome (Dode *et al.*, 2003).

Several transgenic mice have been developed that have been instructive for the understanding of the genetic component of palatal clefts. Several signaling molecules are necessary for initiating palate growth, including Fgf10, expressed in the mesenchyme, and its receptor Fgfr2b, in the epithelium. The activation of Fgfr2b mediates the expression of sonic hedgehog (*Shh*) in the epithelium (Rice *et al.*, 2004). *Msx1* was identified as having a critical role in mediating epithelial-mesenchymal interactions during craniofacial bone and tooth development. The animals that do not express this protein (*Msx1*^{-/-}) have extensive craniofacial defects, including cleft secondary palate and deficiency of alveolar mandible and maxilla, with failure of tooth development and abnormalities of the nasal, frontal, and parietal bones, and malleus in the middle ear (Satokata and Maas, 1994).

In *Tgfβ3*^{-/-} mice, the secondary palate cleft was their only craniofacial defect. Interestingly, in organ culture, the palatal

shelves from these mutated mice were able to fuse in the presence of exogenous Tgfβ3 (Taya *et al.*, 1999). The authors suggested that the one role of Tgfβ3 was to regulate the formation of epithelial filopodia prior to fusion. In a separate study, the medial edge epithelial surfaces of embryonic days 12, 13, and 14 mouse palatal shelves from homozygous null (*Tgfβ3*^{-/-}), heterozygous (*Tgfβ3*^{+/-}), or homozygous normal (*Tgfβ3*^{+/+}) mice were observed with Environmental Scanning Electron Microscopy (ESEM) (Martinez-Alvarez *et al.*, 2000a). These investigators found that, in mice that were heterozygous or homozygous-normal, the MEE had surface bulging. However, the surface bulges were not seen in the *Tgfβ3*^{-/-} embryos (Martinez-Alvarez *et al.*, 2000a). In addition, the MEE was thinner and had fewer apoptotic cells (Martinez-Alvarez *et al.*, 2000a).

The Shuler laboratory examined the phenotype of highly back-crossed (12 generations) *Tgfβ3*^{-/-} animals compared with *Tgfβ3*^{+/+} and *wild-type* mice and found that 100% of *Tgfβ3*^{-/-} newborns had cleft secondary palate, 91.4% complete cleft, and 8.6% partial cleft. In the partial-cleft-palate newborn mice, fusion occurred only between the 2nd and 5th rugae. No epithelium remained in the midline fusion region. In addition, some of the heterozygous animals (8.8% of *Tgfβ3*^{+/-}) and homozygous normal (2.5% of *TGF-β3*^{+/+}) newborns had a failure of fusion between the primary and secondary palates (χ^2 test, $0.1 > p > 0.05$). Failure of fusion of the primary palate with the secondary palate was also identified in *Tgf-β3*^{+/-} adult mice. Third, complete cleft palate was not detected in either *Tgfβ3*^{-/-} or *Tgfβ3*^{+/+} newborns (Cui *et al.*, 2004).

Investigators used another transgenic mouse in a two-component genetic system (*Cre-Flox* or *Cre-Lox*) for marking the progeny of the CNC during tooth and mandible development, using the *Wnt1* promoter. *Wnt1* transgene expression was limited to the migrating neural crest cells that were derived from the developing dorsal central nervous system. The second mouse line carried a deletion of the *Tgfr2* receptor, so that progeny had decreased Tgfβ signaling in only the CNC. The resulting fetuses had a complete cleft of the secondary palate, calvaria agenesis, and other skull defects, with complete phenotype penetrance (Ito *et al.*, 2003). Disruption of the Tgfβ signaling pathway did not negatively affect CNC migration. It was determined that the cleft palate in these mutant mice resulted from decreased cell proliferation due to decreased cyclin D1 expression within the CNC-derived palatal mesenchyme. The MEE of the mutant palatal shelf remained functionally competent for EMT when the palatal shelves were placed in contact *in vitro* (Ito *et al.*, 2003).

This particular mouse is very valuable, since it has added a marker (β -gal) to the CNC with a conditional reporter allele (Ito *et al.*, 2003). Other transgenic mice have been engineered with either this reporter or green fluorescent protein (GFP) derivatives for cell lineage and ablation studies. The advantage of the *Cre/LoxP* system was that the genes could be selectively over- or underexpressed in a single tissue type, rather than the whole animal (Chai *et al.*, 2000).

Several laboratories collaborated on a study using 3 mutant animals in a cross-breeding strategy to explore the role of *Fgf10*^{-/-}, FGF receptor (*Fgfr2B*^{-/-}), and Sonic hedgehog (*Shh*) mutant mice, all of which exhibited cleft palate, to show that *Shh* is a downstream target of Fgf10/Fgfr2b signaling (Rice *et al.*, 2004). They demonstrated that mesenchymally expressed

Fgf10 activated its receptor, Fgfr2b, in the epithelium. Furthermore, they determined that the receptor, Fgfr2b, mediates the epithelial expression of *Shh* that signals back to the mesenchyme to target molecules including *bone morphogenetic protein (Bmp2)* and *patched (Ptc)*. They confirmed this by demonstrating that cell proliferation was decreased in the palatal epithelium and mesenchyme of *Fgfr2b*^{-/-} mice. These results reveal a new role for Fgf signaling in mammalian palate development and demonstrated a coordinated epithelial-mesenchymal interaction essential during the initial stages of palate development (Rice *et al.*, 2004).

During the 2004 General Session of the International Association for Dental Research, several laboratories reported their latest research on either transgenic mice (*Tgfβ3*^{-/-}, Odd-skipped transcription factor 1,2 *Osr2*), naturally occurring mutants (Dancer-Tbx10), or down-regulation of a specific mRNA with antisense oligonucleotides (*snail*) (Bush *et al.*, 2004; Cui *et al.*, 2004). The *Osr2*^{-/-} mice exhibit failure of midface development, including a missing cranial base, open eyelids, and palatal shelves that do not grow vertically or elevate as well as those of wild-type control animals (Lan *et al.*, 2004). An investigation into the proliferation level of the mesenchymal cells in the palatal shelves demonstrated that the *Osr2*^{-/-} mice had 25% fewer dividing cells. The investigators also reported that *Pax9* was down-regulated in the *Osr2*^{-/-} palatal shelves, while *Tgfβ3*, *Msx1*, *Bmp4*, and *Tbx2* were unchanged compared with those in wild-type control mice (Lan *et al.*, 2004).

The *Dancer (Dc)* mouse is a spontaneous mutation that exhibits cleft lip and palate, since the palatal shelves do not elevate, while heterozygote animals are predisposed to teratogen-induced clefting. By the use of PCR, RT-PCR, *in situ* hybridization, and Southern hybridization, the gene responsible for this mutation was identified as the *Tbx10* gene. It was demonstrated that the *Dc* mutation was caused by the insertional translocation of a chromosome 10 fragment into the *Tbx10* first intron. This insertion resulted in the ectopic expression of a mutant-specific *Tbx10* transcript. Transgenic recapitulation of the *Dc* phenotype further confirmed that it is caused by *Tbx10* mis-expression (Bush *et al.*, 2004). In summary, several transgenic mice have been developed that will be useful tools for improving our understanding of important palate and EMT genes. However, each animal must be evaluated to determine if the cleft phenotype is a primary effect of the transgenic manipulation, or a secondary effect caused by other physical factors.

CRANIAL NEURAL CREST EMT

Before the closure of the neural folds in the mammalian head, the induced neural crest cells break away from an embryonic epithelial layer of the dorsal neural tube by changing their shape and properties, from those of typical neuroepithelial cells to those of mesenchymal cells (Fig. 1). In a recent meeting celebrating the contributions of James A. Weston to the understanding of the neural crest, Dr. Weston proposed that the CNC were derived from an early population of non-neuronal ectodermal cells (Erickson, 2004; Weston *et al.*, 2004). The controversy appears to be centered on confusion over what portion of the neural fold gives rise to the neural crest. Weston's group provides evidence that the cells at the edge of the neural fold express E-cadherin and Pdgfrα; therefore, the

ectomesenchyme may be from the non-neuronal epithelium in the neural fold (Weston *et al.*, 2004). This theory is certain to engage many investigators in the next few years. This review will concentrate on the transition from epithelial cell types, since both neuroepithelia and surface ectoderm must complete EMT. (A recent special issue of *Developmental Dynamics* was dedicated to this topic, "Special focus on the neural crest and the contributions of James A. Weston".)

A major difference between cells of the CNC and those of the trunk is that the CNC cells are patterned with level-specific instructions in the head, whereas those of the trunk do not appear to be pre-programmed. In the cranial region, the CNC migrate in streams throughout the cranial mesenchyme, with a level-specific instruction, to reach their final destinations. Extensive transplantation experiments demonstrated that the maintenance of this segmental characteristic is very important in the patterning of head development (Noden, 1983; Couly *et al.*, 1998; Ferguson *et al.*, 2000). Recently, *in vitro* studies have suggested that all neural crest cells have the potential to form skeletal elements (McGonnell and Graham, 2002); however, the CNC may play a key role in organizing the innervation of the hindbrain by the cranial sensory ganglia (Graham *et al.*, 2004).

Many research groups have been interested in investigating neural crest cell differentiation. The CNC cells are multipotent stem-like cells, which respond to temporospatially expressed signals and become 'committed'. Candidate regulators include growth factors—members of the Tgfβ family (Delannet and Duband, 1992), Fgfs (Kinoshita *et al.*, 1993; Baird, 1994), Pdgf (Morrison-Graham *et al.*, 1992), and Wnt (homologous to *Wingless* in *Drosophila*) gene products (Nusse and Varmus, 1992; Augustine *et al.*, 1993). The role of the Tgfβ family in CNC developmental processes has recently been reviewed (Chai *et al.*, 2003). The involvement of several signal transduction molecules and transcription factors has also been reported (Duband *et al.*, 1995).

The paired maxillary processes and mandibular prominences of the first branchial arch give rise mainly to the structures of the upper and lower jaws. The neural crest component of the maxilla derives from the forebrain and midbrain, while that of the mandible arises from the midbrain and hindbrain (rhombomeres 1 and 2). These CNC cells contribute mainly to the following structures in the first branchial arch: palate and maxilla, dermis and fat of skin, dental papilla, Schwann cells of peripheral nerves, melanocytes, and some connective tissue. A recent study demonstrated that the conditional ablation of the *TβrII* gene in the cranial neural crest lineage resulted in clefting of the secondary palate and calvarial defects. The pathogenesis of cleft palate in these mice appears to be related to impairment of cell proliferation (Ito *et al.*, 2003).

PALATAL FUSION

As the precursor of the secondary palate, the lateral palatine processes appear during the 6th week in human embryos. Initially, the 2 palatal shelves grow downward, lateral to the tongue. At this point, the tongue is narrow and tall, almost completely filling the oral-nasal cavity, and reaches the nasal septum. During the 7th week, the 2 palatal shelves dramatically change their positions and elevate to a horizontal position above the dorsum of the tongue (Greene and Pratt, 1976; Shaw

et al., 1991; Johnston and Bronsky, 1995; Long, 1998; Vieira and Orioli, 2001; Murray and Schutte, 2004).

Palatal shelves are composed of predominantly CNC-derived mesenchymal and ectomesenchymal cells covered by craniopharyngeal ectoderm. Initially, 2 epithelial layers cover the surfaces of the developing palatal shelves as a primary boundary (periderm and basal cells). The basal epithelia on the edges of the 2 opposing shelves are defined as medial edge epithelia (MEE) (Figs. 1, 2). During palatal morphogenesis, the MEE of the approximating palatal shelves fuse at the apical borders of the basal cells to form a midline epithelial seam. The epithelial cell seam rapidly disappears and establishes mesenchyme continuity across the intact horizontal palate. At almost the same time as the midline epithelial cells disappear, the epithelia on the nasal aspect of the palate (NE) differentiate into pseudostratified ciliated columnar cells, while those on the oral aspect (OE) become stratified squamous cells. Another structure involved in the formation of the palate is the nasal septum. It reaches the level of the palatal shelves when the latter fuse to form the definitive secondary palate (Ferguson, 1978, 1988; Fitchett and Hay, 1989; Griffith and Hay, 1992; Takigawa and Shiota, 2004).

Several theories have been proposed to explain how these 2 epithelium-covered palatal shelves fuse into one continuous structure. Some consistent features have been observed in various morphological studies. The sequence of events appears to be: loss of the superficial periderm cells, adhesion of the basal cells, basement membrane breakdown, and disappearance of the epithelial cells. One theory proposed that all of the epithelial cells died (Farbman, 1968; Hayward, 1969; Smiley, 1970; Hudson and Shapiro, 1973; Shah and Chaudhry, 1974; Pratt and Martin, 1975; Shah *et al.*, 1991), another proposed that the epithelial cells migrated to the oral or nasal surface (Carette and Ferguson, 1992), and, more recently, it was proposed that the epithelial cells transformed into mesenchymal cells (Fitchett and Hay, 1989; Shuler *et al.*, 1991, 1992) and continued to populate the palate, but as a different cell type.

The cell death theory has been resurrected in 2 publications (Martinez-Alvarez *et al.*, 2000b; Cuervo and Covarrubias, 2004). Apoptotic cells (TUNEL-positive) were observed in the MEE cell population prior to and during seam formation from albino Swiss mice killed during various stages of palatogenesis *in vivo*. In addition, the authors identified macrophages, in the MEE seam, apparently phagocytosing the dying cell population. However, these same images showed that over 80% of the MEE cells were healthy and not TUNEL-positive. In a more recent study, mouse palatal explants were cultured in suspension (Fig. 2D) in the presence of apoptosis enzyme inhibitors, and the palates fused normally (Takahara *et al.*, 2004). The authors suggested that the static culture system (Fig. 2C) used by other groups may contribute to increased cell death. In other experiments, using the static culture system, the MEE were infected with a retrovirus expressing LacZ. Although not all of the MEE cells were infected, the virus experiments clearly showed that some MEE cells died, while other cells transformed into mesenchyme. Some of the retrovirally labeled MEE cells migrated into mesenchyme and not only were found some distance from the midline, but also were TUNEL-negative (Martinez-Alvarez *et al.*, 2000b).

Using the lipophilic cellular markers DiI or CCFSE, several groups have successfully traced the fate of MEE (Griffith and

Hay, 1992; Shuler *et al.*, 1992). A more recent cell lineage study that labeled the CNC with β -gal (*Wnt1-Cre/R26R* mouse) and epithelium with DiI definitely demonstrated that the MEE were maintained as mesenchymal cells (Ito *et al.*, 2003).

The transformation of MEE occurs through many biological events, which have been distinguished into the following sequence: First, the superficial layer of periderm cells sloughed off through an apoptosis mechanism (Martinez-Alvarez *et al.*, 2000b). After the periderm cells died, the underlying basal cells produced desmosomes and adhered, forming a midline seam (Fig. 1) (Ferguson, 1988; Fitchett and Hay, 1989). The midline epithelial seam then lost cell-cell junctions and became mesenchymal. Included in these changes was the loss of the epithelium-specific adhesion molecules E-cadherin and syndecan-1 that were localized to the basolateral surfaces of the MEE at embryonic day 14 in the mouse. Twelve hours later, when a midline seam had formed, syndecan-1 and E-cadherin were still present on the basal and lateral epithelial surfaces, and remained after the seam broke up into epithelial islands. Expression of both molecules was lost simultaneously and abruptly when EMT occurred (Sun *et al.*, 1998a). The cells also switched expression of intermediate filaments from keratin to vimentin (Fitchett and Hay, 1989; Shuler *et al.*, 1991). In addition, the cells began producing enzymes responsible for matrix degradation, including matrix metalloproteinases (Mmp) and tissue inhibitors of metalloproteinases (Timp). These proteins have been identified by gelatin zymography and reverse zymography of MEE. Specifically, Mmp-2 showed a significant elevation during palatal fusion (Morris-Wiman *et al.*, 1999; Mansell *et al.*, 2000). The increase in these degradative enzymes led to the basement membrane breakdown, allowing the transformed cells to become confluent with the palatal shelf mesenchyme (Griffith and Hay, 1992).

As palate morphogenesis proceeded, the MEE cells decreased mitosis (Cui *et al.*, 2003), changed shape as they lost cell-cell junctions, and produced numerous filopodia, extending from the basal surface, that extended between basement membrane breaks (Boyer *et al.*, 2000). The cell movement was driven by re-arrangement of the cytoskeleton and formation of new cell-substratum contacts (Boyer *et al.*, 2000). In a cell lineage study in which the epithelial cells were labeled with CCFSE, the investigators observed CCFSE bodies in the mesenchymal cells, indicating that these cells were basal epithelial in origin (Fig. 1) (Griffith and Hay, 1992).

Several mechanisms have been developed to scale or grade the amount of EMT in palatal specimens, including counting the number of MEE cells (Nawshad and Hay, 2003; Cuervo and Covarrubias, 2004), or developing a scale system (Kang and Svoboda, 2002; Takahara *et al.*, 2004). Our scale system was based upon the characteristics of palatal fusion divided into 5 stages (1-5) according to histo-morphological observations, including confocal analysis of CCFSE, hematoxylin and eosin (H&E)-stained paraffin sections, and laminin immunohistochemical staining (Fig. 3, Table 1) (Kang and Svoboda, 2002). Single optical confocal images obtained from the middle of the tissues were analyzed for fusion. The location, continuity, and intensity of CCFSE-labeled MEE were compared anterior-posteriorly in the horizontal plane (Figs. 3A-3E). Non-fused or partially fused samples had more intense, continuous, or even 2 epithelial layers of CCFSE-labeled cells (Figs. 3A-3C), while the fused palates had very discrete labeling

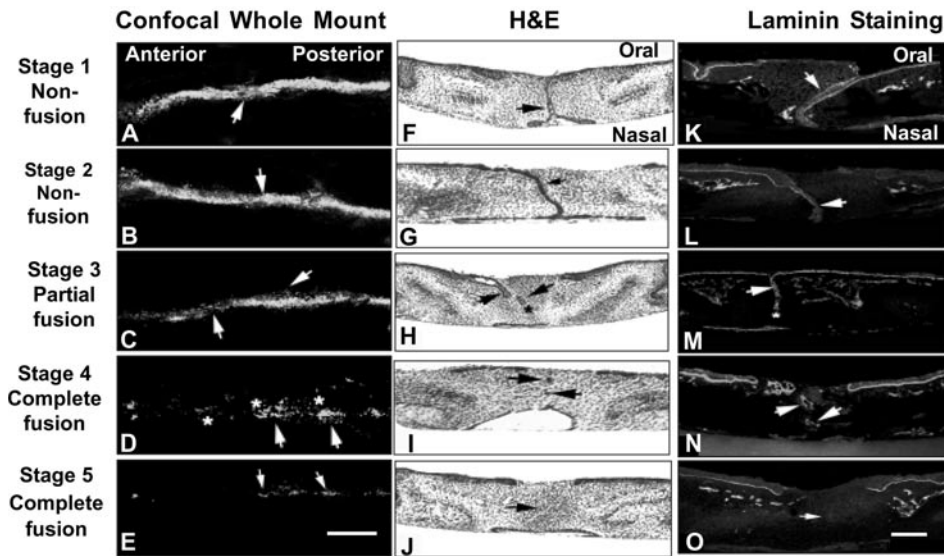
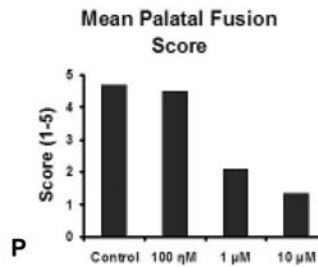


Figure 3. Five stages of palatal fusion *in vitro* as observed from confocal analysis of whole-mount palates stained with CCFSE (A-E), H&E staining (F-J), and laminin immunohistochemical studies (K-O) (Kang and Svoboda, 2002). Characteristics of each stage in the 3 morphological formats are explained in Table 1. Scale bars = 200 μ M in A-E and 160 μ M in F-O. (P) Mean fusion scores of palates after 72 hrs of culture in controls and LY294002 treatment groups. There was no significant difference between controls and 100 η M LY294002-treated tissues. However, the 1- and 10- μ M LY294002-treated palates were significantly different from controls ($p \leq 0.01$).



3O). The amount and continuity of laminin were greater in non- or partially fused samples.

Each cultured palate was scored from 1-5, and the mean fusion score (MFS) was determined for the palates cultured for 72 hrs. The investigators calculated the MFS by multiplying the palate stage (1-5) times the number of embryos for each stage, and dividing the sum by the total number of embryos in that group. Groups with higher fusion scores had MFS closer to 5. Based on this scale, it was determined that different signal transduction inhibitors could have an impact on palatal fusion (Fig. 3P). We have also used the scoring method to determine that nicotine decreased palatal fusion in the mouse palate *in vitro* (Kang and Svoboda, 2003).

LIP FUSION

Chicken upper lip forms by growth and fusion of the bi-maxillary processes of the first

branchial arch. Unlike the chicken secondary palate, the upper lip naturally fuses in the midline. An *in vivo* study with CCFSE and TEM demonstrated that the chicken upper lip fused by EMT mechanisms (Sun *et al.*, 2000). It was shown that, as in the palate, the periderm of the two-layered embryonic epithelium sloughed off before fusion of the basal cells. The authors labeled the cells undergoing programmed cell death by staining the fragmented DNA (TUNEL), followed by ultrastructural analysis, and confirmed that the periderm cells

in the fusion zone (Figs. 3D, 3E), indicating that EMT had occurred in these palates. Palatal samples were also processed as individual cross-sections for H&E staining, and MEE were analyzed in the coronal plane (Figs. 3F-3J). The amount of persistent MEE and degree of mesenchymal confluence were compared between treatment groups. To establish that the basement membrane had completed degradation, the investigators analyzed cross-sections by immunohistochemical localization of laminin, a marker for basal lamina (Figs. 3K-

Table 1. Stages of Palatal Fusion *in vitro*

	CCFSE Labeling	H&E Staining	Laminin Analysis
Stage 1 Non-fusion	Intense CCFSE labeling in the midline. MEE did not thin into a single seam (arrows, Fig. 3A)	Two layers of MEE persistent (arrow, Fig. 3F)	Two layers of basal lamina continue with oral epithelia (arrow, Fig. 3K)
Stage 2 Non-fusion	Two distinctive layers of MEE were NOT observed (arrow, Fig. 3B)	An epithelial seam continuous in the midline (arrow, Fig. 3G)	Laminin reaction beneath the epithelial seam but discontinuous (arrow, Fig. 3L)
Stage 3 Partial fusion	Intense labeling in midline but broken areas observed. Some MEE cells seemed to migrate into mesenchyme (arrows, Fig. 3C)	Mesenchymal confluence was achieved in places (asterisk), but large epithelial islands remained (arrows, Fig. 3H)	Positive laminin was detected next to the remaining MEE (arrow), but not in the confluent area (asterisk, Fig. 3M)
Stage 4 Complete fusion	Islands of MEE were observed (arrows). Discrete CCFSE-stained cells migrated away from the midline (asterisks, Fig. 3D)	Only small epithelial islands remained (arrows, Fig. 3I)	Laminin staining was observed only next to the remaining epithelial islands (arrows, Fig. 3N)
Stage 5 Complete fusion	Only discontinuous CCFSE-stained cells were detected in the fusion zone (arrows, Fig. 3E)	No epithelia persist in the midline (arrow, Fig. 3J)	Laminin was NOT detectable throughout fusion zone (arrow, Fig. 3O)

died. In addition, they determined that the basal cells (lip MEE) transformed into mesenchyme morphologically similar to that of the palate (Sun *et al.*, 2000). Interestingly, the chicken lip does not express *Tgfb* 1-3 and, unlike the mouse or chicken palate, did not require Tgf β for EMT and complete fusion *in vitro*. The authors suggested that factors other than Tgf β , such as *Shh*, could be responsible for EMT and fusion of the upper lip. Shh was observed in the lip epithelium at the time of fusion (Helms *et al.*, 1997). The addition of agents inhibiting Shh, including retinoic acid and Shh antibodies, blocked the normal fusion of maxillary and nasofrontal processes and resulted in bilateral cleft lip in chickens (Helms *et al.*, 1997; Hu and Helms, 1999), indicating that different genes may regulate EMT in specific craniofacial structures.

REGULATION OF EMT

The regulation of EMT is critical during dynamic developmental processes and post-natal homeostasis. In 1989, Hay postulated that the master gene(s) are turned on in epithelia by changes in the environment to initiate EMT (Master Gene theory). These changes in the environment include growth factors, cell adhesion molecules, extracellular matrix, the surface receptors and downstream signal transduction events, and transcription factors (Table 2). Evidence is mounting, in both developmental systems and tumorigenesis studies, that Twist (Kang and Massagué, 2004; Yang *et al.*, 2004), Snail (Cano *et al.*, 2000; Ip and Gridley, 2002; Ohkubo and Ozawa, 2004), and Slug (Bastidas *et al.*, 2004; Del Barrio and Nieto, 2004) may be the transcription factors that control EMT through regulating cell adhesion proteins, although the surface growth factor receptors and subsequent signal transduction pathways may be upstream of the transcription factors (Kang and Massagué, 2004).

The Tgf β super-family includes many small proteins that are multifunctional (controlling growth, migration, and differentiation) during both embryonic development and post-natal tissue homeostasis (Massagué, 1998; Whitman, 1998; Massagué *et al.*, 2000). The cellular responses to Tgf β during craniofacial development and CNC regulation have been extensively studied and recently reviewed (Chai *et al.*, 2003).

During mammalian palate development, *Tgfb* isoforms 1, 2, 3, *TbrII*, and *TbrIII* were detected in the MEE by *in situ* hybridization (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990a,b) or immunohistochemistry (Cui *et al.*, 1998; Cui and Shuler, 2000). Of particular interest was the highly localized expression of *Tgfb3* RNA and, to a lesser extent, that of *Tgfb1* and *Tgfb2* in the MEE and the nasal septum epithelium, which were destined to undergo EMT (Pelton *et al.*, 1990b). Antisense oligodeoxynucleotides or neutralizing antibodies to Tgf β 3, but not Tgf β 1 or Tgf β 2, resulted in the failure of palatal fusion *in vitro* (Brunet *et al.*, 1995). In addition, *Tgfb3* transgenic and knockout mice have cleft palate as their only craniofacial birth defect (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995). When palatal shelves from *Tgfb3* knockout mice were cultured in static organ cultures (Fig. 2C), the midline epithelia failed to go through EMT (Kaartinen *et al.*, 1997; Taya *et al.*, 1999). In addition, although the chicken has a naturally open palate, the cultured chicken palatal shelves fused when Tgf β 3

Table 2. Proteins and Genes That Promote EMT

Cell-surface molecules	α 5 β 1 integrin, T β rl, T β rII, T β rIII, FGFR, EGFR, FGFR, PDGFR α
Growth factors	TGF α and TGF β 1-3 Müllerian-inhibiting factor (MIF) α FGF, FGF10 Neurotrophin-3 (NT-3)
Oncogenes	v-src, c-fos, v-ras, and v-mos
Transcription factors	Sonic hedgehog, LEF1, Twist, Snail/slug Fibroblast-specific factor 1 (FSP1)
Signal transduction	Smads, PI-3 kinase, MAPK, ILK, AKT

was added to the medium (Sun *et al.*, 1998b). Therefore, it has been concluded that Tgf β 3 is an essential growth factor inducing EMT during palatal fusion, indicating that it may be the master gene that stimulates transcription repression of cell-cell proteins. Investigators have proposed that possible mechanisms of Tgf β 3-induced palatal fusion include the regulation of fusion by inducing cell membrane filopodia on MEE prior to shelf contact (Taya *et al.*, 1999). Second, the regulation of extracellular matrix degradation by modulation of the production of tissue inhibitor of metalloproteinase-2 (Timp-2), Mmp13, and Mmp2 has been proposed (Blavier *et al.*, 2001). More recently, it was found that Tgf β 3 is necessary for inhibiting MEE proliferation during EMT (Cui *et al.*, 2003). However, a new study cultured single (unpaired) mouse palatal shelves by suspension and static culture methods and found that MEE cells could disappear throughout the medial edge, even without contact and adhesion to the opposing MEE in suspension culture. MEE cell behavior in the suspension culture demonstrated that apoptosis, EMT, and epithelial cell migration all occurred at various stages of MEE cell disappearance, including the transient formation and disappearance of epithelial triangles and islets (Takigawa and Shiota, 2004).

Several research groups have started to investigate the TGF β 3-stimulated intracellular signaling molecules that are responsible for EMT during palatal fusion. *Smad2* expression was detected during palatal fusion (Cui *et al.*, 2000). In a later investigation, the authors suggested that phosphorylation of Smad2 may be necessary for Tgf β 3 down-regulation of MEE proliferation (Cui *et al.*, 2003). Interestingly, overexpression of *Smad2* in the *Tgfb3*^{-/-} mouse did not completely rescue secondary palate clefts (Cui *et al.*, 2005). There is also evidence, from the studies of mammary epithelial cell culture, that down-regulation of Smad signaling decreased Smad-dependent growth and transcriptional responses; however, the down-regulation did not affect Tgf β -mediated stress fiber formation and EMT (Bhowmick *et al.*, 2001). In another recent study, Hay's group used dominant-negative Smad4 and dominant-negative Lymphoid enhancer factor-1 (LEF1) to demonstrate that Tgf β 3 used Smads to up-regulate synthesis of Lef1 and to activate Lef1 transcription during the induction of palatal EMT. When phospho-Smad2 and -Smad4 were in the nucleus, Lef1 was activated without β -catenin (Nawshad and Hay, 2003; Nawshad *et al.*, 2004a). This work indicates that palatal EMT does not depend on β -catenin (Nawshad and Hay, 2003; Nawshad *et al.*, 2004b) and that Smads can activate Lef1 without β -catenin (Labbe *et al.*, 2000).

As an alternative downstream Tgf β signaling effector, PI-3 kinase has been identified in actin re-organization and Tgf β -mediated EMT (Metzner *et al.*, 1996; Sugiura and Berditchevski, 1999; Bakin *et al.*, 2000). Activated PI-3 kinase phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which recruits its downstream effectors to the plasma membrane. Along with the small GTPases Rac and Rho, PIP₃ activates several serine/threonine kinases, such as 3-phosphoinositide-dependent protein kinases (Pdk) (Alessi *et al.*, 1998; Le Good *et al.*, 1998). Pdk1 activates Pkc (Pullen *et al.*, 1998) and targets protein kinase B (Pkb, also known as Akt) (Burgering and Coffer, 1995), while integrin-linked kinase (ILK), a newly found Pdk, also activates Akt (Hannigan *et al.*, 1997). Upon stimulation, Akt migrates to and anchors the membrane (Andjelkovic *et al.*, 1997). Subsequently, activated Akt detaches from the plasma membrane and translocates into the cytoplasm and nucleus, regulating cell survival, protein synthesis, and cell cycle (Kandel and Hay, 1999). It also appears that PI-3 kinase possesses both lipid kinase and protein kinase activity (Carpenter *et al.*, 1993) and may directly control the activities of individual components of the Ras/Raf/Erk-mitogenic pathway by forming a complex with signal proteins.

The consequences of PI-3 kinase activation are numerous, including effects on cell cycle progression, suspension-mediated apoptosis, cell migration, and alterations in cell-cell adhesion (Roymans and Slegers, 2001). As a downstream effector of Tgf β signaling, PI-3 kinase is involved in actin re-organization, Mmp production, and cell mobility (Metzner *et al.*, 1996; Sugiura and Berditchevski, 1999). A specific inhibitor of PI-3 kinase, LY294002, completely blocked Tgf β -mediated phosphorylation of Smad2, cell migration, and partially blocked EMT in mammary epithelial cell cultures (Bakin *et al.*, 2000). In addition, other experiments demonstrated that Mmp2 production after integrin α 3 β 1 stimulation was PI-3-kinase-dependent (Sugiura and Berditchevski, 1999). Since Mmps are necessary for breaking down basement membrane, it was speculated that blocking PI-3 kinase activity inhibited cell migration and Mmp production, two essential steps of EMT. In addition, the overexpression of a PI-3 kinase downstream effector, ILK, induced anchorage-independent epithelial cell growth, loss of E-cadherin expression, and EMT (Radeva *et al.*, 1997; Wu *et al.*, 1998). ILK was also implicated in TGF β -induced fibroblastic conversion of highly metastatic cells (Janji *et al.*, 1999).

Using static palate organ cultures, we found that, during palatal fusion *in vitro*, EMT was dependent on PI-3 kinase activity within the 72-hour culture time studied (Figs. 1C, 3P) (Kang and Svoboda, 2002). However, it was possible that inhibiting PI-3 kinase delayed but did not completely block EMT, since some of the palates were partially fused (Kang and Svoboda, 2002).

In addition to PI-3 kinase, other signaling pathways can also be activated by Tgf β . An investigation of Tgf β 1-mediated disassembly of epithelial cell-cell junctions demonstrated a link between the Tgf β /Smad pathway and alterations of β -catenin/E-cadherin phosphorylation (Tian and Phillips, 2002). A later study by the same group transiently transfected epithelia with Smad2/4 or Smad3/4 expression vectors, but did not alter cell phenotype (Tian *et al.*, 2003). These results suggested that the Wnt pathway may be a further potential signaling pathway

mediating downstream events following Tgf β receptor binding. As part of the epithelial cytoskeleton, β -catenin binds to E-cadherin. The activity of β -catenin is controlled by a large number of binding partners that affect its stability and localization, which can be modulated by many signaling agents, such as Wnt, Ras, and PI-3 kinase (Espada *et al.*, 1999; Willert *et al.*, 1999). Upon release from the complex, β -catenin relocates into the nucleus and interacts with transcription factors such as T-cell factor/lymphoid enhancer factor-1 (TCF/LEF-1). Stabilized nuclear β -catenin has been shown to induce EMT in tumor cells *in vitro* (Kim *et al.*, 2002). However, it was recently demonstrated that β -catenin does not translocate to the nucleus in palatal MEE (Nawshad and Hay, 2003).

TRANSCRIPTION FACTORS

Throughout this review, transcription factors have been discussed in context with the experimental model, transgenic animals, or signal transduction pathways. It is beyond the scope of this review to provide an exhaustive discussion of transcription factors; therefore, we will concentrate on specific proteins that have been identified specifically in EMT (Table 2).

There are a great many transcriptional partners that affect Tgf- β 's choice of target genes (DNA binding co-factors) and their effect on their transcription (co-activators, co-repressors) (Shi and Massagué, 2003). Cbfa1, Cbfa3, Stat3, and Jun are examples of DNA-binding co-factors, Cbp and P300 are examples of the co-activators, and Tgif, c-Ski, SnoN, and Evi-1 are some of the known co-repressors of the Tgf- β signaling system (Massagué *et al.*, 2000; Massagué, 2003).

There is mounting evidence that Lef1 may be one of the controlling transcription factors that determines EMT cell fate (Nawshad and Hay, 2003). It was recently shown that Lef1 transcription is up-regulated *via* a Smad2/4 mechanism during palatal EMT. Furthermore, using laser capture microscopy, this group demonstrated that mRNA was also quantitatively increased in the MEE cells (Nawshad *et al.*, 2004b).

Another transcription factor, Fsp1, was also shown to be involved in EMT (Okada *et al.*, 1997). Although a murine fibroblast-specific protein, Fsp1 expression was induced in renal proximal tubular epithelial cells when treated with growth factors (Egf/Tgf β) and/or extracellular matrix protein (Collagen I). The epithelia having *de novo* expression of Fsp1 displayed mesenchymal phenotypic morphology and protein expression. In addition, this EMT was abolished when cells were treated with *Fsp1* antisense oligomers.

As stated previously, Twist, Snail, and Slug appear to down-regulate cell-cell adhesion proteins in developmental and tumorigenesis models (Kang and Massagué, 2004). Snail represses transcription of *E-cadherin* and activates mesenchymal differentiation, such as vimentin and fibronectin (Cano *et al.*, 2000; Schlessinger and Hall, 2004; Zhou *et al.*, 2004). Recent experiments show that Snail regulation is balanced between EMT-promoting growth factors and blocking factors, begging the question, Can Snail serve as the master gene, since it regulates cell adhesion molecules?

ECM BREAKDOWN

In addition to changes in growth factors, the modulation of the extracellular environment also includes the maintenance and degradation of ECM, which is mediated, in part, by Mmps and

Timps. Temporospatial expression of Mmps 2, 3, 7, 9, and 13, and Timps 1 and 2, was observed during murine palatal fusion (Morris-Wiman *et al.*, 1999, 2000). In the palatal fusion zone of Tgf β 3-deficient mice, Timp-2 was completely absent; Mmp-2 and Mmp-13 had reduced levels (Blavier *et al.*, 2001). Upon exposure to Mmp inhibitor (BB 3103), the murine palatal shelves failed to fuse in culture (Brown *et al.*, 2002).

Other mechanisms of Tgf β 3 included the regulation of extracellular matrix degradation (Blavier *et al.*, 2001). The investigators compared the expression of several Mmps, including a cell-membrane-associated Mmp (Mt1-Mmp) and Timp-2, in normal and Tgf β 3^{-/-} mice. They found that Mmps and Timp-2 were highly expressed by wild-type MEE. Mmp-13 was expressed both in MEE and in adjacent mesenchyme, whereas gelatinase A (Mmp-2) was expressed by mesenchymal cells neighboring the MEE. In contrast, Tgf β 3^{-/-} mice had complete absence of Timp-2 in the midline and expressed significantly lower levels of Mmp-13 and slightly reduced levels of Mmp-2. In support of a role for Tgf β 3 in regulating matrix breakdown, Mmp-13 expression was strongly induced by Tgf β 3 in palatal fibroblasts. It was also shown that blocking Mmps or exposure of palates to higher concentrations of Timp-2 caused palatal fusion failure. The MEE cells did not transform into mesenchyme, indicating that ECM degradation by Mmps was a necessary step for palatal fusion (Blavier *et al.*, 2001).

CLINICAL/TERATOLOGY

The etiology of cleft palate is generally considered multifactorial; however, a recent study has identified *IRF6*, a transcription factor, as responsible for the autosomal-dominant disorder, Van der Woude syndrome (Murray and Schutte, 2004; Zuccherro *et al.*, 2004), a model for isolated cleft lip and palate. Other candidate genes related to cleft palate include: *TGF α* , *TGF β 2* and *3*, *MSX1*, *B-cell lymphoma 3 (Bcl3)*, *FGFR1*, and the retinoic acid receptor alpha (*RARA*) (Vieira and Orioli, 2001; Murray and Schutte, 2004).

Disturbances at any stage during palate development—*i.e.*, defective palatal shelf growth, failed or delayed shelf elevation, and failure of shelf fusion—can result in cleft palate (Ferguson, 1988; Murray and Schutte, 2004). As one of the most common congenital craniofacial defects, cleft palate occurs in approximately 1 *per* 750 live births in the United States (Fogh-Andersen, 1971; Hay, 1971; Murray and Schutte, 2004). Cleft palate may not be life-threatening, but many functions can be disturbed because of the structures involved, such as feeding, digestion, speech, middle-ear ventilation, hearing, respiration, and facial and dental development. These problems can also cause emotional, psychosocial, and educational problems. In addition, cleft palate is an economic burden, since it costs an average of \$100,000 *per* patient for the entire treatment, amounting to \$697 million *per* year in the United States (*Morbidity and Mortality Weekly Report*, 1995).

Children with oral clefting need to see a variety of specialists who will work together as a team, including surgeons, dentists, audiologists, neurologists, pediatricians, psychologists, speech therapists, ophthalmologists, and otolaryngologists. Treatment usually begins in infancy and often continues through early adulthood, and although different experts focus on the patient's different needs at different stages, the entire team of experts follows through multiple surgeries,

growth, and development. There have been many medical advances in the treatment of cleft palate. A possibility of *in utero* surgical correction has been explored. A recent study has documented that TGF β 3 aids in the 'scarless' repair of cleft palate (Weinzweig *et al.*, 1999, 2002).

CONCLUSION AND FUTURE DIRECTIONS

In summary, we have discussed the experimental models and the current knowledge of EMT mechanisms, especially during craniofacial development. It is the orchestration of cytokines, extracellular matrix, cell-surface proteins, signaling molecules, and transcription factors that makes EMT a masterpiece.

We are just beginning to uncover the myth of this cellular phenotype transition that plays important roles during development and homeostasis. There have been advancements in research tools since the discovery of the EMT phenomenon. Laser-capture microscopy has aided researchers in the investigation of the temporospatial molecular changes of specific cell groups (Nawshad *et al.*, 2004b). Newer transfection techniques have enabled more accurate cell-lineage studies to be conducted. In addition, exciting findings in signaling pathways are being investigated and published daily (Murray and Schutte, 2004). It will be just a matter of time until the pathway leading to EMT will be mapped and understood.

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