



Growth Factors and Signaling Proteins in Craniofacial Development

Robert Spears and Kathy K.H. Svoboda

Regulation of growth and development is controlled by the interactions of cells with each other and the extracellular environment through signal transduction pathways that control the differentiation process by stimulating proliferation or causing cell death. This review will define the common signaling molecules and provide an overview of the general principles of signal transduction events. We also review the signal transduction pathways controlling one specific mechanism found in craniofacial development termed epithelial-mesenchymal transformation (EMT) employed during gastrulation, cranial neural crest migration, and secondary palate formation.

Semin Orthod 11:184–198 © 2005 Elsevier Inc. All rights reserved.

Growth Factors and Signal Transduction

Intracellular signaling is usually triggered by a cell surface event such as a specific protein (ligand) binding to a cell surface receptor to form a receptor-ligand interaction. Cells contacting neighboring cells or their surrounding noncellular tissue are termed cell-cell or cell-extracellular matrix (ECM) contacts (Fig 1A).¹ Interactions of cells with other cells or the ECM can stimulate many reactions, including: increased cell division, cell movement, differentiation, and even programmed cell death (apoptosis). The cell surface binding proteins (receptors) are classified by the protein structure and ligand characteristics. These proteins are usually located on the cell surface in the plasma membrane. Part of the protein may be located outside the cell to interact with the ligand. This part of the proteins is called the extracellular ligand binding domain. Part of the protein will traverse through the membrane, termed the membrane spanning domain, and a portion of the protein will be inside the cell and termed the cytoplasmic domain.

Most modern cell biology textbooks list at least four types of cell surface receptors, including the G protein-coupled

receptors (GPCR, Fig 1A), ion-channel receptors, tyrosine kinase-linked receptors, and receptors with intrinsic enzymatic activity.² The GPCRs are characterized by multiple transmembrane domains (usually seven) that wind the protein in and out of the membrane in a serpentine conformation (Fig 1A, GPCR). The ion-channel receptors are closely related and actually open a membrane channel when the ligand binds. Many cytokine receptors are in the tyrosine kinase-linked class as they lack intrinsic activity; but when the ligand binds, intracellular tyrosine kinases become activated to generate cellular changes. The classic growth factor receptors have kinase activity within the protein (intrinsic) and therefore make up the fourth class of receptors—the receptor tyrosine kinases, or receptor serine/threonine kinases (Fig 1A and C). These receptors usually have one transmembrane domain, and at least two molecules must become closely associated (dimerize) to activate the signal.

In addition to these classic receptor classes, cells can respond to their ECM environment through integrin receptors or proteoglycan receptors such as the syndecan family (Fig 1A and B). Single transmembrane domains with large extracellular and much smaller cytoplasmic domains characterize these receptors. The syndecan molecules have long glycosaminoglycan chains that assist in sequestering the fibroblast growth factors close to the cell membrane.^{3–6} The integrin receptors are heterodimers composed of α and β subunits. The family is very large with at least 25 integrin heterodimers, including 19 α subunits and 8 β subunits identified.⁷ The integrins do not have kinase activity, but on binding to ECM molecules, some associated proteins become activated by autophosphorylation and then phosphorylate (activate) sur-

Department of Biomedical Sciences, Baylor College of Dentistry, Texas A&M Health Science Center, Dallas, TX.

This work was supported by grant sponsors: Baylor Oral Health Foundation; Tobacco Endowment Fund, Texas A&M University System; Grant Number: 304-202850-4013.

Address correspondence to Robert Spears, PhD, Texas A&M University System, Baylor College of Dentistry, Department of Biomedical Sciences, 3302 Gaston Ave, Dallas, TX 75266; Phone: 214-828-8297; Fax: 214-828-8951. E-mail: rspears@bcd.tamhsc.edu

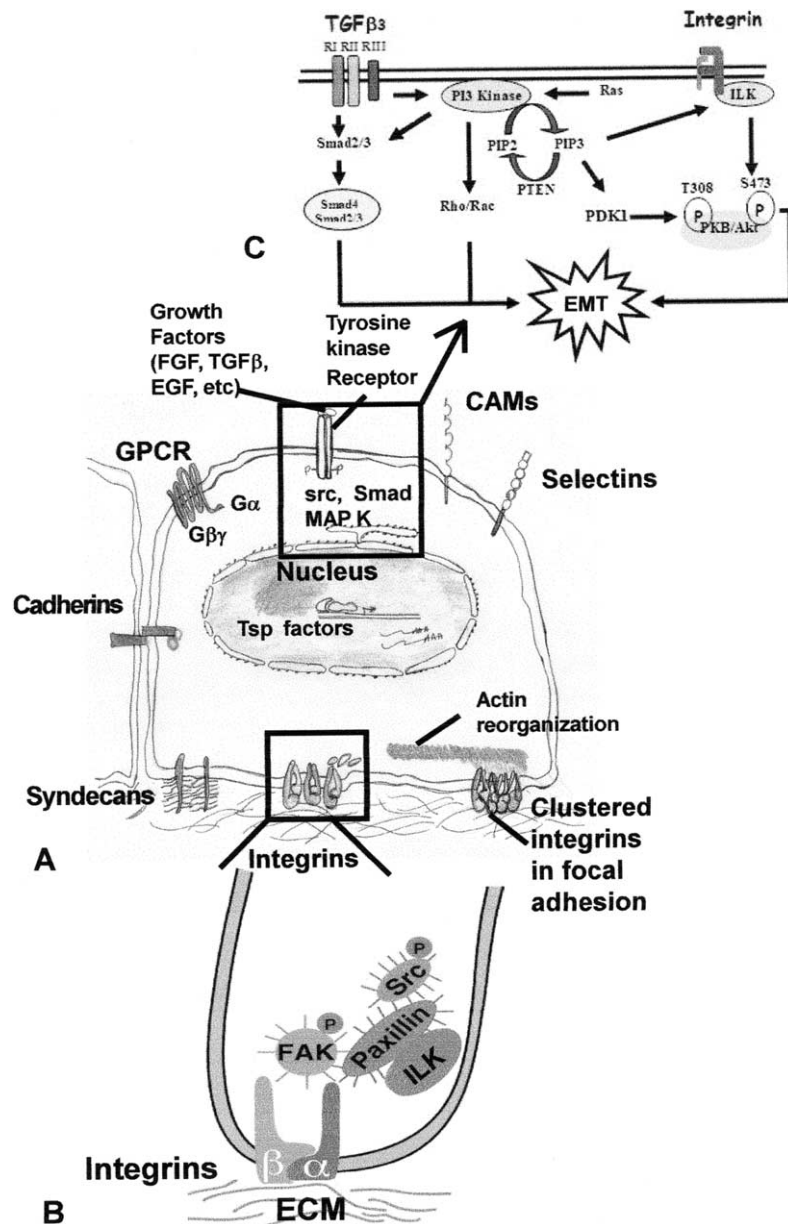


Figure 1 (A) Many different types of receptors are on cells that contribute to the same signal pathways. In this illustration, G protein-coupled receptor (GPCR), receptor tyrosine kinase, cell adhesion molecules (CAMs), selectins, integrins, syndecans, and cadherins are represented with some of their intracellular signal molecules identified. All of the receptor molecules have three common structural features: an extracellular ligand binding domain, a transmembrane domain, and an intracellular cytoplasmic domain. (B) The integrin molecules (lower square) were enlarged to demonstrate that the cytoplasmic domain interacts with other proteins (FAK, paxillin, Src, and ILK). These proteins become phosphorylated when integrins bind to ligands (ECM) and cluster to form focal adhesions. (C) TGF- β receptors are in the tyrosine kinase receptor class (upper square) and on activation signal through several pathways including the Smad and PI3-kinase pathways to stimulate EMT (as discussed in the text).

rounding proteins to generate signals for specific cellular activities that usually change the actin cytoskeleton. One of the first proteins to become phosphorylated is focal adhesion kinase (FAK) (Fig 1B).

Another concept in recent literature is that different signaling molecules may be sequestered in membrane microdomains termed lipid rafts^{8,9} that may facilitate cross talk between different receptors and signaling pathways.^{1,10-16}

Intracellular Signaling Proteins

One basic principle of signal transduction is that the proteins exist in at least two states: activated and inactivated. At this time we know of several methods to turn the signals on and off, including phosphorylation, dephosphorylation, intracellular location, nucleotides (ADP/ATP or GDP/GTP) cycles, and calcium/ion levels (see glossary; Table 2).

GPCR cascade. One of the first signal transduction mechanisms described was the GPCR cascade that generates the classic second messengers cyclic AMP (cAMP), cyclic GMP (cGMP), diacylglycerol (DAG), phosphoinositols, and calcium (Ca^{2+}). When G protein-linked or hormone receptors become activated, they trigger a series of events at the cell surface that cause transient increases in these second messenger molecules.² As with other signaling events, there is a transient increase in the active form of the molecule followed by a rapid decrease to produce a “signal.” Briefly, when the ligand binds to the receptor it causes a change in the protein structure that allows the G-protein α subunit to bind to the cytoplasmic domain of the receptor (Fig 1A, GPCR). This interaction causes the exchange of GDP for GTP on the α subunit and the disassociation of the $\beta\gamma$ subunits from the α subunit. The activated (GTP bound) α subunit interacts with adenylyl cyclase, the membrane-bound enzyme responsible for producing cAMP. After activating adenylyl cyclase, the α subunit reverts to the GDP state and reassociates with the $\beta\gamma$ subunits. Not only is the number of GPCRs very large, but α subunits are also numerous, providing a wide variety of signals to the cell. Once the G proteins are activated, the signal can be amplified by interactions with other proteins.

Classically, adenylyl cyclase produces cAMP that activates other kinases termed the cyclic AMP-dependent kinases (cAPKs), also called protein kinase A (PKA). These kinases can phosphorylate (activate) a number of substrates depending on the specific stimulus to amplify the signal from the cell surface.² One effect of activation is the release of calcium from intracellular storage areas, such as the rough endoplasmic reticulum and mitochondria. Since free calcium levels in cells are maintained at very low levels, the rapid increase in calcium levels from these intracellular organelles has been a way to visualize signal events. Calcium can be labeled with either quantitative ratiometric dyes or single wavelength dyes to monitor these rapid changes in cells following stimulation.¹⁷ Rapid sequestering of the free calcium ions by molecules such as calmodulin that bind several calcium ions turns off the calcium signal.

Phosphorylation and dephosphorylation. Phosphorylation and dephosphorylation provide another mechanism for signal on and off switches. The enzymes that phosphorylate other proteins are called kinases. The common amino acids that become phosphorylated are tyrosine, threonine, and serine. As stated previously, the phosphorylated proteins may be activated by adding phosphate molecules, and deactivated by removing the phosphates, a function usually performed by another enzyme class, the phosphatases. Some proteins can autophosphorylate themselves; once activated, they can phosphorylate surrounding substrates. An example of this type of protein was discussed previously in describing the integrin molecules; focal adhesion kinase (FAK) becomes phosphorylated when integrins bind to their ligand, ECM (ie, collagen, fibronectin, or laminin). Once FAK becomes phosphorylated it will activate or phosphorylate paxillin, an actin-associated protein, and another kinase, Src (Fig 1B).¹⁸ Src can

also start activating surrounding proteins, creating an amplification of the original signal.

To complicate things, some proteins are inactive in the phosphorylated state and become active after dephosphorylation. Therefore, it is important to understand the possible changes in the proteins before starting an investigation. Cells may contain a constant amount of a given protein in a pool, but the protein has to be in an active state to produce a signal that will change the proteins around it.¹⁹ It is important to remember that just because an mRNA for a given protein is expressed; it does not indicate that the protein is produced or that it is activated.

Nucleotide binding. Protein is generally in an inactive state if the ADP or GDP nucleotide is bound and becomes activated when the ATP or GTP is bound. An example of this type of activation is the small G-protein families, Ras and Rho.^{1,12,20-23} These proteins alternate between the GTP-bound active form (on) and the GDP-bound inactive form (off) to regulate other downstream kinases. Many other proteins regulate the “on” and “off” state of the small G proteins. The guanine-nucleotide exchange factors (GEFs) are the “on” signal as they add GTP to the protein. GTP-activating proteins (GAPs) are the “off” signal as they remove a phosphate to deactivate the protein. Guanine-nucleotide dissociation inhibitor proteins (GDIs) sequester the inactive protein in the cytoplasmic pool. We have shown that one of these regulatory proteins (p190RhoGAP; 190 kDa protein that functions as a GAP for Rho) becomes phosphorylated very quickly in embryonic epithelia in response to cells binding ECM.¹⁹ We have also shown that decreasing Rho protein levels or activity decreased other integrin signaling molecules.²⁴

Intracellular location. Some proteins move to specific intracellular structures, such as focal adhesions^{1,25} when they become activated. Paxillin, α -actinin, and talin accumulate at the focal adhesions in both migratory and stationary cells. In addition, integrin molecules become clustered at the focal adhesion of fibroblast cells (Fig 1A) and new evidence indicates that these proteins may act as sensors for mechanical forces.¹⁴

Many proteins move to the plasma membrane when they become activated. The movement to the plasma membrane may take several steps, including release from a cytoplasmic chaperone protein and/or acquiring a lipid tail. The small G proteins (such as Rho) require both the release from the guanine-nucleotide dissociation inhibitor (GDI) protein and a lipid tail to move to the membrane.^{26,27} So, in addition to having a GTP bound to the protein, the protein itself moves to the site of action. Many of the small GTPase proteins (Ras, Raf, Rac) follow similar intracellular translocation patterns on activation.

Other activated proteins move to the nucleus and may act as transcription factors. Examples of this type of intracellular translocation are some of the MAP kinase proteins.²⁸⁻³⁰ MAP kinases can respond to a variety of extracellular signals, including osmotic stress, heat shock, cytokines, and mitogens.³¹ Two of the MAP kinases, the extracellular signal-reg-

ulated kinases (erk-1 and erk-2; also referred to as erk-1/2), translocate to the nucleus after activation to regulate the expression of various transcription factors (Table 1).^{30,31} Activation of the MAP kinase pathways has been identified as a mechanism used by integrins to regulate gene expression leading to cell shape changes during cell spreading or migration,^{10,21,32,33} and as a cross-talk pathway between integrins and growth factors.^{1,10,34}

Sometimes the signal protein needs to bind another protein, a chaperone, before translocating to the nucleus. Transforming growth factor beta (TGF- β) proteins activate a class of proteins called Smads. These growth factors bind to cell surface receptors (T β RI and T β RII) and activate specific Smad proteins (Smad 2 or 3) that then bind to a chaperone protein, Smad 4, before translocating to the nucleus (Fig 1C) to act as transcription factors.³⁵⁻³⁸ Similarly, bone morphogenetic proteins (BMPs) bind to BMP receptors, activate Smads 1 or 5, which bind Smad 4 to translocate to the nucleus. More details concerning this pathway will be discussed later in this review (EMT regulation).

Summary

Intracellular responses to cell surface receptors are complicated and the subject of many active investigations. A number of studies have established reciprocal linkages between ECM-integrins, growth factor signaling, cell-cell adhesion molecules, specialized membrane domains (lipid rafts), and G protein-linked receptors.¹ In addition, cross talk has been established between the ECM and intracellular mitogen-stimulated pathways, the small G proteins, and the phosphoinositols.^{39,40} The cell's microenvironment and the resulting tissue profoundly influence each of these linkages. Thus, for a cell to achieve a differentiated phenotype or respond to microenvironment changes, the ECM molecules and their receptors must integrate both form and function. In contrast, mutated genes and aberrant interactions with the microenvironment may degrade this integration, possibly resulting in malignant transformation or abnormal development.⁴¹ Recently, it has also become apparent that integrins regulate Rho GTPases and vice versa. Integrins and GTPases might therefore be organized into complex signaling cascades that regulate cell behavior.^{1,10,21} In the next section of this review, specific signaling pathways important for craniofacial development will be discussed in detail.

Growth Factors Important in Epithelial/Mesenchymal Interactions

Cell phenotype transformations, from epithelial to mesenchymal (epithelial-mesenchymal transformation, EMT) and vice versa have been well documented in embryonic development, wound healing, and tumor metastasis. Epithelia serve as the boundary between the external environment and the remainder of the organ 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.

Mesenchymal cells are more mobile and surrounded by extracellular matrix. They have anterior-posterior polarity and form only transient contacts with their neighboring cells. During EMT cell phenotype transition, epithelial cells lose cell-cell attachment, break through the basal lamina, become mobile, and express mesenchymal proteins (Fig 2B).

From the mid-1990s and more recently, several reviews were published on EMT in development and pathogenesis.⁴²⁻⁴⁸ EMT and the opposite, mesenchymal-epithelial transformation, occur during normal developmental processes. EMT occurs during gastrulation, one of the earliest developmental events that change the two layered embryonic disc into a three layered embryo. This process involves the invagination of the top layer (epiblast) cells to form the mesoderm⁴⁹ and endoderm germ layers.^{50,51} In addition several other developmental processes such as sclerotome⁵² and cardiac cushion mesenchyme⁵³⁻⁵⁵ development require EMT. In contrast, mesenchymal-epithelial transformations occur in the formation of somites, kidneys, and caudal or secondary neural tube.⁵⁶ This review will concentrate on EMT during the development of craniofacial structures, and specifically on cranial neural crest (CNC) and secondary palate formation.

Cranial Neural Crest

Before the closure of the neural folds in the mammalian head, the neural crest cells break away from an embryonic epithelial layer of the dorsal neural tube by changing their shape and properties from neuroepithelial cells to mesenchymal cells. In a recent meeting celebrating the contributions of James A. Weston to the understanding of neural crest, Weston proposed that the CNC were derived from an early population of non-neuronal ectoderm.^{57,58} While this controversial theory is certain to engage many investigators in the next few years, this review will concentrate on the current theory that CNC are derived from neural epithelium. However, as both ectoderm and neuroepithelium are epithelial cell types, they still have to complete EMT. A recent issue of *Developmental Dynamics* is dedicated to this topic as a special issue subtitled "Special Focus on the Neural Crest and the Contributions of James A. Weston."⁵⁹

A major difference between neural crest cells in the craniofacial region 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 preprogrammed.⁶⁰ In the cranial region, the CNC migrate in diffuse streams throughout the cranial mesenchyme, with a level-specific instruction, to reach their final destinations. These cells have also been referred to as ectomesenchyme in some textbooks.⁶¹ Extensive experiments demonstrated that the maintenance of this segmental characteristic is very important in patterning of head development.⁶²⁻⁶⁴

The CNC cells are multipotent stem-like cells, which respond to temporal-spatially expressed signals and become "committed." Candidate regulators include growth factors—members of the TGF- β family,⁶⁵ fibroblast growth factors

Table 1 Transcription Factors

Cbfa 1	Core-binding factor a1, controls the differentiation of mesenchymal cells into osteoblasts
Dax 1	Nuclear hormone receptor family expressed in the early indifferent gonad
Dlx	Distalless-paired patterning genes (6 members) that closely associated with Hox genes and are involved in morphogenesis of the jaw and inner ear
E12	Transcription activator for MyoD
Egr-1	Early growth response-1, found in bud stage tooth mesenchyme
Emx-2	Homeobox gene that is necessary for corpus collosum formation
En-1 -2	Engrailed-1 and -2, with Pax-1 and -2 are critical for organizing the development of the midbrain and cerebellum on both sides of the isthmus organizer
Eya-1-2	Eyes absent-1, -2—expressed in lens placode, require Pax-6 for expression
GATA-4	Is important in early heart development
Gbx2	Gastrulation brain homeobox 2—expressed in the hindbrain to form midbrain-hindbrain border, necessary for the formation of rhombomeres 1 and 3
Gli-1-3	Zinc finger transcription factors regulating morphogenesis; Gli-1 is released from microtubule complex downstream of shh, whereas Gli-3 is anterior and suppresses shh
Goosecoid	Homeodomain protein expressed in the organizer region of the primitive node; activates chordin, noggin and other genes in the organizer region
HNF-3	Hepatic nuclear factor 3—expressed in early foregut with GATA-4
HNF-3 β	Hepatic nuclear factor 3 β —expressed in the early organizer region with goosecoid and chordin
Hox a-d	Homeobox—containing a-d—patterning genes found in clusters on four chromosomes— necessary in the craniocaudal segmentation of the body
Id	Inhibitor of DNA binding that can form a heterodimer with MyoD and block MyoD dimerization
Krox-20	A segmentation gene that guides the formation of rhombomeres 3 and 5, kreisler and Hoxa-1 are involved in formation of rhombomere 5
Lbx-1	Lim-type homeobox containing genes
Lef-1	Lymphoid enhancer factor-1, involved in Wnt- β -catenin pathway during epithelial-mesenchymal transformation
Lbx-3-4	Expressed in Rathke's primordium with Rpx to form Rathke's pouch
Lim-1	Homeobox containing transcription factor necessary for head development
Lmx-1	Found in limb bud mesoderm—responds to limb ectoderm signaling molecule, Wnt-7a to induce dorsal characteristics; ventral ectoderm produces En-1 to suppress Wnt-7a and Lmx-1
MEF-2	Myocyte enhancer factor-2—important in early heart development
MFH-1	Mesenchyme forkhead-1-winged helix transcription factor—deficiency in mice leads to interruption of the left aortic arch
Msx-1	Expressed in rapidly proliferating mesenchyme at tips of facial primordium, limb buds, and dental lamina, Msx-1 and -2 expressed in tooth bud stage mesenchyme
MyoD	During muscle development myogenic regulatory factors are expressed in sequence (Myf-5, Pax-3, MyoD, myogenin MRF-4)
Nkx2-5	Important in early heart development
Oct-3-4	Homeodomain genes of the POU family; Oct-4 is important in early cleavage stages and is in all blastomeres up to the morula stage
Otx-1-2	Orthodenticle homologue—controls forebrain/midbrain formation, Otx-2 characterizes precursors of 1st arch
Paraxis	Helix-loop-helix transcription factor in somites that mediate mesenchyme to epithelium transformation
Pax-1-9	Paired box-1 to -9—contain a paired domain and entire or partial homeobox domains; Pax genes are important for sense organs and nervous system development; in addition they are involved in tissues that use epithelial-mesenchymal transition in development processes
Pdx-1	Pancreatic duodenal homeobox-1 expressed in pancreatic progenitor cells with Hlx-9
Pit-1	Pituitary-1, member of POU gene family expressed in the pituitary
Pitx-1	Expressed in hindlimb development upstream of Tbx-4; Pitx-2 is expressed in oropharyngeal membrane early in development
Rpx	Rathke's pouch homeobox-containing gene is expressed in Rathke's pouch primordium with Lbx-3 and Lbx-4, stimulated by BMP-4 and FGF-8
SF-1	Steroidogenic factor-1 is expressed in the early indifferent gonad, developing adrenal cortex, pituitary, and hypothalamus
SIP-1	Zinc finger protein that recognizes E-box motifs and found on cells transforming from epithelial to mesenchyme (EMT) and represses E-cadherin

Table 1 Continued

Slug	Zinc finger protein expressed in epiblast cells during gastrulation and neural crest cells at the neural plate stage of development; the cells that express slug transform from epithelial to mesenchymal phenotypes
Snail	Zinc finger protein that recognizes E-box motifs and found on cells transforming from epithelial to mesenchyme (EMT) and represses E-cadherin; closely related to slug
Sox	Large family (over 20) that have a common high-mobility group (HMG) domain that binds to 7 nucleotides on the minor groove of the DNA helix; Sox proteins work with other transcription factors in a large variety of tissues; Sox-9 controls differentiation of mesenchymal cells into precartilag
Sry	Sex-determining region, a member of the Sox gene family found on the Y-chromosome, triggers testis development by inhibiting Dax-1
Tbx-4-5	T-box family, -5 expressed in forelimb; -4 expressed in hind limb with Pitx-1, Tbx-5 also expressed in the dorsal retina
Twist	Basic helix-loop-helix protein that is a regulator of morphogenesis that plays an essential role in EMT including mesoderm development during gastrulation by repressing E-cadherin
Vax-2	Ventral anterior homeobox-2—expressed in the ventral retina
Wt-1	Wilms' tumor suppressor gene-1—expressed in mesonephric ducts—regulates transformation from mesenchyme to epithelium
ZFY	Zinc finger Y

*Compiled from various sources including Carlson.¹³⁸

(FGFs),^{66,67} platelet-derived growth factor (PDGF),⁶⁸ and Wnt gene products^{69,70} (Table 2). The role of the TGF- β family in CNC developmental processes has recently been reviewed.⁷¹ The involvement of several signal transduction molecules and transcription factors have also been reported.⁴⁴

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 upper jaw derives from the forebrain and midbrain, while that of the lower jaw from the midbrain and hindbrain (rhombomeres 1 and 2). These CNC cells mainly contribute 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 removal of the transmembrane signaling receptor (T β RII) gene in only the cranial neural crest lineage resulted in clefting of secondary palate and calvaria defects. The pathogenesis of cleft palate in these mice appears to be related to impairment of cell proliferation.⁷² The normal formation of some derivatives of the first branchial arch, such as palate and lip, needs epithelial-mesenchymal transformation during embryonic remodeling.

Regulation of EMT

Growth factors and signal transduction. The regulation of EMT is critical during dynamic developmental processes and postnatal homeostasis. Hay (1989) postulated that master gene(s) turned on in epithelia by changes in the environment initiate EMT (master gene theory) (Fig 2). Recently several molecules have been identified as possible master genes including the transcription factors Twist, Snail or SIP1 (Table 1). The changes in the environment that may initiate EMT include growth factors, cell adhesion molecules, extracellular matrix, the surface receptors and downstream signal trans-

duction events (Table 2), and transcription factors (Table 1). Epithelial cells are characterized by cell-cell adhesions (adherens junctions, desmosomes, and hemidesmosomes) and an intact basal lamina. During EMT the cells lose expression of cell adhesion proteins, especially E-cadherin, and increase expression of enzymes that break down the basal lamina (Fig 2B).

This review will focus on TGF- β and its downstream signaling molecules primarily during palate development. The TGF- β superfamily includes many small proteins that are multifunctional (controlling growth, migration, and differentiation) during both embryonic development and postnatal tissue homeostasis.⁷³⁻⁷⁵ The cellular responses to TGF- β during craniofacial development and CNC regulation have been extensively studied and recently reviewed.⁷¹

Studies designed to investigate how cells interpret TGF- β signals have identified three types of TGF- β transmembrane signaling receptors: type I, type II (T β RI and T β RII),⁷⁶ and T β RIII (Fig 1C). Types I and II TGF- β receptors have a cytoplasmic serine/threonine kinase domain. TGF- β s initiate signaling by assembling receptor complexes. The initial binding of T β RII to ligand is recognized by T β RI and the formation of ligand/T β RI/T β RII/complex results in the activation of T β RI by T β RII.⁷⁷

Activated T β RI propagates the signal by phosphorylating many downstream effectors, such as Smad proteins (Fig 1C).⁷⁸ The Smad proteins are homologues to the *Drosophila* “mothers against dpp” (mad) proteins and the *Caenorhabditis elegans* Sma proteins. In vertebrates at least nine genes have been found that comprise the Smad family. Different members of the Smad family have different signaling roles. Smads 1, 2, 3, and 5 interact with the receptors and are termed R-Smads. These proteins become phosphorylated by the activated receptors and are transported to the nucleus.^{71,79-81} However, when there are no outside-in signals, R-Smads stay in the cytoplasm partly by binding to the protein SARA

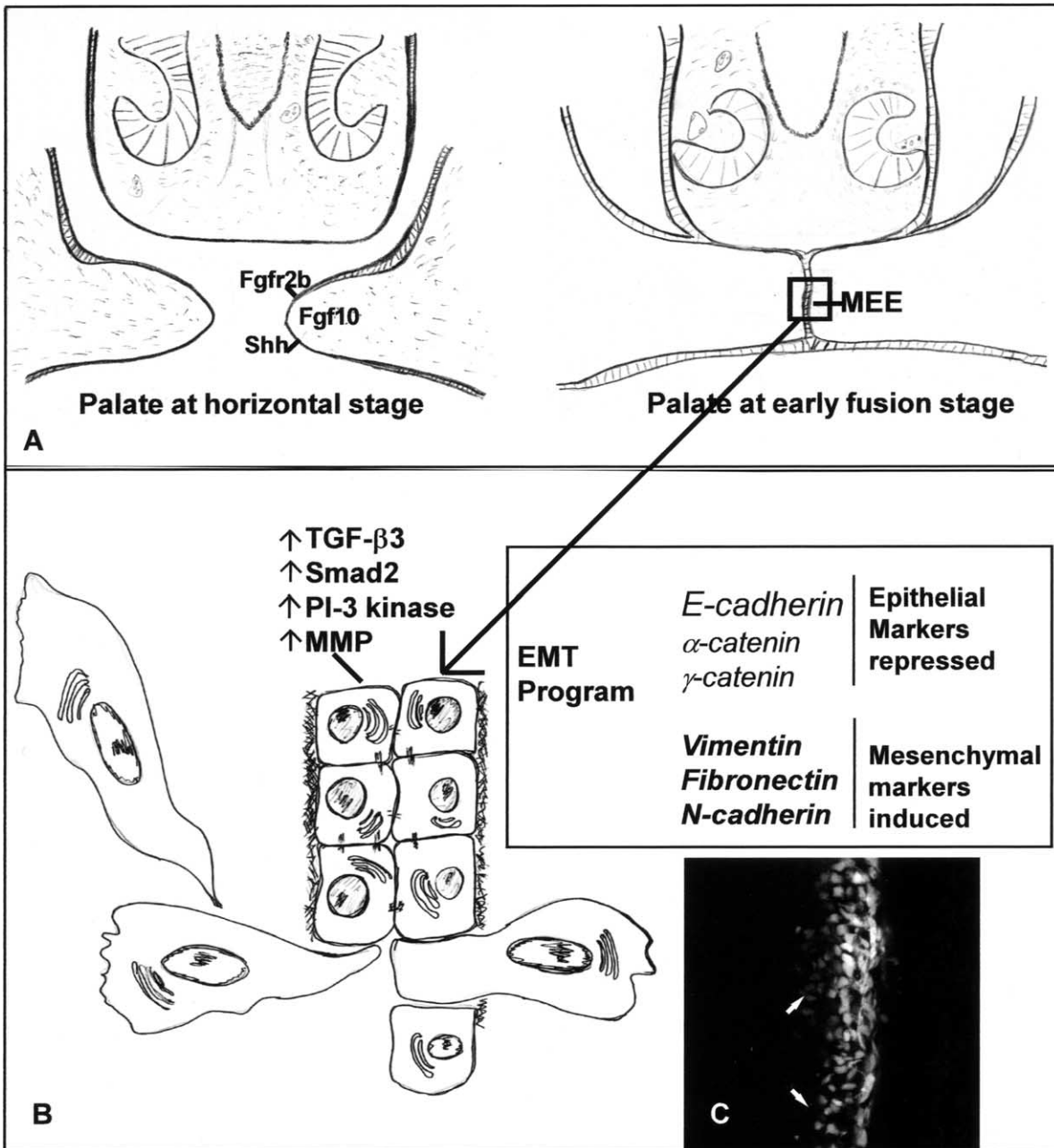


Figure 2 (A) The palatal shelves grow from the maxillary prominence as illustrated in the horizontal and early fusion stages. The outgrowth of the palatal shelves is stimulated by Fgf10 in the mesenchyme that stimulates the Fgfr2b in the epithelium and increases shh that signals back to the mesenchyme.^{64,72} When the palatal shelves meet in the midline, the medial edge epithelial cells (MEE) disappear.⁷² The surface periderm cells progress through programmed cell death (apoptosis), and some basal cells migrate to the oral or nasal epithelium, but other cells change phenotypes and become mesenchymal through a regulated program. (B) The master gene theory proposes that a regulator causes the down regulation of epithelial genes, particularly E-cadherin, and the upregulation of mesenchymal genes such as vimentin, fibronectin and N-cadherin. The cells also degrade the basal lamina which includes type IV collagen and laminin by increasing matrix metalloproteinases (MMP). (C) The change in phenotype can be visualized by labeling the epithelial cells with a fluorescent marker (CCFSE) and then culturing the palatal shelves for 3 days. The labeled marker was visualized in single confocal optical sections in the mesenchymal region where the palatal shelves had recently fused (C, arrows).⁴⁶

(Smad Anchor for Receptor Activation).⁸² Receptor phosphorylation of R-Smads weakens the affinity of R-Smads for SARA and exposes their nuclear import signal. Activation of R-Smads also increases their affinity to Smad4 (Co-

Smad).^{83,84} As a result, the Smad complex translocates into the nucleus, where Smads interact with transcription factors.

The Smad signaling pathway is under the control of many regulatory agents including: the transmembrane protein,

BAMBI, that has an intracellular domain that mimics the structure of a type I receptor and prevents the formation of receptor complexes⁸⁵; the ubiquitin ligase, Smurf1⁸⁶; the antagonistic Smad6 and Smad7,^{87,88} and the oncoproteins Ski/Sno.⁸⁹ In addition, in the nucleus the cellular responses to other signal inputs determine which genes will be recognized by the Smad complex.⁹⁰ Recently the interferon regulatory factor 6 (IRF6) gene was identified as the candidate for the cause of an autosomal dominant form of cleft lip and palate, Van der Woude's syndrome.⁹¹ However, it is not clear how the point mutation in this gene contributes to cleft lip or palate.

Although the Smad pathway has received much attention in the past 5 years, it is now appreciated that the TGF- β -activated receptor complex can also signal through other pathways,⁹² such as those involving the mitogen-activated protein kinases (MAPKs),^{93,94} phosphoinositol-3 kinase (PI-3 kinase) (Fig 1C),^{95,96} and PP2A/p70s6K, though the molecular details of this coupling are still obscure. The relative importance and interplay of these various pathways in the changing responses of cells to TGF- β are just beginning to be probed.

TGF- β family members are essential for EMT during development. The embryonic cardiac cushion has been extensively studied in an organ culture model that demonstrated TGF- β 2-mediated initial endothelial cell-cell separation while TGF- β 3 was required for the cell morphological change that enabled the migration of cells into the underlying extracellular matrix.⁹⁷ These conclusions were based on experiments that used antisense oligodeoxynucleotides or neutralizing antibodies to TGF- β 3 to inhibit EMT in vitro.⁹⁷

Palate development. As shown in Fig 2, the secondary palate forms as an outgrowth of the maxillary prominence. Interestingly, it was recently shown that sonic hedgehog (shh) and the Fgfr2b were also expressed in the early palatal epithelium⁹⁸ and appear to be induced by Fgf10. When this pathway was disrupted in transgenic animals, the palatal processes failed to grow.⁹⁸ The normal palatal shelves elevate and grow toward the midline where they fuse and some of the medial edge epithelial (MEE) cells move into the mesenchyme through the EMT process (Fig 2). The migrating MEE cells can be visualized if the epithelial cells are bathed in a marker that only penetrates the surface epithelium such as carboxy 2,7'-dichlorofluorescein diacetate succinimidyl ester (CCFSE). After the palatal shelves have been organ cultured for 3 days, the label was found in the mesenchymal cells (Fig 2C) demonstrating the labeled cells had progressed through EMT.⁹⁹

During mammalian palate development TGF- β isoforms 1, 2, 3, T β RII, and T β RIII were detected in the medial edge epithelium (MEE) by in situ hybridization¹⁰⁰ and immunohistochemistry.^{101,102} Of particular interest was the highly localized expression of TGF- β 3 RNA, and to a lesser extent that of TGF- β 1 and TGF- β 2 in the MEE and the nasal septum epithelium, which were destined to undergo EMT.¹⁰⁰ Experiments using antisense oligodeoxynucleotides or neutralizing antibodies to TGF- β 3, but not TGF- β 1 or TGF- β 2, resulted

in the failure of palate fusion in vitro.¹⁰³ Further experiments demonstrated that Tgf- β 3 transgenic and knockout mice have cleft palate as their only craniofacial birth defect.^{104,105} When palatal shelves from Tgf- β 3 knockout mice were cultured, the midline epithelia failed to go through EMT.^{106,107} However, overexpressing Smad2 on the Tgf- β 3 null animal partially rescued palatal fusion.¹⁰⁸ In addition, although the chicken has naturally open palate, the cultured chicken palatal shelves fused when TGF- β 3 was added into the medium.¹⁰⁹

It is clear from these experiments that TGF- β 3 is an essential growth factor inducing EMT during palatal fusion. 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 before shelf contact.¹⁰⁷ Second, the regulation of extracellular matrix degradation by modulating the production of tissue inhibitor of metalloproteinase-2 (TIMP-2), MMP13, and MMP2 has been proposed.¹¹⁰ More recently, it was found that TGF- β 3 is necessary for inhibiting MEE proliferation during EMT.¹¹¹

Several research groups have started to investigate the TGF- β 3-stimulated intracellular signaling molecules that are responsible for EMT during palate fusion. Smad2 expression was detected during palatal fusion,¹¹² and it has been suggested that phosphorylation of Smad2 may be necessary for Tgf- β 3 downregulation of MEE proliferation.¹¹¹ Interestingly, overexpression of Smad2 in the TGF- β 3^{-/-} mouse did not completely rescue secondary palate clefts. There is also evidence from the studies of mammary epithelial cell culture that downregulation of Smad signaling decreased Smad-dependent growth and transcriptional responses; however, the downregulation did not affect TGF- β -mediated stress fiber formation and EMT.¹¹³

Understanding the signal transduction pathways can be complicated. For example, we have recently investigated the role of TGF- β signaling during secondary palate formation in a mouse model (Fig 3). We found that an alternative downstream effector to MEE signaling, PI-3 kinase, has been identified as an effector in actin reorganization and TGF- β -mediated EMT.¹¹⁴⁻¹¹⁶ 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 (Fig 1C). Along with the small GTPases Rac and Rho, PIP3 activates several serine/threonine kinases such as 3-phosphoinositide-dependent protein kinases (PDKs).^{117,118} PDK1 activates PKC, p70S6K1,¹¹⁹ and targets Thr308 on protein kinase B (PKB, also known as Akt),¹²⁰ while integrin-linked kinase (ILK), a newly found PDK activates Akt on its Ser473 site.¹²¹ On stimulation, Akt migrates and anchors to the membrane.¹²² Subsequently, activated Akt detaches from the plasma membrane and translocates into the cytoplasm and nucleus, regulating cell survival, protein synthesis, and cell cycle.¹²³ It also appears that PI-3 kinase possesses both lipid and protein kinase activity¹²⁴ 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-medi-

Table 2 Signal Transduction Proteins, Abbreviations, and Definitions

Activin	Signaling protein, member of the TGF- β family, active in mesoderm induction
Angiopoietin-1	Sprouting factor—interacts with Tie-2 (receptor) during angiogenesis
BAMBI	Transmembrane protein, whose intracellular domain resembles the homodimerization interface of a T β RI receptor and prevents the formation of TGF- β receptor complexes
bax	bcl-associated x protein that accelerates cell death (apoptosis)
β -catenin	Binds to E-cadherin in cell-cell junctions, but can be modulated by many signaling agents (Wnt, Ras, and PI-3 kinase) to relocate to the nucleus and interacts with transcription factors (TCF/LEF-1); stabilized nuclear β -catenin has been shown to induce EMT in vitro ¹³³
bcl-2	B cell lymphoma protein that blocks cell death (apoptosis)
BMP-1-9	Bone morphogenetic protein, members of the TGF- β family—induction of the neural plate, skeletal differentiation, and other inductions
CAM	Cell adhesion molecules (neuronal CAM [N-CAM], L-CAM = E-cadherin)
cAMP	Cyclic AMP, second messenger in GPCR signal transduction pathways
cAPKs	Cyclic AMP-dependent kinases also called protein kinase A (PKA)
Cereberus	Signaling factor, Lim-1 and Cereberus-related 1 null mice are headless
cGMP	Cyclic GMP
Chordin	Signaling molecule active in very early development including primitive streak formation, expressed with nodal, cripto, and Vgl; also part of the primitive node (organizer)
Cripto	See chordin description
Cyclops	Signaling molecule expressed in optic primordia to separate optic fields
COL	Collagen—extracellular matrix protein; over 25 members of the family have been identified; cell surface receptors for collagen are integrins
DAG	Diacylglycerol, second messenger for GPCRs
Dia	p140 Diaphanous, substrate for RhoGTP, promotes actin polymerization
dpp	Decapentaplegic-TGF- β family, signaling in limb development
E-cadherin	Cell adhesion molecule found in adherens junctions
ECM	Extracellular matrix includes collagen, fibronectin, proteoglycans, laminin, etc
EGF	Epidermal growth factor
erk	Extracellular signal regulated protein found in the MAP kinase signaling cascade
ET-1	Endothelin-1, signaling molecule secreted by coronary arteries to stimulate conduction system development
FAK	Focal adhesion kinase, associated with integrin receptors and signaling
FGF 1-10,	Fibroblast growth factor 1-10, signaling molecules expressed throughout development; associated with syndecan and FGF receptors I-III
Follistatin	Signaling molecule that works with noggin and chordin (from notochord) to block BMP-4 to induce the formation of the nervous system
GAP	GTPase activating protein in the Rho, Ras Cdc42 protein pathways
Gdf-5	Growth/differentiation factor-5, member of BMP family, active in joint formation downstream of Wnt-14
GDI	Guanine-nucleotide dissociation inhibitor, sequesters RhoGDP and keeps it in an inactive form
GDNF	Glial cell-derived neurotrophic factor, stimulates ureteric bud outgrowth in metanephrogenic blastema
GEF	Guanine exchange factors, convert Rho family proteins from GDP to GTP state
GPCR	G protein-coupled receptors, general description of receptors that require G proteins for propagation of the signal—see glossary.
grb2	Growth factor receptor-bound protein 2, adapter protein for growth factor receptors
HGF	Hepatic growth factor (also scatter factor)
ICE	Interleukin-1 Converting Enzyme
IGF	Insulin growth factor
Ihh	Indian hedge hog, signaling molecule in the sonic hedgehog family
Inhibin	Inhibition of gonadotropin secretion by hypophysis
Integrins	ECM receptors- α and β heterodimers—see glossary
ILK	Integrin-linked kinase
JNK/SAPK	Jun N-terminal kinase also known as SAPK, stress-activated protein kinase
Kinase	Enzymes that phosphorylate other proteins
Lefty	TGF- β family, determination of body asymmetry
LIF	Leukemia inhibitory factor
MAP kinase	Mitogen-activated protein kinase—see glossary
MAPKK	Mitogen-activated protein kinase kinase also known as MEK
MAPKKK	Mitogen-activated kinase-kinase-kinase or MEKK
MIS	Mullerian inhibitory substance, TGF- β family, regression of paramesonephric duct
MMP	Matrix metalloproteinase, large family of enzymes that digest ECM proteins

Table 2 Continued

NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
Nodal	TGF- β family, formation of mesoderm and primitive streak, left-right axial fixation
Noggin	Signaling molecule
Notch	Cell surface receptor activated by Delta or Jagged that inhibits neighboring cell from differentiating into the dominant phenotype; one mechanism used to produce glial cells instead of neurons
NT-3	Neurotrophin 3, member of the nerve growth factor family, necessary for neural crest migration especially in the cardiac outflow tract
PDGF	Platelet-derived growth factor
PDK	3-Phosphoinositide-dependent protein kinases
PKB	Protein kinase B also known as Akt
PKC	Protein kinase C
Ptc	Patched receptor for Shh that inhibits Smo unless bound to Shh—See Shh
Phosphatase	Enzyme that dephosphorylates proteins
PI ₃ kinase	Phosphatidylinositol 3 kinase
PIP2	Phosphatidylinositol (4, 5)-bisphosphate
RA	Retinoic acid
r-Fng	Radical fringe-expressed in the apical ectodermal ridge during early limb development
SARA	Smad anchor for receptor activation, TGF- β signaling
ROCK	Rho associated coiled-coil containing protein kinase also known as p160 Rho kinase
Shh	Sonic hedgehog, signaling molecule that binds to the receptor Ptc (patched) that releases the inhibitory effect of Ptc on Smo; active in primitive node, notochord, floor plate, intestinal portals, zone of polarizing activity, hair and feather buds, ectodermal tips of facial processes, apical ectoderm of 2nd arch, tips of epithelial lung buds, retina, genital tubercle
Smo	Smoothed, integral membrane protein, that activates Gli, a transcription factor
Smad	sma and mad homology signal proteins renamed 1996, act in the TGF- β pathway
Src	A kinase that was first described in the Rous sarcoma virus; however, it is also found in normal cells
TGF- β 1-3	Transforming growth factor, large family of growth factors
T β RI, II, III	Transforming growth factor receptors
TIMP	Tissue inhibitors or metalloproteinases
VEG-f	Vascular endothelial growth factor
Wnt-1	Homologous to Wingless in <i>Drosophila</i> —found in neural ectoderm anterior to isthmic organizer

*Various references including Lodish and colleagues² and Carlson.¹³⁸

ated apoptosis, cell migration, and alterations in cell-cell adhesion.¹²⁵ PI-3 kinase has key regulatory functions and is involved in the development of cancer. The control of PI-3 kinase activity is, therefore, essential for homeostasis.

Many of these cellular changes require the reorganization of actin cytoskeleton. As a downstream effector of TGF- β signaling, PI-3 kinase is involved in actin reorganization, metalloproteinases (MMP) production, and cell mobility.^{114,115} It was demonstrated that LY294002, a specific inhibitor of PI-3 kinase, completely blocked TGF- β -mediated C-terminal phosphorylation of Smad2, cell migration, and partially blocked EMT in mammary epithelial cell culture.¹¹⁶ Since MMPs are necessary for breaking down basement membrane, it was speculated that blocking PI-3 kinase activity inhibited cell migration and MMP production, which are essential processes during 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.^{126,127} ILK was also implicated in TGF- β -induced fibroblastic conversion of highly metastatic cells.¹²⁸

A recent investigation in our laboratory supported the theory that PI-3 kinase was necessary for EMT during secondary palatal development in vitro (E13.5-E16.5).¹²⁹ We demonstrated that the PI-3 kinase inhibitor, LY294002, decreased the mean palatal

fusion score and blocked basal lamina degradation. Control palates fused (Fig 3A, A') and the MEE progressed through EMT while the basal lamina degraded (Fig 3C, C') in culture. Palatal shelves treated with LY294002 had MEE cells in the midline (Fig 3B, B') and the basal lamina still contained laminin (Fig 3D, D') and therefore was not degraded. However, it was possible that inhibiting PI-3 kinase delayed but did not completely block EMT as some of the palates were partially fused¹²⁹ (Fig 3, graph of mean fusion score). Although there is evidence that PI-3 kinase may be downstream of TGF- β , it is also downstream of other growth factors and integrin receptors as mentioned previously.

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.¹³⁰ A later study by the same group transiently transfected epithelia with Smad2/4 or Smad3/4 expression vectors but did not alter cell phenotype.¹³¹ 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

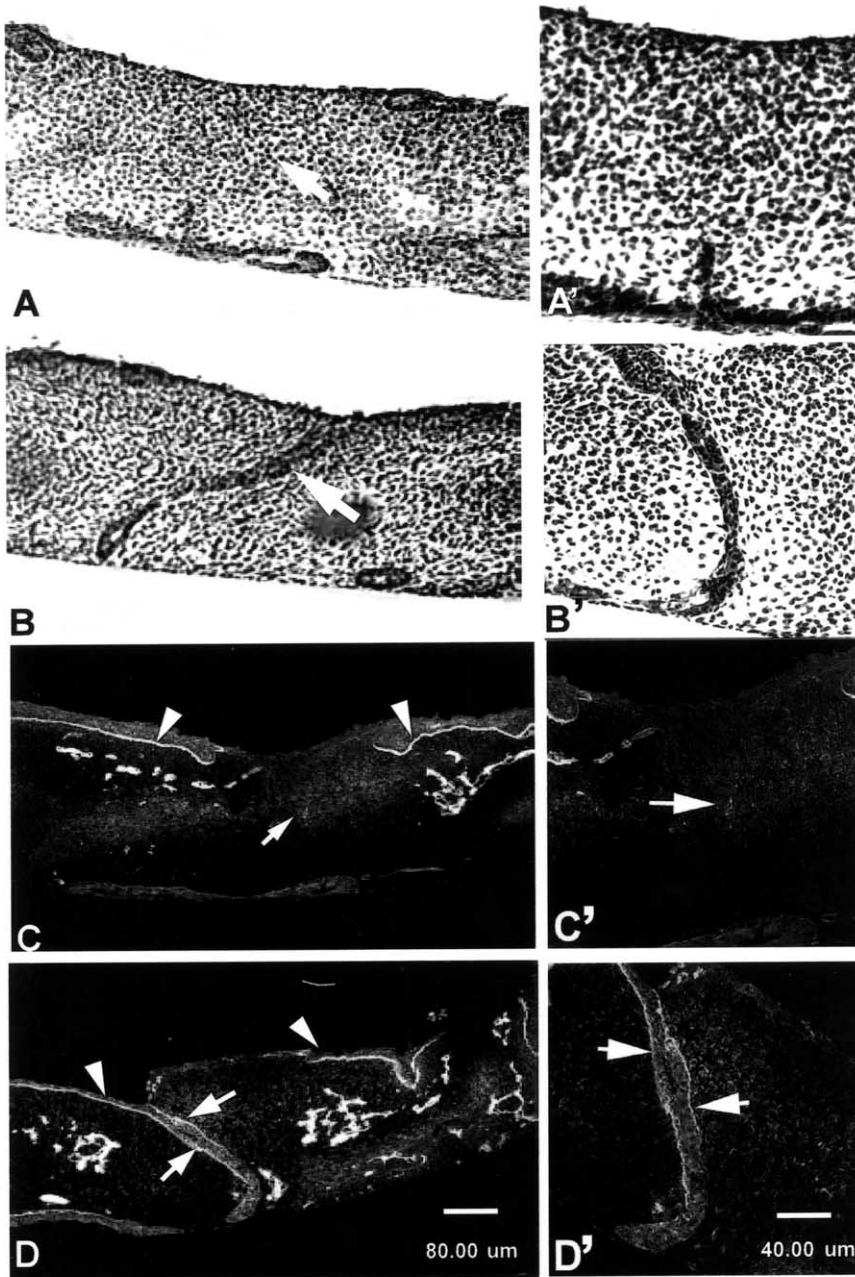
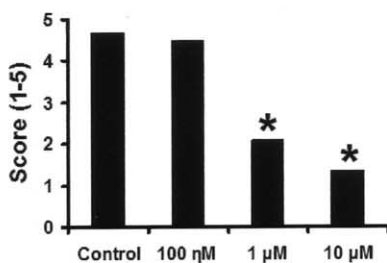


Figure 3 Individual sections of hematoxylin and eosin (H&E; A, A', B, B') and laminin stained (C, C', D, D') from palatal tissues after 72 hours in control (A, C) or 1 μM LY294002-treated (B, D) medium. The palate completely fused in control medium (A, A'). No epithelia were observed in the midline. In the presence of 1 μM LY294002, medial edge epithelia persisted (arrows in B, B'). Laminin was observed beneath the oral surface epithelia in all groups (arrowheads in C, D) and in the mesenchymal areas that may be developing blood vessels. However, laminin was negative in the midline of the control-cultured palate (arrows in C, C'), indicating that the basal lamina had completely degraded in the fused palate. In contrast, laminin was detected in the midline of LY294002-treated groups (arrows in D, D') lateral to two layers of MEE. Scale bar = 80 μm in D (applies to A–D), 40 μm in D' (applies to A'–D').

Mean Palatal Fusion Score



stability and localization, which can be modulated by many signaling agents such as Wnt, Ras, and PI-3 kinase.^{42,132,133} On release from the complex, β -catenin relocates into the nucleus and interacts with transcription factors such as TCF/LEF-1. Sta-

bilized nuclear β -catenin has been shown to induce EMT in vitro.¹³⁴

In addition to changes in growth factors, the modulation of extracellular environment also includes the maintenance and

degradation of ECM, which is mediated in part by metalloproteinases (MMPs) and tissue inhibitors or metalloproteinases (TIMPs). Temporospatial expression of MMPs 2, 3, 7, 9, and 13, and TIMPs 1 and 2 were observed during murine palatal fusion.^{135,136} In the palatal fusion zone of TGF- β -deficient mice, TIMP-2 was completely absent; MMP-2 and MMP-13 had reduced levels.¹¹⁰ On exposure to MMP inhibitor (BB 3103), the murine palatal shelves failed to fuse in culture.¹³⁷

Conclusions and Future Directions

Craniofacial growth and development is a complex and closely regulated process. In recent years, it has become apparent that the interaction between the numerous growth regulatory factors and the affected cells and tissues of the developing human are under tight temporal and spatial regulation.¹³⁸ While a great deal of information has been gained since the first discoveries of growth factors, further studies are required to determine how these factors can be manipulated within a clinical environment to augment treatment. Naturally occurring endogenous signals for craniofacial development are potential therapeutic agents for treatment of various craniofacial anomalies. However, effective and acceptable therapeutic agents with this property are lacking and still remain in the initial stages of exploration.

The actions of growth factors are very complex, with each growth factor having both multiple and different effects on various tissues, as well as the interactions each factor may have on each other. Yet to be determined are the complexities of these effects and how they may be affected by concentration, duration of exposure to the factor, and the developmental stage of the cells or tissue treated. Before growth factor therapy can be effective in clinical settings, a variety of questions will need to be answered, including safety issues, dosage and treatment regimens, temporal concerns, and proper vehicles with which delivery of growth factors to their intended site of action can be achieved. Many growth factors require a prolonged exposure to generate a response, making the choice of delivery vehicle of critical importance.

Despite the limits of current technology, the application of growth factors in craniofacial growth and development shows considerable promise. It is likely that combinations of growth factors may prove useful in some clinical circumstances. The vast potential for growth factors to enhance the treatment of various craniofacial anomalies should not be ignored but, instead, needs further investigation. We are clearly on the threshold of a new era in developing treatment regimens, in which we will be able to regulate the processes governing both normal and abnormal growth and development and perhaps ultimately understand the complexities involved in craniofacial development.

Glossary for Signal Transduction

Receptor Types

G protein-coupled receptors (GPCR): The largest family of receptor molecules. These receptors are characterized by multiple transmembrane domains (usually seven) that wind the protein in and out of the membrane in a serpentine conformation.

Examples include: muscarinic acetylcholine receptors, nicotinic acetylcholine receptors, rhodopsin, β -adrenergic receptor.

Ion-channel receptors: The ion-channel receptors are closely related to GPCRs and actually open a membrane channel when the ligand binds.

Tyrosine kinase receptors: These receptors usually have one transmembrane domain and at least two molecules must dimerize to activate the signal. Examples include: EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; IGFR, insulin growth factor receptor; T β RI and T β RII, TGF- β receptors I and II; FGF, fibroblast growth factor.

Tyrosine kinase-linked class of receptors: These receptors lack intrinsic activity, but when the ligand binds, intracellular tyrosine kinases become activated to generate cellular changes.

Extracellular matrix-associated receptors: Integrin receptors and the syndecan family have single transmembrane domain with a large extracellular and a much smaller cytoplasmic domain. The syndecan molecules have long glycosaminoglycan chains that assist in sequestering the FGF close to the cell membrane.³⁻⁶ The integrin receptors are heterodimers composed of α and β subunits. The family is large with at least 25 integrin heterodimers, including 19 α subunits and 8 β subunits identified.⁷ The integrins do not have kinase activity, but on binding to ECM molecules, associated proteins become activated by autophosphorylation and then phosphorylate surrounding proteins to generate signals to the MAP kinase, Rho, or PI-3 kinase pathways.

Intracellular Signaling Molecules—2nd Messengers

General Definitions

Adapter proteins: Proteins with specific protein-protein domains that hold large multiprotein complexes together. Adapter proteins do not have catalytic activity and do not activate other proteins. They contain different domains that act as docking sites for other proteins such as domains for phosphotyrosine residues (SH2 and PTB domains), proline-rich (SH3 and WW domains), phosphoinositides (PH domains), and the unique sequence with the C-terminal hydrophobic residue (PDZ domains).

Kinase: The enzyme class that phosphorylates amino acids (serine, threonine, and tyrosine) by transferring a phosphoryl group from ATP or GTP to the protein with a specific consensus sequence.

Phosphatase: The enzyme that removes the phosphoryl group from a protein.

GPCR-associated second messenger proteins: $G\alpha\beta\gamma$ disassociate and activate the adenylate cyclase enzyme to produce cyclic AMP (cAMP), cyclic GMP (cGMP), diacylglycerol (DAG), phosphoinositols, and calcium (Ca^{2+}).

Ras-Raf-MAP kinase: The MAP kinase family can respond to a variety of extracellular signals including, osmotic stress, heat shock, cytokines, and mitogens. Upstream signaling leads to an activation of tyrosine kinases that activate G proteins (Ras, Rac, cdc42) via adapter proteins such as grb2 or mSOS1. The G proteins activate Raf, also known as mitogen-activated kinase-kinase-kinase (MAPKKK) or MEKK. Activated Raf stimulates (via serine/threonine phosphorylation) mitogen-activated kinase-kinase (MAPKK) or MEK. MEK stimulates (via tyrosine and threonine phosphorylation) mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein (erk). In the MEKK family, there are several Raf isoforms including raf-1, B-Raf, and A-Raf. There are at least 5 MEKs with MEK 1/2 acting specifically on erk1/2. There are now several MAPK branches including erk1/2, JNK/SAPK, p38, erk-3, and several p38-related homologues.

Phosphoinositol signaling pathway via PI-3 kinase: PI-3 kinase phosphorylates PIP (PI(4)phosphate) or PIP₂ (PI(4,5)bisphosphate) at the D3 position to generate, respectively, PI(3,4)P₂ or PI(3,4,5)P₃.¹³⁸ These phospholipid by-products have been implicated in downstream signaling of cytoskeletal reorganization through interactions with profilin, gelsolin, and Rac. PI-3 kinase signaling is necessary for EMT during palate development.

Small G proteins Rho, Rac, and Cdc42: Alternate between the GTP-bound active form (on) and the GDP-bound inactive form (off). Many other proteins regulate the "on" and "off" state of the small G proteins. The guanine-nucleotide exchange factors (GEFs) are the "on" signal as they add GTP to the protein. GTP-activating proteins (GAPs) are the "off" signal as they remove a phosphate to deactivate the protein. Guanine-nucleotide dissociation inhibitor proteins (GDIs) sequester the inactive protein in the cytoplasmic pool.^{1,12,20-23} Decreasing Rho protein levels or activity decreased other integrin signaling molecules.²⁴

References

- Sastry SK, Burridge K: Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. *Exp Cell Res* 261:25-36, 2000
- Lodish H, Berk A, Matsudaira P, et al: *Molecular Cell Biology*. 4th ed. New York, WH Freeman, 2004
- Rapraeger AC: Heparan sulfate-growth factor interactions. *Methods Cell Biol* 69:83-109, 2002
- McQuade KJ, Rapraeger AC: Syndecan-1 transmembrane and extracellular domains have unique and distinct roles in cell spreading. *J Biol Chem* 278:46607-46615, 2003
- Rapraeger AC, Ott VL: Molecular interactions of the syndecan core proteins [Review]. *Curr Opin Cell Biol* 10:620-628, 1998
- Rapraeger AC: Molecular interactions of syndecans during development [Review]. *Semin Cell Dev Biol* 12:107-116, 2001
- Humphries MJ: Integrin structure. *Biochem Soc Trans* 28:311-339, 2000
- Chapman HA, Wei Y, Simon DI, et al: Role of urokinase receptor and caveolin in regulation of integrin signaling. *Thromb Haemost* 82:291-297, 1999
- Giancotti FG, Ruoslahti E: Integrin signaling. *Science* 285:1028-1032, 1999
- Schwartz MA, Baron V: Interactions between mitogenic stimuli, or, a thousand and one connections. *Curr Opin Cell Biol* 11:197-202, 1999
- O'Neill GM, Fashena SJ, Golemis EA: Integrin signaling: a new Cas(t) of characters enters the stage. *Trends Cell Biol* 10:111-119, 2000
- Ridley A: Rho GTPases: integrating integrin signaling. *Cell Biol* 150: F107-F109, 2000
- Giancotti FG: A structural view of integrin activation and signaling. *Dev Cell* 4:149-151, 2003
- Katsumi A, Orr AW, Tzima E, et al: Integrins in mechanotransduction. *J Biol Chem* 279:12001-12004, 2004
- Yamada KM, Pankov R, Cukierman E: Dimensions and dynamics in integrin function. *Braz J Med Biol Res* 36:959-966, 2003
- Vinogradova O, Vaynberg J, Kong X, et al: Membrane-mediated structural transitions at the cytoplasmic face during integrin activation. *Proc Natl Acad Sci USA* 101:4094-4099, 2004
- Nuccitelli R (ed): *A Practical Guide to the Study of Calcium in Living Cells*. *Methods in Cell Biology*, Vol 40. San Diego, Academic Press, 1994
- Cary LA, Guan JL: Focal adhesion kinase in integrin-mediated signaling. *Front Biosci* 4:D102-D113, 1999
- Svoboda KKH, Orlow DL, Chu CL, et al: ECM stimulated actin bundle formation in embryonic corneal epithelia is tyrosine phosphorylation dependent. *Anat Rec* 254:348-359, 1999
- Settleman J: Getting in shape with Rho. *Nat Cell Biol* 2:E7-E9, 2000
- Schwartz MA, Shattil SJ: Signaling networks linking integrins and Rho family GTPases. *Trends Biochem Sci* 25:388-391, 2000
- Symons M, Settleman J: Rho family GTPases: more than simple switches. *Trends Cell Biol* 10:415-419, 2000
- Bokoch GM: Regulation of cell function by Rho family GTPases. *Immunol Res* 21:139-148, 2000
- Reenstra WR, Orlow DL, Svoboda KK: ECM-stimulated signaling and actin reorganization in embryonic corneal epithelia are Rho dependent. *Invest Ophthalmol Vis Sci* 43:3181-3189, 2002
- Turner CE: Paxillin and focal adhesion signalling. *Nat Cell Biol* 2:E231-E236, 2000
- Read PW, Liu X, Longenecker K, et al: Human RhoA/RhoGDI complex expressed in yeast: GTP exchange is sufficient for translocation of RhoA to liposomes. *Protein Sci* 9:376-386, 2000
- Michaelson D, Silletti J, Murphy G, et al: Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol* 152:111-126, 2001
- Roovers K, Assoian RK: Integrating the MAP kinase signal into the G1 phase cell cycle machinery. *Bioessays* 22:818-826, 2000
- Treisman R: Regulation of transcription by MAP kinase cascades. *Curr Opin Cell Biol* 8:205-215, 1996
- Kortenjann M, Shaw PE: The growing family of MAP kinases: regulation and specificity. *Crit Rev Oncog* 6:99-115, 1995
- Garrington TP, Johnson GL: Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol* 11: 211-218, 1999
- Ridley AJ, Schwartz MA, Burridge K, et al: Cell migration: integrating signals from front to back. *Science* 302:1704-1709, 2003
- Robinson MJ, Cobb MH: Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 9:180-186, 1997
- Schwartz MA: Integrin signaling revisited. *Trends Cell Biol* 11:466-470, 2001
- Giorgio L, Hemmati-Brivanlou A: A molecular basis for Smad specificity. *Dev Dynam* 214:269-277, 1999
- Schiffer M, von Gersdorff G, Bitzer M, et al: Smad proteins and transforming growth factor-beta signaling. *Kidney Int Suppl* 77:S45-S52, 2000
- Zimmerman CM, Padgett RW: Transforming growth factor beta signaling mediators and modulators. *Gene* 249:17-30, 2000
- Massague J: How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 1:169-178, 2000
- Porter JC, Hogg N: Integrins take partners: cross-talk between integrins and other membrane receptors. *Trends Cell Biol* 8:390-396, 1998
- Ossowski L, Aguirre-Ghiso JA: Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr Opin Cell Biol* 12:613-620, 2000

41. Boudreau N, Bissell MJ: Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr Opin Cell Biol* 10:640-646, 1998
42. Hay ED: The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. *Develop Dynam* 233: 706-720, 2005
43. Hay ED, Zuk A: Transformations between epithelium and mesenchyme: normal, pathological, and experimentally induced. *Am J Kidney Dis* 26:678-690, 1995
44. Duband JL, Monier F, Delannet M, et al: Epithelium-mesenchyme transition during neural crest development. *Acta Anat* 154:63-78, 1995
45. Guarino M, Micheli P, Pallotti F, et al: Pathological relevance of epithelial and mesenchymal phenotype plasticity. *Pathol Res Pract* 195: 379-389, 1999
46. Kang P, Svoboda KKH: Epithelial-mesenchymal transformation during craniofacial development. *J Dent Res* 84:678-690, 2005
47. Nawshad A, LaGamba D, Hay ED: Transforming growth factor beta (TGFbeta) signalling in palatal growth, apoptosis and epithelial mesenchymal transformation (EMT). *Arch Oral Biol* 49:675-689, 2004
48. Shuler CF: Programmed cell death and cell transformation in craniofacial development. *Crit Rev Oral Biol Med* 6:202-217, 1995
49. Sanders EJ, Prasad S: Invasion of a basement membrane matrix by chick embryo primitive streak cells in vitro. *J Cell Sci* 92(Pt 3):497-504, 1989
50. Lawson A, Schoenwolf GC: Epiblast and primitive-streak origins of the endoderm in the gastrulating chick embryo. *Development* 130: 3491-3501, 2003
51. Chapman SC, Schubert FR, Schoenwolf GC, et al: Anterior identity is established in chick epiblast by hypoblast and anterior definitive endoderm. *Development* 130:5091-5101, 2003
52. Solursh M, Fisher M, Meier S, et al: The role of extracellular matrix in the formation of the sclerotome. *J Embryol Exp Morphol* 54:75-98, 1979
53. Runyan RB, Markwald RR: Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. *Dev Biol* 95:108-114, 1983
54. Potts JD, Runyan RB: Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta. *Dev Biol* 134:392-401, 1989
55. Boyer AS, Erickson CP, Runyan RB: Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGFbeta signal transduction mechanisms. *Dev Dyn* 214:81-91, 1999
56. Griffith CM, Wiley MJ, Sanders EJ: The vertebrate tail bud: three germ layers from one tissue. *Anat Embryol (Berl)* 185:101-113, 1992
57. Erickson CA: James A. Weston and the JAWsfest: a celebration of his contributions to our understanding of the neural crest. *Dev Dyn* 229:2-4, 2004
58. Weston JA, Yoshida H, Robinson V, et al: Neural crest and the origin of ectomesenchyme: neural fold heterogeneity suggests an alternative hypothesis. *Dev Dyn* 229:118-130, 2004
59. Schoenwolf GC (ed): Special focus on the neural crest and the contributions of James A. Weston, in Schoenwolf GC (ed): *Developmental Dynamics*, Vol 229. New York, Wiley-Liss, 2004
60. Graham A, Begbie J, McGonnell I: Significance of the cranial neural crest. *Dev Dyn* 229:5-13, 2004
61. Nanci A: *Ten Cate's Oral Histology: Development, Structure, and Function*. 6th ed. St Louis, Mosby, 2003
62. Noden DM: The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev Biol* 96:144-165, 1983
63. Couly G, Grapin-Botton A, Coltey P, et al: Determination of the identity of the derivatives of the cephalic neural crest: incompatibility between Hox gene expression and lower jaw development. *Development* 125:3445-3459, 1998
64. Ferguson CA, Tucker AS, Sharpe PT: Temporospatial cell interactions regulating mandibular and maxillary arch patterning. *Development* 127:403-412, 2000
65. Delannet M, Duband JL: Transforming growth factor-beta control of cell-substratum adhesion during avian neural crest cell emigration in vitro. *Development* 116:275-287, 1992
66. Kinoshita Y, Kinoshita C, Heuer JG, et al: Basic fibroblast growth factor promotes adhesive interactions of neuroepithelial cells from chick neural tube with extracellular matrix proteins in culture. *Development* 119:943-956, 1993
67. Baird A: Fibroblast growth factors: activities and significance of non-neurotrophin neurotrophic growth factors. *Curr Opin Neurobiol* 4:78-86, 1994
68. Morrison-Graham K, Schatteman GC, Bork T, et al: A PDGF receptor mutation in the mouse (Patch) perturbs the development of a non-neuronal subset of neural crest-derived cells. *Development* 115:133-142, 1992
69. Nusse R, Varmus HE: Wnt genes. *Cell* 69:1073-1087, 1992
70. Augustine K, Liu ET, Sadler TW: Antisense attenuation of Wnt-1 and Wnt-3a expression in whole embryo culture reveals roles for these genes in craniofacial, spinal cord, and cardiac morphogenesis. *Dev Genet* 14:500-520, 1993
71. Chai Y, Ito Y, Han J: TGF-beta signaling and its functional significance in regulating the fate of cranial neural crest cells. *Crit Rev Oral Biol Med* 14:78-88, 2003
72. Ito Y, Yeo JY, Chytil A, et al: Conditional inactivation of TGFbeta2 in cranial neural crest causes cleft palate and calvaria defects. *Development* 130:5269-5280, 2003
73. Massague J: TGF-beta signal transduction. *Annu Rev Biochem* 67: 753-791, 1998
74. Massague J, Blain SW, Lo RS: TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 103:295-309, 2000
75. Whitman M: Smads and early developmental signaling by the TGF-beta superfamily. *Genes Dev* 12:2445-2462, 1998
76. Sporn MB, Roberts AB: TGF-beta: problems and prospects. *Cell Regul* 1:875-882, 1990
77. Wrana JL, Tran H, Attisano L, et al: Two distinct transmembrane serine/threonine kinases from *Drosophila melanogaster* form an activin receptor complex. *Mol Cell Biol* 14:944-950, 1994
78. Derynck R, Gelbart WM, Harland RM, et al: Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell* 87:173, 1996 (letter)
79. Heldin CH, Miyazono K, ten Dijke P: TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390:465-471, 1997
80. Roberts AB: TGF-beta signaling from receptors to the nucleus. *Microbes Infect* 1:1265-1273, 1999
81. Wrana JL, Attisano L: The Smad pathway. *Cytokine Growth Factor Rev* 11:5-13, 2000
82. Tsukazaki T, Chiang TA, Davison AF, et al: SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* 95:779-791, 1998
83. Candia AF, Watabe T, Hawley SH, et al: Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development* 124: 4467-4480, 1997
84. Engel ME, Datta PK, Moses HL: Signal transduction by transforming growth factor-beta: a cooperative paradigm with extensive negative regulation. *J Cell Biochem Suppl* 31:111-122, 1998
85. Onichtchouk D, Chen YG, Dosch R, et al: Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature* 401:480-485, 1999
86. Podos SD, Hanson KK, Wang YC, et al: The DSmurf ubiquitin-protein ligase restricts BMP signaling spatially and temporally during *Drosophila* embryogenesis. *Dev Cell* 1:567-578, 2001
87. Topper JN, Cai J, Qiu Y, et al: Vascular MADs: two novel MAD-related genes selectively inducible by flow in human vascular endothelium. *Proc Natl Acad Sci USA* 94:9314-9319, 1997
88. Nakao A, Afrakhte M, Moren A, et al: Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 389: 631-635, 1997
89. Stroschein SL, Wang W, Zhou S, et al: Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein [see comments]. *Science* 286:771-774, 1999
90. Watanabe M, Whitman M: The role of transcription factors involved in TGFbeta superfamily signaling during development. *Cell Mol Biol (Noisy-le-grand)* 45:537-543, 1999
91. Zuccherro TM, Cooper ME, Maher BS, et al: Interferon regulatory

- factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* 351:769-780, 2004
92. Wakefield LM, Roberts AB: TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 12:22-29, 2002
 93. Cucina A, Sapienza P, Borrelli V, et al: Nicotine reorganizes cytoskeleton of vascular endothelial cell through platelet-derived growth factor BB. *J Surg Res* 92:233-238, 2000
 94. Engel ME, McDonnell MA, Law BK, et al: Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* 274:37413-37420, 1999
 95. Higaki M, Shimokado K: Phosphatidylinositol 3-kinase is required for growth factor-induced amino acid uptake by vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 19:2127-2132, 1999
 96. Krymskaya VP, Hoffman R, Eszterhas A, et al: TGF-beta 1 modulates EGF-stimulated phosphatidylinositol 3-kinase activity in human airway smooth muscle cells. *Am J Physiol* 273:L1220-L1227, 1997
 97. Boyer AS, Ayerinkas II, Vincent EB, et al: TGFbeta2 and TGFbeta3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart. *Dev Biol* 208:530-545, 1999
 98. Rice R, Spencer-Dene B, Connor EC, et al: Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 113:1692-1700, 2004
 99. Kang P, Svoboda KKH: Nicotine inhibits palatal fusion and modulates nicotinic receptors and the PI-3 kinase pathway in medial edge epithelia. *Orthod Craniofac Res* 6:129-142, 2003
 100. Fitzpatrick DR, Denhez F, Kondaiah P, et al: Differential expression of TGF beta isoforms in murine palatogenesis. *Development* 109:585-595, 1990
 101. Cui XM, Warburton D, Zhao J, et al: Immunohistochemical localization of TGF-beta type II receptor and TGF-beta3 during palatogenesis in vivo and in vitro. *Int J Dev Biol* 42:817-820, 1998
 102. Cui XM, Shuler CF: The TGF-beta type III receptor is localized to the medial edge epithelium during palatal fusion. *Int J Dev Biol* 44:397-402, 2000
 103. Brunet CL, Sharpe PM, Ferguson MW: Inhibition of TGF-beta 3 (but not TGF-beta 1 or TGF-beta 2) activity prevents normal mouse embryonic palate fusion. *Int J Dev Biol* 39:345-355, 1995
 104. Kaartinen V, Voncken JW, Shuler C, et al: Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* 11:415-421, 1995
 105. Proetzel G, Pawlowski SA, Wiles MV, et al: Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 11:409-414, 1995
 106. Kaartinen V, Cui XM, Heisterkamp N, et al: Transforming growth factor-beta3 regulates transdifferentiation of medial edge epithelium during palatal fusion and associated degradation of the basement membrane. *Dev Dyn* 209:255-260, 1997
 107. Taya Y, O'Kane S, Ferguson MW: Pathogenesis of cleft palate in TGF-beta3 knockout mice. *Development* 126:3869-3879, 1999
 108. Cui XM, Shiomi N, Chen J, et al: Overexpression of Smad2 in Tgf-beta3-null mutant mice rescues cleft palate. *Development* 131:193-202, 2004
 109. Sun D, Vanderburg CR, Odierna GS, et al: TGF-beta3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro. *Development* 125:95-105, 1998
 110. Blavier L, Lazaryev A, Groffen J, et al: Tgf-beta3-induced palatogenesis requires matrix metalloproteinases. *Mol Biol Cell* 12:1457-1466, 2001
 111. Cui XM, Chai Y, Chen J, et al: TGF-beta3-dependent SMAD2 phosphorylation and inhibition of MEE proliferation during palatal fusion. *Dev Dyn* 227:387-394, 2003
 112. Cui XM, Chai Y, Ito Y, et al: Expression of TbetaR-I and SMAD2 in embryonic palatal tissues. *J Dent Res* 79:416, 2000
 113. Bhowmick NA, Ghiassi M, Bakin A, et al: Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 12:27-36, 2001
 114. Sugiura T, Berditchevski F: Function of alpha3beta1-tetraspanin protein complexes in tumor cell invasion. Evidence for the role of the complexes in production of matrix metalloproteinase 2 (MMP-2). *J Cell Biol* 146:1375-1389, 1999
 115. Metzner B, Barbisch M, Bachmann F, et al: Evidence of the involvement of phosphatidylinositol 3-kinase in the migration, actin stress fiber formation, and alpha v beta 3-integrin-mediated adherence of human melanoma cells. *J Invest Dermatol* 107:597-602, 1996
 116. Bakin AV, Tomlinson AK, Bhowmick NA, et al: Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 275:36803-36810, 2000
 117. Alessi DR, Kozlowski MT, Weng QP, et al: 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. *Curr Biol* 8:69-81, 1998
 118. Le Good JA, Ziegler WH, Parekh DB, et al: Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* 281:2042-2045, 1998
 119. Pullen N, Dennis PB, Andjelkovic M, et al: Phosphorylation and activation of p70s6k by PDK1. *Science* 279:707-710, 1998
 120. Burgering BM, Coffey PJ: Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376:599-602, 1995
 121. Hannigan GE, Dedhar S: Protein kinase mediators of integrin signal transduction. *J Mol Med* 75:35-44, 1997
 122. Andjelkovic M, Alessi DR, Meier R, et al: Role of translocation in the activation and function of protein kinase B. *J Biol Chem* 272:31515-31524, 1997
 123. Kandel ES, Hay N: The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res* 253:210-229, 1999
 124. Carpenter CL, Auger KR, Chanudhuri M, et al: Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. *J Biol Chem* 268:9478-9483, 1993
 125. Roymans D, Slegers H: Phosphatidylinositol 3-kinase in tumor progression. *Eur J Biochem* 268:487-498, 2001
 126. Wu C, Keightley SY, Leung-Hagesteijn C, et al: Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J Biol Chem* 273:528-536, 1998
 127. Radeva G, Petrocelli T, Behrend E, et al: Overexpression of the integrin-linked kinase promotes anchorage-independent cell cycle progression. *J Biol Chem* 272:13937-13944, 1997
 128. Janji B, Melchior C, Gouon V, et al: Autocrine TGF-beta-regulated expression of adhesion receptors and integrin-linked kinase in HT-144 melanoma cells correlates with their metastatic phenotype. *Int J Cancer* 83:255-262, 1999
 129. Kang P, Svoboda KK: PI-3 kinase activity is required for epithelial-mesenchymal transformation during palate fusion. *Dev Dyn* 225:316-321, 2002
 130. Tian YC, Phillips AO: Interaction between the transforming growth factor-beta type II receptor/Smad pathway and beta-catenin during transforming growth factor-beta1-mediated adherens junction disassembly. *Am J Pathol* 160:1619-1628, 2002
 131. Tian YC, Fraser D, Attisano L, et al: TGF-beta1-mediated alterations of renal proximal tubular epithelial cell phenotype. *Am J Physiol Renal Physiol* 285:F130-F142, 2003
 132. Espada J, Perez-Moreno M, Braga VM, et al: H-Ras activation promotes cytoplasmic accumulation and phosphoinositide 3-OH kinase association of beta-catenin in epidermal keratinocytes. *J Cell Biol* 146:967-980, 1999
 133. Willert K, Shibamoto S, Nusse R: Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. *Genes Dev* 13:1768-1773, 1999
 134. Kim K, Lu Z, Hay ED: Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biol Int* 26:463-476, 2002
 135. Morris-Wiman J, Du Y, Brinkley L: Occurrence and temporal variation in matrix metalloproteinases and their inhibitors during murine secondary palatal morphogenesis. *J Craniofac Genet Dev Biol* 19:201-212, 1999
 136. Morris-Wiman J, Burch H, Basco E: Temporospatial distribution of matrix metalloproteinase and tissue inhibitors of matrix metalloproteinases during murine secondary palate morphogenesis. *Anat Embryol (Berl)* 202:129-141, 2000
 137. Brown NL, Yarram SJ, Mansell JP, et al: Matrix metalloproteinases have a role in palatogenesis. *J Dent Res* 81:826-830, 2002
 138. Carlson BM: *Human Embryology and Developmental Biology*. 3rd ed. Philadelphia, Mosby, 2004.