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REVIEWS–A PEER REVIEWED FORUM

# The Mesenchymal Cell, Its Role in the Embryo, and the Remarkable Signaling Mechanisms That Create It

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**This review centers on the role of the mesenchymal cell in development. The creation of this cell is a remarkable process, one where a tightly knit, impervious epithelium suddenly extends filopodia from its basal surface and gives rise to migrating cells. The ensuing process of epithelial–mesenchymal transformation (EMT) creates the mechanism that makes it possible for the mesenchymal cell to become mobile, so as to leave the epithelium and move through the extracellular matrix. EMT is now recognized as a very important mechanism for the remodeling of embryonic tissues, with the power to turn an epithelial somite into sclerotome mesenchyme, and the neural crest into mesenchyme that migrates to many targets. Thus, the time has come for serious study of the underlying mechanisms and the signaling pathways that are used to form the mesenchymal cell in the embryo. In this review, I discuss EMT centers in the embryo that are ready for such serious study and review our current understanding of the mechanisms used for EMT in vitro, as well as those that have been implicated in EMT in vivo. The purpose of this review is not to describe every study published in this rapidly expanding field but rather to stimulate the interest of the reader in the study of the role of the mesenchymal cell in the embryo, where it plays profound roles in development. In the adult, mesenchymal cells may give rise to metastatic tumor cells and other pathological conditions that we will touch on at the end of the review. *Developmental Dynamics 233:706–720, 2005.***
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**Key words:** Epithelial–mesenchymal transformation; Wnt pathway; other LEF-1 signaling pathways; TGFβ signaling pathways; chordate embryology; stress fibers; somites; primitive streak; E-cadherin

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## INTRODUCTION TO MESENCHYME AND EPITHELIUM

Epithelial–mesenchymal transformation (EMT) is a major embryological mechanism for tissue remodeling that currently is attracting considerable attention to the role of the mesenchymal cell in the embryo. The basic tissue phenotypes in vertebrates are epithelium and mesenchyme (Fig. 1). The EMT can be defined as a process that produces complete loss of epithelial traits by the former epithelial cells accompanied by total acquisition of mesenchymal characteristics, such as vimentin, myosin, invasive motility, and so on. Although EMT evolved among several other animals, the remarkable ability of developing embryos to change one tissue type to the other reached its peak in the vertebrates, where it endowed these embryos with the ability to evolve a vertebral column. This they did from the sclerotome mesenchyme, using EMT to provide fibroblasts and osteoblasts that encircle the neural tube and synthesize extracellular matrix, including the bone and cartilages necessary to construct the vertebral column (Lash et al., 1957). Primitive streak and somite mesenchyme also provide the major source of the cells that produce the vertebrate arms and legs (Ede et al., 1977). Mesenchymal morphology (Trelstad et al., 1967; Hay, 1968) allows these cells to travel to specific targets in the embryo, where they differentiate and/or induce differentiation of other cells. The mesenchy-

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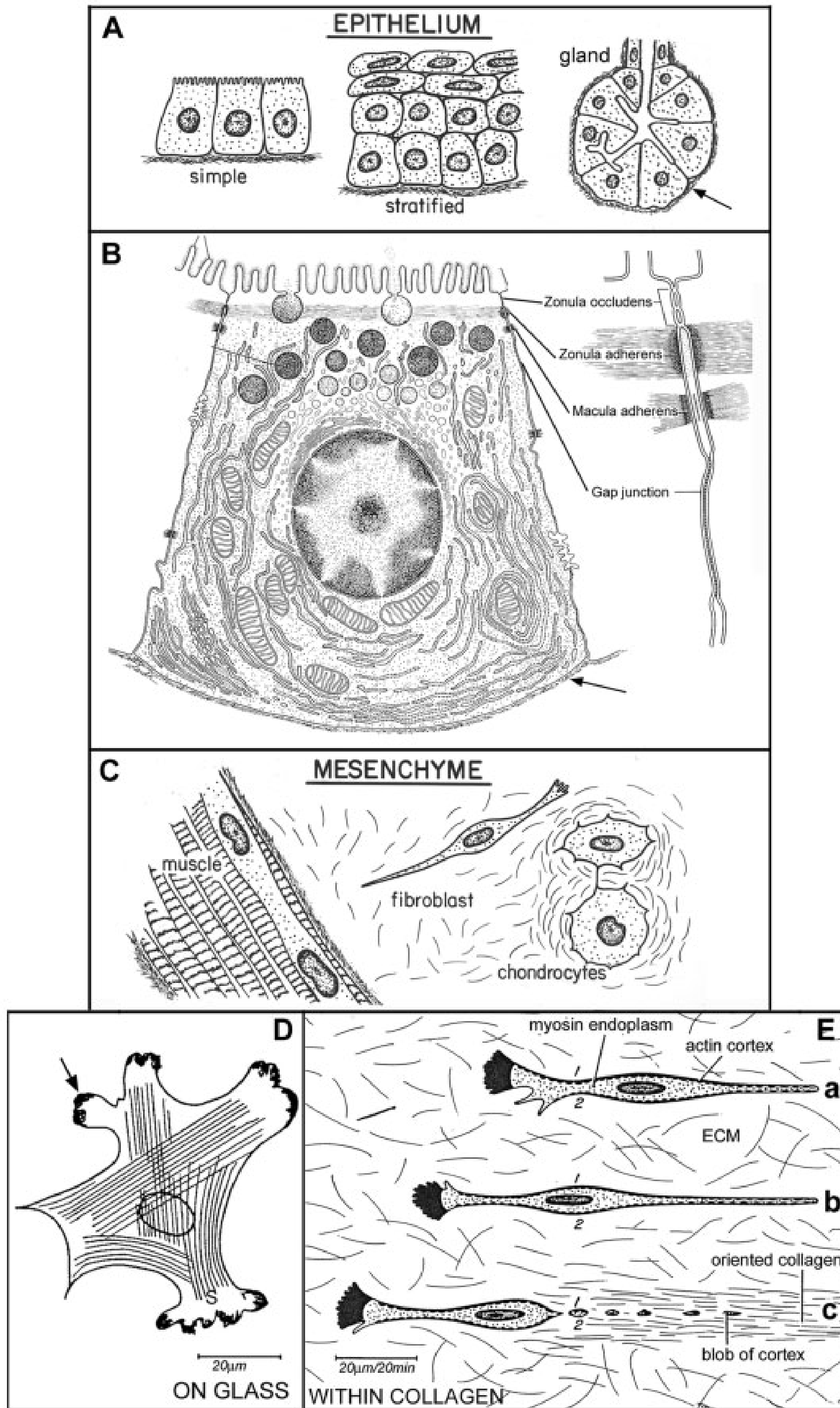



**Fig. 1.** **A,C:** The structure of typical epithelial cells (A) and mesenchymal cells (C) are shown. **B:** Epithelia are rich in adherens junctions containing E-cadherin in complexes with catenins linked to the cytoskeleton. Epithelia on stressed surfaces develop desmosomes (macula adherens). The epithelial cells have apical–basal polarity. The apical side may have microvilli. The basal actin cortex attaches the cytoskeleton to the underlying basal lamina (arrow) that contains collagen and laminin. Secretory epithelial cells polarize the Golgi complexes toward the apical surface from which the secretions exit. Mesenchymal cells are also highly polarized. Active cells have a bipolar morphology. **E:** They leave the epithelium of origin using filopodia on the front end. The back end will eventually detach, producing pear-shaped cells (c). **D:** On two-dimensional substrates, mesenchymal cells lose polarity and motility and produce stress fibers (s) and ruffles (arrow). E: See text for discussion of the mechanism of mesenchymal cell movement through collagen. (A,C: from Hay, 1984, Copyright Alan R. Liss, Inc.; D,E: from Hay, 1991, Copyright, Plenum Press; B: from Fawcett, 1981, Copyright, W.B. Saunders Co.)

mal cell, long neglected, is the subject of this review.

Epithelium is the earliest tissue, and it forms sheets of cells closely attached to each other by adherens junctions (Fig. 1B). These sheets of contiguous cells are attached firmly to an underlying extracellular matrix (ECM) layer (Fig. 1B, arrow) containing type IV collagen and laminin and known as the basement membrane or basal lamina. The close attachment of the lateral surfaces of epithelial cells gives the underlying tissues of the organism desirable protection from outside intruders. Epithelia under tension develop very strong adherens junctions called desmosomes (macula adherens, Fig. 1B) that contain specialized cadherins and catenins that dissociate during EMT (Savagner et al., 1997). Specialized cadherins (Takeichi, 1995) are also found in endothelium (VE-cadherin) and fibroblasts (N-cadherin). Epithelial cells are highly polarized with respect to the apical (free) side. They often secrete glandular products from the apical surface and some also secrete ECM from the basal surface. Basal actin cortex attaches the cells to the underlying ECM by means of integrins (receptors for ECM).

Many types of junctions evolved in the early epithelia, and their most important one, the adherens junction, recruited a remarkable protein named E-cadherin (cad for calcium-dependent) that induces both zonulae adherens junctions and desmosomes (Fig. 1B). Loss of E-cadherin (Cano et al., 2000) results in disappearance of catenins from junctions and loss of all epithelial features. These events led to the emergence of a new migratory cell type, the mesenchymal cell. During EMT, epithelial cells lose apical–basal polarity and acquire front end–back end polarity. The polarity regulatory mechanisms are not fully understood (Harris and Peifer, 2004). Metalloproteinases appear that promote EMT by digesting basement membranes (Lochter et al., 1997). They may also promote the ability of mesenchymal cells to invade ECM (see Egeblad and Werb, 2002). The ability to reside in ECM facilitates the translocation of mesenchymal cells. In vivo, collagen type I and fibronectin are the major ECMs found along EMT pathways (Newgreen and Thiery,

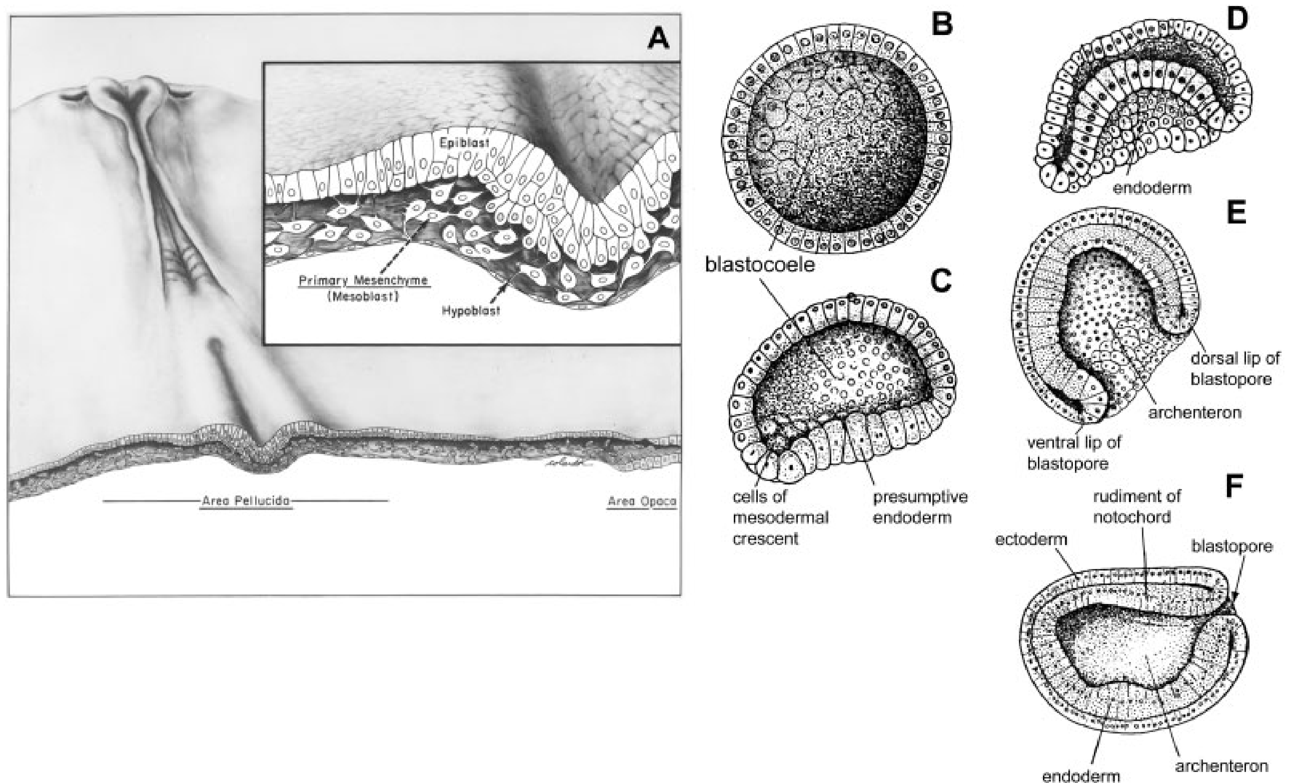



**Fig. 2.** A–F: Gastrulation (formation of the three germ layers) is shown here in higher chordates (A) compared with protochordates (B–F). **A:** Epithelial–mesenchymal transformation (EMT) of the epiblast (inset) produces primitive streak mesenchyme that forms the mesoderm and endoderm of the avian embryo. Gastrulation is mainly mesenchymal. In this 24-hr chick embryo, four epithelial somites have been induced from primitive streak mesenchyme. **B–F:** Amphioxus, on the other hand, is composed mainly of epithelium, and gastrulation is accomplished using epithelium; the endoderm and mesoderm germ layers invaginate (C,D) into the interior of the ball-shaped blastula (B), where they form epithelial sheets that lie against the ectoderm (E,F). Amphioxus forms a gut and mouth without mesenchymal cells but remains very small. (A: from Hay, 1968, Copyright Williams and Wilkins; B–F: from Conklin, 1932, Copyright, Wiley-Liss.)

1980; McCarthy and Hay, 1991). In vivo, collagen modulates the mesenchymal cell shape and cytoskeleton (Tomasek et al., 1982). In the sections to follow, the various attributes of mesenchymal cells will be discussed further, beginning with their ability to invade and move through ECM.

## MECHANISM OF LOCOMOTION

The full-blown mesenchymal cell (Fig. 1E) has no E-cadherin, and the only junctions it makes are transitory gap junctions when colliding with other mesenchymal cells (Trelstad et al., 1967). The typical mesenchymal cell is polarized for cell locomotion, with a trailing pseudopodium and a very active front end that contains the Golgi apparatus. Here, the Golgi complex helps to produce cell membranes for the filopodia that interact with the surrounding three-dimensional (3D) ECM. Mesenchymal cells apparently exit the epithelium of origin by extending filopodia and pseudopodia through the ECM of the epithelial basement membrane to propel the cell into the ECM to which it adheres (Hay, 1985). The trailing pseudopodium leaves blobs of cytoplasm behind in the epithelium and on the ECM (Fig. 1E). The pull of the cell on the ECM sculpts the matrix (Doljanski, 2004).

A probable method of locomotion is that the myosin endoplasm (attached to the cytoskeleton) slides forward along the actin-rich cortex (Fig. 1Ea), which is attached by integrins to the adjacent ECM. For forward motion to be achieved (Fig. 1Eb), the renewing cell cortex apparently becomes fixed to ECM (1, 2, Fig. 1Ea), because parts of it are always left behind with cytoplasm attached to ECM (Fig. 1Ec). The cell becomes bipolar again after a new front end rich in filopodia and actin complexes reforms. These observations are consistent with the hypothesis that myosin is sliding forward and is pulling the cytoplasm along on the actin cortex.

Although not fully explored for EMT as yet, the fixed cortex mechanism of translocation (Hay, 1989) is probably the method by which the transforming cell protrudes out of the epithelium during EMT to enter the ECM. There is evidence that it leaves the back end behind within the epithelium (Bilozur and Hay, 1989). A list of genes that up-regulate to produce this kind of movement could include the following: VIMENTIN, the intermediate filament likely to attach to myosin to slide the endoplasm forward on the actin cortex. However, vimentin is not specific to EMT. It is found in lens and other ocular epithelia, where it performs functions not specific to mesenchyme. ACTIN, smooth muscle actin is present but is not idiopathic for

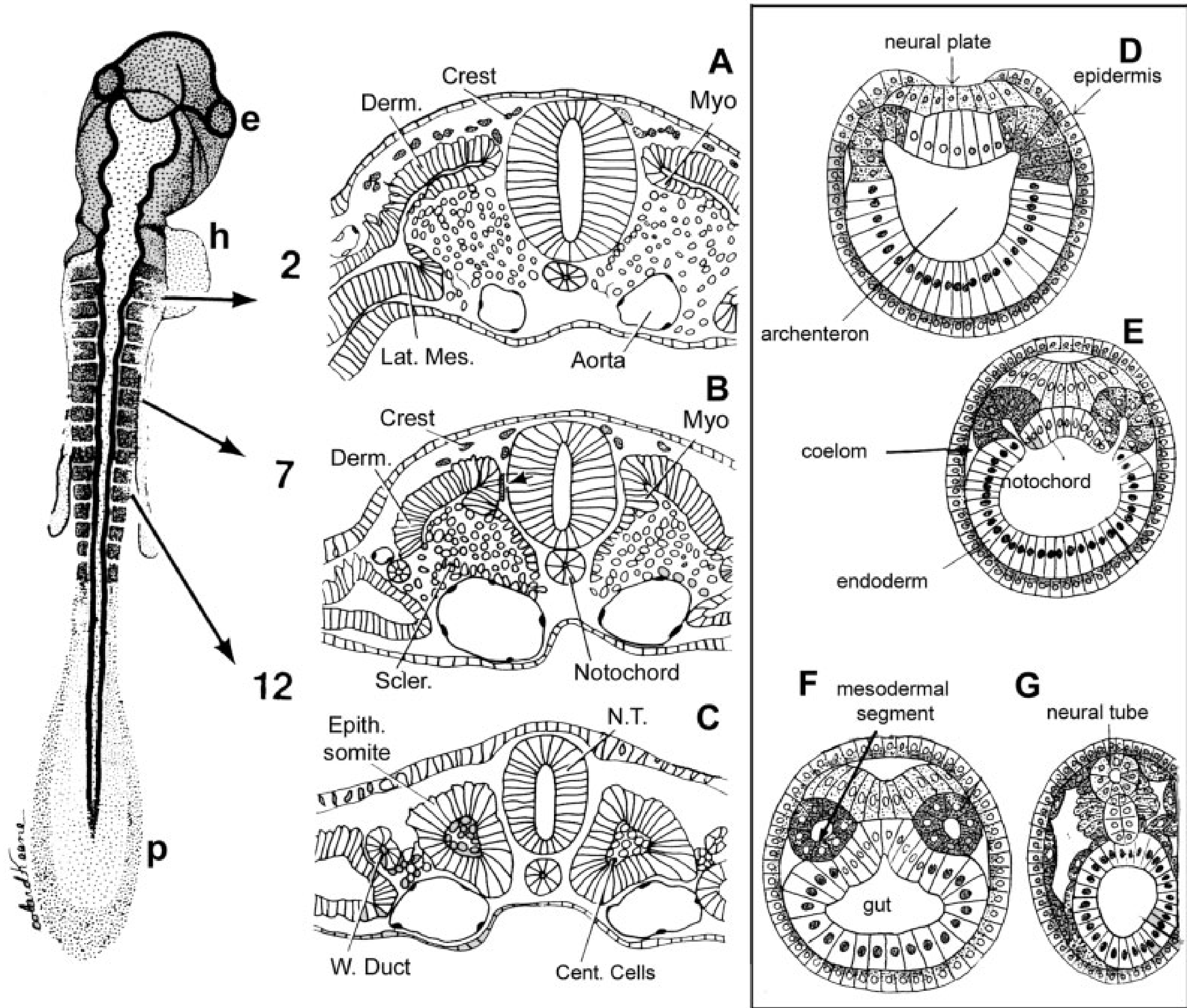



**Fig. 3.** Formation of mesodermal somites in higher and lower chordates. The primitive streak (p) forms a longitudinal crease in vertebrates, which leaves cells behind as it moves posteriorly. The 48-hr chick embryo shown here has formed eyes (e), heart (h), and primitive streak (p). **C:** The newest somites are epithelial. The neural tube (N.T.) invaginated from surface epithelium. **A,B:** Older somites are transforming to mesenchyme. Scler., sclerotome; Derm., dermatome; Myo, muscle; Lat. Mes., lateral mesenchyme. Nuclei of mesenchymal cells are shown. **D–G:** Amphioxus forms "somites" from the archenteron epithelium. F,C: Referred to as mesodermal segments (F), they resemble epithelial somites (C). However, Amphioxus mesodermal segments lack the potential to form mesenchyme. Mesenchyme from true somites allows vertebrates to develop arms and legs, as well as the vertebrate column. (A: from McCarthy and Hay, 1991, Copyright, Int J Dev Biol; D–G: after Hatchek, from Berrill NJ and Karp G, Copyright, McGraw Hill.)

mesenchyme. FIBRONECTIN, but it also is produced by epithelia, as well as fibroblasts. There are, in fact, no specific biochemical markers by which we can define the mesenchyme.

The definition of the mesenchymal cell has to be based on morphology and invasive motility; the cell should have (1) front end–back end polarity, (2) elongate morphology (3) filopodia, and (4) invasive motility. Invasion of a collagen gel[1] is the recommended test of motility and has been used for years (Behrens et al., 1989). The four points in this definition of the mesenchymal cell were endorsed by a vote at the first Boden International Conference on EMT held at Port Douglas, Australia, in October 2003.

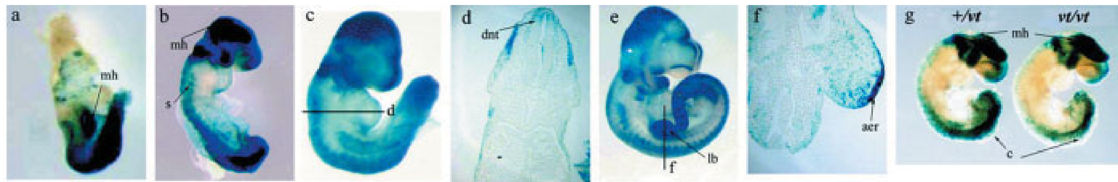
[1]Collagen can be purchased solublilized in acetic acid or be extracted from rat tails in the lab. It is then neutralized to polymerize. The mesenchymal cells are placed on top after gelling. Focus down into the ECM to count the cells that invaded (Behrens et al., 1989). A Boyden chamber should not be used. A simple petri dish should support the collagen gel.

## EMBRYONIC EMT PRODUCES MIGRATING CELLS NOT STRESS FIBERS

An alternate definition for the mesenchymal cell in wide use states that any

cell with its actin aggregated in stress fibers is a mesenchymal cell. Because this definition is often used in the in vitro literature, as the end point of EMT signaling pathways, I will digress from my main theme to explain what is wrong with it. The end point of true EMT in vivo is the activation of genes that create the mechanisms cells use to migrate through 3D ECM (Fig. 1E), an enormously important phenomenon we need to understand.

A total artifact, the stress fiber, can be created by removing mesenchymal cells from the animal and culturing them on

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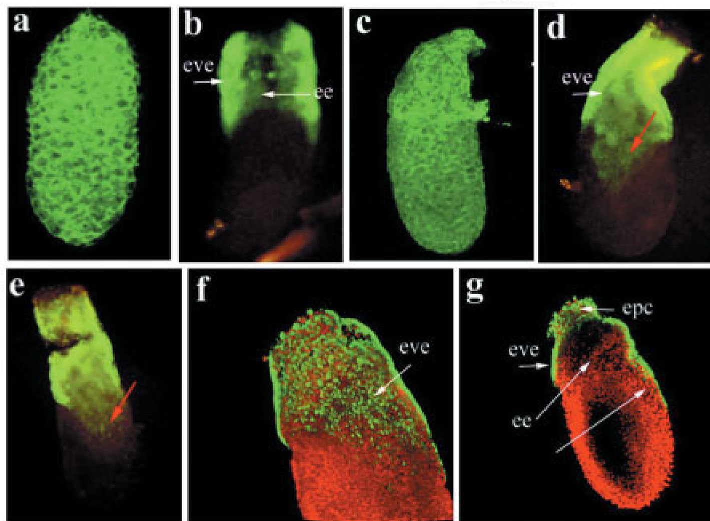


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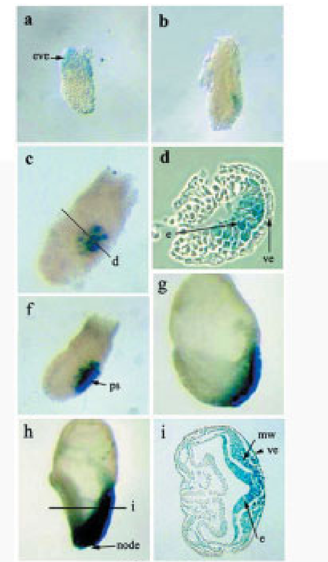


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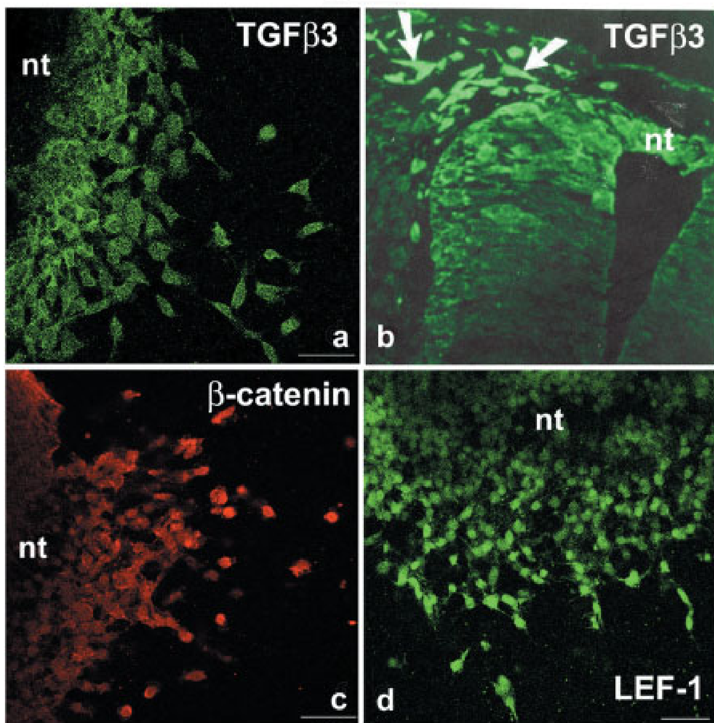


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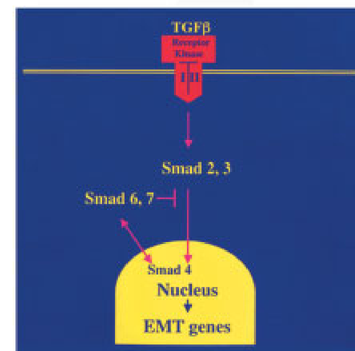


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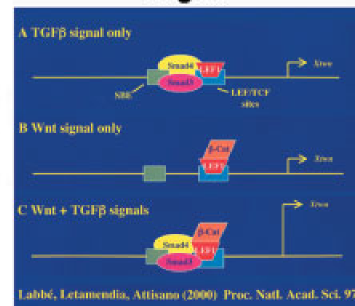



Fig. 9

**Figs. 4–9.**

planer (2D) substrates, such as coverslips. The cell flattens out and loses its elongated, migratory morphology. Most if not all of the myosin and actin molecules needed for cell locomotion, polymerize within the so-called stress fibers under these circumstances (s, Fig. 1D) and, thus, are missing from the cell cortex and endoplasm. It is important not to define the mesenchymal cell on the basis of the presence of stress fibers, because these are abnormal cells that do not actively migrate (Herman et al., 1981; Tomasek et al., 1982). Moreover, the Rho A and MEK pathways (Bhowmick et al., 2001; Roberts, 2002) that produce stress fibers do not produce true EMT in vivo (Nawshad and Hay, 2003). Indeed, Rho GTPases have adverse effects on cell contractility (Aspenstrom et al., 2004). The healthy mesenchymal cell is both a migratory and a secretory cell. It secretes much more collagen and fibronectin than epithelial cells do and is often called a fibroblast, which is a mesenchymal cell that mainly makes fibers. In the next section, we come to another nomenclature problem, the relation of the terms mesenchyme and mesoderm. Mesenchyme refers to cell(s) with the attributes defined above. A mesenchymal cell invades any layer of the body containing ECM. The term mesoderm refers to a layer (derm) of cells in the middle (meso) of the organism. Usually, it is a layer of cells lying between ectoderm (the outside germ layer) and endoderm (the inside germ layer). Because it is the middle layer, mesoderm contains both mesenchyme and epithelia of blood vessels and glands.

## SIGNIFICANT EMT CENTERS IN THE EMBRYO AND THE FATE OF THE MESENCHYME THEY GENERATE

The first and most important EMT in the embryo of the higher vertebrate produces the mesenchyme that condenses to form definitive mesoderm (middle layer of the embryo) and endoderm (inner layer). This process is called gastrulation. It is brought about by the primitive streak (Bellairs, 1986; Stern, 2004), which regresses posteriorly, leaving newly created mesenchymal cells behind along the trunk in large numbers that will form almost all of the mesoderm of the embryo, including some epithelia as well as connective tissue. The presumptive mesoderm and endoderm reside in the epiblast, and they both invaginate as primitive streak mesenchyme into the space between the epiblast and the hypoblast (Fig. 2A).

Gastrulation in the lower chordates is a totally epithelial event. The protochordate Amphioxus (Conklin, 1932; Fig. 2B–F) and amphibians form a blastopore through which presumptive mesodermal and endodermal epithelia invaginate (Fig. 2E). Without a significant source of mesenchyme, the resulting epithelial organism (Fig. 2F) remains small and primitive. Amphioxus somites (mesodermal segment, Fig. 3F) remain epithelial; the early vertebrates that began to produce mesenchyme for somites used it to make a vertebral column and bony appendages. Amphioxus (Fig. 3D–G) forms an epithelial neural tube and dorsal notochord and expresses neural crest genes (Trainor et al., 2003) and some neural crest but does not have the EMT potential of the primitive streak.

Primitive streak mesenchyme migrates anteriorly to form the somites and participate with neural crest mesenchyme in formation of the heart mesoderm. Subsequently, the epithelial endocardium of the presumptive valves of the heart undergoes partial EMT stimulated by transforming growth factors-beta (TGFβs) 2 and 3 to form the cushion mesenchyme (Runyan et al., 1992). Mesenchymal cells from the primitive streak participate in the formation of many epithelial mesodermal organs, such as notochord as well as somites. This process involves mesenchymal–epithelial transformation (MET; Fig. 3C). Because the somites will soon become mainly mesenchymal again in morphology (Fig. 3A,B),

**Fig. 4.** Role of Wnt and transforming growth factor-beta pathways in embryonic epithelial–mesenchymal transformations. **a–g:** Expression of endogenous LEF-1/LacZ transgene in 8- to 10-day-old embryos. Stain for β-galactosidase activity (blue) shows wide distribution of β-catenin/LEF-1 activity in the early mouse embryo. (From Mohamed et al., 2004, Copyright Wiley-Liss.) For abbreviations, see Mohamed et al. (2004).

**Fig. 5.** Role of Wnt and transforming growth factor-beta pathways in embryonic epithelial–mesenchymal transformations. **a–g:** Several hours before the primitive streak forms, specific antibodies detect the accumulation of nonphosphorylated β-catenin (red arrows) in subsets of cells. Another antibody (green) detects total β-catenin. Nuclei are stained red (4′,6-diamidine-2-phenylidole-dihydrochloride [DAPI]). (From Mohamed et al., 2004, Copyright Wiley-Liss.) eve, extraembryonic visceral endoderm. For other abbreviations, see Mohamed et al. (2004).

**Fig. 6.** Role of Wnt and transforming growth factor-beta pathways in embryonic epithelial–mesenchymal transformation. **a–i:** Expression of endogenous LEF-1/LacZ transgene (blue) in gastrulation stage mouse embryos (same method as in Fig. 4). Ages 5.5(a) – 7.75(i) days. (From Mohamed et al., 2004, Copyright Wiley-Liss.) ps, primitive streak; e, ectoderm; node, Henson's node. For other abbreviations, see Mohamed et al. (2004).

**Fig. 7.** Role of Wnt and transforming growth factor-beta (TGFβ) pathways in embryonic epithelial–mesenchymal transformations. **a:** Immunostaining showing expression of TGFβ3 in neural crest cells emigrating from a 2-day-old avian neural tube (nt) cultured on collagen for 2 days. **b:** Section of an avian embryo showing TGFβ3 in neural crest emigrating in vivo at 2 days. **c,d:** Neural tubes set up as in a and stained for β-catenin (c) and LEF-1 (d). (Courtesy of Jia Shi.)

**Fig. 8.** Role of Wnt and transforming growth factor-beta (TGFβ) pathways in embryonic epithelial–mesenchymal transformations (EMTs). Simplified diagram of the TFGβ pathway. The ligand activates receptors I and II, which become involved in phosphorylation of Smad 2 and 3. Smad 4 transports phosphorylated Smad 2 and 3 into the nucleus. Smads 6 and 7 are inhibitory. (Drawn by C. Chui and E. Hay.)

**Fig. 9.** Role of Wnt and transforming growth factor-beta (TGFβ) pathways in embryonic epithelial–mesenchymal transformations. **A:** TGFβ can activate LEF-1 by binding Smads to one end of it. **B:** β-Catenin binds the opposite end from Smads to activate LEF-1. **C:** Attisano and her colleagues (Labbe et al., 2000) showed that Wnt Signals (β-catenin) and TGFβ signals (Smad 3,4) can bind LEF-1 spontaneously to reinforce the total signal. Examples applying these principles in vivo are described in the text. (Drawn by Caroline Chui, based on Labbe et al., 2000, Copyright, National Academy of Sciences.)

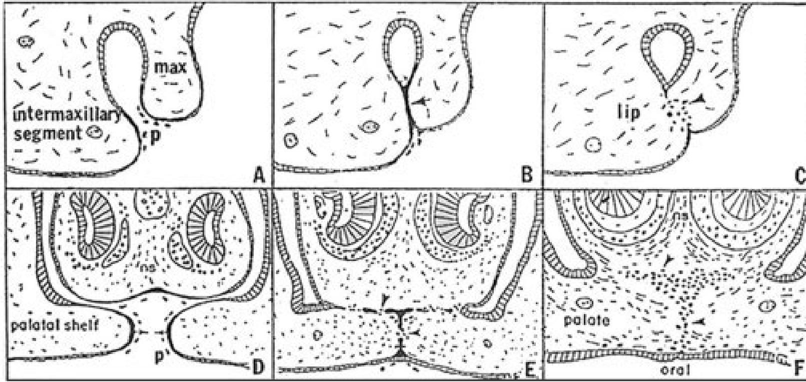



Fig. 10

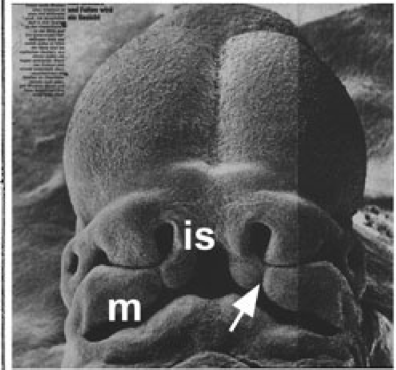
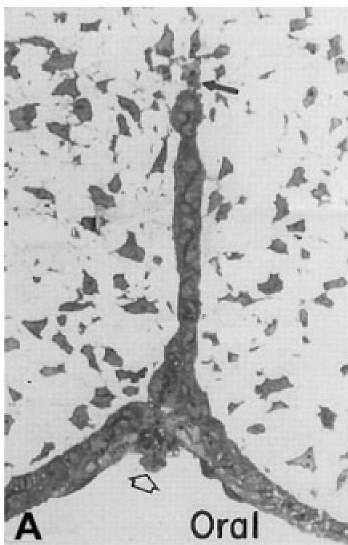
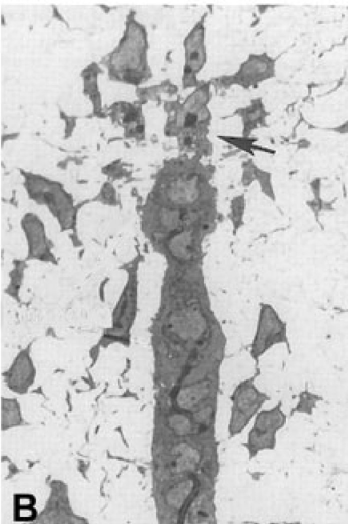



Fig. 11



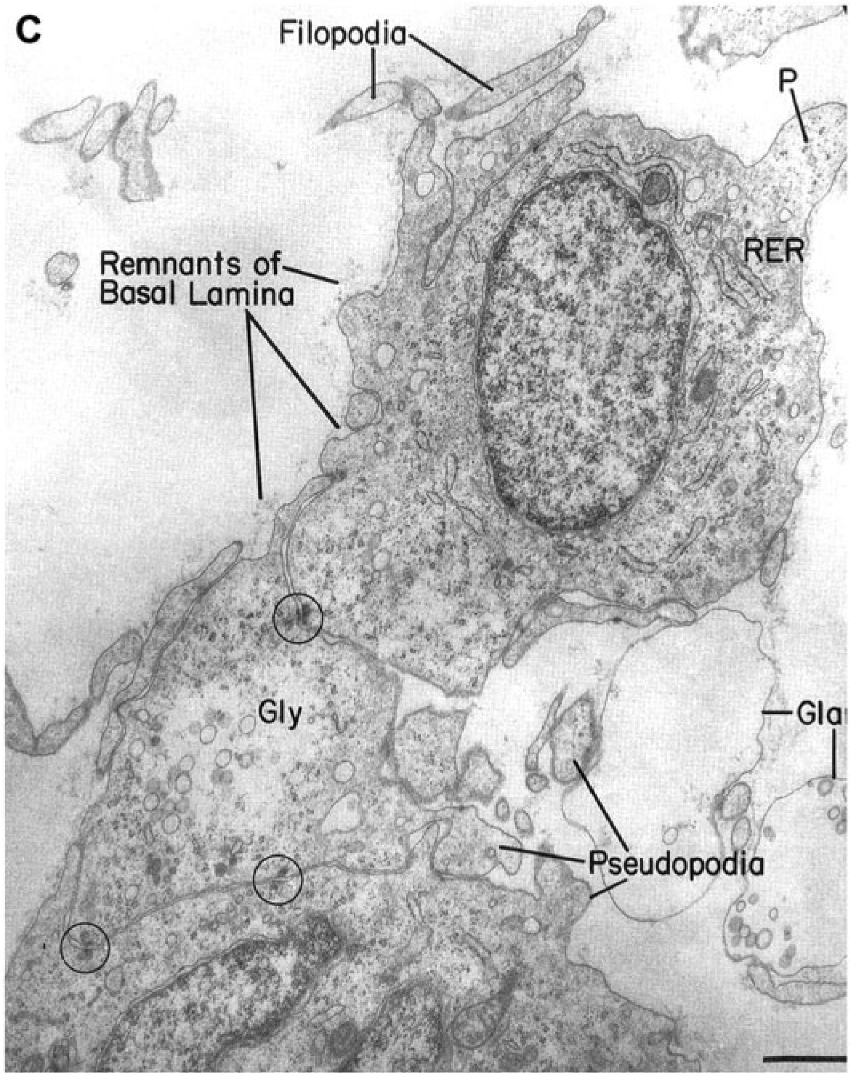




Fig. 12

Figs. 10–12.

one wonders why this transitory epithelial state (Fig. 3C) exists. The explanation probably lies in the evolution of the higher chordates (e.g., birds, mammals) from epithelial chordates (e.g., amphioxus) that have coded basic pattern information within primitive epithelia that the vertebrate embryo has to recreate (Fig. 3D–G) to express its phylogeny. Of interest, it was discovered recently that Wnt6 regulates the epithelialization of the streak-derived segmental plate (sp, Fig. 3) to form the initial somites (Schmidt et al., 2004). These are epithelial somites (Fig. 3C), and they will undergo EMT to form the sclerotome (Scler., Fig. 3B). This structure is the somite mesenchyme that migrates around the neural tube (N.T., Fig. 3C) to form the spinal column of the vertebrates (Lash et al., 1957).

Recently, Mohamed et al. (2004) completed an elegant study showing that the Wnt/β-catenin pathway specifies the cells that form the primitive streak of the mouse embryo. The streak is attached anteriorly to Hensen's node and caudally to the tail (Fig. 4c). Endogenous LEF-1/LacZ was detected by β-galactosidase activity (blue, Fig. 4). Total β-catenin was stained green (Fig. 5) by immunohistochemistry, and nuclei are shown as red using 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI) staining. β-Catenin was first expressed in the extraembryonic visceral endoderm (eve, Fig. 5) and from there spreads to the primitive streak to combine with LEF-1.

Endogenous LEF-1 is widely present in somites (Fig. 4b–e), primitive streak (Fig. 6i), and many other sites in the early embryo (Fig. 4a,f,g). β-Catenin is also widespread at this time, suggesting that the Wnt pathway is being used by numerous early embryonic processes, including neural crest as well as the primitive streak.

The neural crest is not a primitive streak-derived mesenchyme. It originates from the dorsal neural tube epithelium (Fig. 7). The trunk neural crest mesenchyme lacks the potential to produce the type of connective tissue and skeletal structures that evolved with EMT to produce the vertebrate column. However, considerable amounts of craniofacial crest mesenchyme form connective tissue that contributes to the head and face in the vertebrate (Noden, 1986). Part of the skull derives from primitive (p, Fig. 3) streak mesenchyme moving anteriorly into the head. Both the nearby developing heart (h, Fig. 3) and branchial arches also use neural crest to form some or all of their connective tissue, and of course, the neural crest is deeply involved in evolution of the peripheral nervous system.

To form its superficial features, the face of the higher vertebrate is ingenious in its use of local EMT to remove unwanted epithelium (Figs. 10, 11). This use is an amazing evolution of the functions of EMT. The nose forms a nostril by invagination of the outside epithelium inward. How could EMT contribute to such a process? In the case of the nose and lip, the maxillary process (m, Fig. 11) fuses its medial nasal edge epithelium with the epithelium of the intermaxillary segment (is, Fig. 11), after sloughing of the periderm (outer keratinized layer), to produce an epithelial seam (Fig. 10B) that undergoes EMT to achieve mesenchymal confluency (Fig. 10C), while keeping the lip intact (Sun et al., 2000). Failure of this EMT results in bilateral cleft lip, and failure of palate seam EMT results in cleft palate. The roof of the oral cavity forms by EMT of the medial epithelial seam produced by adherence of the palatal shelves on the inner sides of the maxillary processes.

Diagrams of sections of palate shelves show the sloughing of peridermal epithelial cells (depicted by black dots, Fig. 10D–F) as the shelves approach each other. Sloughing of the outer epithelial layer promotes adherence of the basal epithelial cells that form the midline palatal seam (Fig. 10E, arrowhead). The sloughing begins (Fig. 10D, p) when the two nonfused shelves start to move toward each other. The triggering mechanism is unknown. These are very good examples of programmed cell death in the embryo. If all of the periderm sloughs as in vivo (Fitchett and Hay, 1989), no dying cells appear among the basal epithelial cells forming the midline palatal seam (Fig. 12A–C); the midline seam basal epithelial cells do not stain for apoptosis (Nawshad et al., 2004) in vivo. In vitro, inappropriate culture conditions are responsible for much of the reported basal cell death (Takigawa and Shiota, 2004). Palates express Snail (Martinez-Alvarez et al., 2004), which promotes cell survival, as well as EMT (Vega et al., 2004). Both the lip and palate midline epithelial seams transform into typical mesenchymal cells (Fig. 12) with filopodia and bipolar morphology. The mouse palate, but not the avian palate or lip expresses TGFβ3 (Sun et al., 1998b). When palatal E-cadherin and syndecan, a proteoglycan that promotes epithelialization,

**Fig. 10.** Role of epithelial–mesenchymal transformation (EMT) in palate and lip epithelial seams. A–C: Diagrams of lip development. One side of the face is shown. **A,B:** The maxillary process (max, A) fuses with the intermaxillary segment under the nostril to form an epithelial seam (arrow, B). Failure of fusion of the epithelial seam that forms between the intermaxillary segment and maxilla results in cleft lip (arrow, Fig. 11). **C:** Fusion is completed by EMT of the adherent seam (arrowhead). **D–F:** Diagrams of palate development. The palatal shelves grow out from the inner face of the maxillary processes and move across the roof of the mouth (D) to form a seam (E) that transforms to mesenchyme (arrowheads, F) to fuse the palate. (A–C: from Sun et al., 2000, Copyright Academic Press; DF: from Griffith and Hay, 1992, Copyright The Company of Biologists.) For abbreviations, see Griffith and Hay (1992).

**Fig. 11.** Role of epithelial–mesenchymal transformation in palate and lip epithelial seams. Scanning electron photomicrograph of a developing human head showing the maxillary process (m) fusing with the intermaxillary segment (is). Arrow indicates site of cleft lip. (Author unknown.)

**Fig. 12.** Role of epithelial–mesenchymal transformation (EMT) in palate and lip epithelial seams. **A:** Light micrograph of a palate seam breaking up into mesenchyme, magnified further in **B** to show the transforming cell (arrow) at the tip of the breaking seam. **C:** Similar cell viewed by TEM showing its attachment to the seam by desmosomes (circles) (Fig. 12A–C, Fitchett and Hay, 1989; Copyright, Academic Press). Scale bar = 100 nm.

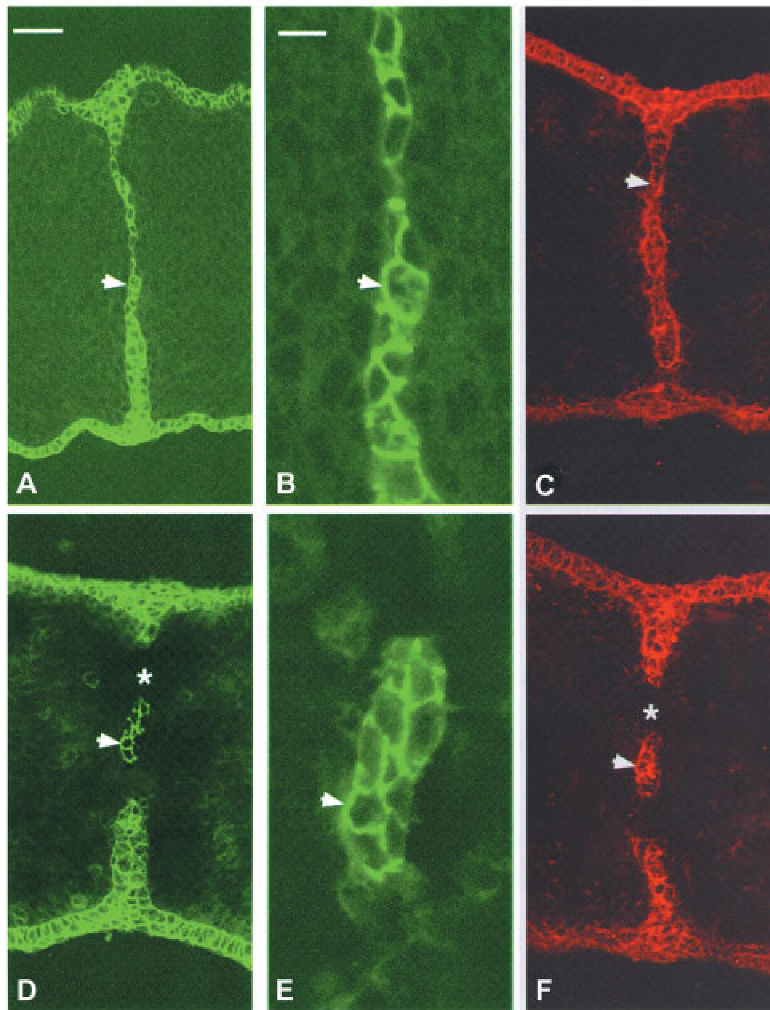



Fig. 13

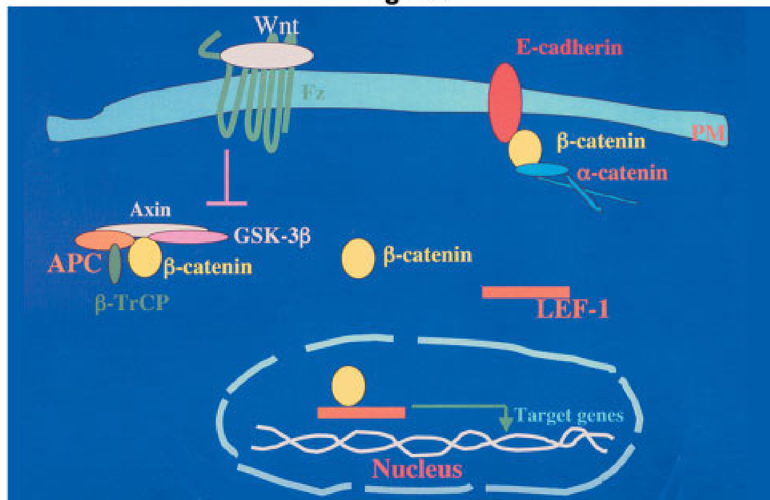



Fig. 14

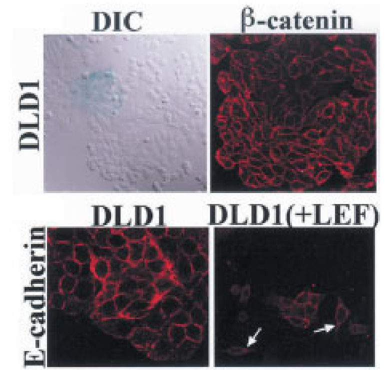



Fig. 15

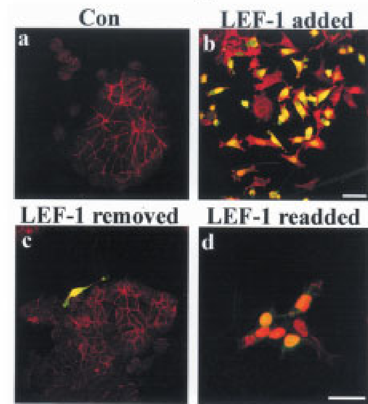



Fig. 16

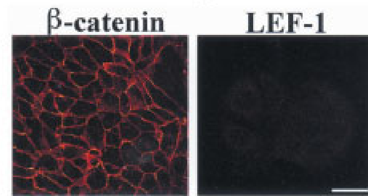



Fig. 17

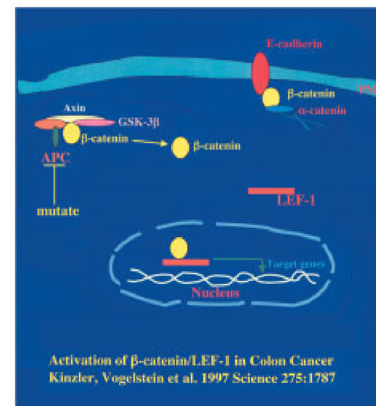



Fig. 18

Figs. 13–18.

simultaneously down-regulate (Fig. 13; Sun et al., 1998a), mesenchymal cells are released whose migration through the palate can be traced by 1,1′, di-octadecyl-3,3,3′,3′,-tetramethylindo-carbocyanine perchlorate (DiI; Shuler et al., 1992) or carboxyfluorescein (Griffith and Hay, 1992; Sun et al., 1998b). Desmosomes (circles, Fig. 12) disappear during palatal EMT, as reported for Slug-induced EMT elsewhere (Savagner et al., 1997).

## SIGNALING PATHWAYS IN DEVELOPMENT

The reader is referred to the Warkany Lecture by Gerhart (1999) for a penetrating assessment of the simultaneous evolution of signaling pathways among vertebrates, other chordates, and unicellular eukaryotes. Only five signal pathways are widely used in early embryonic development: the Wnt pathway, the TGFβ family (including TGFβ1-3, bone morphogenetic protein (BMP), Nodal, Activin, and Dorsalin), the Hedgehog family, the receptor tyrosine kinase (RTK) pathway, and the Notch pathway. Only approximately a dozen more are added to the next stage of development (Gerhart, 1999). The early pathways contribute to embryo organizers, including the dorsal lip of the amphibian, and also are used to induce cell proliferation, cell differentiation, secretion, motility, and transcription. These signaling pathways are conserved from pre-Cambrian times. Protein components of the responses date back 2 billion years (Gerhart, 1999).

Thus, it is no surprise to see that the Wnt and TGFβ pathways are the major pathways used in early embryonic EMT and that Notch may also have roles in EMT. RTK ligands, such as fibroblast growth factor (FGF) and epidermal growth factor (EGF), promote EMT (Boyer and Thiery, 1993). The Wnt, Hedgehog, and Notch pathways are involved in production of somite mesenchyme for the vertebral column. The reader is referred to the following reviews for additional discussion of Notch and Hedgehog pathways (Johnson and Scott, 1998; Artavanis-Tsakonas et al., 1999; Kopan, 2002; Zavadil et al., 2004).

## WNT AND TGFB SIGNALING PATHWAYS INTERACT TO MEDIATE EMBRYONIC AND NEOPLASTIC EMT

The Wnt signaling pathway activates LEF-1 by providing a source of β-catenin to induce its transcriptional activity (Fig. 14). Wnt glycoproteins, such as Wnt1 and Wnt3a bind to the frizzled receptor (Fz, Fig. 14) that in turn produces an inhibitor, disheveled, to knockout the serine–threonine kinase, GSK-3β that phosphorylates cytoplasmic β-catenin to send it to the APC-rich ubiquitin pathway that destroys it. The surviving cytoplasmic β-catenin rescued by Wnt (Fig. 14) can now be transported to the nucleus by the existing LEF-1 (Kim and Hay, 2001) to activate LEF-1 transcription. Garcia-Castro et al. (2002) reported that Wnt6 produced in ectoderm overlying the neural tube signals the avian neural tube to activate neural crest formation, but Wnt1 may also play a role. The β-catenin/LEF-1 pathway can be activated without Wnt if some other mechanism stabilizes β-catenin, e.g., mutated APC in tumors (Fig. 18; Kinzler and Vogelstein, 1997). β-Catenin itself is not signaling EMT genes (de Melker et al., 2004); its role is to activate LEF-1 for target gene transcription. Addition of LEF-1 DNA in an adenovirus (Fig. 15, Kim et al., 2002) down-regulates E-cadherin (Fig. 15) and transforms the epithelial cells to mesenchyme (Fig. 16). If LEF-1 is removed, they revert to epithelial cells (Fig. 16). The DLD1 epithelial cells have abundant endogenous β-catenin that activates the exogenous LEF-1.

TGFβ2 and β3 use Smad 4 to transport Smad 2 and Smad 3 (Fig. 8) into the nucleus to activate EMT (see Roberts, 2002; Nawshad et al., 2004, for reviews). Members of the TGFβ superfamily exhibit neural crest-inducing abilities. BMP4 and 7 play a definite role in the forming crest (Garcia-Cas-

**Fig. 13.** Role of E-cadherin and the Wnt pathway in epithelial–mesenchymal transformation (EMT). **A–C:** Immunostaining of adjacent frozen sections of the same palatal seam for E-cadherin (A,B) and syndecan (C) at embryonic day (E) 14 plus 12 hr in culture. **D–F:** A similar seam at E14 plus 18 hr (D, E are stained for E-cadherin and F for syndecan). B and E are magnifications of E-cadherin–rich areas at the arrows in A and D, respectively. D,F: E-cadherin and syndecan, an epithelial proteoglycan, simultaneously disappear during breaks of the seam (asterisks) that are giving rise to mesenchyme. E-cadherin and syndecan totally disappear from areas of EMT. (From Sun et al., 1998a, Copyright, Int J Dev Biol.) Scale bars = 50 mm in A (applies to A,C,D,F), in B (applies to B,E).

**Fig. 14.** Role of E-cadherin and the Wnt pathway in epithelial–mesenchymal transformation. Diagram of the Wnt pathway. Wnt raises β-catenin levels by inhibiting its destruction, allowing β-catenin to activate LEF-1. See text for further description. (Drawn by C. Chui and E. Hay, unpublished.)

**Fig. 15.** Role of E-cadherin and the Wnt Pathway in epithelial–mesenchymal transformation. DLD1 colon carcinoma cells stained for β-catenin and E-cadherin show an epithelial pattern. Addition of LEF-1 destroys expression of E-cadherin (arrows, see also Fig. 14, lower right). (From Kim et al., 2002, Copyright Elsevier Science Ltd.)

**Fig. 16.** Role of E-cadherin and the Wnt Pathway in epithelial–mesenchymal transformation (EMT). **a,b:** LEF-1 added to the control (con) DLD1 cells (a) transforms the epithelial cells to mesenchyme (b). **c,d:** Removal of LEF-1 reverses this effect (c), but LEF-1 readded (d) reverts them to EMT. Yellow stain, LEF-1 in nuclei. (From Kim et al., 2002, Copyright Elsevier Science Ltd.)

**Fig. 17.** Role of E-cadherin and the Wnt Pathway in epithelial–mesenchymal transformation. Normal (corneal) epithelium does not express LEF-1 but does express β-catenin on lateral surfaces where adherens junctions have formed E-cadherin/β-catenin complexes. (From Kim et al., 2002, Copyright Elsevier Science Ltd.)

**Fig. 18.** Role of E-cadherin and the Wnt Pathway in epithelial–mesenchymal transformation. In colon carcinoma, the APC mutation results in an excess of β-catenin, which promotes activation of the β-catenin/LEF-1 pathway without Wnt. (Drawn by C. Chui and E. Hay. Unpublished diagram.)

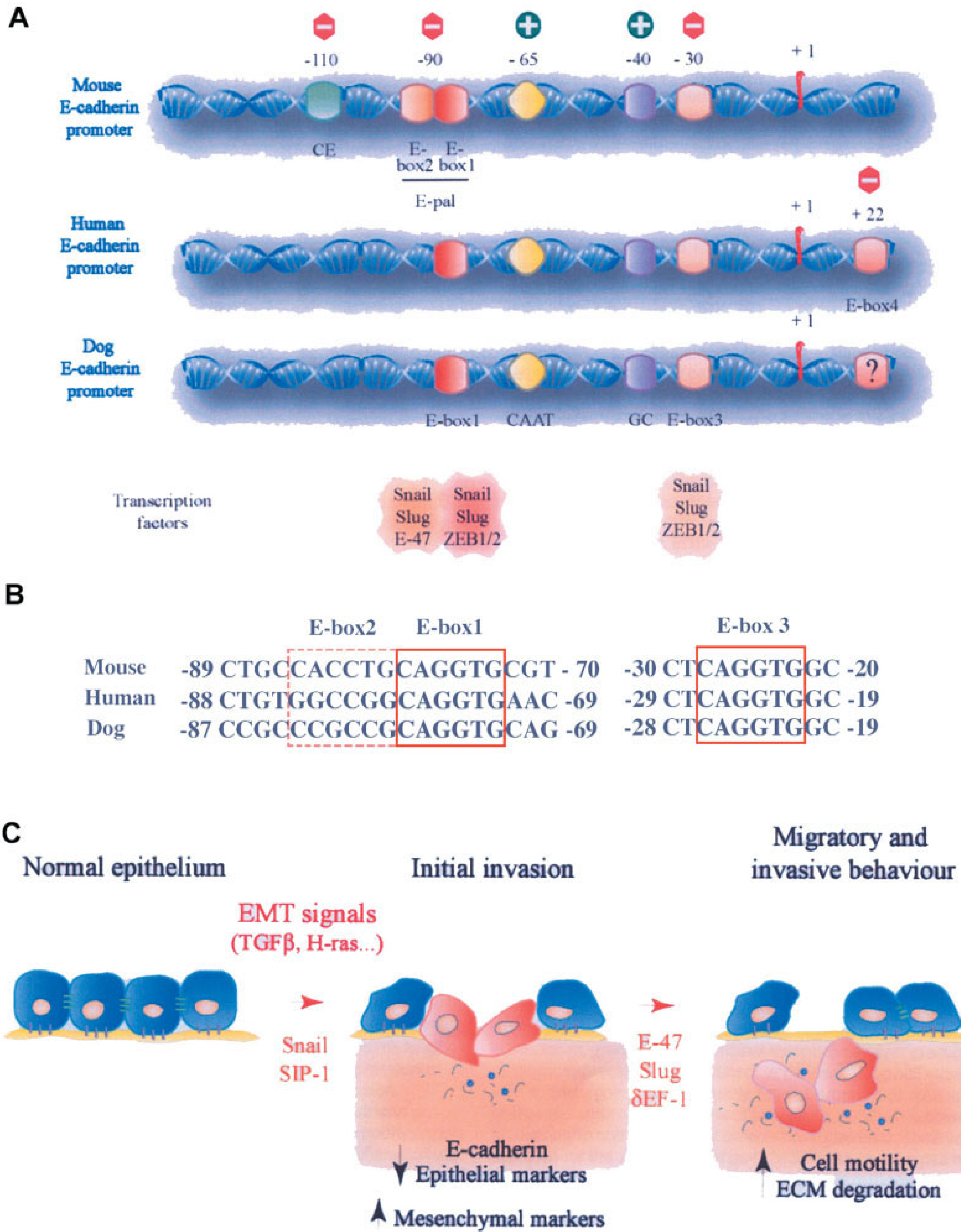



**Fig. 19.** Interaction of repressors with the E-cadherin promoter. **A:** Diagrams of mouse, human, and canine E-cadherin promoters. The E-cadherin promoter control elements exert either a positive or negative effect on E-cadherin expression. The CAAT, GC, and E-box1/E-box3 are conserved, but E-box2 is only present in the mouse and E-box4 only in the human. Binding of the repressor transcription factors to the different E-boxes is shown where known (Snail, Slug, E-47, ZEB1/2). SIP-1 is same as ZEB2. The binding locations of Twist and LEF-1 are not known at this time. **B:** Sequences of the E-boxes present in the proximal region of the mouse, dog, and human E-cadherin promoters are shown. **C:** Speculative diagram of a model in which several repressors (e.g., Snail and SIP-1) participate in the turn on of mesenchymal markers (effectors) after shut down of the E-cadherin epithelial marker by Snail and SIP-1. Other repressors (e.g., Slug, E-47) may contribute to sustained E-cadherin repression to create a flow of invasive mesenchymal cells and extracellular matrix degradation. (From Peinado et al., 2004, Copyright Int J Dev Biol.) EMT, epithelial–mesenchymal transformation; TGFβ, transforming growth factor-beta.

tro et al., 2002). Transforming neural crest cells also stain intensely for TGFβ3 (Fig. 7a,b), as well as LEF-1 (Fig. 7d), and β-catenin (Fig. 7c). There are data implicating slug in neural crest transformation and migration (Nieto et al., 1994). (See Garcia-Castro et al., 2002, LaBonne et al., 2000, Luo et al., 2003, for further review of genes expressed by forming neural crest.)

Heart development uses several TGFβs in the chick and mouse embryo, including TGFβ2 and TGFβ3 (Runyan et al., 1992; Camenisch et al., 2002; Liebner et al., 2004; Timmerman et al., 2004). TGFβ2 influences the levels of Snail and VE-cadherin (see Takeichi, 1995, for review of other cadherins). There is overlap with Wnt pathways in the heart (Liebner et al., 2004), β-Catenin is required for EMT in mammalian heart cushion EMT, whereas the Notch gene seems to be required for endocardial EMT (Timmerman et al., 2004). Palates null for Notch are cleft, suggesting a role for Notch in palate EMT (Jiang et al., 1998), but the overall role of Notch in embryonic EMTs is not clear at this time.

TGFβ3 both up-regulates LEF-1 synthesis and activates its transcriptional function in the palate. A study of the mechanism used by TGFβ3 to induce EMT of the palatal seam, revealed that the cytoplasm of the medial edge epithelial seam contained abundant LEF-1 and β-catenin, but surprisingly no β-catenin entered the nucleus to activate LEF-1 (Nawshad and Hay, 2003). Sense and antisense β-catenin ODNs were added to try to force the system to use β-catenin/LEF-1. However, the palate seam cells would not import the abundant β-catenin into nuclei. Nevertheless, abundant LEF-1 entered the nuclei and palatal EMT prospered as long as Smad 4 and phosphorylated Smad 2 or 3 are also present in the nucleus (Fig. 14). DNSmad totally inhibited transport and activation of LEF-1. The surprising answer, already predicted by Labbe et al. (2000), was that the Smad 2/4 heterodimer (Fig. 9) binds as strongly to LEF-1 as β-catenin and is just as effective in activating LEF-1 transcription. Palate medial edge seam EMT is completely independent of β-catenin, RhoA, and MEK pathways (Nawshad and Hay, 2003), but PI-3 kinase plays a role in TGFβ signaling (Kang and Svoboda, 2002).

## MECHANISMS THAT PRODUCE MIGRATING MESENCHYMAL CELLS

MET is the antithesis of EMT. Over the years, it has been demonstrated over and over again that transfection of mesenchymal cells with plasmids carrying the E-cadherin gene reverts mesenchyme to epithelium (Nagafuchi et al., 1987; Mege et al., 1988; Chen and Obrink, 1991; Behrens et al., 1992; Birchmeier and Birchmeier, 1994; Hay, 1995; Takeichi, 1995; Vanderburg and Hay, 1996. Transfection of invasive corneal fibroblasts with the E-cadherin gene causes their dramatic transformation from mesenchyme to stratified epithelia with desmosomes (Vanderburg and Hay, 1996).

It gradually has seemed more and more likely that EMT is the direct result of down-regulation of E-cadherin gene expression. Indeed, Behrens et al. (1989) and many others demonstrated that epithelial cells acquire invasive qualities as a result of loss of E-cadherin–mediated adhesion. In an in vitro model of mammary cells, Eger et al. (2000) showed that Fos up-regulates β-catenin and LEF-1 activity, followed by down-regulation of E-cadherin to activate EMT. This finding implicates LEF-1, a transcription factor usually activated by β-catenin, in the down-regulation of E-cadherin (Fig. 15) and the subsequent up-regulation of target genes for EMT. Tan et al. (2001) reported that the integrin-linked kinase ILK also stimulates LEF-1 and down-regulates E-cadherin to induce EMT. Kim et al. (2000) used LEF-1 adenovirus infection (Fig. 16) to show that β-catenin/LEF-1 directly down-regulates E-cadherin and transforms DLD1 cells to mesenchyme. Dominant-negative LEF-1 inhibits this change. Kang and Massague (2004) concluded that down-regulation of E-cadherin is not sufficient for initiating EMT, because E-cadherin could not restore the epithelial phenotype in cells overexpressing Twist (Yang et al., 2004). In the experiments reviewed above, however, E-cadherin transfection of fibroblasts consistently converts invasive mesenchymal cells to epithelium (see above and Hay, 1995, for review). It is likely that the presence of Twist, a repressor of E-cadherin, prevented E-cadherin epithelial function in Yang's experiments.

The discovery and isolation of repressors of E-cadherin was first reported by Cano et al. (2000) and Nieto et al. (2002), who showed that Snail and Slug, members of a zinc-finger transcription factor family, cause EMT in MDCK cells by repressing E-cadherin expression by means of binding to its promoter (Fig. 19A). Nieto and Cano discovered that Snail family molecules interact with the E2 box of the E-pal element (Fig. 19A,B) in the E-cadherin promoter to totally repress the E-cadherin gene (Peinado et al., 2004). Cano previously had analyzed cadherin promoters with Birchmeier and Behrens (see Faraldo et al., 1997) and showed that E-cadherin expression in carcinomas prevented invasiveness.

Most of the repressors (transcription factors, Fig. 19A) that cause EMT down-regulate the E-cadherin promoter (Fig. 19A,B). These repressors are Snail (Nieto, 2002), Zeb1, Sip1 (Comijn et al., 2001), Twist (Yang et al., 2004), E12/E47 (Perez-Moreno et al., 2001; Balos et al., 2003), Slug (Bolos et al., 2002), and LEF-1 (Kim et al., 2002). One might ask how could a negative event like turn off of the E-cadherin gene cause EMT in embryos and tumors? Such an event would release β-catenin from adherens junctions, which could activate the Wnt pathway to induce EMT by means of LEF-1. However, this Wnt mechanism presumably does not affect the other repressors. Various authors have suggested that turn off of E-cadherin activates the repressors to stimulate EMT. This idea assumes that by down-regulating E-cadherin, these molecules overcome E-cadherin's inhibitory effects on them (Fig. 19C) and now can turn on mesenchymal genes, such as those for vimentin, actin, myosin, bipolar morphology, and invasiveness (Peinado et al., 2003, 2004; Vega et al., 2004; mesenchymal markers, Fig. 19). Compatible with this idea is the fact that Snail is expressed

by fibroblasts and Slug by migrating neural crest cells (Nieto et al., 1994). Snail binds to the E-boxes (Fig. 19) of the E-cadherin promoter to inactivate E-cadherin, and thus induces embryonic EMTs, such as *Xenopus* neural crest (LaBonne and Bronner-Fraser, 2000). Snail and Twist are expressed in vivo in embryos, in cells transforming to mesenchyme (Soo et al., 2002). Not unexpectedly, the major signaling pathways for EMT in embryos and tumors, Wnt and TGFβ, up-regulate the E-cadherin repressors. For example, TGFβ family members stimulate synthesis of Snail (Peinado et al., 2003) and LEF-1 (Nawshad and Hay, 2003).

## PERSPECTIVES

Thus, it is likely that EMT has evolved as the result of the evolution of anti–E-cadherin transcription factors, such as these repressors (Snail, Slug, Twist, LEF-1, and so on). One could imagine that, over millions of years, several of the early signaling pathways acquired the ability to regulate EMT effector molecules and to use them to challenge the limitations of the sedentary epithelial cells to diversify the structure of living organisms. The two signaling pathways exhibiting the most flexibility (Wnt and TGFβ) gained control of early embryogenesis. The possibility that additional TGFβ pathways in the embryo might also evolve the ability to interact with Wnt pathways to use Smad 2/4 to activate LEF-1 along with or instead of β-catenin was predicted by Attisano's discovery (Labbe et al., 2000) that Smads can bind to one end of the LEF-1 molecule and β-catenin to the other. Similar interactions between Wnt and TGF-β signaling pathways during formation of Spemann's organizer have been reported by Nishita et al. (2000). TGFβ3-dependent embryonic EMTs using β-catenin/LEF-1 include neural crest and heart. Mouse palate uses Smad 2/4 to activate as well as synthesize LEF-1 (Nawshad et al., 2004). Mouse heart uses the Wnt EMT pathway (Liebner et al., 2004) and the avian heart uses both TGFβ2 and β3 as ligands for EMT in the formation of valve and septal mesenchymal cells. TGFβ2 and β3 have distinct roles to play in the chick and mouse heart as does slug, VE-cadherin, and Wnt (Camenisch et al., 2002). The Wnt pathway has been shown to be active in primitive streak EMT (Mohamed et al., 2004) and somite MET (Schmidt et al., 2004), and these two systems are candidates to use TGFβ as well. Further studies of these productive embryonic EMT and MET systems will surely reveal interactions with other early signaling pathways, such as Notch and the RTK pathways, that may regulate E-cadherin levels in the embryo.

In closing, it should be pointed out that, in the aging animal, the mechanisms that activate embryonic mesenchymal transformation may induce spreading of cancers through the ECM and cause several other pathological conditions, such as excessive fibrosis (Zeisberg and Kalluri, 2004). Many epithelia transform to mesenchyme when they are suspended within 3D collagen (Greenburg and Hay, 1982, 1986, 1988). For further discussion of EMT pathology, the reader is referred to reviews by Kinzler and Vogelstein (1997), Tan et al. (2001), Thiery (2002), Yang et al. (2004), and Kang and Massague (2004). The E-cadherin repressors Twist (especially) and Snail are involved heavily in activating neoplastic EMT (Soo et al., 2002; Nieto, 2002; Peinado et al., 2003; Yang et al., 2004). The TGFβ family is well known for producing metastatic tumors in the adult using Smad 2-, 3-dependent pathways for activating EMT mechanisms (Oft et al., 1998). The β-catenin/LEF-1 complex activates without Wnt in colon cancer (Kinzler and Vogelstein, 1997) because of mutations to APC that allow abundant amounts of β-catenin to be available for activating LEF-1–based cell metastasis. Because the basic EMT mechanism, repression of E-cadherin by LEF-1, is turned on by both TGFβ and Wnt signaling pathways, agents against LEF-1, as well as Snail, Twist, and other repressors, should be developed to try to prevent the inappropriate down-regulation of E-cadherin that can cause tumor metastasis in the adult.

A difficult question that remains to be researched is how can these cells that carry negative repressors of the E-cadherin gene be stimulated by the inhibition of E-cadherin to produce the effector molecules that create the amazing physiology of the mesenchymal cell. And even more profound questions await the developmental biologist. In the embryo, the mesenchymal cell moves with purpose away from its somite to follow signals emanating from distinct organs that induce it to become a fibroblast, chondroblast, or osteoblast. Or it may itself signal the epithelial cells of a developing organ using some of the pathways we have considered in this article for the cross-talk.

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