

TGF β ₃ signaling activates transcription of the LEF1 gene to induce epithelial mesenchymal transformation during mouse palate development

Ali Nawshad and Elizabeth D. Hay

Department of Cell Biology, Harvard Medical School, Boston, MA 02115

Epithelial mesenchymal transformation (EMT) of the medial edge epithelial (MEE) seam creates palatal confluence. This work aims to elucidate the molecular mechanisms by which TGF β ₃ brings about palatal seam EMT. We collected mRNA for PCR analysis from individual transforming MEE cells by laser microdissection techniques and demonstrated that TGF β ₃ stimulates lymphoid-enhancing factor 1 (LEF1) mRNA synthesis in MEE cells. We show with antisense β -catenin oligonucleotides that up-regulated LEF1 is not activated by β -catenin in palate EMT. We ruled out

other TGF β ₃ targets, such as RhoA and MEK1/2 pathways, and we present evidence using dominant-negative Smad4 and dominant-negative LEF1 showing that TGF β ₃ uses Smads both to up-regulate synthesis of LEF1 and to activate LEF1 transcription during induction of palatal EMT. When phospho-Smad2 and Smad4 are present in the nucleus, LEF1 is activated without β -catenin. Our paper is the first to show that the Smad2,4/LEF1 complex replaces β -catenin/LEF1 during activation of EMT in vivo by TGF β ₃.

Introduction

During the fusion of mouse embryo palatal shelves, some of the cells of the outer layers of the opposed medial edge epithelia (MEE) slough off, allowing the lateral surfaces of the underlying epithelial layers to contact each other and to adhere to form a midline seam. This seam subsequently transforms from epithelium completely to mesenchyme (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler et al., 1992). The formation of the MEE seam activates as yet unknown signals that endow component epithelial cells with a competence to respond to TGF β ₃ to create confluence of the palatal shelves by epithelial mesenchymal transformation (EMT). One cause of the birth defect, cleft palate, is failure in the EMT step of palate development, although failure of the other steps in palatogenesis, such as palatal growth, elevation, and adherence between paired shelves can also cause clefts. TGF β ₃ signaling has been known for sometime to be essential for palatogenesis (Karttinen et al., 1995; Proetzel et al., 1995), as it activates EMT in the MEE seam (Sun et al., 1998; Cui and Shuler, 2000). However, TGF β ₃ is not essential for adherence of the MEE seam (Sun et al.,

1998), which is brought about by E-cadherin-dependent desmosomes.

The evidence for EMT of the MEE seam consists of cytological descriptions and cell-tracing experiments showing that the adherent seam breaks up into islands that become mesenchyme (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler et al., 1992; Sun et al., 1998, 2000). The source of the mesenchymal cells could be identified with transmission electron microscopy (TEM) by the remnants of epithelium carried on their surfaces as they detached, e.g., desmosomes (Fitchett and Hay, 1989). The transforming epithelial cells send out filopodia that disrupt the basement membrane. The cells become elongated in cell shape and migrate into preexisting connective tissue, acquiring all the cytological features of mesenchymal cells (Fitchett and Hay, 1989). Griffith and Hay (1992) used carboxyfluorescein (CCFSE) for TEM, which is taken up by epithelia and packaged into insoluble isolation bodies. The latter allow labeled cells transforming from the MEE seam to be identified as epithelial in origin by TEM (Griffith and Hay, 1992). The advantage of CCFSE is that it is never transferred from cell to cell (Sun et al., 2000). Thus, elongated cells with mesen-

Address correspondence to Dr. Elizabeth D. Hay, Dept. of Cell Biology, Harvard Medical School, 220 Longwood Ave., B-1, Room 342, Boston, MA 02115-6092. Tel.: (617) 432-1651. Fax: (617) 432-0407. email: ehay@hms.harvard.edu

Key words: LEF1 transcription; Smad2; palate confluence; β -catenin; Smad pathways

Abbreviations used in this paper: AS, antisense; C3, *Clostridium botulinum* C3; DN, dominant negative; EMT, epithelial mesenchymal transformation; LCM, laser capture microdissection; LEF1, lymphoid-enhancing factor 1; MEE, medial edge epithelium.

chymal morphology derived from MEE are easily identified by TEM some distance from the midline in confluent palates (Griffith and Hay, 1992). Shuler et al. (1992) used *Dil* to document this transformation by light microscopy.

Thus far, the only major signaling pathway that has been consistently shown to be directly involved in transformation of epithelium to mesenchyme is β -catenin/lymphoid-enhancing factor 1 (LEF1; Hay, 2003; Kim et al., 2002), but whether or not β -catenin will activate LEF1 depends on specific isoforms of LEF/TCF transcription factor that contain an alternative COOH-terminal "E" tail (Atcha et al., 2003). This restriction raises the possibility of LEF1 activation by other non- β -catenin mechanisms. Recent papers by Labbe et al. (2000) and Nishita et al. (2000) have shown that LEF1 can also be activated by other factors, such as Smads. Another transcription factor that has been implicated is Snail by repressing E-cadherin (Cano et al., 2000). Previous works showed that activation of β -catenin/LEF1 is associated with c-Fos-induced EMT in mammary cells (Eger et al., 2000; El-Tanani et al., 2001) and with ILK-mediated EMT (Novak et al., 1998), as well as with metastasis in carcinomas (Morin et al., 1997; El-Tanani et al., 2001). Kim et al. (2002) demonstrated that LEF1 administered in an adenovirus directly induces EMT in colon carcinomas, and we show here that LEF1 is present in the epithelium of maxillary processes preparing to undergo EMT. LEF^{-/-} mice have severe craniofacial deformities (Duan et al., 1999), suggesting that the LEF1 gene plays an important role in cranial embryogenesis.

TGF β is a secreted cytokine that has a diversity of biological effects including pivotal roles during embryonic development (Nakajima et al., 1994; Boyer et al., 1999). Induction of EMT by TGF β has been studied in vitro in many different epithelial cells types, including mouse mammary cell lines (Miettinen et al., 1994; Piek et al., 1999) and human keratinocytes (Zavadil et al., 2001). In vivo, TGF β plays a role in cardiac valve induction and correlates with EMT (Runyan et al., 1992). The TGF β homodimer signals through the Smad pathway using transmembrane serine/threonine kinase receptors designated as TGF β type I (T β RI) and type II (T β RII) receptors (Lin et al., 1992; Wrana et al., 1992; Lutz and Knaus, 2002). Phospho-Smad3 is not present in the MEE (Cui et al., 2003), but phospho-Smad2 and 4 are well represented. Under ideal conditions, Smad2 phosphorylation and transport into the nucleus by Smad4 (Abdollah et al., 1997) is promoted by TGF β receptors (Itoh et al., 2003), early endosomes (Panopoulou et al., 2002), and Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998; Itoh et al., 2002). PI3 kinase, which can modulate SARA via FYVE, has also been shown to be involved in palatal EMT (Kang and Svoboda, 2002). In the nucleus, Smads bind to a specific DNA site (GTCTAGAC) and cooperate with various transcription factors in regulating target gene expression (Ten Dijke et al., 2002). The induced Smad2/Smad4 heteromeric complexes in polyamine-deficient cells are able to bind to this specific DNA site, suggesting that Smads mediate transcriptional activation (Liu et al., 2003).

Non-Smad pathways have also been implicated in TGF β signaling (Bhowmick et al., 2001; Roberts, 2002). There is

evidence in vitro that TGF β induces mesenchyme-like cells containing actin stress fibers independently of Smads, using the Ras-Raf-MEK-ERK (Mulder, 2000) and RhoA-Rac-MAPK-JNK-Jun pathways (Bhowmick et al., 2001; Roberts, 2002). However, production of stress fibers does not define EMT (Hay, 1995). Yu et al. (2002) demonstrated that TGF β signaling through MAPK/MEK does not initiate EMT. Kaartinen et al. (2002) reported that activation of the RhoA kinase pathway is not sufficient for palatal EMT. TGF β -induced EMT was found to require T β R, and thus to use Smad (Itoh et al., 2003). By inhibiting RhoA and MEK pathways, we show that they are not involved in palatal EMT.

Our major objective is to elucidate the roles of TGF β_3 and LEF1 during palatal EMT. We show that TGF β_3 acts via LEF1 to induce mouse palatal EMT not only by up-regulating LEF1 mRNA, but also by transcriptional activity of LEF1. Without Smads, palatal EMT cannot be achieved by LEF1. There is little or no involvement of β -catenin in the activation of palatal EMT by LEF1. This paper provides the first evidence of a Smad-activated LEF1 mechanism driving EMT in development. It is tempting to hypothesize that TGF β_3 -initiated Smad-dependent LEF1 pathways will prove to be the major regulators of embryonic EMT.

Results

First, we established that normal mouse (CF1) palates are completely confluent at 60 h (Fig. 1, A–D). Other mouse strains, such as Swiss Webster, usually take 72 h for complete transformation (Cui and Shuler, 2000). During normal palate development in vitro or in vivo in the CFW1 mouse, opposed palatal shelves adhere by 12 h after contact to form the 2–3-cell-wide MEE seam (Fig. 1 A, arrow) that breaks up into islands (Fig. 1 B) during transformation into mesenchyme. The transformation is well underway by 48 h (Fig. 1 C) and is complete by 60 h, when all cells in the MEE have achieved typical mesenchymal characteristics (Fig. 1 D).

The role of TGF β_3 in palatal EMT does not involve its non-Smad pathways

It has been proved experimentally that it is the EMT step of avian palatogenesis that requires TGF β_3 (Sun et al., 1998). As expected, treatment of mouse palates with blocking TGF β_3 antibody does not affect the cadherin-dependent adherence of the MEE seam, but does prevent its transformation to mesenchyme (Fig. 1, E and F; arrows). TGF β_3 -blocking antibody inhibits epithelial cell transformation to mesenchyme by preventing binding of endogenous TGF β_3 to the receptors that initiate EMT signals. The result is that the MEE fails to transform.

In addition to Smad pathways, TGF β has recently been shown to induce EMT in vitro by two non-Smad pathways, RhoGTPase-Rac-MEK-JNK (Bhowmick et al., 2001) and Ras-Raf-MEK1/2-ERK (Janda et al., 2002). We used *Clostridium botulinum* C3 (hereafter referred to as C3) to disrupt RhoA by inducing ADP ribosylation of RhoA protein at Asn41, which inactivates downstream signaling of Rho GTPases (Zubiaur et al., 1995). We used MEK1/2 inhibitor U0126 (Favata et al., 1998) to explore a possible role of this

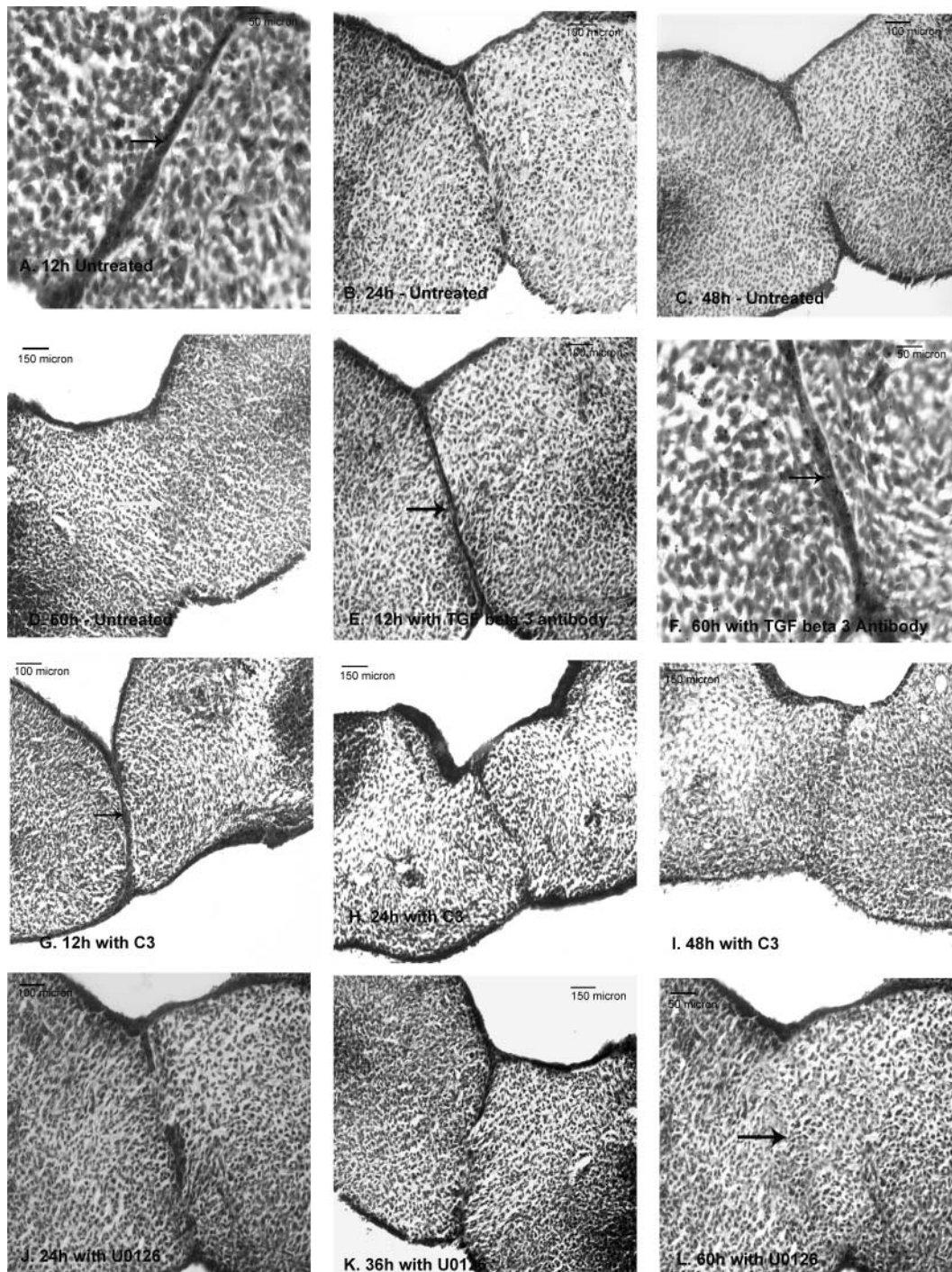


Figure 1. Effect of inhibitors of $TGF\beta_3$, RhoA, and MEK pathways on palatal EMT. Hematoxylin and eosin-stained sections of palates fixed at different stages of palatogenesis (A–D) and after treatment with $TGF\beta_3$ -blocking antibody (E and F), C3 (G–I) and U0126 (J–L). (A–D) Normal palatal shelves adhere to form the MEE seam by 12 h (A, arrow). Then, at 24 h the seam starts to break into small islands of epithelial cells (B). By 48 h, a few remnants of MEE are left (C). EMT is complete by 60 h (D, arrow). (E and F) Palates treated with anti- $TGF\beta_3$ antibody (2 ng/ μ L) form a seam (E, arrow) by 12 h, but do not transform the MEE by 60 h (F, arrow). (G–I) Palates treated with C3, a RhoA inhibitor, form an MEE by 12 h (G, arrow) that transforms into mesenchyme by 24–48 h (H and I). The palates reach complete confluence by 60 h (not depicted). (J–L) Palates treated with U0126, a MEK1/2 inhibitor, form MEE seams that also undergo transformation (J, 24 h; K, 36 h) to complete EMT at 60 h (L, arrow). There were no effects of RhoA inhibitor (C3) or MEK inhibitor (U0126) on normal palatogenesis.

pathway. With C3 or U0126, palatal shelves form MEE by 12 h (Fig. 1, G and J) and proceed (Fig. 1, H, I, and K) to complete the EMT by 60 h (Fig. 1 L), the same as normal palates (Fig. 1, A–D). Thus, MEK1/2 and RhoA have no detectable effects on $TGF\beta_3$ -mediated palatogenesis.

We evaluated the effects of these inhibitors by quantitating morphological data (Fig. 2). Fig. 2 shows the number of cells in the MEE seam at different times (12–60 h) of palate development. They were counted in cross sections of frozen palates using Leica software for laser capture microdissection

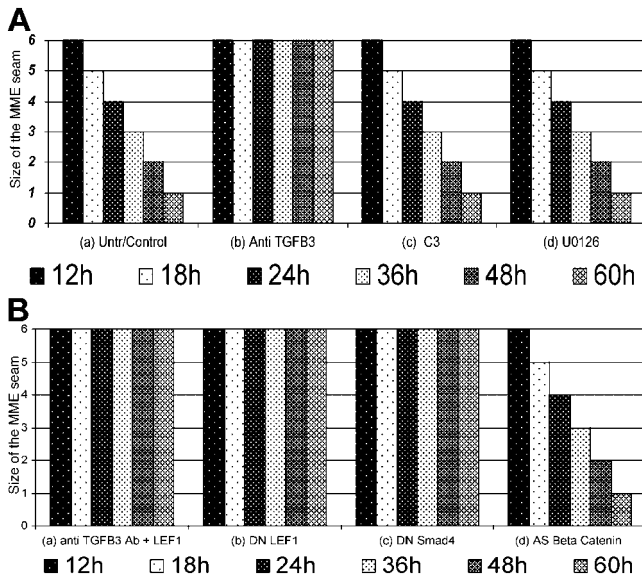


Figure 2. Quantification of disappearance of cells from epithelial seam during EMT. Using LCM on 8- μ m-thick frozen sections from the middle one third of the palate, we estimated cell numbers in each section of the MEE seam from 12 to 60 h after the beginning of the culture. On the ordinate, we estimated seam transformation by the number of cells remaining in it. Stage 1, 0 cells (confluence); stage 2, 1–19 cells; stage 3, 20–39 cells; stage 4, 40–59 cells; stage 5, 60–76 cells; stage 6, untransformed seam (75 cells). Palates were treated from the beginning of culture with the following reagents: TGF β ₃-blocking antibody, TGF β ₃-blocking antibody plus LEF1, RhoA inhibitor C3, MEK1/2 inhibitor U0126, DN LEF1 virus, DN Smad4 adenovirus, and AS β -catenin. A (a) is the untreated control. Length of time is indicated by the decoration inside the graph bars. Untreated/control palates are confluent by 60 h (A, a), and palates treated with anti-TGF β ₃ antibody do not transform (A, b). Treatment with C3 (A, c), U0126 (A, d), and AS β -catenin (B, d) have no effect on MEE seam transformation. LEF1 does not rescue blockage by anti-TGF β ₃ antibody (B, a). Treatment with DN LEF1 (B, b) or DN SMAD4 (B, c) inhibits transformation.

(LCM; see Materials and methods). A section of untransformed seam contains 62–96 epithelial cells with an average of 76. We staged palatogenesis according to the number of epithelial cells left in sections of the seam (see legend for Fig. 2). The untreated control is shown in Fig. 2 A (a). The intact seam is stage 6 at 12 h; 60–76 cells remain in the seam at 18 h, stage 5; 40–59 cells at 24 h, stage 4; 20–39 cells at 36 h, stage 3; 1–19 cells at 48 h, stage 2; and no cells, representing complete transformation (confluence), at 60 h, stage 1.

C3 (Fig. 2 A, c) and U0126 (Fig. 2 A, d) are seen in this graph to have no effect on palatal EMT. The timing of stages 1–6 is exactly the same as the control (Fig. 2 A, a). The data shown here also confirm the role of TGF β ₃ in palatal EMT. Treatment with TGF β ₃ antibody arrests the MEE seam at stage 6 (Fig. 2 A, b). These data strongly support the idea that endogenous TGF β ₃ does not cause EMT in this system by non-Smad pathways.

β -Catenin remains in the cytoplasm during palatal EMT

Next, we tested the hypothesis that TGF β ₃ induces palatal EMT via the β -catenin/LEF1 or Wnt pathway, because it has recently been shown to directly mediate a number of EMTs (Kim et al., 2002). We found that the MEE does not

transform at all in presence of dominant-negative (DN) LEF1 (Fig. 2 B, b; remain at stage 6), indicating that the β -catenin/LEF1 pathway is needed for palatal EMT.

To examine these interrelations, we analyzed the effects of TGF β ₃ on LEF1 mRNA synthesis and subsequent transport of β -catenin/LEF1 to the nucleus. To our surprise, we detected no β -catenin in the palatal nuclei before, during, or after EMT in untreated palates (Fig. 3, A and B), nor in palates treated with either LEF1 (Fig. 3, C–F) or DN Smad4 (Fig. 3 G), indicating β -catenin paucity characterizes normal palates. Because immunofluorescence is not a quantitative method, we cannot rule out the presence of very small amounts of β -catenin in these nuclei. However, the data are clear cut enough to support the hypothesis that palatal LEF1 transcriptional activity depends on Smads, not β -catenin (Labbe et al., 2000).

To explore the possible role of β -catenin, which normally is responsible for activation of LEF1 transcription, in palatogenesis, we treated normal palates undergoing EMT with sense and antisense (AS) β -catenin oligonucleotide and found that AS β -catenin significantly reduces β -catenin protein levels compared with sense (Fig. 3, H and I). However, AS β -catenin has no inhibitory effects on EMT at all (Fig. 2 B, d). Thus, normal palate does not use β -catenin for EMT.

LCM and quantification of LEF1 mRNA during palatal EMT

To study the process further by which TGF β ₃ up-regulates LEF1 during palatal EMT, we used a new technique, LCM, to obtain cells from the MEE seams to measure their metabolism by highly sensitive real-time PCR. We demonstrated that LEF1 gene expression reaches a peak in MEE cells at the onset of EMT during palate development. Endogenous LEF1 mRNA is expressed at 12 h (Fig. 4 A, a) and gradually increases to a peak by 36 h of incubation, dropping at 48 h before complete confluence at 60 h, when no MEE epithelial cells remain. The high level of LEF1 mRNA expression at 36 h is at the high point of transformation by the normal epithelial seam. Even though LEF1 levels fall after 36 h, enough is present for cells to complete transformation. When normal palates were treated with exogenous LEF1 virus, the expression of LEF1 mRNA by MEE cells increased over the control to reach peak by 24 h instead of 36 h, and decreased by 48 h (Fig. 4 A, b).

By quantitative PCR, we also show that palates treated with blocking antibodies to TGF β ₃, DNLEF1, and DN Smad4 do not express LEF1 mRNA (Fig. 4 A, c and d; Fig. 4 B, c). MEE seams treated with TGF β ₃-blocking antibody (Fig. 1, E and F; Fig. 2 A, b), DN LEF1 (Fig. 2 B, b), or DN Smad4 (Fig. 2 B, c) remain in stage 6, i.e., they do not transform. Inhibition of the endogenous expression of LEF1 mRNA (Fig. 4 A, c and d; Fig. 4 B, c) results in no transformation of MEE seam.

As LEF1 mRNA synthesis and seam EMT are inhibited by TGF β ₃-blocking antibody, DN LEF1, or DN Smad4, we expected that exogenous LEF1 would rescue inhibition of palatal EMT by TGF β ₃-blocking antibody (Fig. 2 B, a), but it did not. This was a surprise because the DN LEF1 results (Fig. 2 B, b; Fig. 5 E) indicate that LEF1 is required for EMT. Correlations indicate that LEF1 mRNA peaks at 36 h

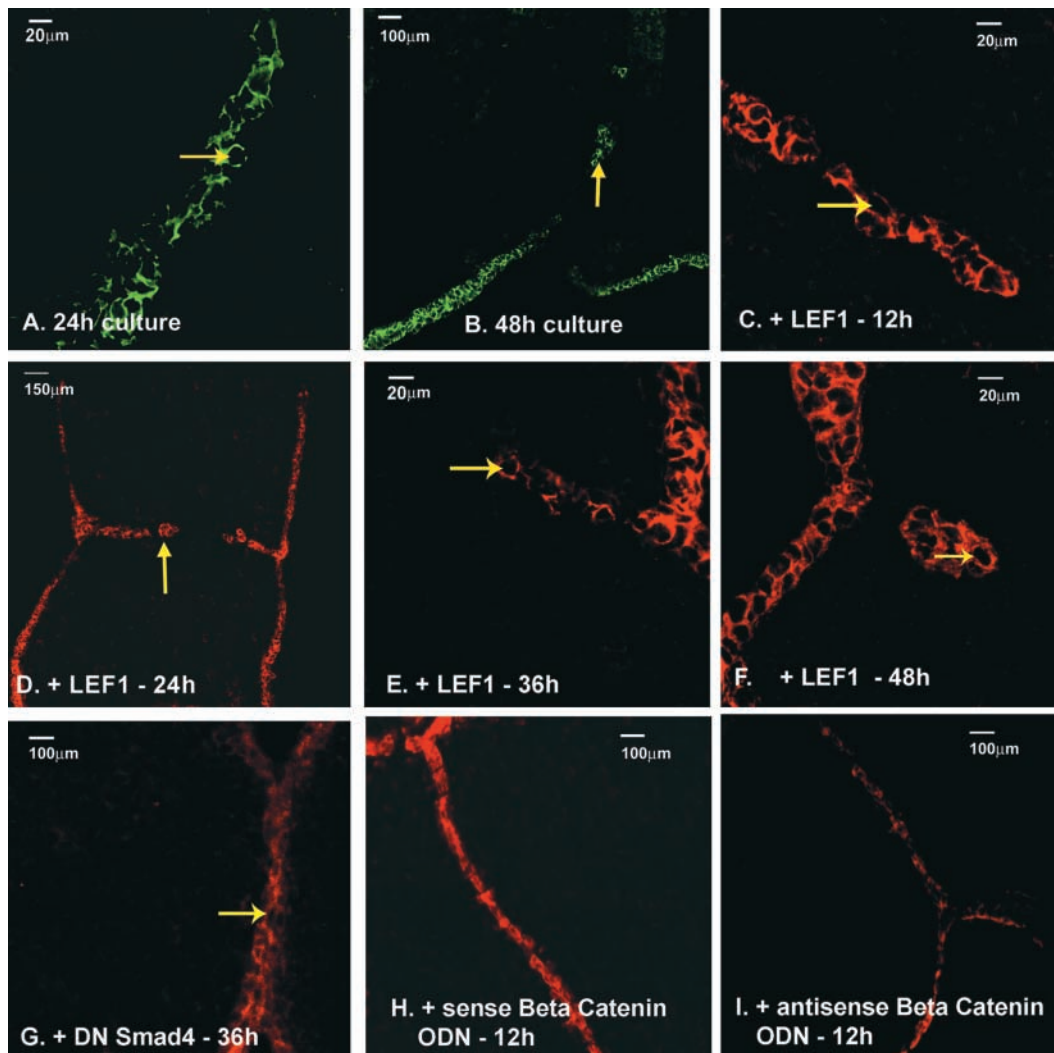


Figure 3. Expression of β -catenin in treated and/or untreated palates during different stages of palatogenesis. A and B are stained with a fluorescein antibody (green) that recognizes β -catenin. C–I are stained with a rhodamine antibody (red) that recognizes β -catenin because some of the probes used there (e.g., LEF1) contained GFP. (A and B) β -Catenin expression can be detected by immunofluorescence in untreated paired palates. β -Catenin is located on the cell surface and/or cytoplasm of the MEE and transforming epithelial cells (A, 24 h; B, 48 h), but not in nuclei (arrows). (C–F) Administration of the LEF1 adenovirus fails to up-regulate β -catenin in the nuclei (arrows) of the transforming seams (C, 12 h; D, 24 h; E, 36 h; F, 48 h). (G) Administration of DN Smad4 adenovirus also does not affect pattern of β -catenin expression as compared with normal palatal MEE (E). Rhodamine-conjugated β -catenin antibody staining reveals expression of β -catenin only within the cytoplasm. (H and I) AS β -catenin oligonucleotide (I) significantly inhibits β -catenin expression, but sense treatment does not (H). There is no inhibitory effect of these oligodeoxynucleotides on EMT, because palatal EMT is not dependent on β -catenin (see text).

and remains significantly up-regulated until EMT is completed at 60 h (Fig. 4 A, a). The amount of LEF1 mRNA is substantial (Fig. 4 B, a). This result can be explained by a need for a constant source of phospho-Smad2 not only to up-regulate levels of LEF1, but also to activate LEF1 transcription (Attisano and Tuen Lee-Hoeflich, 2001) in order to bring about EMT during palatogenesis.

We also used real-time quantitative PCR techniques to compare the effects of Smad-independent signaling on LEF1 mRNA levels during the EMT phase of palatogenesis when non-Smad pathways are inhibited. Inhibition of RhoA with C3 (Fig. 4 B, d) and MEK1/2 with U0126 (Fig. 4 B, e) did not alter normal LEF1 mRNA levels or affect palatal EMT. AS β -catenin also has no effect on LEF1 mRNA (Fig. 4 B, b), confirming that it is not involved in normal palatal EMT.

LEF1 and Smads interact to mediate the effects of $TGF\beta_3$ on palatal EMT

It is well within the realm of possibility that many natural EMTs, regulated by $TGF\beta_3$, use Smad/LEF1 instead of β -catenin complexes to activate LEF1. That this is so for the palate is shown by the following data. We showed that AS β -catenin does not inhibit normal MEE transformation (Fig. 2 B, d; Fig. 5, A–D). Thus, β -catenin is not normally used in palatal EMT. We confirmed that morphology of the AS β -catenin-treated palates is the same as normal palates (Fig. 5, A–D). They reach confluence by EMT at exactly the same time as normal palates (Fig. 1, A–D; Fig. 2 A, c and d). Because β -catenin has no role in palatal EMT, it is not a reasonable candidate for the necessary interactions with LEF1 during activation of LEF1 transcription. Palate EMT must be using Smad/LEF1, which already has

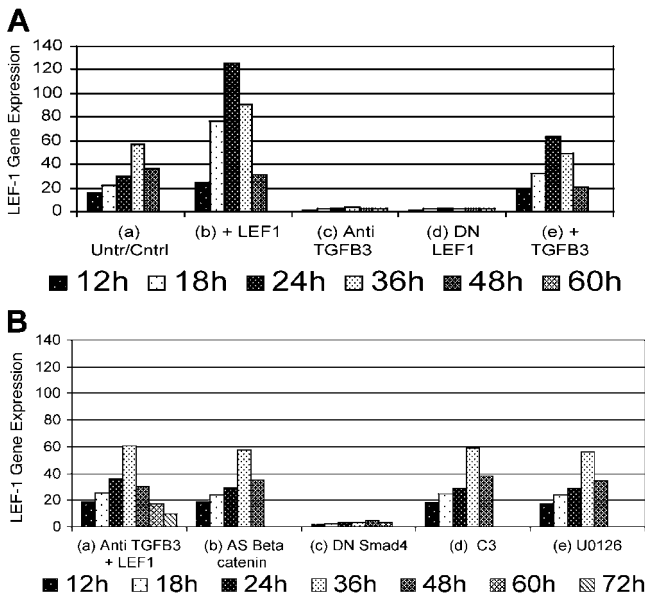


Figure 4. LEF1 gene expression (real-time quantitative PCR). LEF1 mRNA expression was measured by highly sensitive real-time PCR in LCM-dissected MEE cells from frozen sections. (A) Palates were treated with exogenous LEF1 virus, anti-TGF β ₃ antibody, DN LEF1 virus, and exogenous recombinant TGF β ₃. (B) Palates were treated with exogenous LEF1 virus plus anti-TGF β ₃-blocking antibody, AS β -catenin oligodeoxynucleotide, DN Smad4 adenovirus, C3, a Rho inhibitor, and U0126, a MEK1/2 inhibitor. Length of time is indicated within each graph bar, as in Fig. 2. (A, a) In the untreated palates (Untr/Cntrl), LEF1 expression peaks at 36 h, at which time EMT also peaks. (A, b) In presence of exogenous LEF1, there is a large increase in LEF1 mRNA expression that peaks at 24 h instead of 36 h. (A, c) TGF β ₃ inhibition by blocking antibody causes complete inhibition of LEF1 mRNA expression and EMT (A, d) DN LEF1 causes palates to cease endogenous LEF1 mRNA expression and remain untransformed. (A, e) Exogenous TGF β ₃ causes LEF1 mRNA and protein to up-regulate and reach a peak at 24 h without significantly hastening the EMT process. (B, a) Exogenous LEF1 DNA increases LEF1 mRNA, but the seam remains untransformed if palates are treated with TGF β ₃-blocking antibody. (B, b) AS β -catenin did not affect LEF1 mRNA expression as compared with the control (A, a). (B, c) DN Smad4 inhibits LEF1 mRNA expression and MEE remains untransformed. (B, d) RhoA inhibitor C3 had no effect on normal LEF1 mRNA expression or EMT, (B, c) and neither did MEK1/2 inhibitor U0126 (B, e).

been shown by others to activate LEF1 transcription (Labbe et al., 2000).

To restore EMT in TGF β ₃-blocked palates treated with LEF1 (Fig. 2 B, a), active TGF β ₃ and LEF1 are required. We showed here that TGF β ₃ only promotes palatal EMT through Smads and LEF1-dependent pathways. Thus, the addition of TGF β ₃ undoubtedly adds the Smads that are needed to activate LEF1. We showed that palates treated with either DN LEF1 or DN Smad4 adenovirus do not undergo EMT (Fig. 2 B, b and c), and LEF1 mRNA expression is significantly reduced (Fig. 4 A, d; Fig. 4 B, c). The palatal shelves adhere to form normal MEE, but the EMT process is completely abolished by DN Smad4, and MEE remain untransformed (Fig. 5 F). These palates recover completely when phospho-Smads are available to interact with LEF1. This interaction has been documented and analyzed (Labbe et al., 2000), and is the only known way that LEF1

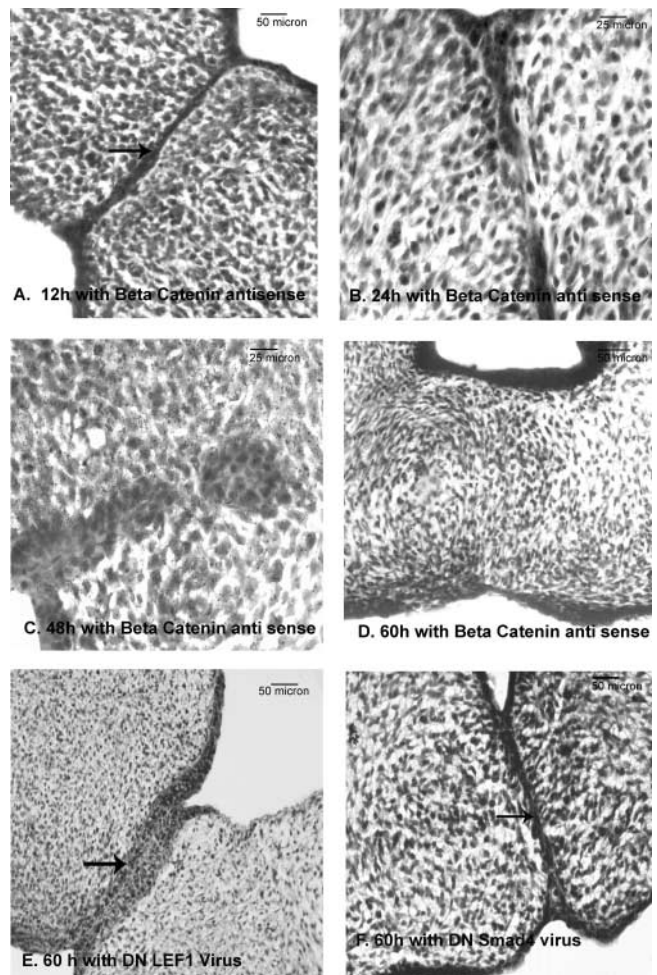


Figure 5. Palates treated with DN LEF1 and DN Smad4 do not undergo EMT, but normal EMT follows treatment with AS β -catenin oligodeoxynucleotide. Hematoxylin and eosin staining of palate sections 12–60 h after treatment DN LEF1 and DN Smad4 adenoviruses or 60 h with AS β -catenin from the beginning of culture. (A–D) Developing palates 12 h after treatment with AS β -catenin have formed an MEE seam (A, black arrow), and EMT is underway by 24 h (B) and 48 h (C) after treatment. Confluence is reached by 60 h (D, white arrow) without β -catenin. Thus, palatal EMT does not normally use β -catenin/LEF1, and in some other way activates LEF1 transcription. (E and F) DN LEF1 (E) and DN Smad4 (F) treated palates fail to transform the MEE to mesenchyme by 60 h (black arrows). Thus, palatal EMT requires active LEF1 and active Smads.

transcription can be activated in the absence of β -catenin. Moreover, we show below that phospho-Smad2 must be in the nucleus (Fig. 6) to activate LEF1 transcription, where it interacts with LEF1.

Smad2 is activated and present in the nucleus during palatal EMT

Phospho-Smad2, an important signaling molecule of the TGF β ₃ pathway, can easily be detected in the nucleus of normal MEE seam cells by antibodies to phospho-Smad2 protein (Fig. 6 A, arrow), but phospho-Smad3 is not present (Cui et al., 2003). Phospho-Smad2 remains in the MEE cytoplasm when palates are treated with DN Smad4 (Fig. 6 B, arrow), because active Smad4 is not available to

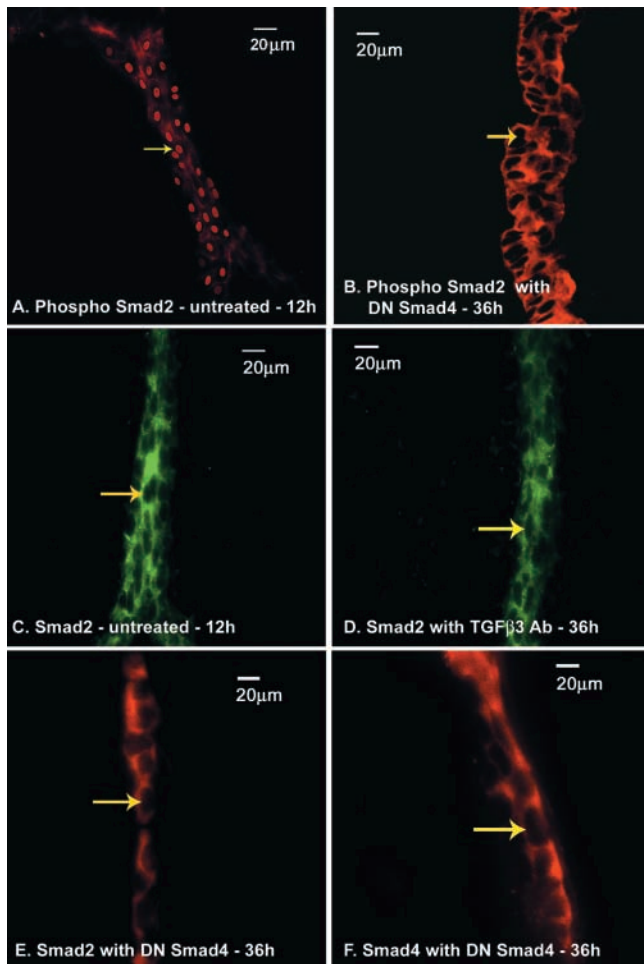


Figure 6. Immunolocalization of Smad2, phospho-Smad2, and Smad4 in untreated and treated (TGF β ₃-blocking antibody and DN Smad4) palates. (A) Phospho-Smad2 is intensely present in the nuclei of normal palatal MEE (12 h), indicating it is translocated by Smad4 into the nucleus (arrow) during normal palatogenesis. (B) Phospho-Smad2 in MEE of palates treated (36 h) with DN Smad4 is located in the cytoplasm and no nuclear localization occurs (arrow), confirming that Smad4 is required for nuclear translocation of phospho-Smad2. (C) Unphosphorylated Smad2 is located in the cytoplasm of MEE cells not in the nucleus (arrow) of palates lacking Smad4 after 12 h of culture. (D) Smad2 is also located in cytoplasm of the MEE cells (36 h), but not in the nucleus (arrow) in the presence of blocking TGF β ₃, confirming that TGF β ₃ is required for the phosphorylation of Smad2. (E) Smad2 in MEE of palates (36 h) treated with DN Smad4 is located in the cytoplasm of the cell, but not in the nucleus (arrow). At this late phase, the MEE is still untransformed. (F) DN Smad4 inhibits the transport of Smad4 itself to the nucleus. Thus, nuclear Smad2/4 is not available to activate LEF1, and these palates do not transform.

transport it to the nucleus (Fig. 6 C, arrow). As expected, when treated with blocking TGF β ₃ antibody, palates do not express phospho-Smad2 nuclear proteins (unpublished data), and only cytoplasmic Smad2 protein is present in the MEE cells (Fig. 6 D). A similar pattern (absence from the nucleus) was always observed in DN Smad4-treated palates (Fig. 6 E). Smad4 is also in the cytoplasm (Fig. 6 F) when palatal shelves are treated with DN Smad4, effectively inhibiting Smad4 nuclear translocation and EMT, confirming that the Smad2–Smad4 complex is responsible

for TGF β ₃-mediated LEF1 mRNA synthesis and activation during palatal EMT.

Discussion

In this work, we investigate the nature of the TGF β ₃-initiated signaling pathways that bring about EMT of the palatal MEE. By PCR, we found that TGF β ₃ up-regulates LEF1 mRNA, and by administering DN LEF1, that LEF1 is required for TGF β ₃ to induce palatal EMT. However, infection with an adenovirus expressing LEF1 that induces EMT in β -catenin-rich DLD1 cells (Kim et al., 2002) does not rescue MEE EMT in palates blocked with anti-TGF β ₃. Thus, we found that TGF β ₃ needs to be present in addition to LEF1 to bring about EMT in these palates.

The discussion will focus on proposed mechanisms for this newly described phenomenon (Labbe et al., 2000), whereby the TGF β ₃ ligand turns on a series of events activating T β Rs and Smads, to up-regulate LEF1 synthesis and transcription, thereby causing palatal epithelial cells to transform into mesenchymal cells. Thus, confluence by EMT of a fusing embryonic organ in situ can be achieved in a highly sophisticated manner.

LEF1 promotion of palatal EMT and LEF1 mRNA is dependent on TGF β ₃ signaling

This is the first paper reporting that LEF1 mRNA is up-regulated in the MEE during palatal EMT. Expression of the LEF1 transcription factor is increased as EMT proceeds and reaches a peak between 24 and 36 h. The mRNA level then gradually falls, and the transformation is complete by 60 h. We used LCM on an embryonic organ (palate) to dissect individual MEE cells for analysis of LEF1 gene expression and quantification with real-time PCR. This is an important new technology that permits single cells to be microdissected as a pure population for chemical analysis from frozen sections of embryonic anlage.

To understand the unexpected enigma mentioned above that inhibition of palatal EMT by adding TGF β ₃-blocking antibody is not rescued by addition of pure LEF1, it is necessary to consider the mechanisms cells use to activate LEF1 transcription that might be in use by the palate MEE. In tumors and cell lines, it is common for β -catenin to activate LEF1/TCF transcription (Morin et al., 1997; Eger et al., 2000; Kim et al., 2002), but an astonishing variant of this mechanism has recently been reported by Labbe et al. (2000) and Nishita et al. (2000). Smads, which can only be activated by TGF β , have been shown to activate LEF1 transcription. Thus, MEE disappearance by EMT could be dependent on both TGF β ₃ and LEF1 expression. Once TGF β ₃ ligands are bound to an appropriate receptor regime, the MEE seam is the target for TGF β ₃ signaling involving Smads2/4 and LEF1. Here, we show that both synthesis and transcription of LEF1 are activated by Smads as predicted by Labbe et al. (2000) and Nishita et al. (2000)

Smads, not β -catenin, activate LEF1 mRNA synthesis and transcription during palatal EMT

β -Catenin is an important partner in LEF1 transcriptional factor activity, and when LEF1 is bound to β -catenin, LEF1

transports it to the nucleus (Behrens et al., 1996; Kim and Hay, 2001). Interaction of LEF1 with nuclear β -catenin is known to effect Wnt signaling by stimulating the activity of LEF1/TCF transcription factors (Eastman and Grosschedl, 1999). β -Catenin is such an important component of LEF1 transcriptional activity in a variety of EMTs in vitro and in vivo (Novak et al., 1998; Eger et al., 2000; El-Tanani et al., 2001) that we expected to find it well represented in the nuclei of palate cells using LEF1 for EMT.

It came as surprise, then, to find no detectable β -catenin in the nuclei of transforming MEE cells. Abundant β -catenin is localized in the cytoplasm and cell surface during all stages of normal palate development, but treatment with exogenous LEF1 did not move it to the nucleus. Even if a minute amount of β -catenin is present in MEE nuclei that is not detectable by immunofluorescence, it is unlikely that significant β -catenin signaling through LEF1 could transform in the MEE cells. Next, we looked at the possibility that normal palates are not using β -catenin-activated LEF1 to transform the MEE to mesenchyme. We treated palates with AS β -catenin and found it did not inhibit palatal EMT. Thus, palates must be using some other activator for LEF1.

Smads are the obvious candidates to interact with LEF1 to activate its transcription (Labbe et al., 2000). We used DN Smad4 to show that Smads are indeed essential for activation of LEF1 leading to palatal EMT. Moreover, we showed by immunohistochemistry that phospho-Smad2 is present in nuclei of the normal MEE cells during EMT at the time that LEF1 is up-regulated by TGF β ₃. DN Smad4 abolishes all nuclear Smads. We conclude that TGF β ₃-mediated cell signaling during palatogenesis is not β -catenin dependent, and that LEF1 transcriptional activity is solely Smad dependent during palatal EMT.

Considering the wide-spread and successful use of β -catenin to activate the LEF1 transcription factor, one might ask why use Smads instead? There may be disadvantages in using β -catenin to activate LEF1 in some systems (Ishitani et al., 2003). If β -catenin is bound with LEF1, TGF β may not be able to dissociate the complex (Sasaki et al., 2003). It may be that in vivo, it is easier to control LEF1 with Smads during TGF β ₃ signaling in embryos, leaving β -catenin under separate control in Wnt pathways. In the adult, while TGF β signaling can be involved in carcinogenesis (Oft et al., 1998; Cohen, 2003), the β -catenin pathway is plagued by malignant mutations.

TGF β ₃ promotes Smad pathway, not Smad-independent pathways, in the MEE seam

Smads can only be activated by members of the TGF β superfamily using specific receptors (Piek et al., 1999; Dennler et al., 2002). TGF β induces phosphorylation of Smad2, which is known to activate the LEF1 transcription factor (Labbe et al., 2000; Nishita et al., 2000). Itoh et al. (2003) have demonstrated that TGF β -induced EMT requires intact T β RI, and thus is dependent on Smad pathway. Our data indicate that only Smad-dependent pathways regulate palatal EMT. Phospho-Smad2 is strongly expressed in the nuclei of the transforming MEE. When palates are treated with DN Smad4, Smad2 remains inactive in the cytoplasm because its nuclear translocation depends on shuttle by Smad4. With

TGF β ₃-blocking antibody or DN Smad4, there is no expression of phospho-Smad2/4 in the MEE nuclei, raising the possibility that LEF1 mRNA up-regulation depends on both phospho-Smad2 and phospho-Smad4. Nishita et al. (2000) found that both Smad3 and Smad4 activated LEF1 transcriptional activity in the systems they analyzed. In the palate, Smad2 substitutes for Smad3 (Cui et al., 2003).

Although the Smad2/4 pathway is the only one likely to be involved in palatal EMT, several investigators recently have proposed TGF β ₃ signals EMT by Smad-independent signaling. These pathways are RhoA-Rac-MEK-JNK (Yu et al., 2002) and/or Ras-Raf-MEK-ERK (Mulder, 2000). C3, a specific inhibitor of RhoA kinase, blocks RhoA-Rac pathway (Kaartinen et al., 2002; Smith et al., 2003). U0126 completely inhibits MEK1/2, which takes place further downstream (Kretschmar et al., 1999), and abolishes the pathway involving Ras-Raf-MEK (Adnane et al., 2002). The RhoA pathway of TGF β has been shown to activate in palates, but not to induce significant EMT (Bhowmick et al., 2001; Kaartinen et al., 2002). Another paper concluded that TGF β ₁ only induces EMT through the RhoA pathway (Bhowmick et al., 2001). We found that treatment of palates with inhibitors of RhoA (C3) and MEK1/2 (U0126) does not affect LEF1 mRNA expression or the palate EMT. The fact that DN Smad4 inhibits palatal EMT also indicates that the Smad-dependent TGF β ₃ signaling is required for activation of palatal EMT.

In summary, this work has investigated the remarkable cooperative roles of TGF β ₃ and LEF1 in bringing about EMT in palatogenesis. The discovery that LEF1 is required for palatal EMT is completely new. Also, for the first time we present evidence that TGF β ₃ up-regulates LEF1 mRNA and that Smads, not β -catenin, activate LEF1 in this embryonic EMT. Our analysis of the palate extends the results of Labbe et al. (2000) and Nishita et al. (2000), and makes it tempting to speculate that embryos in general might use the TGF β pathway to turn on EMT via LEF1. This finely tuned pathway may not be subject to the extensive mutations that frequently occur in β -catenin/LEF1 pathways.

Materials and methods

40 timed pregnant CF1 mice (Charles River Laboratories) were anesthetized with peritoneal Avertin (2,2,2-tribromoethanol with Tert-amyl alcohol; Sigma-Aldrich). Palatal shelves from 300 mouse embryos at a stage (E14) of palate development before fusion of the shelves were dissected under sterile conditions and placed in pairs on TSO-Agarose in organ culture dishes. Tissues were incubated at 37°C in a humidified gas mixture (5% CO₂ and 95% air), and the TSO medium was changed every 24 h.

Before and after placing palatal shelves together in pairs, they were treated with the following molecules alone or in combination: (1) GFP full-length LEF1 adenovirus. Full-length LEF1 with GFP was subcloned into pAdEasy™ EGFP plasmid expressing full-length active LEF1 and GFP under different CMV promoters using the adenovirus construction kit supplied by Dr. B. Vogelstein (The Johns Hopkins School of Medicine, Baltimore, MD). Generation of active LEF1 virus and DN LEF1 virus was as described previously (He et al., 1998), and all virus probes were carefully tested for activity (Kim et al., 2002). Palates transfected with 200 μ l of 1.0 μ g/ml LEF1 adenovirus in TSO buffer express LEF1 and undergo EMT. (2) GFP DN LEF1 adenovirus. A LEF1 construct coding aa 1–373 in the EVR-FL9b plasmid was the gift of Dr. Marian Waterman (University of California, Irvine, Irvine, CA). A Kozak sequence, KPN1 restriction site, and start site were inserted upstream of the LEF1 5' amino acid, and a stop codon and Xba restriction site were added downstream of aa 313 (bp 939) to delete the NLS of B box, resulting in failure in nuclear transportation. The

PCR fragment was inserted into pAdEasy™ EGFP plasmid. The resulting construct was recombined with adenoviral genome using the Vogelstein kit. Palates were transfected as above. All viruses were replication defective. (3) GFP DN Smad4 adenovirus. A recombinant adenovirus expressing a DN mutant Smad4 (AdMSmad4, pCMV5/Smad4) (1–514), a gift of Dr. D.M. Simeone (University of Michigan, Ann Arbor, MI), containing a COOH-terminal truncated DN Smad4 gene, was subcloned into the adenovirus vector. The DN Smad4 construct was bluntly ligated into the EcoRV site of the shuttle vector (pAdTrack) as described by He et al. (1998). Recombination was confirmed using multiple restriction enzyme digest analyses. The linearized recombinant plasmid was packaged into infectious adenoviral particles by transfecting HEK 293 cells using LipofectAMINE™ PLUS reagent, and the recombinant adenovirus was harvested after 7–10 d. Recombinant adenoviruses were screened for expression of the introduced genes by fluorescent microscopy. (4) 2 ng/μl anti-mouse TGFβ₃-blocking antibody (R&D Systems). (5) 2 ng/μl recombinant TGFβ₃ (R&D Systems). (6) AS β-catenin oligonucleotides 5'-GGAGTTAACCACAACAGGCGAGTCC-3' and control sense 5'-CCT-GACGGACAACCAATTGAGG-3', as described by Haertel-Wiesmann et al. (2000), used by Brunet et al. (1995), were tested in our laboratory. (7) 15 μM MEK1/2 inhibitor UO126 (Cell Signaling). (8) RhoA inhibitor C3 (Calbiochem). Palates were treated with 10 μg/ml LipofectAMINE™ for 2 h before C3 treatment. The C3 coenzyme (30 μM), a toxin derived from *C. botulinum*, is an ADP ribosyltransferase that catalyzes mono-ADP ribosylation of the small GTP-binding proteins Rho (A, B, and C) at asparagine 41 (Wilde et al., 2002). Because ADP ribosylation occurs in the putative effector region of Rho, this modification interferes with correspondent Rho GTPase-dependent signaling pathways (Wilde et al., 2002). C3 is widely used in cell biology as a convenient tool to specifically inactivate Rho proteins (Borbiev et al., 2000).

Palatal shelves were routinely incubated 1 h before placing them together, and the medium was then changed every 24 h. Adenovirus, AS β-catenin, and C3 treatments (Lu et al., 2001) in single palates were continued for 48 h. Palates were fixed in Bouin's fixative before embedding in paraffin or cryomold tissue freezing medium, TBS (Triangle Biomedical Sciences) for sections. We only collected prime sections from the middle one third of the palate.

Evaluation of adenovirus

All our adenovirus experiments were based on earlier published experiments (Kim and Hay, 2001) in which DLD1 cells exposed to 200 μl/ml full-length LEF1 virus were shown to undergo EMT promptly and to express LEF1 (Kim and Hay, 2001). Previously, we did tests to confirm the titration and dilution, and optimized the protocol. Adenovirus treatment continued in single palatal shelves until GFP protein is visualized by microscopy confirming viral protein production, and then shelves were placed together for further incubation. Most cultured palates were collected at 12–60 h. We found that the virus does not hurt the cells, and there are no morphological changes due to viral infection.

The LEF1 construct used for the DN virus probe specifically lacks its B box for nuclear export. The DN LEF1 plasmid was also administered in 200 μl/ml virus and effectively blocked LEF1 mRNA expression (Fig. 4 A, d). The DN Smad4 virus, which lacks COOH terminus for its nuclear translocation, abolished both Smad4 and Smad2 from the nuclei of the palate MEE cells. Palatal MEE cells were stained with Smad4 antibody to evaluate effects of DN Smad4 (Zhang et al., 2001), which shows that protein expression is exclusively in the cytoplasm of the MEE cells (Fig. 6 F). Both DN LEF1 and DN Smad4 significantly reduce LEF1 expression (Fig. 4 A, d; Fig. 4 B, c).

Evaluation of chemical inhibitors

Two chemical inhibitors were used at concentrations recommended by the manufacturer that had been shown to eliminate RhoA by C3 (Lu et al., 2001) and MEK1/2 with UO126 (Chow et al., 2001). To optimize dose and confirm the effects of TGFβ₃-blocking antibody, recombinant TGFβ₃, AS β-catenin, and UO126 and C3, we stained the palatal sections with TGFβ₃ antibody (Santa Cruz Biotechnology, Inc.), anti-β-catenin antibody (Sigma-Aldrich), phospho-p44/42 MAPK (Thr202/Tyr204) E10 antibody (Cell Signaling), and anti-RhoA antibody, respectively.

LCM

To study the EMT process in palatal MEE, uncontaminated by existing mesenchyme, analysis of pure epithelial and mesenchymal cell populations is necessary. We isolated epithelial cells dissected from the MEE of 300 pairs of palates by highlighting individual cells from the intact MEE and transforming MEE using Leica software. (1) Cutting and staining. The block is at-

tached to the chuck in the cryostat with OCT™. 8-μm sections were cut onto special slides (Leica) coated with plastic foil. We used HistoGene™ LCM staining (Arcturus) for frozen sections. HistoGene™ stains nuclei bright blue without degrading nucleic acids and yields high quality RNA. Stained sections were immediately processed for LCM. (2) Dissection. Using built-in software (IM1000) of Leica LCM, we were able to highlight MEE cells and transforming epithelial cells. Highlighted cells were cut using a laser beam (7.2 mW at 30 Hz) and collected in sterile plastic tubes. After microdissection (roughly 1,000 cells from each sample), the collecting tube containing cells in lysis buffer (β-ME and RLT; 50 μl buffer with 0.04% proteinase K, 10 mM Tris-HCL, pH 8.0, 1 mM EDTA, and 1% Tween 20) is capped and homogenized. RNeasy® mini kit (QIAGEN) was used for total RNA extraction and purification. Extracted RNA was purified and quantified by spectrophotometry. All 260/280-nm ratios were above 1.8–2.2.

Real-time PCR

Total RNA samples of epithelial cells from different stages of palatogenesis were prepared and reverse transcribed into cDNA, and real-time PCR was performed as described by Scanlan et al. (2002).

If the ΔC_T values are the same, no change in the amount of the LEF1 gene in the transforming palatal MEE cells relative to single untransformed palatal MEE cells has occurred. If a difference between the two ΔC_T values is observed, the amount of the LEF1 gene molecule has increased or decreased, and this value is incorporated into a Microsoft® Excel program to generate statistics and graphs representing actual value of X, which is equal to 2^{-ΔΔC_T}. Graph bar represents quantitative and numerical average value of X (n = 300), where X = -2^{ΔC_T} [ΔC_T = {C_T^{LEF1}_{palate} - C_T^{GAPDH}_{palate}} - {C_T^{LEF1}_{single} - C_T^{GAPDH}_{single}}]. LEF1: sense, 5'-CCCACACGGACAGTGACCTA-3'; antisense, 5'-TGGGCTCCTGCTCCTTTCT-3'. TaqMan® probe: 5'-FAM-TGCACGTGAAGCCTCAACACGAACA-VIC-3'; 5'-FAM-AGCCACATCGCTCAGACACCATGG-VIC-3'. GAPDH: sense, 5'-CCTGTTTCGACAGTCAGCCG-3'; antisense, 5'-CGACCAATCCGTTGACTCC-3'. Immunofluorescence staining was used as follows: rabbit polyclonal anti-β-catenin antibody (1:1,000; Sigma-Aldrich), goat polyclonal anti-Smad2 antibody (1:100; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-phospho-Smad2 antibody (1:100; Cell Signaling; recognizes endogenous Smad2 of Ser 465 and 467 phosphorylation); and rabbit polyclonal anti-Smad4 antibody (1:200; Santa Cruz Biotechnology, Inc.).

Sections are blocked for 3 h at RT with 2% normal goat serum or 5% normal rabbit serum, depending on secondary antibody in PBS, and then incubated with primary antibody (e.g., mouse monoclonal anti-β-catenin antibody at 1:1,000) at 4°C overnight. The titers of the primary antibodies are optimized by trial and error. Sections are washed in PBS three times before they are incubated at RT with secondary antibody (1:200) for 2 h. Rhodamine (Pierce Chemical Co.) was conjugated for β-catenin antibody and phospho-Smad2 antibody or fluorescence (Pierce Chemical Co.) for β-catenin antibody and Smad2 antibody. Sections were then washed three times in PBS followed by three times in de-ionized water and were mounted in Vectashield® (Vector Laboratories). A microscope (ES1000; Nikon) was used to image rhodamine- and fluorescein-conjugated antibody by fluorescence.

We thank Dr. Bjorn R. Olsen for allowing us to use the Leica LCM microscope at the Forsyth Institute (Harvard School of Dental Medicine, Boston, MA); Dr. M. Waterman for plasmids coding LEF1 cDNA; Ms. Toni Burbidge and Mr. Damian LaGamba (Harvard Medical School, Boston, MA) for excellent technical assistance; and Dr. D.M. Simeone for DN Smad4 plasmid.

This research was supported by a grant from the National Institute of Dental and Craniofacial Research (R01-DE11142) from the U.S. Public Health Service to E.D. Hay.

Submitted: 4 June 2003

Accepted: 5 November 2003

References

- Abdollah, S., M. Macias-Silva, T. Tsukazaki, H. Hayashi, L. Attisano, and J.L. Wrana. 1997. TβRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J. Biol. Chem.* 272:27678–27685.
- Adnane, J., E. Seijo, Z. Chen, F. Bizouarn, M. Leal, S.M. Sebti, and T. Munoz-Antonia. 2002. RhoB, not RhoA, represses the transcription of the transforming growth factor β type II receptor by a mechanism involving activator protein 1. *J. Biol. Chem.* 277:8500–8507.

- Atcha, F.A., J.E. Munguia, T.W. Li, K. Hovanes, and M.L. Waterman. 2003. A new β -catenin-dependent activation domain in T cell factor. *J. Biol. Chem.* 278:16169–16175.
- Attisano, L., and S. Tuen Lee-Hoeflich. 2001. The Smads. *Genome Biol.* 2:REVIEWS3010.
- Behrens, J., J.P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier. 1996. Functional interaction of β -catenin with the transcription factor LEF-1. *Nature.* 382:638–642.
- Bhowmick, N.A., M. Ghiassi, A. Bakin, M. Aakre, C.A. Lundquist, M.E. Engel, C.L. Arteaga, and H.L. Moses. 2001. Transforming growth factor- β 1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol. Biol. Cell.* 12:27–36.
- Borbiev, T., S. Nurmukhambetova, F. Liu, A.D. Verin, and J.G. Garcia. 2000. Introduction of C3 exoenzyme into cultured endothelium by lipofectamine. *Anal. Biochem.* 285:260–264.
- Boyer, A.S., C.P. Erickson, and R.B. Runyan. 1999. Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGF β signal transduction mechanisms. *Dev. Dyn.* 214: 81–91.
- Brunet, C.L., P.M. Sharpe, and M.W. Ferguson. 1995. Inhibition of TGF- β 3 (but not TGF- β 1 or TGF- β 2) activity prevents normal mouse embryonic palate fusion. *Int. J. Dev. Biol.* 39:345–355.
- Cano, A., M.A. Perez-Moreno, I. Rodrigo, A. Locascio, M.J. Blanco, M.G. del Barrio, F. Portillo, and M.A. Nieto. 2000. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* 2:76–83.
- Chow, S., H. Patel, and D.W. Hedley. 2001. Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors. *Cytometry.* 46:72–78.
- Cohen, M.M., Jr. 2003. TGF β /Smad signaling system and its pathologic correlates. *Am. J. Med. Genet.* 116A:1–10.
- Cui, X.M., and C.F. Shuler. 2000. The TGF- β type III receptor is localized to the medial edge epithelium during palatal fusion. *Int. J. Dev. Biol.* 44:397–402.
- Cui, X.M., Y. Chai, J. Chen, T. Yamamoto, Y. Ito, P. Bringas, and C.F. Shuler. 2003. TGF- β 3-dependent SMAD2 phosphorylation and inhibition of MEE proliferation during palatal fusion. *Dev. Dyn.* 227:387–394.
- Dennler, S., M.J. Goumans, and P. ten Dijke. 2002. Transforming growth factor β signal transduction. *J. Leukoc. Biol.* 71:731–740.
- Duan, D., Y. Yue, W. Zhou, B. Labeled, T.C. Ritchie, R. Grosschedl, and J.F. Engelhardt. 1999. Submucosal gland development in the airway is controlled by lymphoid enhancer binding factor 1 (LEF1). *Development.* 126:4441–4453.
- Eastman, Q., and R. Grosschedl. 1999. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr. Opin. Cell Biol.* 11:233–240.
- Eger, A., A. Stockinger, B. Schaffhauser, H. Beug, and R. Foisner. 2000. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of β -catenin and upregulation of β -catenin/lymphoid enhancer binding factor-1 transcriptional activity. *J. Cell Biol.* 148:173–188.
- El-Tanani, M.K., R. Barraclough, M.C. Wilkinson, and P.S. Rudland. 2001. Regulatory region of metastasis-inducing DNA is the binding site for T cell factor-4. *Oncogene.* 20:1793–1797.
- Favata, M.F., K.Y. Horiuchi, E.J. Manos, A.J. Daulerio, D.A. Stradley, W.S. Feeser, D.E. Van Dyk, W.J. Pitts, R.A. Earl, F. Hobbs, et al. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273:18623–18632.
- Fitchett, J.E., and E.D. Hay. 1989. Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Dev. Biol.* 131:455–474.
- Griffith, C.M., and E.D. Hay. 1992. Epithelial-mesenchymal transformation during palatal fusion: carboxyfluorescein traces cells at light and electron microscopic levels. *Development.* 116:1087–1099.
- Haertel-Wiesmann, M., Y. Liang, W.J. Fantl, and L.T. Williams. 2000. Regulation of cyclooxygenase-2 and periostin by Wnt-3 in mouse mammary epithelial cells. *J. Biol. Chem.* 275:32046–32051.
- Hay, E.D. 1995. An overview of epithelio-mesenchymal transformation. *Acta. Anat. (Basel).* 154:8–20.
- Hay, E.D. 2003. Rise and fall of epithelial phenotype. In EMT Concept and Examples from the Vertebrate Embryo. P. Savagner, editor. Landes Bioscience, Georgetown, TX. In press.
- He, T.C., S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA.* 95:2509–2514.
- Ishitani, T., J. Ninomiya-Tsuji, and K. Matsumoto. 2003. Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/ β -catenin signaling. *Mol. Cell Biol.* 23:1379–1389.
- Itoh, F., N. Divecha, L. Brocks, L. Oomen, H. Janssen, J. Calafat, S. Itoh, and P. ten Dijke. 2002. The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF- β /Smad signalling. *Genes Cells.* 7:321–331.
- Itoh, S., M. Thorikay, M. Kowanetz, A. Moustakas, F. Itoh, C.H. Heldin, and P. ten Dijke. 2003. Elucidation of Smad requirement in transforming growth factor- β type I receptor-induced responses. *J. Biol. Chem.* 278:3751–3761.
- Janda, E., K. Lehmann, I. Killisch, M. Jechlinger, M. Herzig, J. Downward, H. Beug, and S. Grunert. 2002. Ras and TGF β cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J. Cell Biol.* 156:299–313.
- Kaartinen, V., J.W. Voncken, C. Shuler, D. Warburton, D. Bu, N. Heisterkamp, and J. Groffen. 1995. Abnormal lung development and cleft palate in mice lacking TGF- β 3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* 11:415–421.
- Kaartinen, V., L. Haataja, A. Nagy, N. Heisterkamp, and J. Groffen. 2002. TGF β 3-induced activation of RhoA/Rho-kinase pathway is necessary but not sufficient for epithelio-mesenchymal transdifferentiation: implications for palatogenesis. *Int. J. Mol. Med.* 9:563–570.
- Kang, P., and K.K. Svoboda. 2002. PI-3 kinase activity is required for epithelial-mesenchymal transformation during palate fusion. *Dev. Dyn.* 225:316–321.
- Kim, K., and E.D. Hay. 2001. New evidence that nuclear import of endogenous β -catenin is LEF-1 dependent, while LEF-1 independent import of exogenous β -catenin leads to nuclear abnormalities. *Cell Biol. Int.* 25:1149–1161.
- Kim, K., Z. Lu, and E.D. Hay. 2002. Direct evidence for a role of β -catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biol. Int.* 26:463–476.
- Kretschmar, M., J. Doody, I. Timokhina, and J. Massague. 1999. A mechanism of repression of TGF β /Smad signaling by oncogenic Ras. *Genes Dev.* 13: 804–816.
- Labbe, E., A. Letamendia, and L. Attisano. 2000. Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor- β and wnt pathways. *Proc. Natl. Acad. Sci. USA.* 97:8358–8363.
- Lin, H.Y., X.F. Wang, E. Ng-Eaton, R.A. Weinberg, and H.F. Lodish. 1992. Expression cloning of the TGF- β type II receptor, a functional transmembrane serine/threonine kinase. *Cell.* 68:775–785.
- Liu, X., F.Q. Wen, T. Kobayashi, S. Abe, Q. Fang, E. Piek, E.P. Bottinger, A.B. Roberts, and S.I. Rennard. 2003. Smad3 mediates the TGF- β -induced contraction of type I collagen gels by mouse embryo fibroblasts. *Cell Motil. Cytoskeleton.* 54:248–253.
- Lu, J., T.E. Landerholm, J.S. Wei, X.R. Dong, S.P. Wu, X. Liu, K. Nagata, M. Inagaki, and M.W. Majesky. 2001. Coronary smooth muscle differentiation from proepicardial cells requires rhoA-mediated actin reorganization and p160 rho-kinase activity. *Dev. Biol.* 240:404–418.
- Lutz, M., and P. Knaus. 2002. Integration of the TGF- β pathway into the cellular signalling network. *Cell. Signal.* 14:977–988.
- Miettinen, P.J., R. Ebner, A.R. Lopez, and R. Derynck. 1994. TGF- β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J. Cell Biol.* 127:2021–2036.
- Morin, P.J., A.B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, and K.W. Kinzler. 1997. Activation of β -catenin-Tcf signaling in colon cancer by mutations in β -catenin or APC. *Science.* 275:1787–1790.
- Mulder, K.M. 2000. Role of Ras and Mapks in TGF β signaling. *Cytokine Growth Factor Rev.* 11:23–35.
- Nakajima, Y., E.L. Krug, and R.R. Markwald. 1994. Myocardial regulation of transforming growth factor- β expression by outflow tract endothelium in the early embryonic chick heart. *Dev. Biol.* 165:615–626.
- Nishita, M., M.K. Hashimoto, S. Ogata, M.N. Laurent, N. Ueno, H. Shibuya, and K.W. Cho. 2000. Interaction between Wnt and TGF- β signalling pathways during formation of Spemann's organizer. *Nature.* 403:781–785.
- Novak, A., S.C. Hsu, C. Leung-Hagesteijn, G. Radeva, J. Papkoff, R. Montesano, C. Roskelley, R. Grosschedl, and S. Dedhar. 1998. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and β -catenin signaling pathways. *Proc. Natl. Acad. Sci. USA.* 95:4374–4379.
- Of, M., K.H. Heider, and H. Beug. 1998. TGF β signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr. Biol.* 8:1243–1252.
- Panopoulou, E., D.J. Gillyooly, J.L. Wrana, M. Zerial, H. Stenmark, C. Murphy, and T. Fotsis. 2002. Early endosomal regulation of Smad-dependent signaling in endothelial cells. *J. Biol. Chem.* 277:18046–18052.

- Piek, E., A. Moustakas, A. Kurisaki, C.H. Heldin, and P. ten Dijke. 1999. TGF- β type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J. Cell Sci.* 112:4557–4568.
- Proetzel, G., S.A. Pawlowski, M.V. Wiles, M. Yin, G.P. Boivin, P.N. Howles, J. Ding, M.W. Ferguson, and T. Doetschman. 1995. Transforming growth factor- β 3 is required for secondary palate fusion. *Nat. Genet.* 11:409–414.
- Roberts, A.B. 2002. The ever-increasing complexity of TGF- β signaling. *Cytokine Growth Factor Rev.* 13:3–5.
- Runyan, R.B., J.D. Potts, and D.L. Weeks. 1992. TGF- β 3-mediated tissue interaction during embryonic heart development. *Mol. Reprod. Dev.* 32:152–159.
- Sasaki, T., H. Suzuki, K. Yagi, M. Furuhashi, R. Yao, S. Susa, T. Noda, Y. Arai, K. Miyazono, and M. Kato. 2003. Lymphoid enhancer factor 1 makes cells resistant to transforming growth factor β -induced repression of c-myc. *Cancer Res.* 63:801–806.
- Scanlan, M.J., C.M. Gordon, B. Williamson, S.Y. Lee, Y.T. Chen, E. Stockert, A. Jungbluth, G. Ritter, D. Jager, E. Jager, et al. 2002. Identification of cancer/testis genes by database mining and mRNA expression analysis. *Int. J. Cancer.* 98:485–492.
- Shuler, C.F., D.E. Halpern, Y. Guo, and A.C. Sank. 1992. Medial edge epithelium fate traced by cell lineage analysis during epithelial-mesenchymal transformation in vivo. *Dev. Biol.* 154:318–330.
- Smith, P.G., C. Roy, Y.N. Zhang, and S. Chaudhuri. 2003. Mechanical stress increases RhoA activation in airway smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* 28:436–442.
- Sun, D., C.R. Vanderburg, G.S. Odierna, and E.D. Hay. 1998. TGF β 3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro. *Development.* 125:95–105.
- Sun, D., C.M. Griffith, and E.D. Hay. 2000. Carboxyfluorescein as a marker at both light and electron microscope levels to follow cell lineage in the embryo. *Methods Mol. Biol.* 135:357–363.
- Ten Dijke, P., M.J. Goumans, F. Itoh, and S. Itoh. 2002. Regulation of cell proliferation by Smad proteins. *J. Cell. Physiol.* 191:1–16.
- Tsukazaki, T., T.A. Chiang, A.F. Davison, L. Attisano, and J.L. Wrana. 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGF β receptor. *Cell.* 95:779–791.
- Wilde, C., H. Barth, P. Sehr, L. Han, M. Schmidt, I. Just, and K. Aktories. 2002. Interaction of the Rho-ADP-ribosylating C3 exoenzyme with RalA. *J. Biol. Chem.* 277:14771–14776.
- Wrana, J.L., L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X.F. Wang, and J. Massague. 1992. TGF β signals through a heteromeric protein kinase receptor complex. *Cell.* 71:1003–1014.
- Yu, L., M.C. Hebert, and Y.E. Zhang. 2002. TGF- β receptor-activated p38 MAP kinase mediates Smad-independent TGF- β responses. *EMBO J.* 21:3749–3759.
- Zavadil, J., M. Bitzer, D. Liang, Y.C. Yang, A. Massimi, S. Kneitz, E. Piek, and E.P. Bottinger. 2001. Genetic programs of epithelial cell plasticity directed by transforming growth factor- β . *Proc. Natl. Acad. Sci. USA.* 98:6686–6691.
- Zhang, L., K. Graziano, T. Pham, C.D. Logsdon, and D.M. Simeone. 2001. Adenovirus-mediated gene transfer of dominant-negative Smad4 blocks TGF- β signaling in pancreatic acinar cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280:G1247–G1253.
- Zubiaur, M., J. Sancho, C. Terhorst, and D.V. Faller. 1995. A small GTP-binding protein, Rho, associates with the platelet-derived growth factor type- β receptor upon ligand binding. *J. Biol. Chem.* 270:17221–17228.