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PEA3 transcription factors are expressed in tissues undergoing branching morphogenesis and promote formation of duct-like structures by mammary epithelial cells in vitro

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Abstract

The genetic program that controls reciprocal tissue interactions during epithelial organogenesis is still poorly understood. Erm, Er81 and Pea3 are three highly related transcription factors belonging to the Ets family, within which they form the PEA3 group. Little information is yet available regarding the function of these transcription factors. We have previously used in situ hybridization to compare their expression pattern during critical stages of murine embryogenesis [Oncogene 15 (1997), 937; Mech. Dev. 108 (2001), 191]. In this study, we have examined the expression of PEA3 group members during organogenesis of the lung, salivary gland, kidney, and mammary gland. In all of these developmental settings, we observed a tight correlation between branching morphogenesis and the expression of specific members of the PEA3 group. To assess the functional relevance of these findings, Erm and Pea3 were overexpressed in the TAC-2.1 mammary epithelial cell line, which has the ability to form branching duct-like structures when grown in collagen gels. We found that overexpression of Erm and Pea3 markedly enhances branching tubulogenesis of TAC-2.1 cells and also promotes their invasion into a collagen matrix. Collectively, these findings suggest that the differential expression of PEA3 group transcription factors has an important role in the regulation of branching morphogenesis and raise the question of their implication in branching signaling.
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Keywords: PEA3; Transcription factors; Branching morphogenesis; Mammary development; Mammary cells; Expression analysis; Mouse

Introduction

The development of epithelial organs, including lung, kidney, salivary glands, and mammary gland, is orchestrated by sequential and reciprocal tissue interactions. An essential event in organogenesis is branching morphogenesis, the process by which a primitive epithelial bud bifurcates and extends to form arborized ducts and/or acinar structures. It has long been known that, in most developing organs, branching morphogenesis is dependent on continuous interactions between an epithelial anlage and the adjacent mesenchyme (Grobstein, 1956). However, the underlying molecular mechanisms have only recently begun to be elucidated. Thus, a number of studies have led to the identification of mesenchyme-derived diffusible messengers that promote the elongation and branching of epithelial tubes (see, for example, Bellusci et al., 1997; Montesano et al., 1991; Sakurai et al., 2001; Vega et al., 1996). An additional, crucial determinant of branching morphogenesis is the spa-

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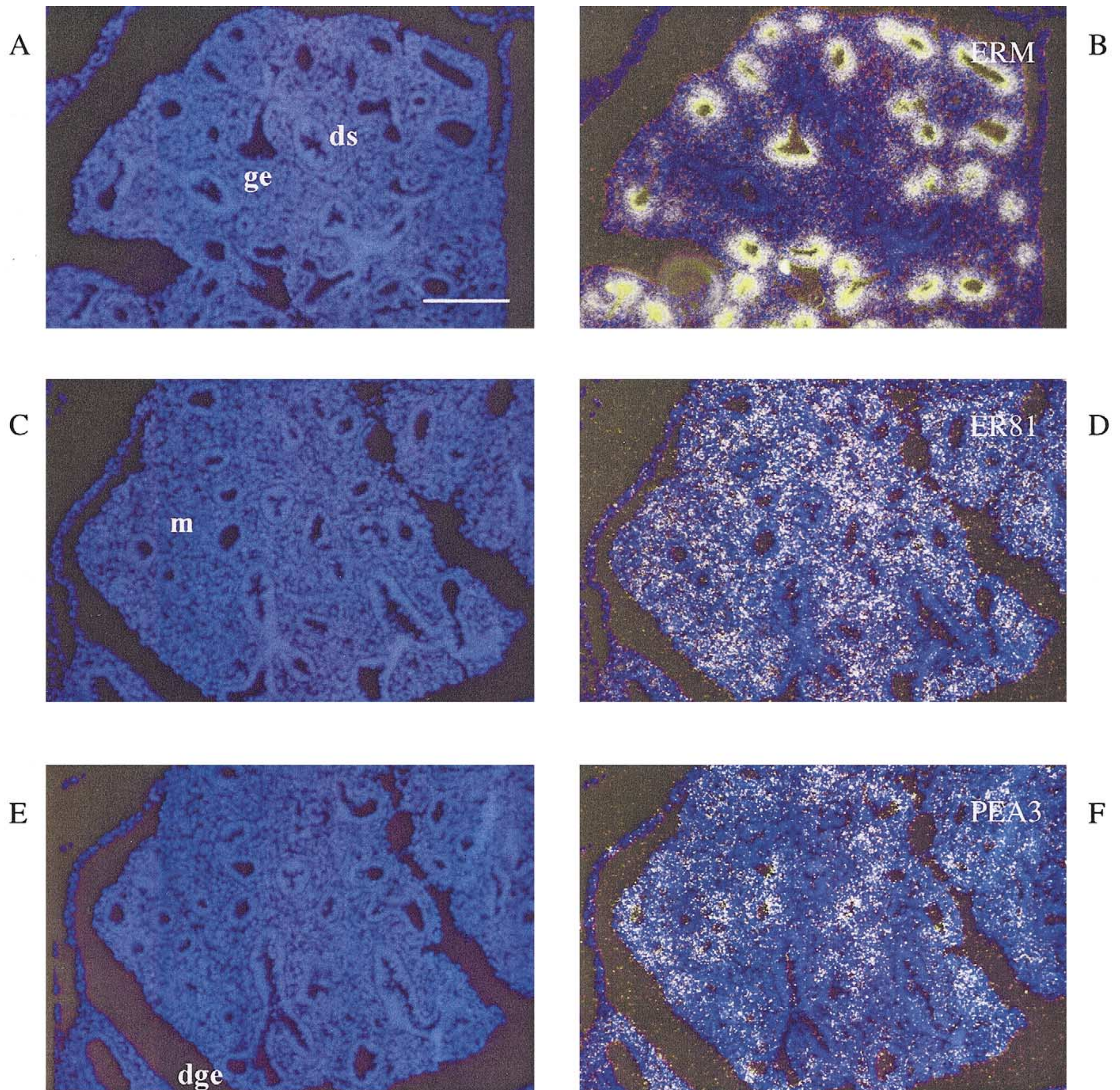


Fig. 1. Expression of *erm* (B), *er81* (D), and *pea3* (F) in sections of lung from a 15.5-day embryo. Darkfield views and Hoechst staining. (A, C, E): Hoechst staining (control). Bar, 200 μm. *Erm* is expressed in the distal growing epithelium (ge) but not in differentiated stalks (ds). *Er81* expression is observed in the mesenchymal compartment (m). *Pea3* is selectively expressed in the distal growing part of the ducts (dge).

tially and temporally coordinated expression of tissue-specific transcription factors (Ekblom, 1996; Keijzer et al., 2001; Thesleff et al., 1995).

Transcription factors belonging to the Ets family are thought to play an important role in the regulation of epithelial–mesenchymal interactions (Neve et al., 1998; Maroulakou and Bowe, 2000). Ets transcription factors are characterized by a conserved region of approximately 85 amino acids, named the ETS domain, which defines a sequence-specific helix–turn–helix DNA-binding motif (Graves and Petersen, 1998; Wasylyk et al., 1993). Divergence rate analysis of the different ETS domains indicates that the *ets* gene family can be subdivided into different groups (Graves and Petersen, 1998). Within the Ets family, the transcription factors Pea3/E1AF, Er81/ETV1, and Erm/ETV5 have been assigned to the same PEA3 group according to the divergence rate analysis of the ETS domain. These proteins are very closely related, as they share more than 95% sequence identity in the DNA binding domain and more than 60% in the amino- and carboxy-terminal regions

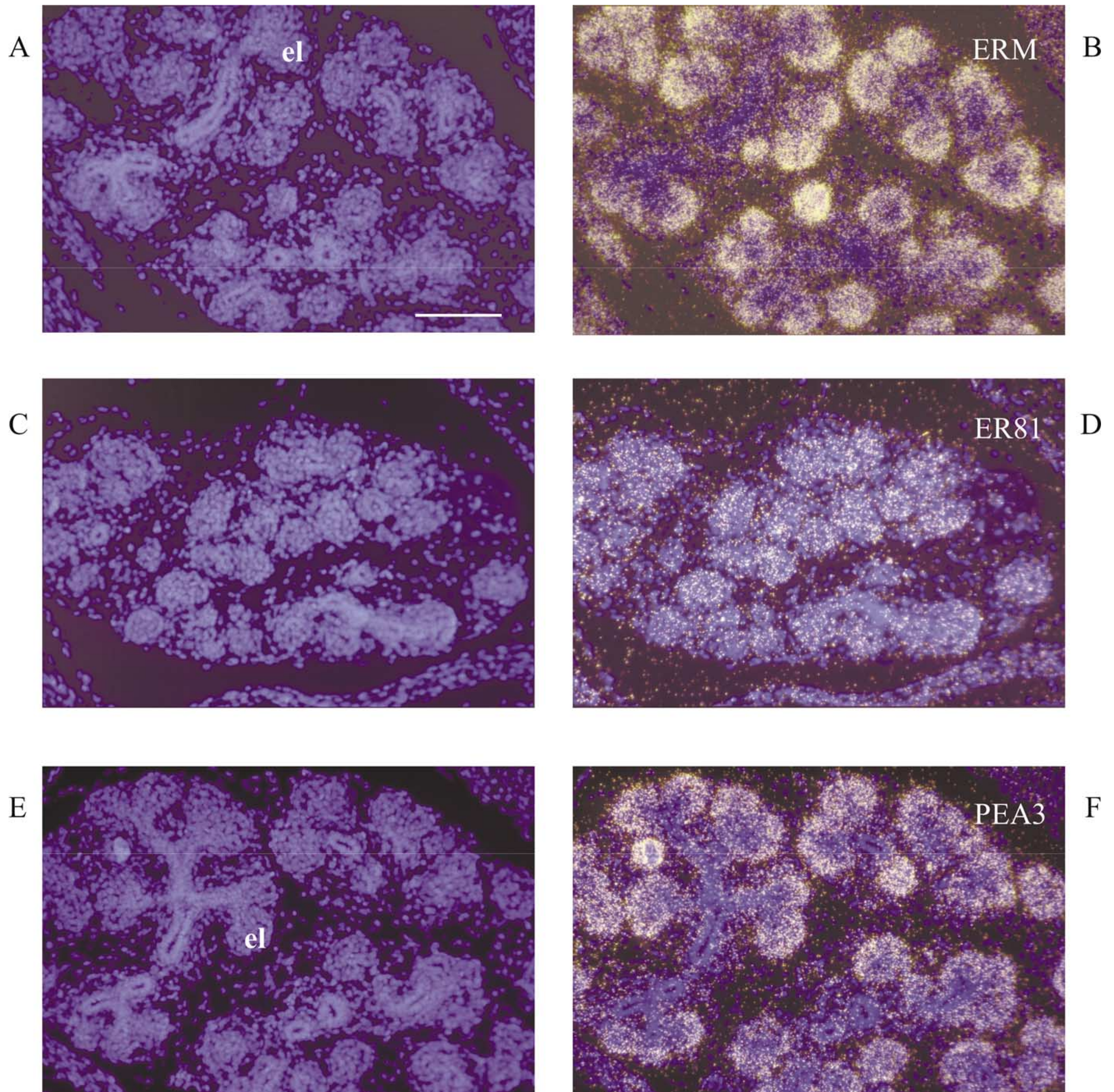


Fig. 2. Expression of *erm* (B), *er81* (D), and *pea3* (F) in sections of salivary glands from a 15.5-day embryo. Darkfield views and Hoechst staining. (A, C, E): Hoechst staining (control). Bar, 200 μm. *Erm* and *pea3* are expressed in the distal part of the growing epithelial lobules (el), whereas *er81* is expressed in the condensed mesenchymal cells around growing epithelial buds.

known to be transcriptional activating domains (de Launoit et al., 2000; Laget et al., 1996).

In situ hybridization studies performed at various stages of murine embryogenesis have shown that *pea3, er81,* and *erm* genes exhibit both patterns of coexpression and patterns of differential expression in developing parenchymal organs, which suggests that their protein products fulfill overlapping as well as specific roles in epithelial morphogenesis (Chotteau-Lelievre et al., 1997, 2001). Interestingly, Pea3 is overexpressed in oncogene-induced mouse mammary tumors (Trimble et al., 1993) and metastatic human breast cancer cells (Baert et al., 1997). In addition, overexpression of Pea3 in the MCF-7 breast cancer cell line induces the transcription of MMP-9 and promotes the formation of metastases in nude mice (Kaya et al., 1996). These findings suggest that, when aberrantly regulated, PEA3 group transcription factors can also contribute to tumorigenesis.

The objective of the present study was twofold. The first objective was to comparatively assess the spatiotemporal expression patterns of *erm, er81,* and *pea3* genes during branching morphogenesis of lung, kidney, salivary glands, and mammary gland. The second objective was to determine, using an in vitro assay of branching morphogenesis, whether overexpression of PEA3 group members in mammary epithelial cells promotes the formation of arborized duct-like structures. We report here that PEA3 group transcription factors present a characteristic expression pattern associated with processes of branching morphogenesis and that this pattern is similar in different epithelial organs. In addition, we show that overexpression of Erm and Pea3 promotes the formation of duct-like structures by mammary epithelial cells in vitro. Collectively, these findings support the involvement of Pea3 transcription factors in the regulation of branching morphogenesis.

Materials and methods

In situ hybridization

Embryos were obtained from natural matings of OF1 mice. Embryonic day 0.5 (E 0.5) was assigned the day the vaginal plug was found.

In situ hybridization was performed on tissue sections of mouse embryos or mammary gland at various developmental stages using murine *erm, er81,* and *pea3* riboprobes as previously reported (Chotteau-Lelievre et al., 1997).

Northern blot analysis

Cells were washed in 1× PBS and then lysed in RNA plus (Quantum) as described by the manufacturer. Total RNA (10 μg) was loaded and separated on 1.2% agarose/formaldehyde/MOPS gels and then transferred onto nitrocellulose membranes (Hybond C extra, Amersham, Pharmacia Biotech). cDNA fragments of *erm, er81,* and *pea3* (Chotteau-Lelievre et al., 1997) were used as probes after [^{32}P]CTP labeling using the Megaprime DNA labeling system (Amersham, Pharmacia Biotech). Prehybridization and hybridization were carried out at 42°C in a solution of 3× SSC, 5× Denhardt's solution, 50% formamide, and 20 μg/ml of denatured salmon sperm DNA. Washes were performed at 50°C using 2× SSC and 0.1% SDS.

Plasmids

pcDNA3.1 vector (InVitrogen) containing full-lengh cDNA of *erm* and *pea3* was used to generate stable cell lines. For the transactivation assays, the promoting regions of human ICAM-1 gene (de Launoit et al., 1998; Voraberger et al., 1991) and the TORU element (Wasylyk et al., 1990) cloned upstream to the luciferase gene were used.

Cell culture

Wild type TAC-2.1 cells (Soriano et al., 1996), a clonal subpopulation of the TAC-2 murine mammary epithelial cell line (Soriano et al., 1995), and their derivatives (see below) were cultured on collagen-coated tissue culture flasks (Falcon, Becton-Dickinson and Co., San Jose, CA) in high glucose DMEM (GIBCO BRL, Life Technologies, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS, GIBCO), penicillin (110 IU/ml), and streptomycin (110 μg/ml).

Transient transfection

TAC 2.1 cells were seeded at 70% confluence and transfected by incubating with a DNA polyethylenimine (PEI 10 μM, Euromedex) complex (500 ng DNA: 2 μl PEI) for 6 h in serum-free medium (OptiMEM, Life Technologies). Several independent experiments were performed in triplicate. Cell lysates were prepared 36 h later for the luciferase (Promega) and β-galactosidase (Tropix Inc, Galactolight) activities by following the manufacturer's instructions and using a monolight 2010 Luminometer (Turner Designs, Sunnyvale, CA). The measured luciferase activity levels were corrected by using the cotransfected pSG5 LacZ activity as an internal control.

Establishment of stable cell lines

TAC 2.1 cells were transfected with pcDNA-*erm,* pcDNA-*pea3,* or pcDNA3.1 vectors under the same conditions used for transient transfection (see above). Transfected cells were selected for resistance to active Geneticin (G418, Boehringer Mannheim) at 0.4 mg/ml. Resistant clones were isolated and screened for *erm* and *pea3* expression, respectively, by Northern blot and transactivation activity.

In vitro assay of branching morphogenesis

Wild type TAC-2.1 cells and their derivatives were suspended at 1 × 10^4 cells/ml in 2 ml collagen gels cast in 35-mm dishes (Becton Dickinson Labware) as described (Soriano et al., 1995). After the collagen had gelled, 2 ml complete medium (high glucose DMEM + 10% FCS) with or without 20 ng/ml recombinant human hepatocyte growth factor (HGF; a generous gift of Dr. Ralph Schwall, Genentech, South San Francisco, CA) was added. Media and treatments were renewed every 2 or 3 days. After 9 days, the cultures were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and three randomly selected fields (measuring 2.2 mm × 3.4 mm) per experimental condition in each of three separate experiments were photographed under bright-field illumination with a Nikon Diaphot TMD inverted photomicroscope. The total length of cords in each colony was measured with a Qmet 500 image analyser (Leica, Cambridge, UK). Values for cord length obtained

from the largest colonies are an underestimate, since in these colonies, a considerable proportion of cords were out of focus and therefore could not be measured. The median value of total cord length per photographic field was determined in each of three separate experiments per condition.

Assay of lumen formation

The ability of TAC-2.1 cell lines to form widely patent lumina was evaluated under conditions that promote the formation of cyst-like structures, i.e., in hydrocortisone-supplemented cultures (Soriano et al., 1995). The cells were suspended in collagen gels at 2×10^4 cells/ml in serum-free DMEM/F12 (1:1) medium with ITS+ supplement (Becton Dickinson), 1 μg/ml hydrocortisone, and 50 ng/ml cholera toxin. After 9 days, the cultures were fixed as above and 150 randomly selected colonies per experimental condition were examined in each of 3 separate experiments by using a Nikon Diaphot TMD inverted photomicroscope and scored as cysts when containing a single wide lumen (Soriano et al., 1995). Data were expressed as mean percentage of cysts, and statistical significance was determined by using the Student's unpaired *t* test.

Collagen gel invasion assay

TAC-2.1 cell lines were seeded onto the surface of collagen gels cast in 35-mm dishes (4×10^5 cells/dish) and incubated in 2 ml complete medium (high glucose DMEM + 10% FCS) supplemented with 20 ng/ml epidermal growth factor (EGF). After 9 days, the cultures were fixed as above, and 3 randomly selected fields (measuring 1.0×1.4 mm) per experimental condition in each of 3 separate experiments were photographed at a focal level of 20 μm beneath the surface monolayer using the 10× phase contrast objective of a Nikon Diaphot TMD inverted photomicroscope. Invasion was quantified as described previously (Pepper et al., 1992; Soriano et al., 2000) by determining the total length of all cellular structures that had penetrated beneath the surface monolayer. Data are expressed as mean total cord length ± S.E.M. (in mm), and statistical significance was determined by using the Student's unpaired *t* test.

Processing for light and electron microscopy

Collagen gel cultures were fixed as described above, extensively rinsed in cacodylate buffer, and cut into 3×3-mm fragments. These were postfixed in 1% osmium tetroxide in Veronal acetate buffer for 45 min and further processed as described (Montesano et al., 1991). Two-dimensional cultures in collagen-coated dishes were postfixed in 1% osmium tetroxide for 20 min, dehydrated, and embedded in situ in a thin layer of Epon 812. After polymerization, the Epon disk was removed from the plastic dish and cut into small blocks, which were reembedded in the same resin in flat Teflon moulds to obtain sections perpendicular to the culture plane. Semithin (1-μm-thick) sections were cut with an LKB ultramicrotome (LKB Instruments, Gaithersburg, MD), stained with 1% methylene blue, and photographed under transmitted light using an Axiophot photomicroscope (Carl Zeiss, Oberkaden, Germany). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Results

Expression of erm, er81, and pea3 during the development of glandular organs

We have previously shown that PEA3 group transcription factors are expressed in restricted areas of the developing embryo where morphogenetic tissue interactions are occurring (Chotteau-Lelievre et al., 1997). The objective of the present study was to carry out a detailed comparative analysis of the expression patterns of the PEA3 group transcription factors in a number of organogenetic processes that are dependent on different types of tissue interactions.

Endodermal–mesodermal interactions

At E9.5, there is the first evidence of emergence of the laryngotracheal groove that forms an anteriorly directed diverticulum of the pharyngeal region of the primitive foregut. This entoblastic diverticulum subsequently gives rise to the epithelium of the upper and lower respiratory airways. Lung development is initiated when bilateral epithelial tracheal buds sprout from the primitive gut endoderm into surrounding mesoderm. The tracheal bud elongates until it penetrates the splanchnic mesoderm, where it is induced to branch repeatedly. The pseudoglandular stage of lung development is characterized by branching morphogenesis, which results from combined growth of epithelial ducts and cleft formation. Then, the developing lung undergoes repetitive dichotomous branching to form the primitive bronchial tree. This stage is followed by further ramification of the tubules and their differentiation into two different morphological entities: the proximal part develops into the bronchial portion of the adult lung, while the distal part evolves into tubules and terminal sacs. The epithelium forms the conducting and respiratory airways of the adult lung, whereas the mesoderm becomes the lung stroma. *Erm* and *pea3* genes were expressed in the lung bud epithelium from E10.5 to E17.5. During the branching process that generates the bronchi, *erm* was expressed in the distal growing epithelium but not in differentiated stalks. At the same stages, *er81* expression was observed in the mesenchymal compartment, whereas *pea3* was selectively expressed in the distal growing part of the ducts (Fig. 1). At E17.5–E18, the bronchi are considerably distended and display numerous ramifications. The lung structure suddenly changes. The alveo-

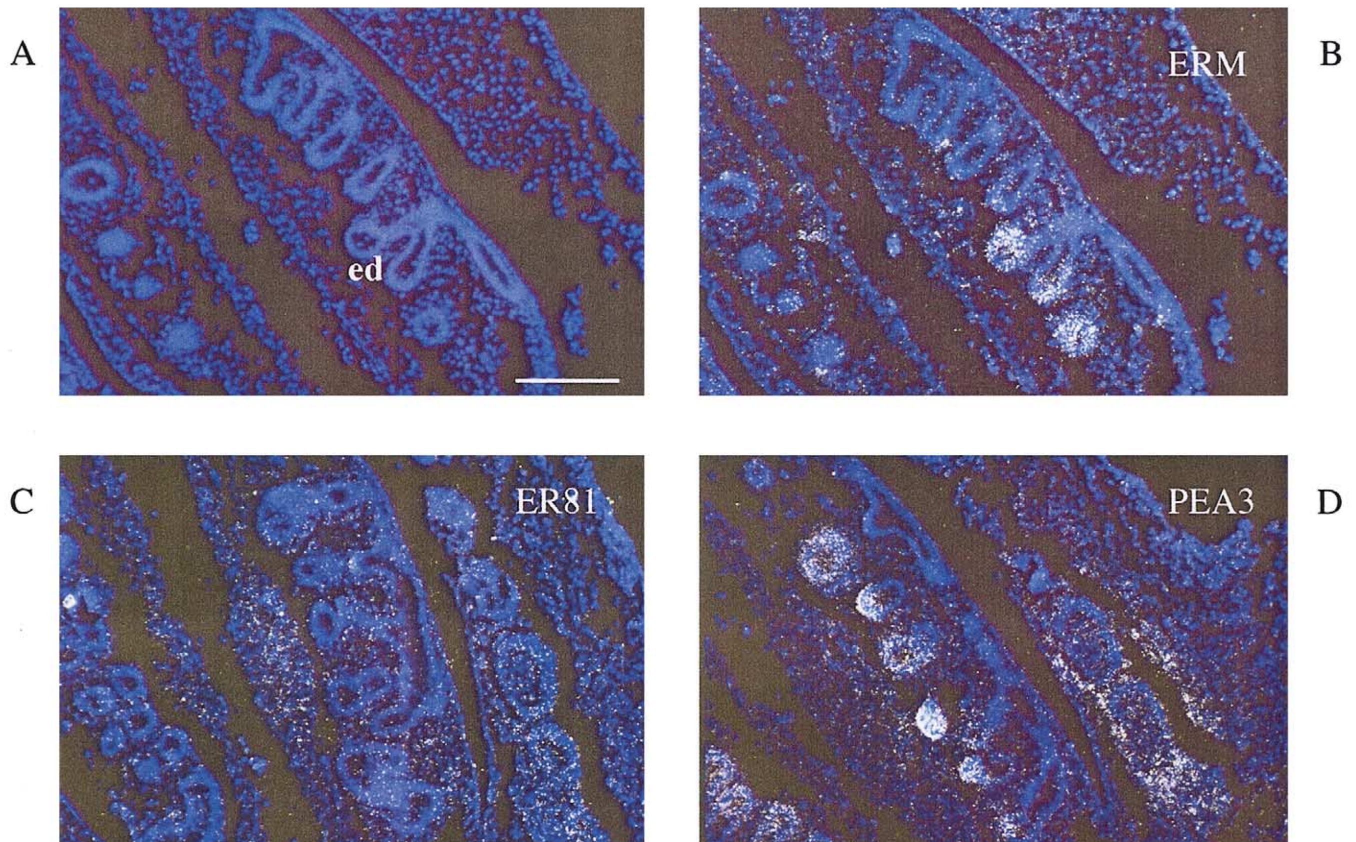


Fig. 3. Expression of *erm* (B), *er81* (C), and *pea3* (D) in sections of mesonephros from a 9.5-day embryo. Darkfield views and Hoechst staining. (A) Hoechst staining (control). Bar, 200 μm. *Erm* and *pea3* are expressed at the tip of the epithelial duct (ed) of the collecting Wollfian's duct. *Er81* expression is observed in the mesenchymal compartment.

lar-lining cells, which were initially cuboidal in shape, acquire a squamous morphology. During this evolution, the expression of *erm* became restricted to the mesenchyme, *er81* remained expressed in the mesenchyme, and *pea3* displayed a weak level of expression in this compartment (data not shown).

Salivary glands emerge at E11 of gestation as an invagination of part of the mouth floor epithelium into the mandible mesenchyme. The epithelial bud then gives rise to bulbous epithelial lobules. Narrow internal clefts provide a support for elongation of separated lobules tightly wrapped up by mesenchymal cells. *Erm* and *pea3* genes were highly expressed in the bulbous epithelial lobules (Fig. 2). As branching occurred, the expression was restricted to the distal part of these growing lobules. No labeling was observed in the more differentiated epithelial stalks. *Er81* was evenly expressed in the condensed mesenchymal cells around growing epithelial buds (Fig. 2). By E17.5, tubule formation is clearly apparent. As for lung development, *erm* and *pea3* were expressed in the distal part of the branching epithelium and also in the flanking mesenchyme (data not shown). At later stages, the expression of the three genes became restricted to the mesenchymal compartment (data not shown).

Mesodermal–mesodermal interactions

The urogenital system derives from the intermediate mesoderm and undergoes extensive transformation to generate the epithelial ducts and tubules that take part in the morphogenesis of the successive kidneys. At E9.5, the pronephric duct arises in the rostral intermediate mesoderm, migrates caudally, and induces the formation of nephric tubules. *pea3* and *erm* were expressed in the collecting Wolffian duct (Fig. 3). At E10 p.c., mesonephric differentiation takes place and ducts growing in the mesonephric blastema connect to the Wolffian duct that protrudes at the surface of the genital ridge. During the initial step of mesonephric differentiation under Wolffian duct control, mesonephric tubules emerge from the intermediate mesoderm. Both *erm* and *pea3* were expressed at the tip of the epithelial ducts. *Er81* was never expressed in the tubules but lightly in the mesenchyme (Fig. 3). The metanephros, i.e., the definitive kidney, results from the invasion of metanephric blastema by ureters. The ureters bud out from the posterior Wolffian duct, contact the metanephric mesenchyme, and increase in length. The mesenchymal and ureteric components interact in a reciprocal induction: the mesenchyme induces the ureteric buds to branch and grow. At the tips of these branches, the ureteric buds induce the loose mesen-

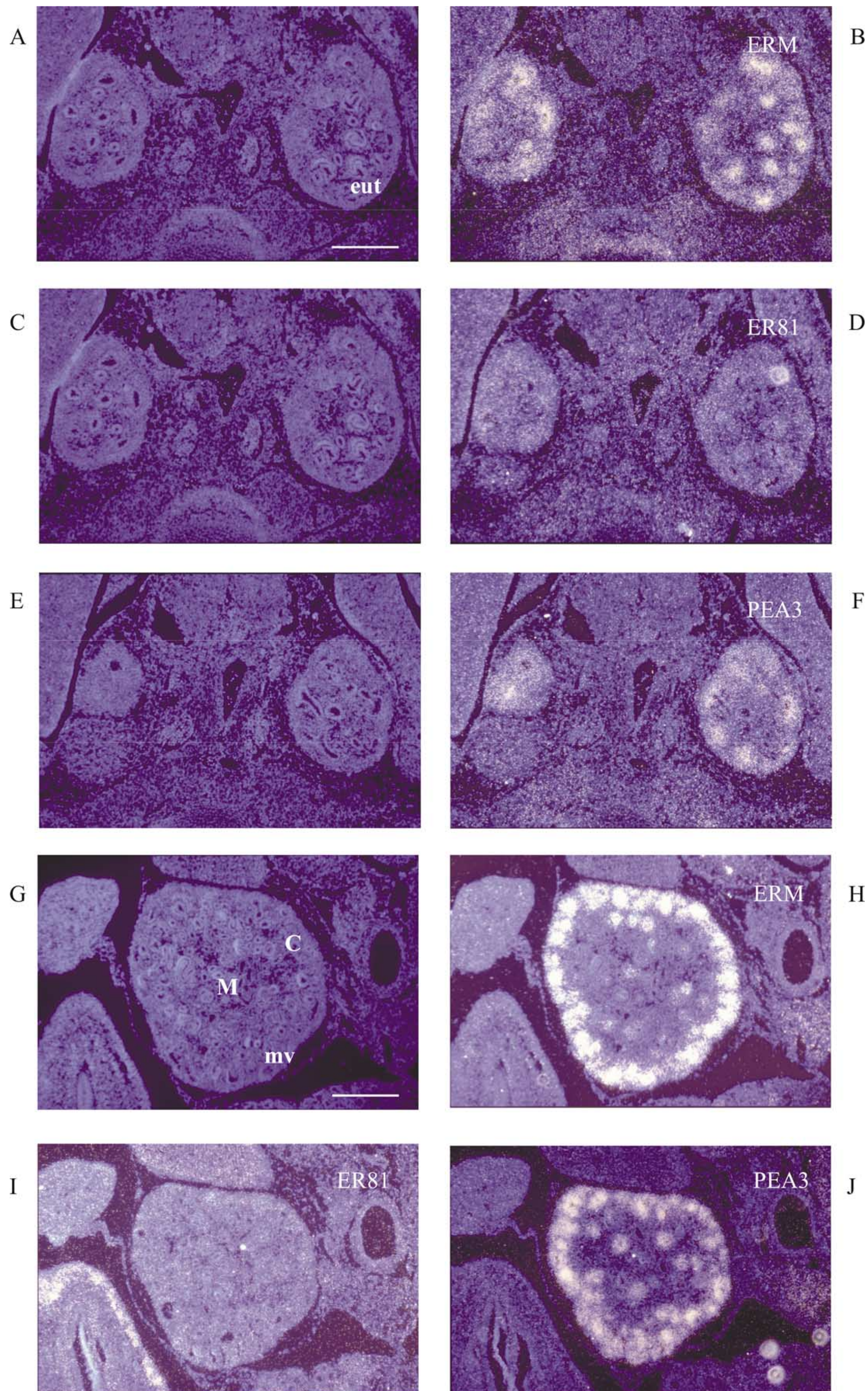


Fig. 4. Expression of *erm* (B, H), *er81* (D, I), and *pea3* (F, J) in sections of kidney from a 12.5 (A–F)- and 15.5 (G–J)-day embryos. Darkfield views and Hoechst staining. (A, C, E, G) Hoechst staining (control). Bars, 200 μm. *Erm* and *pea3* are expressed in the growing epithelial ureteric tubules (eut) of a 12.5-day embryo (B, F). During subsequent differentiation of the kidney, *erm* and *pea3* are expressed in the metanephric vesicles (mv), first in the cortical (C) and medullary (M) regions and later only in the cortex (H, J). *Er81* expression is slightly observed in the mesenchymal compartment (D, I).

chyme to condense into epithelium-like cell aggregates. A connection is established between the ureteric bud and newly formed epithelial vesicles. Ducts of mesenchymal origin will yield the secretory part of the nephrons (including glomeruli), while the branched ureteric buds will evolve into collecting ducts. The branching of ureteric buds occurs at E11.5 so that E12.5 embryos display some growing ureteric tubules within metanephrogenic mesenchyme. *Erm* and *pea3* were expressed both in growing epithelial ureteric tubules and in the adjacent mesenchyme, whereas *er81* was slightly expressed in the mesenchyme (Fig. 4A–F).

During subsequent differentiation of the kidney (E13.5–E16.5), the primitive glomeruli are initially dispersed throughout the cortical as well as the medullary region, but later, tend to be concentrated in the outer region of the kidney, the future cortex. During these stages, the expression of PEA3 group transcription factors exhibited a more restricted distribution. Specifically, *erm* and *pea3* were expressed at sites of interactions between metanephrogenic mesenchyme and epithelial tubules, first in the cortical and medullary regions and later only in the cortex. The expression corresponded to the formation of the metanephric vesicles, which represent the initial step in glomeruli formation, and was present in the epithelium and the surrounding mesenchymal condensation. The mesenchymal expression of *er81* decreased progressively until E17.5. Later, undifferentiated mesenchyme disappeared from the medullar region, and *er81* expression was dramatically reduced in this region (Fig. 4G–J).

From E18 until birth, expression of *erm* and *pea3* decreased concomitantly with the differentiation of the glomeruli and was restricted to the subcapsular region of the cortex, where poorly differentiated metanephrogenic cap tissue is still abundant. At later stages, *erm* and *pea3* were expressed weakly and homogeneously in the mesenchymal compartment (data not shown).

Ectodermal–mesodermal interactions

The mammary gland is composed of two main components: the parenchymal epithelium, which originates from ectodermal cells, and the stroma (adipose and connective tissue), which derives from the mesoderm. Mammary gland development begins in the embryo with the emergence of the mammary anlage that gives rise to primary and secondary ducts. We have previously reported that *erm, er81,* and *pea3* are expressed in 15.5-day female embryo in the epithelial buds of the mammary gland (Chotteau-Lelievre et al., 1997). In this study, we characterize the expression of these genes during the postnatal development of the gland, i.e., during the phases of pregnancy, lactation, and involution.

At birth, mammary gland consists of a primary duct connected to the nipple and 15–20 branching ducts embedded in the fat pad. From approximately 4 weeks postpartum, the ducts grow and ramify under the influence of ovarian hormones, forming an arborized tree. Following the onset of pregnancy, in response to the sustained elevated levels of estrogen and progesterone, ductal elongation and branching resume, and clusters of alveoli bud off from the growing ducts. During the second half of gestation, alveolar morphogenesis is followed by the structural and functional differentiation of alveolar epithelial cells in preparation for milk fat and protein secretion, which takes place during lactation. After weaning, the mammary gland involutes rapidly, ultimately leaving only a rudimentary ductal system and a few remaining alveoli (reviewed by Daniel and Silberstein, 1987). Expression of the 3 PEA3 members was assessed by Northern blot, RT-PCR, and in situ hybridization analysis of mammary gland tissue during puberty (D26-28-32-35-45), pregnancy (D2-6-15), lactation (D6), and involution (D1-3). No signal was observed by Northern blot, probably due to the sensitivity level of this technique. Multiplex RT-PCR showed expression of the 3 genes, and particularly *erm* and *pea3*, throughout all stages of mammary gland development. *Er81* was only slightly detected in the pubescent gland (data not shown). In situ hybridization reveals expression of *erm* and *pea3,* but not of *er81,* in epithelial ducts (particularly in the terminal end buds) from the onset of puberty (D26, Fig. 5) until D35, that is, during stages in which extensive branching occurs. No signal was detected for the 3 genes in the other stages studied (i.e., pregnancy, lactation, and involution), possibly because of the low sensitivity of in situ hybridization. Because of this limitation, we have been unable to assess the expression of *erm* and *pea3* in the lateral (alveolar) buds that develop during pregnancy (in glands of pubescent mice, only a few lateral buds are transiently formed during each oestrous cycle).

TAC-2.1 mammary epithelial cells overexpressing Erm or Pea3 have an enhanced capacity to form branching duct-like structures and to invade collagen gels

The in situ hybridization studies described above suggested the involvement of PEA3 group transcription factors in the control of branching morphogenesis. To test this hypothesis directly, we stably overexpressed Erm and Pea3 in a murine mammary epithelial cell line (TAC-2.1 cells) that has the ability to form branching duct-like structures in collagen gels (Soriano et al., 1995). For each construction, several clones were selected for overexpression of the transgene by Northern blot analysis (Fig. 6). As negative controls, 10 cell lines that had been transfected with pcDNA3.1 containing no insert were isolated and the clone named TAC/pc7 was used as a control. The function of overexpressed genes was assessed by the capacity of the selected clones (TAC/Erm1; TAC/Erm14; TAC/Pea3/8; and TAC/Pea3/48) to activate the transcription of a luciferase reporter gene cloned upstream to the human ICAM-1 promoter (de Launoit et al., 1998; Voraberger et al., 1991) or the TORU element (Wasylyk et al., 1990). These promoters are known to be actively transactivated by these Ets family members

(Defossez et al., 1997). As shown in Fig. 7, TAC/Erm1, TAC/Erm14, TAC/Pea3/8, and TAC/Pea3/48 presented a significant capacity of transactivation compared with either wild type TAC-2.1 or mock-transfected TAC/pc7 cells.

The ability of transfected TAC-2.1 cells to form branching duct-like structures was assessed both in the absence and in the presence of hepatocyte growth factor (HGF), a potent tubulogenic stimulus for these cells (Soriano et al., 1995). When suspended in collagen gels in the absence of HGF, TAC-2.1 cells transfected with the empty pcDNA3.1 plasmid (TAC/pc7 cells) formed small, poorly branched colonies (Fig. 8A). Addition of 20 ng/ml HGF induced the development of elongated cords with multiple branch points (Fig. 8B). The behavior of TAC/pc7 cells was therefore similar to that observed previously with wild type TAC-2 cells (Soriano et al., 1995). Strikingly, TAC-2.1 clones overexpressing Erm (TAC/Erm1 and TAC/Erm14 cells) instead formed long branched cords, even in the absence of exogenous HGF (Fig. 8C and E). In addition, cord elongation and branching in cultures of Erm-transfected cells was further enhanced by treatment with HGF (Fig. 8D and E). As a result, following incubation with HGF, cultures of Erm-expressing cells contained a considerably more extensive system of arborized duct-like structures than cultures of TAC/pc7 cells (Fig. 8B, D, and E). A similar increase in both spontaneous and HGF-induced cord formation was observed in TAC-2.1 clones overexpressing Pea3 (Fig. 8F). In contrast, ectopic expression of Er81 (which is expressed predominantly in the mesenchymal compartment), did not elicit significant changes in the tubulogenic properties of TAC-2.1 cells (data not shown).

Having found that TAC/Erm and TAC/Pea3 cells form long branching cords extending into the collagen matrix, we wished to determine whether these cells are endowed with invasive properties. To this end, we cultured wild-type and transfected TAC-2.1 cells on the surface of three-dimensional collagen gels and subsequently evaluated their ability to penetrate into the underlying matrix by focusing below the surface of the gel. When grown on a collagen gel for 9 days, TAC/pc7 cells formed a cobblestone-like monolayer and remained exclusively confined to the surface of the gel (Fig. 9A). In contrast, under the same experimental conditions, TAC/Erm1 and TAC/Erm14 cells invaded the underlying collagen matrix as branched cord-like structures (Fig. 9B–E). A comparatively less conspicuous invasion of collagen gels was qualitatively observed in clones TAC/Pea3/8 and TAC/Pea3/48 (results not shown).

TAC-2.1 cells overexpressing Erm or Pea3 exhibit a partially transformed phenotype

The finding that Erm-expressing TAC-2.1 cells (and to a lesser extent Pea3-expressing TAC-2.1 cells) possess invasive properties prompted us to investigate whether these cells exhibited phenotypic traits (such as loss of contact-mediated inhibition of proliferation) that are characteristic of transformed cells. When seeded in tissue culture dishes at saturation density and grown for 7 days, TAC/pc7 cells formed a contact-inhibited cobblestone-like monolayer (Fig. 10A and C). In marked contrast, under the same culture conditions, TAC/Erm1 and TAC/Erm14 cells acquired an elongated shape and grew in a disordered criss-cross pattern resulting in cell multilayering, as confirmed by examination of thin sections perpendicular to the bottom of the dishes (Fig. 10B and D). Cellular overlapping was also observed in postconfluent cultures of TAC/Pea3/8 and TAC/Pea3/48 cells. To determine whether cell multilayering was associated with the ability to proliferate under anchorage-independent conditions, TAC-2.1 cell lines were grown in agarose gels for 2 weeks. Under these conditions, none of the cell lines was able to form multicellular colonies (results not shown). These findings show that, although TAC-2.1 cells overexpressing Erm or Pea3 are less sensitive to contact-mediated inhibition of cell growth, they remain dependent on cell-substratum adhesion for proliferation.

Finally, since loss of normal tissue organisation is a hallmark of malignant transformation, we examined whether overexpression of PEA3 group transcription factors could affect the ability of TAC-2.1 cells to form well-organized alveolar-like structures in hydrocortisone-supplemented cultures (Soriano et al., 1995). When grown in collagen gels in the presence of hydrocortisone and cholera toxin, TAC/pc7 cells formed spheroidal cysts enclosing a widely patent lumen (Fig. 11A) delimited by a palisade of cubic epithelial cells (Fig. 11B), as previously observed with nontransfected TAC-2.1 cells (Soriano et al., 1995). In contrast, the vast majority of colonies formed by Erm-transfected cells consisted of compact cell aggregates containing only focal lumen-like spaces (Fig. 11C–E). A comparatively less marked impairment of lumen formation was qualitatively observed in cells overexpressing Pea3 (not shown).

Discussion

Epithelial–mesenchymal interactions are inducive interactions (also called secondary inductions) occurring between cells or tissues of the same or different embryonic origin. These interactions govern the development of glandular organs, such as lung, salivary glands, pancreas, mammary glands, and kidney. Although the fate of the epithelium is autonomously determined, the epithelial cells cannot undergo a normal development in the absence of a mesenchymal signal. While the importance of epithelial–mesenchymal interactions in organogenesis is well established, the molecular mechanisms involved in the mediation of inducive signals are poorly understood.

We report here that the PEA3 group members are highly expressed during the development of glandular epithelia, and particularly during processes of branching morphogen-

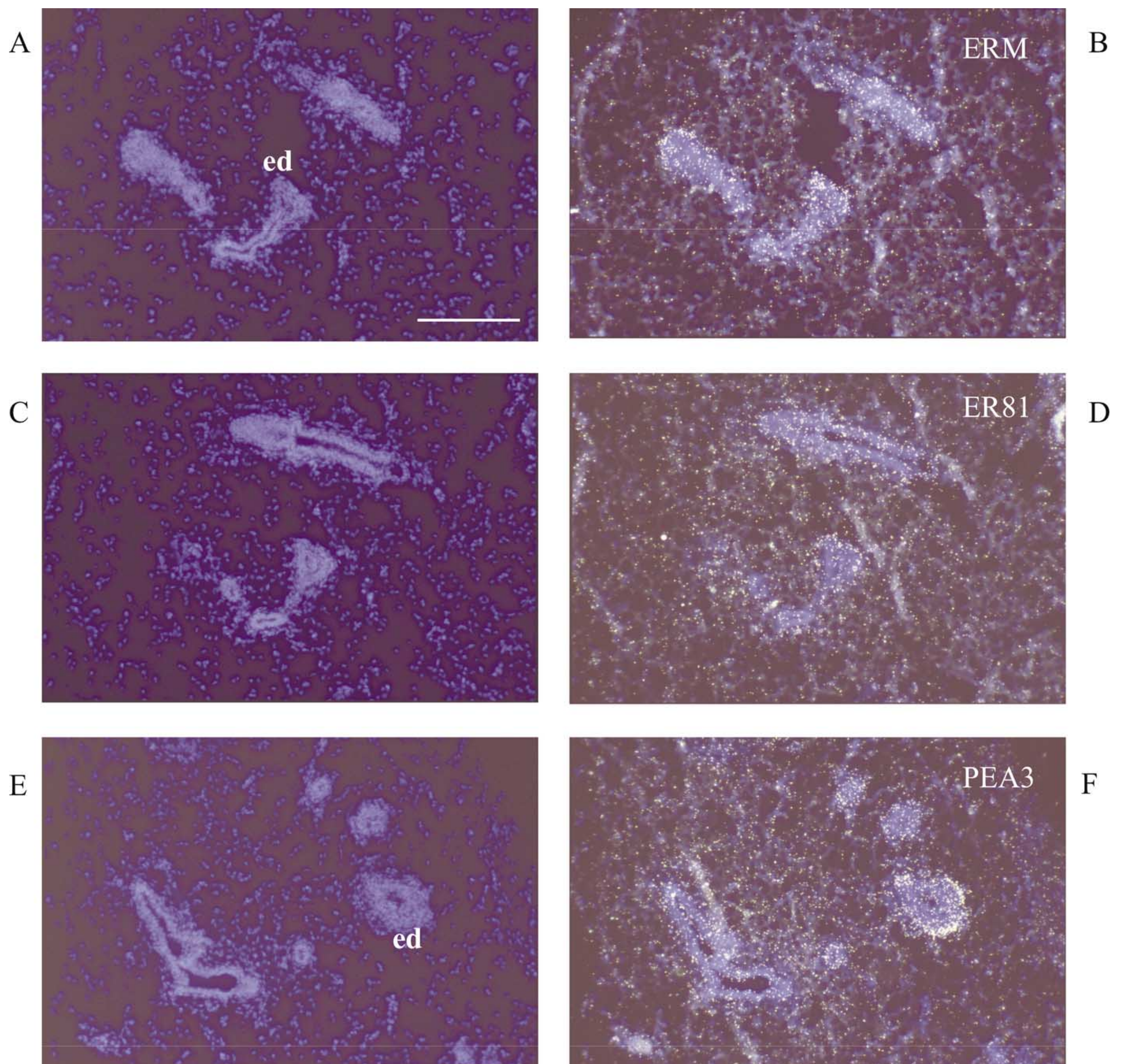


Fig. 5. Expression of *erm* (B), *er81* (D), and *pea3* (F) in sections of mammary gland from pubescent mice (26 days old). Darkfield views and Hoechst staining. (A, C, E) Hoechst staining (control). Bar, 200 μm. *Erm* and *pea3* are expressed in epithelial ducts (ed).

esis. Interestingly, while individual members of the PEA3 group are differentially expressed during these events, the overall pattern of expression of PEA3 group members is very similar in the different organs examined, i.e., lung, salivary glands, kidney, and mammary gland. Specifically, irrespective of the context in which branching morphogenesis takes place, a recurrent pattern of expression was observed for each of the *pea3*-related genes. Thus, *erm* is consistently expressed in the distal portions of epithelial buds, where branching morphogenesis occurs and DNA synthesis is highest (Goldin and Opperman, 1980). *Erm* is also expressed, albeit less strongly, in the adjacent mesenchyme. *Pea3* localizes in a more restricted region of the growing epithelium and is also expressed at a lesser extent in the surrounding mesenchyme. These PEA3 members are expressed not only during mesenchyme-induced branching morphogenesis, but also during the conversion of mesenchyme to epithelium, as observed during kidney development. A signal originating from the ureteric epithelium instructs the condensed mesenchyme to convert into an epithelium, which eventually gives rise to the glomeruli as well as to proximal and distal tubules. We observed a

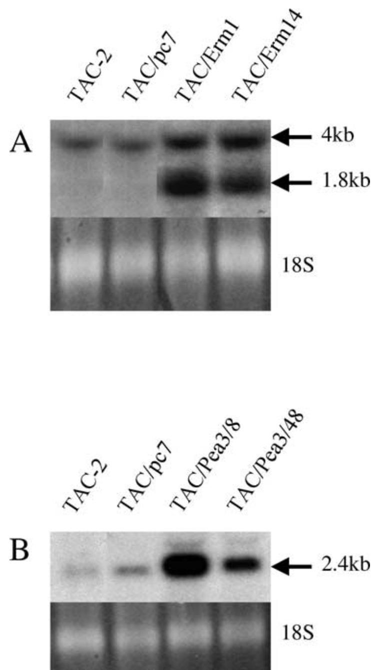


Fig. 6. Northern analysis of *erm* (A) and *pea3* (B) transgene expression in TAC/pc7, TAC/Erm1, TAC/Erm14, TAC/Pea3/8, TAC/Pea3/48, and parental TAC-2.1 cells. The lower panel shows ethidium bromide staining of 18S RNA. In (A), the signals at 4 and 1.7 kb represent endogenous and transgenic *erm* mRNA expression, respectively. In (B), endogenous and exogenous *pea3* mRNA expressions are not distinguishable and generate a signal at approximately 2.4 kb. The overexpression of exogenous *pea3* is demonstrated by a comparatively higher signal.

correlation between the conversion phenomenon and the expression of *erm* and *pea3* genes. The highest level of expression is first seen throughout the undifferentiated condensed mesenchyme of the metanephric vesicles and later in the cap tissue (just subjacent to the capsule of the kidney), as well as in the epithelium of developing glomeruli. In these different organs, *er81* is exclusively expressed at a uniform level in mesenchymal cells. The expression of *erm* and *pea3* decreases dramatically in parallel with epithelial differentiation. The expression pattern of *erm* and *pea3* genes therefore closely correlates with the occurrence of branching morphogenesis and is likely to control underlying cellular processes, such as extracellular matrix remodeling, migration, and proliferation.

A large number of reports have evidenced the transcriptional regulation of MMP encoding genes by members of the ETS family (Gutman and Wasylyk, 1990; Sato and Seiki, 1993; Trojanowska, 2000; Wasylyk et al., 1991). Of special relevance to our field of investigations is the report that E1AF, the human homologue of Pea3, upregulates the expression of different MMP: MMP-1, MMP-3, MMP-9/gelatinase B (Higashino et al., 1995), and MT1-MMP (Habelhah et al., 1999). This probably accounts for the metastatic potential of Pea3-expressing cell lines (Shindoh et al., 1996) (Habelhah et al., 1999; Hida et al., 1997a,b; Kaya et al., 1996). In addition, it was shown using different models

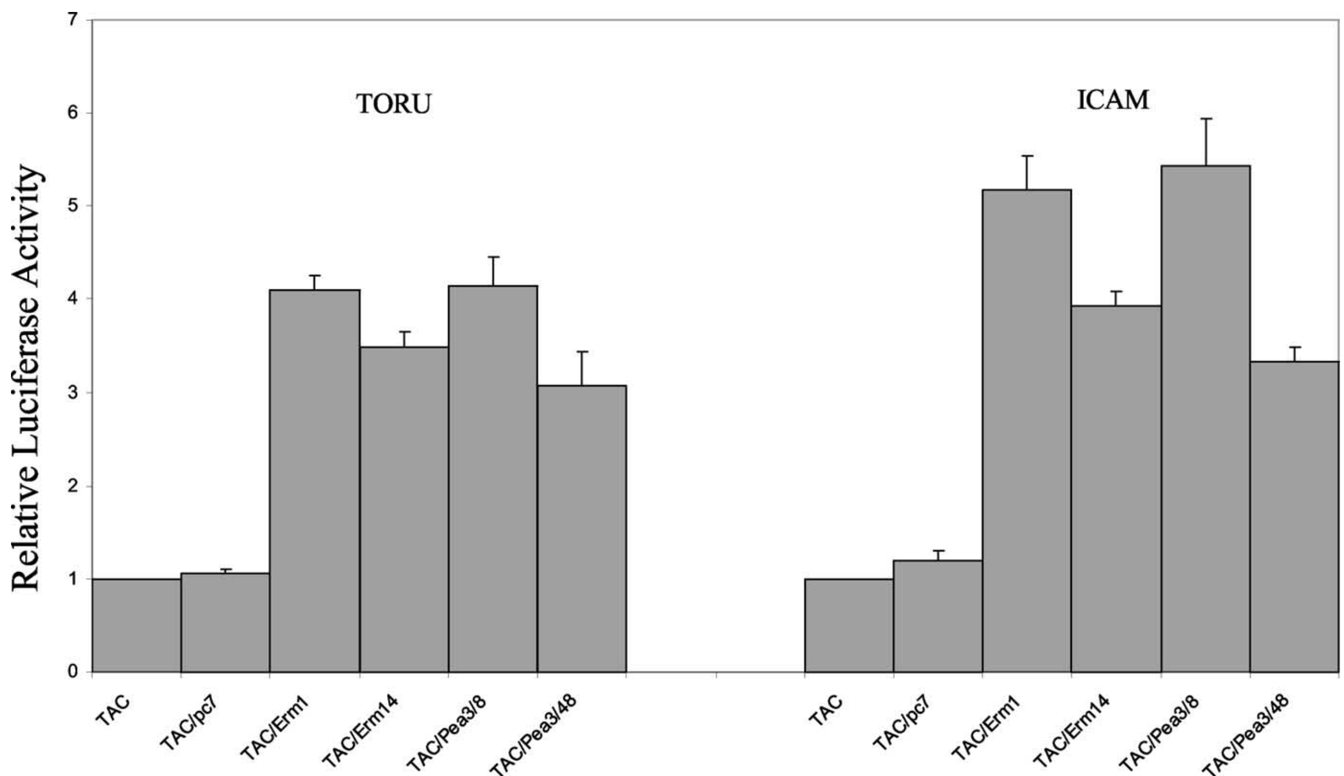
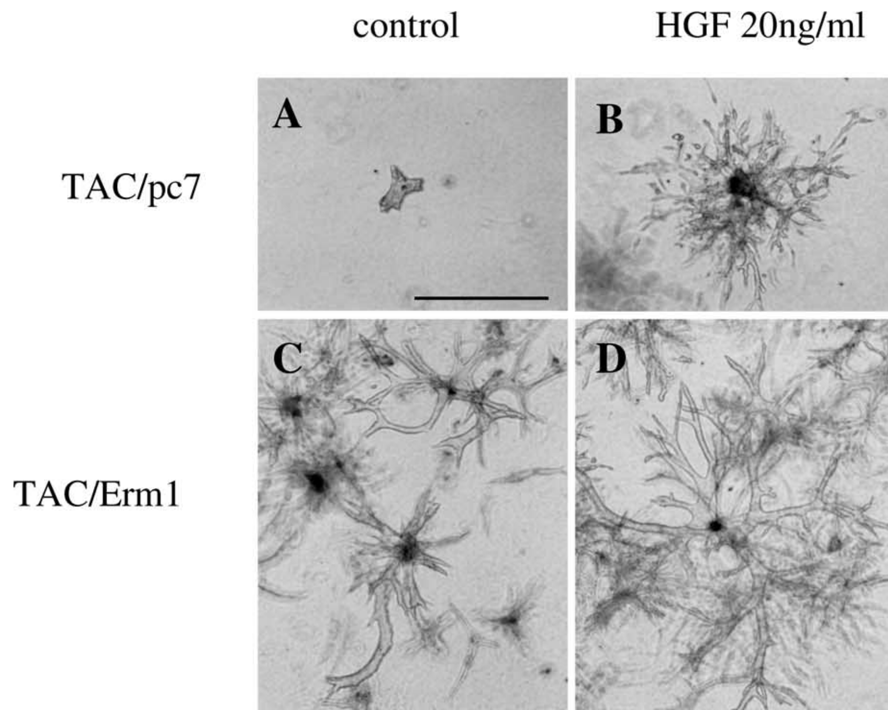


Fig. 7. Transactivation assays of TORU element and human ICAM-1 promoter in parental and transfected TAC-2.1 cells. Cells were transiently transfected with 3XTORU-LUC (Wasylyk et al., 1990) or ICAM-LUC (de Launoit et al., 1998; Voraberger et al., 1991) reporter vectors and pSG-LacZ vector to normalize for transfection efficiency. Luciferase relative activity was indicated after normalization with the β-galactosidase activity.



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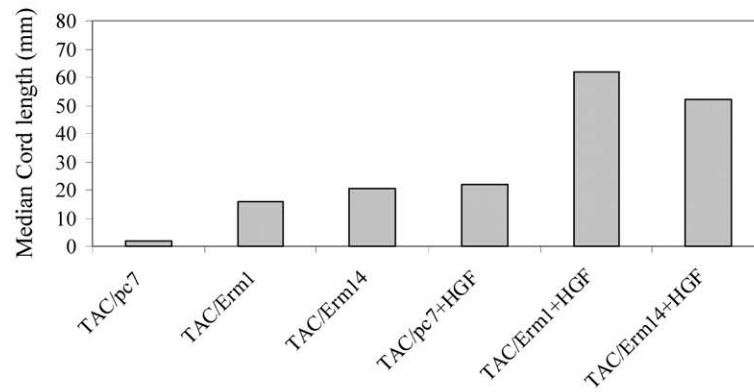

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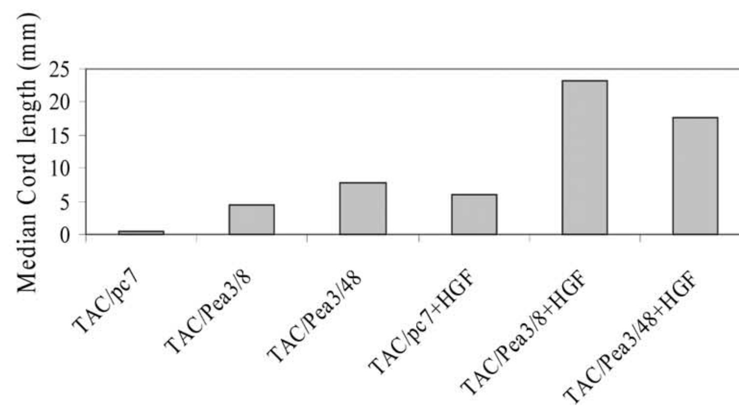

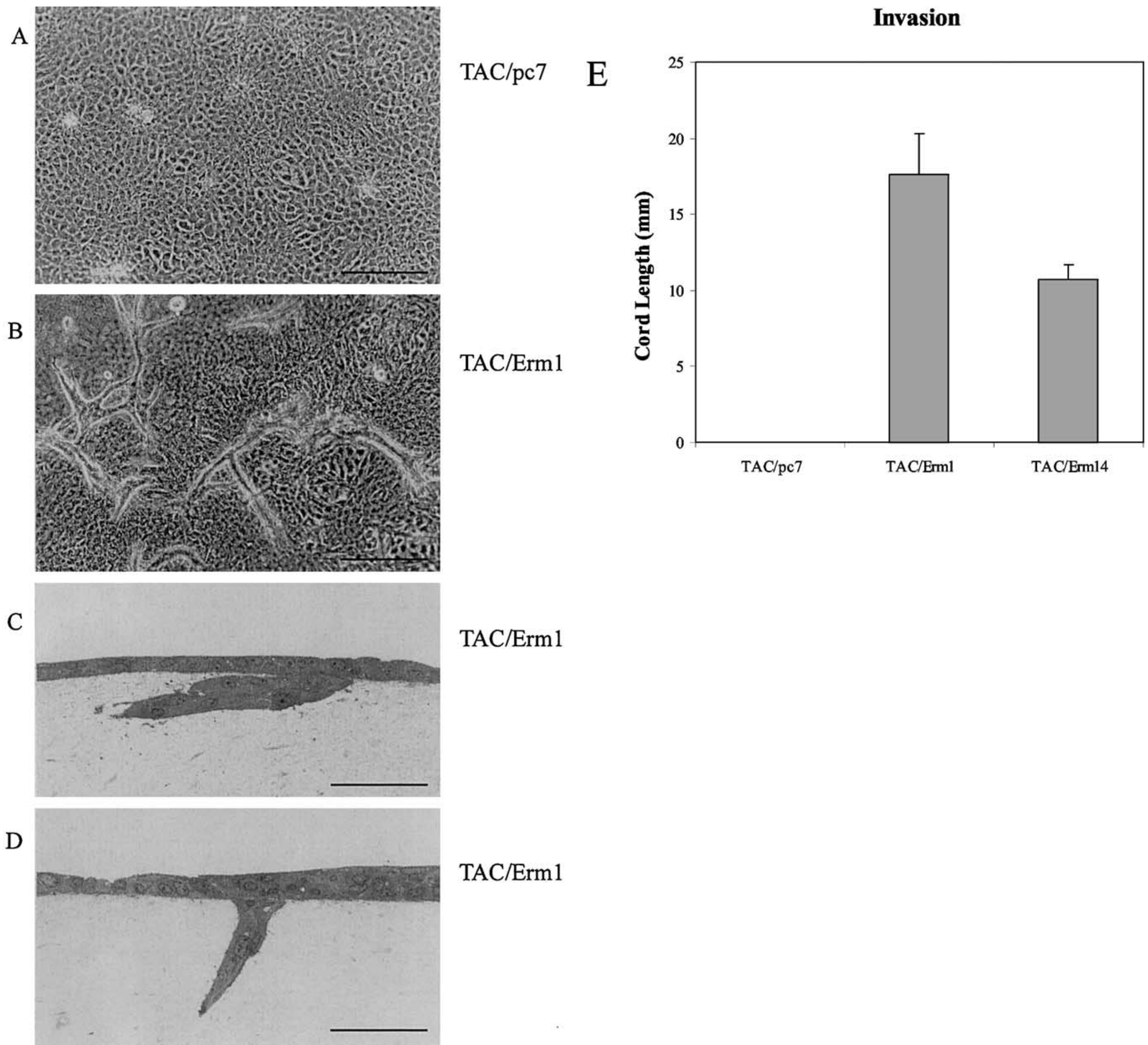

Fig. 9. Overexpression of Erm confers an invasive phenotype to TAC-2.1 cells. Cells were seeded onto the surface of a collagen gel and grown for 9 days in the presence of 20 ng/ml EGF. TAC/pc7 cells form a monolayer on the surface of the gel (A). In contrast, TAC/Erm1 cells invade the underlying matrix as branching cords (B) oriented either parallel (C) or perpendicular (D) to the gel surface. (A, B) Phase-contrast microscopy; bars, 200 μm. (C, D) Semithin sections perpendicular to the surface of the gel; bars, 50 μm. (E) Quantitative analysis of collagen matrix invasion. Invasion was quantitated as described in Materials and methods by determining the mean total length ± S.E.M. (in mm) of all invading structures present in a photographic field 20 μm beneath the surface monolayer.

that uPA expression is upregulated through PEA3/AP1 sites located in its promoter (Eandi et al., 2001; Fafeur et al., 1997; Ried et al., 1999). It is therefore conceivable that the PEA3 transcription factors facilitate branching morphogenesis, at least in part, by inducing the expression of MMP and uPA, which in turn remodel the extracellular matrix. An

Fig. 8. Overexpression of Erm and Pea3 promotes branching tubulogenesis by TAC-2.1 cells. Cells were grown in collagen gels for 9 days in the absence (A, C) or the presence (B, D) of 20 ng/ml HGF. In the absence of exogenously added growth factor, TAC/pc7 cells form small irregularly shaped colonies with minimal branching (A). Addition of HGF induces the formation of numerous branching cords (B). Untreated TAC/Erm1 cells form long branching cords similar to those formed by HGF-treated TAC/pc7 cells (C). When incubated with HGF, TAC/Erm1 cells form a system of arborized duct-like structures that is considerably more extensive than that seen in HGF-treated TAC/pc7 cells (D). Bar, 500μm. (E, F) Quantitative analysis of tubulogenesis in Erm- and Pea3-transfected TAC-2.1 clones. Columns represent the median values of cord length per photographic field (three fields per conditions in each of three separate experiments).

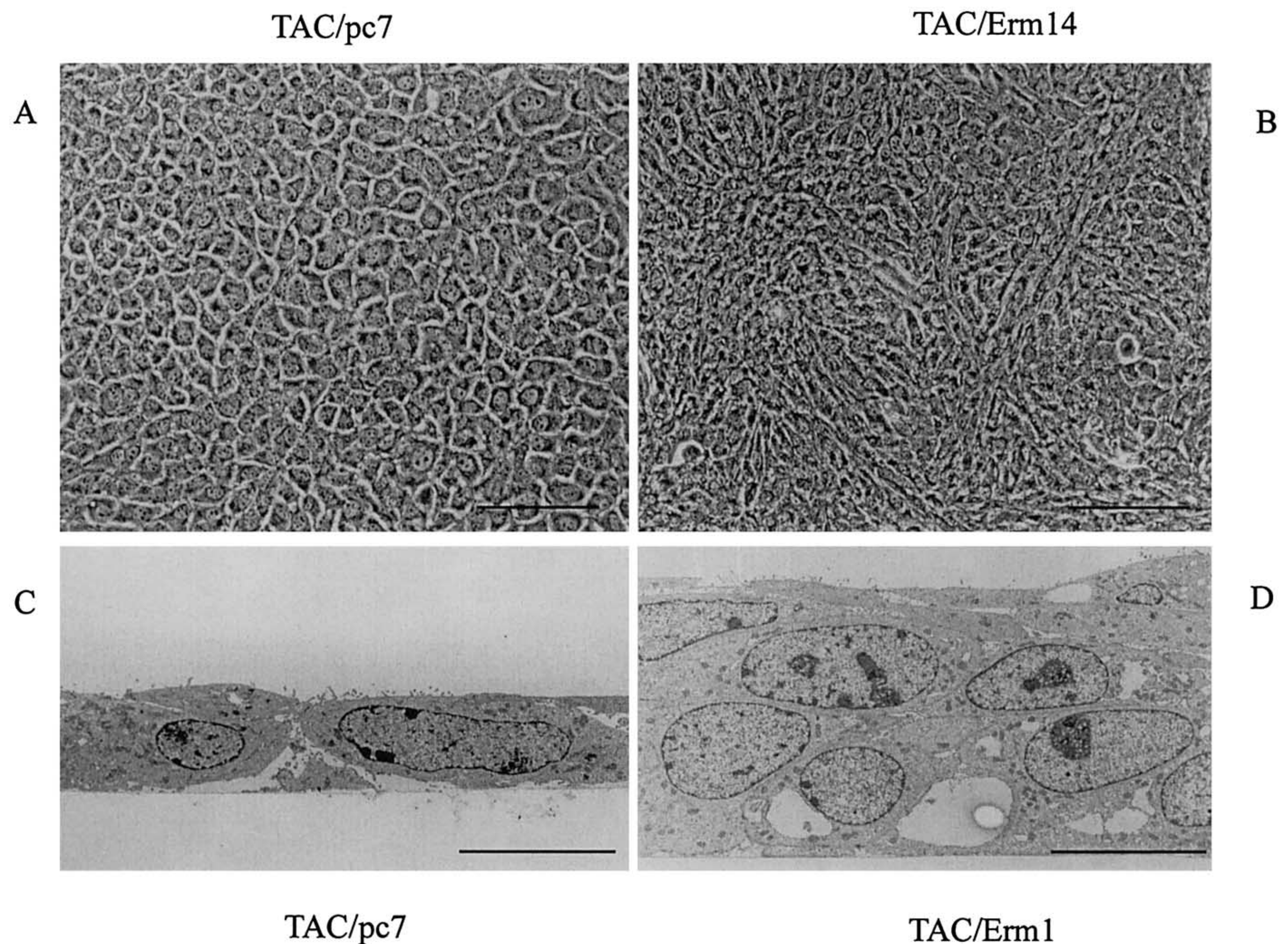

Fig. 10. Loss of contact-mediated inhibition of cell proliferation in Erm-overexpressing TAC-2.1 cells. Cells seeded in collagen-coated dishes at saturating cell density (3×10^5 cells/ml) were incubated with 20 ng/ml EGF for 7 days. Whereas TAC/pc7 cells form a contact-inhibited cobblestone-like monolayer (A, C), TAC/Erm1 and TAC/Erm14 cells grow in a criss-cross pattern and pile up forming multiple layers (B, D). (C, D) Thin sections perpendicular to the bottom of the dishes. Bars: (A, B) 100 μm; (C, D) 10 μm.

additional mechanism of action of PEA3 group members could involve the modulation of cell adhesion molecules, which are essential determinants of epithelial morphogenesis. In this respect, it is highly relevant that Ets proteins (Erm and Ets1) have been reported to modulate the expression of the cell adhesion molecule ICAM-1 (de Launoit et al., 1998) and that Ets Binding Sites are important in regulation of ICAM-1 promoter (Roebuck et al., 1995).

Having established that PEA3 group transcription factors are preferentially expressed at sites of epithelial–mesenchymal interactions associated with the branching of epithelial buds, we next investigated whether their overexpression alters the morphogenetic properties of mammary epithelial cells grown in a three-dimensional extracellular matrix environment. We found that expression of Erm and Pea3 in TAC-2.1 mammary epithelial cells induces the formation of branching duct-like structures to an extent comparable to that observed following incubation with the tubulogenic factor HGF (Soriano et al., 1995). Notably, addition of HGF to collagen gel cultures of TAC-2.1 cell lines expressing Erm or Pea3 results in the development of a network of duct-like structures that is considerably more extensive than that found in mock-transfected TAC-2.1 cells. These findings are consistent with the increased mobility observed in Pea3 transfected lung cancer cells upon HGF treatment (Hiroumi et al., 2001) and raise the intriguing possibility that Erm and Pea3 cooperate with HGF in the induction of mammary gland ductal morphogenesis. Interestingly, Pea3 has recently been shown to be upregulated in murine mammary epithelial cells in response to Wnt-1 (Howe et al., 2001), which we have found previously promotes branching morphogenesis of TAC-2.1 cells (Uyttendaele et al., 1998). It is therefore conceivable that the branching morphogenetic activity of Wnt-1 is mediated in part through the induction of Pea3. The mechanisms whereby Erm and Pea3 promote the formation of branching duct-like structures by TAC-2.1

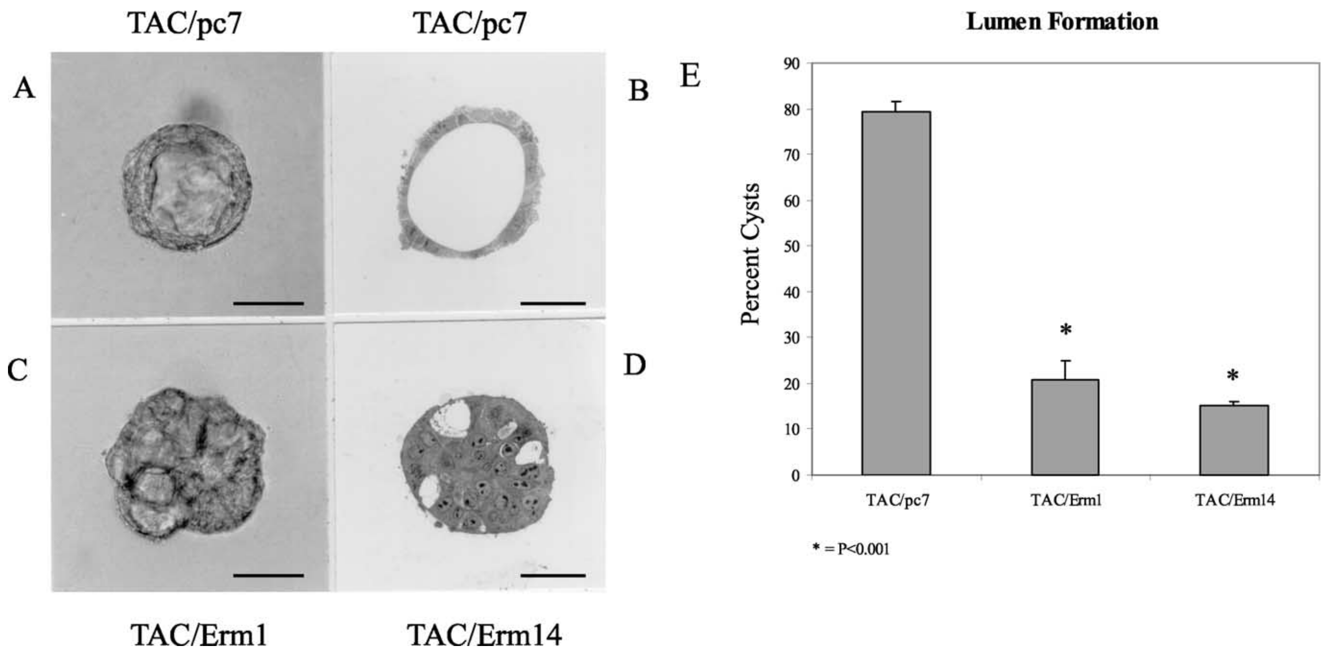

Fig. 11. Overexpression of Erm disturbs hydrocortisone-induced lumen formation by TAC-2.1 cells. Cells were suspended in collagen gels in serum-free medium and incubated with 1 μg/ml hydrocortisone and 50 ng/ml cholera toxin for 9 days. Under these conditions, TAC/pc7 cells form spheroidal cysts containing a widely patent lumen (A) delimited by a palisade of cubic epithelial cells (B). In contrast, TAC/Erm1 and TAC/Erm14 cells form solid colonies containing small focal lumina (C, D). (A, C) Bright field microscopy. Bars, 50 μm; (B, D) Semithin sections. Bars, 25 μm. (E) Quantitative analysis of lumen formation. A total of 150 randomly selected colonies per experimental condition were examined in each of 3 separate experiments and scored as cysts when containing a single wide lumen. Data were expressed as mean percentage of cysts ± S.E.M., and statistical significance was determined by using the Student's unpaired *t* test.

cells are not known, but may involve the release of morphogenetic factors, the production/activation of extracellular proteinases, the deposition of specific extracellular matrix components or altered expression of their receptors, and increased cell survival or proliferation. With respect to the latter possibility, however, it is relevant to underscore that the vast majority of colonies which form in long-term collagen gel cultures of wild type TAC-2 cells consist of irregularly shaped cell aggregates devoid of tubular extensions (Soriano et al., 1995). This finding indicates that an increase in cell number, while likely contributing to the extension of existing tubes, is not by itself sufficient to promote epithelial branching. Studies are in progress to identify the target genes that may mediate the observed effects of PEA3 group transcription factors.

The findings that *erm* and *pea3* are strongly expressed in epithelial cells undergoing morphogenesis in vivo and stimulate the formation of branching cords by TAC-2.1 mammary epithelial cells in vitro supports the involvement of these transcription factors in the development of parenchymal organs. However, we have also shown that Erm, and to a lesser extent Pea3, confer on TAC-2.1 cells certain characteristics of transformed cells. These include the capacity to invade the underlying matrix when seeded on the surface of a collagen gel, the loss of contact-mediated inhibition of cell proliferation, as well as an impaired ability to form polarized, lumen-containing multicellular structures.

How can these apparently paradoxical findings be reconciled? It is highly relevant in this context to recall that a number of biological processes required for normal morphogenesis can also contribute to tumorigenesis when inappropriately reactivated during postnatal life. Thus, morphogenetic cell rearrangements involve transient down-modulation of cell adhesion and polarity, increased cell motility, and expression of balanced proteolytic activity, allowing spatially and temporally restricted extracellular matrix invasion. When disregulated, however, these processes may facilitate tumor progression by disrupting normal epithelial architecture and promoting uncontrolled invasion of surrounding tissues (Birchmeier et al., 1996; Thiery, 2002). In accord with these notions, we propose that PEA3 group transcription factors play a dual role in the regulation of epithelial cell behavior. Transient expression of Erm and Pea3 in developing glandular tissues may facilitate branching tubulogenesis, owing in part to their ability to induce matrix-degrading proteinases. On the other hand, sustained high level expression of these transcription factors may endow epithelial cells with pronounced invasive properties and interfere with the physiological mechanisms responsible for cell polarization and differentiation, thereby contributing to tumorigenesis. It will be important to determine in future studies whether Erm- and Pea3-overexpressing TAC-2.1 cells are tumorigenic in vivo.

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