The Estrogen-dependent c-JunER Protein Causes a Reversible Loss of Mammary Epithelial Cell Polarity Involving a Destabilization of Adherens Junctions

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Abstract. Members of the epidermal growth factor (EGF) receptor family are known to be specifically involved in mammary carcinogenesis. As a nuclear target of activated receptors, we examined c-Jun in mammary epithelial cells. For this, we used a c-JunER fusion protein which was tightly controlled by estrogen. Activation of the JunER by hormone resulted in the transcriptional regulation of a variety of AP-1 target genes. Hormone-activated JunER induced the loss of epithelial polarity, a disruption of intercellular junctions and normal barrier function and the formation of irregular multilayers. These changes were completely reversible upon hormone withdrawal. Loss of epithelial polarity involved redistribution of both apical and basolateral proteins to the entire plasma membrane. The redistribution of E-cadherin and β-catenin was accompanied by a destabilization of complexes formed between these two proteins, leading to an enrichment of β-catenin in the detergent-soluble fraction. Uninduced cells were able to form three-dimensional tubular structures in collagen I gels which were disrupted upon JunER activation, leading to irregular cell aggregates. The JunER-induced disruption of tubular structures was dependent on active signaling by growth factors. Moreover, the effects of JunER could be mimicked in normal cells by the addition of acidic fibroblast growth factor (aFGF). These data suggest that a possible function of c-Jun in epithelial cells is to modulate epithelial polarity and regulate tissue organization, processes which may be equally important for both normal breast development and as initiating steps in carcinogenesis.

MAMMARY epithelial cells are able to extensively modulate their phenotype when the mammary gland changes its size and functional activity during pregnancy, lactation, and weaning. These phenotypical changes are regulated by numerous hormones and growth factors as well as by interactions between cells (Gabelman and Emerman, 1992; Oka et al., 1991; Streuli et al., 1991). Most of them involve alterations in epithelial polarity.

Polarized epithelial cells are structurally and functionally divided into several domains that contain distinct sets of proteins and lipids (Edidin, 1993; Simons et al., 1992). The apical domain faces the lumen of the mammary gland and is the site of milk protein secretion during lactation. The lateral domain is responsible for the close interaction between neighboring cells via different junctional complexes such as tight junctions, adherens junctions, gap junctions, and desmosomes (Woods and Bryant, 1993). These complexes contain specific and/or common proteins which are responsible for the intercellular connections. Examples are ZO-1, a protein associated with tight junctions (Balda et al., 1993), and E-cadherin/uvomorulin, a transmembrane homophilic adhesion molecule that binds catenins and interacts with the cytoskeleton through proteins such as fodrin to form the adherens junctional complex (for review see Birchmeier and Behrens, 1994; Fleming et al., 1993). And finally, the basal membrane contacts the surrounding mesenchyme. The apical domain is separated from the basolateral part of the cell by a circumferential ring of tight junctions, which is also responsible for the sealing of the intercellular space (Mandel et al., 1993). Thus, monolayers of epithelial cells form an effective barrier between the luminal and the interstitial compartments of the mammary gland.

During breast cancer development, the phenotype of mammary epithelial cells including cellular interactions, state of polarity, and response to proliferation and differentiation control is altered, eventually resulting in highly tumorigenic and invasive cell types (Birchmeier and Behrens, 1994; Fish and Molitoris, 1994). Several protein families, acting at different stages in signal transduction cascades induced by extracellular signals, have been implicated

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in mammary carcinogenesis. Overexpression of the growth factor TGFα in transgenic mice leads to altered mammary gland development and neoplasia (Matsui et al., 1990). Amplification and/or mutational activation of receptor tyrosine kinases including Neu/c-ErbB-2 (Dougall et al., 1994) and EGF receptor (Jardines et al., 1993) is found in about one third of human breast tumors and is correlated with poor prognosis. Moreover, members of the FGF receptor (FGFR)[1] family are amplified in another set of human breast carcinomas (Adnane et al., 1991), stressing the important role of receptor tyrosine kinases in malignancies of the mammary gland.

Ligand-activated growth factor receptors transmit their signals to the nucleus by activating components of various intracellular signaling pathways, eventually leading to changes in gene expression (for review see Kazlauskas, 1994). Blockage of growth factor–induced signal transduction causes a growth arrest in mammary epithelial cells (Stampfer et al., 1993) and overexpression of components of the Ras pathway severely affects differentiation and proliferation of these cells in vivo and in vitro (Cha et al., 1994; Daly et al., 1994; Jehn et al., 1992; Morrison and Leder, 1994).

Several members of the AP-1 family of transcription factors, including c-Fos and c-Jun, are nuclear targets of these signal transduction pathways (Deng and Karin, 1993; Derijard et al., 1994; Engelberg et al., 1994). AP-1 proteins form homo- and/or heterodimeric complexes and bind to a common sequence element known as the TPA-responsive element (TRE; for review see Woodgett, 1990). c-Fos induces severe changes of epithelial polarity in mammary epithelial cells (Jehn et al., 1992; Reichmann et al., 1992). However, c-Jun and c-Fos are subject to different regulatory mechanisms (Minden et al., 1994; Rapp et al., 1994). c-Jun is the nuclear target of ErbB-2–induced (Ben-Levy et al., 1994) and other Ras/MAP kinase–dependent signals (Derijard et al., 1994) and its activity is required for oncogenic transformation (Brown et al., 1993). We therefore were interested to study the potential role of this oncoprotein in the modulation of the normal, polarized phenotype of epithelial cells and in breast carcinogenesis.

In this paper, we show that activation of an estrogen-dependent c-JunER fusion protein in mouse mammary epithelial cells causes a reversible loss of epithelial polarity. The tight contacts between cells are loosened, leading to irregular multilayers on permeable supports and formation of solid cords instead of hollow ducts in collagen gels. In addition, the E-cadherin/β-catenin complex is disrupted, and its components are redistributed. This does not involve tyrosine phosphorylation of β-catenin, which has been implicated in the loss of cell adhesiveness after v-src transformation (Behrens et al., 1993; Hamaguchi et al., 1993). Supporting the idea that activated c-JunER mimics the physiological effects of endogenous c-Jun, the phenotype is thoroughly dependent on growth factors. Moreover, we can induce a similar phenotype in collagen gels by the addition of acidic fibroblast growth factor (aFGF).

1. *Abbreviations used in this paper*: aFGF, acidic fibroblast growth factor; CAT, chloramphenicol acetyltransferase; DPPIV, dipeptidyl peptidase IV; E2, β-estradiol; ER(HBD), estrogen receptor (hormone-binding domain); FGFR, fibroblast growth factor receptor; PAI-1, plasminogen activator inhibitor-1; TER, transepithelial electrical resistance; TRE, TPA-responsive element; UPA, urokinase-type plasminogen activator.

Materials and Methods

Cells and Cell Culture

EpH4 cells (H4) were generated by infecting spontaneously immortalized Ep1 cells (Reichmann et al., 1992) with the empty retroviral vector pHMW and subcloning the cells in the presence of 115 U/ml hygromycin B (Calbiochem Corp., La Jolla, CA). Clones obtained built up polarized monolayers when grown on permeable filters and exhibit high transepithelial electrical resistance (TER), as well as polarized expression of apical and basal epithelial marker proteins (López-Barahona et al., 1995). They form three-dimensional, tubular structures when cultured in collagen I gels and other extracellular matrices. Cells were routinely cultured in DMEM supplemented with 5% FCS (Boehringer Ingelheim) and 10 mM Hepes, pH 7.3, as described (Reichmann et al., 1992). β-Estradiol (E2; Sigma Chem. Co., St. Louis, MO) was used at a concentration of 10^{-6} M. Ethanol was used as a solvent control (−E2). For culture on permeable filters, cells were counted in a CASY 1 (Schärfe System), and 2.5-3 × 10^5 cells seeded on cell culture inserts (Falcon 3090). MDCK ts v-*src* cells were cultured as described (Behrens et al., 1993).

Culture in Collagen I Gels

Cells were seeded in collagen I (Sigma Chem. Co.) gels as described (Reichmann et al., 1992) at 2 × 10^4 cells/ml with the following modifications. After the collagen gels had polymerized, they were overlaid with serum-free mammary epithelial cell basal medium (PromoCell) supplemented with 0.4% bovine pituitary extract (BPE; PromoCell), 0.04 IU/ml insulin, 10^{-6} M isoproterenol, 25 ng/ml hydrocortisone and 2.5 ng/ml TGFα.

Retroviral Infection

Retroviral vectors pMV-7 and pMV-ER(HBD) and pMV-c-*jun* have been described previously (Bergers et al., 1994; Kirschmeier et al., 1988). pMV-*jun*ER was constructed by fusing a 990-bp PstI–BspHI *c-jun* fragment from pMV-c-*jun* together with an oligonucleotide, restoring the complete cDNA of *c-jun*, in-frame into the PstI–BamHI sites upstream of the hormone-binding domain in pMV-ER(HBD). GP+E packaging cells (Markowitz et al., 1988) were transfected with the respective constructs as described (Reichmann et al., 1992) and selected in 0.8 mg/ml G418. For retroviral infection, epithelial H4 were cocultured for 2 d with resistant virus-producing GP+E cells pretreated with 10 μg/ml mitomycin C for 5 h. Epithelial cell clones were selected in 1 mg/ml G418, isolated, and expanded as described (Reichmann et al., 1992).

Chloramphenicol Acetyltransferase Assay

Clones of the GP+E packaging line expressing the respective retroviral constructs were transfected by calcium phosphate coprecipitation with reporter plasmids. These were vectors containing the *CAT* gene fused to parts of the human collagenase promoter either lacking the TRE (−63 Col-CAT) or containing one TRE (−73 Col-CAT) or a pentamerized binding site in front of the minimal thymidine kinase promoter ($p(-73/-65)_5$ tk-CAT). Generation of the reporter plasmids is described elsewhere (Yang-Yen et al., 1990). The cells were washed and scraped off the culture dish in PBS on ice, pelleted, resuspended in PBS, and spun in an Eppendorf centrifuge at top speed for 20 s. After resuspension in 100 μl 0.25 M Tris/HCl, pH 7.5, they were disrupted by three freeze/thaw cycles and cell debris was pelleted (5 min, 14,000 rpm). The protein concentration of the supernatant was determined and extracts containing identical amounts of protein were subjected to CAT assay.

RNA Preparation and Northern Blot Analysis

Total RNA was prepared essentially as described (Puissant and Houdebine, 1990). Briefly, after guanidinium thiocyanate extraction and isopropanol precipitation the pellets were resuspended in 1 ml 4 M LiCl to solubilize copurified polysaccharides. RNA was pelleted, resuspended in 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.5% SDS and precipitated with ethanol. 10 μg of RNA was separated in 1.5% agarose and transferred onto Gene Screen™ membranes (New England Nuclear, Boston, MA). Single

stranded DNA probes were radioactively labeled with ^{32}P by using an Oligolabeling Kit (Pharmacia LKB Biotechnology, Piscataway, NJ) and hybridized at 65°C in 7% SDS, 0.5 M NaPhosphate, pH 7.0, 1 mM EDTA. Membranes were washed in 1% SDS, 40 mM NaPhosphate at 65°C and autoradiographed. Probes used were *fra-1* (Bergers et al., 1995), *UPA* (urokinase-type plasminogen activator), *PAI-1* (plasminogen activator inhibitor 1) (Reichmann et al., 1992), and mouse *junB* and *junD*.

Immunoprecipitation and Subcellular Fractionation

For immunoprecipitation, cells were grown on filter culture inserts (75 mm, Costar Corp., Cambridge, MA), scraped off the filters in 1% Triton X-100 in L-CAM assay buffer, supplemented with protease inhibitors and 0.2 mM sodium orthovanadate as described by Behrens et al. (1993), extracted 1 h on ice and aliquots containing identical amounts of protein subjected to immunoprecipitation. Precipitates were resuspended in sample buffer and analyzed by immunoblotting as described below.

Subcellular fractionation of cells grown on filters (75 mm, Costar) was performed essentially as described by Navarro et al. (1993). Fractions soluble and insoluble in 2.5% NP-40 were analyzed by immunoblotting as described below.

Whole Cell Protein Extracts and Immunoblotting

Cells were scraped off the culture dish in 1× Laemmli SDS gel sample buffer, boiled for 5 min and proteins were separated in 6–15% SDS-polyacrylamide gradient gels. For immunoblots the proteins were transferred onto Nitrocellulose BA 85 (Schleicher & Schuell, Keene, NH) which was subsequently blocked with 3% BSA (Sigma) in PBS, 1 mM EDTA and 0.05% Tween 20 (block buffer). The antibody reaction was performed in block buffer containing 0.02% NaAzide for at least 2 h. Filters were washed three times for 10 min in 50 mM Tris/HCl, pH 8.0, 0.1 M NaCl and 0.1% Tween 20 (wash buffer), incubated with horseradish peroxidase–conjugated secondary antibody ($1:10^5$) in 5% milk powder in PBS (freshly prepared) for 30 min, washed three times and subjected to immunodetection using ECL Western blotting reagents (Amersham Corp.). All steps were performed at room temperature. Stripping of blots was performed according to the manufacturer's specifications.

Densitometry

To quantify data from Western blots and combined immunoprecipitation Western analysis, films were scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). In each case, data from two independent experiments were scanned twice and the values obtained averaged. Standard deviations were <10% in all cases.

Immunofluorescence

Cells grown on filters were fixed and permeabilized either with acetone/methanol 1:1 at −20°C for 3 min or with 3% paraformaldehyde (PFA) in 0.2 M Hepes, pH 7.3, 1 mM $CaCl_2$, 1 mM $MgCl_2$, followed by 0.5% Triton X-100 in the same buffer. The filters were cut into pieces for staining with several antibodies, blocked with 0.2% gelatin in PBS for 30 min, incubated with primary antibodies in PBS/gelatin for 60 min, washed four times for 10 min and incubated with secondary antibodies for 30 min. After three washes for 5 min, filter pieces were mounted with Mowiol in PBS, covered with coverslips and subjected to confocal microscopy.

Microscopy

Cells grown either on permeable filters or in collagen I gels were prefixed 10 min in 3% PFA in 0.2 M Hepes, pH 7.3, at room temperature. Cells were further fixed in 8% PFA, 0.2 M Hepes, pH 7.3, for 30–60 min on ice. For ultrastructural studies cells were postfixed with 1% osmium tetroxide in PBS, pH 7.2, for 1 h on ice, block stained with 1% aqueous uranyl acetate for 1 h, dehydrated in ethanol at room temperature and finally embedded in Epon. Ultrathin sections (50–70 nm thick) were stained with uranyl acetate and lead citrate and viewed in a Philips CM10 transmission electron microscope at 60 kV. For scanning electron microscopy, samples were fixed and block stained as described above, dehydrated in isopropanol, and critical point-dried from carbon dioxide. The specimens were sputter-coated with 6 nm gold-palladium and inspected in a Hitachi S-800 scanning electron microscope. For immunocytochemistry samples were dehydrated in ethanol at progressively lower temperatures, embedded in Lowicryl HM20 and polymerized at −35°C by UV light (Schwarz et al., 1993). Ultrathin sections were mounted on coverslips (Schwarz, 1994). After blocking unspecific binding sites with 0.5% BSA and 0.2% gelatin in PBS, sections were incubated with rabbit anti–β-catenin antibodies and subsequently with Cy3-labeled goat anti–rabbit IgG. To visualize Cy-3 fluorescence at an improved signal to noise ratio, a bandpass filter set for Cy 3 (Chroma High Q No. F41-007, AF -Analysentechnik, Tübingen, FRG) was used instead of a conventional TRITC filter. The labeled sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei in immunofluorescence microscopy.

For confocal microscopy cells were stained by immunofluorescence as mentioned above and visualized by use of a 600 confocal fluorescence microscope fitted with a confocal imaging system (Bio-Rad, England). The distance between two scanning planes was 0.2 μm.

Transepithelial Electrical Resistance Measurements

Cells were grown on filter culture inserts (Falcon 3090). The functional integrity of tight junctions was assayed by measuring the TER of cell layers using the Millicell Electrical Resistance System (Millipore Corp., Bedford, MA) and the electrode system Endohm-24 (World Precision Instruments, New Haven, CT). TER values were calculated after subtraction of the very low background of blank inserts.

Antibodies

Rabbit antiserum to c-Jun was a gift from Dr. M. Nicklin (Royal Hallamshire Hospital, Sheffield). Mouse monoclonal antibody to the hormone-binding domain of the human estrogen receptor (F3) has been described (Ali et al., 1993). Mouse monoclonal antibody against the apical protein dipeptidyl peptidase IV (DPPIV) was kindly provided by Dr. M. Pierres (Centre d'Immunologie de Marseille-Luminy). Rat monoclonal antibody (mAb1622) to ZO-1, a tight junctional marker protein, was obtained from Chemicon Intl., Inc. (Temecula, CA). Rabbit antiserum specific for the 84-kD fragment of E-cadherin was kindly provided by Dr. R. Kemler (Max-Planck-Institut für Immunbiologie, Freiburg) and polyclonal antibody to β-catenin was a kind gift from Dr. J. Behrens (Max-Delbrück-Zentrum für Molekulare Medizin, Berlin) and Dr. S. Schneider (Max-Planck-Institut für Entwicklungsbiologie, Tübingen). Anti-phosphotyrosine mouse monoclonal clone 4G10 was purchased from UBI and affinity-purified labeled secondary antibodies were obtained from Amersham. Cy-3–labeled antibodies were from Jackson ImmunoResearch Labs (West Grove, PA).

Results

Expression of a Functionally Active c-JunER Protein in Epithelial Cells

To analyze the potential effects of c-Jun in mammary epithelial cells, we constructed retroviral expression vectors containing a fusion protein of c-Jun with the hormone-binding domain of the human estrogen receptor (pMV-*jun* ER). Retroviral vectors expressing bona fide c-Jun (pMV-c-*jun*), the hormone-binding domain of the estrogen receptor [ER(HBD)] alone [pMV-ER(HBD)] or only the neomycin resistance gene as a selectable marker (pMV-7) were used as controls (Fig. 1 *A*; see Materials and Methods). For production of viruses these vectors were transfected into the fibroblastic packaging line GP+E (Markowitz et al., 1988). These cells were also used to test the estrogen-dependent transactivation function of the retrovirally transduced conditional c-JunER protein (also referred to as JunER). For this, GP+E fibroblasts expressing JunER or ER(HBD) were transiently transfected with reporter plasmids containing the CAT gene fused to parts of the human collagenase promoter, containing no TRE, one TRE, or the pentamerized AP-1–binding site (Yang-Yen et al., 1990). The cells were cultivated in the absence or presence of E2. JunER-expressing fibroblasts trans-

fected with reporter constructs containing TRE(s) showed very high CAT activity in the presence but not in the absence of hormone (Fig. 1 *B*). As expected, transfection with the construct lacking the TRE lead to only basal transcription levels even after E2 addition. Furthermore, only basal levels of CAT activity was detected in cells expressing the ER(HBD) with all the reporter constructs used (Fig. 1 *B*), confirming the protein's lack of transactivation potential.

Next, mammary epithelial cells capable of full epithelial polarization (H4 cells, see Materials and Methods) were infected with the retroviruses shown in Fig. 1 *A*. G418-resistant clones (H4 pMV, H4 c-jun, H4 ER(HBD), and H4 junER, respectively) were tested for RNA and protein expression by Northern analyses and immunoblots. mRNAs of the expected lengths (data not shown) and proteins of the expected sizes (Fig. 1 *C*) were detected in the infected cells. The JunER protein (M_r = 70–75) reacted with antibodies against c-Jun and against the hormone-binding domain of the human ER (F3; Ali et al., 1993). As expected, c-Jun (M_r = 39; both endogenous and retroviral) failed to react with F3 antibody, whereas the ER(HBD) polypeptide (M_r = 33) was exclusively detected with F3 (Fig. 1 *C*). In addition, the ER-specific antibody also reacted with endogenous estrogen receptor (mER; M_r = 66; Parker et al., 1993).

Expression and localization of JunER in epithelial cells plus or minus estradiol was also checked by indirect immunofluorescence (Fig. 1 *D*). The JunER protein was diffusely expressed throughout the cytoplasm and the nucleus in the absence of E2 but showed clear nuclear accumulation in the presence of E2. Similar levels of nuclear c-Jun were expressed in H4 c-jun clones, but in a hormone-independent fashion (data not shown). Thus, the retrovirally infected cells expressed the expected proteins in substantial amounts and in the appropriate subcellular compartments.

Regulation of AP-1 Target Genes by JunER in Epithelial Cells

Having shown in fibroblasts that c-JunER behaved like a

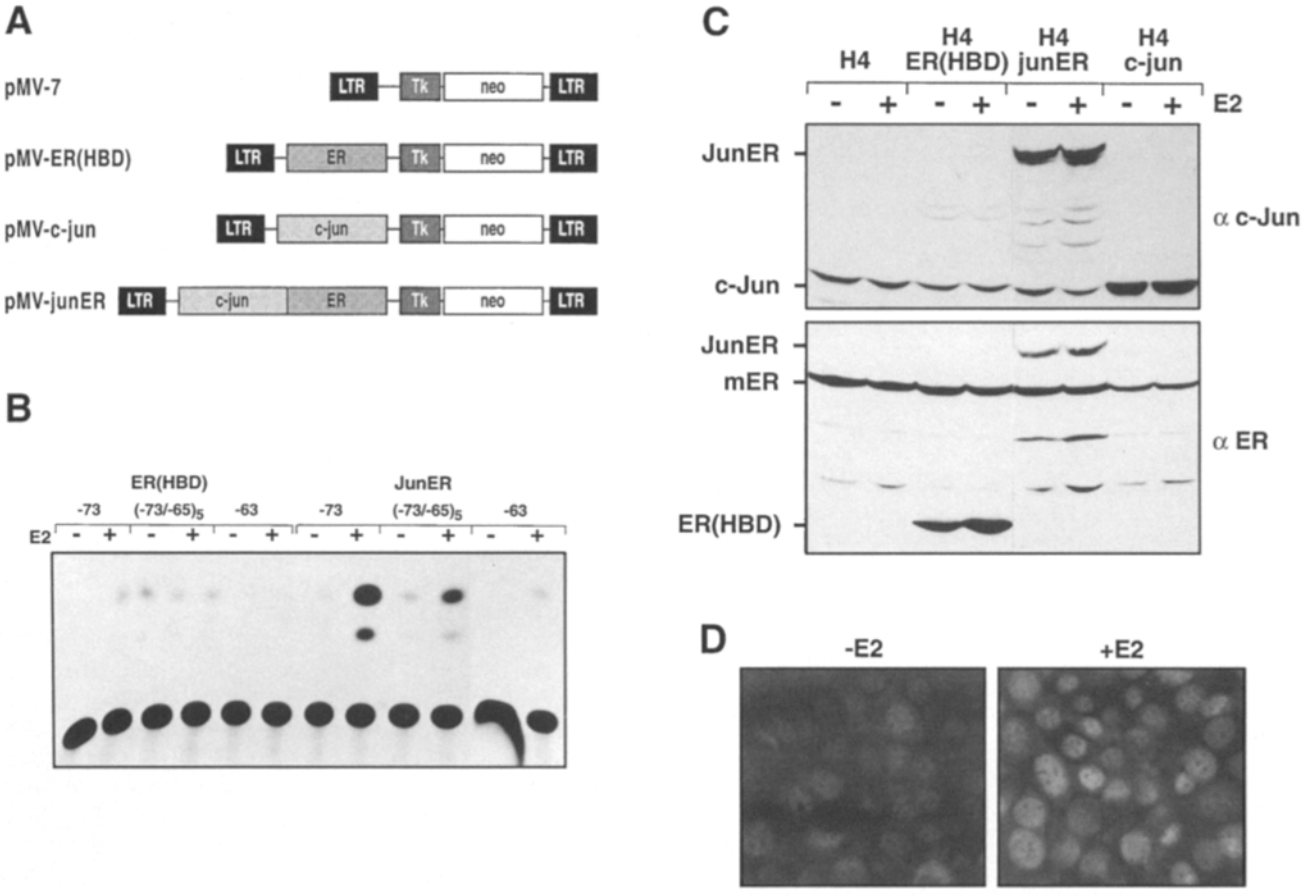


Figure 1. Structure, biological activity, and expression of retroviral vectors used. (*A*) Structures of the retroviral vectors pMV-7, pMV-ER(HBD), pMV-c-*jun*, and pMV-*jun*ER. LTR, long terminal repeat. (*B*) Virus producing fibroblasts expressing the fusion protein JunER or ER(HBD) were transiently transfected with the reporter constructs −73 Col-CAT (−73), p(−73/−65)$_5$ tk-CAT [(−73/−65)$_5$] and −63 Col-CAT (−63). The cells were grown in the presence (+) or absence (−) of E2 for 1 d. Cell extracts containing equal protein amounts were analyzed for CAT expression. (*C*) Epithelial H4 cells were infected with the various retroviral constructs and clones were grown in the absence (−) and presence (+) of E2 for 2 d. Protein lysates of cells were analyzed by SDS-PAGE and immunoblots using antibodies to c-Jun (α–c-Jun) and to ER(HBD) (α ER). (*D*) H4 junER cells were cultivated without (−E2) or with (+E2) hormone, immunostained with α–c-Jun and subjected to confocal microscopy (horizontal section through the nuclei).

hormone-inducible transcription factor, it was of interest to determine in epithelial cells, if known AP-1 target genes were regulated by JunER in a hormone-dependent fashion. Such genes include other members of the AP-1 family (Bergers et al., 1995; Rozek and Pfeifer, 1993) as well as proteases and their inhibitors (Hu et al., 1994; Knudsen et al., 1994; Lee et al., 1993; Reichmann et al., 1992). H4 cells expressing JunER, ER(HBD) or no exogenous protein were cultivated in the presence or absence of E2 for 2 h, as well as for 1, 2, or 4 d. Total RNA prepared from these cells was then studied by Northern blot analysis.

Two interesting findings were obtained. First, mRNA expression of several AP-1–regulated genes, including *junB, fra-1* (Bergers et al., 1995), the protease *UPA* and its inhibitor *PAI-1,* strongly decreased with time in the absence of an active JunER (Fig. 2, *left panel*). This suggested an effect of cell density and/or epithelial polarization on the expression of these genes. In contrast, the expression of *junD* mRNA (Li et al., 1992; Ryder et al., 1989) was comparatively high on day 4 (Fig. 2, *left panel*). Loss of expression of these genes with time in culture was clearly an intrinsic property of the parental cell line, since it also occurred in H4 and H4 ER(HBD) cells, regardless of the presence or absence of hormone (data not shown).

More importantly, JunER activated or repressed most of these genes in a hormone-dependent fashion. Activation of JunER caused a strong repression of *junB* as soon as 2 h after hormone addition, which was not observed in control parental H4 cells (Fig. 2, *right panel*). This *junB* downregulation was also visible after longer times of E2 induction (not shown). A similar, albeit much weaker, downregulation by JunER was seen continuously for *junD* from day 1. In contrast, a strong E2-dependent upregulation of *fra-1, UPA,* and *PAI-1* mRNAs was seen at day 2 in the JunER-expressing cells (Fig. 2, *right panel*). At this time point, expression of these genes had already been abolished in the absence of an active JunER by the density-dependent mechanism described above (Fig. 2, *left panel*). Again, this effect was not observed in control cells (H4, Fig. 2; H4 ER(HBD), not shown). In summary, our results show that several genes known to be regulated by AP-1 proteins were indeed subject to regulation in the epithelial cells by activation of JunER.

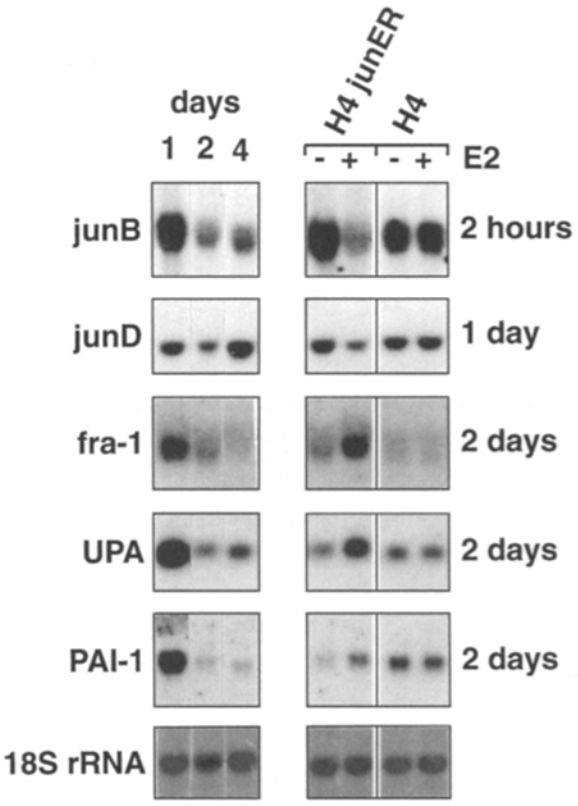


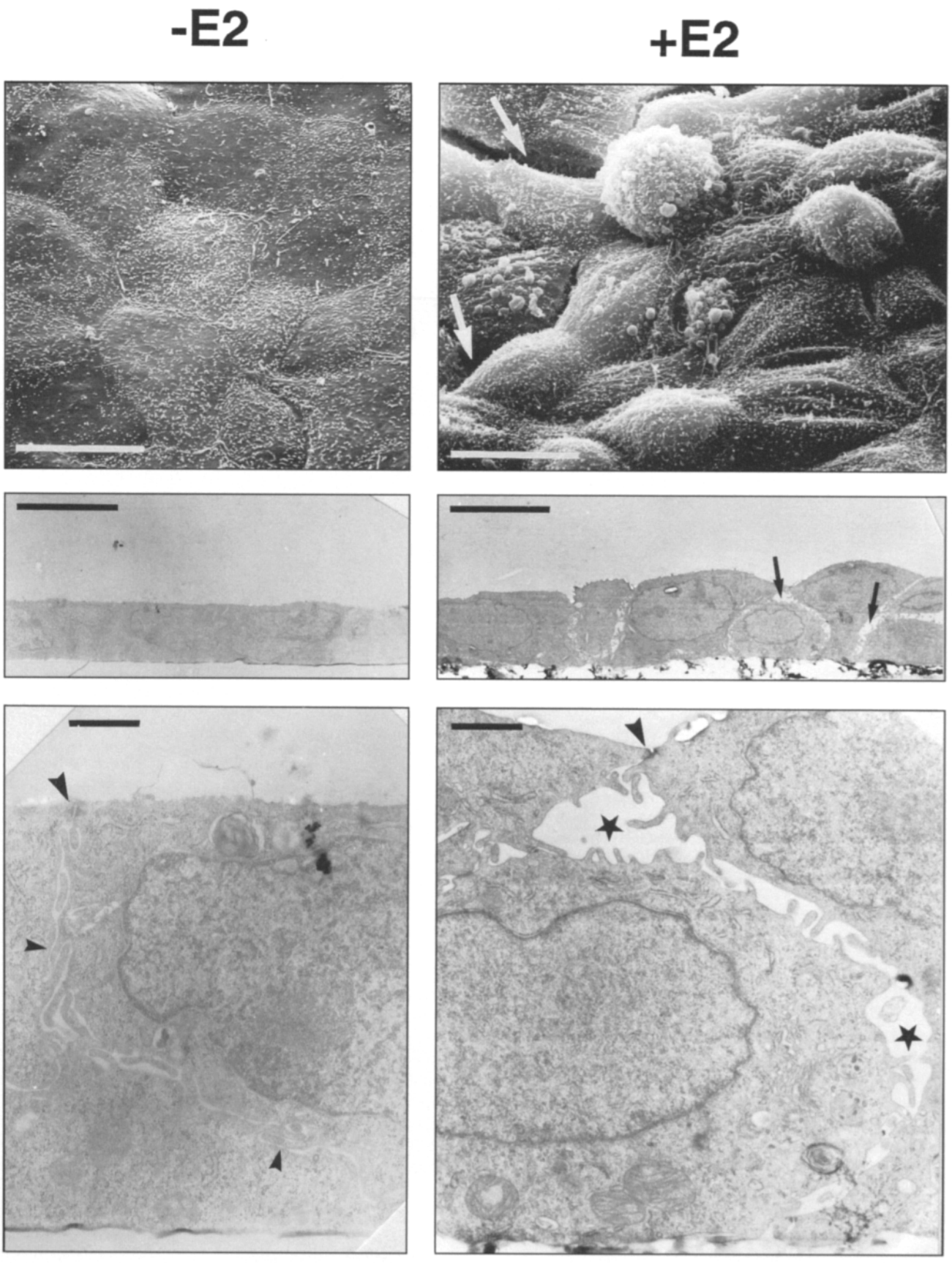
Figure 2. Regulation of AP-1 target genes by activated JunER. H4 junER cells and control H4 cells were grown with or without E2 for various time periods (2 h; 1, 2, and 4 d). For the 2-h incubation period, the cells were kept in culture for 1 d before induction and then treated with hormone (+) or left untreated (−) for 2 h. Total RNA was analyzed on Northern blots. The left panel shows the density dependent decrease in mRNA levels (RNAs from H4 junER cells grown in the absence of E2 for 1, 2, and 4 d). In the right panel, JunER-induced down- or upregulation of genes is shown at the earliest time point, at which these effects were clearly visible. For visualization of *junB*- mRNA downregulation, H4 junER cells were treated (+) or not treated with E2 (−) for 2 h. *junD* mRNA downregulation was evident after 1 d and upregulation of *fra-1, UPA* and *PAI-1* mRNAs was visible after 2 d. Note that RNA levels do not change in H4 control cells upon E2 treatment for identical time periods. 18 S rRNA confirms equal loading (±E2 for 2 d is shown in right panel).

Activation of JunER in Polarized Epithelial Cells Causes Loosening of Cell Contacts and Redistribution of Apical and Basolateral Proteins

Having demonstrated that the JunER protein was regulating target gene expression in the epithelial cells in a hormone-dependent fashion, we next wanted to analyze possible effects of the activated JunER protein on the state of epithelial polarization. Analysis of another conditional AP-1 protein, c-FosER, had already shown that its activation by E2 disrupted epithelial polarity and even caused epithelial-mesenchymal transition (Reichmann et al., 1992). Therefore, we sought to determine whether or not the effects of the c-Fos and c-Jun fusion proteins were distinct.

H4 junER and control parental H4 cells were grown on permeable filter supports for 4 d in the absence and presence of E2. Morphological analysis by scanning (SEM) and transmission (TEM) electron microscopy showed that uninduced H4 junER (Fig. 3, −E2), as well as control H4 cells in the presence or absence of E2 (data not shown) formed monolayers of tightly connected cells. Microvilli were expressed only at the apical surface. In contrast, the E2-activated JunER induced a dissociation of tight cell contacts, cell rounding, and growth in multilayers (Fig. 3, +E2). The addition of hormone also caused a distribution of microvilli expression over the entire cell surface. Examination by TEM at higher magnification revealed that tight junctions appeared to be reduced in number and morphologically abnormal after JunER activation. Extensive interdigitations of lateral plasma membranes as observed between neighboring, uninduced control cells were completely absent in the E2-treated H4 junER cells leaving large gaps and clefts between the loosely contacting cells.

To investigate if these morphological alterations were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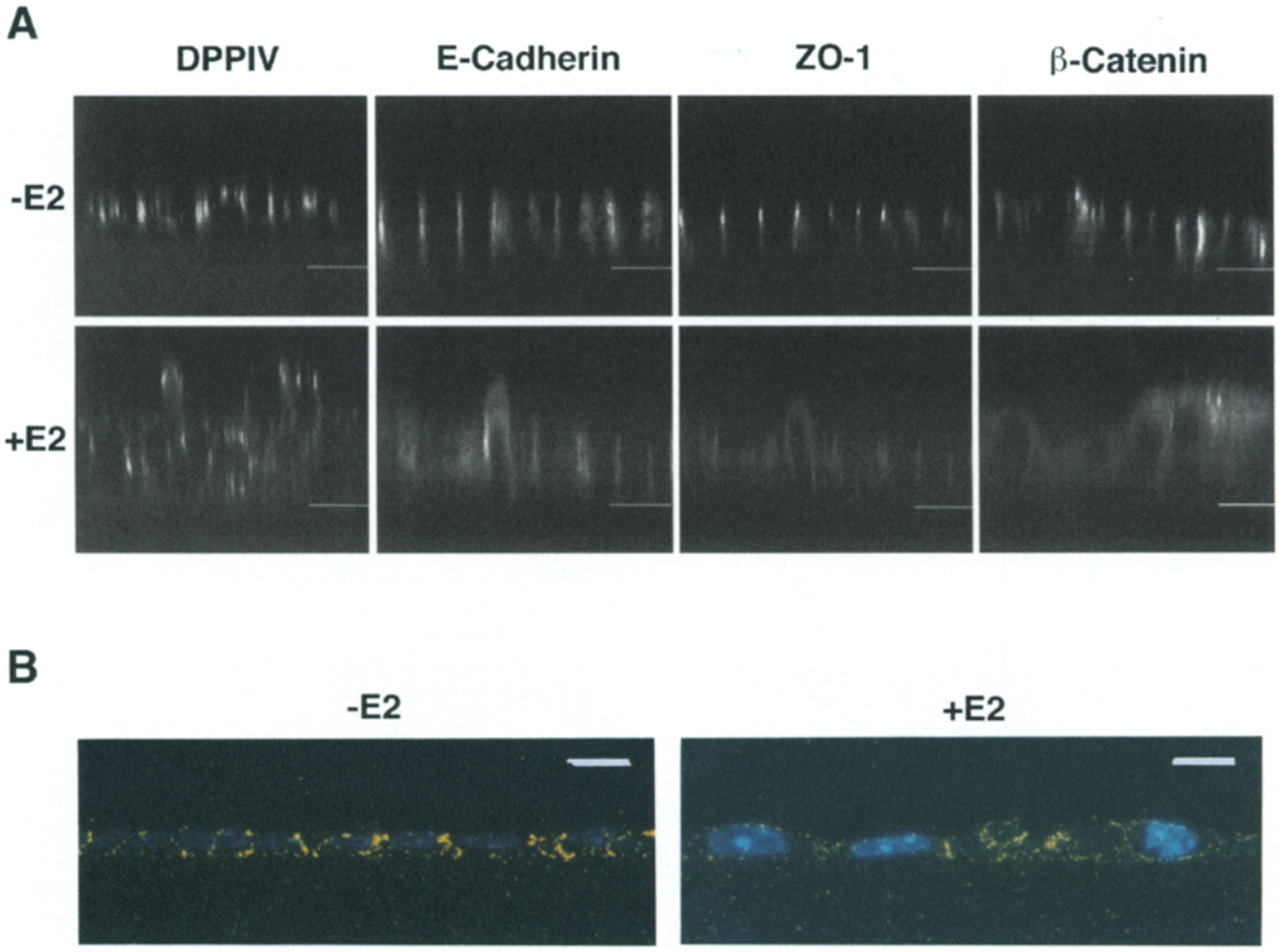


Figure 4. The polarized expression of basolateral and apical proteins is disrupted by JunER activation. (*A*) H4 junER were grown on filters without (−E2) or with E2 (+E2) as described in Fig. 3, fixed and stained by immunofluorescence for DPPIV, E-cadherin, ZO-1, and β-catenin. The staining was visualized as vertical sections employing a confocal microscope. To improve the resolution of fluorescence signals, we extended the Z-axis to double the actual height of the cells (compare to *B*). Positions of filter membranes are indicated by white lines. (*B*) Ultrathin Lowicryl HM20 sections of such filters were stained for β-catenin (*yellow*) and nuclei visualized with DAPI (*blue*). Due to a different nuclear geometry, the nuclei in *B* appear bigger in the presence of estradiol than in its absence. Bars: 10 μm.

accompanied by a loss of epithelial polarity, the distribution of apical and basolateral/junctional proteins was investigated. Cells grown on porous supports were analyzed for the localization of such proteins using indirect immunofluorescence and confocal microscopy (Fig. 4 *A*). The apical marker DPPIV (Casanova et al., 1991) was restricted to the apical membrane domain in JunER cells in the absence of E2, while it became redistributed over the entire membrane after E2 treatment (Fig. 4 *A*). Staining of the junctional proteins E-cadherin (Gumbiner and McCrea, 1993; Kemler, 1993), β-catenin (Gumbiner and McCrea, 1993; Kemler, 1993), and ZO-1 (Madara et al., 1993) demonstrated that all of these proteins were restricted to the lateral cell membrane in the absence of E2. ZO-1 was specifically concentrated at the sites of tight junctions (Fig. 4 *A*). Upon JunER activation, all the basolateral proteins were completely redistributed. β-Catenin and ZO-1 were barely detectable by immunofluorescence in the E2-treated cells (Fig. 4 *A*). Again, the JunER-induced formation of irregular multilayers and rounding up of cells was clearly visible (see also Fig. 3).

For β-catenin, the results of confocal microscopy were confirmed by fluorescence microscopy (Fig. 4 *B*) or immunoelectron microscopy (data not shown) of ultrathin Low-

Figure 3. Activation of JunER in epithelial cells induces loosening of cell contacts and multilayer formation. 2.5×10^5 H4 junER cells were seeded on permeable filter membranes and grown either with (+E2) or without (−E2) hormone for 4 d. The cells were then fixed and prepared for TEM and SEM. Uninduced cells form monolayers of polarized and tightly connected cells with apical microvilli and massive interdigitations (*small arrowheads, left bottom panel*). In the presence of E2, the cells form irregular multilayers with enlarged intercellular spaces (arrows and asterisks in right panels). Still, we can detect occasional but atypical structures possibly resembling disrupted tight junctions (compare *large arrowheads* in *bottom panels*). Bars: (*upper and middle panels*) 10 μm; (*bottom panels*) 1 μm.

icryl sections. We could detect basolateral expression of β-catenin in the JunER cells without E2 treatment. After E2 addition, β-catenin was redistributed to the whole cell surface of the loosely connected irregular cells. No alterations of the polarized expression of all tested proteins were observed in control parental and H4 ER(HBD) cells irrespective of hormone (not shown). We conclude that E2-activated JunER effectively induces loss of epithelial polarity in mammary epithelial cells.

JunER-induced Loss of Epithelial Polarity Disrupts Tubular Morphogenesis in Collagen I Gels

To determine whether the c-Jun effects observed in epithelial cells were physiologically relevant, we sought to elucidate if the loss of cell adhesion and epithelial polarity induced by activated JunER would have an effect on the formation of glandlike structures that can be obtained in collagen I gels (see Materials and Methods). Within 2–3 wk in the absence of E2, H4 junER cells formed three-dimensional, branching tubular structures with clearly visible lumina (Fig. 5 *A*, day 0, *arrows*) that closely resembled the ducts of the mammary gland in vivo. 24 h after the addition of E2 (day 1), these lumina were disrupted (*arrows*), leading to cordlike structures. The tight contacts between the cells appeared to be loosened. Even after 4 d of hormone treatment we did not observe cellular invasion into the extracellular matrix (Fig. 5 *A*, day 4). TEM micrographs from ultrathin sections through such branched structures showed that H4 junER cells without E2 formed tubules of tightly connected cells. In contrast, the structures after hormone addition represented irregular cords of loosely connected cells without any lumen (Fig. 5 *B*). Cells growing on top of the gels exhibited exactly the same phenotypes as cells grown on filter supports (see Fig. 3). No alterations of tubular morphogenesis could be induced by E2 in control H4 and H4 ER(HBD) cells (data not shown).

Staining of such ultrathin sections with β-catenin antibody revealed that the adherens junctional protein was strictly basolaterally expressed in the glandlike tubules under normal conditions (Fig. 6). By immunogold electron microscopy of corresponding serial sections (Schwarz et al., manuscript in preparation), the seemingly apical fluorescence of occasional cells (Fig. 6) could be shown to localize to lateral contacts between two neighboring cells, the apical to basal axes of which were differing from the sectional plane. In contrast, hormonal activation of JunER caused β-catenin redistribution in the cells forming disorganized cords (Fig. 6). We conclude that activated c-JunER also inhibits tubular morphogenesis of mammary epithelial cells.

Hormonally Activated JunER Mimics the Activation of Endogenous c-Jun by Cellular Signaling Pathways

c-Jun transcriptional activity is regulated by protein phosphorylation as a consequence of growth factor receptor signaling (Minden et al., 1994; Nikolakaki et al., 1993; Smeal et al., 1994). We therefore examined whether c-JunER activity was still dependent on growth factor–induced signals. H4 junER cells were precultured in serum-free collagen I gels in the presence of growth factors (BPE and TGFα) required for cell growth and formation of branching tubules (Fig. 7 *A*). After branching tubules had formed, the growth factors were withdrawn for 1 d, and then E2 was added in the absence of growth factors. Even after 4 d of E2 treatment, the formed tubules maintained their lumina and did not collapse (Fig. 7 *A*). Withdrawal of factors had no visible adverse effects on cell viability, but solely inhibited further growth, as seen in control cultures lacking both growth factors and estrogen (Fig. 7 *A*).

Having shown that JunER, similar to bona fide c-Jun, requires upstream cellular signals to affect epithelial polarity in collagen I gels, we wanted to study whether growth factor receptors that are known to signal via c-Jun could act on epithelial polarity in a similar fashion as hormone-activated JunER. Using the same conditions that were employed for JunER activation, parental H4 cells were grown in collagen I gels (Fig. 7 *B*, 0 h) and subsequently treated with various growth factors. Strikingly, aFGF (5 ng/ml) in the presence of heparin (Spivak-Kroizman et al., 1994) was able to induce a phenotype almost identical to that seen with activated JunER (Fig. 7 *B*). Within 48 h of growth factor treatment preformed lumina (*long arrows*) collapsed. Heparin alone (control) did not induce any phenotypical changes (*short arrows*).

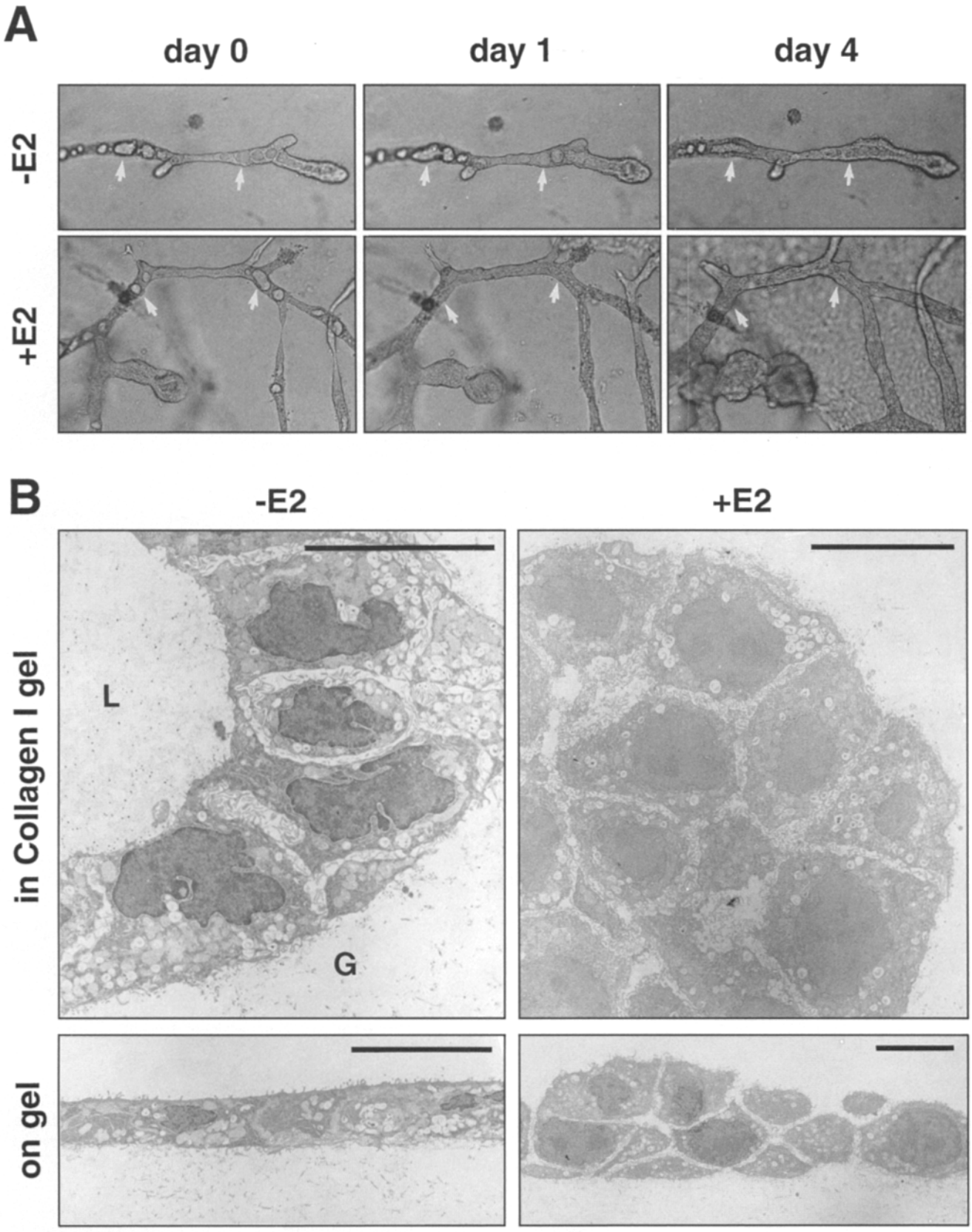
We conclude from these data that the presence of external upstream signals is a prerequisite for the E2-induced activation of the chimeric JunER protein. Moreover, aFGF affects the mammary epithelial phenotype similar to activated JunER, presumably by activating endogenous c-Jun.

Loss of Epithelial Polarity and Junctional Integrity Caused by JunER Is Fully Reversible after Hormone Withdrawal

In earlier work (Reichmann et al., 1992), we demonstrated that the time span during which a conditional c-FosER was activated was of crucial importance to its effect in mammary epithelial cells: loss of epithelial polarity induced by a short-term activation for 30–60 min was fully reversible, while long-term activation of c-FosER resulted in an irreversible epithelial-mesenchymal transition (Reichmann et al., 1992). Since c-Fos exhibits its transactivation activity in heterodimeric complexes with c-Jun, we were interested to learn whether epithelial cells expressing JunER would behave similarly or differently to Ep-fosER cells regarding reversibility of the induced alterations.

A rapid and quantitative method to assess the state of epithelial polarity and the integrity of epithelial cell monolayers seeded on porous supports is to measure TER (see Materials and Methods; Cereijido et al., 1993; Mandel et al., 1993). As an indication for the progressive sealing of tight junctions, the TER of H4 junER cells continued to

Figure 5. Activated JunER induces disruption of tubular morphogenesis in collagen I gels. (*A*) Cells were seeded in collagen I gels at a density of 2×10^4 cells/ml and kept in culture for three weeks until branching, tubular structures had developed (day 0). The gels were further cultivated with (+E2) or without hormone (−E2) for 4 d and the same structures photographed 1 d (day 1) and 4 d (day 4) after



hormone addition. Note the E2-induced disruption of lumina (*arrows*) in glandlike multicellular structures and the inability of the cells to invade the gel, even after 4 d of JunER activation. (*B*) On day 4 these gels were processed for EM. Histological staining of ultrathin Epon sections confirms the loss of lumen in hormone-treated cells. *L*, lumen; *G*, gel. Cells growing on top of the gels show the same phenotypes as on filters. Bars: 10 μm.

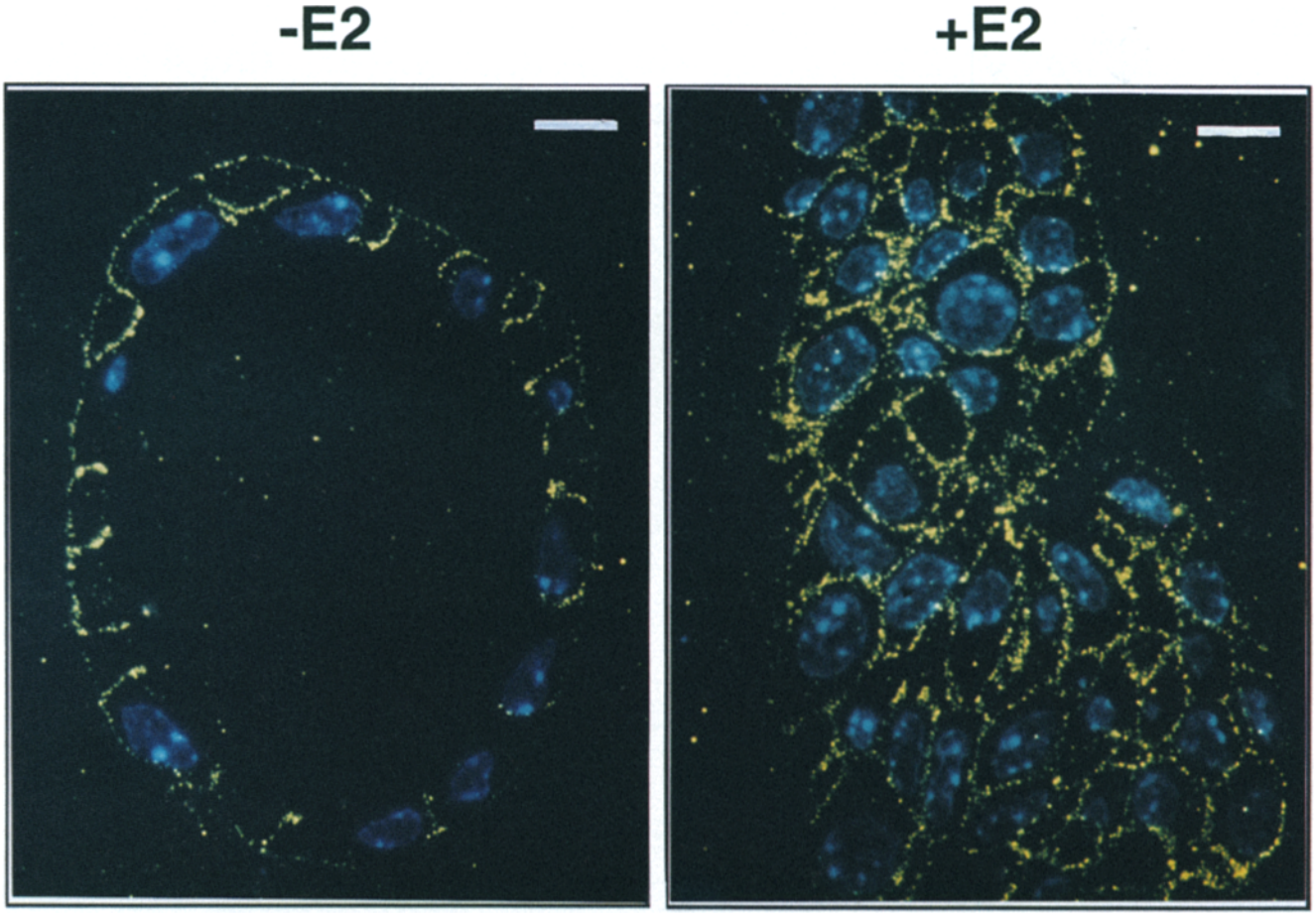


Figure 6. β-Catenin labeling of sections through collagen gel-grown cell structures. H4 junER structures grown in collagen I gels were treated with (+E2) or without (−E2) hormone for 4 d, fixed, and embedded in Lowicryl HM20. Ultrathin sections were immunostained with a β-catenin–specific antibody and counterstained with DAPI (see legend to Fig. 4). β-Catenin is restricted to the basolateral sites of the cell monolayer enclosing a lumen (−E2) and completely redistributed over the whole membrane in cells arranged in irregular cords (+E2). Bars: 10 μm.

increase in the absence of E2 (Fig. 8 *A*, *open symbols*), similar to that of control H4 ER(HBD) and H4 parental cells both with or without E2 (data not shown). Parallel filters with H4 junER cells were maintained in the absence of E2 for 5 d, allowing a strongly elevated TER to develop and thereafter treated with hormone (Fig. 8 *A*, *arrow*, +E2). Similar to c-FosER (Reichmann et al., 1992), activation of the JunER oncoprotein disrupted the already formed functional tight junctions, as indicated by the decline of TER after E2 addition (Fig. 8 *A*, *filled symbols*).

In contrast, activation of JunER by addition of E2 and the constitutive expression of c-Jun in H4 c-jun cells (data not shown) completely inhibited the formation of functional tight junctions, as shown by the constantly low TER of the respective filter cultures (Fig. 8 *B*, *filled symbols*). This inhibition was completely reversible. When H4 junER cells were grown in the presence of hormone for 5 d and thereafter in the absence of hormone, (Fig. 8 *B*, *arrow*, −E2), the cells were still able to reach a TER value (*open symbols*) similar to that of uninduced control cells (compare to Fig. 8 *A*).

Similar as in FosER cells treated with a short pulse of E2 (Reichmann et al., 1992), the reversibility of the JunER induced loss of polarity could be confirmed by analyzing apical and basolateral polarization markers by immunofluorescence and confocal microscopy. All the proteins seen to be uniformly distributed to the whole plasma membrane in hormone-treated H4 junER cells became relocalized in a strictly apical or basolateral fashion, respectively, when the cells were switched to E2-free medium for one week (data not shown). Hence, even after 5 d of E2 treatment, H4 junER cells were still able to fully rebuild polarized monolayers and to reform functional tight junctions upon hormone withdrawal.

As a more stringent test for the ability of H4 junER cells to regain full epithelial polarity after hormone withdrawal, we examined if the cords of hormone-induced H4 junER cells formed in collagen gels after E2 treatment could rearrange to form tubules of polarized cells when E2 was washed out of the collagen gels. JunER-expressing cells were cultivated in gels in the absence of hormone to enable branching and lumen formation. Subsequently, the tubular structures were disrupted by the addition of E2 for 1–4 d and then kept without estrogen for another 7–9 d to check for repolarization and tubular reorganization. Within this time period the cells were able to reform tubular

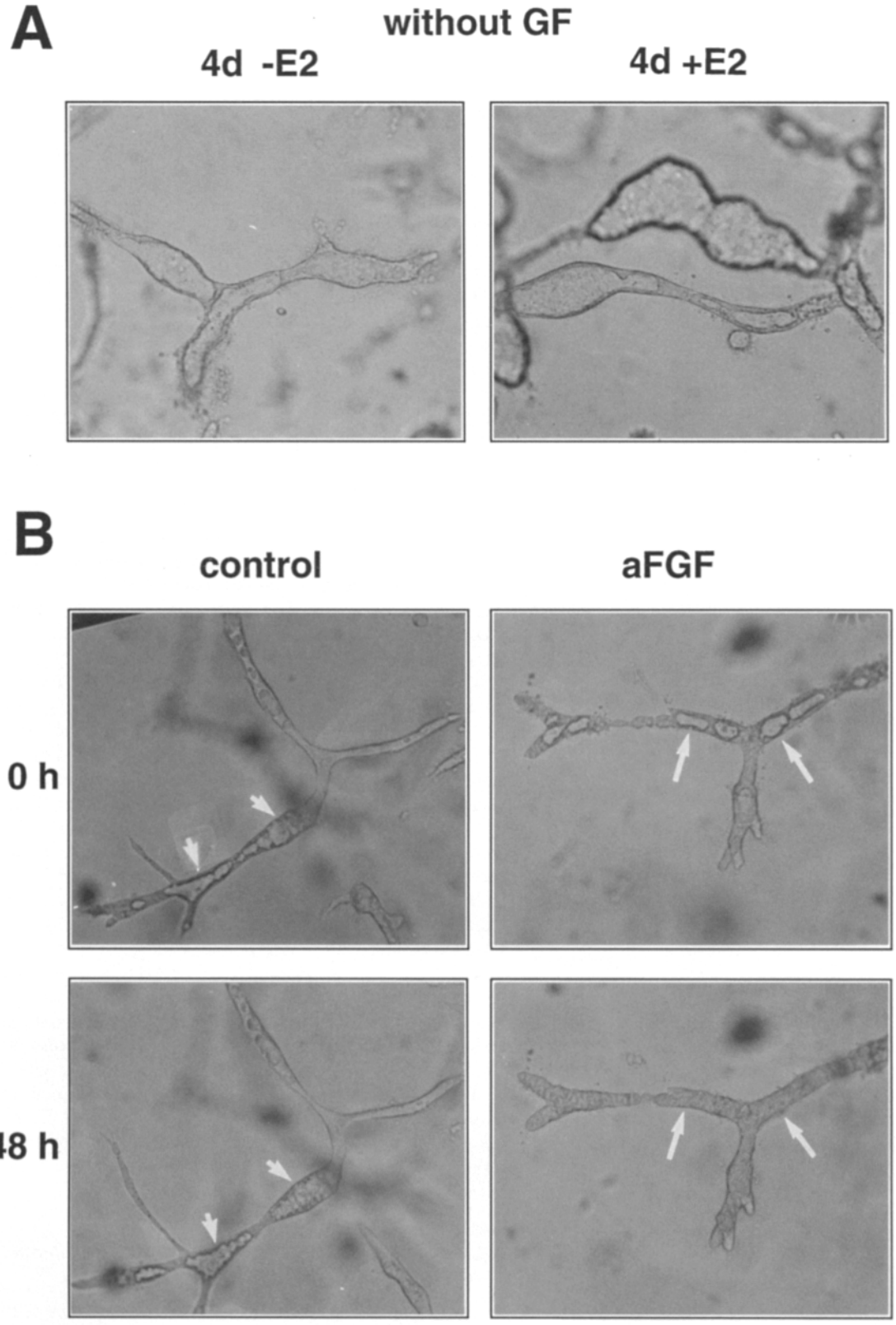


Figure 7. (*A*) JunER-induced disruption of tubular morphogenesis is inhibited by the lack of growth factors. JunER-expressing cells were precultured in collagen gels for 12 d. TGFα and BPE (see Materials and Methods) as a source of other growth factors were withdrawn for 24 h (without GF). Subsequently, E2 (+E2) or ethanol (−E2) were added for another 4 d in the absence of BPE/TGFα. Note the preservation of lumina even in the presence of E2. (*B*) aFGF mimics JunER-induced loss of epithelial polarity in parental H4 cells. H4 cells were cultured in collagen I gels for 11 d, photographed (0 h), and treated with heparin/aFGF (aFGF) or heparin alone (control) for 48 h. The branching tubules obtained (*top right panel*, lumina indicated by long arrows) collapsed in the aFGF-treated cultures (*bottom right, long arrows*). Lumina were retained in the cultures receiving only heparin (*left panels*; lumina indicated by short arrows).

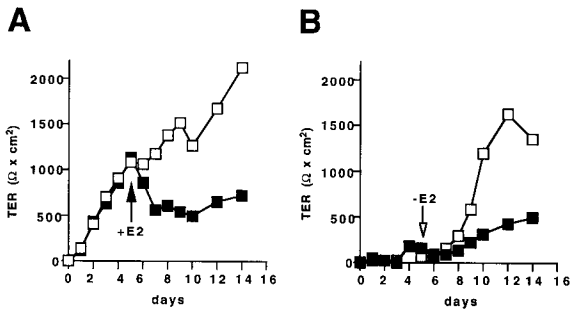


Figure 8. (*A*) Function of preformed tight junctions is destroyed by activated JunER. H4 junER cells were grown on filters without E2 to enable formation of functional tight junctions as determined by TER measurements (see Materials and Methods). On day 5 (*arrow*, +E2) E2 was added to one culture. Note progressive increase of TER in the absence of estradiol (*open squares*), while E2 addition (*filled squares*) causes the TER to rapidly decrease to levels obtained in cells grown continuously in E2 (compare to panel *B*, *filled squares*). (*B*) The inhibition of tight junction functionality is reversible. H4 junER cells cultured in the presence of E2 for 5 d are unable to develop a high TER. After hormone withdrawal on day 5 (*empty squares, arrow*, −E2) the cells gained a high TER (comparable to cells continuously grown in the absence of E2, see panel *A*, *empty squares*). This indicates that the cells are still able to reform functional tight junctions. The other cell aliquot grown further in the presence of E2 failed to develop a high TER even after 14 d.

structures of polarized monolayers (Fig. 9, *top panels*). In agreement with the marker expression of repolarized cells on filters, cells within these tubules exhibited strictly basolateral β-catenin expression again (Fig. 9, *bottom panels*). If reverted H4 junER cells were allowed to form tubules in collagen gels without E2, these could be again induced to collapse by E2 (data not shown). We conclude from these data that the loss of epithelial polarity induced by JunER is completely reversible at any stage.

Reversible Loss of Epithelial Polarity Caused by Activated JunER Is Associated with Reduced Stability of E-Cadherin/β-Catenin Complexes

The JunER-induced loss of epithelial polarity in mammary epithelial cells is obviously associated with the redistribution and/or loss of various junctional proteins such as E-cadherin and β-catenin (see Figs. 4 and 6). Since cell adhesion is known to be initiated by adherens junctions (Balda et al., 1993; Fleming et al., 1993; Marrs et al., 1993; Watabe et al., 1994), we sought to further characterize the JunER effects on the adherens junctional complex.

First, we investigated whether the expression of the E-cadherin and β-catenin proteins was downregulated upon prolonged E2 treatment (4 d), as has been reported for E-cadherin after long-term c-FosER activation (Reichmann et al., 1992). In Western blots using whole cell lysates we detected no or only minor differences in E-cadherin and β-catenin levels (data not shown). Since the immunofluorescence data suggested that the latter protein was partially redistributed to the cytoplasm (see Fig. 4), uninduced and induced cells were grown on filters and subjected to differential extraction using 2.5% NP-40 (Navarro et al., 1993). The detergent-insoluble (i) proteins being tightly associated with the cyto- and/or membrane-skeleton, and the soluble (s) fractions were separated by SDS-PAGE and analyzed for their content of E-cadherin and β-catenin by immunoblots (Fig. 10 *A*). The blots obtained were then quantified by densitometry (see Materials and Methods). The ratio of soluble to insoluble (s:i) E-cadherin was not altered in any of the cells tested. About two-thirds of the protein were found in the insoluble fraction (*s:i* = 0.6) in JunER or control cells either treated or not treated with E2.

A different behavior was seen for β-catenin. Fig. 10 *A* clearly shows that hormone activation of c-JunER caused a strong decrease in insoluble β-catenin. These changes could be quantified by calculating the ratio soluble/insoluble β-catenin, each normalized to the respective E-cadherin levels. This normalized ratio of soluble to insoluble β-catenin increased from 3.7 in untreated JunER cells to 8.0 in cells treated with estradiol. No such increase was seen in control H4 ER(HBD) cells (Fig. 10 *A*), where the respective values were 3.3 in treated cells and 3.5 in cells not treated with hormone.

To determine whether the enhanced solubility of β-catenin was correlated with a change of complex formation between E-cadherin and β-catenin, we performed coimmunoprecipitation-Western studies. H4 junER or control cells were grown on filters, treated or not treated with E2 for 4 d, and extracted. Similar amounts of protein were subjected to immunoprecipitation with an antibody against E-cadherin, separated by SDS-PAGE blotted for immunodetection with antibodies to E-cadherin and β-catenin, and quantified by densitometry as above. Again the amount of E-cadherin did not grossly change upon hormone addition (Fig. 10 *B*). Strikingly, however, coimmunoprecipitated β-catenin was diminished in induced H4 junER cells as compared to untreated and control cells (Fig. 10 *B*). The ratio β-catenin/E-cadherin in the immunoprecipitated complex (see Materials and Methods) decreased 2.9-fold in hormone-treated H4 junER cells, in comparison to untreated cells. In control cells, this ratio remained essentially constant (H4 ER(HBD), 0.9-fold difference; Ep fosER cells, 1.1-fold difference). The MDCK ts v-*src* cells (used as a positive control for β-catenin phosphorylation, see Fig. 10 *B* and below) could not be analyzed in this fashion, since the E-cadherin antibody did not cross-react to the dog protein under denaturing conditions.

We conclude from these experiments that the regulatory protein β-catenin is in part released from its complex with E-cadherin and is shifted from a detergent-insoluble to a more soluble state after JunER activation (see Discussion).

JunER-induced Changes in β-Catenin/E-Cadherin Interaction Do Not Involve Alterations in β-Catenin Tyrosine Phosphorylation

Recently, several groups have reported the deregulation of E-cadherin–mediated adhesiveness via tyrosine phosphorylation of β-catenin (Behrens et al., 1993; Hamaguchi et al., 1993; Sommers et al., 1994; Kinch et al., 1995). Since JunER induced similar changes, i.e., a loss of intercellular adhesion and a redistribution of these two proteins without changing their expression levels, we determined the state of β-catenin tyrosine phosphorylation in the H4

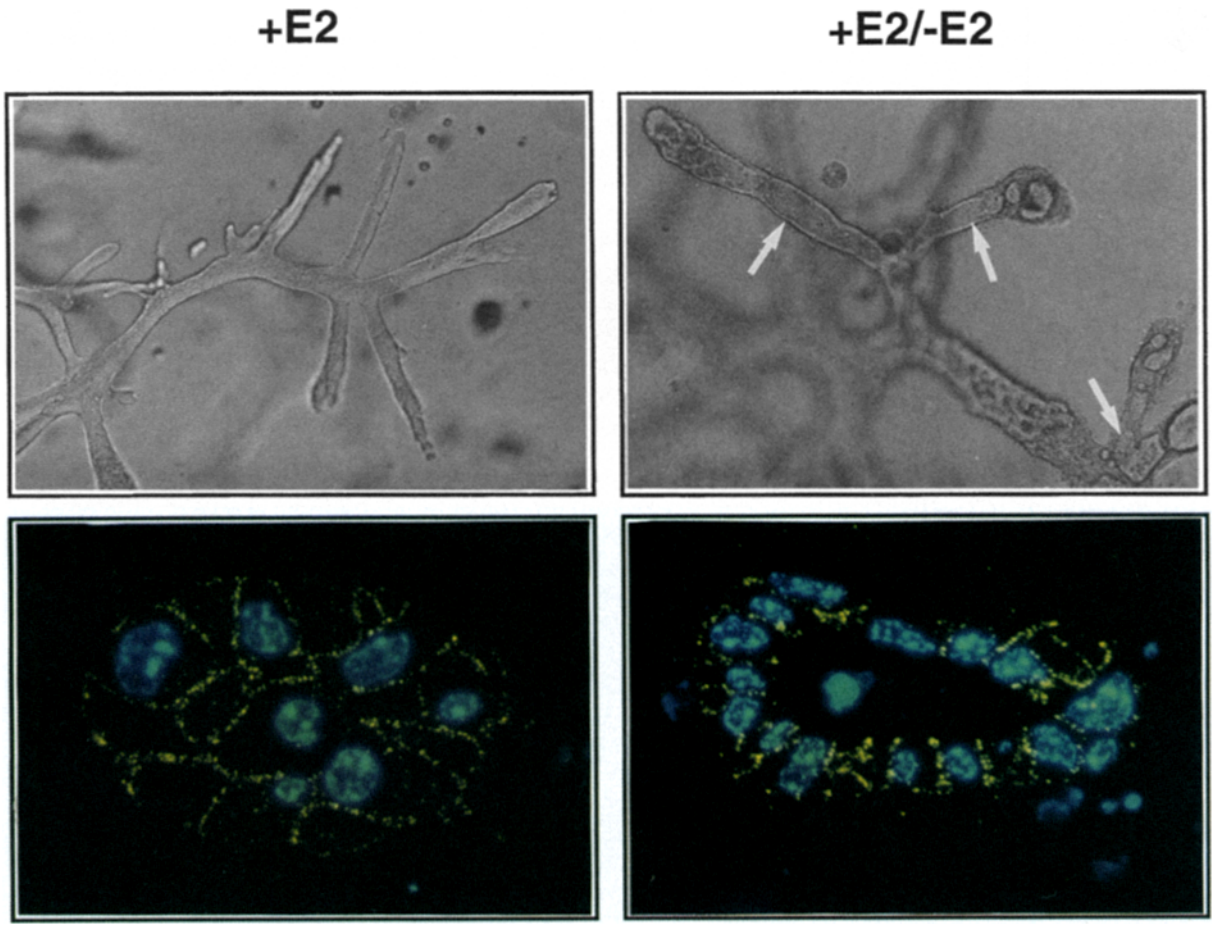


Figure 9. The JunER-induced loss of polarity in tubular structures is reversible. H4 junER cells were grown in collagen gels until tubular structures had formed. Subsequently, E2 was added for 2 d to induce luminal collapse and redistribution of β-catenin to the entire cell surfaces (*+E2*). The structures were withdrawn from hormone and further cultivated in the absence of hormone under the appropriate conditions for 7 d (*+E2/−E2*). The structures obtained were photographed (*top panels*). Ultrathin sections from the fixed structures were then prepared and subjected to on-section labeling (see legend to Fig. 6). Note the reformation of lumina (*arrows*) and regaining of basolateral expression of β-catenin in the structures after E2 withdrawal.

junER cells. As a positive control, ts v-src–expressing MDCK cells (Behrens et al., 1993) were analyzed after cultivation at the permissive (35.5°C) and the nonpermissive (40.5°C) temperature. In none of the samples derived from JunER cells before or after E2 treatment, or from the various control cells (including Ep fosER cells) any phosphorylation of β-catenin on tyrosine could be detected (Fig. 10 *B*). The MDCK ts v-src control cells, on the other hand, showed the expected strong tyrosine phosphorylation of β-catenin at the permissive temperature, but not at 40.5°C (Fig. 10 *B*). This negative result was verified after extensive overexposure of the blot (data not shown). Thus, β-catenin tyrosine phosphorylation does not seem to be involved in the observed JunER-induced release of β-catenin from E-cadherin and/or the cyto-/membrane-skeleton.

Discussion

In this paper, mouse mammary epithelial cells expressing a conditional JunER fusion protein have been employed. This system is expected to reflect the regulation of epithelial polarity in response to signal transduction induced by growth factor receptors. The data obtained show that JunER reversibly modulates cellular polarity and tubular morphogenesis in mammary epithelial cells and implicate c-Jun as a nuclear effector of extracellular signaling in the regulation of mammary epithelial polarity.

JunER, a Hormone-inducible Transcription Factor Acting on AP-1 Target Genes

Estrogen receptor fusion proteins have been successfully used for the analysis of many other transcription factors in different cell types (Briegel et al., 1993; Reichmann et al., 1992). JunER-induced transactivation (~50-fold) of a reporter gene via TRE(s) was fully dependent on E2 in fibroblasts, while ER(HBD) alone failed to transactivate. Importantly, estradiol (E2) did not elicit any effects in parental epithelial cells (H4) or cells expressing ER(HBD) under the given conditions, although these cells express the endogenous estrogen receptor (Fig. 1 *C*). Therefore, clones expressing JunER could be selected and expanded in the absence of JunER activity, induced to fully polarize, and then exposed to the activated oncoprotein by addition of E2. Similar to findings with the conditional transcrip-

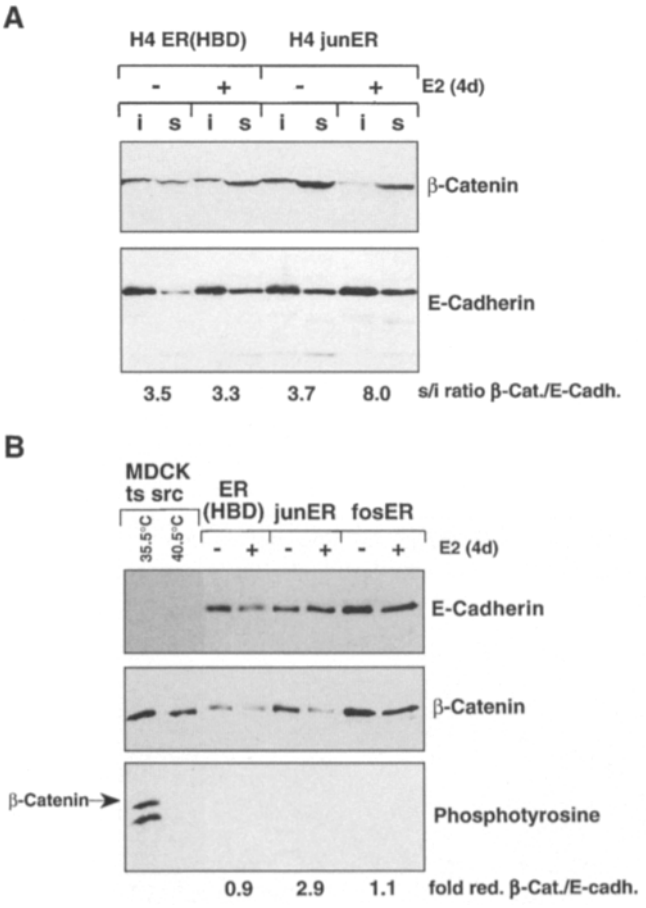


Figure 10. JunER destabilizes the E-cadherin/β-catenin complex. (*A*) H4 junER and H4 ER(HBD) cells were cultivated on filters with (+) or without (−) E2 for 4 d. They were then differentially extracted with 2.5% NP-40. The detergent-soluble (s) and -insoluble (i) fractions were analyzed by SDS-PAGE and immunoblotting. Upon scanning by densitometry, the s:i ratios of β-catenin normalized to E-cadherin were calculated (see numbers at the bottom of lanes). About one third of β-catenin is not extractable from H4 junER cells without E2, while almost all of the protein is found in the soluble fraction after the addition of hormone, thus raising the s:i ratio to 8.0. No increase in s:i is seen in the control H4 ER(HBD) cells (Note that less protein was loaded from the soluble fraction of H4 ER(HBD) −E2). (*B*) H4 ER(HBD) and H4 junER clones were treated as described in *A*. Extracts were then immunoprecipitated with antiserum against E-cadherin. MDCK ts src cells grown at the permissive (35.5°C) or the restrictive (40.5°C) temperature and Ep fosER cells ∓E2 were used as controls. Immunoprecipitates were analyzed on immunoblots using the same α–E-cadherin antibody (*top panel*). After stripping, the blots were reprobed with an antibody to β-catenin (*middle panel*) as well as with a phosphotyrosine antibody (*bottom panel*). Note that while E-cadherin expression does not change (less protein was loaded in lane *4*), a strongly reduced amount of β-catenin is coimmunoprecipitated in H4 junER cells +E2. Densitometry shows that the ratio β-catenin/E-cadherin is 2.9-fold reduced in the hormone-treated cells (compared to the untreated controls), whereas no such difference is seen in the control cells. Note also that the murine E-cadherin-specific antiserum is useful for immunoprecipitation in the canine cells but is nonreactive on the denatured proteins in immunoblots.

tion factor GATA-2/ER (Briegel et al., 1993), JunER was predominantly nuclear when activated by E2 in epithelial cells, whereas most of the protein was cytoplasmic in control cells not treated with hormone, supposedly due to interactions with heat shock proteins (Pratt and Welsh, 1994).

As expected, activated JunER was able to regulate AP-1 target gene expression in epithelial cells. The most prominent effect was seen on *junB*, which was repressed almost sevenfold after only 2 h of hormone treatment. JunB is known to counteract c-Jun activity (Deng and Karin, 1993) and its expression is correlated with the differentiation of various cell types including epithelial cells (Schlingensiepen et al., 1993; Wilkinson et al., 1989). Thus, downregulation of the negative effector JunB by c-Jun may prevent expression of a fully polarized epithelial phenotype. The (much weaker) downregulation of *junD* mRNA, a third member of the Jun family (Ryder et al., 1989) is consistent with the expression of JunD in quiescent (Ryder et al., 1989) and terminally differentiated cells (Li et al., 1992). Interestingly, JunD has recently been shown to antagonize fibroblast growth and transformation by activated Ras (Pfarr et al., 1994).

Other genes affected by JunER were the AP-1 target genes *fra-1*, the protease *UPA* and its specific inhibitor *PAI-1*. The expression of these genes was strongly affected by cell density and, consequently, by the status of epithelial polarity. While we found a high expression level in sparse cells, it was drastically reduced when the cells became dense enough to build up epithelial polarity. In line with its ability to prevent epithelial polarization, E2-activated JunER maintained high expression of *fra-1*, *UPA*, and *PAI-1* mRNAs at cell densities where little or almost no mRNA was detected in the untreated controls. Supporting the significance of our data, upregulation of *UPA* and *PAI-1* has been correlated with a loss of epithelial cell adhesion (Frixen and Nagamine, 1993) and a degree of malignancy in breast carcinomas (Reilly et al., 1992).

Does JunER act in the same fashion as unmodified c-Jun? There are several observations that argue in favor of this idea. First, cloning and expansion of JunER-expressing clones in the presence of hormone gave rise to a nonpolarized cell type similar to that obtained by constitutively overexpressing bona fide c-Jun (unpublished data). These cells may have undergone cellular progression step(s) in addition to overexpression of c-Jun. Alternatively, JunER may induce epithelial-mesenchymal transition in cells at cloning density but not in a layer of already polarized cells.

Furthermore, JunER seems to respond to the same cellular regulatory mechanisms as c-Jun. c-Jun is posttranslationally regulated by phosphorylation of several Ser and Thr residues, which either positively (Derijard et al., 1994; Minden et al., 1994) or negatively (Lin et al., 1992; Nikolakaki et al., 1993) affect its transactivation potential. c-Jun function is also regulated by interaction with other nuclear factors such as nuclear hormone receptors (for review see Pfahl, 1993) and other negative regulators (Treier et al., 1994). Interestingly, hydrocortisone at higher concentrations (250 ng/ml) was able to repress the JunER-induced effects on tubular morphogenesis (not shown). In addition, omission of growth factors from the collagen gels inhibited the JunER-induced destruction of polar epithelial organization. This suggests that, similar to c-Jun, up-

stream signaling from ligand-activated growth factor receptors is required for JunER function. In line with this, aFGF could induce the same loss of tubular morphogenesis in normal H4 cells lacking an exogenous c-Jun(ER). Thus, we assume that endogenous c-Jun normally regulates epithelial polarity, if properly activated by extracellular signals. Nevertheless, our data do not rule out that upstream signaling by serum factors may also alter the function of the ER(HBD) in the fusion protein (Gabelman and Emerman, 1992).

Reversible Loss of Epithelial Polarity by Hormone Activation of JunER

The results of this paper demonstrated that the activated JunER protein severely altered epithelial polarity. JunER caused a loosening of intercellular contacts and a redistribution of apical markers, e.g., DPPIV (Casanova et al., 1991) and numerous basolateral markers to the entire cell surface. Redistribution of the tight junction protein ZO-1 was correlated with a loss of functional tight junctions as determined by strongly reduced TER and by the clearly diminished numbers and abnormal structure of tight junctions detected by electron microscopy. Since the lumina of branched tubules formed in collagen gels are thought to be maintained by apical secretion, changes in the integrity of tight junctions induced by active JunER may cause apically secreted material to leak out into the gel and thus cause a destruction of already formed lumina. In addition, the JunER-induced loss of polarity would also interfere with directed apical secretion per se (Streuli et al., 1991). Interestingly, changes in the production and apical secretion of milk proteins have already been reported to be induced by the oncogenes *ras*, *mos*, *src*, and *neu* (Dougall et al., 1994; Jehn et al., 1992), all of which are able to activate c-Jun and other AP-1 proteins.

The formation of tight junctions as well as the polarized distribution of cell surface proteins is dependent on the integrity of adherens junctions (Marrs et al., 1993, 1995; Watabe et al., 1994). Tight junction sealing is regulated by an E-cadherin–induced signal via protein kinase C (Balda et al., 1993; Lewis et al., 1995). Thus, loss of functional tight junctions may be a consequence of impaired adherens junctions. This agrees with our finding that all proteins of the adherens junctions (E-cadherin, α-, β-catenin, and fodrin) were redistributed to the entire cell surface, both on filters and in collagen I gels. Furthermore, redistribution of E-cadherin is known to be involved in the destabilization of cellular junctions in a number of human breast tumors (Oka et al., 1991).

Activation of JunER causes the loss of high TER, polarized expression of apical and basolateral markers and the ability to form tubular structures in collagen gels. All of these parameters could be completely restored in the cells several days after hormone withdrawal, confirming that the JunER-induced effects were fully reversible. This indicates that activation of JunER does not repress epithelial marker genes and induces mesenchymal ones, as does c-FosER (Reichmann et al., 1992), but rather affects mechanisms involved in the maintenance of epithelial polarity. Interestingly, loss of epithelial polarity after estrogen induction was a fast process, apparently complete in 24 h or less, while restoration of polarity after estrogen withdrawal was slow, requiring 5–9 d depending on the parameter assayed. The reasons for this slow kinetics of repolarization are unknown at present. Since a second E2-induced depolarization of the repolarized cells occurred as fast and complete as the initial one, epigenetic changes or alterations in c-JunER expression can be ruled out as explanations for the slow repolarization.

Regulation of the E-Cadherin/β-Catenin Complex

Downregulation of E-cadherin is involved in carcinogenesis and tumor cell invasiveness (Birchmeier and Behrens, 1994; Frixen and Nagamine, 1993). Its adhesive function is dependent on the association with several cytoplasmic proteins named catenins (Gumbiner and McCrea, 1993; Watabe et al., 1994). β-Catenin is more directly interacting with E-cadherin than α-catenin (Aberle et al., 1994; Kawanishi et al., 1995). The stability of β-catenin/E-cadherin complexes was strongly reduced after E2 induction, contrary to the reduced cell adhesion observed in other cell types occurring without effects on catenin association (Behrens et al., 1993; Sommers et al., 1994).

As a possible regulatory event in catenin function, we have investigated tyrosine phosphorylation of β-catenin. In other systems, tyrosine hyperphosphorylation has been correlated to inactivation of cadherin-dependent cell adhesion (Behrens et al., 1993; Hamaguchi et al., 1993; Sommers et al., 1994) and was induced by growth factors (Hoschuetzky et al., 1994). Nevertheless, we failed to detect elevated phosphotyrosine levels in β-catenin, when epithelial polarity was disrupted by c-JunER. Very recently, Kinch et al. (1995) demonstrated that tyrosine phosphorylation of several proteins including β-catenin was elevated in Ras-transformed human breast epithelial cells, causing the disruption of β-catenin/E-cadherin complexes as well as enhanced solubility of both E-cadherin and β-catenin. Possibly, oncogenic activation of Ras not only activates c-Jun, but also other pathways that cause tyrosine phosphorylation of β-catenin, increased solubility of E-cadherin, and transformation. None of the latter effects were induced by activating JunER, indicating that c-Jun induces only a subset of the effects caused by oncogenic Ras in mammary epithelial cells.

In line with our results, PDGF- or aFGF-induced changes in epithelial cell adhesion do not involve phosphorylation of β-catenin (Boyer et al., 1992; Hoschuetzky et al., 1994). Acidic FGF-induced scattering, epithelial-mesenchymal transition, and redistribution of E-cadherin in NBT-II bladder carcinoma cells did neither affect steady state levels nor the solubility of this protein. Together with the observation that aFGF was able to mimic the JunER-induced effects in parental H4 cells, these results suggest that c-Jun may be one of the nuclear effectors of aFGF signaling in epithelial cells.

Another level at which epithelial polarity may be regulated is the association of adherens junctions with the cytoskeleton. Our finding that β-catenin protein dissociates from E-cadherin and accumulates in the soluble fraction after JunER activation is in line with the observed correlation between enhanced solubility of β-catenin and its dissociation from E-cadherin reported by Kinch et al. (1995).

In this respect, it is interesting to note that APC, the product of a tumor suppressor gene which is mutated in a familial form of adenomatous polyposis coli and other intestinal tumors, strongly interacts with β-catenin. Both APC and E-cadherin bind to the same central core domain of β-catenin which contains the *Arm*-like repeats (for review see Polakis, 1995). Thus, APC possibly competes for β-catenin binding to E-cadherin (Hülsken et al., 1994; Rubinfeld et al., 1993). Very recently, nonmutated APC was proposed to be involved in degradation of the cytoplasmic form of β-catenin (Munemitsu et al., 1995), also suggesting that the soluble form of this protein plays an important role in the deregulation of epithelial cells.

A Role for c-Jun in Epithelial Cell Behavior and Breast Carcinogenesis?

It is obvious that a certain plasticity of epithelial polarity is required in case of cell division, organ development, and remodeling, as well as wound healing (Birchmeier and Birchmeier, 1994; Nelson, 1992). Likewise, this plasticity may be aberrantly regulated in carcinogenesis (Fish and Molitoris, 1994; Reichmann, 1994). Our major reason to focus on c-Jun as a regulator of epithelial polarity was its likely function as a downstream effector integrating the signals from many different growth factor receptors that regulate epithelial cell behavior.

Why are the JunER-induced changes distinct from those induced by c-FosER (Reichmann et al., 1992)? One possibility is that the set of genes regulated by JunER is clearly distinct from those affected by c-FosER. For instance, c-FosER, but not JunER, induces tissue-type plasminogen activator, collagenase I, and stromelysin I (Reichmann et al., 1992; and data not shown). A possible reason for this is that c-Jun target genes important for transformation are regulated by heterodimers of c-Jun with transcription factors of the ATF/CREB family (van Dam et al., 1993, 1995), as has been shown in the case of *PAI-1* regulation (Knudsen et al., 1994).

In line with our findings, it has been recently reported that upregulation of c-Jun or JunB is neither sufficient to cause tumors nor to induce progression of a mild form of fibromatosis to a fully malignant tumor phenotype (Bossy-Wetzel et al., 1992). Thus, c-Jun is probably unable to cause tumors by itself, but rather induces a certain flexibility in the polarized phenotype of mammary epithelial cells, perhaps by reversibly changing the expression of a limited number of AP-1–responsive genes. In line with this, *c-jun* mRNA accumulates in mouse mammary gland epithelial cells at regions of intensive organ remodeling during the first day(s) after delivery when the gland is preparing for lactation (Oft, M., and H. Beug, unpublished results).

Although unable to cause tumors, JunER may mimic early steps in breast cancer development, i.e., the loss of tight tissue organization. Our finding that aFGF induced the same changes in tubular morphogenesis as JunER corresponds to the selective expression of a variant of FGFR-2 at branching points of bronchioles in the developing mammalian lung epithelium (for review see Mason, 1994). Moreover, a dominant negative FGFR was able to completely block branching morphogenesis and epithelial differentiation of the mouse lung (Peters et al., 1994). Finally, members of the FGFR family have been found to be amplified in a subset of human mammary tumors (Adnane et al., 1991). This suggests that aberrant FGF action via c-Jun can give rise to tumor development. Very recently, elevated levels of c-Jun expression leading to enhanced AP-1 activity in mouse epidermal cell lines rendered these cells more sensitive to tumor promoting agents (Dong et al., 1994), supporting the hypothesis that c-Jun is involved in tumor initiation.

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References

Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J. Cell Sci.* 107:3655–3663.

Adnane, J., P. Gaudray, C.A. Dionne, G. Crumley, M. Jaye, J. Schlessinger, P. Jeanteur, D. Birnbaum, and C. Theillet. 1991. BEK and FLG, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. *Oncogene.* 6:659–663.

Ali, S., Y. Lutz, J.P. Bellocq, M.P. Chenard-Neu, N. Rouyer, and D. Metzger. 1993. Production and characterization of monoclonal antibodies recognizing defined regions of the human estrogen receptor. *Hybridoma.* 12:391–405.

Balda, M.S., L. Gonzalez-Mariscal, K. Matter, M. Cereijido, and J.M. Anderson. 1993. Assembly of the tight junction: the role of diacylglycerol. *J. Cell Biol.* 123:293–302.

Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M.M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol.* 120:757–766.

Ben-Levy, R., H.F. Paterson, C.J. Marshall, and Y. Yarden. 1994. A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3302–3311.

Bergers, G., P. Graninger, S. Braselmann, C. Wrighton, and M. Busslinger. 1995. Transcriptional activation of the fra-1 gene by AP-1 is mediated by regulatory sequences in the first intron. *Mol. Cell. Biol.* 15:3748–3758.

Bergers, G., A. Reikerstorfer, S. Braselmann, P. Graninger, and M. Busslinger. 1994. Alternative promoter usage of the Fos-responsive gene Fit-1 generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1176–1188.

Birchmeier, W., and J. Behrens. 1994*a*. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim. Biophys. Acta.* 1198:11–26.

Birchmeier, W., and C. Birchmeier. 1994*b*. Mesenchymal-epithelial transitions. *Bioessays.* 16:305–307.

Bossy-Wetzel, E., R. Bravo, and D. Hanahan. 1992. Transcription factors junB and c-jun are selectively up-regulated and functionally implicated in fibrosarcoma development. *Genes Dev.* 6:2340–2351.

Boyer, B., S. Dufour, and J.P. Thiery. 1992. E-cadherin expression during the acidic FGF-induced dispersion of a rat bladder carcinoma cell line. *Exp. Cell Res.* 201:347–357.

Briegel, K., K.C. Lim, C. Plank, H. Beug, J.D. Engel, and M. Zenke. 1993. Ectopic expression of a conditional GATA-2/estrogen receptor chimera arrests erythroid differentiation in a hormone-dependent manner. *Genes Dev.* 7: 1097–1109.

Brown, P.H., R. Alani, L.H. Preis, E. Szabo, and M.J. Birrer. 1993. Suppression of oncogene-induced transformation by a deletion mutant of c-jun. *Oncogene.* 8:877–886.

Casanova, J.E., Y. Mishumi, Y. Ikehara, A.L. Hubbard, and K.E. Mostov. 1991. Direct apical sorting of rat liver dipeptidylpeptidase IV expressed in Madin-Darby canine kidney cells. *J. Biol. Chem.* 266:24428–24432.

Cereijido, M., L. Gonzalez-Mariscal, R.G. Contreras, J.M. Gallardo, R. Garcia-Villegas, and J. Valdes. 1993. The making of a tight junction. *J. Cell Sci. Suppl.* 17:127–132.

Cha, R.S., W.G. Thilly, and H. Zarbl. 1994. N-nitroso-N-methylurea-induced rat mammary tumors arise from cells with preexisting oncogenic Hras1 gene

mutations. *Proc. Natl. Acad. Sci. USA.* 91:3749–3753.
Daly, R.J., M.D. Binder, and R.L. Sutherland. 1994. Overexpression of the Grb2 gene in human breast cancer cell lines. *Oncogene.* 9:2723–2727.
Deng, T., and M. Karin. 1993. JunB differs from c-Jun in its DNA binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes Dev.* 7:479–490.
Derijard, B., M. Hibi, I.H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R.J. Davis. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell.* 76:1025–1037.
Dong, Z., M.J. Birrer, R.G. Watts, L.M. Matrisian, and N.H. Colburn. 1994. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc. Natl. Acad. Sci. USA.* 91:609–613.
Dougall, W.C., X. Qian, N.C. Peterson, M.J. Miller, A. Samanta, and M.I. Greene. 1994. The neu-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene.* 9:2109–2123.
Edidin, M. 1993. Patches and fences: probing for plasma membrane domains. *J. Cell Sci. Suppl.* 17:165–169.
Engelberg, D., C. Klein, H. Martinetto, K. Struhl, and M. Karin. 1994. The UV response involving the Ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. *Cell.* 77:381–390.
Fish, E.M., and B.A. Molitoris. 1994. Alterations in epithelial polarity and the pathogenesis of disease states. *N. Engl. J. Med.* 330:1580–1588.
Fleming, T.P., Q. Javed, J. Collins, and M. Hay. 1993. Biogenesis of structural intercellular junctions during cleavage in the mouse embryo. *J. Cell Sci. Suppl.* 17:119–125.
Frixen, U.H., and Y. Nagamine. 1993. Stimulation of urokinase-type plasminogen activator expression by blockage of E-cadherin–dependent cell–cell adhesion. *Cancer Res.* 53:3618–3623.
Gabelman, B.M., and J.T. Emerman. 1992. Effects of estrogen, epidermal growth factor, and transforming growth factor-α on the growth of human breast epithelial cells in primary culture. *Exp. Cell Res.* 201:113–118.
Gumbiner, B.M., and P.D. McCrea. 1993. Catenins as mediators of the cytoplasmic functions of cadherins. *J. Cell Sci. Suppl.* 17:155–158.
Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. p60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:307–314.
Hoschuetzky, H., H. Aberle, and R. Kemler. 1994. β-Catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J. Cell Biol.* 127:1375–1380.
Hu, E., E. Mueller, S. Oliviero, V.E. Papaioannou, R. Johnson, and B.M. Spiegelman. 1994. Targeted disruption of the c-*fos* gene demonstrates c-*fos*–dependent and –independent pathways for gene expression stimulated by growth factors or oncogenes. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3094–3103.
Hülsken, J., W. Birchmeier, and J. Behrens. 1994. E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. *J. Cell Biol.* 127: 2061–2069.
Jardines, L., M. Weiss, B. Fowble, and M. Greene. 1993. neu(c-erbB-2/HER2) and the epidermal growth factor receptor (EGFR) in breast cancer. *Pathobiology.* 61:268–282.
Jehn, B., E. Costello, A. Marti, N. Keon, R. Deane, F. Li, R.R. Friis, P.H. Burri, F. Martin, and R. Jaggi. 1992. Overexpression of Mos, Ras, Src, and Fos inhibits mouse mammary epithelial cell differentiation. *Mol. Cell. Biol.* 12: 3890–3902.
Kawanishi, J., J. Kato, K. Sasaki, S. Fujii, N. Watanabe, and Y. Niitsu. 1995. Loss of E-cadherin-dependent cell–cell adhesion due to mutation of the β-catenin gene in a human cancer cell line, HSC-39. *Mol. Cell. Biol.* 15:1175–1181.
Kazlauskas, A. 1994. Receptor tyrosine kinases and their targets. *Curr. Opin. Genet. Dev.* 4:5–14.
Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* 9:317–321.
Kinch, M.S., G.J. Clark, C.J. Der, and K. Burridge. 1995. Tyrosine phosphorylation regulates the adhesion of ras-transformed breast epithelia. *J. Cell Biol.* 130:461–471.
Kirschmeier, P.T., G. M. Housey, M.D. Johnson, A.S. Perkins, and I.B. Weinstein. 1988. Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. *DNA.* 7:219–225.
Knudsen, H., T. Olesen, A. Riccio, P. Ungaro, L. Christensen, and P.A. Andreasen. 1994. A common response element mediates differential effects of phorbol esters and forskolin on type-1 plasminogen activator inhibitor gene expression in human breast carcinoma cells. *Eur. J. Biochem.* 220:63–74.
Lee, J.S., D. von der Ahe, B. Kiefer, and Y. Nagamine. 1993. Cytoskeletal reorganization and TPA differently modify AP-1 to induce the urokinase-type plasminogen activator gene in LLC-PK1 cells. *Nucleic Acids Res.* 21:3365–3372.
Lewis, J.E., P.J. Jensen, K.R. Johnson, and M.J. Wheelock. 1995. E-cadherin mediates adherens junction organization through protein kinase C. *J. Cell Sci.* 108:3615–3621.
Li, L., J.C. Chambard, M. Karin, and E.N. Olson. 1992. Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino terminus of Jun can mediate repression. *Genes Dev.* 6:676–689.
Lin, A., J. Frost, T. Deng, T. Smeal, N. al Alawi, U. Kikkawa, T. Hunter, D. Brenner, and M. Karin. 1992. Casein kinase II is a negative regulator of c-Jun DNA binding and AP-1 activity. *Cell.* 70:777–789.
López-Barahona, M., I. Fialka, J.M. González-Sancho, M. Asunción, M. González, T. Iglesias, J. Bernal, H. Beug, and A. Muñoz. 1995. Thyroid hormone regulates stromelysin expression, protease secretion and the morphogenetic potential of normal, polarized mammary epithelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1145–1155.
Madara, J.L., S. Carlson, and J.M. Anderson. 1993. ZO-1 maintains its spatial distribution but dissociates from junctional fibrils during tight junction regulation. *Am. J. Physiol.* 264:1096–1101.
Mandel, L.J., R. Bacallao, and G. Zampighi. 1993. Uncoupling of the molecular "fence" and paracellular "gate" functions in epithelial tight junctions. *Nature (Lond.).* 361:552–555.
Markowitz, D., S. Goff, and A. Bank. 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* 62:1120–1124.
Marrs, J.A., E.W. Napolitano, C. Murphy-Erdosh, R.W. Mays, L.F. Reichardt, and W.J. Nelson. 1993. Distinguishing roles of the membrane-cytoskeleton and cadherin mediated cell–cell adhesion in generating different Na^+,K^+-ATPase distributions in polarized epithelia. *J. Cell Biol.* 123:149–164.
Marrs, J.A., C. Andersson-Fisone, M.C. Jeong, L. Cohen-Gould, C. Zurzolo, I.R. Nabi, E. Rodriguez-Boulan, and W.J. Nelson. 1995. Plasticity in epithelial cell phenotype: modulation by expression of different cadherin cell adhesion molecules. *J. Cell Biol.* 129:507–519.
Mason, I.J. 1994. The ins and outs of fibroblast growth factors. *Cell.* 78:547–552.
Matsui, Y., S.A. Halter, J.T. Holt, B.L. Hogan, and R.J. Coffey. 1990. Development of mammary hyperplasia and neoplasia in MMTV-TGF α transgenic mice. *Cell.* 61:1147–1155.
Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R.J. Davis, G.L. Johnson, and M. Karin. 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science (Wash. DC).* 266:1719–1723.
Morrison, B.W., and P. Leder. 1994. neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2–initiated tumors. *Oncogene.* 9:3417–3426.
Munemitsu, S., I. Albert, B. Souza, B. Rubinfeld, and P. Polakis. 1995. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor gene. *Proc. Natl. Acad. Sci. USA.* 92:3046–3050.
Navarro, P., E. Lozano, and A. Cano. 1993. Expression of E- or P-cadherin is not sufficient to modify the morphology and the tumorigenic behavior of murine spindle carcinoma cells. Possible involvement of plakoglobin. *J. Cell Sci.* 105:923–934.
Nelson, W.J. 1992. Regulation of cell surface polarity from bacteria to mammals. *Science (Wash. DC).* 258:948–955.
Nikolakaki, E., P.J. Coffer, R. Hemelsoet, J.R. Woodgett, and L.H. Defize. 1993. Glycogen synthase kinase 3 phosphorylates Jun family members in vitro and negatively regulates their transactivating potential in intact cells. *Oncogene.* 8:833–840.
Oka, T., M. Yoshimura, S. Lavandero, K. Wada, and Y. Ohba. 1991. Control of growth and differentiation of the mammary gland by growth factors. *J. Dairy Sci.* 74:2788–2800.
Peters, K., S. Werner, X. Liao, S. Wert, J. Whitsett, and L. Williams. 1994. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3296–3301.
Pfahl, M. 1993. Nuclear receptor/AP-1 interaction. *Endocr. Rev.* 14:651–658.
Pfarr, C.M., F. Mechta, G. Spyrou, D. Lallemand, S. Carillo, and M. Yaniv. 1994. Mouse JunD negatively regulates fibroblast growth and antagonizes transformation by ras. *Cell.* 76:747–760.
Polakis, P. 1995. Mutations in the APC gene and their implications for protein structure and function. *Curr. Opin. Genet. Dev.* 5:66–71.
Pratt, W.B., and M.J. Welsh. 1994. Chaperone functions of the heat shock proteins associated with steroid receptors. *Semin. Cell Biol.* 5:83–93.
Puissant, C., and L.M. Houdebine. 1990. An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Biotechniques.* 8:148–149.
Rapp, U.R., J. Troppmair, T. Beck, and M.J. Birrer. 1994. Transformation by Raf and other oncogenes renders cells differentially sensitive to growth inhibition by a dominant negative c-jun mutant. *Oncogene.* 9:3493–3498.
Reichmann, E. 1994. Oncogenes and epithelial cell transformation. *Semin. Cancer Biol.* 5:157–165.
Reichmann, E., H. Schwarz, E.M. Deiner, I. Leitner, M. Eilers, J. Berger, M. Busslinger, and H. Beug. 1992. Activation of an inducible c-FosER fusion protein causes loss of epithelial polarity and triggers epithelial-fibroblastoid cell conversion. *Cell.* 71:1103–1116.
Reilly, D., L. Christensen, M. Duch, N. Nolan, M.J. Duffy, and P.A. Andreasen. 1992. Type-1 plasminogen activator inhibitor in human breast carcinomas. *Int. J. Cancer.* 50:208–214.
Rozek, D., and G.P. Pfeifer. 1993. In vivo protein-DNA interactions at the c-jun promoter: preformed complexes mediate the UV response. *Mol. Cell. Biol.* 13:5490–5499.
Rubinfeld, B., B. Souza, I. Albert, O. Muller, S.H. Chamberlain, F.R. Masiarz, S. Munemitsu, and P. Polakis. 1993. Association of the APC gene product with β-catenin. *Science (Wash. DC).* 262:1731–1734.
Ryder, K., A. Lanahan, E. Perez-Albuerne, and D. Nathans. 1989. jun-D: a

third member of the jun gene family. *Proc. Natl. Acad. Sci. USA.* 86:1500–1503.
Schlingensiepen, K.H., R. Schlingensiepen, M. Kunst, I. Klinger, W. Gerdes, W. Seifert, and W. Brysch. 1993. Opposite functions of jun-B and c-jun in growth regulation and neuronal differentiation. *Dev. Genet.* 14:305–312.
Schwarz, H. 1994. Immunolabelling of ultrathin resin sections for fluorescence and electron microscopy. *In* Electron Microscopy. ICEM 13-Paris. B. Jouffrey and C. Colliex, editors. Les Editions de Physique, Les Ulis, France. Vol. 3. pp. 255–256.
Schwarz, H., A. Müller-Schmid, and W. Hoffmann, 1993. Ultrastructural localization of ependymins in the endomeninx of the brain of the rainbow trout: possible association with collagen fibrils of the extracellular matrix. *Cell Tissue Res.* 273:417–425.
Simons, K., P. Dupree, K. Fiedler, L.A. Huber, T. Kobayashi, T. Kurzchalia, V. Olkkonen, S. Pimplikar, R. Parton, and C. Dotti. 1992. Biogenesis of cell-surface polarity in epithelial cells and neurons. *Cold Spring Harbor Symp. Quant. Biol.* 57:611–619.
Smeal, T., M. Hibi, and M. Karin. 1994. Altering the specificity of signal transduction cascades: positive regulation of c-Jun transcriptional activity by protein kinase A. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:6006–6010.
Sommers, C.L., E.P. Gelmann, R. Kemler, P. Cowin, and S.W. Byers. 1994. Alterations in β-catenin phosphorylation and plakoglobin expression in human breast cancer cells. *Cancer Res.* 54:3544–3552.
Spivak-Kroizman, T., M.A. Lemmon, I. Dikic, J.E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlessinger, and I. Lax. 1994. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell.* 79:1015–1024.
Stampfer, M.R., C.H. Pan, J. Hosoda, J. Bartholomew, J. Mendelsohn, and P. Yaswen. 1993. Blockage of EGF receptor signal transduction causes reversible arrest of normal and immortal human mammary epithelial cells with synchronous reentry into the cell cycle. *Exp. Cell Res.* 208:175–188.
Streuli, C.H., N. Bailey, and M.J. Bissell. 1991. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell–cell interaction and morphological polarity. *J. Cell Biol.* 115:1383–1395.
Treier, M., L.M. Staszewski, and D. Bohmann. 1994. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the δ domain. *Cell.* 78:787–798.
van Dam, H., M. Duyndam, R. Rottier, A. Bosch, L. de Vries-Smits, P. Herrlich, A. Zantema, P. Angel, and A.J. van der Eb. 1993. Heterodimer formation of c-Jun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 12: 479–487.
van Dam, H., D. Wilhelm, I. Herr, A. Steffen, P. Herrlich, and P. Angel. 1995. ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1798–1811.
Watabe, M., A. Nagafuchi, S. Tsukita, and M. Takeichi. 1994. Induction of polarized cell–cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J. Cell Biol.* 127:247–256.
Wilkinson, D.G., S. Bhatt, R.P. Ryseck, and R. Bravo. 1989. Tissue-specific expression of c-jun and junB during organogenesis in the mouse. *Development.* 106:465–471.
Woodgett, J.R. 1990. Fos and jun: two into one will go. *Semin. Cancer Biol.* 1: 389–397.
Woods, D.F., and P.J. Bryant. 1993. Apical junctions and cell signalling in epithelia. *J. Cell Sci. Suppl.* 17:171–181.
Yang-Yen, H.F., J.C. Chambard, Y.L. Sun, T. Smeal, T.J. Schmidt, J. Drouin, and M. Karin. 1990. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein–protein interaction. *Cell.* 62:1205–1215.