

TGF- β Induced Transdifferentiation of Mammary Epithelial Cells to Mesenchymal Cells: Involvement of Type I Receptors

Päivi J. Miettinen, Reinhard Ebner, Alfredo R. Lopez,* and Rik Derynck

Departments of Growth and Development, and Anatomy, Programs in Cell Biology and Developmental Biology, University of California at San Francisco, San Francisco, California 94143-0640; and *Hematology/Oncology Section 111H, VA Medical Center, University of California at San Francisco, San Francisco, California 94121

Abstract. The secreted polypeptide transforming growth factor- β (TGF- β) exerts its multiple activities through type I and II cell surface receptors. In epithelial cells, activation of the TGF- β signal transduction pathways leads to inhibition of cell proliferation and an increase in extracellular matrix production. TGF- β is widely expressed during development and its biological activity has been implicated in epithelial-mesenchymal interactions, e.g., in branching morphogenesis of the lung, kidney, and mammary gland, and in inductive events between mammary epithelium and stroma.

In the present study, we investigated the effects of TGF- β on mouse mammary epithelial cells in vitro. TGF- β reversibly induced an alteration in the differentiation of normal mammary epithelial NMuMG cells from epithelial to fibroblastic phenotype. The change in cell morphology correlated with (a) decreased expression of the epithelial markers E-cadherin, ZO-1, and desmoplakin I and II; (b) increased expression of mesenchymal markers, such as fibronectin; and (c) a fibroblast-like reorganization of actin fibers. This phenotypic differentiation displays the hallmarks

of an epithelial to mesenchymal transdifferentiation event.

Since NMuMG cells make high levels of the type I TGF- β receptor Tsk7L, yet lack expression of the ALK-5/R4 type I receptor which has been reported to mediate TGF- β responsiveness, we evaluated the role of the Tsk7L receptor in TGF- β -mediated transdifferentiation. We generated NMuMG cells that stably overexpress a truncated Tsk7L type I receptor that lacks most of the cytoplasmic kinase domain, thus function as a dominant negative mutant. These transfected cells no longer underwent epithelial to mesenchymal morphological change upon exposure to TGF- β , yet still displayed some TGF- β -mediated responses.

We conclude that TGF- β has the ability to modulate E-cadherin expression and induce a reversible epithelial to mesenchymal transdifferentiation in epithelial cells. Unlike other transdifferentiating growth factors, such as bFGF and HGF, these changes are accompanied by growth inhibition. Our results also implicate the Tsk7L type I receptor as mediating the TGF- β -induced epithelial to mesenchymal transition.

TRANSFORMING growth factor- β (TGF- β) is a secreted growth and differentiation factor that exists as three different isoforms, and exerts a variety of biological activities depending on the target cell type (Roberts and Sporn, 1991; Derynck, 1994a). Following interaction with its cell surface receptors, TGF- β has the ability to stimulate or inhibit cell proliferation. The antiproliferative activity is best documented in epithelial cells, whereas the growth stimulatory effect occurs mainly in cells of mesenchymal ori-

gin such as fibroblasts. TGF- β also increases the expression of several extracellular matrix proteins and integrin receptors for extracellular matrix components, and inhibits the proteolytic degradation of this matrix (Edwards et al., 1987; Roberts et al., 1988; Heino et al., 1989; Ignatz et al., 1989). TGF- β furthermore affects cell differentiation, which may be related to its ability to modulate cell interaction with the extracellular matrix. Among mesenchymal cells, TGF- β is known to inhibit adipocyte differentiation, stimulate chondrocyte formation and inhibit or stimulate myoblast and osteoblast differentiation depending on the physiological conditions and stage of differentiation of the specific cell type (Roberts and Sporn, 1991; Derynck, 1994a). Many members of the large TGF- β superfamily, including the bone morphogenetic factors (BMPs) and the activins, also strongly affect mesenchymal differentiation pathways, thus making it

Address all correspondence to Rik Derynck, Dept. of Growth and Development, University of California at San Francisco, San Francisco, CA 94143-0640. Tel.: (415) 476-7322. Fax: (415) 476-1499.

1. *Abbreviations used in this paper:* BMP, bone morphogenetic factor; TGF- β , transforming growth factor- β ; TPA, 12-*o*-tetradecanoyl-phorbol 13-acetate.

possible that during development, differentiation of these cell types may be orchestrated by the interplay of TGF- β -related factors (Kingsley, 1994).

During development, TGF- β expression is often detected in both epithelial and mesenchymal cells (Heine et al., 1987). Most notably, TGF- β is frequently localized at sites of interaction between the epithelium and mesenchyme, suggesting a functional involvement for TGF- β in epithelial-mesenchymal interactions. In this context, TGF- β may specifically modulate the branching morphogenesis of several organs such as the lung, kidney, and mammary gland (Silberstein and Daniel, 1987; Heine et al., 1990; Robinson et al., 1991; Rogers et al., 1993). All three TGF- β isoforms are expressed in the developing mouse mammary gland (Robinson et al., 1991). Their expression patterns are somewhat overlapping, since each isoform is produced by both mammary epithelial and stromal cells. Exogenous TGF- β inhibits both formation and growth of ductal buds and stimulates production of extracellular matrix (Silberstein and Daniel, 1987; Daniel et al., 1989; Silberstein et al., 1990; Robinson et al., 1991). Moreover, overexpression of active TGF- β 1 in the mammary gland of transgenic mice results in the formation of a hypoplastic ductal tree (Pierce et al., 1993). This further suggests a role for TGF- β as a regulator of ductal growth.

How TGF- β exerts its variety of activities at the receptor level is at present largely unknown. Chemical cross-linking of radiolabeled TGF- β to cell surface TGF- β -binding proteins has revealed the existence of up to nine different "receptors" (Massagué, 1992). Among these, the types I, II, and III TGF- β receptors are most frequently detected in the surface of many different cell types. The type III receptor, also called betaglycan, is a cell surface proteoglycan that presumably does not have any signaling activity (Lopez-Casillas et al., 1991; Wang et al., 1991). However, it may enhance presentation of TGF- β to the type II receptor (Lopez-Casillas et al., 1993; Moustakas et al., 1993). In contrast, the type II and type I receptors mediate most, if not all TGF- β signaling (Laiho et al., 1990). Molecular cloning and characterization of the *in vitro* kinase activity has shown that both receptor types are transmembrane serine/threonine kinases. Only one type II TGF- β receptor has been cloned and shown to bind TGF- β without the requirement of another receptor or detectable accessory protein (Lin et al., 1992). In contrast, cDNAs for several type I receptors have been characterized, including Tsk7L (Ebner et al., 1993a), ALK-5/R-4/ESK-2 (Franzén et al., 1993; He et al., 1993; Bassing et al., 1994a; Tomoda et al., 1994), and TSR-1 (Attisano et al., 1993) receptors. The ability of these type I receptors to bind TGF- β depends on the coexpression of the type II TGF- β receptor (Ebner et al., 1993a; Bassing et al., 1994b). The type II receptor can physically interact with the type I receptor, and in this way provides ligand specificity and affinity (Wrana et al., 1992; Ebner et al., 1993b; ten Dijke et al., 1994). Thus, in the presence of activin type II receptor, the Tsk7L type I receptor binds activin and, in the presence of TGF- β type II receptors, it binds TGF- β (Ebner et al., 1993b). The relative contributions of the type I and II receptors to the multiple activities of TGF- β remain unclear. We have previously proposed that in epithelial cells, the type II receptor, presumably in combination with the type I receptor, mediates the antiproliferative effect of TGF- β , and that

the TGF- β -induced increase in fibronectin and PAI-1 synthesis as well as c-jun expression is mediated through the type I receptor (Chen et al., 1993). However, Wieser et al. (1993) have reported that the transcriptional and antiproliferative responses to TGF- β require fully functional forms of both type I and II receptors. Whereas several type I receptors bind TGF- β when coexpressed with the type II TGF- β receptor (ten Dijke et al., 1994), only one of these, namely ALK-5/R-4, has been shown to restore TGF- β responsiveness in a mutant mink lung epithelial cell line lacking type I receptors (Bassing et al., 1994a; Carcámo et al., 1994; ten Dijke et al., 1994). No TGF- β mediated functional responsiveness has as yet been documented for the type I receptor Tsk7L (Ebner et al., 1993a).

In the present study, we have investigated the effect of TGF- β on mouse mammary epithelial cells *in vitro*. TGF- β inhibited proliferation of these cells and concomitantly induced an epithelial to fibroblastic transdifferentiation. This reversible morphological transdifferentiation was accompanied by changes in expression patterns for specific epithelial markers such as E-cadherin, and suggests a role for TGF- β as a differentiation factor e.g. at sites of epithelial to mesenchymal transitions. Since the NMuMG epithelial cells lack ALK-5/R-4 type I receptors for TGF- β but express high levels of Tsk7L type I receptors, we used this TGF- β dependent transdifferentiation to evaluate the involvement of Tsk7L in TGF- β -induced signal transduction. Inhibition of the functional expression of Tsk7L receptors by overexpressing a dominant negative Tsk7L truncated receptor resulted in inhibition of the TGF- β -induced phenotypic differentiation suggesting, that Tsk7L is involved in the transdifferentiation process.

Materials and Methods

Cell Lines and Culture Conditions

Mouse mammary gland epithelial cells, NMuMG (CRL 1636; American Type Culture Collection [ATCC], Rockville, MD), were obtained from Dr. M. Bernfield (Harvard Medical School, Boston, MA) and grown in H-21 DMEM (4.5 g glucose/l) supplemented with 7.5% fetal calf serum, 10 μ g/ml insulin, 100 μ g/ml penicillin, and 100 U/ml streptomycin. Mink lung (Mv1Lu) epithelial cells (CCL 64; ATCC) and MDCK kidney epithelial-like cells (CCL 34; ATCC) were cultured in H-16 DMEM (3.0 g glucose/l) supplemented in 10% fetal calf serum, whereas COMMA-ID cells (obtained from Dr. M. Bissell, Lawrence-Berkeley Labs., Berkeley, CA) were grown in DME/F-12 with 5% fetal calf serum and 5 μ g/ml insulin. All cell lines were grown at 37°C in 5% CO₂ and passaged every third day following 0.05% trypsin-versene treatment. Treatments with TGF- β 1 (or TGF- β 2 or activin) were initiated on day 2.

For trypan blue staining, cells grown with or without TGF- β were trypsinized and combined with their medium to include the detached dead cells. They were then spun down and mixed with an equal volume of 0.4% trypan blue. The proportion of viable cells (nonstained) was determined in a counting chamber. To visualize apoptotic cells, cells were grown on coverslips and subsequently fixed in 70% ethanol, treated with RNaseA and stained with propidium iodide (Sigma Chem. Co., St. Louis, MO).

Experiments with antisense and sense strand oligonucleotides to the mouse Tsk7L type I and the mouse type II TGF- β receptor were performed in serum-free media supplemented with ITS (GIBCO BRL, Gaithersburg, MD). The oligonucleotide sequences were: Tsk7L antisense 5'-TACTCC-ATCGACCATTGTATA-3' and sense 5'-TATACAATGGTCGATGGAGTA-3'; mouse type II receptor antisense 5'-CAGCCCCGACCCATGGCAGC-3' and sense 5'-GCTGCCATGGGTCGGGGGCTG-3'. All sequences span the ATG start codon. The oligonucleotides (10 μ M) were added to NMuMG cell cultures daily starting from day 1. TGF- β treatments were started on day 2.

Reagents

Recombinant TGF- β 1, TGF- β 2, and activin were obtained from Genentech (South San Francisco, CA) and used at 2–20 ng/ml. Protein kinase C inhibitors H7 (final concentration 50 μ M; LC Chemicals, Woburn, MA), HA1004 (final concentration 50 μ M; LC Chemicals), bisindolylmaleimide (final concentration 1–100 μ M; LC Chemicals), calphostin C (final concentration 0.5 μ M; LC Chemicals), chelerytrine (final concentration 5 μ M; Calbiochem-Novabiochem, La Jolla, CA), and staurosporine (final concentration 20 nM; Sigma Chem. Co.) were added either 5 min before or after TGF- β 1 stimulations. 12-*o*-tetradecanoyl-phorbol 13-acetate (TPA, final concentration 1–1000 ng/ml; Sigma Chem. Co.) was included in the media for 2 or 24 h before TGF- β 1 treatment.

Cell Proliferation Assay

NMuMG cells were grown at a density of 5×10^4 cells/well (50% confluency) in 24-well plates. After 24 h, the medium was changed to serum-free medium and TGF- β 1 (2 ng/ml) was added to the medium. After 21-h treatment, cells were incubated with 2 μ Ci of methyl- 3 H-thymidine (NEN-DuPont, Boston, MA) for an additional 3 h. Cells were then washed twice with ice-cold PBS, precipitated in 10% trichloroacetic acid, and counted for radioactivity. Experiments were done in triplicate and the results are represented as the mean value.

Immunohistochemistry and Western Blot Analysis

The following antibodies were used: mouse anti-desmoplakins 1 and 2, (69-542; ICN Biomedicals, Inc., Costa Mesa, CA), mouse anti-vimentin (5255; Sigma); rat anti-E-cadherin (U-3254; Sigma); rabbit anti-fibronectin (F-3648; Sigma); mouse anti-vinculin (9131; Sigma); rat anti-ZO-1 (MAB 1520; Chemicon Intl. Inc., Temecula, CA); mouse anti-cytokeratin 18, a gift from Professor I. Virtanen (University of Helsinki). Cells were grown on glass coverslips and stimulated with TGF- β 1 for 24–48 h. They were then fixed either in ice-cold acetone-methanol for 10 min (mouse anti-desmoplakins 1 and 2, mouse anti-vimentin) or 3% paraformaldehyde for 30 min (rat anti-E-cadherin, rabbit anti-fibronectin, mouse anti-vinculin, rat anti-ZO-1) before application of primary antibodies. Bound antibodies were detected using fluorochrome-conjugated secondary antibodies (FITC- or TRITC-conjugated donkey anti-mouse-IgM, donkey anti-rat, goat anti-rabbit, goat anti-mouse from Jackson Biochemicals, West Grove, PA) and analyzed by fluorescence microscopy. Actin was detected using FITC-conjugated phalloidin (from Dr. T. Mitchison, University of California, San Francisco) after fixation in 3% paraformaldehyde and permeabilization in 0.5% Triton X-100.

For Western analysis, cells cultured with or without TGF- β 1 were lysed in 2% SDS, 50 mM Tris, pH 7.4, 2 mM EDTA with 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 1 mM PMSF. Equal protein amounts were denatured in reducing Laemmli sample buffer, run in 7.5% SDS-polyacrylamide gel at 175V (Laemmli, 1970), and electroblotted to nitrocellulose filters (Towbin et al., 1979). Nonspecific binding to the filter was blocked by incubating the membranes overnight in 3% bovine serum albumin and 0.1% Tween 20 in Tris-buffered saline. The membrane-bound antibodies to E-cadherin, fibronectin, vimentin, vinculin, and ZO-1 were visualized with alkaline-conjugated secondary antibodies (Jackson) and nitroblue tetrazolium substrate (Kilkegaard-Poulsen, Gaithersburg, MD). For E-cadherin immunoblotting analysis, the cell lysates were also incubated with concanavalin-A and the concanavalin-A bound fraction was used for Western blot.

Northern Hybridization

RNA was extracted from confluent 10-cm plates of untreated or TGF- β -treated NMuMG cells using Ultraspec according to the manufacturer's instructions (Biotecx Laboratories Inc., Houston, Texas). 30- μ g RNA samples were electrophoretically separated on a 1.3% agarose-formaldehyde gel and transferred to Hybond-N+ (Amersham Corp., Arlington Heights, IL) according to standard procedures (Maniatis et al., 1982). After transfer, the filters were stained with 0.04% methylene blue to confirm equal loading and integrity of the RNA samples. Hybridization probes were prepared by radiolabeling the cDNAs using the random priming method. Northern analyses were done using cDNAs for E-cadherin (gift from Dr. M. Takeichi, Kyoto University, Japan), fibronectin (Schwarzbauer et al., 1983), Tsk7L type I receptor (Ebner et al., 1993a), and ALK-5/R4 type I receptor (a PCR fragment containing the cytoplasmic kinase domain). Filters were hybridized overnight at 42°C with 1×10^6 cpm/ml of each cDNA probe in hy-

bridization buffer (50% formamide, 0.5% SDS, 5 \times Denhardt's solution, 0.15 mg/ml salmon sperm DNA, washed in 0.1 \times SSC, 0.1% SDS at 52°C for 1 h and exposed for autoradiography. To detect mRNA for the ALK-5/R4 type I receptor, we used low-stringency washes, i.e., 0.5 \times SSC, 0.1% SDS at 42°C for 1 h.

Generation of Stable Transfected Cell Clones

The expression plasmid for the cytoplasmically truncated form of the Tsk7L (Tsk7L-DN) with a COOH-terminal Flag epitope tag has been described previously (Ebner, 1993a). This truncated form has Glu¹⁵⁵ as the last amino acid of the receptor thus lacking the GS domain (amino acids 191–195), which is conserved among type I receptors. NMuMG cells were transfected with Tsk7L-DN together with an expression plasmid containing the neomycin resistance gene using Lipofectamine following the manufacturer's instructions (GIBCO BRL, Grand Island, NY). Transfected cell clones surviving neomycin selection (G418 700 μ g/ml, GIBCO BRL) were screened by nonradioactive Northern blot analysis (Engler-Blum et al., 1993) with some modifications. First, RNA isolated using Ultraspec (Biotecx) was separated in 1.3% agarose formaldehyde gel and capillary blotted onto Hybond-N+ membrane (Maniatis et al., 1982). Full-length Tsk7L cDNA probe was random prime labeled with digoxigenin-dCTP (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instructions. Northern blots were prehybridized in 20% SDS, 0.25 M Na₂HPO₄ (pH 7.2), 1 mM EDTA, 0.5% blocking buffer (Boehringer Mannheim) at 68°C for 1 h and with 2.5 ng/ml of labeled probe for additional 14–18 h. The blots were next washed in 0.1% SDS, 0.1 \times SSC at 68°C for 3 \times 20 min. The bound probe was detected with alkaline-phosphatase-conjugated anti-digoxigenin antibody and Lumiphos-created chemiluminescence (Boehringer Mannheim).

Biotinylation of Cell Surface Proteins and Immunoprecipitation

Cell surface proteins including the TGF- β receptors were biotinylated as described (Lopez-Casillas et al., 1993). Briefly, near-confluent cells (wild-type NMuMG, Tsk7L-DN clones, or NMuMG transiently transfected with Tsk7L-DN) were washed with Krebs-Ringer buffer containing 20 mM Hepes, pH 7.2. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce Chemical, Rockford, IL) was then added to the cells at 1 mg/ml in Krebs-Ringer buffer and incubated 2 h at 4°C. The biotinylation reaction was quenched by adding 10 mM glycine, 0.4 M sucrose, 10 mM Tris-HCl (pH 7.4), 1.5 mM CaCl₂, 5 mM MgCl₂. The cells were then scraped into lysis buffer (25 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, 0.5 mM EGTA, 0.1% BSA, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 1 mM PMSF) and subjected to immunoprecipitation with anti-Flag M2 antibody (4 μ g/ml; IBI, Eastman Kodak, New Haven). After addition of rabbit anti-mouse IgG bound to Protein-A (Pharmacia LKB Nuclear, Gaithersburg, MD), the immunoprecipitates were washed twice in lysis buffer without BSA and proteinase inhibitors and twice in 0.25 mM Tris-HCl (pH 7.5), 0.15 mM NaCl. The samples were then run in 15% SDS-polyacrylamide gel under reducing conditions, electrotransferred to nitrocellulose and incubated overnight in 0.5% Tween 20, 1 M glucose, 10% glycerol, 3% BSA, 1% skimmed milk in PBS to block nonspecific binding of streptavidin. The biotinylated proteins were detected using streptavidin-conjugated peroxidase and the ECL system (Amersham Corp.).

¹²⁵I-TGF- β Crosslinking

Near-confluent cells were washed in binding buffer (Krebs-Ringer with 20 mM Hepes and 0.5% BSA) and then incubated with 50 pM ¹²⁵I-TGF- β (Amersham Corp.) in binding buffer for 4 h at 4°C and cross-linked as described (Wang et al., 1991). Cells were then scraped in 1 ml binding buffer, centrifuged, and resulting pellets were further resuspended in 60 μ l of lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 1% Triton X-100 with 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 1 mM PMSF). After centrifugation, the soluble fraction was mixed with 5 \times Laemmli sample buffer containing 25% β -mercaptoethanol and separated on 12.5% SDS-polyacrylamide gels. After fixation in 50% methanol, 10% acetic acid, the gel was exposed for autoradiography. In some experiments, wild-type NMuMG cells and Tsk7L-DN clones were transiently transfected with an expression plasmid for the mouse type II TGF- β receptor (Lawler et al., 1994) using Lipofectamine.

Results

TGF- β Induces an Epithelial to Mesenchymal Differentiation in NMuMG Cells

TGF- β is a known inhibitor of cell proliferation for a variety of cell types, including epithelial cells. NMuMG mammary epithelial cells were tested for their response to TGF- β . This immortalized cell line, derived from a normal mammary gland is not transformed as assessed from its behavior in cell culture and does not form malignant tumors in vivo. Microscopic examination of the cells surprisingly revealed a drastic alteration in cell shape following exposure to TGF- β . Whereas the untreated NMuMG cells displayed the cuboidal appearance characteristic of epithelial cells, treatment with TGF- β induced a phenotypic change which made the cells elongated and spindle-shaped, i.e., characteristic of fibroblasts (Fig. 1). The effect was dose dependent and was already apparent at a concentration of 0.5 ng/ml, but was faster and more pronounced at higher concentrations of TGF- β (up to 10 ng/ml). However, the maximum effect was already reached at 2 ng/ml of TGF- β . The phenotypic change in the differentiation state was evident within 16–18 h after addition of TGF- β . TGF- β 2 also induced the same phenotypic alteration in differentiation state. TGF- β 3 was not available for our

experiments. However, activin A, another member of the TGF- β superfamily, had no effect on the morphology of NMuMG cells at varying concentrations (up to 20 ng/ml). The differentiation towards fibroblast-like phenotype required only a brief exposure to TGF- β , as low as 5 min. Finally, this effect of TGF- β was reversible, since removal of TGF- β from the culture media restored the epithelial phenotype within 2 d.

The proliferative response to NMuMG cells was tested in a [3 H]thymidine incorporation assay, indicative of DNA synthesis and cell proliferation. These experiments were done not only in serum-containing but also in serum-free medium to exclude the effects due to serum-derived TGF- β s and other growth factors. The DNA synthesis of NMuMG cells treated with 2 ng/ml of TGF- β 1 was reduced by 60% (Fig. 2 A) both in the presence and absence of serum. The proliferation of these cells as measured by counting the cell number was similarly decreased (Fig. 2 B) without any sign of increased cell death or apoptosis as analyzed by trypan blue and propidium iodide staining (data not shown). To evaluate whether the TGF- β -induced epithelial to fibroblastic differentiation was restricted to NMuMG cells or was a more general response of epithelial cells, we also tested several other epithelial cell lines. Several other cell lines tested, the lung epithelial Mv1Lu cells and the kidney epithe-

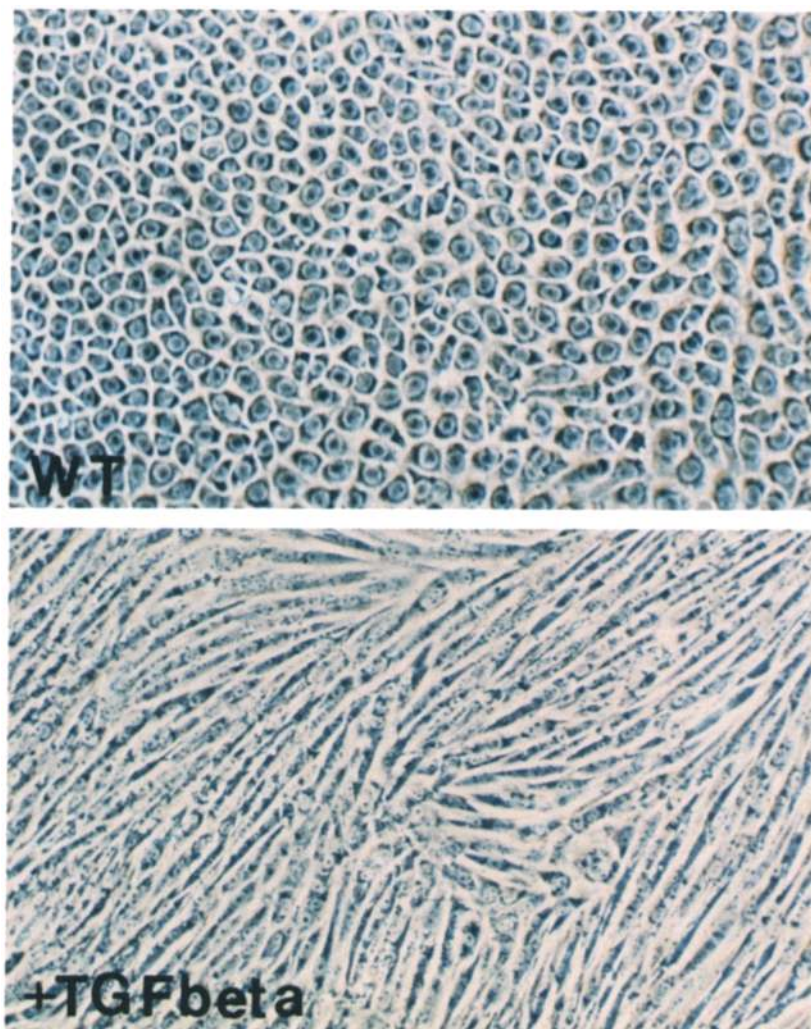


Figure 1. TGF- β induces an epithelial to mesenchymal differentiation in normal mammary epithelial NMuMG cells. Untreated NMuMG cells exhibited a cuboidal, epithelial cell phenotype (WT), while 36-h treatment with TGF- β 1 (2 ng/ml) induced a fibroblastic transition resulting in an elongated, spindle cell morphology.

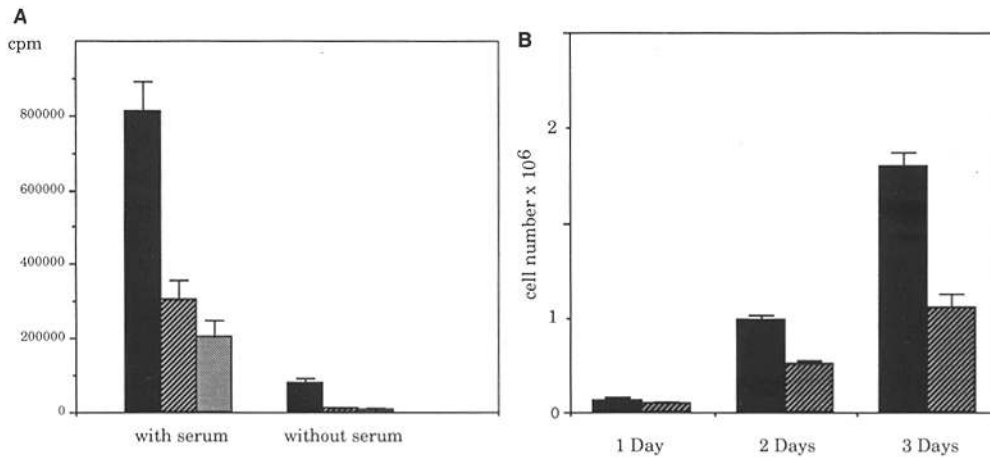


Figure 2. Growth inhibition of NMuMG cells after TGF- β treatment. (A) NMuMG cells were treated with TGF- β 1 (2 ng/ml or 10 ng/ml) for 24 h. Their rate of DNA synthesis was assayed by measuring the uptake of [3 H]thymidine at 21 h after initiation of TGF- β treatment. Serum-free media was used to exclude effects due to serum-derived TGF- β and other growth factors. As shown, TGF- β was growth inhibitory both in the presence and absence of serum. ■, CO; ▨, 2 ng/ml TGF- β ; ▩, 10 ng/ml TGF- β . (B) NMuMG

cells were plated in 24-well plates at a density of 50,000 cells/well. TGF- β 1 treatment (2 ng/ml) was started on the following day. Cells were then trypsinized and counted daily starting at 24 h after initiation of TGF- β treatment. The number of cells in the TGF- β -treated wells was approximately half of the control wells, indicative of the antiproliferative effect of TGF- β .

lial MDCK cells at low density and mammary epithelial COMMA-ID cells, all responded to TGF- β with a similar change in morphology, although the cells were generally less spindle-shaped even at higher concentrations of TGF- β (data not shown).

Characterization of the Alteration in Differentiation State

To further characterize the shape change of the NMuMG cells following treatment with TGF- β , we tested whether the change in morphology correlated with the disappearance of epithelial markers and appearance of mesenchymal cell markers. As epithelial markers, we examined the expression of desmoplakins 1 and 2, E-cadherin, vinculin, and ZO-1 by immunofluorescence. To characterize the fibroblastic character of the TGF- β -treated cells, we used immunofluorescence to determine the expression of fibronectin, vimentin and the characteristic intracellular pattern of actin fibers. For this purpose, NMuMG cells were grown on coverslips with or without TGF- β and stained with various antibodies to the above mentioned cytoskeletal proteins.

In untreated NMuMG cells, E-cadherin was located at the cell surface and appeared as a continuous line at the boundaries between neighboring cells, characteristic for epithelial cells. Following TGF- β treatment, this epithelial pattern disappeared and the immunostaining was more diffuse and drastically decreased or was absent in the fibroblastic cells (Fig. 3, A and B). Without TGF- β treatment, the immunoreactivity pattern for ZO-1, a tight junction protein, was very similar to the epithelial E-cadherin immunofluorescence staining. As in other epithelial cells, the ZO-1 protein was localized as a continuous line at the cell-cell boundaries. However, upon TGF- β 1 treatment, the ZO-1 staining decreased and displayed a somewhat coarser, punctate-like pattern at the cell boundaries (Fig. 3, C and D). Also, the staining patterns for desmoplakins, vinculin (Fig. 3, E-H) and actin were characteristic of epithelial cells and resembled the localization of E-cadherin and ZO-1 at the boundaries between the cells. Following TGF- β treatment and fibroblast-like cell differentiation, the immunoreactivity for desmo-

plakin disappeared (Fig. 3 F), and the vinculin staining changed from a peripheral pattern to a punctate staining (Fig. 3 H). In epithelial cells, actin is connected with and becomes part of the zonula adherens and, accordingly, actin immunoreactivity was detectable cortically in NMuMG cells before addition of TGF- β . Following TGF- β treatment, actin was reorganized as longitudinal stress fibers in the spindle-shaped, elongated cells, characteristic of fibroblastic cells (Fig. 4, A and B). We also verified the subcellular localization of cytokeratin 18 (Fig. 4, C and D), which in untreated NMuMG cells has the characteristic epithelial staining pattern, but displays an altered distribution following TGF- β treatment. Finally, the immunofluorescence staining patterns of fibronectin and vimentin expression were determined. Following TGF- β treatment, the staining for both increased in intensity. Fibronectin was seen as a network surrounding the elongated cells (Fig. 4 F), while the vimentin filaments were organized as a longitudinal meshwork in the cells (Fig. 4 H).

Western analysis was also performed to analyze the expression levels of E-cadherin, fibronectin, vimentin and vinculin in NMuMG cells before and after TGF- β -induced differentiation. In accordance with the immunohistochemical staining, the expression level of E-cadherin decreased, whereas fibronectin expression increased (Fig. 5). The levels of vimentin and vinculin expression remained similar in TGF- β -treated and -untreated cells. Thus, the TGF- β -induced changes in expression pattern of these two proteins visualized by immunofluorescence more likely reflect a reorganization of the cytoskeleton than an increase in expression. To assess the expression level of the glycosylated fraction of E-cadherin, which presumably correlates with the expression on the cell surface E-cadherin, Western blot analysis was also done using cell lysates which were preabsorbed to concanavalin A. Similar to the analyses of total cell lysates, TGF- β treatment resulted in a decrease in the level of concanavalin A-bound E-cadherin (data not shown). Finally, analysis of the mRNA levels by Northern analysis showed a TGF- β -induced (2 ng/ml) decrease in E-cadherin mRNA and an increase in fibronectin mRNA (Fig. 6), in accordance with the corresponding protein levels.

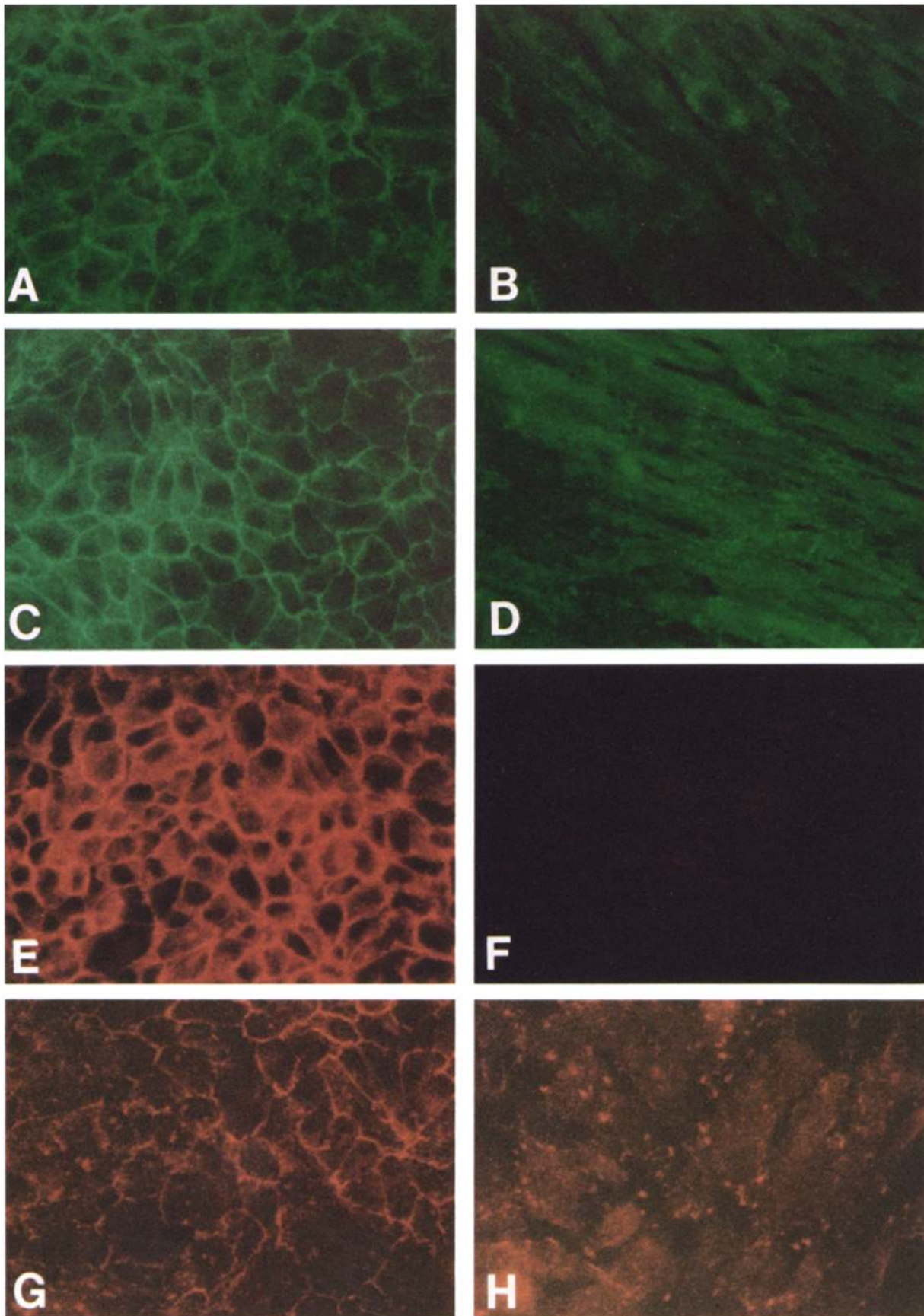


Figure 3. Decreased expression of E-cadherin, ZO-1, desmoplakins 1 and 2 and vinculin after TGF- β treatment of NMuMG cells. Cells were grown on coverslips in the absence (A, C, E, and G) or presence of TGF- β 1 (B, D, F, and H). After fixation and permeabilization, they were stained with antibodies to E-cadherin (A and B), ZO-1 (C and D), desmoplakins 1 and 2 (E and F) and vinculin (G and H). In untreated cells, all these cytoskeletal proteins were located cortically as a continuous line at the cell boundaries. However, upon TGF- β -induced phenotypic differentiation, E-cadherin and desmoplakins disappeared, ZO-1 immunoreactivity was decreased and vinculin changed from a peripheral rim-like staining pattern to a punctate cytoplasmic one.

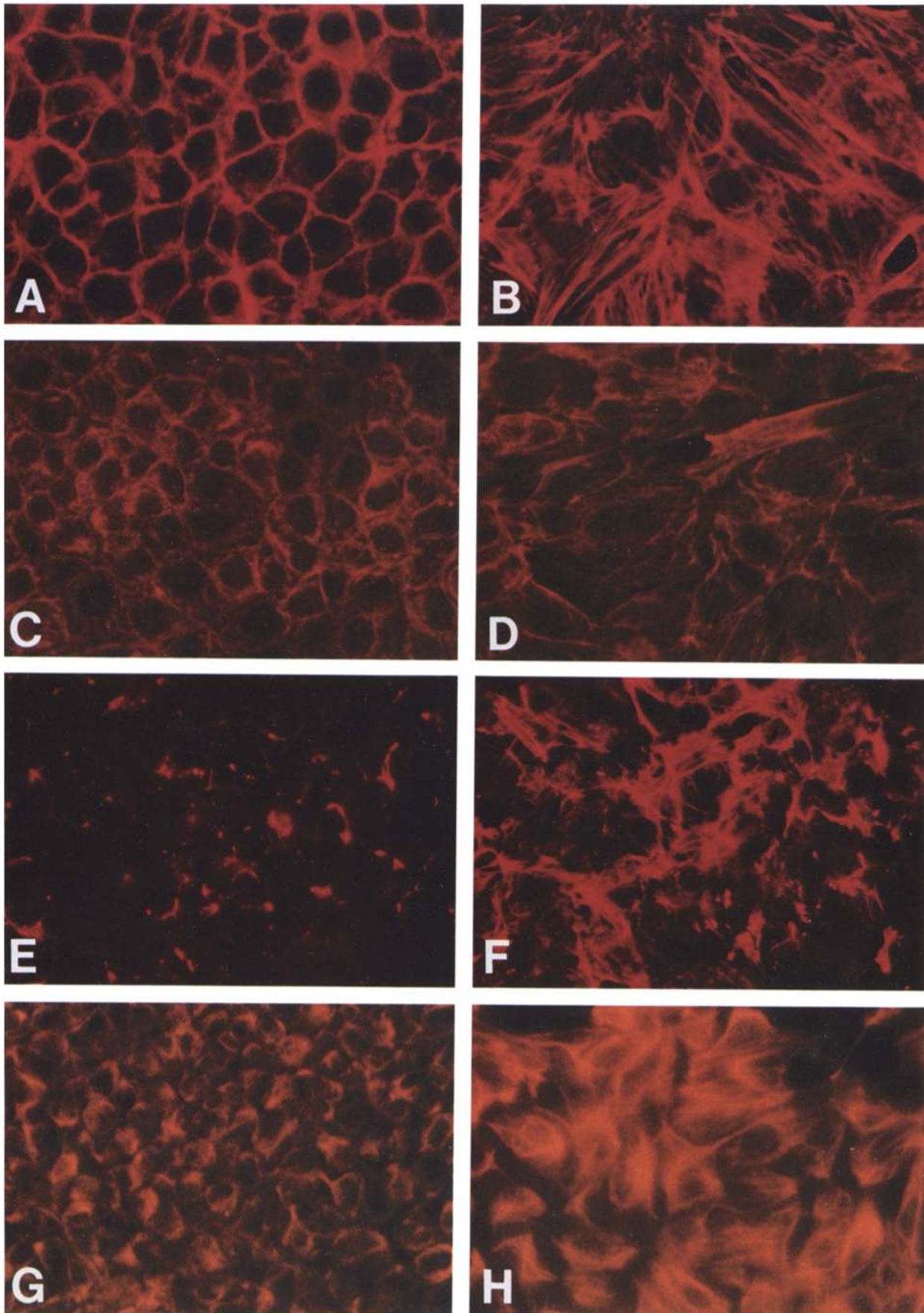


Figure 4. Localization of actin, cytokeratin 18, fibronectin and vimentin in NMuMG cells after TGF- β treatment. Cells were grown on coverslips without (A, C, E, and G) or with TGF- β 1 (B, D, F, and H). After fixation and permeabilization, they were stained with antibodies to actin (A and B), cytokeratin 18 (C and D), fibronectin (E and F) and vimentin (G and H). In untreated cells, actin was localized cortically (A) but was reorganized into stress fibers during fibroblast-like differentiation (B). Cytokeratin 18 changed from a ring-like staining (C) to an irregular meshwork across the cells (D). Fibronectin immunoreactivity was strongly increased upon fibroblastic transition (F), while vimentin was mainly reorganized to a network across the cells (H).

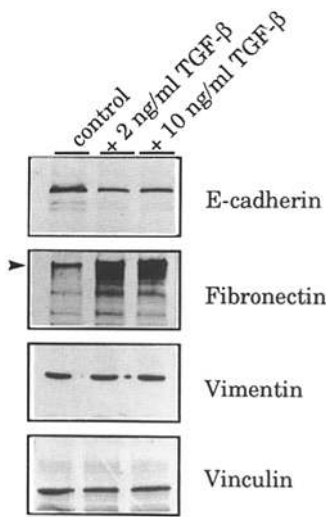


Figure 5. Western analysis of NMuMG cell lysates after TGF- β treatment. NMuMG cells cultured without or with TGF- β 1 (2 ng/ml and 10 ng/ml) were lysed, and the proteins were run in 7.5% SDS-polyacrylamide gel and electroblotted to nitrocellulose filters. Western analysis showed a decrease in E-cadherin expression whereas fibronectin expression (arrow indicates the 220-kD band) was increased. There was no detectable change in vimentin or vinculin expression.

Inhibition of TGF- β -induced Differentiation using Protein Kinase Inhibitors

Because the type I and type II TGF- β receptors are transmembrane serine/threonine kinases, we evaluated the effect of several kinase inhibitors on the TGF- β -induced change in differentiation. Among these, we used (a) H7, which inhibits mainly protein kinase C and, at higher concentrations, protein kinases A and G; (b) HA1004, a protein kinase A inhibitor; (c) staurosporin, a general protein kinase C inhibitor; and (d) bisindolylmaleimide, calphostin C and chelerythrine which are known to inhibit protein kinase C isoforms α , β 1, β 2, and γ . The inhibitors were added 5 min before or after the initiation of TGF- β treatment. H7 (50 μ M) and staurosporin (20 nM) were able to abolish or partially inhibit the epithelial to fibroblastic differentiation. In contrast, HA1004, at concentrations (50 μ M) that block protein kinase A activity, had no effect suggesting that the inhibition seen with H7 may be mediated through an inhibition of a protein kinase C. Most likely PKC- α , - β , or - γ are not involved, since bisindolylmaleimide, calphostin C or chelerythrine did not inhibit the transition in differentiation state. We then tested whether the phorbol ester TPA, an activator of protein kinase C, could induce the phenotypic differentiation by itself. Our results showed that TPA alone was not able to induce the mesenchymal differentiation and that depletion of cellular PKC storage by long term (24 h) TPA pretreatment did not inhibit the TGF- β -induced phenotypic change (data not shown).

H7- and HA1004-treated NMuMG cells were also evaluated for their expression patterns of E-cadherin, fibronectin and ZO-1 using immunofluorescence (Fig. 7). Surprisingly, although H7 inhibited the TGF- β -induced fibroblastic phenotype, the decreased E-cadherin staining pattern was very

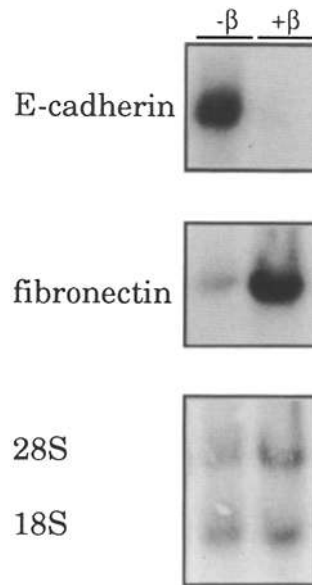


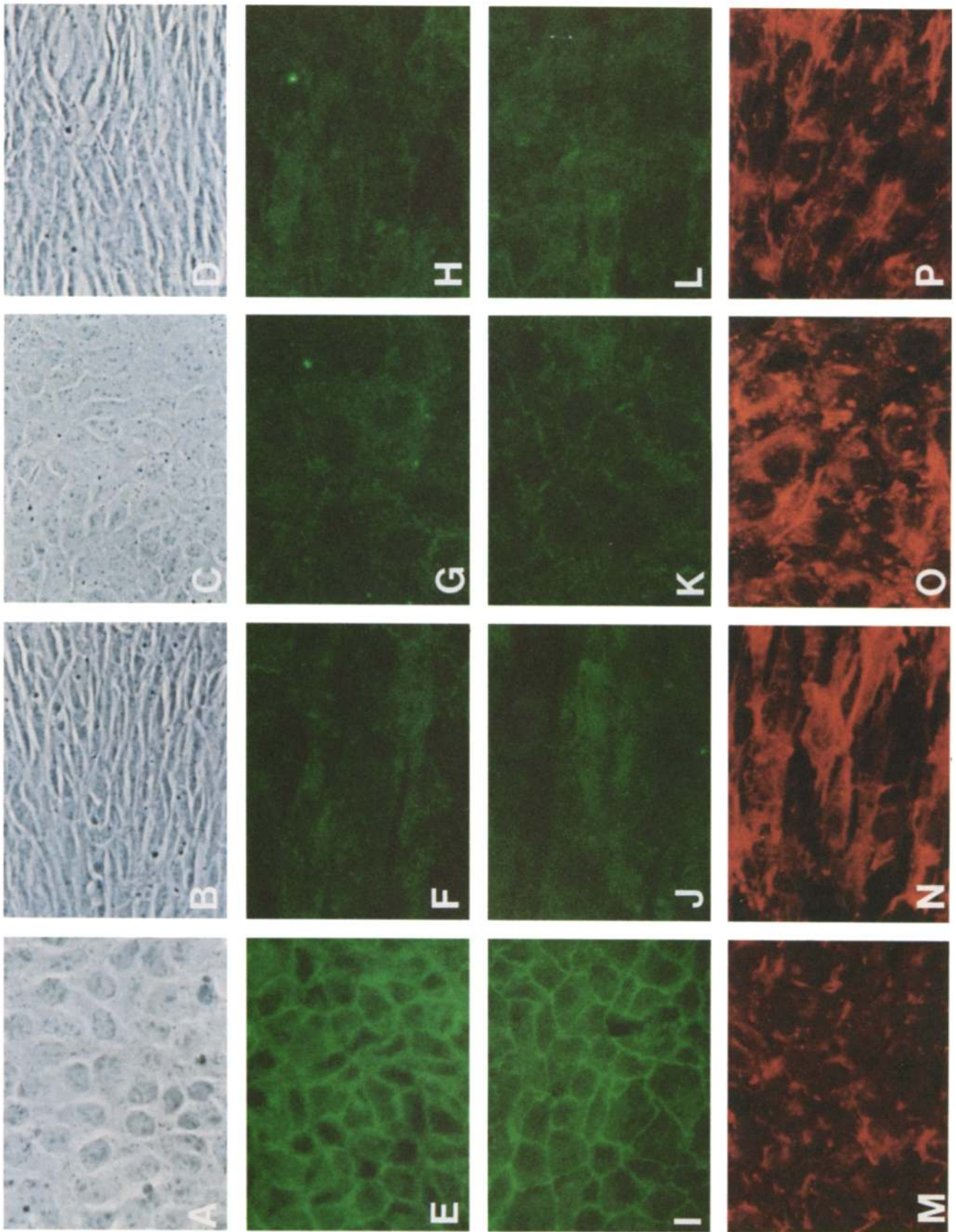
Figure 6. Northern analysis of E-cadherin and fibronectin mRNA expression in NMuMG cells. RNA samples (30 μ g) from untreated (- β) and TGF- β 1-treated (+ β ; 2 ng/ml) cells were subjected to Northern analysis. As shown, TGF- β down-regulated E-cadherin and increased fibronectin mRNA expression, which correlates with the immunohistochemical results. 28S and 18S were stained with methylene blue to verify equal RNA loading.

similar to the TGF- β -treated cells in the absence of H7. Furthermore, ZO-1 expression in TGF- β -treated cells in the presence of H7 also showed a pattern similar to the one in the absence of H7: ZO-1 was localized very coarsely and dot-like at the cell-cell boundaries contrary to the expected fine continuous tight-junctional expression in the untreated NMuMG epithelial cells. Finally, fibronectin immunoreactivity in the presence of H7 was increased after TGF- β treatment, but it was detectable mainly in the cytoplasm instead of in the extracellular matrix outside the cells as seen in the absence of H7.

Characterization of Cells Expressing a Dominant-negative Form of the Tsk 7L Type I Receptor

NMuMG cells make high levels of the TGF- β /activin type I receptor Tsk7L and, in fact, served as the mRNA source for the original Tsk7L cDNA cloning (Ebner et al., 1993a). However, we were not able to detect mRNA levels for the type I receptor ALK-5/R-4/ESK-2 using either Northern hybridization or reverse transcriptase-PCR amplification (data not shown). Thus, we used NMuMG cells to evaluate the role of the Tsk 7L type I receptor in the TGF- β -mediated epithelial to fibroblastic differentiation. NMuMG cells were treated with antisense oligonucleotides for the Tsk7L type I and the type II receptor or with the corresponding sense oligonucleotides. Only the Tsk7L antisense oligonucleotides inhibited the TGF- β -induced fibroblastic phenotype, whereas the other oligonucleotides had no effect on the cell shape (data not shown). Because of the possibility that some nonspecific effects may be associated with the exogenous use

Figure 7. Effect of protein kinase inhibitors H7 and HA1004 on TGF- β 1-induced transdifferentiation. Untreated NMuMG cells (A, E, I, and M), cells treated with TGF- β (B, F, J, and N), 50 μ M H7 plus TGF- β (C, G, K, and O), or 50 μ M HA1004 plus TGF- β (D, H, L, and P) were grown on coverslips and stained with antibodies to E-cadherin (E-H), ZO-1 (I-L), and fibronectin (M-P). H7 inhibited the TGF- β -induced transdifferentiation (C), whereas HA1004 did not (D). Although H7-plus TGF- β -treated cells looked epithelial, their E-cadherin and ZO-1 expression was down-regulated and fibronectin expression was up-regulated. Note that much of fibronectin immunoreactivity is intracellularly (P), instead of extracellular localization as seen in cells treated with TGF- β only (N).



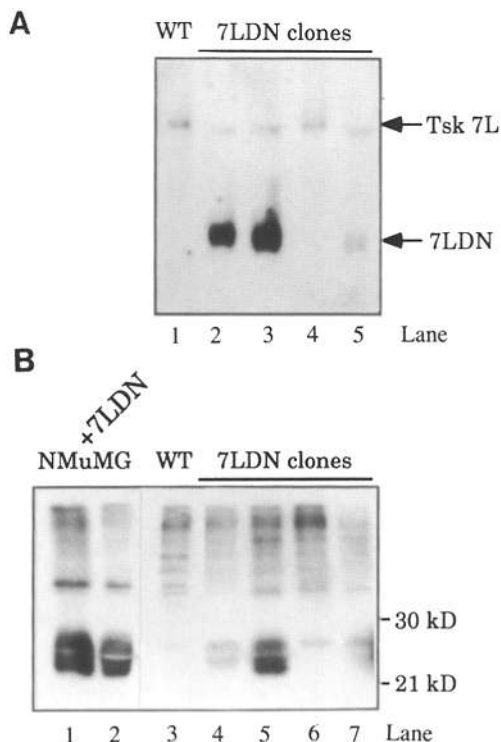


Figure 8. Characterization of cells expressing a truncated form of Tsk7L type I receptor. NMuMG cells were stably transfected with an expression vector for Tsk7L-DN lacking the cytoplasmic kinase domain. This truncated receptor also carries a carboxy-terminal FLAG epitope tag. **(A)** Transfected clones selected for neomycin resistance were subjected to Northern analysis. As shown, wild type (WT) (lane 1) and stably transfected clones (lanes 2–5) all express the full-length Tsk7L mRNA (upper arrow), while Tsk7L-DN mRNA expression is seen in three clones (lanes 2, 3, and 5, lower arrow). Among the Tsk7L-DN transfected clones two high level expressing clones (clones 1 and 2, lanes 2, 3), one low level expressing clone (clone 6, lane 5) and one non-expressing clone (clone 5, lane 4) were chosen for further analyses. **(B)** Cell surface proteins from wild type (WT, lane 3) and Tsk7L-DN-expressing clones (lanes 4–7) were biotinylated and immunoprecipitated with anti-FLAG antibody. The order of the different clones in lanes 4–7 is identical as for lanes 2–5 in **A**. As positive controls, we used NMuMG cells transiently transfected with the Tsk7L-DN expression plasmid using the Lipofactamine® (lane 1) or calcium phosphate (lane 2) methods. All Tsk7L-DN mRNA expressing clones expressed the 20–28-kD FLAG immunoreactive, truncated Tsk7L receptor at their cell surface. Their relative expression level of the truncated receptor correlated with the mRNA expression level. A similar 20–28-kD FLAG-immunoreactive protein was precipitated from the transiently transfected NMuMG cells. Occasionally a faint band of 28 kD was seen in the wild-type cells (lane 3), but was considered as nonspecific.

of antisense oligonucleotides, we wanted to confirm these results by overexpressing a cytoplasmically truncated form of the Tsk7L receptor. This mutant receptor lacks its cytoplasmic kinase domain and most of the spacer domain that follows the transmembrane region and by analogy with the truncated type II receptor (Brand et al., 1993; Chen et al., 1993) is expected to exert a dominant negative inhibition on the signaling of the endogenous Tsk7L receptor. To allow specific immunoprecipitation of the truncated Tsk7L-DN receptor, an eight-amino acid FLAG epitope tag was added

to its C-terminal end. NMuMG cells were transfected with an expression plasmid for this truncated receptor and stable cell clones expressing Tsk7L-DN were established. The expression levels of Tsk7L-DN in the stable clones were first analyzed by Northern analysis. Two clones expressing high levels of Tsk7L-DN mRNA and one expressing this mRNA at low level, as well as one negative control clone were chosen for further analyses (Fig. 8 **A**). To verify the expression of Tsk7L-DN at the cell surface, the cell surface proteins were biotinylated and subsequently immunoprecipitated with an anti-FLAG antibody specific for Tsk7L-DN. The stable NMuMG clones expressing high levels of Tsk7L-DN mRNA showed a FLAG-immunoreactive band of 20–28 kD, which was also seen, albeit at lower levels, in the clone expressing a low level of Tsk7L-DN mRNA (Fig. 8 **B**). NMuMG cells transiently transfected with Tsk7L-DN were used as positive controls. They expressed a FLAG-immunoreactive protein of the same size as the stable clones, consistent with the predicted size of the FLAG-tagged truncated Tsk7L. As expected, there is some heterogeneity in size because of the glycosylated nature of the receptor (data not shown). Untransfected NMuMG cells occasionally showed a band in the same size range. However, its intensity was considerably weaker and it was therefore considered nonspecific.

The clones were also analyzed by immunohistochemical staining using the anti-FLAG monoclonal antibody. Clones expressing Tsk7L-DN mRNA and the corresponding FLAG-immunoreactive truncated receptor protein, also displayed a specific staining with anti-FLAG antibody at the cell surface, consistent with the membrane location of truncated Tsk7L. Untransfected NMuMG cells and neomycin-resistant control clones lacking Tsk7L-DN mRNA had a weak background staining in the cytoplasm (data not shown).

Cross-linking of the cell surface receptors using ^{125}I -TGF- β showed only a low level of ligand binding to the overexpressed truncated Tsk7L receptor in the transfected clones (data not shown). This was expected since ligand binding to the type I receptors depends on the coexpression of the type II receptor (Ebner et al., 1993a; Bassing et al., 1994; ten Dijke et al., 1994) and the expression level of the type II receptors is low in NMuMG cells (Ebner et al., 1993a). However, transient coexpression of a transfected type II receptor in these Tsk7L-DN clones resulted in a higher level of ^{125}I -TGF- β cross-linking to the truncated Tsk7L type I receptor (data not shown), which is in agreement with the required cooperativity for ligand binding.

The transfected NMuMG clones expressing the truncated Tsk7L type I receptor were then tested for their phenotypic responsiveness to TGF- β . In contrast to the control clone and the parental untransfected NMuMG cells which underwent an epithelial to fibroblastic phenotypic change in response to TGF- β , the clones expressing high levels of the truncated Tsk7L receptor did not alter their phenotype in response to TGF- β or showed only a mild responsiveness (Fig. 9). The transfected clone expressing a low level of Tsk7L-DN mRNA and protein showed an intermediate phenotypic change in response to TGF- β .

The stable clones expressing the Tsk7L-DN receptor were also analyzed for the effect of TGF- β treatment on several epithelial/mesenchymal markers using immunofluorescence. Although the cell morphology did not become fibroblastic in these transfected cells, TGF- β affected the expression of sev-

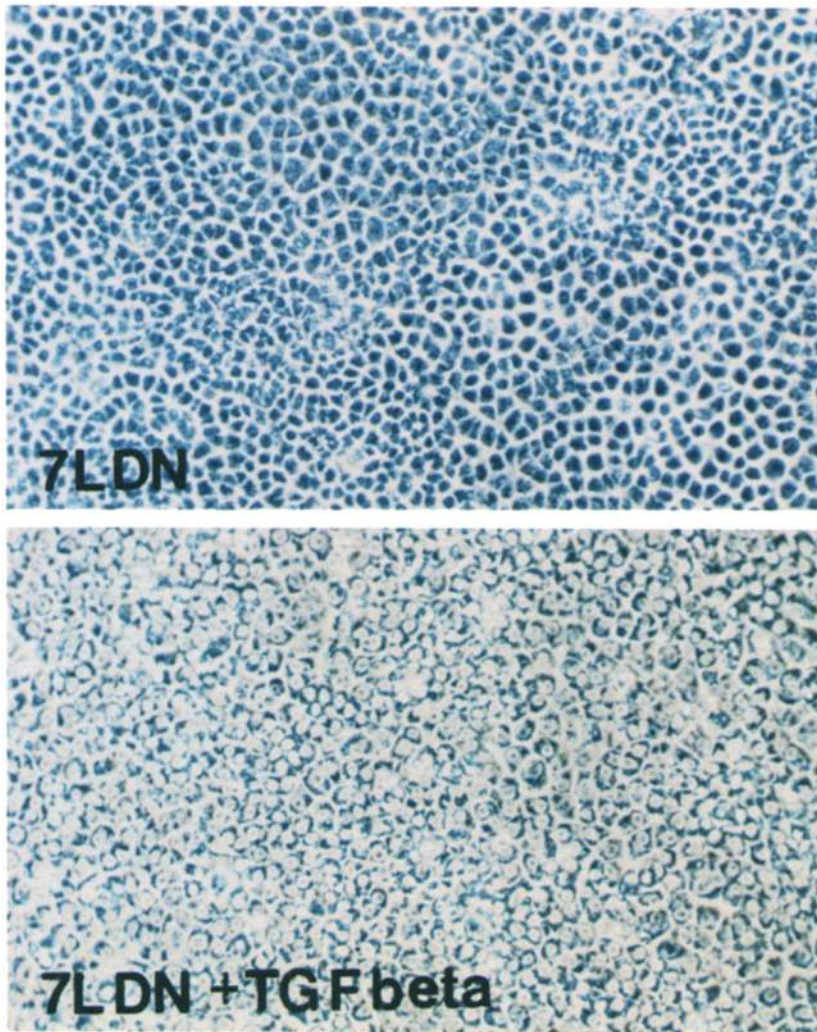


Figure 9. Dominant negative effect of expression of the Tsk7L-DN type I receptor on the TGF- β -induced epithelial to mesenchymal differentiation. When stably transfected Tsk7L-DN clones were treated with TGF- β (2 ng/ml) for 36 h, no fibroblastic differentiation was seen in contrast to the parental NMuMG cells (see Fig. 1).

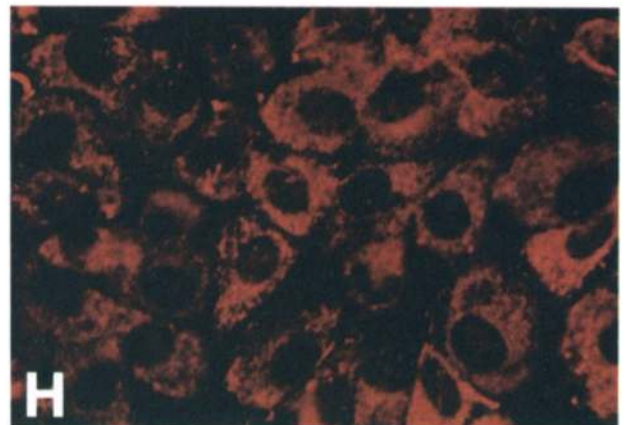
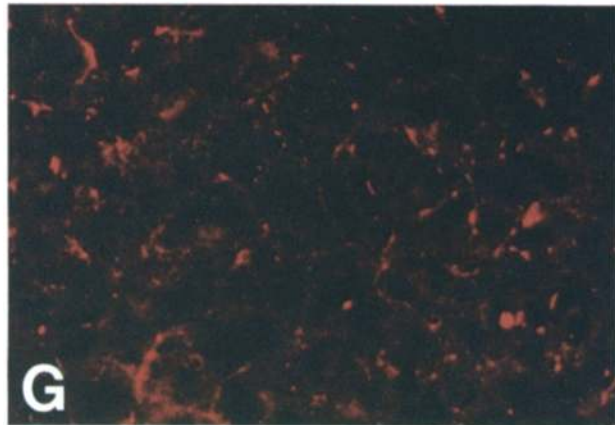
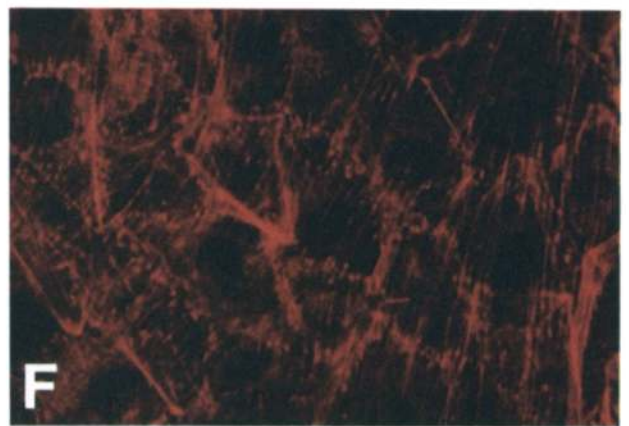
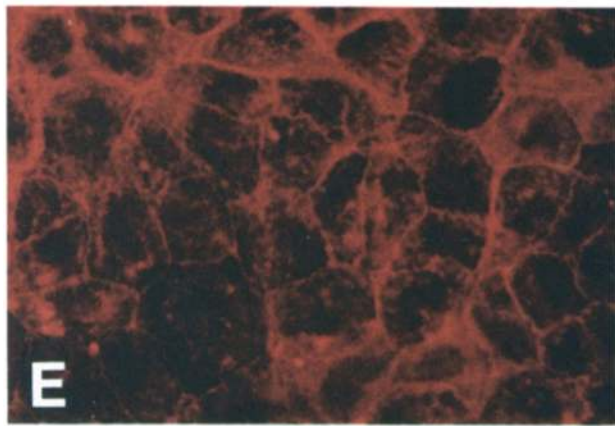
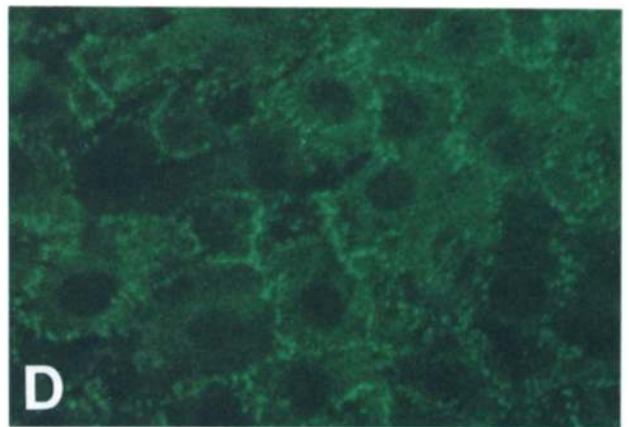
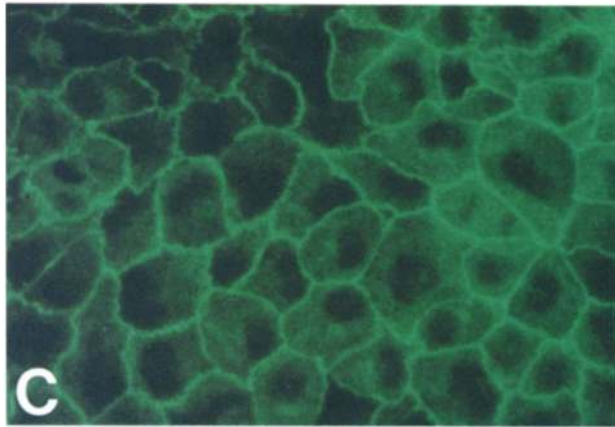
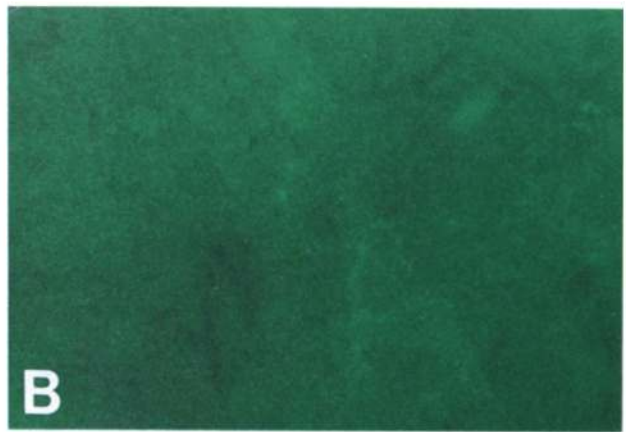
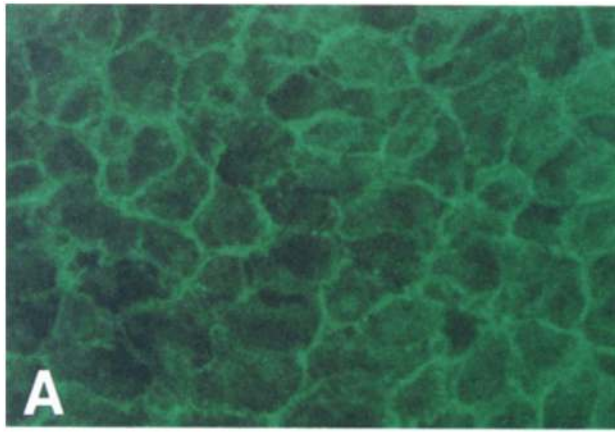
eral marker proteins (Fig. 10). In the absence of TGF- β , the expression patterns of the markers in the Tsk7L-DN expressing clones were very similar to the untransfected cells. Following TGF- β treatment, E-cadherin immunoreactivity at the cell boundaries disappeared in the Tsk7L-DN transfected cells, similarly to the change in the untransfected NMuMG cells (Fig. 10, *A* and *B*). Also, TGF- β induced a change in the localization of ZO-1 protein in the Tsk7L-DN-transfected cells, which resembled the pattern in H7-treated, untransfected NMuMG cells in the presence of TGF- β , i.e., ZO-1 was located in a punctated fashion at the cell boundaries (Fig. 10, *C* and *D*) contrary to the fine, continuous staining seen in nontreated cells. Furthermore, actin was distributed both cortically and as stress fibers in TGF- β -treated, Tsk7L-DN-expressing cells (Fig. 10, *E* and *F*) and vinculin was present as intensely staining dots in the cell membrane of these cells (data not shown). The vinculin immunolocalization was similar to TGF- β -treated wild-type NMuMG cells, although the intensity of the staining was more pronounced. Finally, immunoreactivity for fibronectin was increased in response to TGF- β and localized intracellularly (Fig. 10, *G* and *H*). Little fibronectin was detectable outside the cells in contrast to untransfected NMuMG cells. We also analyzed the expression levels of E-cadherin and fibronectin using Western analysis of cell lysates from untreated and TGF-

β -treated Tsk7L-DN clones. These results correlated with the immunohistochemical data, i.e., the level of E-cadherin was decreased and fibronectin expression was increased following exposure of the Tsk7L-DN clones to TGF- β . Also, Northern analyses showed that E-cadherin mRNA was decreased and fibronectin mRNA was increased in these cells after TGF- β treatment (data not shown).

Finally, we tested the effect of TGF- β on the proliferation rate of the transfected cells expressing the truncated Tsk7L type I receptor. [3 H]thymidine incorporation was measured to assess the inhibition of DNA synthesis by TGF- β . Our results showed that the transfected clones expressing the truncated Tsk7L receptor were growth inhibited by TGF- β , similar to the parental NMuMG cells (Fig. 11). The extent of TGF- β -induced growth inhibition was somewhat higher in the high level Tsk7L-DN-expressing clones than in the untransfected cells.

Discussion

In this study, we show that mouse mammary epithelial cells undergo a reversible transition to a mesenchymal phenotype upon TGF- β treatment. Besides the change in cell morphology, this transition is characterized by downregulation of the expression of the epithelial markers E-cadherin, ZO-1, and



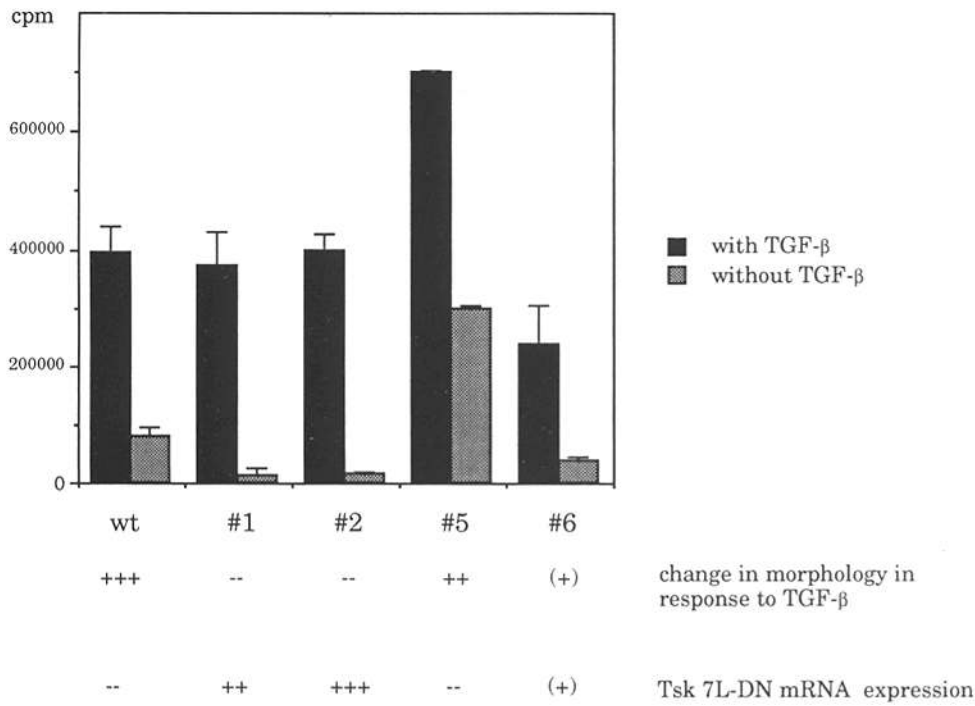


Figure 11. Proliferation and differentiation characteristics of Tsk7L-DN clones. Stably transfected clones expressing Tsk7L-DN mRNA and protein (Fig. 8, A and B) were analyzed for their growth response to TGF- β treatment in serum-free medium. Their rate of DNA synthesis was assayed by measuring uptake of [3 H]-thymidine at 21 h after initiation of TGF- β treatment. Independent of their ability to undergo the TGF- β -induced epithelial-mesenchymal transdifferentiation, all Tsk7L-DN clones were growth inhibited by TGF- β .

desmoplakins I and II. In addition, there is an increased expression of the mesenchymal marker fibronectin and a reorganization of actin stress fibers and vimentin from an epithelial to a fibroblastic pattern. Since the cellular phenotype before and after TGF- β treatment is clearly distinguishable and the resulting fibroblast-like cells originate from the epithelial cells, we conclude that this TGF- β -induced process fulfills the criteria of transdifferentiation (Eguchi and Kodama, 1993). An elongation of human mammary epithelial cells in the presence of TGF- β has previously been noticed, but no further characterization was carried out (Hosobuchi and Stampfer, 1989). Several other growth factors have earlier been shown to induce an epithelial to fibroblastic differentiation. TGF- α and acidic FGF induce a fibroblastic differentiation of bladder epithelial carcinoma cells, as based on their change in morphology and the differential expression of several of the same markers as used in our current study (Gavrilovic et al., 1990; Valles et al., 1990). Furthermore, overexpression of scatter factor/HGF and its tyrosine kinase receptor, c-met, is able to induce a transition from fibroblastic to an epithelial phenotype in 3T3 fibroblast-derived tumor cells (Tsarfaty et al., 1994). All these factors are mitogenic growth factors and mediate their effects following binding to transmembrane tyrosine kinase receptors, in contrast to TGF- β , which signals through serine/threonine kinase receptors and is growth inhibitory. The current demonstration that TGF- β induces an epithelial to mes-

enchymal transdifferentiation is not only restricted to the mammary epithelial cell line tested, but was also apparent in other epithelial cell lines.

Several epithelial cell types can be induced to transform to mesenchymal cells by extracellular matrix e.g., retinal pigmented epithelial cells (Eguchi and Okada, 1973; Greenburg and Hay, 1986), notochordal epithelial cells and thyroid epithelial cells (Greenburg and Hay, 1988). Epithelial to mesenchymal conversion of defined cell types also takes place during development. As an example, embryonic ectodermal cells of the posterior epiblast differentiate into mesenchymal cells which subsequently migrate away from their original site (Burdsal et al., 1993). How such transitions are induced in vivo is as yet unclear, but it is conceivable that localized extracellular matrix proteins and growth factors are involved. Our current findings emphasize the possibility that TGF- β may be involved in epithelial to mesenchymal transdifferentiation during development and organ morphogenesis. In addition, TGF- β is capable to transdifferentiate endothelial cells of atrioventricular canal (Potts and Runyan, 1989) and fat-storing cells of liver to mesenchymal-like cells (Bachem et al., 1993). The ability of TGF- β to induce mesenchymal differentiation of epithelial cells may have a considerable importance during development and organ morphogenesis. Specifically, with respect to mammary epithelial cells, TGF- β has the ability to inhibit ductal branching morphogenesis (Silberstein and Daniel, 1987).

Figure 10. Effect of TGF- β treatment on E-cadherin, ZO-1, actin and fibronectin expression in Tsk7L-DN clones. Cells were grown on coverslips without (A, C, E, and G) or with TGF- β 1 (B, D, F, and H). After fixation and permeabilization, they were stained with antibodies to E-cadherin (A and B), ZO-1 (C and D), actin (E and F), and fibronectin (G and H). In the absence of TGF- β , E-cadherin, ZO-1, and actin were located cortically as a continuous line, similar to wild-type NMuMG cells (Figs. 3 and 4). However, TGF- β treatment resulted in a disappearance of E-cadherin immunoreactivity (B) and a decreased ZO-1 staining (D), even though the cell morphology remained epithelial. Also, actin was again reorganized into stress fibers resembling fibroblasts (F). Fibronectin expression was increased in the presence of TGF- β , but was mainly localized intracellularly instead of extracellularly (H).

This was also recently illustrated in transgenic mice in which directed overexpression of active TGF- β 1 in the mammary gland inhibited duct formation (Pierce et al., 1993). The inhibition of branching morphogenesis by TGF- β together with its ability to regulate extracellular matrix deposition and the currently described activity as a reversible inducer of epithelial to mesenchymal transdifferentiation imply a complex role for TGF- β as a physiological regulator of mammary gland function.

The mechanistic basis for the TGF- β -induced epithelial to mesenchymal transdifferentiation of NMuMG cells is unclear. Considering the postulated role of E-cadherin in the maintenance of the epithelial phenotype (Nagafuchi et al., 1987; Matsuzaki et al., 1990), it is conceivable that downregulation of its expression and localization at the cell boundaries may play an important role in the transdifferentiation phenomenon both during development and carcinogenesis. This possibility is substantiated by the observation that neutralizing anti-E-cadherin antibodies induce a change in epiblast cells from an epithelial to mesenchymal morphology, strongly suggesting that disruption of E-cadherin function results in their acquisition of phenotypic characteristics of mesoderm (Burdal et al., 1993). Furthermore, loss of E-cadherin expression can also lead to dedifferentiation and invasiveness of carcinoma cells, and overexpression of E-cadherin in poorly differentiated carcinoma cell lines inhibits invasiveness (Behrens et al., 1989; Frixen et al., 1991; Sommers et al., 1992). However, the decreased E-cadherin expression in the TGF- β -treated NMuMG cells may not be the driving factor that induces complete transdifferentiation since the TGF- β -treated, Tsk7L-DN-expressing NMuMG cells look epithelial, yet have decreased E-cadherin expression. Finally, our results also illustrate the ability of TGF- β to inhibit E-cadherin expression. No growth and differentiation factors have previously been shown to modulate E-cadherin expression. Thus, TGF- β may play a role in the regulation of E-cadherin synthesis *in vivo* and could thus have a functional significance for the downregulation of E-cadherin expression during normal and tumor development.

Little is known about the TGF- β receptor-mediated signaling events following ligand binding (Derynck, 1994b). TGF- β mediates its activities through the type I and II receptors, which are both transmembrane serine/threonine kinases. At least several of the TGF- β responses, e.g., induction of c-jun, fibronectin, and plasminogen activator inhibitor 1 as well as the antiproliferative effect of TGF- β , are inhibited by the protein kinase inhibitor H7 (Ohtsuki and Massagué, 1992). We now show that the TGF- β -induced transdifferentiation of NMuMG cells can be inhibited by H7 and staurosporine, but not by HA1004, suggesting an involvement of a protein kinase C. This would be consistent with the observed translocation of protein kinase C to the cell membrane in astrocytes in the presence of TGF- β (Robertson et al., 1988). However, bisindolylmaleimide and calphostin C, which inhibit the protein kinase isoforms PKC- α , - β I, - β II, and - γ , failed to inhibit transdifferentiation, and the phorbol ester TPA, which activates several PKC isoforms, did not induce transdifferentiation. This suggests that other PKC isoforms, such as the TPA-resistant PKC- ζ or - λ might be involved or that, even though required, activation of PKC alone is not sufficient for the induction of trans-

differentiation. The increased intracellular immunoreactivity for fibronectin in H7-treated cells and in the Tsk7L-DN clones following TGF- β treatment is in agreement with previous findings showing that fibronectin matrix assembly is modulated by PKC-mediated phosphorylation (Somers and Mosher, 1993). Thus, TGF- β -mediated signaling could directly modulate the cytoskeletal organization. It is obviously also possible that the inhibitors we have used directly block the kinase activities of the type I or type II TGF- β receptors. However, this is unlikely since *in vitro* kinase assays indicate that Tsk7L type I receptor and type II receptor autophosphorylation are not inhibited by H7 (Lawler, S., R. M. Maruoka, and R. Derynck, unpublished results). Taken together, our results using protein kinase inhibitors indicate the involvement of at least one kinase activity downstream from the TGF- β receptors and raise the possibility of an involvement of a protein kinase C in the TGF- β -signaling pathway.

As shown in this report, NMuMG cells undergo an epithelial to mesenchymal transdifferentiation upon exposure to TGF- β , but not activin. Because these cells express a high level of Tsk7L type I receptors, but not ALK-5/R4/ESK-2, we evaluated the role of Tsk7L in the transdifferentiation. Using antisense oligonucleotides specific for Tsk7L and by overexpressing a truncated kinase-negative form of Tsk7L that functions as a dominant negative mutant, we were able to abolish this TGF- β -mediated change in cell morphology. These results strongly suggest that the type I receptor Tsk7L may be important for the transdifferentiation process. By far the most likely explanation is that Tsk7L functions as a TGF- β receptor that mediates this response, a possibility reinforced by the binding of TGF- β to both the full-size and truncated Tsk7L (Ebner et al., 1993a,b; data not shown). It cannot, however, be formally excluded that TGF- β induces the synthesis of another TGF- β -related factor which exerts its transdifferentiating activity through Tsk7L.

Although the Tsk7L-DN stable clones did not undergo fibroblastic transdifferentiation, they were still growth inhibited by TGF- β and showed an increased fibronectin synthesis and decreased E-cadherin and ZO-1 immunostaining when exposed to TGF- β . This difference in inhibition of the responses can as yet not be fully explained based on the current limited data on receptor activation, especially considering the multiplicity of type I receptors. The type II receptor has clearly been demonstrated necessary for the antiproliferative effect of TGF- β in epithelial cells (Chen et al., 1993), and is thought to cooperate with a kinase-active type I receptor for this activity (Wieser et al., 1993). However, the fact that growth inhibition and upregulation of fibronectin synthesis by TGF- β are maintained in the Tsk7L-DN clones, would argue that either functional Tsk7L receptors are not required for these activities or that another type I receptor mediates these responses to TGF- β . Other type I receptors, such as TSR-1, that can bind TGF- β in the presence of the type II receptor could be involved in these responses in NMuMG cells. Furthermore, TGF- β -mediated gene activation in NMuMG cells could also happen independently of growth inhibition as shown in other cell lines (Zentella et al., 1991; Geiser et al., 1992; Fafeur et al., 1993; Stampfer et al., 1993). Alternatively, the dominant negative inhibition may not be complete allowing a residual level of Tsk7L receptor function, or the truncated Tsk7L could nonspecific-

cally inhibit an otherwise functional receptor complex. However, the fact that Tsk7L-DN selectively abolishes only one of the TGF- β -mediated pathways, i.e., the transdifferentiation, argues against these possibilities. Obviously, extensive characterization of the signaling activities and the physiological interactions between the type II and the different type I receptors will be required before we will be able to understand how the TGF- β responsiveness and the diversity of activities induced by TGF- β is determined for a particular cell line and cell type. Nonetheless, our data support a role for the Tsk7L type I receptor in the TGF- β -mediated transdifferentiation of mammary epithelial cells.

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