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TGF-β type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells

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SUMMARY

The capacities of different transforming growth factor-β (TGF-β) superfamily members to drive epithelial to mesenchymal transdifferentiation of the murine mammary epithelial cell line NMuMG were investigated. TGF-β1, but not activin A or osteogenic protein-1 (OP-1)/bone morphogenetic protein-7 (BMP-7), was able to induce morphological transformation of NMuMG cells as shown by reorganisation of the actin cytoskeleton and relocalisation/downregulation of E-cadherin and β-catenin, an effect that was abrogated by the more general serine/threonine kinase and protein kinase C inhibitor, staurosporine. TGF-β1 bound to TGF-β type I receptor (TβR-I)/ALK-5 and TβR-II, but not to activin type I receptor (ActR-I)/ALK-2. Activin A bound to ActR-IB/ALK-4 and ActR-II, and BMP-7 bound to ActR-I/ALK-2, BMP type I receptor (BMPR-I)/ALK-3, ActR-II and BMPR-II. TGF-β1 and BMP-7 activated the Smad-binding element $(SBE)_4$ promoter with equal potency, whereas activin A had no effect. Transfection of constitutively active (CA)-ALK-4 activated the 3TP promoter to the same extent as TGF-β1 and CA-ALK-5 indicating that activin signalling downstream of type I receptors was functional in NMuMG cells. In agreement with this, activin A induced low levels of plasminogen activator inhibitor I expression compared to the high induction by TGF-β1. In contrast to activin A and BMP-7, TGF-β1 strongly induced Smad2 phosphorylation. Consistent with these findings, TGF-β1 induced the nuclear accumulation of Smad2 and/or Smad3. In addition, NMuMG cells transiently infected with adenoviral vectors expressing high level CA-ALK-5 exhibited full transdifferentiation. On the other hand, infections with low level CA-ALK-5, which alone did not result in transdifferentiation, together with Smad2 and Smad4, or with Smad3 and Smad4 led to transdifferentiation. In conclusion, TGF-β1 signals potently and passes the activation threshold to evoke NMuMG cell transdifferentiation. The TGF-β type I receptor (ALK-5) and its effector Smad proteins mediate the epithelial to mesenchymal transition. Activin A does not induce mesenchymal transformation, presumably because the number of activin receptors is limited, while BMP-7-initiated signalling cannot mediate transdifferentiation.

Key words: Actin cytoskeleton, Phenotypic transformation, SMAD, TGF-β receptor, Transdifferentiation

INTRODUCTION

Transforming growth factor (TGF)-β, which is the prototypic member of the TGF-β superfamily, exerts a broad range of biological activities. It has an antiproliferative effect primarily on cells of epithelial origin, whereas it promotes growth of mesenchymal cell types. It plays pivotal roles during embryonic development where it is involved in patterning the embryo and inducing cell differentiation and organogenesis (reviewed by Roberts and Sporn, 1990; Whitman, 1998). Several members of the TGF-β superfamily can modulate mesenchymal cell fates, including development of myoblasts, chondrocytes, or osteoblasts (Roberts and Sporn, 1990; Kingsley, 1994). Furthermore, TGF-β superfamily members might be involved in epithelial-mesenchymal differentiation processes such as nephrogenesis (Rogers et al., 1993), branching morphogenesis of the lungs and mammary glands (Silberstein and Daniel, 1987; Heine et al., 1990; Robinson et al., 1991; Hogan and Yingling, 1998), and budding morphogenesis of limbs, feathers and genitalia (Hogan, 1999). Processes that are beneficial and required for proper embryonic development frequently form the basis for pathological conditions in adults. Improper TGF-β signalling is often correlated with pathogenesis, like tumourigenesis (reviewed by Hata et al., 1998). Although TGF-β acts as a tumor suppressor during early benign stages of skin tumourigenesis, it potently aggravates the malignancy at later stages. This is accompanied by an epithelial to mesenchymal cell transition, which occurs during transformation of squamous carcinoma to invasive spindle cell carcinoma (Cui et al., 1996; Portella et al., 1998).

Members of the TGF-β superfamily, including TGF-βs, activins and bone morphogenetic proteins (BMPs), mediate their pleiotropic effects by signalling through transmembrane serine/threonine kinase type I and type II receptors. Upon ligand-induced heteromeric complex formation between type II receptors and type I receptors, type I receptors become phosphorylated by the type II receptors (Wrana et al., 1994) and activate downstream signalling components among which Smad proteins play a pivotal role (reviewed by Heldin et al., 1997; Massagué, 1998). The Smad family can be subdivided into (i) receptor-activated Smads (R-Smads) which include Smad2 and Smad3 for TGF-β and activin signalling, and Smad1, Smad5 and Smad8 for BMP signalling, (ii) common-mediator Smads (Co-Smads) like Smad4, and (iii) inhibitory Smads (Anti-Smads), including Smad6 and Smad7 (reviewed by Heldin et al., 1997; Massagué, 1998). R-Smads contain a conserved SSXS phosphorylation motif in their very C-termini, of which the last two serine residues become phosphorylated following interaction with activated type I receptors. Phosphorylation of R-Smads results in their heteromerisation with Smad4 followed by nuclear translocation and regulation of gene transcription in association with other transcription factors (reviewed by Heldin et al., 1997; Massagué, 1998).

The mammary epithelial cell line NMuMG has been shown to undergo mesenchymal transdifferentiation following TGF-β stimulation (Miettinen et al., 1994). Here, we investigated whether other TGF-β family members, including activin A and BMP-7, share this property with TGF-β, and we further addressed signalling by these polypeptide factors in NMuMG cells at the molecular level. Activin A failed to induce morphological transformation, presumably due to low activin receptor numbers. BMP-7 did not induce transdifferentiation but induced a transcriptional response in these cells. We observed that TGF-β1-induced transdifferentiation correlated with Smad2 phosphorylation, Smad2 and/or Smad3 nuclear translocation and specific Smad-sensitive transcriptional activation. In addition, overexpression of a constitutively active TGF-β type I receptor (ALK-5), by means of adenoviral infection, resulted in transdifferentiation in a dose-dependent manner. Using the same system, we show that low dose of CA-ALK-5 receptor that cannot lead to transdifferentiation on its own, when supplied in combination with either Smad2 plus Smad4 or with Smad3 plus Smad4 can cause transdifferentiation. Thus, TGF-β1-mediated transdifferentiation of NMuMG cells is mediated through activation of ALK-5 and its downstream Smad effector proteins.

MATERIALS AND METHODS

Reagents and constructs

TGF-β1 was obtained from Dr N. Ferrara at Genentech (San Francisco, CA, USA), activin A was a gift from Dr Y. Eto at Ajinomoto Company (Kawasaki, Japan), osteogenic protein 1 (OP-1)/bone morphogenetic protein 7 (BMP-7), BMP-2, cartilage-derived morphogenetic protein-1 (CDMP-1) and CDMP-2 were provided by Dr K. Sampath at Creative Biomolecules (Hopkinton, MA, USA). The p3TP-Lux promoter-reporter construct containing three sets of tetradecanoyl phorbol acetate-responsive elements together with part of the human plasminogen activator inhibitor (PAI)-1 promoter was obtained from J. Massagué (Memorial Sloan-Kettering Cancer Center, NY, USA). The $(SBE)_4$-Lux reporter which contains 4 repeats of the CAGACA sequence identified as a Smad-binding element in the JunB promoter (Jonk et al., 1998) was obtained from Dr S. Itoh (Ludwig Institute for Cancer Research, Uppsala, Sweden). Constitutively active (CA) type I receptor constructs (CA-ALK-1, CA-ALK-2, CA-ALK-3, CA-ALK-4, CA-ALK-5, CA-ALK-6) were previously described by Nakao et al. (1997). The adenoviral vectors encoding β-galactosidase, C-terminally haemaglutinin (HA) epitope tagged CA-ALK-5, and N-terminally Flag-tagged Smad2, Smad3 and Smad4 were provided by Dr K. Miyazono (Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan) and are described by Fujii et al. (1999).

Cell culture

Mouse NMuMG breast epithelial tumour cells, obtained from American Type Culture Collection (Manassas, VA, USA), were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 10 μg/ml insulin, 100 units/ml penicillin and 50 μg/ml streptomycin. Cells were grown in a 5% CO_2-atmosphere at 37°C.

Polyclonal and monoclonal antibodies

Preparation of antisera against ALK-1 (RRQ), ALK-2 (RRN), ALK-3 (KSI), ALK-4 (RVY), ALK-5 (VPN), ALK-6 (DET), ActR-II (ARC), ActR-IIB (RKP), BMPR-II (SMN and NRR), TβR-II (DRL), Smad2 (SED), and phosphorylated Smad2 (PS2) have been described previously (Franzén et al., 1993; ten Dijke et al., 1994; Rosenzweig et al., 1995; Nakao et al., 1997; Persson et al., 1998; Piek et al., 1999). Mouse monoclonal anti-E-cadherin and anti-β-catenin antibodies were purchased from Transduction Laboratories, Lexington, KY, USA. Two monoclonal antibodies (mAb), one which recognises specifically Smad1 (mAb A-4, sc-7965) and one that recognises primarily Smad3 but also Smad2 (mAb H-2, sc-7960) were a generous gift from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies against the haemaglutinin and Flag epitopes were purchased from Boehringer Mannheim (Mannheim, Germany) and Sigma (St Louis, MO, USA) respectively.

Transdifferentiation of NMuMG cells and fluorescence microscopy of the actin cytoskeleton, E-cadherin, β-catenin and Smad proteins

NMuMG cells were seeded at a density of 2×10^4 cells/cm^2 on 22 × 22 mm glass coverslips coated with 0.1% gelatin. The next day, cells were stimulated with activin A, BMP-7, or TGF-β1 in the presence of 10% FBS. Inhibition of TGF-β1-mediated transdifferentiation of NMuMG cells was tested with the more general serine/threonine kinase and protein kinase C (PKC) inhibitor staurosporine (Sigma, St Louis, MO, USA), the PKC-α, -β1, -β2, -γ isoform-specific inhibitor bisindolyl-maleimide I (Calbiochem-Novabiochem Corp., La Jolla, CA, USA), the MEK inhibitor PD98059 (Biomol, Research Laboratories, Inc., Plymouth Meeting, PA, USA), the specific PI3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002; Biomol, Plymouth Meeting, PA, USA), the PI3-kinase inhibitor, wortmannin (Calbiochem-Novabiochem Corp. La Jolla, CA, USA) or the Src-specific inhibitor PP1 (Calbiochem-Novabiochem Corp., La Jolla, CA, USA).

After 36 hours of stimulation, cells were processed for direct fluorescence of the actin cytoskeleton with 0.25 μM tetramethylrhodamine B isothiocyanate (TRITC)-conjugated-phalloidin (Sigma, St Louis, MO, USA) as previously described (Moustakas and Stournaras, 1999). For indirect immunofluorescence, specimens were incubated with a 200-fold dilution of mouse monoclonal anti-E-cadherin or anti-β-catenin antibody, 1,000-fold dilution of mouse monoclonal anti-Smad1 or anti-Smad2/3 antibodies, and 500-fold dilution of mouse monoclonal anti-haemaglutinin (HA) or anti-Flag antibodies (for detection of adenovirally encoded proteins) followed by incubation with a 100-fold diluted TRITC-conjugated or fluorescein isothiocyanate (FITC)-

conjugated goat anti-mouse IgG antibodies (Dako-PattsA/S, Glostrup, Denmark) as previously described (Moustakas and Stournaras, 1999). In double staining experiments, indirect immunofluorescence preceded the direct fluorescent phalloidin staining. All specimens were mounted on glass slides with fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and were observed on an Olympus Vanox-T model AHBT microscope equipped with an AH2-RFL epifluorescent illumination unit. Photomicrographs were obtained with a 35 mm Olympus (C-35AD-4) camera on Kodak P400 black and white film.

Receptor affinity cross-linking studies

Activin A, BMP-7, and TGF-β1 were iodinated to comparable specific activity by the chloramine-T method as described by Frolik et al. (1984). Affinity binding and cross-linking of iodinated growth factors to receptors on NMuMG cells, followed by immunoprecipitation with receptor-specific antisera, was performed as previously described (Piek et al., 1997).

Transient transfection studies

NMuMG cells were seeded at a density of 2×10^4 cells/cm^2 in 6-well tissue culture plates. The next day transient transfections were performed using the Fugene6 transfection reagent, following the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany). Cells were transfected with 1 μg 3TP-Lux reporter construct or 0.5 μg $(SBE)_4$-Lux reporter construct. For ligand-induced reporter assays, 1 μg pcDNA3 was added, while for analysis of reporter activity induced by CA type I receptors, 1 μg plasmid carrying the respective receptor cDNAs was co-transfected. After 24 hours, cells were stimulated for 20 hours with activin A, BMP-7 and TGF-β1. In all transfections the β-galactosidase expression plasmid pCH110 (Pharmacia, Uppsala, Sweden) served as an internal control to correct for transfection efficiency. β-Galactosidase activity was measured in 100 mM Na_2HPO_4/NaH_2PO_4 (pH 7.3), 1 mM $MgCl_2$, 100 mM 2-mercaptoethanol and 0.67 mg/ml *o*-nitrophenyl-galactopyranoside (Sigma, St Louis, MO, USA). The data are presented as bar-graphs with average values and standard errors derived from triplicate measurements from at least two independent experiments.

Transient adenoviral infection studies

Adenoviral stocks were maintained and titred in 293T cells as described by Fujii et al. (1999) and their titre ranged between 2.5 and 9×10^8 plaque forming units (pfu) per millilitre. Establishment of optimal infection conditions of NMuMG cells was performed using a β-galactosidase-encoding virus and staining of fixed cell monolayers with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as described by Fujii et al. (1999). Under optimal conditions, more than 90% of the cells were infected as determined by the blue, β-galactosidase-positive staining. Routine infections were performed at a multiplicity of infection (MOI) of 100 with single viruses. This corresponds to 1×10^2 pfu per cell. Titration experiments used MOIs from 5 to 500 and in multiple vector co-infections the described MOI of the individual virus was used but the total viral MOI was always kept at 150 maximum.

NMuMG cells were seeded at a density of 5×10^4 cells/cm^2 in 24-well tissue culture plates (light microscopy and X-Gal staining assays) or at a density of 5×10^5 cells/cm^2 on glass coverslips positioned in 6-well plates (direct fluorescence and indirect immunofluorescence assays). The next day the culture medium was changed to DMEM containing 5% FBS 1 hour prior to the infection. Cells were infected at the appropriate MOI for 12 hours and then washed and fed fresh 5% FBS-DMEM. Cells were observed every 8 hours and finally assayed 48-60 hours post-infection.

PAI-1 assays

NMuMG cells, grown to 90% confluence, were rinsed in PBS and medium was changed to methionine- and cysteine-free MCDB 104 (SVA, Uppsala, Sweden) containing 0.1% bovine serum albumin (BSA). Subsequently, activin A, BMP-7 or TGF-β1 were added for two hours, followed by labelling for two hours with 1 μCi/ml [^{35}S]methionine/[^{35}S]cysteine (Amersham, Buckinghamshire, UK). Thereafter, PAI-1 production was assayed as previously described (Yamashita et al., 1995).

Endogenous E-cadherin, β-catenin and Smad2/3 and adenovirally encoded receptor and Smad detection by western blotting

Normal NMuMG cells or adenovirus-infected cells were grown to 90% confluence. Cells were rinsed in PBS and serum-starved for three hours in DMEM containing 0.1% BSA, followed by addition of 2 nM activin A, 5.7 nM BMP-7, or 400 pM TGF-β1. After stimulation for 0, 60 minutes or 36 hours cells were put on ice, rinsed with PBS and lysed in solubilisation buffer (125 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, 1.5% Trasylol, and 1% Triton X-100) for 40 minutes. Cell lysates were quantified by Bradford analysis for protein content and extracts representing equal total protein amounts were separated by sodium dodecyl sulfate (SDS)-gel electrophoresis using an 8.5% (Smad2 phosphorylation analysis) or a 7% (all other experiments) polyacrylamide gel, followed by wet-transfer of the proteins to Hybond-C extra nitrocellulose membranes (Amersham, Buckinghamshire, UK). Non-specific binding of proteins to the membranes was blocked in TBS-T buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween 20) containing 3% BSA. The primary Smad antibodies were diluted 1,000-fold, the E-cadherin antibody was diluted 500-fold, the β-catenin antibody was diluted 1,000-fold, the anti-HA antibody 300-fold and the anti-Flag antibody 500-fold in TBS-T and secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amersham, Buckinghamshire, UK) was used at a 10,000-fold dilution in TBS-T. Detection was performed by enhanced chemiluminescence (ECL).

RESULTS

TGF-β1, but not activin A or BMP-7, induces morphological transdifferentiation of NMuMG cells

The potencies of different members of the TGF-β superfamily to induce phenotypic transformation of the epithelial breast tumour cell line NMuMG was investigated. Staining of the actin cytoskeleton with TRITC-phalloidin revealed the characteristic epithelial architecture in control cuboidal cells with strong cortical and diffuse or punctate cytoplasmic actin staining (Fig. 1A). Treatment with TGF-β1 clearly resulted in transdifferentiation from the epithelial to a fibroblastic morphology, which was also evident by phase contrast microscopy (data not shown). TRITC-phalloidin fluorescence showed that the epithelial architecture was completely shifted into a prominent fibroblastic organisation of the actin cytoskeleton, with distinct stress fibres emanating from well-formed focal adhesions (Fig. 1B). The fibroblastic cells were usually arranged in parallel arrays, in line with the arrangement of their stress fibres. In agreement with the observations by Miettinen et al. (1994), transdifferentiation was observed from 16 hours after stimulation with concentrations as low as 20 pM TGF-β1, and this process could be fully reversed when TGF-β was effectively removed from the culture medium either by co-incubation with a neutralising antibody against TGF-β or by exposure of cells to serum-free medium (data not shown). Previous studies (Miettinen et al., 1994) showed that TGF-β2 also induced phenotypic transformation of NMuMG cells,

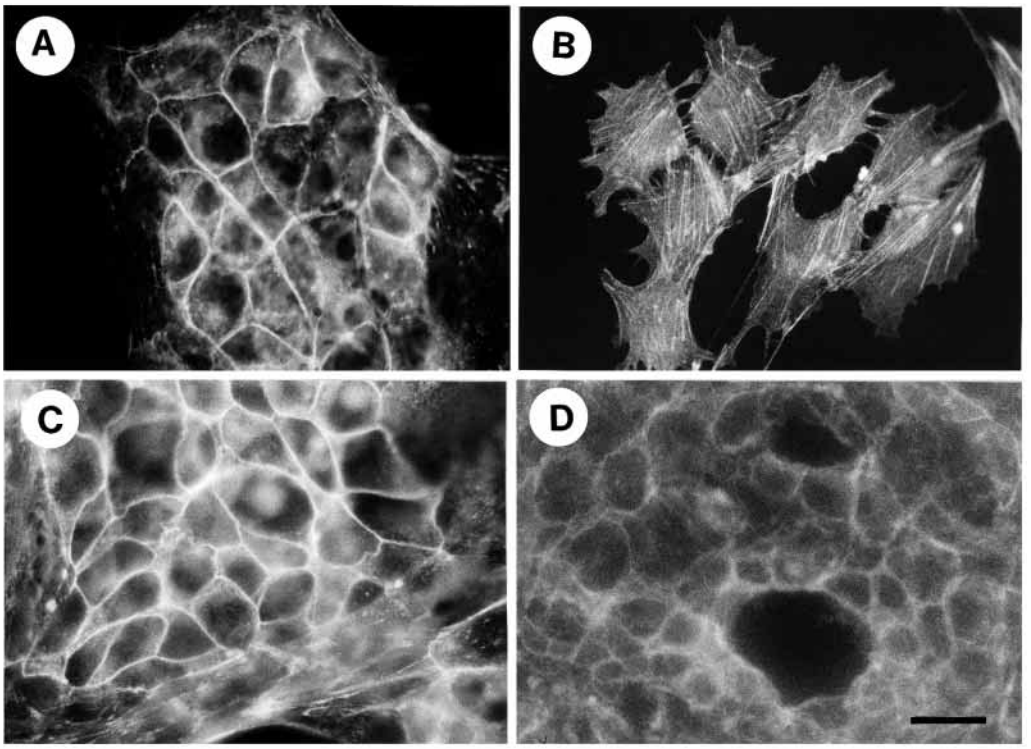

Fig. 1. Organisation of the actin cytoskeleton in normal and transdifferentiated NMuMG cells. Cells were stimulated for 36 hours with solvent or with specific ligand, followed by direct staining of the actin cytoskeleton using TRITC-phalloidin as described in Materials and Methods. Control cells (A), 100 pM TGF-β1-treated cells (B), 20 nM activin A-treated cells (C), 28.5 nM BMP-7-treated cells (D). Bar, 10 μm.

while 800 pM activin A was unable to do so. In the present study, a panel of TGF-β superfamily members was tested. In contrast to TGF-β1, none of the other members tested in excessive amounts, including 20 nM activin A, 5.7 nM BMP-2, 28.5 nM BMP-7, 5.7 nM cartilage-derived morphogenetic protein-1 (CDMP-1) or 5.7 nM CDMP-2, induced transdifferentiation of NMuMG cells (Fig. 1C,D, and data not shown). Thus, only TGF-β is able to induce transdifferentiation of NMuMG cells as illustrated by reorganisation of the actin cytoskeleton.

Downregulation and relocalisation of E-cadherin and β-catenin accompany TGF-β1-mediated transdifferentiation of NMuMG cells

E-cadherin was used as an additional marker to follow epithelial cell differentiation. Control epithelial cells exhibited the characteristic cuboidal E-cadherin staining, which was fully overlapping with the actin staining (compare Fig. 2A with Fig. 1A). This is in agreement with the well-established association of E-cadherin with the cortical actin cytoskeleton. In the transdifferentiated cells, E-cadherin staining was decreased and localised in areas of cell-cell adhesion between fibroblastic cells, whereas it was completely absent from areas of the plasma membrane that made no contacts with adjacent cells (Fig. 2B, note that exposure time for this panel is 10-fold longer than in 2A). We did not observe co-localisation of E-cadherin with the actin stress fibres in the fibroblast-like cells, but weak E-cadherin staining was observed in some, but not all, focal adhesions (compare Fig. 2B with Fig. 1B). The significant downregulation of the steady state E-cadherin levels in the transdifferentiated cells was verified by western analysis of total detergent soluble cell extracts (Fig. 2C). Staining of the cells for β-catenin showed an overlapping distribution of this protein with E-cadherin in the epithelial state as expected (Fig. 2D). In contrast, in the transdifferentiated fibroblastic state, β-catenin levels decreased but not as dramatically as for E-cadherin (Fig. 2E and 2F) while β-catenin also relocated to those cell-cell contact areas between neighbouring fibroblasts while exhibiting a finger-like pattern on the cell membrane (Fig. 2E). Thus, transdifferentiation of NMuMG cells is characterised by the stress-fibre architecture of the actin cytoskeleton, and by relocation and reduced expression of E-cadherin and β-catenin.

Effects of protein kinase inhibitors on morphological transdifferentiation induced by TGF-β1 in NMuMG cells

To explore the possible signalling cascades involved in TGF-β1-mediated epithelial to mesenchymal transdifferentiation of NMuMG cells, a series of protein kinase and phospholipid kinase inhibitors were used and morphological analysis of the cells by actin staining followed (Fig. 3A-J). In addition to the more general serine/threonine kinase and protein kinase C inhibitor staurosporine and the protein kinase C α, β1, β2, γ isoform-inhibitor bisindolyl-maleimide I, which were previously analysed (Miettinen et al., 1994), we also tested the MEK1 inhibitor PD98059, the PI-3 kinase inhibitors LY294002 and wortmannin, and the Src family tyrosine kinase inhibitor PP1. However, with the exception of staurosporine and bisindolyl-maleimide I, none of the inhibitors exhibited a major effect on cell morphology and actin architecture of control or 200 pM TGF-β1-stimulated NMuMG cells (Fig. 3A-J and data for wortmannin not shown). Interestingly, bisindolyl-maleimide I did not interfere with morphological transdifferentiation, but it had a reproducible effect on the actin cytoskeleton of the fibroblastic cells as stress fibres appeared extremely thin or punctate, while strong actin staining was evident in the perinuclear space in a strongly punctate or

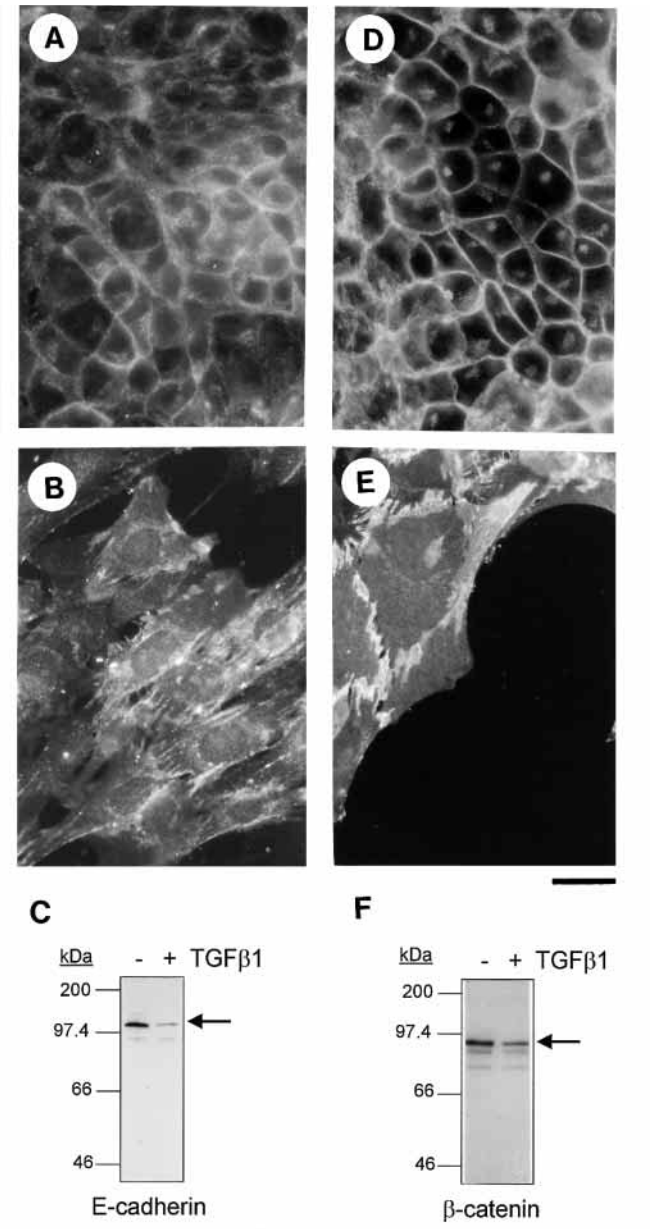

Fig. 2. Localisation and expression of E-cadherin and β-catenin in normal and transdifferentiated NMuMG cells. Cells were stimulated for 36 hours with solvent or TGF-β1, followed by indirect immunofluorescence against E-cadherin or β-catenin as described in Materials and Methods. Control cells (A and D), 100 pM TGF-β1-treated cells (B and E). E-cadherin immunofluorescence (A and B), β-catenin immunofluorescence (D and E). Bar, 10 μm. Western blot analysis (C and F) of control (-) and transdifferentiated after 36 hours treatment with TGF-β1 (+) cell extracts was performed as described in Materials and Methods. Duplicate aliquots of the same extracts were electrophoresed and analysed with the E-cadherin (C) and β-catenin (F) antibodies. Arrows mark the position of the relevant protein band and bars indicate the position of relative molecular mass standards (in kDa).

vesiculated form (Fig. 3D). In contrast, addition of staurosporine, a more general serine/threonine kinase and protein kinase C inhibitor, resulted in a relative disruption of cell-cell adhesion and a slight disorganisation of the cortical actin cytoskeleton in both control and TGF-β1-treated cells, and no evidence of morphological transdifferentiation could be observed in the presence of TGF-β1 (Fig. 3A,B). Thus, our data exclude a role for signal transduction pathways involving MEK1, PI3-kinase or Src-tyrosine kinases in TGF-β1-mediated phenotypic transformation of NMuMG cells, and in the organisation of actin stress fibres of the fibroblastic cells (Fig. 3E-J).

Identification of receptors for TGF-β1, activin A and BMP-7 in NMuMG cells

In view of the absence of an effect of activin A or BMP-7 on transdifferentiation of NMuMG cells, we investigated whether the cells expressed receptors for activin A, BMP-7, and TGF-β1. Affinity cross-linking studies on the NMuMG cells using 200 pM ^{125}I-TGF-β1, 500 pM ^{125}I-activin A, or 850 pM ^{125}I-BMP-7, followed by receptor-immunoprecipitation revealed that ^{125}I-TGF-β1 bound to ALK-5 and TβR-II, but not to ALK-2, ActR-II or ActR-IIB (Fig. 4A). In addition, receptor immunoprecipitation with ALK1-specific antibodies after ^{125}I-TGF-β1-affinity labelling did not result in any detectable complexes (data not shown). ^{125}I-Activin A formed heteromeric complexes only with ALK-4 and ActR-II and it did not bind to ALK-2 or ActR-IIB (Fig. 4B), while ^{125}I-BMP-7 interacted with ALK-2, ALK-3, ActR-II and BMPR-II but not with ALK-6 or ActR-IIB (Fig. 4C). Thus, the specificity of binding of ligands to type II and type I receptors on the cell surface of NMuMG cells is consistent with previous analyses of these receptors in other cell types (Franzén et al., 1993; Yamashita et al., 1995; Macías-Silva et al., 1998). TGF-β1 associates with ALK-5 to propagate downstream signalling, activin A interacts with ALK-4, and BMP-7 binds to ALK-2 and ALK-3 in NMuMG cells. It must be noted that ^{125}I-activin A and ^{125}I-BMP-7, with comparable specific activity as ^{125}I-TGF-β1, were not only used at higher concentrations than TGF-β1, but visualisation of ^{125}I-activin A and ^{125}I-BMP-7 cross-linked receptors also required significantly longer exposure times of the autoradiograms compared to the detection of ^{125}I-TGF-β1-bound receptors. Therefore, the expression of activin A and BMP-7 receptors compared to TGF-β1 receptors appeared to be very low.

TGF-β1 is more potent than activin A in activating Smad-responsive luciferase reporters and inducing PAI-1 production in NMuMG cells

In a broad range of cell types, TGF-β and activin are potent inducers of endogenous plasminogen activator inhibitor (PAI)-1 production, as well as of the 3TP-Lux reporter that is driven by three TPA-responsive elements and part of the PAI-1 promoter. In addition, the $(SBE)_4$-Lux reporter which contains 4 repeats of the CAGACA sequence which was identified as a Smad binding element in the JunB promoter, is a suitable read-out system for TGF-β, activin, as well as BMP signalling (Jonk et al., 1998). Thus, these systems were used to address the signalling capacities of activin A, BMP-7 and TGF-β1 in NMuMG cells. The $(SBE)_4$-Lux reporter was clearly induced by both TGF-β1 and BMP-7, whereas activin A had no effect (Fig. 5A). On the other hand, while only TGF-β1 was potent enough to induce the 3TP-Lux reporter, over-expression of constitutively active (CA)-ALK-4 or CA-ALK-5 also resulted in 3TP-Lux reporter activation (Fig. 5B). In contrast, all other

tested constitutively active type I receptors, CA-ALK-1, CA-ALK-2, CA-ALK-3, CA-ALK-6, failed to support 3TP-Lux reporter activation. In agreement with the reporter assays, TGF-β1 potently induced PAI-1 protein expression, compared to the slight induction mediated by activin A, while BMP-7 had no effect (Fig. 5C). These results indicate that the activin A signal transduction pathway is not impeded downstream of activated type I receptors. The finding that activin A is less potent than TGF-β1 could be explained by the lower expression level of receptors for activin A compared to TGF-β1 (Fig. 4).

TGF-β1 efficiently induces Smad2 phosphorylation and mediates nuclear translocation of Smad2 and/or Smad3

Using our previously characterised PS2 antiserum which specifically recognises the phosphorylated SSVS motif of Smad2 (Piek et al., 1999), we investigated the levels to which activin A, TGF-β1 and BMP-7 were able to induce phosphorylation of Smad2, a downstream effector molecule of ALK-4 and ALK-5. As observed in the western blot analysis presented in Fig. 6, TGF-β1, in contrast to excess activin A or BMP-7, potently induced phosphorylation of Smad2. Apparently, the potencies of activin A and TGF-β1 to induce transdifferentiation (Fig. 1) and gene expression (Fig. 5) correlated to their capacities to activate Smad2 and reflect the relative receptor levels on the cell surface (Fig. 4).

To monitor the subcellular distribution of endogenous Smads in NMuMG cells we used immunofluorescence analysis (see Materials and Methods). Smad1 was localised throughout the cell in a diffuse and punctate pattern in control NMuMG cells and this pattern was not altered after stimulation with TGF-β1 from 30 minutes to 36 hours (not shown). In contrast, a monoclonal antibody, which recognises both endogenous Smad2 and Smad3 (Fig. 7C), detected a quantitative translocation of Smad2 and/or Smad3 into the nuclei of NMuMG cells in response to TGF-β1 (Fig. 7A,B). In the resting state, NMuMG cells exhibited a punctate cytoplasmic pattern with occasional weak nuclear staining using this antibody (Fig. 7A). Upon stimulation with TGF-β1, no detectable cytoplasmic staining could be observed and the fluorescence accumulated in the nuclei (Fig. 7B). The fully transdifferentiated NMuMG cells showed predominantly nuclear staining and weaker cytoplasmic fluorescence which must result from the continuous presence of TGF-β1 in the medium during the course of the 36 hour incubation (data not shown). The combined results suggest activation and nuclear accumulation of Smad2 and/or Smad3, but not Smad1, during the response of NMuMG cells to TGF-β1.

The constitutively active TβR-I/ALK-5 receptor potently induces NMuMG transdifferentiation

In order to obtain firm evidence for the role of the ALK-5 receptor in NMuMG transdifferentiation, we made use of the efficient transient overexpression system of adenoviral vectors (Miyake et al., 1996). We specifically tested viruses expressing CA-ALK-5(T204D) or β-galactosidase as a negative control. Upon infection of NMuMG monolayers, the control β-galactosidase adenovirus did not alter the growth or morphological characteristics of the NMuMG cells at MOIs up to 400 (Fig. 8A, and data not shown). For most experiments, MOI of 100 or 150 was chosen

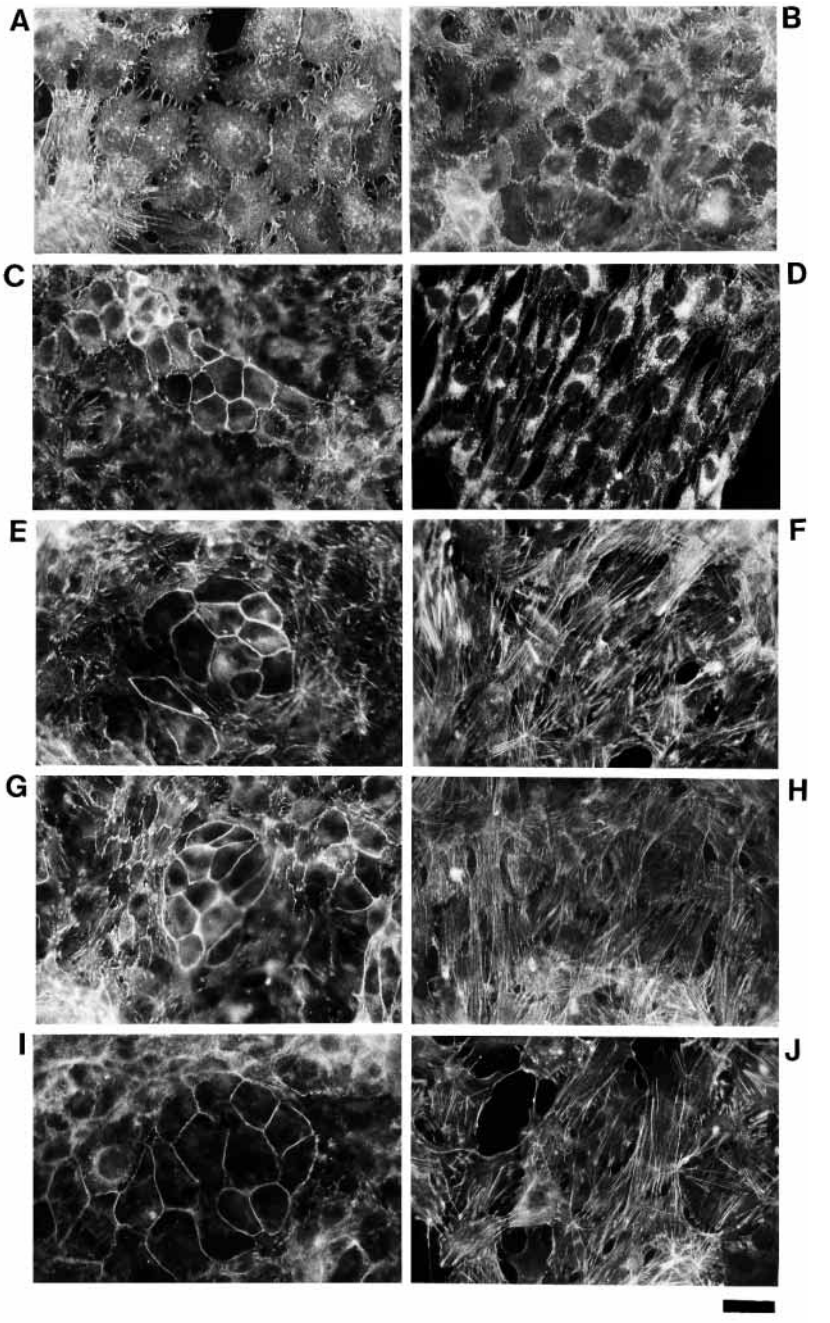

Fig. 3. Effect of protein kinase inhibitors on TGF-β1-induced transdifferentiation of NMuMG cells. NMuMG cells were stimulated for 36 hours with kinase inhibitors in the absence or presence of TGF-β1, followed by direct staining of the actin cytoskeleton using TRITC-phalloidin as described in Materials and Methods. Control cells (A,C,E,G,I), 100 pM TGF-β1-treated cells (B,D,F,H,J), 10 nM staurosporine (A,B), 5 μM bisindolyl-maleimide I (C,D), 10 μM PD 98059 (E,F), 2.8 μM LY 294002 (G,H), 50 nM PP1 (I,J). Bar, 10 μm.

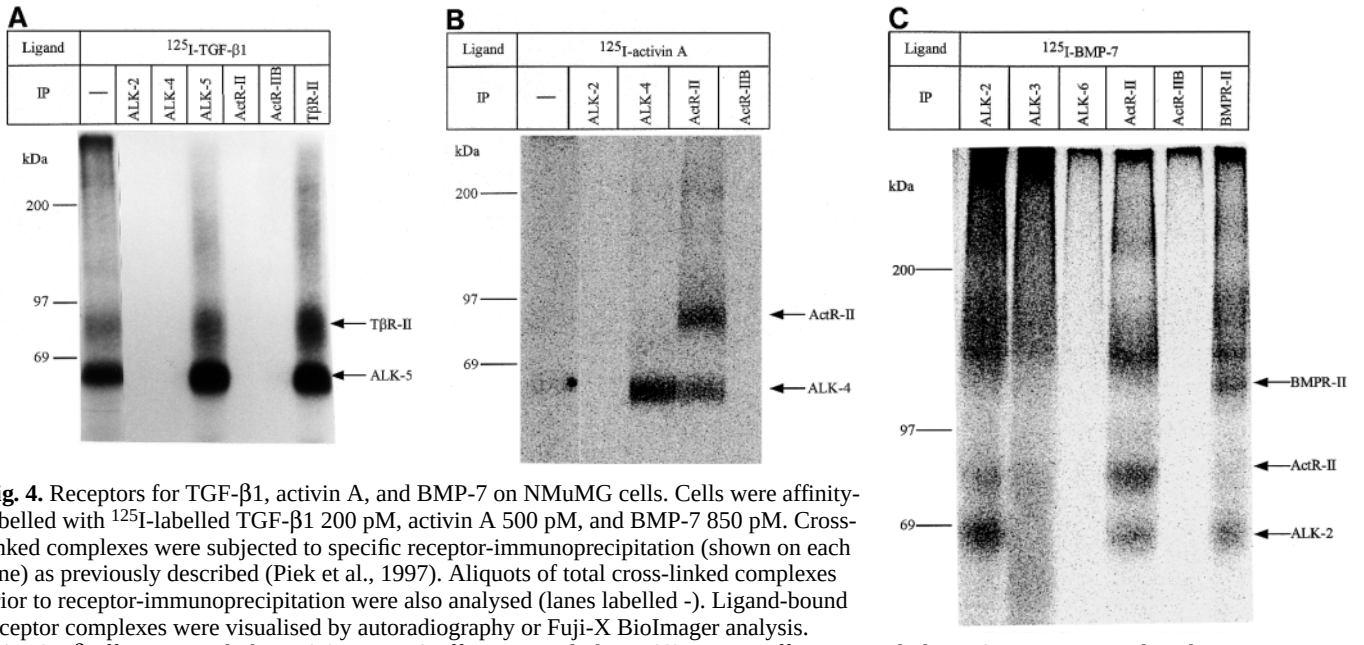

Fig. 4. Receptors for TGF-β1, activin A, and BMP-7 on NMuMG cells. Cells were affinity-labelled with ^{125}I-labelled TGF-β1 200 pM, activin A 500 pM, and BMP-7 850 pM. Cross-linked complexes were subjected to specific receptor-immunoprecipitation (shown on each lane) as previously described (Piek et al., 1997). Aliquots of total cross-linked complexes prior to receptor-immunoprecipitation were also analysed (lanes labelled -). Ligand-bound receptor complexes were visualised by autoradiography or Fuji-X BioImager analysis. (A) TGF-β affinity cross-linking, (B) activin A affinity cross-linking, (C) BMP-7 affinity cross-linking. Arrows point to the relevant immunoprecipitated affinity-labelled receptors. The position of relative molecular mass standards (in kDa) is also indicated with bars to the left of each panel.

as optimal and in situ β-galactosidase staining proved that over 90% of the cells became infected under these conditions (data not shown). In such β-galactosidase-expressing cells TGF-β1 was able to induce transdifferentiation with the same potency and kinetics as in the uninfected cells (compare Fig. 8 with Fig. 1B). Infection with the CA-ALK-5 virus resulted in a dose-dependent, ligand-independent transdifferentiation of the infected cells (Fig. 8C,D). MOI of 50 or higher was required to observe full NMuMG transdifferentiation in over 90% of the culture (data not shown and Fig. 8D), whereas at lower MOIs stress fibres were becoming more prominent and dense, an event that preceded complete morphological transformation to fibroblasts (data not shown). Under the conditions used for the infection experiments the first signs of transdifferentiation on individual cells were observed 12 hours post-infection (at a MOI of 50 or higher) but the phenomenon was established in the majority of the cultured cells only after 24 hours post-infection (data not shown). We conclude that exogenously supplied activated ALK-5 is sufficient to drive NMuMG transdifferentiation, which is in agreement with the preceding analysis of the endogenous receptors and their signalling specificity.

The TGF-β pathway Smad effectors synergise with the TβR-I/ALK-5 receptor to regulate NMuMG transdifferentiation

To examine the relative involvement of the signalling effectors lying downstream of the type I receptor ALK-5, namely Smad2, Smad3 and Smad4 in NMuMG transdifferentiation, we also exploited the adenoviral infection system described above. In this case we tested viruses expressing Smad2, Smad3 and Smad4 in addition to the CA-ALK-5 virus. Infection with viruses expressing Smad2, Smad3 or Smad4, each one alone, did not lead to NMuMG transdifferentiation (data not shown). Co-infection with these viruses in pairs including one R-Smad and the co-Smad, Smad4, resulted in enhancement of the stress fibres in the epithelial cells but not in phenotypic transformation (Fig. 8E for Smad3 and Smad4 and data not shown for Smad2 and Smad4). Finally co-infection with three viruses, CA-ALK5 at low MOI 20, which by itself does not result in appreciable transdifferentiation (Fig. 8C), Smad3 at MOI 50 and Smad4 at MOI 50 gave rise to mature fibroblasts in over 90% of the infected culture (Fig. 8F). A qualitatively similar, albeit less efficient transdifferentiation result was obtained under the same triple infection conditions but when the Smad2 virus was used in the place of Smad3 (data not shown). The kinetics of transdifferentiation under the triple infection protocol were essentially the same as described above for CA-ALK-5 (data not shown). The observed transdifferentiation under these conditions was not the result of excessive adenoviral infection as the total viral MOI was kept below 150 and the expression levels of each viral protein were roughly equivalent (Fig. 8G). Furthermore, co-infection experiments with multiple adenoviruses encoding the same proteins used in this study have been previously reported to result in undetectable alterations of the expression levels or the biological potencies of individual proteins (Fujii et al., 1999). Thus, both TGF-β pathway effector complexes, Smad2 plus Smad4 and Smad3 plus Smad4 synergise with CA-ALK-5 with different efficiencies towards the transdifferentiation response.

DISCUSSION

The breast tumour cell line NMuMG is a suitable model system to study epithelial to mesenchymal transdifferentiation mediated by TGF-β1. We observed that, of all the TGF-β superfamily members tested, only TGF-β1 was capable of inducing transdifferentiation and reorganising the actin

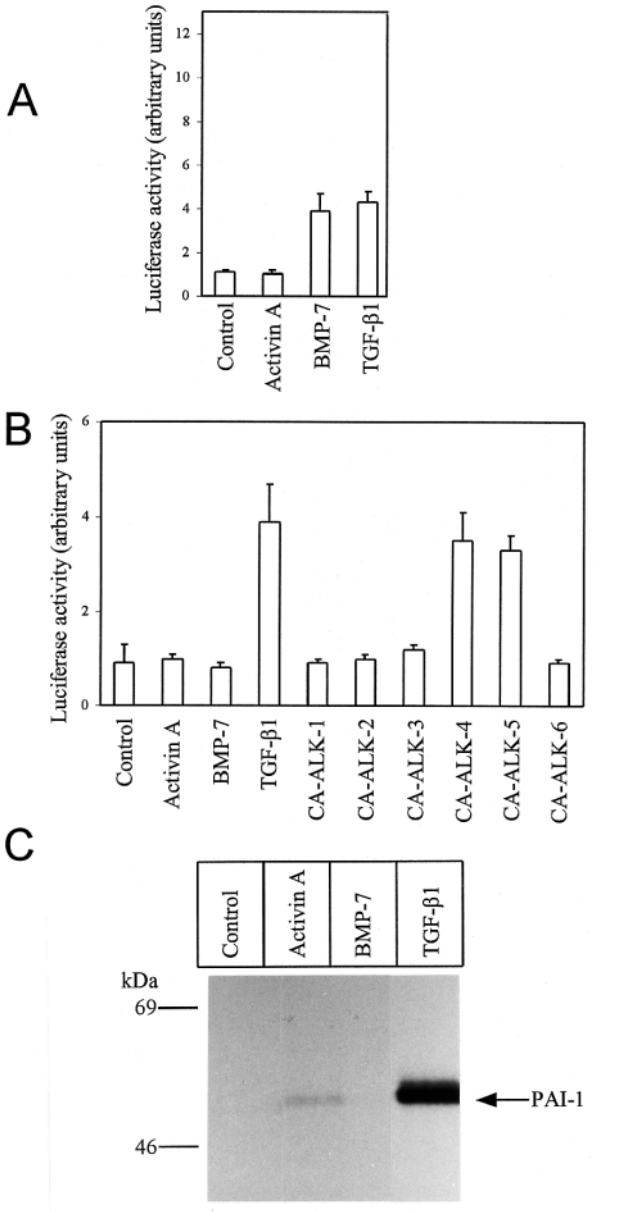

Fig. 5. Analysis of the transcriptional activation potencies of activin A, BMP-7 and TGF-β1 in NMuMG cells. (A) $(SBE)_4$-Lux reporter activation. NMuMG cells seeded in 6-well dishes were transiently transfected with 0.5 μg $(SBE)_4$-Lux reporter construct, 100 ng β-galactosidase expression plasmid pCH110, and 1 μg pcDNA3 vector, as described in Materials and Methods. After 24 hours, cells were stimulated without or with 2 nM activin A, 5.7 nM BMP-7, or 400 pM TGF-β1. Luciferase activity was measured after 20 hours, and values are corrected for transfection efficiency as measured by β-galactosidase activity. (B) 3TP-Lux reporter activation. Transient transfections were performed as described in A, with the exception of using 1 μg 3TP-Lux reporter construct instead of $(SBE)_4$-Lux reporter in the presence or absence of 1 μg constitutively active (CA) type I receptor (ALK) plasmids, as described in Materials and Methods. (C) Induction of PAI-1 protein synthesis. NMuMG cells were stimulated for 2 hours with 2 nM activin A, 5.7 nM BMP-7, or 400 pM TGF-β1, followed by metabolic labelling for 2 hours with [^{35}S]methionine/[^{35}S]cysteine. Extracellular matrix proteins were extracted from the tissue culture dish and separated on an 8% SDS polyacrylamide gel. The position of the PAI-1 protein is indicated by arrow and bars represent relative molecular mass standards (in kDa).

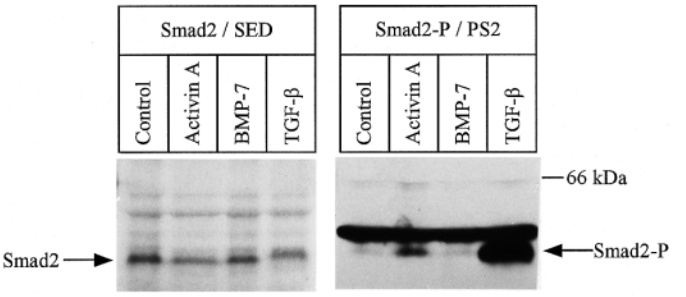

Fig. 6. Phosphorylation of Smad2 by activin A and TGF-β1 in NMuMG cells. Cells were serum-starved for 3 hours, followed by stimulation for 60 minutes with 2 nM activin A, 5.7 nM BMP-7 or 200 pM TGF-β1. Western blotting was performed as described in Materials and Methods, using the Smad2 antibody (SED) and the phosphorylated Smad2 (SMAD2-P) antibody (PS2). Arrows point to the two Smad2 protein species and bars indicate the position of relative molecular mass standards (in kDa).

cytoskeleton from a cortical arrangement to the formation of stress fibres. This mesenchymal transition was characterised by decreased expression and relocalisation of E-cadherin and β-catenin and was fully blocked by staurosporine. TGF-β1 was found to bind primarily to TβR-II and ALK-5, but not to ALK-2. Induction levels of PAI-1 protein synthesis, 3TP-Lux and $(SBE)_4$-Lux reporter activity by TGF-β1 and activin A correlated with Smad2 phosphorylation levels and Smad2 and/or Smad3 nuclear accumulation, most probably reflecting the relative cell surface receptor levels for these two polypeptide factors. Full transdifferentiation could be elicited by transient overexpression of high levels of adenovirally-encoded CA-ALK-5 receptor or by low levels of adenoviral Smad2 plus Smad4 or Smad3 plus Smad4 together with very low levels of CA-ALK-5, in a synergistic manner.

During carcinogenesis, epithelial cell types can undergo mesenchymal transformation to a fibroblast-like phenotype, thereby gaining the possibility to migrate and metastasise. TGF-β has been shown to play an important role in the mesenchymal transition of epithelial cells (Miettinen et al., 1994; Oft et al., 1996, 1998) and in the malignant progression of fibroblast-like squamous carcinoma cells to highly invasive spindle cell carcinomas during in vivo skin carcinogenesis (Caulin et al., 1995; Cui et al., 1996; Portella et al., 1998). The fact that TGF-β1 potently induces PAI-1 protein expression along with mesenchymal transdifferentiation in NMuMG cells supports this idea. The present analysis corroborates the previous findings and further emphasises that mesenchymal transition is not a general property of all TGF-β superfamily members, rather it is a highly specific function of TGF-β1 (Fig. 1).

Malignant progression of epithelial cells is often accompanied by reduced E-cadherin expression levels (Miettinen et al., 1994; Caulin et al., 1995; Portella et al., 1998). Interestingly, although the overall E-cadherin levels are reduced upon transdifferentiation of NMuMG cells, E-cadherin exhibits a distinct redistribution to plasma membrane areas that preserve extensive cell-cell adhesion (Fig. 2B). The same applies to β-catenin although its downregulation is not as dramatic as that for E-cadherin (Fig. 2E,F). One can speculate that these remnants of the epithelial cell architecture in the

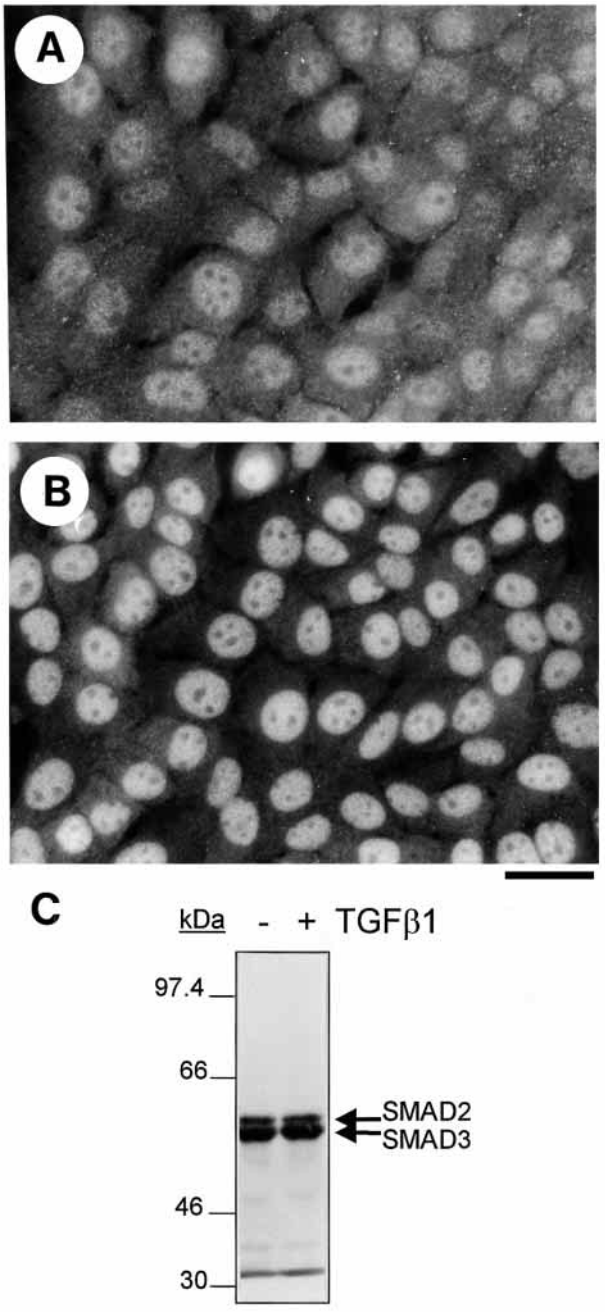

Fig. 7. Subcellular distribution of endogenous Smad2 and/or Smad3 in NMuMG cells. Cells were stimulated for 1 hour with solvent or TGF-β1, followed by indirect immunofluorescence against Smad2 and/or Smad3 as described in Materials and Methods. Control cells (A) and 100 pM TGF-β1-treated cells (B) are shown. Bar, 10 μm. C indicates the specificity of the antibody used in immunofluorescence experiments by showing a representative western blot of normalised total extracts from NMuMG cells treated with solvent (- lane) or with 100 pM TGF-β1 for 36 hours (+ lane) when cells transdifferentiated fully. Western blot analysis was performed as described in Materials and Methods using the same monoclonal antibody against Smad2 and/or Smad3 as in A and B. The positions of the two Smad proteins are indicated with arrows and bars indicate the position of relative molecular mass standards (in kilo-Daltons (kDa)). It must be noted that the detected total Smad2 and Smad3 levels do not vary between the two conditions tested.

fibroblast-like phenotype could be one reason for the reversible nature of the transdifferentiation phenomenon in epithelial cells (Miettinen et al., 1994; Oft et al., 1996; Portella et al., 1998). In other words, the remaining E-cadherin and associated molecules (such as β-catenin) may be part of the signalling network that will revert the morphological phenotype back to the epithelial state after prolonged (more than 48 hours) incubation post-TGF-β1-treatment (Miettinen et al., 1994).

It is of interest that only the more general serine/threonine kinase and protein kinase C inhibitor staurosporine could significantly interfere with the transdifferentiation process (Fig. 3A,B), as previously reported (Miettinen et al., 1994). It should be noted that the general morphology of the epithelial cell population changes upon staurosporine addition, since cells lose their tight contacts. Possibly, appropriate cell-cell and cell-matrix interactions are required to assure cellular transformation. On the other hand, as the early step in signal transduction by TGF-β1 is the activation of its cognate serine/threonine kinase receptors, the experiments presented here cannot exclude a direct inhibitory effect of staurosporine on cell surface receptor activity. One must note, however, that two previous reports disfavour the latter possibility (Ohtsuki and Massagué, 1992; Miettinen et al., 1994). The effect of bisindolyl-maleimide I on the actin cytoskeleton of the transdifferentiated cells (Fig. 3C,D) might suggest that proper stress-fibre formation is no prerequisite for mesenchymal transformation, and the observed actin cytoskeleton redistribution could be one of the facets of the transdifferentiated phenotype. The results suggest a role for protein kinase C isoforms in the proper organisation of the stress fibres in the fibroblastic cells. In addition, the specific inhibitors against MEK1, PI3-kinase, and Src family kinases failed to interfere with the transdifferentiation process or with organised stress fibre formation (Fig. 3E-J), although the latter kinases are known to participate in cytoskeleton-associated signalling or organisation of the actin cytoskeleton per se (Seidel-Dugan et al., 1992; Wennström et al., 1994). The present data favour a distinct mechanism, which is initiated by TGF-β and does not primarily depend on any of these kinases.

Miettinen et al. (1994) have proposed the involvement of ALK-2 in the transdifferentiation induced by TGF-β1, based on the observations that (i) ALK-2 expression was high while ALK-5 could not be detected by northern blot analysis or PCR, (ii) antisense ALK-2 oligonucleotides and (iii) overexpression of a dominant negative ALK-2 mutant abrogated TGF-β1-mediated transdifferentiation of NMuMG cells. ALK-2 has been characterised as a type I receptor for BMP-7, signalling through Smad1 and Smad5 (Yamashita et al., 1995; Macías-Silva et al., 1998; Chen and Massagué 1999), while ALK-5 is the established type I receptor for TGF-β signalling (Franzén et al., 1993; Wrana et al., 1994). We detected ALK-2, ALK-3, ActR-II and BMPR-II expression in NMuMG cells following ^{125}I-BMP-7-affinity cross-linking and receptor immunoprecipitation studies (Fig. 4C). Comparable studies using ^{125}I-TGF-β1 revealed abundant expression of ALK-5 and TβR-II, but there were no indications for interaction of TGF-β1 with ALK-2 (Fig. 4A). We could also not detect ^{125}I-TGF-β1-cross-linked proteins corresponding to the size of ALK-1, which is a TGF-β type I receptor in endothelial cells, slightly larger in size than ALK-5, and which mediates signalling through Smad1 and Smad5 (Macías-Silva et al.,

1998; Chen and Massagué, 1999). A clonal variation in the NMuMG cell lines that were used may underlie the discrepancies in our findings and those by Miettinen et al. (1994). Finally, ^{125}I-activin A associated with ALK-4 and ActR-II on the cell surface of NMuMG cells (Fig. 4B). Thus, for the cells used in our studies, TGF-β1 triggers transdifferentiation most likely by signalling through ALK-5, as confirmed by CA-ALK-5 overexpression, and the levels of this receptor are significantly higher than those of the activin A and BMP-7 receptors on the NMuMG cell surface.

Activin and TGF-β exert many overlapping activities in vitro. The kinase domains of ALK-4 and ALK-5 share high sequence similarity and the L45 loop, which is a major determinant of Smad-binding specificity, is identical in ALK-4 and ALK-5 (Feng and Derynck, 1997; Chen et al., 1998; Persson et al., 1998; Chen and Massagué, 1999), resulting in common signalling propagation that involves Smad2 and Smad3. In a few cell systems, however, differential effects exerted by TGF-β and activin or Smad2 and Smad3 have been observed (Nishihara et al., 1993; Torii et al., 1996; Link and Nishi, 1997; Labbé et al., 1998; Shimizu et al., 1998), although the mechanisms underlying differential signalling are not clear. In NMuMG cells, differential signalling by activin A and TGF-β1 seems to reflect the levels of expression of the respective cell surface receptors (Fig. 4), resulting in differential activation levels of the signal transduction pathways induced by these two ligands.

The morphological transformation analysis correlated well to the differential transcriptional activation potencies by the three growth factors as observed for activation of Smad-sensitive reporters and PAI-1 protein induction (Fig. 5). Whereas both $(SBE)_4$-Lux and 3TP-Lux reporters were activated by TGF-β1, they remained insensitive to activin A (Fig. 5A,B), and only the $(SBE)_4$-Lux reporter was activated by BMP-7. In addition, only CA-ALK4 and CA-ALK5, but none of the other activated type I receptors tested, induced the 3TP-Lux reporter. These results emphasise that responsiveness of a target gene to TGF-β family members is dependent on the type of Smads (and/or other signal transduction components) that become activated and that can specifically transactivate the promoter of the target gene, whereby different genes are controlled by different activation thresholds. Thus, in terms of the transdifferentiation process, the present analysis suggests that the differential effects observed for activin A, BMP-7 and TGF-β1 are due to the fact that only the signalling cascade activated by TGF-β1 is both potent and specific enough to drive mesenchymal transformation of NMuMG cells.

An issue of interest is whether the Smad effectors are directly involved in the transdifferentiation process. We observed that TGF-β1, in contrast to activin A and BMP-7, potently induced phosphorylation of Smad2 (Fig. 6). An issue of great importance is whether the other R-Smad of the TGF-β pathways, namely Smad3, is also activated in the NMuMG cells. Despite continuous efforts to address this question using

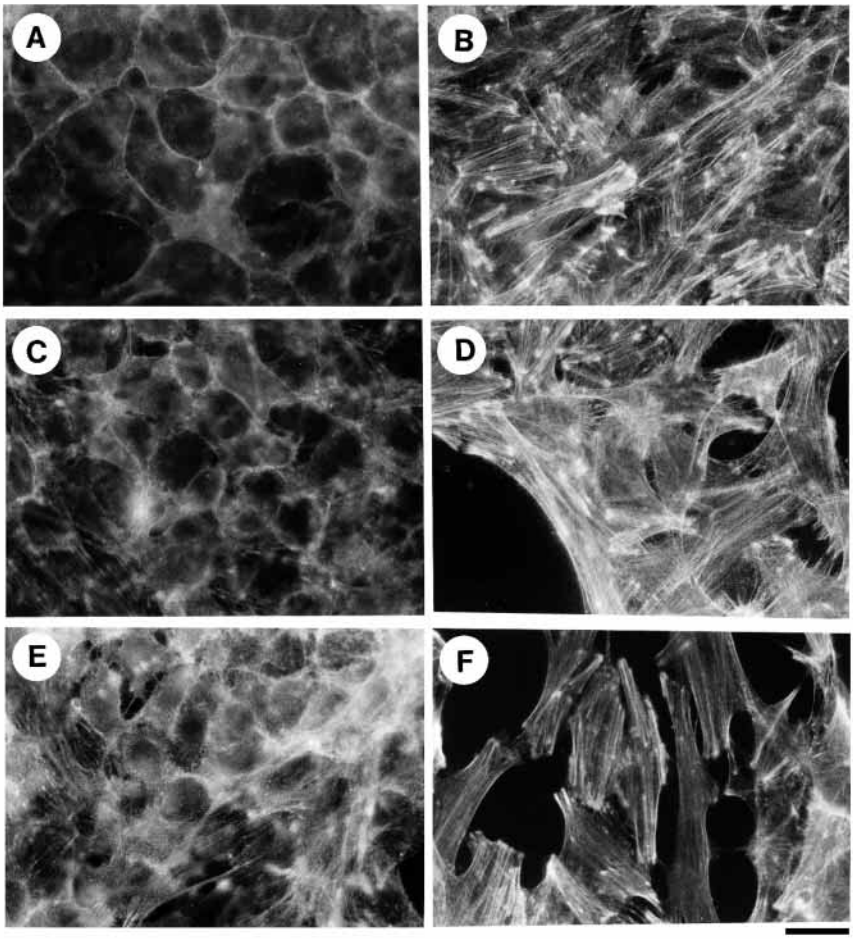

Fig. 8. Organisation of the actin cytoskeleton of adenovirally infected NMuMG cells. Cells were infected with adenoviruses expressing: β-galactosidase at MOI 150 (mock-infection condition) (A and B), constitutively active (CA) ALK-5 receptor at MOI 20 (C) and MOI 150 (D), Smad3 plus Smad4 at MOI 50 each (E) and CA-ALK-5 at MOI 20 plus Smad3 plus Smad4 at MOI 50 each (F). Twelve hours post-infection, cells were treated with solvent (A,C-F) or with 100 pM TGF-β1 for 36 hours (B) and then fixed and processed for actin direct fluorescence as described in Materials and Methods. It must be noted that the MOI 20 CA-ALK5 virus (alone shown in C) synergises with the Smad3 and Smad4 viruses (alone shown in E) to achieve full transdifferentiation (F). G shows a representative western blot of normalised total cell extracts from infected cells with adenoviruses expressing the proteins listed on top of the panel, and performed as described in Materials and Methods. HA refers to the carboxyterminal haemaglutinin epitope of the CA-ALK-5 receptor and F refers to the aminoterminal Flag epitope of the Smad proteins. Duplicate extracts were resolved on the same gel and the two parts of the membrane were probed with each antibody as indicated below the panel. Arrows mark the positions of the analysed proteins and bars indicate the position of relative molecular mass standards (in kDa).

various antibodies, we have not yet obtained conclusive results. However, the Smad3 adenovirus data (Fig. 8) underscore the importance of Smad3 in the process of transdifferentiation and strongly suggest that Smad3, like Smad2, may become activated in the NMuMG cells after stimulation by TGF-β1. TGF-β1 also induced the nuclear accumulation of Smad2 and/or Smad3, but not of Smad1 (Fig. 7, and data not shown). The failure to demonstrate Smad1 activation by TGF-β1 in the transdifferentiated cells strongly suggests that ALK-1 and/or ALK-2 receptor signalling cannot explain the observed phenotypic changes since these two receptors are known to signal via Smad1 (Macías-Silva et al., 1998; Chen and Massagué, 1999). Although the Smad2-phosphorylation levels correspond to the potencies of activin A and TGF-β1 to mediate signalling in NMuMG cells, the above experiments do not firmly establish whether Smad activation can fully account for the transdifferentiation process. For this reason an adenovirus-based overexpression and high efficiency infection protocol was chosen (Fig. 8), which proved to be reliable in the analysis of differentiation in other cell systems (Fujii et al., 1999). Using this approach it was possible to demonstrate that overexpression of activated ALK-5 receptor was sufficient to induce full NMuMG transdifferentiation in a ligand-independent and dose-dependent manner (Fig. 8C,D). In the present study only the ALK-5 receptor was tested but in future experiments the potency of the other type I receptors of the superfamily must be analysed in the NMuMG transdifferentiation system. With respect to the question of Smad protein involvement in transdifferentiation, the data of Fig. 8E,F support a clear role for the Smad3/Smad4 combination. The Smad2/Smad4 combination is also effective in leading to NMuMG transdifferentiation, however, not as efficiently as the Smad3/Smad4 combination (data not shown). The mechanistic implications underlying the observed difference are the topic of future analysis. Surprisingly, overexpression of each protein alone did not result in any detectable transdifferentiation or actin cytoskeleton reorganisation (data not shown). This indicates that despite the high efficiency expression of the individual infected proteins (Fig. 8G) reconstitution of the signalling pathway with a pair of an R-Smad and a co-Smad in addition to activation by receptor is required in a synergistic fashion to achieve full transdifferentiation (Fig. 8F). This finding leaves open the possibility of additional signalling components that become activated by the type I receptor and can co-operate with Smads to elicit full phenotypic transformation. Thus, it is anticipated that further analysis by means of the adenoviral expression system will provide clear answers to the issue of signalling pathway specificity at both the type I receptor and Smad effector level. In addition, the role of other signal transduction molecules that have been reported to be involved in transdifferentiation and actin reorganisation processes, like Rho family members and Ras (Qui et al., 1995, 1997; Oft et al., 1996, 1998), deserves detailed future analysis.

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