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Induction of an Epithelial to Mesenchymal Transition in Human Immortal and Malignant Keratinocytes by TGF-β1 Involves MAPK, Smad and AP-1 Signalling Pathways

Maria Davies,[1]* Max Robinson,[1] Emily Smith,[2] Suzy Huntley,[1] Stephen Prime,[1] and Ian Paterson[1]

[1]Department of Oral and Dental Science, University of Bristol, Bristol, BS1 2LY, United Kingdom
[2]Department of Physiology, The Medical School, University of Birmingham, Edgbaston, B15 2TT, United Kingdom

Abstract Recent data indicate that transforming growth factor-β1 (TGF-β1) can act to promote tumour progression in the late stages of carcinogenesis. The mechanism by which this occurs is unknown although a ligand-induced epithelial–mesenchymal transition (EMT) is thought to be important. In this study, we demonstrate that active Ras is required for TGF-β1-induced EMT in human keratinocytes and that epidermal growth factor (EGF) can substitute for mutant Ras. EMT was reversed by the removal of TGF-β1. Under conditions of TGF-β1-induced EMT, cells were growth inhibited by the ligand resulting in G_1 arrest. In cells containing normal Ras, TGF-β1-activated ERK and p38 mitogen-activated protein kinases (MAPKs), and levels of activation were further increased by co-treatment with EGF. Inhibition of MAPK pathways and Smad2/3 signalling blocked the induction of EMT by TGF-β1. Further, inhibition of the AP-1 transcriptional complex by [6]-Gingerol, or by the ectopic expression of JDP2, blocked TGF-β1-induced EMT and conversely, stimulation of AP-1 by 12-O-tetradecanoylphorbol 13-acetate (TPA) substituted for EGF in the induction of EMT by TGF-β1 in cells containing normal Ras. The presence of oncogenic Ras, the treatment of cells with EGF, or the treatment of cells with TPA to activate AP-1, potentiated TGF-β1-induced Smad-dependent transcription, an effect that was attenuated by the inhibition of MAPKs and AP-1. The results demonstrate that active Ras and TGF-β1 co-operate to reversibly induce EMT in human keratinocytes by mechanisms that involve MAPKs, Smad2/3 and AP-1. Further we demonstrate that MAPK/AP-1 signalling enhances Smad transcriptional activity under conditions associated with TGF-β1-induced EMT. J. Cell. Biochem. 95: 918–931, 2005.

Key words: human keratinocyte; AP-1; EMT; TGF-β1; Smads; MAPK

The process of epithelial to mesenchymal transition (EMT), in which epithelial cells acquire fibroblastoid features, is a fundamental process of embryogenesis, wound healing and cancer invasion/metastasis [Thiery, 2003]. The mechanisms responsible for EMT are not fully understood but involve a delocalisation of the cell membrane protein E-Cadherin, a loss of cytokeratin expression and the induction of vimentin. Transforming growth factor-β (TGF-β)-induced EMT was originally described in cells of murine origin and whilst it has been reported in a limited number of human cell lines [Geng et al., 1999; Janji et al., 1999; Zavadil et al., 2001; Yi et al., 2002], the mechanisms by which this phenomenon occur are poorly understood. The development of additional human cell systems to examine TGF-β1-induced EMT is important not only because cells that have undergone TGF-β-induced EMT are more invasive and motile [Oft et al., 1996; Bakin et al., 2000; Iglesias et al., 2000; Lehmann et al., 2000; Santibanez et al., 2000; Bhowmick et al., 2001a] but also, the pro-oncogenic effects of TGF-β in late stage carcinogenesis have been attributed to this phenomenon [Wakefield and Roberts, 2002].

TGF-β signal transduction is activated following ligand binding to the type II TGF-β receptor (TβR-II) and, after heteromerisation and

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*Correspondence to: Dr. Maria Davies, Department of Oral and Dental Science, University of Bristol, Lower Maudlin Street, Bristol, BS1 2LY, UK.
E-mail: maria.davies@bristol.ac.uk

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transphosphorylation of the type I TGF-β receptor (TβR-I), signal propagation occurs by phosphorylation of the receptor-specific (R) Smads (Smad2 and Smad3). The phosphorylated R-Smads then oligomerise with Smad4, translocate to the nucleus and regulate the transcription of target genes [Massague, 1998]. The association of the Smad complexes with transcription factors and transcriptional co-activators/co-repressors in the nucleus further regulates transcriptional control by TGF-β. It is now recognised that TGF-β1 can also activate mitogen-activated protein kinases (MAPKs) [Hartsough and Mulder, 1995; Yamaguchi et al., 1995; Atfi et al., 1997; Hanafusa et al., 1999; Sano et al., 1999; Bhowmick et al., 2001b] but the exact pathways which are stimulated are thought to be cell type specific. The AP-1 transcriptional complex is a primary target of a number of MAPK pathways and it has been shown that AP-1 components can interact directly with Smad3 [Zhang et al., 1998; Peron et al., 2001; Verrecchia et al., 2001] suggesting that AP-1 may be central to cross-talk between Smad and MAPK pathways. Whilst AP-1 is required for epidermal growth factor (EGF)-induced activation of Rho-like GTPases, cytoskeletal rearrangements, motility and in vitro invasion in epithelial cells [Malliri et al., 1998], its role in TGF-β-induced EMT has not been examined.

The purpose of the present study was to determine whether EMT was induced by TGF-β1 in human keratinocytes and to investigate which signal transduction pathways were associated with this phenomenon. We used a well-characterised cell line derived from human oral squamous cell carcinoma that contains mutant Ha-*ras* (codons 13 and 61) [Yeudall et al., 1993; Prime et al., 1994] and also, a series of human skin keratinocytes transfected with mutant Ha-*ras* (codon 12) [Boukamp et al., 1990; Mueller et al., 2001]. We demonstrate that active Ras is essential for TGF-β1-induced EMT and that inhibitors of MAPKs, Smad2/3 and AP-1 can block this phenomenon. Further, we show that MAPK/AP-1 function to potentiate Smad-dependent transcription under conditions that drive TGF-β1-induced EMT.

MATERIALS AND METHODS

Cell Culture

Details of the spontaneously immortalised human skin keratinocyte cell line (HaCaT) and the mutant c-Ha-*ras*-transfected HaCaT cell clone II-3 have been reported previously [Boukamp et al., 1990; Mueller et al., 2001]. Similarly, the characteristics of the malignant human oral keratinocyte cell line containing mutant Ha-*ras* (H357) has been described previously [Yeudall et al., 1993]. All of the cell lines are growth-inhibited by TGF-β1 [Game et al., 1992; Prime et al., 1994], albeit by different degrees (HaCaT, H357—marked growth inhibition; II-3—modest growth inhibition). The cell lines were cultured in standard medium consisting of Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1, Invitrogen, Paisley, UK) containing 10% (v/v) foetal bovine serum, 0.6 mg/ml L-glutamine and 0.5 μg/ml hydrocortisone as previously described [Prime et al., 1994]. The Ha-*ras* transfected cell line II-3 was cultured in media containing 400 μg/ml geneticin sulphate (G418). AM12 cells were maintained in DMEM containing 10% (v/v) foetal bovine serum and 0.6 mg/ml L-glutamine.

Characteristics of EMT

For the immunocytochemical studies, cells were seeded onto glass coverslips in culture dishes, grown to 40% confluence and treated with either human recombinant TGF-β1 (R&D Systems, Abingdon, UK; 0.1–3 ng/ml) or EGF (Sigma, Poole, UK; 100 ng/ml), or 12-O-tetradecanoylphorbol 13-acetate (TPA; 100 ng/ml) or a combination of TGF-β1 and either EGF or TPA, in standard media containing 1% or 10% (v/v) FBS for 48–72 h. Control dishes received an equivalent volume of growth factor diluent. Following fixation in acetone (10 min, room temperature), immunocytochemical staining for E-Cadherin (mouse monoclonal, 1:500; BD Transduction Laboratories, Franklin Lakes, NJ) and vimentin (mouse monoclonal clone 9, 1:20; Dako Cytomatic, Buckinghamshire, UK) was undertaken using standard procedures.

Those cell lines that underwent EMT in response to TGF-β1 were either transiently transfected with the AP-1 repressor *JDP2* [Aronheim et al., 1997] or treated with the specific chemical inhibitors of MEK (10 μM UO126; Promega, Southampton, UK), p38 MAPK (10 μM SB203580; Merck Biosciences, Nottingham, UK), JNK (10 μM SP600125; Biomol International LP, Devon, UK) and AP-1 activation (100 μM [6]-Gingerol; Calbiochem, Nottingham, UK). The MAP kinase inhibitors

have been widely used to study EMT and did not inhibit TGF-β1-induced Smad2 phosphorylation at the concentrations used in this study (data not shown). Transfection of *JDP2* (2 μg) or the vector control were carried out 24 h prior to TGF-β1 treatment using FuGene 6 (Roche, East Sussex, UK), according to the manufacturers instructions.

In all experiments, the media were replenished and the cells treated with TGF-β1 and/or EGF and/or chemical inhibitors daily for the duration of the experiment (48 or 72 h).

Growth Inhibition In Vitro

Cells were grown to 30% confluence in standard medium containing 10% (v/v) FBS. Following replacement of the culture medium with medium containing 1% (v/v) FBS, the cells were treated with or without TGF-β1 (0.1–0.5 ng/ml). Cells were cultured for a further 48 or 72 h and culture medium, with or without ligand, was replaced every 24 h. Cell numbers were determined using a Coulter counter (Coulter Z series; Beckman Coulter, Buckinghamshire, UK).

Analysis of Cell Cycle Distribution

Cells were cultured in the absence or presence of 3 ng/ml TGF-β1 for 72 h in medium containing 10% (v/v) FBS. Cell pellets (1×10^6) were resuspended in 1 ml ice cold 70% ethanol and frozen at −20°C for 7–14 days. Twenty-four hour prior to analysis, the cells were recovered by centrifugation, resuspended in 1 ml PBS containing 20 μg/ml propidium iodide and 15 μg/ml Rnase A and then incubated in the dark at 37°C for 30 min, followed by overnight incubation at 4°C. Cells were analysed on a FACSCalibur flow cytometer (Becton Dickenson, Oxfordshire, UK) and the data analysed using ModFit software (Verity Software, Topsham, ME).

Western Analysis

To examine the expression of N- and E-Cadherin, HaCaT cells were cultured in the presence or absence of TGF-β (3 ng/ml) with or without EGF (100 ng/ml) and cells containing mutant Ras were treated with TGF-β1 (3 ng/ml) with or without the p38 inhibitor SB203580 (10 μM) for 24–72 h in medium containing 1% (v/v) FBS. In studies to examine MAPK activation, HaCaT cells were cultured in the presence or absence of TGF-β1 (3 ng/ml) with or without EGF (100 ng/ml), and H357 cells were cultured in presence or absence of TGF-β1 (3 ng/ml) with or without MAP kinase inhibitors (UO126, SB203580, SP600125, all 10 μM) in medium containing 1% (v/v) FBS for 10 min to 24 h. The levels of activated ERK 1/2, p38 and JNK were determined by standard immunoblot techniques. The primary antibodies used were anti-phospho-ERK clone pTEpY (1:1000, 4°C, overnight; Promega), anti-phospho-p38 Thr180/Tyr182 (1:1000, 4°C, overnight; Cell Signaling, Beverly, MA), anti-phospho-c-jun Ser63 antibody II (1:500, 4°C, overnight; Cell Signaling), anti-E-Cadherin (1:1500, 1 h, room temperature; BD Transduction Laboratories), and anti-N-Cadherin clone 3B9 (1:500, 4°C, overnight; Zymed Laboratories, San Francisco, CA). The secondary antibodies used were goat anti-mouse horseradish peroxidase and goat anti-rabbit horseradish peroxidase (both 1:1000, 1 h, room temperature, Derbio, Northumberland, UK).

Plasmids

Ligand-induced transcriptional activation was measured using the luciferase reporters 3TP-lux (containing TGF-β-responsive elements of the *PAI-1* and *collagenase* promoters), SBE_4-luc (a Smad3/4-specific response element) [Zawel et al., 1998], distal element (DE)-luc (a Smad2/4 specific response element when co-transfected with *mixer*) [Pierreux et al., 2000] and AP-1-luc (containing 7 AP-1 binding elements).

The retroviral vector pLPCX, and the expression vector containing the C-terminal truncated dominant-negative *Smad3* cDNA (pLPCX-*Smad3 ΔC*) were gifts of Dr. Rik Derynck and have been described previously [Choy et al., 2000]. The retroviral construct expressing *JDP2* in pBabepuro was a gift from Dr Ami Aronhein (Institute of Technology, Israel).

Reporter Assays

Cells were transfected with 0.5 μg reporter plasmid and 0.5 μg of the Renilla control vector pRL-TK (Promega) and incubated for 6 h. TGF-β1 or diluent control, (0.1–2 ng/ml), with or without EGF (100 ng/ml) and with or without the chemical inhibitors (UO126, SB203580, SP600125, all 10 μM; [6]-Gingerol, 100 μM), was added to the media and the cells incubated for a further 20 h. Luminescence was measured, and luciferase values normalised to control

for transfection efficiency using readings obtained for the co-transfected control vector as described previously [Griffiths et al., 1998]. In experiments to determine whether the ectopic expression of JDP2 altered ligand-induced transcriptional activation of 7AP-1-luc and/or SBE_4-luc, cells were transfected with 2 μg plasmid 24 h prior to performing the reporter assay. In experiments to determine whether TPA could enhance TGF-β1-induced transcriptional activation of SBE_4-luc, cells were treated with or without TPA (100 μg/ml) 24 h prior to transfection and again at the time of TGF-β1 treatment.

Abrogation of Smad Signalling

To generate stable cell populations expressing Smad3ΔC, the amphotrophic cell line AM12 was first transfected with pLPCX-Smad3ΔC or pLPCX alone using standard techniques and the cells selected in 3 μg/ml puromycin 24 h after transfection. To generate retroviruses, stably transfected AM12 cells were grown to 80% confluence in a 75 cm^3 flask and, following three washes in PBS, 8 ml culture medium without puromycin was added to each flask and the cells incubated overnight. The conditioned medium was filtered through a 0.45 μm filter to remove cell debris and following the addition of polybrene (8 μg/ml, Sigma), was applied immediately to H357 and II-3 cells which had been grown to 30% confluence. Twenty-four hour after infection, the culture medium was replenished with standard medium containing 10% (v/v) FBS and after a further 24 h, selection was applied (0.5 μg/ml puromycin). Clonal populations of stably transduced cells (II-3-Smad3ΔC and H357-Smad3ΔC) were isolated and the level of expression of Smad3ΔC was determined by Western analysis for Flag using anti-FlagM2 (1:2000, 1 h room temperature; Sigma) and standard immunoblot techniques (data not shown).

RESULTS

TGF-β1-Mediated EMT is Dependent on Ras Signalling and Occurs in Cells That Undergo Ligand-Induced Growth Inhibition

The *ras* transfected clone II-3 and the human malignant oral keratinocyte cell line H357, both containing mutant ras, showed marked EMT after treatment with 3 ng/ml TGF-β1 for 48–72 h. By contrast, TGF-β1-induced EMT was not evident in the parent HaCaT cell line containing normal ras. EMT was characterised by the redistribution of E-Cadherin from a cell membrane to cytoplasmic location and the emergence of positive vimentin staining (Fig. 1A). Western blot analysis of E-Cadherin and N-Cadherin protein levels showed that TGF-β1 (3 ng/ml, 24–72 h) caused a reduction in E-Cadherin and an increase in N-Cadherin in all cells irrespective of the presence of mutant Ras and the ability of the cells to undergo ligand-induced EMT (data not shown). In order to determine whether EGF could substitute for the presence of mutant ras and facilitate TGF-β1-induced EMT, HaCaT cells were treated with the vehicle control, TGF-β1, EGF or both ligands. EMT was only seen in the presence of both TGF-β1 (10 ng/ml) and EGF (100 ng/ml; Fig. 1B); treatment of cells with 10 ng/ml EGF was not sufficient to facilitate TGF-β1-induced EMT.

Prolonged treatment of cells containing mutant *ras* with TGF-β1 (3 ng/ml, every 2 days for 21 days) resulted in an up-regulation of TGF-β1 mRNA, continued cellular proliferation and the adoption of a more spindle-like morphology. Following the removal of exogenous TGF-β1, endogenous ligand expression, the growth inhibitory response and the cellular morphology returned to normal (data not shown). The data show that EMT was reversible.

The cell lines used in this study have been shown previously to be growth-inhibited by TGF-β1 [Game et al., 1992; Prime et al., 1994]. To confirm that the cells that underwent TGF-β1-induced EMT were still responsive to the growth inhibitory effects of the ligand, we performed flow cytometric analysis on HaCaT (normal *ras*) and II-3 (mutant *ras*) cells following treatment with TGF-β1 (72 h). Both cell lines exhibited a similar cell cycle profile before and after treatment with TGF-β1 even though II-3 cells had undergone EMT, with the percentage of cells in the G_0/G_1 phase increasing from 45%–49% to 68%–75%. Together, the data demonstrate that TGF-β1 can still cause a growth arrest in cells undergoing EMT.

Involvement of MAPK and Smad Pathways in EMT

To determine whether MAPK pathways were activated during TGF-β1-induced EMT, HaCaT cells were treated with TGF-β1 in the presence or absence of EGF and MAPK activation

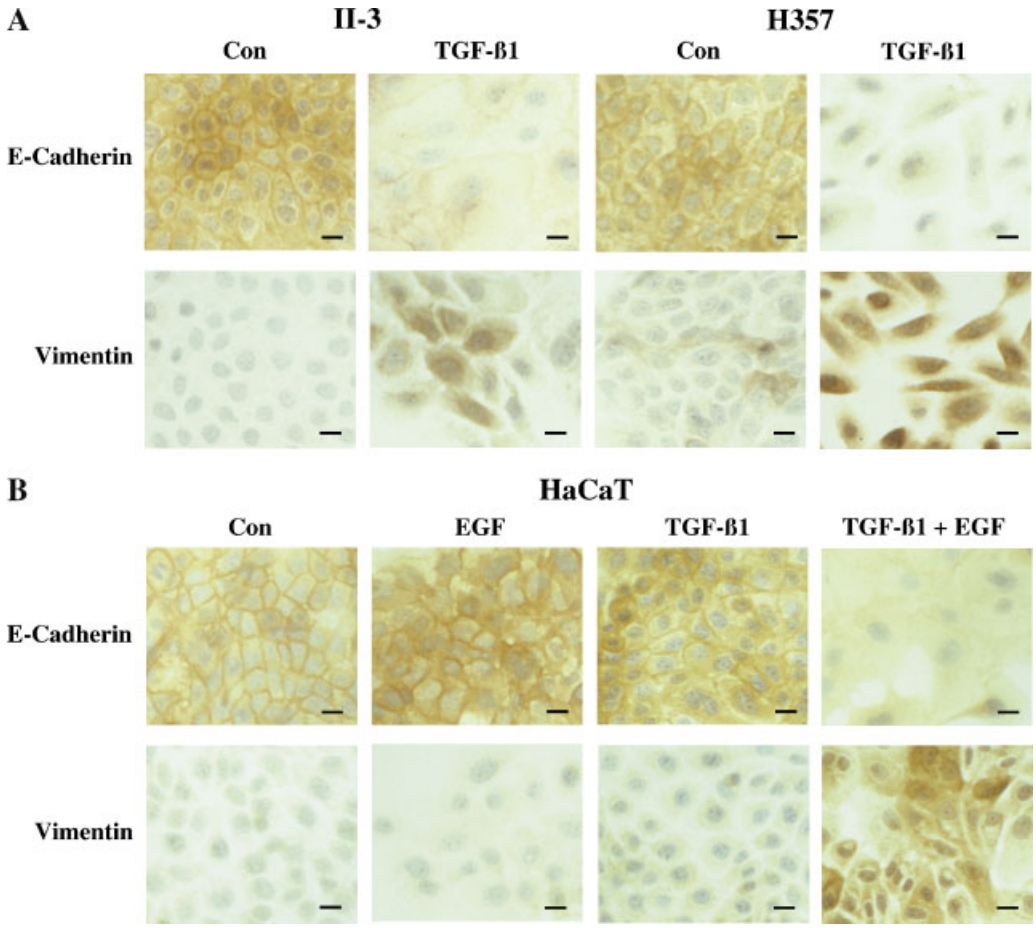

Fig. 1. TGF-β1-induced EMT is dependent on Ras signaling. **A**: Expression of E-Cadherin and vimentin in II-3 and H357 following treatment of cells with vehicle control or TGF-β1 (3 ng/ml) for 72 h. **B**: Expression of E-Cadherin and vimentin in HaCaT following treatment of cells with vehicle control, EGF (100 ng/ml), TGF-β1 (3 ng/ml) or a combination of both ligands for 72 h. Bar = 20 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

assessed by Western blot analysis using phospho-specific antibodies. The results demonstrated that both ERK and p38 were activated by TGF-β1 and that the level of activation was increased in the presence of EGF (Fig. 2A). Phospho-JNK was undetectable (10 min to 24 h) in all conditions by Western blotting. The chemical inhibitors of MEK (UO126), p38 (SB203580) and JNK (SP600125) were used to determine whether MAPK pathways were involved in TGF-β1-induced EMT. The specificity of the MAPK inhibitors used was confirmed by Western blot analysis (Fig. 2B). All of the inhibitors blocked TGF-β1-induced EMT in both H357 and II-3; cells treated with each of the agents in the presence of TGF-β1 looked similar to the controls and were of polygonal morphology, had E-Cadherin localised to the cell membrane and were essentially vimentin negative. The inhibition of TGF-β1-induced EMT by SP600125 in II-3 cells and by UO126 in H357 cells is shown in Figure 2C,D.

Clones of II-3 and H357 transduced with *Smad3ΔC* were examined for expression of Flag by Western analysis. Clones expressing the highest levels of Flag (H357-Smad3ΔC-C4, H357-Smad3ΔC-C14 and II-3-Smad3ΔC-C8) were selected for further study. The levels of Smad3ΔC expressed by the clones were sufficient to both inhibit the transcriptional activation of reporter constructs (3TP-lux and SBE_4-luc; data not shown) and attenuate ligand-mediated growth inhibition (Fig. 3A). Following TGF-β1 treatment (0.1–0.5 ng/ml, 72 h), EMT was inhibited in clones of both II-3 and H357 cells containing *Smad3ΔC*, as compared to vector controls; the data for H357-Smad3ΔC-C14 is shown in Figure 3B.

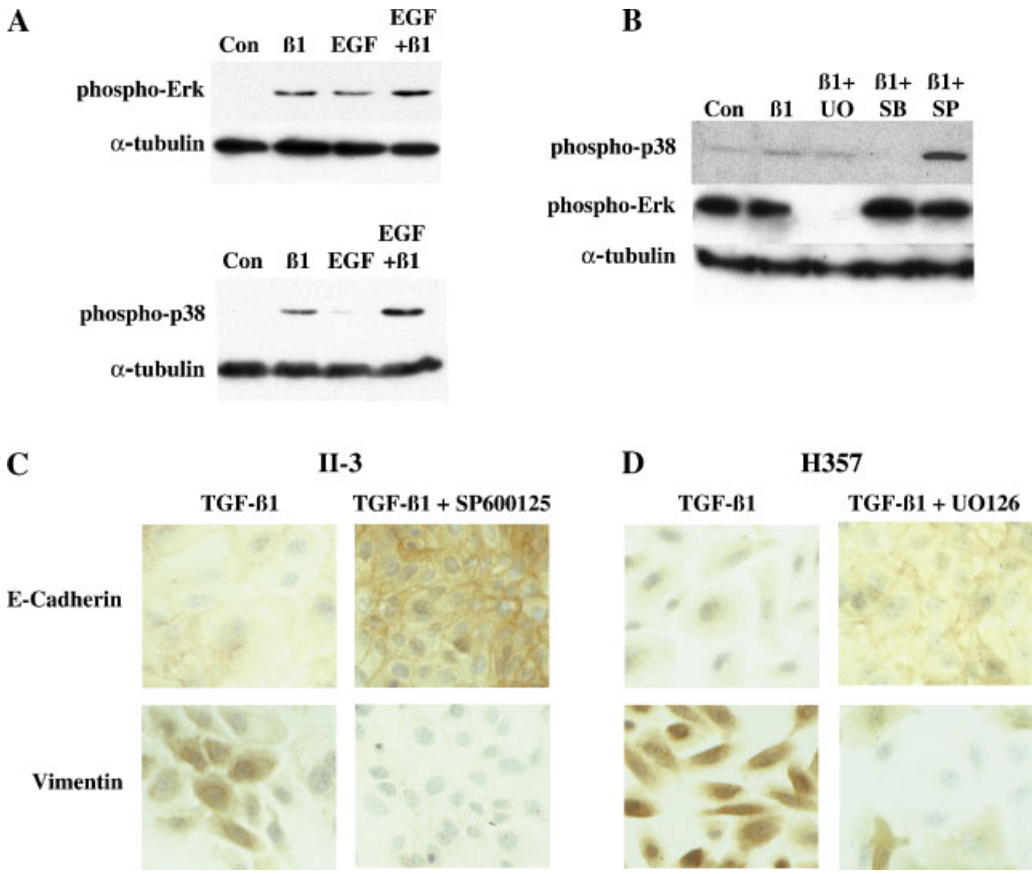

Fig. 2. Involvement of MAP kinase pathways in TGF-β1-induced EMT. **A**: Western blot analysis of the levels of phosphorylated ERK and p38 following treatment of HaCaT cells with or without TGF-β1 (3 ng/ml) in the presence or absence of EGF (100 ng/ml) for 24 h. **B**: Western blot analysis of the levels of phosphorylated ERK and p38 following treatment of H357 cells in the presence of TGF-β1 3 ng/ml in the presence or absence of the MAP kinase inhibitors [UO126 (UO), SB203580 (SB), SP600125 (SP); all 10 μM] for 30 min. All blots were re-probed with α-tubulin to confirm equal loading. **C**: Immunocytochemical analysis of E-Cadherin and vimentin expression in II-3 following treatment of cells with TGF-β1 (3 ng/ml) or TGF-β1 in combination with the JNK inhibitor SP600125 (10 μM) for 48 h. **D**: Immunocytochemical analysis of E-Cadherin and vimentin expression in H357 following treatment of cells with TGF-β1 (3 ng/ml) or TGF-β1 in combination with the MEK inhibitor UO126 (10 μM) for 48 h. Bar = 20 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The degree of transcriptional activation of the SBE_4-luc reporter induced by TGF-β1 in cells containing mutant ras was consistently higher than that seen in cells containing normal ras (Fig. 4A). To determine whether this observation was attributable to the presence of active Ras, HaCaT cells were treated with TGF-β1, EGF or a combination of both ligands. TGF-β1, but not EGF resulted in an increase of Smad3/4-dependent transcription, but the combination of TGF-β1 with EGF resulted in the potentiation of SBE_4-luc activation (Fig. 4B). Similar results were obtained with the Smad2/4-dependent reporter, DE-luc (Fig. 4C).

To determine whether the potentiation of TGF-β1-induced Smad transcription by EGF was due to MAPK signalling, the reporter assays were repeated in the presence of MAPK inhibitors. Each inhibitor significantly reduced the transcriptional activation of SBE_4-luc induced by TGF-β1 in H357 cells (Fig. 5A) and, in HaCaT cells, reduced the activation of SBE_4-luc induced by the combined treatment of TGF-β1 and EGF to levels induced by TGF-β1 alone (Fig. 5B).

Taken together, the results demonstrate that not only are MAPK and Smad pathways involved in TGF-β1-induced EMT in human keratinocytes but, also, MAPK signalling enhances Smad2/3-dependent transcription under conditions that drive TGF-β1-induced EMT.

Role of AP-1 in TGF-β1-Induced EMT

As the transcription factor AP-1 is a known primary target of MAPK pathways, we used a

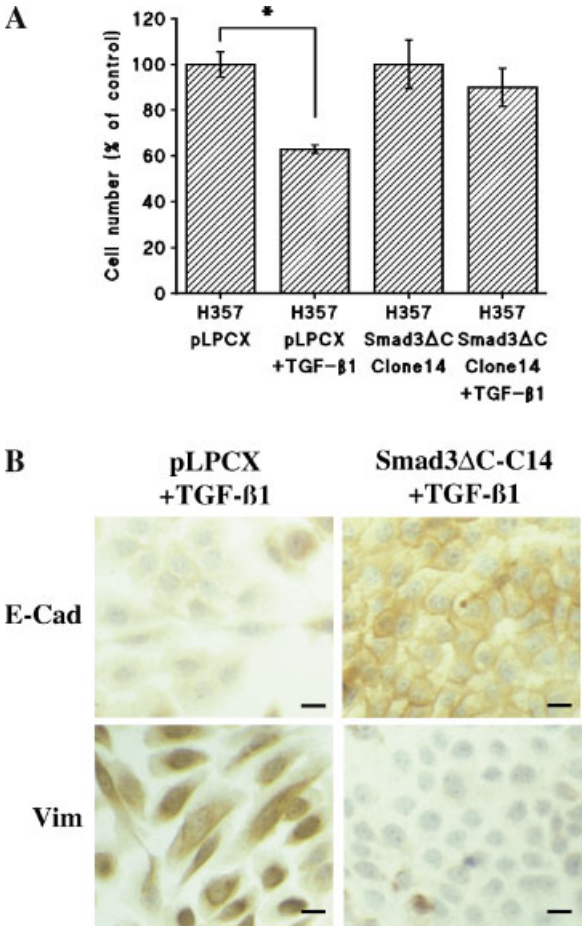

Fig. 3. TGF-β1-induced EMT requires the activity of receptor Smads. **A**: Growth inhibition of cells stably transfected with Smad3ΔC (H357-Smad3ΔC-C14) or pLPCX vector control (H357-pLPCX) following treatment with vehicle control or TGF-β1 (0.1 ng/ml) for 72 h. Values represent the mean ± SD. *, $P < 0.05$ TGF-β1 significantly inhibited the growth of H357-pLPCX but not H357-Smad3ΔC-C14. **B**: Expression of E-Cadherin and vimentin in H357-pLPCX and H357-Smad3ΔC-C14 following treatment of cells with TGF-β1 (0.1 ng/ml) for 72 h. Bar = 20 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

variety of strategies to examine the role of AP-1 in TGF-β1-mediated EMT.

Experiments using [6]-Gingerol, a chemical inhibitor of AP-1 [Bode et al., 2001], demonstrated that it could both inhibit EGF-induced activation of the 7AP-1-luc reporter in HaCaT cells (Fig. 6A), and TGF-β1-induced EMT in II-3 cells (Fig. 6B). Using another approach to inhibit AP-1 activity, we examined whether the ectopic expression of the endogenous inhibitor of Jun, *JDP2*, could inhibit TGF-β1-induced EMT in cell lines containing mutant Ras (H357, II-3). Expression of *JDP2* inhibited both TGF-β1-induced activation of the 7AP-1-luc reporter (Fig. 7A) and TGF-β1-induced EMT in both H357 and II-3 compared to vector only controls; the data for II-3 is shown in Figure 7B.

We next examined whether stimulation of AP-1 with TPA could substitute for EGF in enhancing TGF-β1-induced activation of Smad signalling and in the induction of EMT. The results demonstrated that TPA potentiated TGF-β1-induced transcriptional activation of the SBE_4-luc reporter (Fig. 8A) and co-operated with TGF-β1 to induce EMT (Fig. 8B) in HaCaT cells.

Based on our observation that MAPK pathways potentiate TGF-β1-dependent Smad transcription, we examined whether the AP-1 complex was involved in this process. The results demonstrated that treatment of II-3 cells with [6]-Gingerol reduced TGF-β1-induced SBE_4-luc activation (Fig. 9A), and that ectopic expression of *JDP2* in HaCaT cells inhibited the potentiation of TGF-β1-induced SBE_4-luc activation by EGF (Fig. 9B).

DISCUSSION

TGF-β-induced EMT has been described predominantly in murine cell systems and is thought to be uncommon in cells of human origin [Brown et al., 2004], an observation that can be explained, albeit in part, by differences between murine and human cells [Rangarajan and Weinberg, 2003]. The present study examined the conditions under which human keratinocytes undergo TGF-β1-induced EMT. We demonstrate that treatment of keratinocytes containing mutant *ras* (H357, II-3) with TGF-β1 resulted in EMT, as characterised by a delocalisation of E-Cadherin and an up-regulation of vimentin expression. By contrast, HaCaT cells containing normal *ras* did not undergo EMT in response to TGF-β1 alone, but did undergo this transition in the presence of both TGF-β1 and high concentrations (100 ng/ml) of EGF. Lower concentrations of EGF (10 ng/ml) did not facilitate TGF-β1-induced EMT, despite the fact that HaCaT cells are growth stimulated by this concentration of EGF [Game et al., 1992]. The data show that an active Ras pathway is an important prerequisite of TGF-β1-induced EMT in human keratinocytes. In the present study, TGF-β1 down-regulated E-Cadherin and up-regulated N-Cadherin protein expression irrespective of the presence of mutant Ras and the ability of the cells to

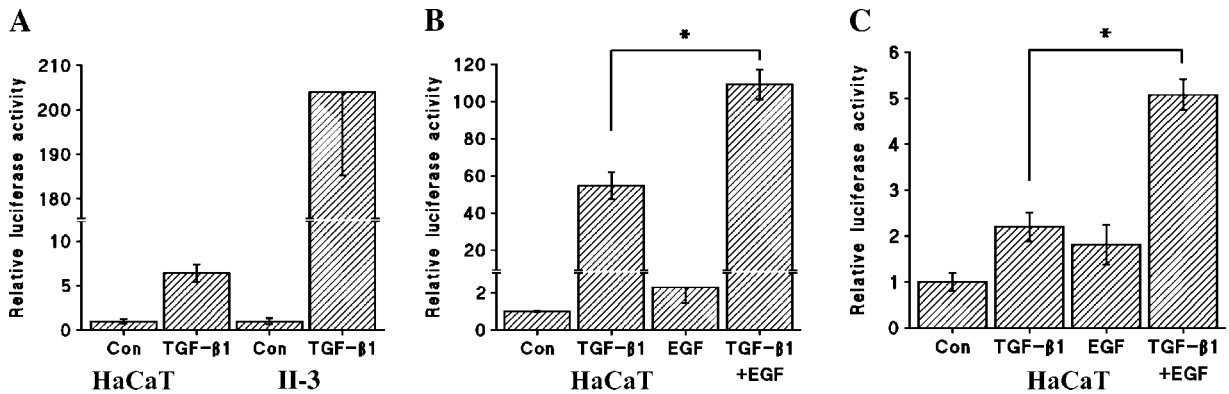

Fig. 4. Potentiation of TGF-β-induced Smad signaling by EGF. **A**: Activation of the SBE_4-luc reporter in HaCaT cells (normal Ras) and II-3 cells (mutant Ras) following treatment with TGF-β1. **B**: Potentiation of TGF-β1-induced SBE_4-luc activity by EGF in HaCaT cells. **C**: Potentiation of TGF-β1-induced DE-luc activity by EGF in HaCaT cells. Concentrations of ligands used were 1 ng/ml TGF-β1 and 100 ng/ml EGF in all experiments. All values represent the mean ± SD. *, $P < 0.05$, EGF significantly elevated TGF-β1-induced activation of Smad-dependent reporter constructs.

undergo EMT. Further, co-treatment of HaCaT cells with EGF and TGF-β1 did not enhance the up-regulation of N-Cadherin and the blocking of TGF-β1-induced EMT with the chemical inhibitor of p38 (SB203580) did not prevent the up-regulation of N-Cadherin (unpublished observations). These data demonstrate that alterations in the levels of E- and N-Cadherin per se do not reflect TGF-β1-induced EMT in human keratinocytes. Our findings contrast with those of Grande et al. [2002] who showed that co-stimulation with EGF was required to induce a transient increase in N-Cadherin during TGF-β1-induced EMT in porcine thyrocytes and highlight the species and cell type differences in the mechanisms responsible for TGF-β-induced EMT.

H357 and II-3 cells containing mutant *ras* and HaCaT cells containing wild type Ras have been shown previously to be growth inhibited by TGF-β1 [Game et al., 1992; Prime et al., 1994]. Cell cycle analysis of II-3 cells in the present study confirmed that the cells remained in G_1 arrest under conditions that induced EMT, indicating that the growth inhibitory response to TGF-β1 was retained during this morphological transition. In addition, we showed that EMT was reversible after removal of TGF-β1, results that are consistent with current thinking that epithelial phenotypic plasticity is a "transient" event that may facilitate motility and metastatic dissemination of tumour cells [Thiery and Chopin, 1999; Gotzmann et al., 2004].

In the present study, TGF-β1 induced the activation of ERK and p38 MAPKs in HaCaT cells and co-treatment of cells with EGF enhanced the activation of these MAPKs but the phosphorylation of JNK could not be detected by Western blot analysis. However, specific inhibitors of MEK, p38 and JNK all blocked TGF-β1-induced EMT, indicating that activation of all three pathways was required for EMT. These inhibitors have been widely used in other studies at similar or higher concentrations [Zavadil et al., 2001; Yu et al., 2002; Yue et al., 2004] and, at the concentrations used in the present study, the inhibitors did not inhibit the phosphorylation of Smad2 by TGF-β1. The results provide convincing evidence for the involvement of MAPK signalling in TGF-β1-induced EMT and are in broad agreement with the findings of others [Lehmann et al., 2000; Ellenrieder et al., 2001; Zavadil et al., 2001; Bakin et al., 2002; Gotzmann et al., 2002; Yu et al., 2002]. Having demonstrated the involvement of MAPKs in TGF-β1-induced EMT, we then determined whether the major effectors of TGF-β signal transduction, the Smad proteins, played a role in mediating this phenomenon. To specifically inhibit Smad signalling, we expressed a dominant-negative *Smad3* cDNA (*Smad3ΔC*) [Choy et al., 2000], known to interfere with the phosphorylation of both Smad2 and Smad3 [Tian et al., 2003], in cells containing mutant Ras. Expression of Smad3ΔC in H357 and II-3 inhibited Smad-dependent transcription, TGF-β1-induced growth inhibition and blocked EMT. The results of this study, therefore, demonstrate that TGF-β1-induced EMT involves Smad-dependent pathways; our findings are in agree-

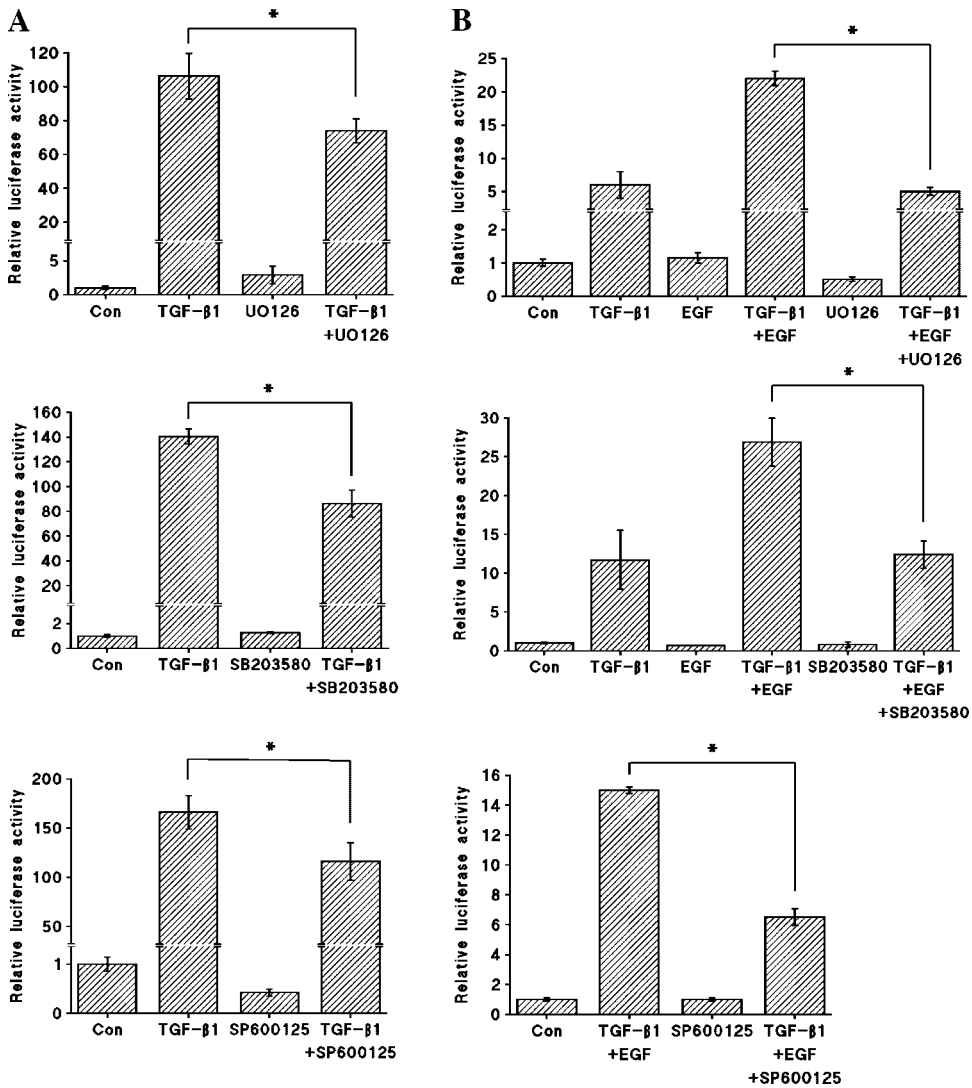

Fig. 5. Inhibition of TGF-β1-induced activation of SBE_4-luc by inhibitors of MAPKs. **A**: SBE_4-luc activity in H357 cells following treatment with TGF-β1 (1 ng/ml), the MAPK inhibitors UO126, SP600125, and SB203580 or TGF-β1 in combination with the MAPK inhibitors. **B**: SBE_4-luc activity in HaCaT cells following treatment with TGF-β1 (1 ng/ml), EGF (100 ng/ml) or a combination of the ligands with or without the MAPK inhibitors UO126, SP600125, and SB203580. The concentrations of the MAPK inhibitors were 10 μM in all experiments. All values represent the mean ± SD. *, $P < 0.05$, MAPK inhibitors significantly inhibited TGF-β1-induced activation of SBE_4-luc.

ment with the limited number of studies using cells of human origin [Ellenrieder et al., 2001; Tian et al., 2003]. It is cautionary to note, however, that Smad-independent pathways may also be involved. For example, Peinado et al. [2003] have shown that TGF-β1 down-regulates E-Cadherin, a marker of EMT, by increasing the levels of the transcriptional repressor Snail in a Smad-independent manner.

Interestingly, the results of this study show that MAPKs function to enhance Smad2/3-dependent transcription during TGF-β1-induced EMT. TGF-β1 activated the Smad-dependent reporter SBE_4-luc to a greater extent in H357 and II-3 compared to HaCaT cells and further, EGF potentiated TGF-β1-induced activation of both the SBE_4-luc (Smad3/4) and DE-luc (Smad2/4) reporters in HaCaT cells; this effect was blocked by the MAPK inhibitors. Our results are supported by previous observations showing that EGF enhanced Smad3 transactivation and TGF-β1-induced activation of SBE_4-luc in rat hepatocytes [Peron et al., 2001]. Oft et al. [2002] have reported a similar synergy between Ras and Smads at the level of transcriptional activation during EMT, in cells of

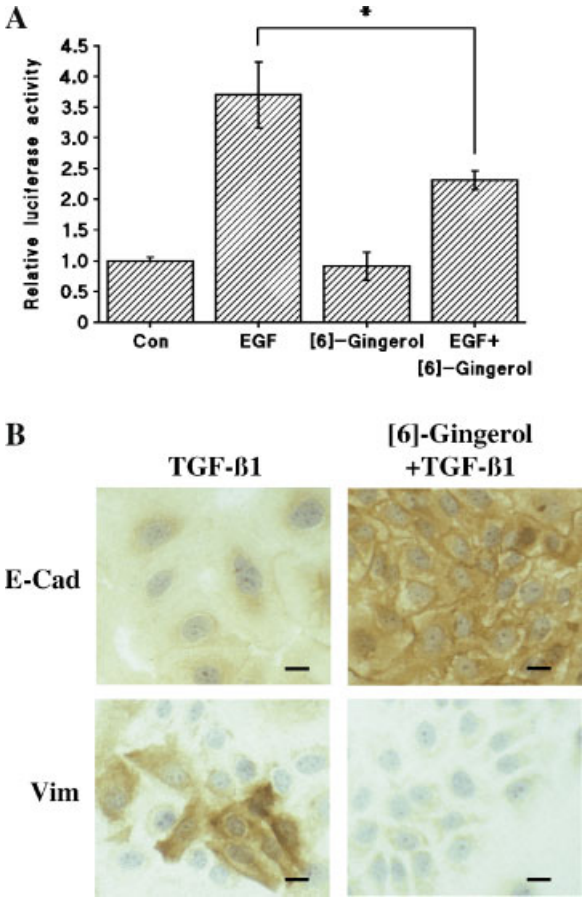

Fig. 6. AP-1 activity is required for TGF-β1-induced EMT. **A**: 7AP-1-luc activity in HaCaT cells following treatment with EGF (100 ng/ml), [6]-Gingerol (100 μM), or a combination of EGF and [6]-Gingerol. All values represent the mean ± SD. *, $P < 0.05$, [6]-Gingerol significantly inhibited EGF-induced activation of 7AP-1-luc. **B**: Expression of E-Cadherin and vimentin in II-3 following treatment of cells with TGF-β1 (1 ng/ml) or TGF-β1 in combination with [6]-Gingerol (100 μM) for 48 h. Bar = 20 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

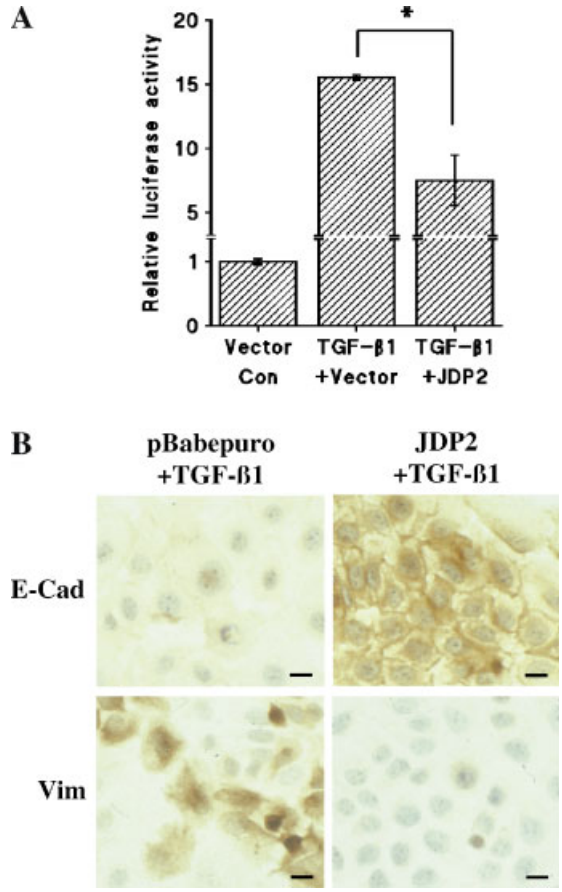

Fig. 7. Inhibition of the AP-1 protein c-jun blocks TGF-β1-induced EMT. **A**: 7AP-1-luc activity in II-3 cells following transient transfection of *JDP2* or vector control and treatment with TGF-β1 (2 ng/ml). Values represent the mean ± SD. *, $P < 0.05$, *JDP2* significantly reduced TGF-β1-induced activation of 7AP-1-luc. **B**: Expression of E-Cadherin and vimentin in II-3 cells transiently transfected with pBabepuro vector control or *JDP2* and treated with TGF-β1 (3 ng/ml). Bar = 20 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

murine origin, by a mechanism involving the sequential elevation of H-Ras and Smad2 levels. However, in the present study, the ability to undergo TGF-β1-induced EMT was not associated with elevated basal levels of phosphorylated Smad2 (unpublished observations).

The role of the AP-1 transcriptional complex in EMT has received little attention in the past [Kustikova et al., 1998; Hulboy et al., 2001] and, as far as we are aware, there are no data relating to the function of AP-1 in TGF-β1-induced EMT. The results of the present study provide strong evidence to support a role of AP-1 in TGF-β1-induced EMT. First, [6]-Gingerol, a phytochemical compound known to inhibit the activation of AP-1 [Bode et al., 2001], blocked AP-1 and Smad-dependent reporter activity and inhibited TGF-β1-induced EMT. Second, transfection of a naturally occurring antagonist of c-jun (*JDP2*) [Aronheim et al., 1997] into cells containing mutant *ras* inhibited the activation of an AP-1-dependent reporter and blocked TGF-β1-induced EMT. And, third, stimulation of AP-1 by TPA substituted for EGF in the induction of EMT by TGF-β1 in cells containing normal Ras and potentiated TGF-β1-induced activation of SBE_4-luc. It is possible that the effects of [6]-Gingerol, *JDP2* and TPA may not be confined to AP-1 but taken together, these results provide strong evidence to support a role for AP-1 in TGF-β1-induced EMT. Further confirmation of these findings would

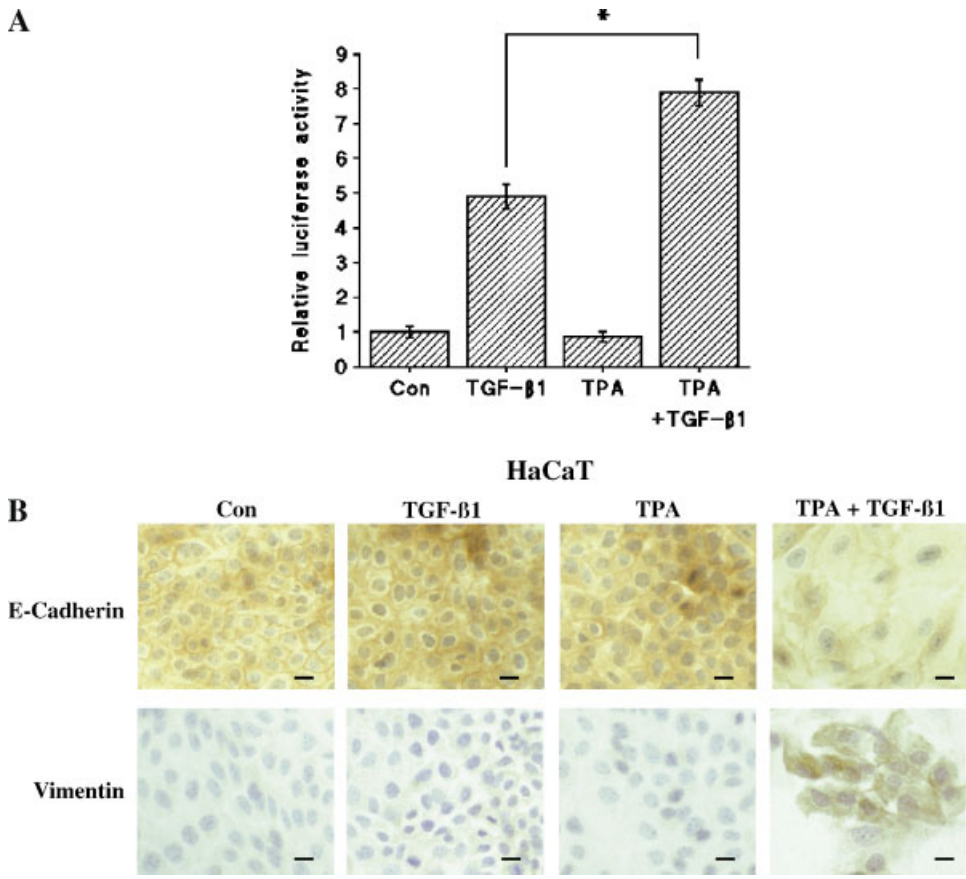

Fig. 8. TPA can substitute for active Ras in TGF-β1-induced EMT. **A**: SBE_4-luc activity in HaCaT cells treated with TGF-β1 (0.1 ng/ml) in the presence or absence of TPA (100 μg/ml). All values represent the mean ± SD. *, $P < 0.05$, TPA significantly elevated TGF-β1-induced activation of SBE_4-luc. **B**: Expression of E-Cadherin and vimentin in HaCaT following treatment of cells with vehicle control, TGF-β1 (1 ng/ml), TPA (100 μg/ml) or TPA in combination with TGF-β1 for 72 h. Bar = 20 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

require the silencing of AP-1 components using siRNA and/or anti-sense expression constructs.

Support for a role of AP-1 in EMT comes from studies where it has not only been shown that c-Jun is required for the re-organisation of the actin cytoskeleton in epithelial cells that over-express the EGF receptor [Malliri et al., 1998] but also, over-expression of Fra-1 in epithelioid adenocarcinoma cells results in morphological changes that resemble EMT [Kustikova et al., 1998]. Hulboy et al. [2001] have also shown that the induction of EMT in murine carcinoma cells is associated with changes in the AP-1 complex from the transcriptionally inactive JunB/Fra-2 dimer to the more active JunD/Fra-2 complex. In the present study, whilst the inhibition of AP-1 blocked the potentiation of TGF-β1-induced activation of Smad-dependent reporters by EGF, we were unable to demonstrate any differences in TGF-β1-induced AP-1 binding to the 12-O-tetradecanoyl-13-acetate-responsive gene promoter element (TRE) in cells containing mutant *ras* as compared to those with normal *ras*, using electrophoretic mobility shift assays (unpublished observations). However, these results do not exclude the possibility that the AP-1 complex acts indirectly to facilitate TGF-β1-induced EMT. For example, it has been shown that EGF, acting via AP-1, can potentiate TGF-β1-induced activation of SBE_4-luc in rat hepatocytes without enhancing binding to either the TRE or SBE [Peron et al., 2001]. The results of the present study and those of Peron et al. [2001] contrast with the findings of Verrecchia et al. [2000, 2001] who show that Jun proteins inhibit Smad-dependent transcription. The effects of TGF-β1, however, are cell type specific, in that the peptide acts as an inhibitor of epithelial cells such as keratinocytes and hepatocytes [Peron et al., 2001; present study] and a stimulator of cells of mesenchymal origin such

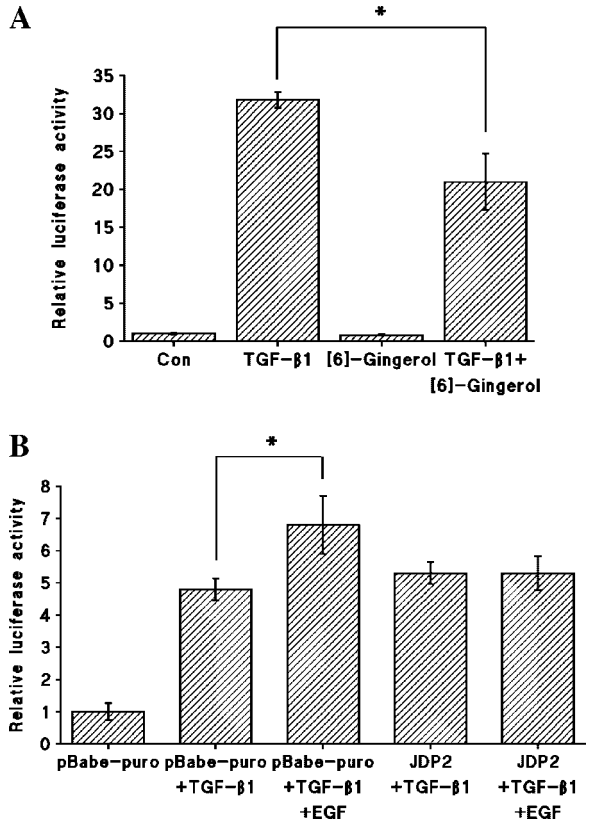

Fig. 9. Inhibition of TGF-β1-induced activation of SBE_4-luc by inhibitors of AP-1. **A**: SBE_4-luc activity in II-3 cells following treatment with TGF-β1 (1 ng/ml), [6]-Gingerol (100 μM) or TGF-β1 in combination with [6]-Gingerol. *, $P < 0.001$ [6]-Gingerol significantly inhibited TGF-β1-induced activation of SBE_4-luc. **B**: SBE_4-luc activity in HaCaT cells transiently transfected with *JDP2* or vector control following treatment with TGF-β1 (0.1 ng/ml). With or without EGF (100 ng/ml) *, $P < 0.05$ EGF significantly increased TGF-β1-induced activation of SBE_4-luc in cells transiently transfected with pBabepuro but not *JDP2*. All values represent the mean ± SD.

as dermal fibroblasts [Verrecchia et al., 2000, 2001].

It has been proposed that activation of the AP-1 complex would sequester transcriptional co-activators such as p300 and PCAF which, in turn, would inhibit Smad-dependent transcription [Verrecchia et al., 2000, 2001]. Intriguingly, we have unpublished data to show that the expression of p300 and PCAF are up-regulated in HaCaT cells treated with TPA or EGF plus TGF-β1, an effect that can be blocked by the inhibition of MAPK signalling. Similar observations have been made previously [Suh et al., 2003]. The results raise the possibility that the AP-1 complex may enhance Smad signalling by acting indirectly to induce the expression of recognised transcriptional activators and, in so doing, facilitate TGF-β1-induced EMT. This proposal is consistent with the findings of Tian et al. [2003] who showed that over-expression of Smad3 in breast cancer cells caused the delocalisation of E-Cadherin and TGF-β1-induced EMT.

It is now well recognised that TGF-β functions as a tumour suppressor in the early stages of tumorigenesis but later, acts to promote metastases and the induction of an EMT is thought to be one mechanism by which TGF-β can facilitate tumour cell dissemination. The present study describes TGF-β1-induced EMT in human keratinocytes and provides evidence that MAPK and Smad pathways, together with the AP-1 complex, are involved in this process. TGF-β signalling is considerably altered during epithelial tumour progression, such that TGF-β receptor expression is frequently reduced and tumour cells become less sensitive to the growth inhibitory effects of the ligand but the integrity of the signalling pathway essentially remains intact [Roberts and Wakefield, 2003]. A complete understanding of the specific pathways associated with EMT, particularly with respect to the cross talk between Smad-dependent and -independent pathways, will be essential if inhibitors are to be developed that block the process of TGF-β-induced metastatic dissemination of tumour cells.

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